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

Kyoko Sasaki,^{1,6} Yoshikazu Nagao,² Yoshihiro Kitano,³ Hideaki Hasegawa,^{1,2} Hiroaki Shibata,¹ Masaaki Takatoku,⁴ Satoshi Hayashi,⁵ Keiya Ozawa,⁴ and Yutaka Hanazono^{1,7}

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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¹ Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan.

² Department of Agriculture, Utsunomiya University, Tochigi, Japan.

³ Department of Surgery, National Center for Child Health and Development, Tokyo, Japan.

⁴ Division of Hematology, Department of Internal Medicine, Jichi Medical School, Tochigi, Japan.

⁵ Department of Obstetrics and Gynecology, National Center for Child Health and Development, Tokyo, Japan.

⁶ Department of Plastic and Reconstructive Surgery, Faculty of Medicine, University of Tokyo, Tokyo, Japan.

⁷ Address correspondence to: Dr. Yutaka Hanazono, Associate Professor, Head, Division of Regenerative Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi, Tochigi 329-0498, Japan. E-mail: hanazono@jichi.ac.jp.

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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 ATC TG-3' and 5'-GGC TGT GAC AAA GTC ACA TGG-3', and the inner primer set was 5'-GTC TGG ATT TCA TCC ATC TG-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 ATC TG-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-

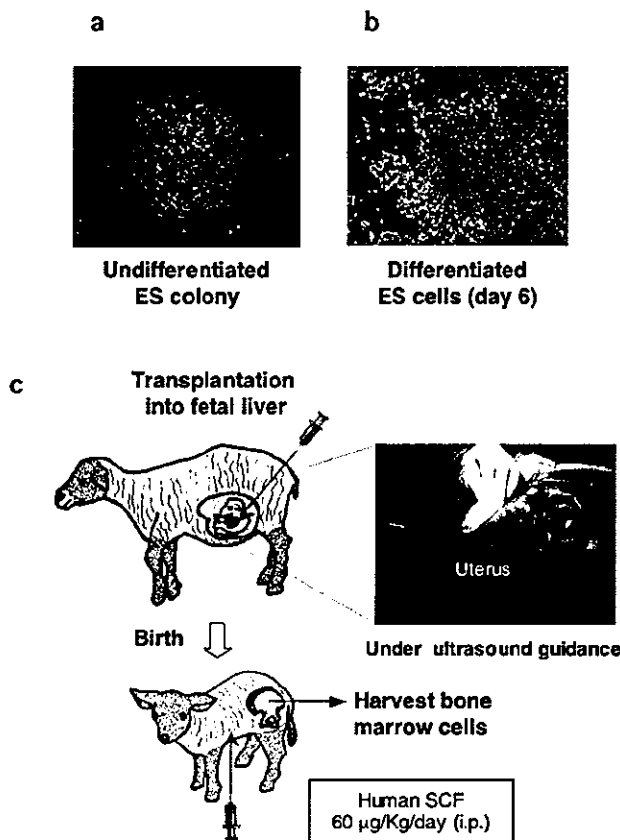


FIGURE 1. In utero transplantation of day 6 cynomolgus embryonic stem (ES)-derived cells. (a) Undifferentiated cynomolgus ES cells were maintained on mouse embryonic fibroblast feeder cells. (b) For hematopoietic induction, undifferentiated ES cells were placed on murine stromal OP9 cells in the presence 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 month	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 month	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-

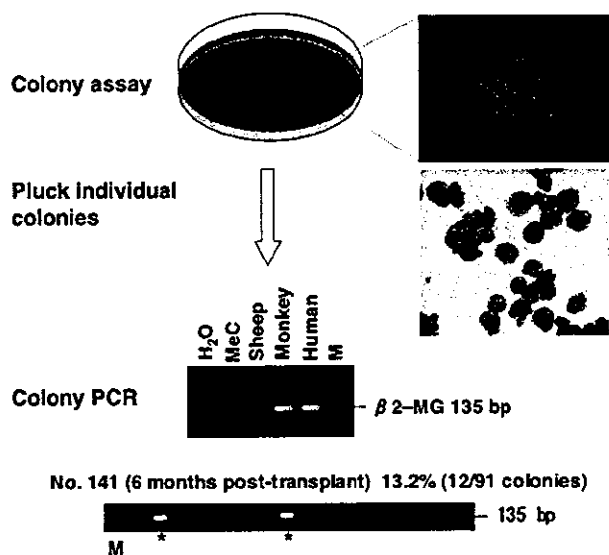


FIGURE 2. Assessment of cynomolgus hematopoiesis in sheep after birth. Bone-marrow cells were harvested from lambs and placed in methylcellulose. Hematopoietic colonies were thus formed. A cytospin specimen (stained with the Wright-Giemsa method) of plucked myeloid colonies demonstrated mature neutrophils. 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.

