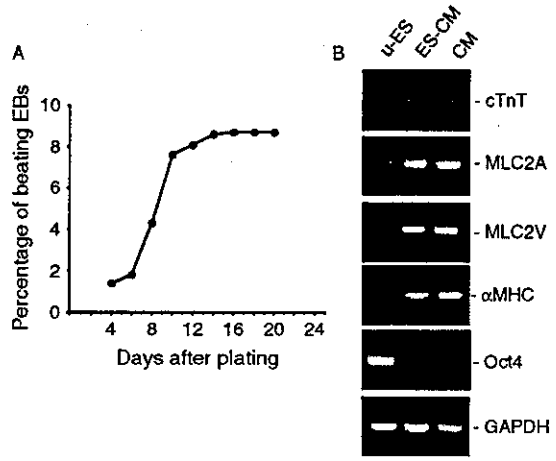


Figure 1. Differentiation of cardiomyocytes from cynomolgus ES cells. (A) Cumulative ratio of EBs derived from cynomolgus ES cells containing spontaneously contracting areas during differentiation. (B) Expression of specific cardiac markers. RNA samples from undifferentiated ES cells (u-ES), contracting area of EBs (ES-CM), and cynomolgus monkey cardiac tissues (CM) analyzed by RT-PCR for the expression of cardiac-specific markers: cTnT, MLC-2A, MLC-2V and αMHC. Oct-4 is undifferentiated ES cell marker. GAPDH served as internal standard

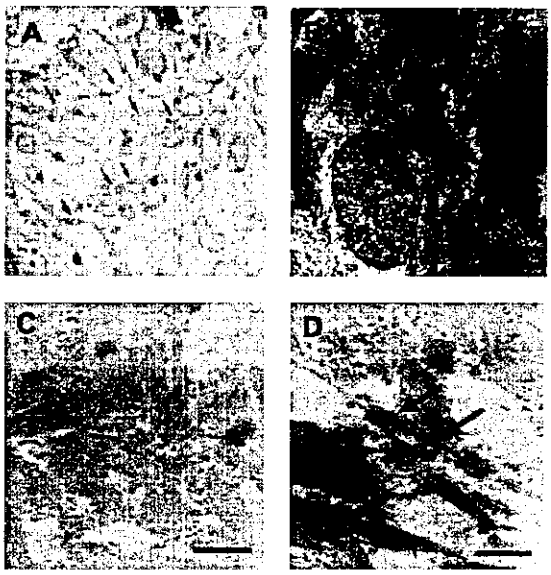


Figure 2. Morphological analysis of contracting EBs 14 days after plating. (A) High power light microscopy image stained with toluidine blue. Myofibers were observed (arrows). Magnification x1000. (B) Low power transmission micrograph revealed sarcomeric organization (arrowheads) and desmosomes (arrows). Scale bar: 2 μm. (C, D) High power transmission micrograph. Sarcomeric organization (C) and desmosomes (D, arrows) are evident. Scale bar: 500 nm

or CV-11 974 (Figure 3). We also confirmed the expression of cTnI and cardiac myosin in the contracting EBs by immunohistochemical staining (Figures 4B and 4E). Taken together, these results indicate that cynomolgus ES cells differentiate into cardiomyocytes *in vitro*.

Transgene expression with a SIV-based lentiviral vector

We used SIV vectors encoding the EGFP gene under the control of the CMV promoter to examine gene transduction in cardiomyocytes derived from ES cells. We detected EGFP expression in cultures by 5 days and this was maintained for at least 28 days (data not shown). Most staining of the transduced cells overlapped with cTnI or cardiac myosin (Figures 4C and 4F). The ratio (%) of EGFP-positive cells among cTnI-positive cells (4276 cells in eight samples) reached 97.1 ± 1.8% at 14 days after transduction (Figure 5). Cardiac differentiation and contractile function were not significantly altered in infected cultures.

Transplantation of cardiomyocytes derived from cynomolgus ES cells

We further investigated whether cardiomyocytes derived from cynomolgus monkey ES cells can survive in the rat myocardial infarction model myocardium. Transplanted cells transduced with the EGFP-SIV vector were identified in myocardial tissue section by green fluorescence, while cells stained with the cardiac-specific marker cTnI were identified by red fluorescence. The myocardial tissue co-expressed cTnI and EGFP 14 days after cell transplantation (Figure 6). In addition, the cTnI- and EGFP-expressed cells were normally stained with DAPI. These results confirmed that cardiomyocytes derived from transplanted ES cells survived in the injured myocardium.

Discussion

The present study demonstrates that cynomolgus monkey ES cells can differentiate into cardiomyocytes *in vitro*. Cardiomyocytic nature was confirmed by (1) the expression of cardiomyocyte-specific molecular markers such as cTnT, MLC-2A, MLC-2V, and αMHC, (2) the ultrastructural features of sarcomeric organization and desmosomes, and (3) intracellular calcium transience. Our cynomolgus ES cells formed contracting areas 3 days after EBs were plated. This point is in between the 1 day for murine ES cells and the 5–8 days for human ES cells after plating [2], reflecting the fetal developmental periods of these three species.

Transplantation of viable cardiomyocytes has emerged as a potential new therapy with which to treat the injured myocardium. Various types of cells including fetal and neonatal cardiomyocytes [18–20], skeletal myoblasts [21–23], and bone marrow cells [24,25] have been used as donor cells. However, the source of these cells might be limited and insufficient for clinical purposes. In

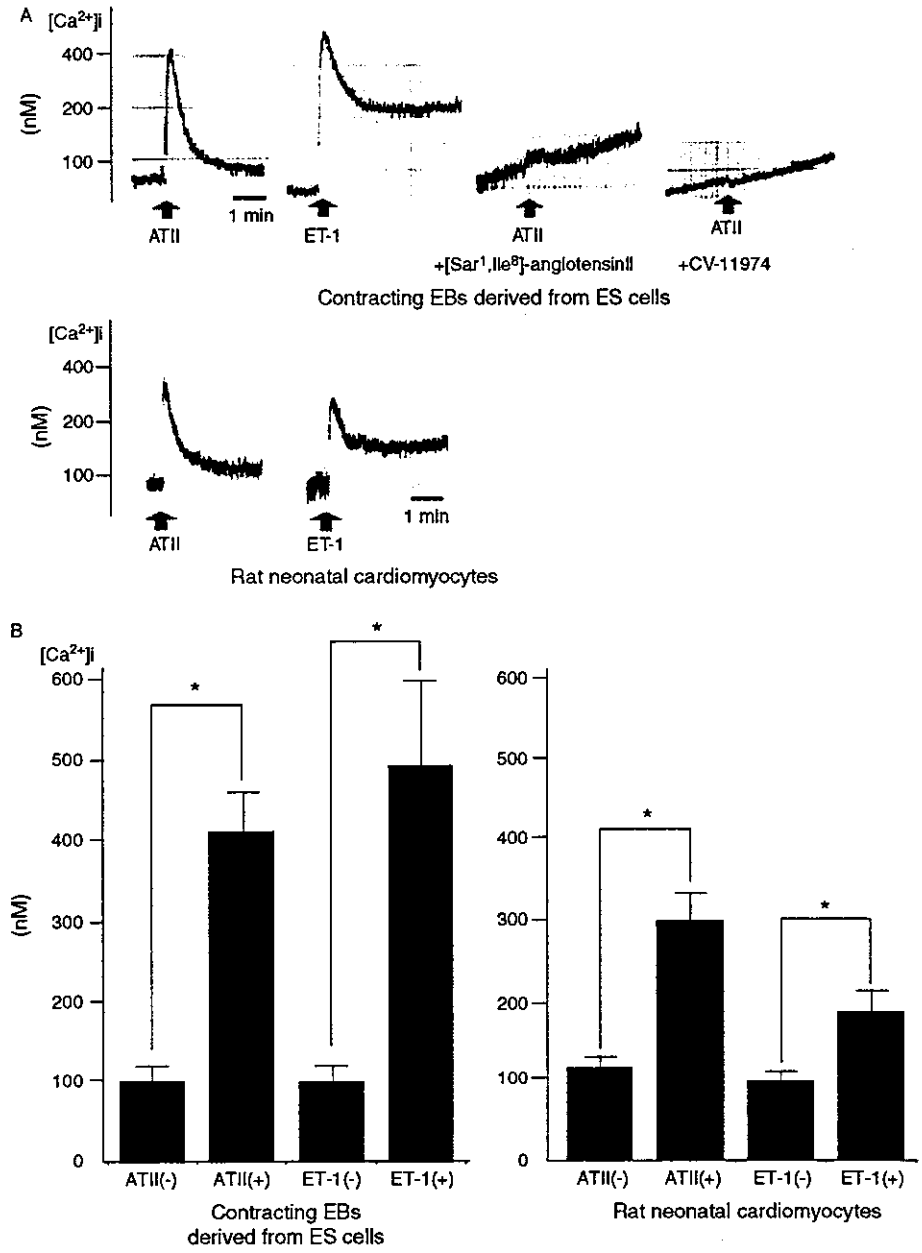


Figure 3. Functional analysis. Intracellular Ca^{2+} transience ($[Ca^{2+}]i$) of contracting EBs and rat neonatal cardiomyocytes determined by fura-2 fluorescence. (A) Typical Ca^{2+} transience appeared in response to 100 nM angiotensin II (ATII) and endothelin-1 (ET-1) in both cell types. The angiotensin-II-stimulated Ca^{2+} transience was inhibited by treatment with [Sar^1,Ile^8]-angiotensin II (non-selective antagonist) or CV-11974 (angiotensin II type 1 receptor selective antagonist) in contracting EBs. (B) Bar graph shows mean ± SD (n = 40). *$p < 0.05$

addition, significant proportions of transplanted cells die after transplantation [26]. Since ES cells have a potent proliferative capacity, cardiomyocytes derived from ES cells are good candidates for cell transplantation therapy [4,27–29]. Cardiomyocytes derived from murine ES cells survive after intracardiac implantation [30]. We have shown here that cardiomyocytes derived from primate ES cells can also survive in the myocardium of myocardial infarction rats.