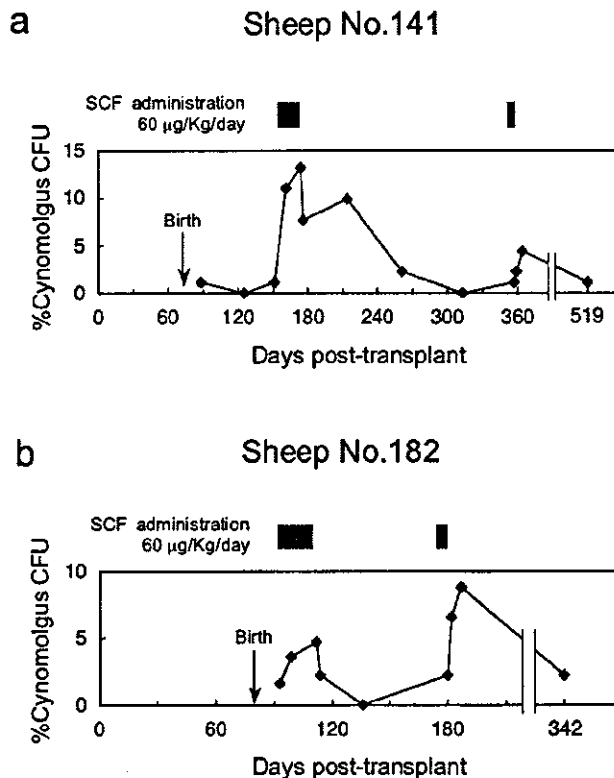


FIGURE 3. Time course of hematopoietic chimerism in the sheep receiving human SCF. (a) In sheep No. 141, human SCF was intraperitoneally administered at 60 $\mu\text{g}/\text{kg}$ once a day from day 156 posttransplantation for 18 days. SCF administration was then stopped and tried again from day 352 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).

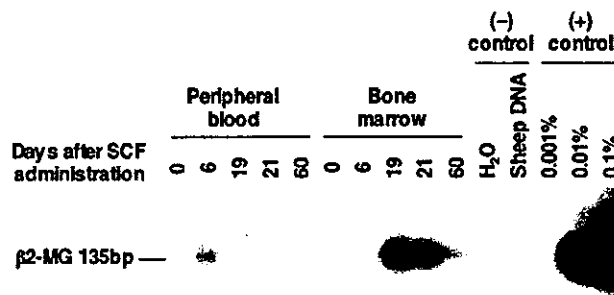


FIGURE 4. Detection of cynomolgus cells in the circulation. DNA was extracted from whole peripheral blood or bone-marrow nucleated cells after birth and subjected to cynomolgus-specific $\beta 2$ -microglobulin ($\beta 2$ -MG) PCR, and Southern blot analysis. Data before and after SCF administration 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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RESEARCH ARTICLE

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

K Sasaki^{1,2}, M Inoue³, H Shibata¹, Y Ueda³, S-i Muramatsu⁴, T Okada¹, M Hasegawa³, K Ozawa¹ and Y Hanazono¹

¹Center for Molecular Medicine, Jichi Medical School, Minamikawachi, Tochigi, Japan; ²Department of Plastic and Reconstructive Surgery, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan; ³DNAVEC Corporation, Tsukuba, Ibaraki, Japan; and ⁴Department of Neurology, Jichi Medical School, Minamikawachi, Tochigi, Japan

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).

Correspondence: Dr Y Hanazono, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi, Tochigi 329-0498, Japan

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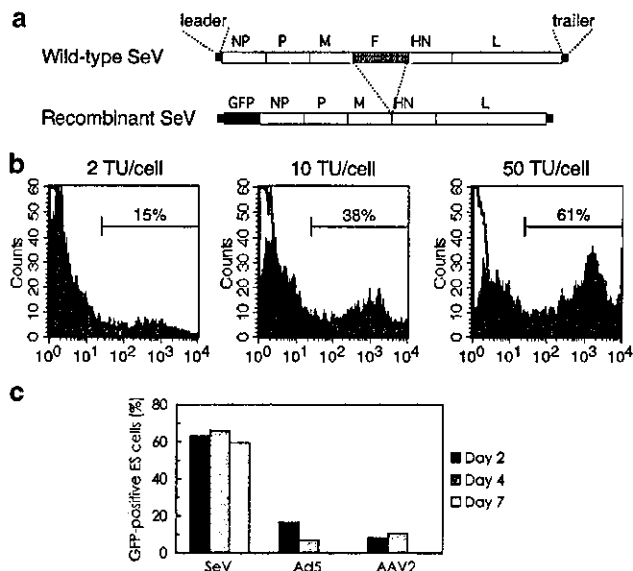


Figure 1 High-level transgene expression in cynomolgus ES cells after infection with SeV vector. (a) Schematic diagrams of the wild-type SeV genome and recombinant F-defective SeV carrying the GFP gene (SeV vector in this study). The SeV genome is 15 384 nucleotides long and its genes (NP, P, M, F, HN, 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

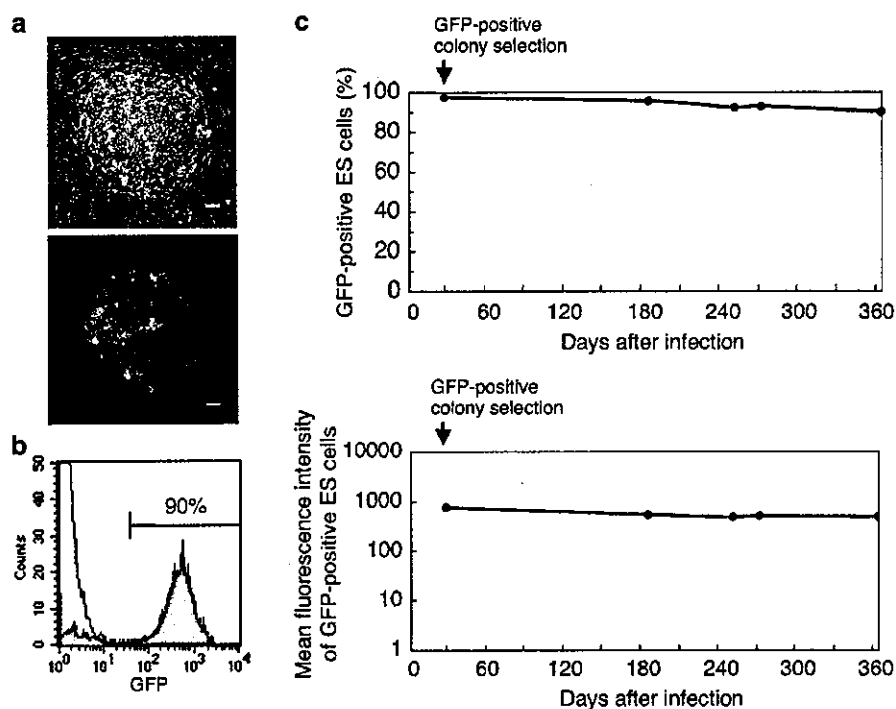


Figure 2 Stable SeV-mediated transgene expression in cynomolgus ES cells. Fluorescent ES cell colonies were plucked under a fluorescent microscope once at 1 month after infection and the cells were further propagated. (a) Phase-contrast (upper) and fluorescence (lower) images of a cynomolgus ES cell 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).

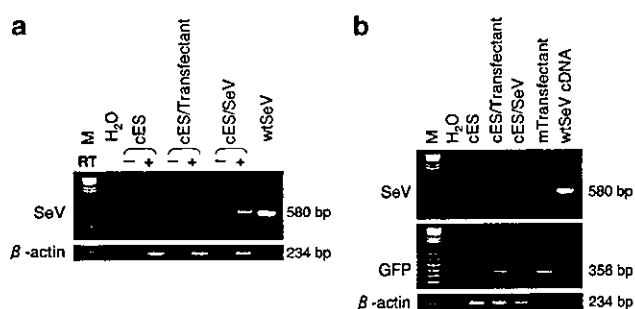


Figure 3 DNA-independent replication and transcription of SeV vector. Total cellular RNA and DNA were extracted from cynomolgus ES cells at day 284 after infection with the SeV vector. RNA-PCR (a) and DNA-PCR (b) for the SeV RNA genome or GFP sequence were conducted. The cynomolgus β -actin sequence 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.