To enhance the effects of cell transplantation therapy, gene modification of the donor cells might be useful for treating cardiac diseases. Angiogenic agents such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) might be candidates for gene transfer as they attenuate myocardial ischemia in patients with ischemic heart disease when administrated either into myocardium as a naked plasmid [31] or into coronary artery as a recombinant protein [32]. We used

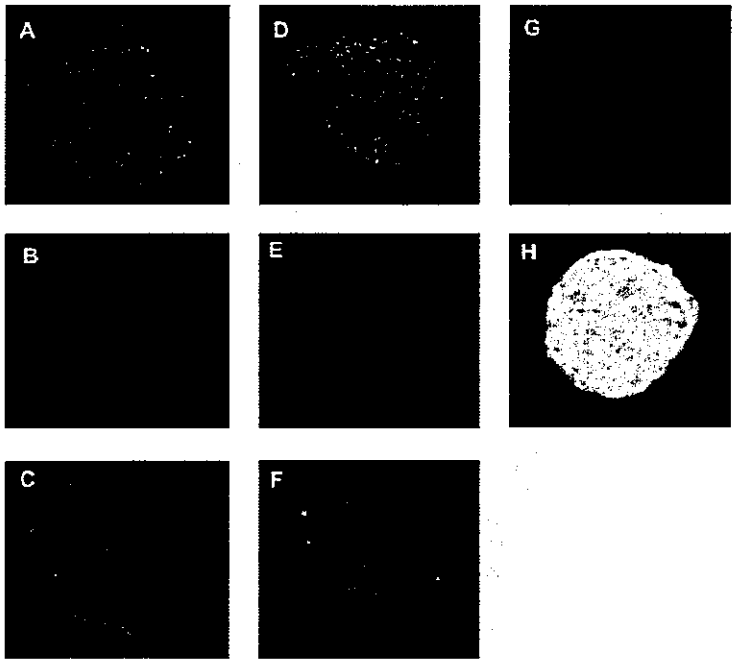


Figure 4. Immunohistochemical staining and EGFP expression. Cardiomyocytes derived from cynomolgus ES cells were transduced with EGFP-SIV vector. Contracting areas were isolated and stained with DAPI (A, D), cTnI (B) and cardiac myosin (E), then EGFP expression (C, F) was identified by fluorescent microscopy. Control staining in which mouse non-specific IgG was used as a primary antibody (G) and phase-contrast photograph (H). Magnification ×100

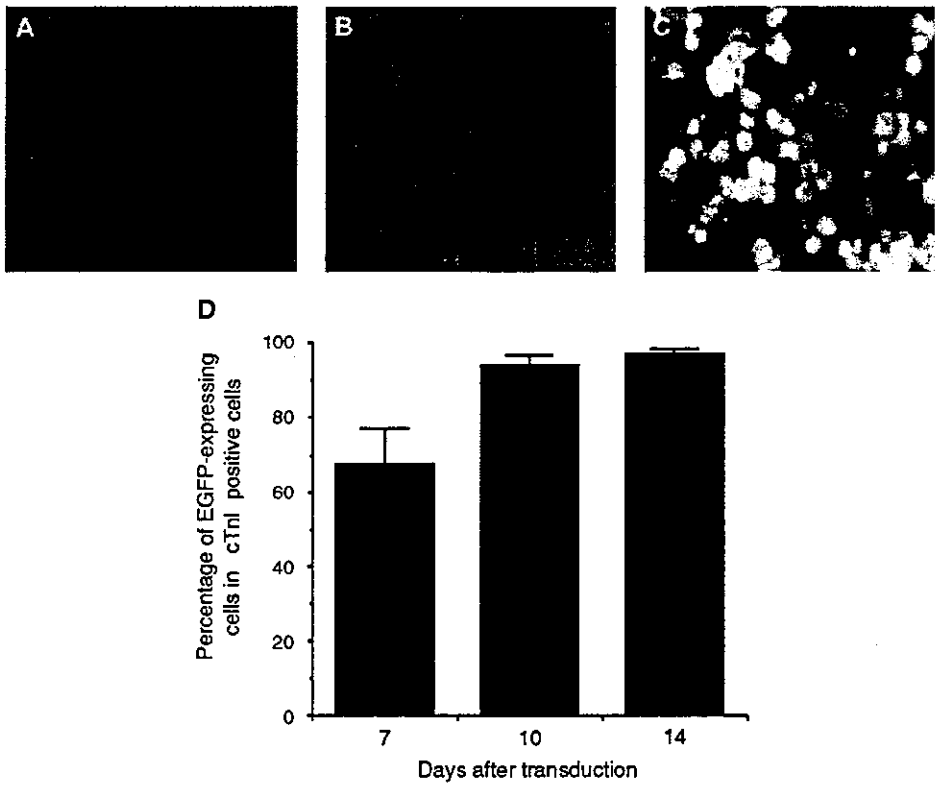


Figure 5. Transgene expression using SIV-based lentivirus vector. Cardiomyocytes derived from cynomolgus ES cells were transduced with EGFP-SIV vector. Contracting areas were isolated, trypsinized and stained with cTnI (A), then EGFP expression (B) was identified by fluorescent microscopy. (C) B merged with A. Magnification ×200. (D) Ratio (%) of cells expressing EGFP in cTnI-positive cells. Values are means ± SD of eight independent experiments

Figure 6. Expression of cTnI and EGFP in myocardium transplanted with cardiomyocytes derived from ES cells. Cardiomyocytes derived from cynomolgus ES cells transduced with the EGFP-SIV vector were implanted into injured myocardium. (A) DAPI staining, (B) cTnI staining, and (C) EGFP expression. Bar: 50 μm

a lentiviral vector based on SIV derived from the African green monkey (SIVagm) [17]. Lentiviral vectors based on either human immunodeficiency virus type 1 (HIV-1) or SIVagm are the only gene delivery vehicles that can efficiently transduce primate ES cells [14,33]. SIVagm-based vectors could offer safety advantages over those based on HIV-1 in human gene therapy. SIVagm is non-pathogenic in its natural host and in experimentally inoculated macaque monkeys, whereas HIV-1 causes severe pathogenicity in humans. In addition to the low homology of sequences between HIV-1 and SIVagm, most viral sequences were removed from our SIVagm vectors. Thus, this vector is unlikely to generate replication-competent virus by recombination between the two types of viruses in humans.

We efficiently and stably expressed EGFP in cardiomyocytes derived from cynomolgus ES cells using a lentiviral vector system based on SIV. Furthermore, we demonstrated that the implanted EGFP-positive cardiomyocytes derived from ES cells survived in the injured rat myocardium. Cell transplantation together with lentivirus-mediated gene modification offers considerable potential as a new therapeutic approach to treating cardiac diseases.

Acknowledgements

We thank Naomi Takino, Chieko Muramatsu, and Yuki Onuma for excellent technical assistance. This study was supported by Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Science, Sports and Culture, the Japanese Government; by a Grant-in-Aid for the Development of Innovative Technology, the Kanae Foundation for Life and Socio-Medical Science; and by funds from The Cell Science Research Foundation.