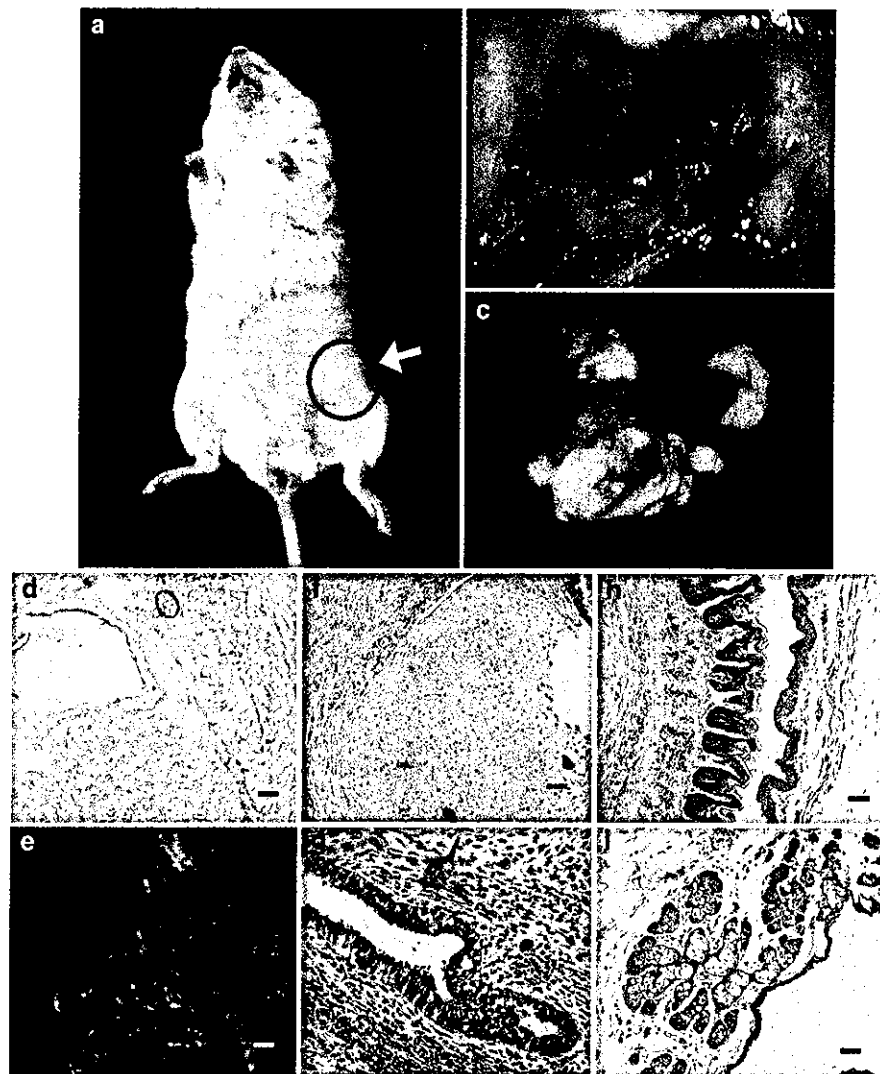


Figure 4 Pluripotency of SeV-infected cynomolgus ES cells. Tumors formed in NOD-SCID mice after inoculation of the SeV-infected cynomolgus ES cells (a). The tumor was fluorescing ((b), bright field; (c), dark field). Fluorescence was observed uniformly in the tumor under a fluorescent microscope ((d), 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