References

1. Anversa P, Fitzpatrick D, Argani S, *et al.* Myocyte mitotic division in the aging mammalian rat heart. *Circ Res* 1991; **69**: 1159–1164.
2. Kehat I, Kenyagin-Karsenti D, Snir M, *et al.* Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* 2001; **108**: 407–414.
3. Xu C, Police S, Rao N, *et al.* Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circ Res* 2002; **91**: 501–508.
4. Boheler KR, Czyz J, Tweedie D, *et al.* Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circ Res* 2002; **91**: 189–201.
5. Kehat I, Gepstein A, Spira A, *et al.* High-resolution electrophysiological assessment of human embryonic stem cell-derived cardiomyocytes: a novel in vitro model for the study of conduction. *Circ Res* 2002; **91**: 659–661.
6. 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**: 273–279.
7. Cherry SR, Biniszkiewicz D, van Parijs L, *et al.* Retroviral expression in embryonic stem cells and hematopoietic stem cells. *Mol Cell Biol* 2000; **20**: 7419–7426.
8. Grez M, Akgun E, Hilberg F, *et al.* Embryonic stem cell virus, a recombinant murine retrovirus with expression in embryonic stem cells. *Proc Natl Acad Sci U S A* 1990; **87**: 9202–9206.
9. Loh TP, Sievert LL, Scott RW. Evidence for a stem cell-specific repressor of Moloney murine leukemia virus expression in embryonal carcinoma cells. *Mol Cell Biol* 1990; **10**: 4045–4057.
10. Eiges R, Schuldiner M, Drukker M, *et al.* Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells. *Curr Biol* 2001; **11**: 514–518.
11. Blomer U, Naldini L, Kafri T, *et al.* Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J Virol* 1997; **71**: 6641–6649.
12. Kafri T, Blomer U, Peterson DA, *et al.* Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat Genet* 1997; **17**: 314–317.
13. Miyoshi H, Takahashi M, Gage FH, *et al.* Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. *Proc Natl Acad Sci U S A* 1997; **94**: 10319–10323.
14. Asano T, Hanazono Y, Ueda Y, *et al.* Highly efficient gene transfer into primate embryonic stem cells with a simian lentivirus vector. *Mol Ther* 2002; **6**: 162–168.
15. Kamitani T, Ikeda U, Muto S, *et al.* Regulation of Na, K-ATPase gene expression by thyroid hormone in rat cardiocytes. *Circ Res* 1992; **71**: 1457–1464.
16. Okada K, Ishikawa S, Saito T. Enhancement of intracellular sodium by vasopressin in spontaneously hypertensive rats. *Hypertension* 1993; **22**: 300–305.
17. 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.
18. Scorsin M, Marotte F, Sabri A, *et al.* Can grafted cardiomyocytes colonize peri-infarct myocardial areas? *Circulation* 1996; **94**: II337–340.
19. Li RK, Jia ZQ, Weisel RD, *et al.* Cardiomyocyte transplantation improves heart function. *Ann Thorac Surg* 1996; **62**: 654–660; discussion 660–661.
20. Reinecke H, Zhang M, Bartosek T, *et al.* Survival, integration, and differentiation of cardiomyocyte grafts: a study in normal and injured rat hearts. *Circulation* 1999; **100**: 193–202.
21. Yoon PD, Kao RL, Magovern GJ. Myocardial regeneration. Transplanting satellite cells into damaged myocardium. *Tex Heart Inst J* 1995; **22**: 119–125.

22. Chiu RC, Zibaitis A, Kao RL. Cellular cardiomyoplasty: myocardial regeneration with satellite cell implantation. *Ann Thorac Surg* 1995; **60**: 12–18.
23. Menasche P, Hagege A, Scorsin M, *et al.* Autologous skeletal myoblast transplantation for cardiac insufficiency. First clinical case. *Arch Mal Coeur Vaiss* 2001; **94**: 180–182.
24. Tomita S, Li RK, Weisel RD, *et al.* Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation* 1999; **100**: II247–256.
25. Orlic D, Kajstura J, Chimenti S, *et al.* Bone marrow cells regenerate infarcted myocardium. *Nature* 2001; **410**: 701–705.
26. Zhang M, Methot D, Poppa V, *et al.* Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. *J Mol Cell Cardiol* 2001; **33**: 907–921.
27. Metzger JM, Lin WI, Johnston RA, *et al.* Myosin heavy chain expression in contracting myocytes isolated during embryonic stem cell cardiogenesis. *Circ Res* 1995; **76**: 710–719.
28. Maltsev VA, Wobus AM, Rohwedel J, *et al.* Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. *Circ Res* 1994; **75**: 233–244.
29. Hescheler J, Fleischmann BK, Lentini S, *et al.* Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis. *Cardiovasc Res* 1997; **36**: 149–162.
30. Klug MG, Soonpaa MH, Koh GY, *et al.* Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. *J Clin Invest* 1996; **98**: 216–224.
31. Losordo DW, Vale PR, Hendel RC, *et al.* Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia. *Circulation* 2002; **105**: 2012–2018.
32. Udelson JE, Dilsizian V, Laham RJ, *et al.* Therapeutic angiogenesis with recombinant fibroblast growth factor-2 improves stress and rest myocardial perfusion abnormalities in patients with severe symptomatic chronic coronary artery disease. *Circulation* 2000; **102**: 1605–1610.
33. Pfeifer A, Brandon EP, Kootstra N, *et al.* Delivery of the Cre recombinase by a self-deleting lentiviral vector: efficient gene targeting in vivo. *Proc Natl Acad Sci U S A* 2001; **98**: 11 450–11 455.

0041-1337/03/7607-1061/0
TRANSPLANTATION

Vol. 76, 1061–1067, No. 7, October 15, 2003
Printed in U.S.A.

ENGRAFTMENT AND TUMOR FORMATION AFTER ALLOGENEIC IN UTERO TRANSPLANTATION OF PRIMATE EMBRYONIC STEM CELLS[1]

TAKAYUKI ASANO,[3,5] NAOHIDE AGEYAMA,[7] KOICHI TAKEUCHI,[4] MIKIO MOMOEDA,[6] YOSHIHIRO KITANO,[8] KYOKO SASAKI,[2,5] YASUJI UEDA,[9] YUTAKA SUZUKI,[10] YASUSHI KONDO,[10] RYUZO TORII,[11] MAMORU HASEGAWA,[9] SHIGEO OOKAWARA,[4] KIYONORI HARII,[5] KEIJI TERAO,[7] KEIYA OZAWA,[3] AND YUTAKA HANAZONO[2,12]

Background. **To achieve human embryonic stem (ES) cell-based transplantation therapies, allogeneic transplantation models of nonhuman primates would be useful. We have prepared cynomolgus ES cells genetically marked with the green fluorescent protein (GFP). The cells were transplanted into the allogeneic fetus, taking advantage of the fact that the fetus is so immunologically immature as not to induce immune responses to transplanted cells and that fetal tissue compartments are rapidly expanding and thus providing space for the engraftment.**

Methods. **Cynomolgus ES cells were genetically modified to express the GFP gene using a simian immunodeficiency viral vector or electroporation. These cells were transplanted in utero with ultrasound guidance into the cynomolgus fetus in the abdominal cavity (n=2) or liver (n=2) at the end of the first trimester. Three fetuses were delivered 1 month after transplantation, and the other, 3 months after transplantation. Fetal tissues were examined for transplanted cell progeny by quantitative polymerase chain reaction and in situ polymerase chain reaction of the GFP sequence.**

Results. **A fluorescent tumor, obviously derived from transplanted ES cells, was found in the thoracic cavity at 3 months after transplantation in one fetus. However, transplanted cell progeny were also detected (~1%) without teratomas in multiple fetal tissues. The cells were solitary and indistinguishable from surrounding host cells.**

Conclusions. **Transplanted cynomolgus ES cells can be engrafted in allogeneic fetuses. The cells will, however, form a tumor if they "leak" into an improper space such as the thoracic cavity.**

Because human embryonic stem (ES) cell lines have dual abilities to proliferate indefinitely and differentiate into multiple tissue types (*1,2*), human ES cell-based transplantation therapies are considered to hold great promise in the treatment of a variety of diseases and injuries. To address the safety and efficacy of these therapies, allogeneic transplantation models of large animals, especially nonhuman primates, would be useful. However, there have been no reports on the transplantation of primate ES cells or their derivatives into allogeneic hosts thus far. There are two major reasons for this. First, the efficient and stable marking of primate ES cells has been difficult. It is necessary to distinguish transplanted allogeneic ES cell progeny from surrounding host cells. Second, the immune rejection of transplanted cells must be circumvented for a sustained engraftment. The cells would otherwise be cleared by immune responses.

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

The early gestational fetus may be a good recipient with which to circumvent immune rejection, because the immune system is so immature as not to induce an immune response. Furthermore, in the animal fetus, "space" would be relatively available as compared with the adult, because of the rapid expansion of the fetal tissue compartments. Thus, transplanted cells could be engrafted without any conditioning of

[1] This study was supported in part by a Grant-in-Aid for the Development of Innovative Technology and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

[2] Division of Regenerative Medicine, Jichi Medical School, Tochigi, Japan.
[3] Division of Genetic Therapeutics, Jichi Medical School, Tochigi, Japan.
[4] Department of Anatomy, Jichi Medical School, Tochigi, Japan.
[5] Department of Plastic and Reconstructive Surgery, University of Tokyo, Tokyo, Japan.
[6] Department of Obstetrics and Gynecology, University of Tokyo, Tokyo, Japan.
[7] Tsukuba Primate Center, National Institute of Infectious Diseases, Ibaraki, Japan.
[8] Department of Surgery, National Center for Child Health and Development, Tokyo, Japan.
[9] DNAVEC Research Inc., Ibaraki, Japan.
[10] Discovery Research Laboratory and Safety Research Laboratory, Tanabe Seiyaku Co., Ltd., Osaka, Japan.
[11] Research Center for Animal Life Science, Shiga University of Medical Science, Shiga, Japan.
[12] Address correspondence to: Yutaka Hanazono, M.D., Ph.D., Associate Professor, Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi, Kawachi, Tochigi 329-0498, Japan. E-mail: hanazono@jichi.ac.jp.

Received 22 February 2003.
Revision Requested 9 May 2003. Accepted 12 June 2003.
DOI: 10.1097/01.TP.0000090342.85649.81

recipients such as irradiation or immunosuppressive treatment. This strategy was indeed successful in achieving human and sheep chimeras (up to 10–30% human blood cells) after in utero human hematopoietic stem cell transplantation into fetal sheep (*7,8*). The engraftment of human mesenchymal stem cells in fetal sheep has also been reported (*9*). In addition, the engraftment of human hematopoietic stem cells was observed in the setting of in utero transplantation in other animals including nonhuman primates (*10,11*) and canines (*12*). In the present study, we have transplanted nonhuman primate (cynomolgus macaque) ES cells stably expressing GFP into allogeneic fetuses in utero and examined the in vivo fate of the transplanted cells using GFP as a genetic tag.

MATERIALS AND METHODS

Vectors

An SIV vector carrying the enhanced GFP (Clonetech, Palo Alto, CA) was used for transduction of cynomolgus ES cells (*3*). The vector was constructed as previously reported (*13*). The vector was self-inactivating because it was constructed with a deletion of the U3 region in the 3' long terminal repeat and was pseudotyped with the vesicular stomatitis virus glycoprotein. The GFP gene was driven by the cytomegalovirus promoter.

A plasmid expressing the enhanced GFP (pCE-EGFP) was used for transfection of cynomolgus ES cells (*6*). The plasmid was derived from pEGFP-1 (Clontech) and contains the human elongation factor 1 alpha promoter to express GFP.

Genetically Modified Cynomolgus ES Cells

Cynomolgus ES cells (CMK10) were transduced using the SIV vector at 10 transducing units per cell as previously described (*3*). The transduced cells were cultured in an undifferentiated state for more than 1 month before transplantation. A subline stably expressing GFP (CMK6G) was established from the parental cynomolgus ES cell line (CMK6) by electroporation of the plasmid pCE-EGFP as previously described (*6*).

All cynomolgus ES cells were cultured on a mouse embryonic fibroblast (MEF) feeder layer as previously described (*14*). MEF cells were obtained from fetal BALB/c mice that have the H-2^d haplotype (Clea, Tokyo, Japan). Cynomolgus ES cells were dissociated from MEF cells using 0.05% trypsin/phosphate-buffered saline (PBS), washed with Hanks' balanced salt solution (Gibco, Gaithersburg, MD), resuspended in 0.4 mL of Hanks' balanced salt solution, and used for transplantation.

Flow Cytometry

The expression of GFP by ES cells was assessed before transplantation by flow cytometry using a FACScan (Becton Dickinson, Franklin Lakes, NJ) with excitation at 488 nm and fluorescence detection at 530±30 nm. The co-cultured BALB/c MEF feeder cells could be distinguished from cynomolgus ES cells by using the phycoerythrin-conjugated mouse anti-mouse $H2K^d$ monoclonal antibody (PharMingen, San Diego, CA), which does not react to cynomolgus cells but reacts to BALB/c cells.

Animals and Transplant Procedures

Four pregnant cynomolgus monkeys aged 18 years (IA10-1, IA10-2, IH10-1, and IH6G-1) were obtained by mating monkeys and were bred in the Tsukuba Primate Center (Ibaraki, Japan) as previously described (*15*). The animals were certified free of intestinal parasites and were seronegative for simian type-D retrovirus, herpes virus B, varicella zoster-like virus, measles virus, and SIV (*16*). All animals were individually housed.

Monkeys were anesthetized by intramuscular administration of ketamine hydrochloride (Ketalar, 10 mg/kg; Sankyo, Tokyo, Japan). Genetically modified cynomolgus ES cells ($3.6–4.8\times10^6$ cells/fetus, approximately 2.0×10^8 cells/kg) were injected into the fetal abdominal cavity (IA10-1's fetus [IA10-1ft] and IA10-2ft) or liver (IH10-1ft and IH6G-1ft) through a 23-gauge needle using an ultrasound-guided technique at approximately the end of the first trimester (Table 1). The full term is 165 days. The weight of the fetus at the time of transplantation was estimated at 20 g (*17*). All surgical procedures and postoperative care of animals were performed in accordance with the Rules for Animal Care and Management of Tsukuba Primate Center (*18*) and Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan (*19*). The protocol of experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Tissue Sampling

Three of the four pregnant monkeys (IA10-1, IA10-2, and IH10-1) underwent a cesarean section 1 month after transplantation. The other monkey (IH6G-1) underwent a cesarean section 3 months after transplantation. All operations were conducted under isoflurane (A.D.S.1000; Shin-ei, Tokyo, Japan)-induced general anesthesia. Each of the pregnant monkeys carried one fetus. The sex of the fetus was confirmed by examining the morphology of external genitalia. The three fetuses (IA10-1ft, IA10-2ft, and IH10-1ft) delivered at 1 month after transplantation were cannulated through the umbilical vessel, and umbilical blood was collected. The other fetus (IH6G-1ft) delivered 3 months after transplantation was cannulated through the intracardiac cavity, and fetal blood was collected. After collection of the blood, the fetuses were irrigated with natural saline for mercy killing and fetal blood was completely washed out.

Fetal tissues of brain, lung, heart, thyroid, thymus, liver, spleen, kidney, small intestine, skeletal muscle, and cartilage were collected from each of the fetuses. Tissue samples were fixed for 4 hr at 4°C in 4% paraformaldehyde in PBS and embedded in paraffin. Tissue

TABLE 1. Allogeneic in utero transplantation of genetically modified cynomolgus ES cells

Fetus	IA10-1ft	IA10-2ft	IH10-1ft	IH6G-1ft
Sex	Male	Female	Male	Male
Transplanted ES cell line	CMK10	CMK10	CMK10	CMK6G
% GFP expression	53.5	43.4	43.5	91.4
Copy number of GFP gene per cell	1.89	1.89	1.89	1.00
Number of transplanted cells	3.6×10^6	3.9×10^6	4.8×10^6	3.9×10^6
Gestational age				
At transplantation	61 (0.37)	54 (0.33)	50 (0.30)	49 (0.30)
At delivery	90 (0.55)	88 (0.53)	84 (0.51)	150 (0.91)
Days of incubation	29 (0.18)	34 (0.21)	34 (0.21)	101 (0.61)
Injection site	Abdominal cavity	Abdominal cavity	Liver	Liver

Values in parentheses represent ratios of days to the full term (165 days).

samples were also embedded in the OCT compound (Sakura, Tokyo, Japan) and frozen in liquid nitrogen and stored at −80°C. In addition, raw samples were frozen in liquid nitrogen and stored at −80°C for subsequent cellular DNA extraction.

Quantitative polymerase chain reaction (PCR)

Cellular DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). To assess the fraction of transplanted cell progeny in each fetal tissue, quantitative DNA PCR for the GFP sequence was performed using 250 ng of DNA as template. The GFP standards consisted of DNA extracted from the MGirL22Y cells (which contain one copy of the GFP sequence per cell) serially diluted with control cynomolgus genomic DNA. The negative control was DNA extracted from naive cynomolgus peripheral blood. PCR of the β-actin sequence was also performed to document initial DNA amounts of samples. The primer set for the GFP sequence was 5'-CGT CCA GGA GCG CAC CAT CTT C-3' and 5'-GGT CTT TGC TCA GGG CGG ACT-3'. The primer set for the β-actin sequence was 5'-CAT TGT CAT GGA CTC TGG CGA CGG-3' and 5'-CAT CTC CTG CTC GAA GTC TAG GGC-3'. Each PCR cycle consisted of four steps (denaturing, annealing, extension, and data acquisition). Amplification conditions for the GFP sequences were 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and 80°C for 15 sec with 40 cycles. Amplification conditions for the β-actin sequence were 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and 75°C for 15 sec with 40 cycles. The final step was inserted to suppress the generation of primer dimers. The temperature of the final step was optimized for each PCR.

PCR was performed in the presence of SYBR Green using the ABI PRISM 7700 sequence detection system (Perkin-Elmer, Foster, CA) with buffers, nucleotides, and Taq polymerase from the QuantiTect SYBR Green PCR Kit (Qiagen) as directed. All quantitative PCRs were certified with each run to yield a linear amplification in the range of the intensity of the positive control series (0.0001–1.0 copy/cell, correlation coefficient >0.98). The threshold cycle (Ct) value of the GFP sequence was normalized based on the Ct value of the internal control β-actin sequence in the same sample. To calculate the fraction (%) of transplanted cell progeny in each tissue, the copy number of the GFP gene per tissue cell was divided by the copy number of the GFP gene per transplanted cell. Amplification products were also analyzed on 2% agarose gel followed by ethidium-bromide staining.

In Situ PCR

In situ detection of transplanted cell progeny was performed by amplifying the GFP sequence as previously reported (*20*). The primer sequences were the same as used for the quantitative PCR described above. The reaction mixture consisted of 420 μM dATP, 420 μM dCTP, 420 μM dGTP, 378 μM dTTP, 42 μM digoxigenin-labeled dUTP (Roche, Mannheim, Germany), 0.8 μM of each GFP primer, 4.5 mM $MgCl_2$, PCR buffer (Mg^{2+} free), and 4 U of Takara Taq DNA polymerase (Takara, Tokyo, Japan). Slides were covered with the Takara Slide Seal for in situ PCR (Takara). PCR was performed using the PTC100 Peltier Thermal Cycler (MJ Research, Watertown, MA) with the following conditions: 94°C for 1 min and 55°C for 1 min with 15 cycles. After amplification, cover seals were lifted off and slides were washed with PBS at room temperature three times each for 5 min.