(Biomeda, 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

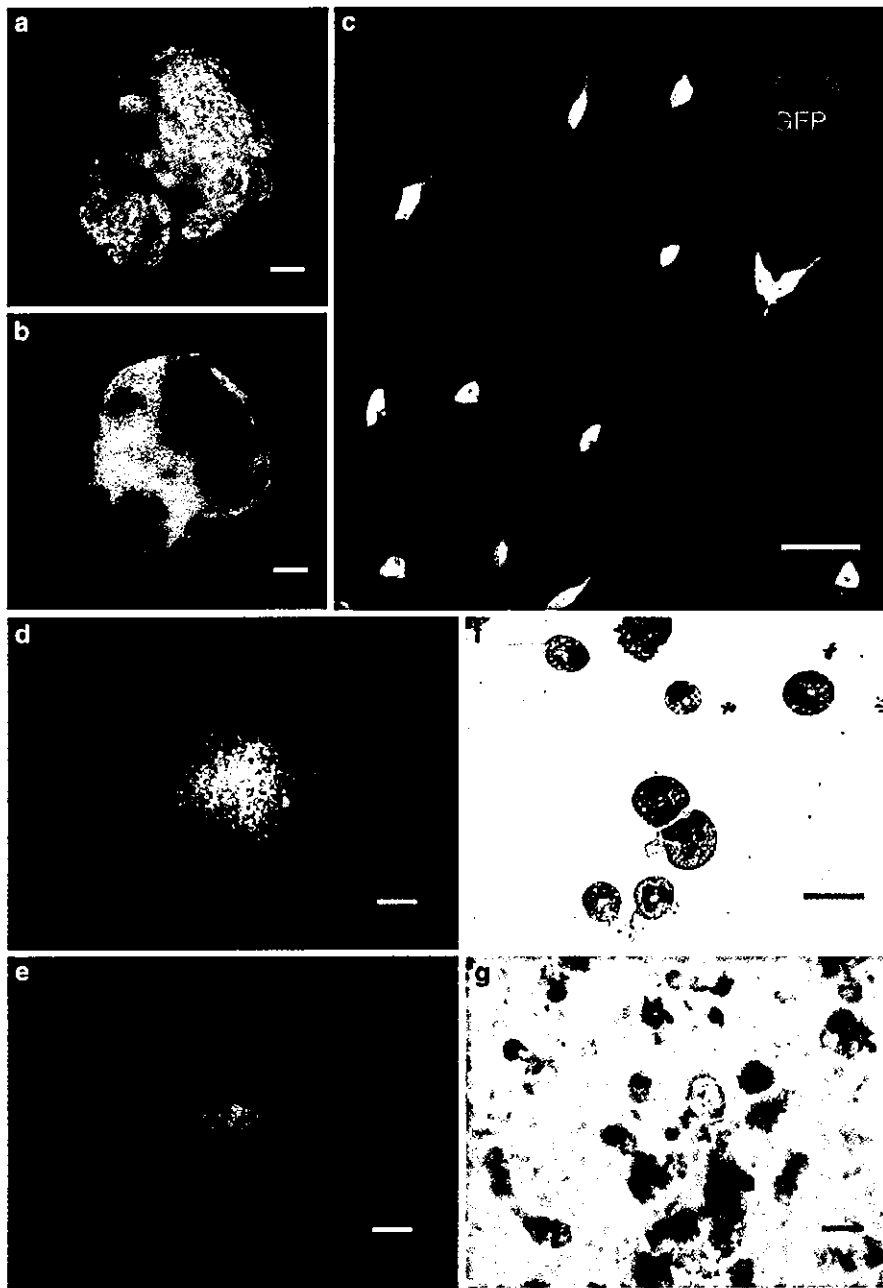


Figure 5 Stable transgene expression during differentiation. A day-20 cystic embryoid body was observed under a fluorescent phase-contrast microscope, confirming that the embryoid body was fluorescing ((a), bright field; (b), dark field). After infection with the SeV vector, fluorescent cynomolgus ES 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

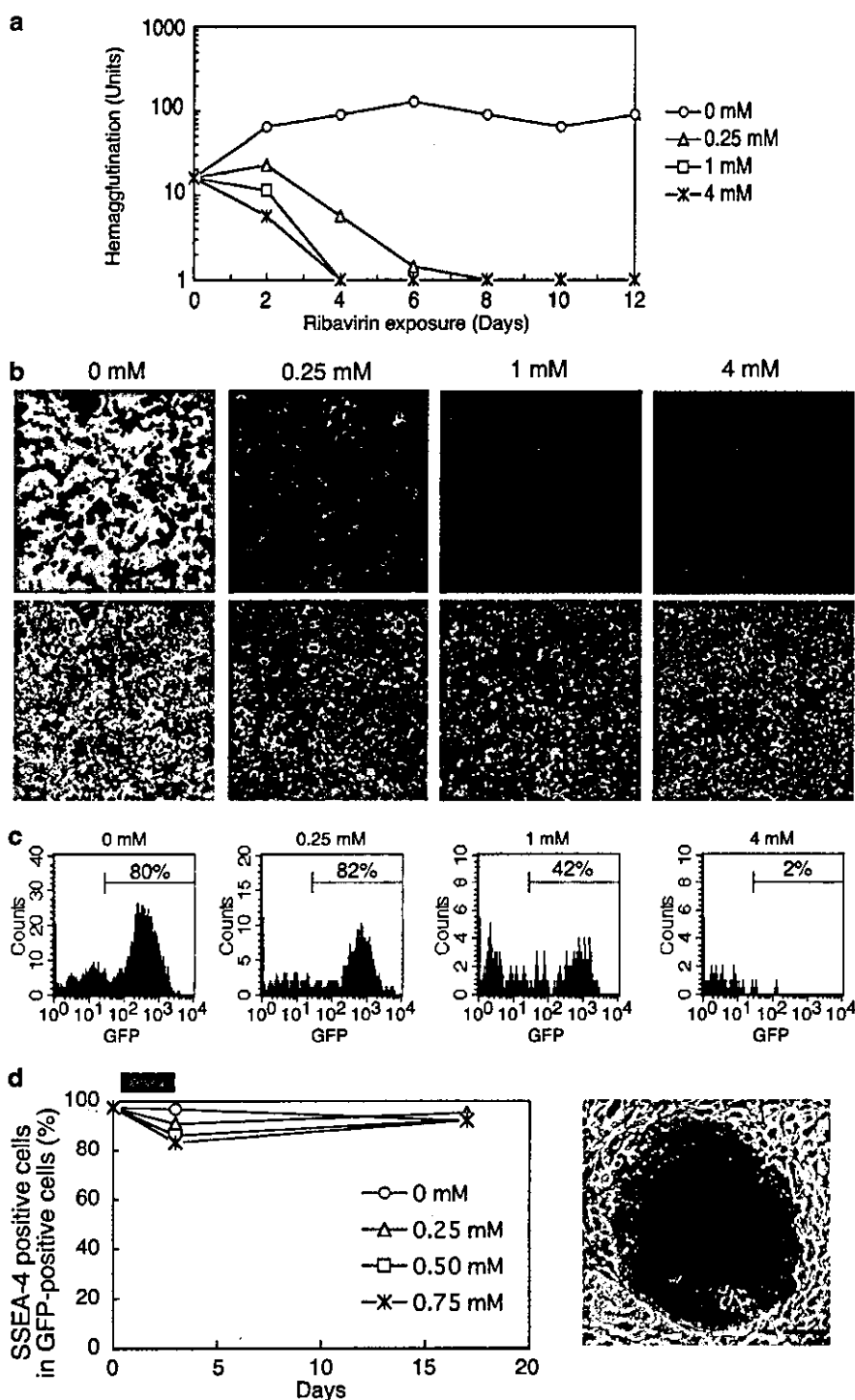


Figure 6 Ribavirin-regulated transgene expression. (a) A rhesus kidney cell line (LLC-MK2) was infected with the SeV vector at 3 TU/cell. Ribavirin was started at various concentrations on day 2 after the infection. The formation of viral particles in the infected LLC-MK2 cells was examined by the hemagglutination 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

H-2d (SF1-1.1; PharMingen, San Diego, CA, USA), which does not react to cynomolgus cells but does react to BALB/c cells.

Teratoma formation

Cynomolgus ES cells (approximately 10^6 cells per site) were injected subcutaneously into the hind leg of 6- to 8-week-old nonobese diabetic/severe combined immunodeficient mice (Jackson Laboratory, Bar Harbor, ME, USA). The resulting tumors (usually 9–12 weeks after the injection) were dissected and fixed in 4% paraformaldehyde. For histological analysis, samples from the tumors were embedded in paraffin and stained with hematoxylin and eosin. To observe GFP fluorescence, samples were embedded in OTC compound (Sakura, Zoeterwoude, Netherlands), frozen, sectioned, and examined under a fluorescence microscope.

Hematopoietic differentiation

The mouse bone marrow stromal cell line OP9 was maintained in α -modified minimum essential medium (Invitrogen) supplemented with 20% FCS as described previously.²⁸ For induction of hematopoietic differentiation, ES cells were seeded onto a mitomycin C-treated confluent OP9 cell layer in six-well plates. Medium to support the differentiation was described elsewhere.²⁹ Cells at day 18 were placed in Methocult GF+ media (StemCell Technologies, Vancouver, Canada) at 1×10^4 and 1×10^5 cells per plate and clonogenic hematopoietic colonies were produced. After 14 days, individual colonies were removed and spun onto glass slides. Cells were stained with the Wright-Giemsa method. The nitro blue tetrazolium (NBT, Sigma) reduction test was performed on the cells as a granulocyte functional assay according to a previously described method.³⁰

Neural differentiation

The induction of neural differentiation was carried out as described previously.³¹ Day-4 embryoid bodies were plated onto tissue culture dishes and nestin-positive cells were selected in DMEM/F12 medium supplemented with 5 μ g/ml of insulin (Sigma), 50 μ g/ml of transferrin (Sigma), 30 nM selenium chloride (Sigma), and 5 μ g/ml of fibronectin (Sigma) for 5 days. Cells were then trypsinized and plated in polyornithine-coated dishes (15 μ g/ml) and expanded in N2 medium³² supplemented with 1 μ g/ml of laminin (Sigma) and 10 μ g/ml of basic fibroblast growth factor (bFGF; Roche, Basel, Switzerland) for 6 days. Differentiation was induced by removal of bFGF. To confirm the neural differentiation, cells were stained with anti-human MAP-2. Briefly, cells were fixed in 4% paraformaldehyde in PBS and incubated with anti-human MAP-2 (HM-2; Sigma; diluted 1:4000) and then by Alexa Fluor 594-labeled antibody (diluted 1:500; Molecular Probe, Eugene, OR, USA). The samples were examined under a fluorescence microscope.