The digoxigenin incorporated-DNA fragments were detected using horseradish peroxidase (HRP)-conjugated rabbit F(ab') anti-digoxigenin antibody (Dako, Glostrup, Denmark) followed by histochemical staining. Briefly, slides were incubated at 37°C for 3 hr with the HRP-conjugated rabbit F(ab') anti-digoxigenin antibody solution diluted (1:100) in blocking solution (2% bovine serum albumin and 5% normal horse serum in PBS) and rinsed with PBS at room temperature three times each for 5 min. Slides were then stained for HRP using the Vector SG substrate kit (Vector, Burlingame, CA). The reaction was terminated by transferring slides into a water bath upon development of a dark blue or black color (usually requiring 3–10 min to develop the signal). Finally, sections were counterstained with the Kernechtrot that stains nucleotides, washed with water, mounted in glycerol, and examined under a light microscope.

RESULTS

In Utero Transplantation

Genetically modified cynomolgus ES cells that stably express GFP were obtained either by transduction with an SIV vector carrying the GFP gene or by transfection with a GFP-expressing plasmid.

The transduction of cynomolgus ES cells (CMK10) using the SIV vector was conducted once at 10 transducing units per cell. The transduction efficiency was about 50% (Table 1). Each transduced cell was estimated to contain 1.89 copies of the GFP gene by quantitative PCR (data not shown). The transduced ES cells were cultured in an undifferentiated state for more than 1 month before transplantation to confirm that GFP expression was stable. These cells were used for transplantation (to IA10-1ft, IA10-2ft, and IH10-1ft; Table 1) without further enrichment of GFP-positive cells. Thus, the fraction of GFP-positive cells remained about 50% at transplantation.

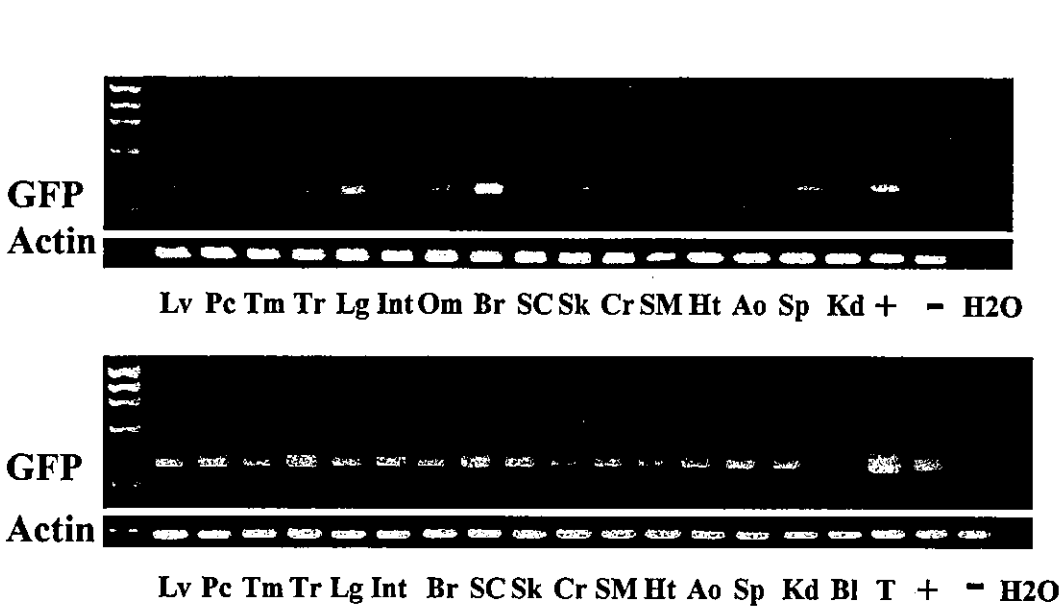
On the other hand, a cynomolgus ES subline (CMK6G) was a cloned, stable transfectant expressing the GFP gene. It was established from the parental cynomolgus ES cell line (CMK6) by electroporation of the GFP-expressing plasmid. Virtually all (>90%) CMK6G cells expressed the GFP gene at transplantation (to IH6G-1ft; Table 1). Each ES cell contained one copy of the GFP gene (data not shown).

The GFP-expressing cynomolgus ES cells ($3.6–4.8\times10^6$ cells/fetus, approximately 2.0×10^8 cells/kg) were transplanted in utero into four allogeneic fetuses in the abdominal cavity (IA10-1ft and IA10-2ft) or liver (IH10-1ft and IH6G-1ft) under ultrasound guidance at approximately the end of the first trimester (Table 1). There were no complications during the pregnancies of all four mother monkeys. The three fetuses (IA10-1ft, IA10-2ft, and IH10-1ft) that received CMK10 cells were delivered by cesarean section 1 month after transplantation. The other fetus (IH6G-1ft), which re-

TABLE 2. Fractions (%) of transplanted cell progeny in fetal tissues

Fetus	IA10-1ft	IA10-2ft	IH10-1ft	IH6G-1ft
Lv-Liver	0.00	0.00	0.01	1.24
Pc-Pancreas	0.10	0.00	0.01	1.54
Tm-Thymus	0.01	0.00	0.00	0.01
Tr-Thyroid	0.02	0.00	0.01	0.34
Lg-Lung	0.01	0.00	0.02	0.12
Int-Small intestine	0.00	0.25	0.00	0.09
Om-Omentum	1.23	0.00	0.02	N.D.
Br-Brain	0.01	0.00	0.02	0.08
SC-Spinal cord	0.02	0.14	0.01	0.38
Sk-Skin	0.02	0.01	0.03	0.18
Cr-Cartilage	0.02	0.05	0.00	0.82
SM-Skeletal muscle	0.01	0.00	0.00	1.04
Ht-Heart	0.00	0.00	0.01	0.83
Ao-Aorta	0.03	0.00	0.00	0.16
Sp-Spleen	0.05	0.00	0.01	0.24
Kd-Kidney	0.00	0.00	0.00	1.20
Bl-Blood	ND	ND	ND	0.21

ND, not determined.



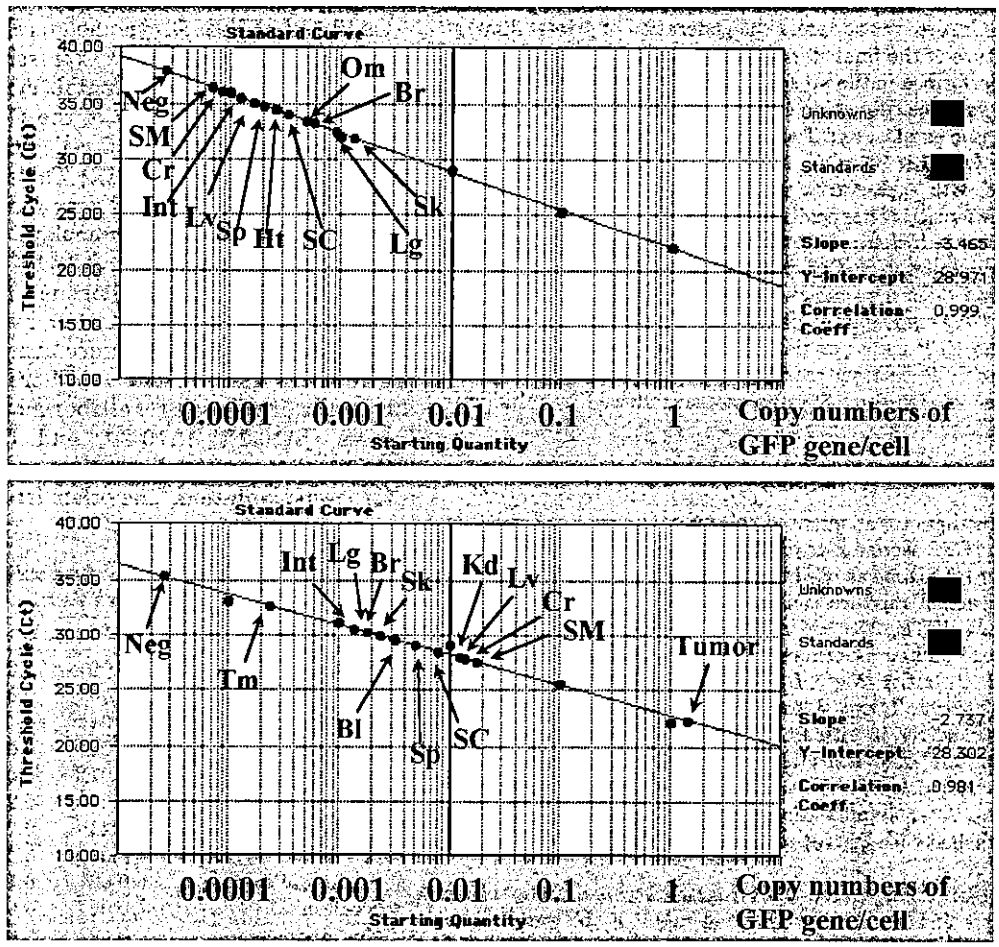


FIGURE 1. Detection of transplanted cell progeny by PCR of the GFP sequence. (a) Gels represent the samples from IH10-1ft at 1 month (34 days) after transplantation (*upper*) and IH6G-1ft at 3 months (101 days) after transplantation (*lower*). PCR of the β-actin sequence was simultaneously performed as controls. +, CMK6G; −, naive cynomolgus cells; T, tumor. Tissue abbreviations are listed in Table 2. (b) Standardized amplification curves of the quantitative PCR for the GFP sequence are shown. Curves represent the PCR results of IH10-1ft at 1 month (34 days) after transplantation (*upper*) and IH6G-1ft at 3 months (101 days) after transplantation (*lower*). Black dots represent the standards and grey dots represent each fetal tissue. Neg, negative control (naive cynomolgus cells).

ceived CMK6G cells, was delivered by cesarean section 3 months after transplantation, just 2 weeks before the expected delivery date.