DNA-PCR

DNA-PCR for the SeV genome and GFP sequences was carried out as follows. DNA was extracted using the QIAamp DNA mini kits (Qiagen, Hilden, Germany) and 250 ng was used for each PCR with ExTaq (Takara, Shiga, Japan). Amplification conditions were 30 cycles of 94°C for 1 min, a variable annealing temperature (noted

below) for 1 min, and 72°C for 1 min. The amplified products were run on 2% agarose gel and visualized by ethidium bromide staining. Primer sequences, annealing temperatures and product sizes were as follows: the SeV vector genome sequence: 5'-AGA GAA CAA GAC TAA GGC TAC C-3' and 5'-ACC TTG ACA ATC CTG ATG TGG-3' (55°C, 580 bp); the GFP sequence: 5'-CGT CCA GGA GCG CAC CAT CTT C-3' and 5'-GGT CTT TGC TCA GGG CGG ACT-3' (60°C, 356 bp). the cynomolgus β -actin sequence: 5'-CAT TGT CAT GGA CTC TGG CGA CGG-3' and 5'-CAT CTC CTG CTC GAA GTC TAG GGC-3' (60°C, 234 bp).

RNA-PCR

RNA-PCR for the SeV RNA genomic sequence was carried out as follows. Total RNA was extracted using RNA STAT-60 (Tel-Test, Friendswood, TX, USA). Reverse transcription was conducted by using Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). The product (250 ng) after the reverse transcription was used for the subsequent PCR as described above.

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Method — Intra-bone marrow transplantation of hematopoietic stem cells in non-human primates: long-term engraftment without conditioning

KYOJI UEDA^{1,5}, YUTAKA HANAZONO^{1,*}, NAOHIDE AGEYAMA², HIROAKI SHIBATA^{1,2}, SATOKO OGATA¹, YASUJI UEDA³, TOSHIAKI TABATA³, SUSUMU IKEHARA⁴, MASAFUMI TANIWAKI⁵, MAMORU HASEGAWA³, KEIJI TERAOKA² and KEIYA OZAWA^{1,†}

¹Center for Molecular Medicine, Jichi Medical School, Tochigi 329-0498, Japan

²Tsukuba Primate Center, National Institute of Infectious Diseases, Ibaraki 305-0843, Japan

³DNAVEC Corporation, Ibaraki 305-0856, Japan

⁴First Department of Pathology, Kansai Medical University, Osaka 570-8506, Japan

⁵Division of Hematology and Oncology, Department of Medicine, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan

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Abstract—It has recently been reported that bone marrow cells can efficiently engraft without marrow conditioning when implanted directly into the bone marrow cavity (intra-bone marrow transplantation, iBMT) in mice. We have successfully examined the efficacy of autologous iBMT in a cynomolgus monkey model in conjunction with an *in vivo* expansion of transplanted cells by a selective amplifier transgene (Ueda *et al.*, 2004) and provide here the detailed parameters of our iBMT method. We injected retrovirally-marked autologous CD34⁺ cells directly into the non-conditioned marrow cavity of the femur and humerus after gently irrigating the cavity with saline. This transplant procedure was safely performed without pulmonary embolism. Gene-marked cells were not detectable in the peripheral blood at one hour and one day after iBMT as assessed by sensitive PCR, indicating that iBMT hardly generated a systemic delivery of transplanted cells. On the other hand, 2 to 30% of clonogenic hematopoietic colonies produced from the implanted marrow were gene-marked at 6–12 months after iBMT. Our iBMT method for non-human primates is thus discussed in terms of long-lived hematopoietic stem/progenitor cells, bone marrow niche and long-term engraftment after iBMT without myeloablative conditioning.

Keywords: gene marking; hematopoietic stem cell; intra-bone marrow transplantation; non-conditioning; non-human primate.

*Corresponding author. E-mail: hanazono@jichi.ac.jp

†Corresponding author. E-mail: kozawa@jichi.ac.jp

INTRODUCTION

One major barrier to effective hematopoietic stem cell (HSC) gene therapy is the necessity for myeloablation before transplantation. Current myeloablative conditioning regimens are associated with high systemic toxicity, and potential damage to marrow stroma possibly resulting in impaired engraftment (Plett *et al.*, 2002). In some disorders such as X-linked severe combined immunodeficiency, however, transplanted genetically-corrected cells increased in number to a therapeutic level without prior conditioning due to a naturally-occurring selective growth advantage (Cavazzana-Calvo *et al.*, 2000). When gene-modified cells do not have such a growth advantage, it is difficult to obtain therapeutic levels of corrected cells *in vivo* after transplantation without prior conditioning (Malech *et al.*, 1997; Dunbar *et al.*, 1998). Conditioning treatment of reduced intensity also results in insufficient engraftment of gene-modified cells in non-human primates (Huhn *et al.*, 1999; Rosenzweig *et al.*, 1999).