In Vivo Detection of Transplanted Cell Progeny

Fetal tissues were collected as listed in Table 2. To detect transplanted cell progeny in fetal tissues, we extracted cellular DNA from each tissue and performed PCR of the GFP transgene sequence. Figure 1a shows representative results at 1 month (IH10-1ft, upper panel) and at 3 months (IH6G-1ft, lower panel) after transplantation. Transplanted cell progeny were widely distributed in multiple fetal tissues. The fraction of transplanted cell progeny in each tissue was estimated at 0.01 to 1% by quantitative PCR for the GFP sequence (Fig. 1b; the same monkeys as those in Fig. 1a). The limit of detection was 1 in 10,000 cells (0.01%). The quantitative PCR results of all the four monkeys are summarized in Table 2.

The transplanted cell distribution considerably varied among individual animals, as was the case with a previous report using human mesenchymal stem cells (*9*). The fractions of transplanted cell progeny in IH6G-1ft that received CMK6G cells were one-log higher than those in the other fetuses that received CMK10 cells (Table 2). IA10-1ft and IA10-2ft received cells in the abdominal cavity, and large numbers of transplanted cell derivatives were detected in tissues in the abdominal cavity such as the omentum (IA10-1ft, 1.23%) and small intestine (IA10-2ft, 0.25%). IH10-1ft and IH6G-1ft received cells in the liver, and considerable numbers of transplanted cell derivatives were detected in tissues distant from the injection site (liver) such as skin (IH10-1ft, 0.03%; IH6G-1ft, 0.18%), lung (IH10-1ft, 0.02%; IH6G-1ft, 0.12%), and brain (IH10-1ft, 0.02%; IH6G-1ft, 0.08%).

We then examined the tissue sections by in situ PCR, which amplified the GFP sequence. Figure 2 shows successful in situ detection of transplanted ES cell derivatives in the small intestine (a), spinal cord (b and d), and liver (c). All cells detected were solitary and indistinguishable from surrounding host cells by microscopic examination. Unfortunately, we could not further characterize the cells by staining specific surface markers, because the procedures for the in situ PCR impaired cell membranes. We tried to detect GFP fluorescence under a fluorescent microscope but were hampered by the high autofluorescence of tissue samples. We also tried to immunostain the samples using anti-GFP antibody (Clontech), but GFP-specific immunostaining could not be detected.

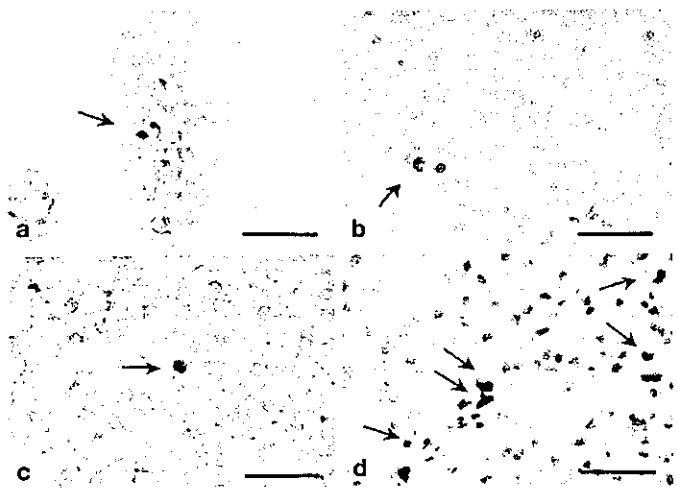


FIGURE 2. Detection of transplanted cell derivatives by in situ PCR of the GFP sequence. Typical GFP sequence-positive cells (*arrows*). (a) A villus of small intestine at 1 month after transplantation (IA10-2ft). (b) Spinal cord at 1 month after transplantation (IA10-2ft). (c) Liver at 3 months after transplantation (IH6G-1ft). (d) Spinal cord at 3 months after transplantation (IH6G-1ft). Bars=50 μm.

Tumor Formation

Although no tumors were observed in the fetuses delivered at 1 month after transplantation, a cystic tumor of 4×3×2.5 cm was observed in the thoracic cavity in the fetus delivered at 3 months after transplantation (Fig. 3a). Both lungs remained deflated after the tumor resection (Fig. 3b). The tumor was derived from transplanted ES cells, because GFP fluorescence in the tumor was clearly observed under a fluorescent scope (Fig. 3, c and d) and because the GFP gene was detected by in situ PCR (Fig. 3e). The tumor consisted of many types of epithelial cells (Fig. 3f), but cells derived from other embryonic germ cell layers were rare. Thus, strictly speaking, the tumor may not be a teratoma but a teratoma-related tumor, because teratomas should consist of all three embryonic germ layer cells. Of note, no tumor was found in any other tissues of the animal even at the injection site (liver), although transplanted cell derivatives were detected at the site by in situ PCR (Fig. 2c).

DISCUSSION

Transplanted ES cell progeny were detected in multiple fetal tissues at approximately 1% or higher at 3 months after transplantation. They were even observed in the central nervous system, which was distant from the injection site. All cells detected were solitary and indistinguishable from surrounding host cells by microscopic examination. The fusion of transplanted ES cells with preexisting host cells may in part account for such a change of phenotype. Although the cell fusion frequency was recently optimized up to 1% under special conditions (heat-shocked epithelial cells vs. mesenchymal stem cells) (*21*), cell fusion usually occurs at one per 10^4 to 10^6 cells (*22,23*). Therefore, it is unlikely that all the detected cells were attributable to fusion events in our study. The transplanted cell fraction is considerably high, given that relatively limited numbers of ES cells were transplanted into the early gestational fetus. Transplanted ES cells might effectively compete with host cells to achieve significant expansion after transplantation.

To deliver transplanted cells to fetal organs, direct injection of cells into the circulation (such as intracardiac injection or injection by umbilical vein) might be more effective than intrahepatic or intraperitoneal injection (*24*). Unfortunately, cynomolgus monkey fetuses at the first trimester were so small (hearts were about 1 cm long) that such injection methods seemed difficult even under the finest ultrasound guidance. We have tried intrahepatic injection as well as intraperitoneal injection. We have performed intrahepatic injection for two fetal monkeys (in this study) and they were

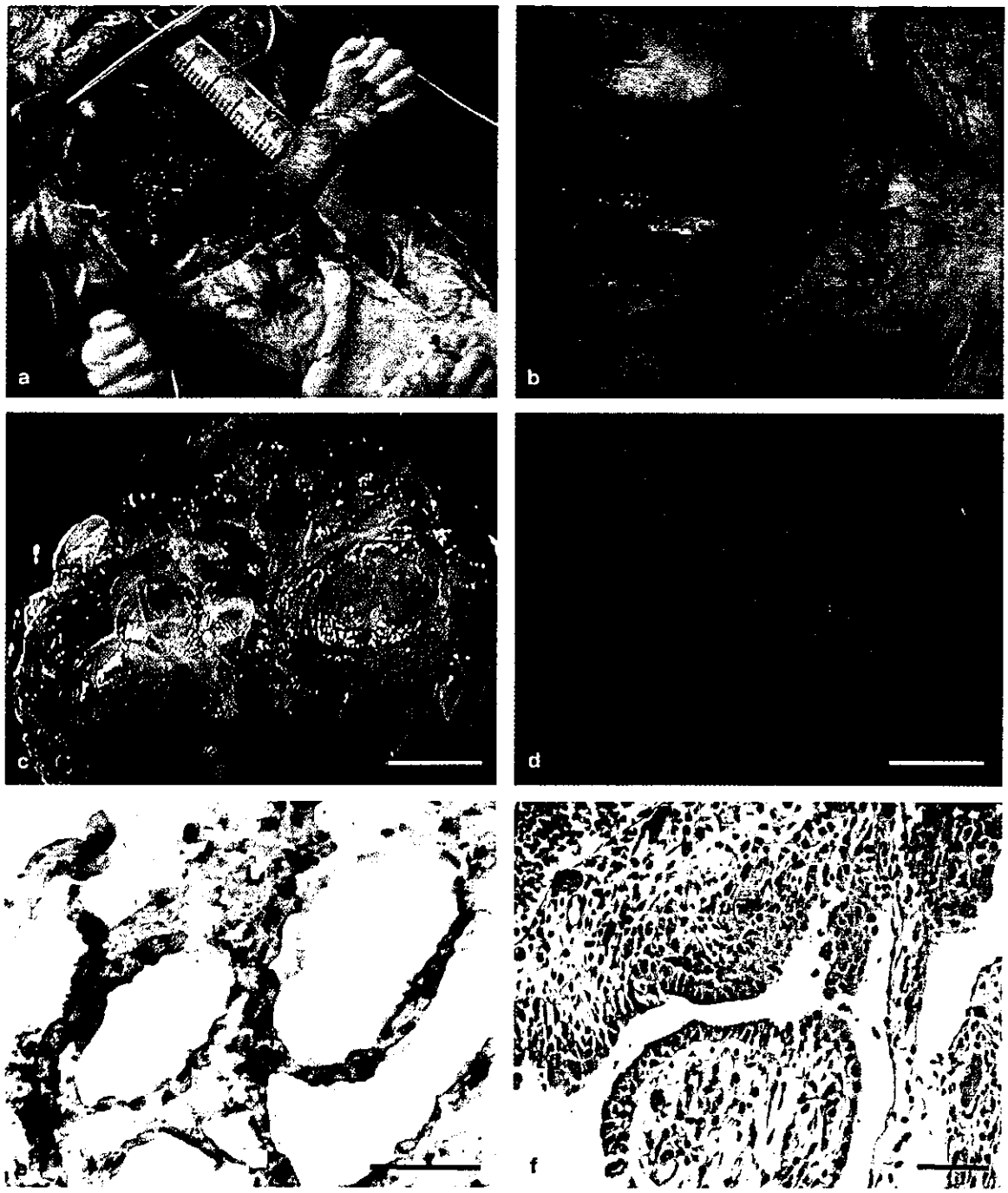


FIGURE 3. **Tumor formation in a cynomolgus fetus transplanted with allogeneic ES cells. (a) A tumor (4×3×2.5 cm) was detected in the thoracic cavity at 3 months after transplantation (IH6G-1ft). (b) Both lungs remained deflated after the tumor resection. The tumor was observed in a bright field (c) and in a dark field (d) under a fluorescent scope. Bars in c and d=1 cm. (e) The GFP gene was detected in the tumor cells by in situ PCR. (f) The tumor mainly consisted of epithelial cells (hematoxylin-eosin staining). Bars in e and f=50 μm.**

alive until delivery. We have also performed intrahepatic injection for nine fetal sheep (in another series of experiments), and only two of them resulted in abortion (our unpublished data). The abortion rate was not higher than that by the intraperitoneal injection. Thus, the intrahepatic approach was as safe as the intraperitoneal one.

It is well known that primate ES cells form teratomas after transplantation into immunodeficient mice (*1,2*). In the present study, a tumor was found in the thoracic cavity of a cynomolgus fetus at 3 months after the transplantation of allogeneic ES cells. The tumor was obviously derived from the transplanted ES cells, because it expressed GFP. To our knowledge, this is the first report of teratoma-related tumor formation in primates after allogeneic transplantation of primate ES cells. From this observation, it is obvious that successful therapeutic use of ES cell-derived donor cells would

require the generation of essentially pure differentiated cell cultures. No tumor was found, however, at other sites including the injection site. In explanation of the tumor site (the thoracic cavity), some ES cells might have been directly injected or leaked into the thoracic cavity during the transplantation procedure, although we carefully targeted the cells inside the liver under ultrasound guidance. Of note, no tumor was detected in any of the fetuses delivered at 1 month after transplantation. One month might be too short to allow teratoma formation. In fact, we have observed teratoma formation at 9 to 12 weeks after transplantation into immunodeficient (NOD/SCID) mice (our unpublished data).

From our observation, it is likely that there was "space" in the expanding fetal tissues that was available for homing and engraftment of transplanted ES cells. The allogeneic fetal host generated space in each tissue from which ES cells could obtain proper growth and regulatory signals. Thus, when ES cells are lodged in such a proper space, they can be engrafted and adapted. On the other hand, when transplanted cells "leak" into improper spaces such as the thoracic cavity (from which the cells cannot obtain proper signals), then the cells may form tumors.

ES cell-based transplantation into adult hosts requires immunosuppression of the host for the sustained engraftment of transplanted cells (*25,26*). In this study, ES cells were transplanted into four different fetal hosts and transplanted cell progeny were detected in all the animals without immunosuppressive treatment. In addition, we found a large teratoma-related tumor 3 months after transplantation in the fetus without immune rejection. These results suggest that the fetuses were tolerant of the transplanted ES cells. Another group has reported that transplantation of human peripheral blood stem cells into rhesus monkey fetuses resulted in the generation of chimeric rhesus infants (*9*), although Lindton et al. (*27*) have reported that the primate fetus may be more immune competent than experimental animals. If transplanted ES cells were to become permanent components of the host body without forming teratomas, we would be able to obtain immunocompetent adult recipients that are tolerant of grafts of the same ES cell origin. This would provide ideal primate recipients for ES cell-based transplantation studies.

Acknowledgments. The authors thank Y. Ochiai (Department of Anatomy, Jichi Medical School, Tochigi, Japan) for tissue preparation. The authors also thank K. Ueda and M. Tanaka for their assistance.

REFERENCES

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998; 282: 1145.
2. Reubinoff BE, Pera MF, Fong C, et al. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000; 18: 399.
3. Asano T, Hanazono Y, Ueda Y, et al. Highly efficient gene transfer into primate embryonic stem cells with a simian lentivirus vector. Mol Ther 2002; 6: 162.
4. Lois C, Hong EJ, Pease S, et al. Germline transmission and tissue-specific expression of transgenesis delivered by lentiviral vectors. Science 2002; 295: 868.
5. Pfeifer A, Ikawa M, Dayn Y, et al. Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc Natl Acad Sci USA 2002; 99: 2140.
6. Takada T, Suzuki Y, Kondo Y, et al. Monkey embryonic stem cell lines expressing green fluorescent protein. Cell Transplant 2002; 11: 631.
7. 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.
8. Zanjani ED, Pallavicini MG, Flake AW, et al. Engraftment and long term expression of human fetal hemopoietic stem cells in sheep following transplantation in utero. J Clin Invest 1992; 89: 1178.
9. 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.
10. Harrison MR, Slotnick RN, Crombleholme TM, et al. In-utero transplantation of fetal liver haemopoietic stem cells in monkeys. Lancet 1989; 2: 1425.
11. Tarantal AF, Goldstein O, Barley F, et al. Transplantation of human peripheral blood stem cells into fetal rhesus monkeys (*Macaca mulatta*). Transplantation 2000; 69: 1818.
12. Omori F, Lutzko C, Abrams-Ogg A, et al. Adoptive transfer of genetically modified human hematopoietic stem cells into preimmune canine fetuses. Exp Hematol 1999; 27: 242.
13. 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.
14. 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.
15. Honjo S, Cho F, Terao K. Establishing the cynomolgus monkey as a laboratory animal. Adv Vet Sci Comp Med 1984; 28: 51.
16. Buchl SJ, Keeling ME, Voss WR. Establishing specific pathogen-free (SPF) nonhuman primate colonies. ILAR J 1997; 38: 22.
17. Tarantal AF. Interventional ultrasound in pregnant macaques: embryonic/fetal applications. J Med Primatol 1990; 19: 47.
18. Honjo S. The Japanese Tsukuba Primate Center for Medical Science (TPC): an outline. J Med Primatol 1985; 14: 75.
19. Primate Society of Japan. Guiding principles for animal experiments using nonhuman primates. Primate Res 1986; 2: 111.
20. Hasse AT, Retzel EF, Staskus KA. Amplifications and detection of lentiviral DNA inside cells. Proc Natl Acad Sci USA 1990; 87: 4971.
21. Spees JL, Olson SD, Ylostalo J, et al. Differentiation, cell fusion, and nuclear fusion during ex vivo repair of epithelium by human adult stem cells from bone marrow stroma. Proc Natl Acad Sci USA 2003; 100: 2397.
22. Ying QL, Nichols J, Evans EP, et al. Changing potency by spontaneous fusion. Nature 2002; 416: 545.
23. 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.
24. Westgren M, Ek S, Bui T, et al. Tissue distribution of transplanted fetal liver cells in the human fetal recipient. Am J Obstet Gynecol 1997; 176: 49.
25. Reubinoff BE, Itsykson P, Turetsky T, et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol 2001; 19: 1134.
26. Zhang SC, Wernig M, Duncan ID, et al. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 2001; 19: 1129.
27. Lindton B, Markling L, Ringden O, et al. Mixed lymphocyte culture of human fetal liver cells. Fetal Diagn Ther 2000; 15: 71.

0041-1337/03/7607-1068/0
TRANSPLANTATION

Vol. 76, 1068–1073, No. 7, October 15, 2003
Printed in U.S.A.