It has been reported that bone marrow cells can efficiently engraft without marrow conditioning when implanted directly into the bone marrow cavity in mice (Zhong *et al.*, 2002). Using the iBMT method, human cord blood cells are able to engraft more efficiently in bone marrow of sublethally irradiated immunodeficient mice as compared to the intravenous transplantation method (Mazurier *et al.*, 2003; Yahata *et al.*, 2003; Wang *et al.*, 2003). These observations raise the possibility that transplanted HSCs could engraft after iBMT without marrow conditioning in primates, although iBMT has been shown to be equivalent to intravenous infusion in patients under standard conditioning with cyclophosphamide and either total body irradiation or busulfan (Hagglund *et al.*, 1998). We have successfully examined this possibility in a cynomolgus monkey model in conjunction with an *in vivo* expansion of transplanted cells by a selective amplifier transgene (Ueda *et al.*, 2004) and provide here the detailed parameters of our iBMT method.

MATERIALS AND METHODS

Animals

Cynomolgus monkeys (*Macaca fascicularis*) were housed and handled in accordance with the rules for animal care and management of the Tsukuba Primate Center and the guiding principles for animal experiments using non-human primates formulated by the Primate Society of Japan. The animals were certified free of intestinal parasites and seronegative for simian type-D retrovirus, herpes virus B, varicella-zoster-like virus, and measles virus. The protocol of experimental procedures was approved by the animal welfare and animal care committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Collection of CD34⁺ cells

Peripheral blood or bone marrow cells were collected by leukapheresis or by aspiration from iliac bones, respectively (Ageyama *et al.*, 2002). From harvested cells, a leukocyte fraction was obtained after erythrocyte lysis with ACK buffer (155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA; Wako, Osaka, Japan). CD34⁺ cells were concentrated using magnet beads conjugated with anti-human CD34 (clone 561; Dynal, Lake Success, NY, USA) which cross-reacts to cynomolgus CD34 (Shibata *et al.*, 2003). The purity of CD34⁺ cells ranged from 90 to 95% as assessed with another anti-human CD34 (clone 563; PharMingen, San Diego, CA, USA) which cross-reacts to cynomolgus CD34 (Shibata *et al.*, 2003).

Retroviral transduction

We used the PLI non-expression retroviral vector, which contains non-translated *neo^R* and *β-gal* sequences (Heim *et al.*, 2000). The titer of viral supernatant was 1×10^6 genomic copies per ml, as assessed by RNA dot blot. CD34⁺ cells were cultured at a starting concentration of $1-5 \times 10^5$ cells/ml in fresh vector supernatant of PLI with recombinant human (rh) stem cell factor (SCF; Biosource, Camarillo, CA, USA), rh thrombopoietin (Kirin, Tokyo, Japan) and rh Flt-3 ligand (Research Diagnostics, Flanders, NJ, USA) each at 100 ng/ml in dishes coated with $20 \mu\text{g}/\text{cm}^2$ of RetroNectin (Takara, Shiga, Japan). Every 24 hours, nonadherent cells were collected, spun down, resuspended in fresh vector supernatant and cytokines, and then returned to the same RetroNectin-coated dishes. After 96-hours transduction, cells were washed and the culture was continued (Dulbecco's modified Eagle's medium [Invitrogen, Carlsbad, CA, USA] containing 10% fetal calf serum [Invitrogen]) for two additional days in the same RetroNectin-coated dishes in the presence of rhSCF alone (Takatoku *et al.*, 2001).

Intra-bone marrow transplantation (iBMT)

Cynomolgus monkeys were anesthetized. Two needles were inserted into both ends of the femur and humerus (Kushida *et al.*, 2002) and iBMT was processed as detailed in the Results section. Upon completion of the iBMT, the needle holes were sealed with bone wax (Lukens, Reading, PA, USA).

Measuring intraosseous pressure

A pressure transducer DX-360 (Nihon Koden, Tokyo, Japan) was connected to an intra-femur/humerus needle via a tight-fitting polyethylene tube filled with normal saline (Lucht *et al.*, 1981; Harper *et al.*, 1991). The transducer was calibrated so that the position of the inserted needle tip was a zero level (0 mmHg). Intraosseous pressure was measured and recorded on a polygraph LEG-1000 (Nihon Koden).