LIVER TRANSPLANTATION FROM MAASTRICHT CATEGORY 2 NON-HEART-BEATING DONORS

ALEJANDRA OTERO,[1] MANUEL GÓMEZ-GUTIÉRREZ,[1] FRANCISCO SUÁREZ,[1] FRANCISCO ARNAL,[2] ANTÓN FERNÁNDEZ-GARCÍA,[3] JAVIER AGUIRREZABALAGA,[1] JOSÉ GARCÍA-BUITRÓN,[3] JOAQUÍN ALVAREZ,[4] AND RAFAEL MÁÑEZ[3,5]

Background. **The demand for liver transplantation has increasingly exceeded the supply of cadaver donor organs. Non-heart-beating donors (NHBDs) may be an alternative to increase the cadaver donor pool.**

Methods. **The outcome of 20 liver transplants from Maastricht category 2 NHBDs is compared with 40 liver transplants from heart-beating donors (HBDs). After unsuccessful cardiopulmonary resuscitation (CPR), cardiopulmonary support (CPS) with simultaneous application of chest and abdominal compression (n=6), and cardiopulmonary bypass (CPB; n=14), which was hypothermic (n=7) or normothermic (n=7), were used to preserve the organs from NHBDs. Factors that may influence the outcome of livers from Maastricht category 2 NHBDs were also investigated.**

Results. **With a minimum follow-up of 2 years, actuarial patient and graft survivals with livers from Maastricht category 2 NHBDs were 80% and 55%, respectively. Transplantation of organs from these donors was associated with a significantly higher incidence of primary nonfunction, biliary complications, and more severe initial liver dysfunction compared with livers from HBDs. Graft survival was 83% in livers from NHBDs preserved with CPS and 42% in those maintained with CPB. No graft failed if the duration of warm ischemia did not exceed 130 min with CPR or CPS, and if the period of CPB did not surpass 150 min when this method was used after CPR, regardless if it was hypothermic or normothermic.**

Conclusion. **Livers from Maastricht type 2 NHBDs may be used for transplantation if the period of warm ischemia during CPR or CPS does not exceed 130 min. Hypothermic or normothermic CPB after CPR preserves liver viability for an additional 150 min.**

Liver transplantation is the treatment of choice for patients with end-stage liver disease, with 1- and 5-year patient survival rates in excess of 80% and 70%, respectively. However, not every patient who needs and is a candidate for liver transplantation benefits from this therapy. For every three patients in the United States who received a liver transplantation, approximately one patient died while waiting for an organ in 2001 (*1*). Spain has the highest rate of cadaver organ donors in the world. However, 9% of the candidates for a liver transplantation died in 2001 before they were allocated for an organ (*2*).

One of the alternatives to increase the number of organs available for transplantation is the use of non-heart-beating donors (NHBDs). These donors have been classified as uncontrolled and controlled in function according to whether the circulatory arrest occurs unplanned or after planned withdrawal of life support, respectively (*3*). However, in addition to how the circulatory arrest takes place, other aspects should be taken in consideration to ensure the ethics of the procedure and the viability of organs. This is the goal of the Maastricht classification system, which groups NHBDs into four categories: dead on arrival to the hospital and not resuscitated (category 1); unsuccessful resuscitation (category 2); withdrawal of life support (category 3); and cardiac arrest while brain dead (category 4) (*4*). Uncontrolled NHBDs are in Maastricht categories 1 and 2, and controlled NHBDs are in categories 3 and 4.

Kidneys, livers, and, exceptionally, lungs from controlled or Maastricht categories 3 and 4 NHBDs have been successfully transplanted (*3, 5–8*), although liver graft survival may be reduced compared with organs from heart-beating donors (HBDs) (*6, 7*). However, the use of these donors in Spain is particularly difficult because the law does not encompass the transplantation of organs from Maastricht category 3 NHBDs. Uncontrolled NHBDs have an even higher potential than controlled NHBDs to augment the number of organs from cadaver donors. Transplantation of organs from Maastricht category 1 NHBDs is difficult because the uncertainty of the cardiac arrest may lead to ischemia times incompatible with the viability of organs. However, in Maastricht category 2 NHBDs, this information is available, discarding those potential NHBDs with prolonged periods of circulatory arrest without cardiopulmonary resuscitation (CPR). Kidneys from these donors have been transplanted with a rate of success that in most cases did not differ from Maastricht category 3 NHBD and HBD grafts (*9–12*).

The major hurdle in the transplantation of kidneys from controlled or uncontrolled NHBDs is the high incidence of delayed graft function resulting from prolonged warm ischemia, which cannot be prevented by measures such as the "in situ" perfusion of the abdominal organs (*13*). Total body cooling and normothermic recirculation through cardiopulmonary bypass (CPB) have been associated with a shorter and reduced frequency of delayed graft function, respectively, after kidney transplantation (*14*). The improved viability of kidneys from NHBDs with these methods has been attributed to an augment in the perfusion pressure and oxygenation of organs (*15, 16*).

[1] Liver Transplant Unit, Hospital Juan Canalejo, La Coruña, Spain.
[2] Pathology Division, Hospital Juan Canalejo, La Coruña, Spain.
[3] Transplantation Division, Hospital Juan Canalejo, La Coruña, Spain.
[4] Transplantation Unit, Hospital Clínico San Carlos, Madrid, Spain.
[5] Address correspondence to: Rafael Máñez, M.D., Ph.D., Transplantation Division; Hospital Juan Canalejo, Xubias de Arriba, 84; 15006 La Coruña; Spain. E-mail: manez@canalejo.org.
Received 16 January 2003. Accepted 17 June 2003.
DOI: 10.1097/01.TP.0000085043.78445.53

Leclerc G, Gal D, Takeshita S, et al.: 1992. Percutaneous arterial gene transfer in a rabbit model. Efficiency in normal and balloon-dilated atherosclerotic arteries. J Clin Invest 90:936–944.

Mann MJ, Whittemore AD, Donaldson MC, et al.: 1999. Ex vivo gene therapy of human vascular bypass grafts with E2F decoy: the PREVENT single-centre, randomised, controlled trial. Lancet 354:1493–1498.

Morishita R, Gibbons GH, Ellison KE, et al.: 1993a. Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia. Proc Natl Acad Sci USA 90:8474–8478.

Morishita R, Gibbons GH, Kaneda Y, et al.: 1993b. Novel in vitro gene transfer method for study of local modulators in vascular smooth muscle cells. Hypertension 21:894–899.

Nabel EG: 1995. Gene therapy for cardiovascular disease. Circulation 91:541–548.

Nabel EG: 2002. CDKs and CKIs: Molecular targets for tissue remodeling. Nat Rev Drug Discov 1:587–598.

Nabel EG, Plautz G, Boyce FM, et al.: 1989. Recombinant gene expression in vivo within endothelial cells of the arterial wall. Science 244:1342–1344.

Nicklin SA, Reynolds PN, Brosnan MJ, et al.: 2001. Analysis of cell-specific promoters for viral gene therapy targeted at the vascular endothelium. Hypertension 2001:38:65–70.

Paddison PJ, Caudy AA, Bernstein E, et al.: 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev 16:948–958.

Panetta CJ, Miyauchi K, Berry D, et al.: 2002. A tissue-engineered stent for cell-based vascular gene transfer. Hum Gene Ther 13:433–441.

Plautz G, Nabel EG, Nabel GJ: 1991. Introduction of vascular smooth muscle cells expressing recombinant genes in vivo. Circulation 83:578–583.

Ross R: 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362:801–809.

Taniyama Y, Tachibana K, Hiraoka K, et al.: 2002. Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. Gene Ther 9:372–380.

Tanner FC, Boehm M, Akyürek LM, et al.: 2000. Differential effects of the cyclin-dependent kinase inhibitors $p27^{(Kip1)}$, $p21^{(Cip1)}$, and $p16^{(Ink4)}$ on vascular smooth muscle cell proliferation. Circulation 101: 2022–2025.

Tanner FC, Yang ZY, Duckers E, et al.: 1998. Expression of cyclin-dependent kinase inhibitors in vascular disease. Circ Res 82: 396–403.

Tirlapur UK, Konig K: 2002. Targeted transfection by femtosecond laser. Nature 418: 290.

Tsui LV, Camrud A, Mondesire J, et al.: 2001. p27-p16 fusion gene inhibits angioplasty-induced neointimal hyperplasi and coronary artery occlusion. Circ Res 89:323–328.

Wills KN, Mano T, Avanzini JB, et al.: 2001. Tissue-specific expression of an anti-proliferative hybrid transgene from the human smooth muscle alpha-actin promoter suppresses smooth muscle cell proliferation and neointima formation. Gene Ther 8: 1847–1854.

Xia H, Mao Q, Paulson HL, et al.: 2002. siRNA-mediated gene silencing in vitro and in vivo. Nature Biotech 20:1006–1010.

Yang X, Atalar E, Li D, et al: 2001. Magnetic resonance imaging permits in vivo monitoring of catheter-based vascular gene delivery. Circulation 104:1588–1590.

PII S1050-1738(02)00255-4 TCM

Genetic Manipulation of Primate Embryonic and Hematopoietic Stem Cells with Simian Lentivirus Vectors

Yutaka Hanazono,* Takayuki Asano, Yasuji Ueda, and Keiya Ozawa

During the past several years, many articles have described how human embryonic stem (ES) cells and adult hematopoietic stem cells (HSCs) can differentiate into cardiac muscle, blood vessels, and various other types of cells. The articles raised the expectation that these stem cells may become useful for the treatment of a variety of diseases, including cardiovascular diseases. Genetic manipulation of ES cells and HSCs would be important for such future applications of the cells. Until now, retroviral vectors have been used primarily for stable expression of transgenes in murine ES cells and HSCs. Because murine models may not predict reliably the biology of ES cells and HSCs in humans, we have utilized primate ES cells and HSCs as targets of gene transfer. We have shown that primate ES cells and HSCs can be transduced efficiently with lentiviral vectors derived from the simian immunodeficiency virus, and that the high transgene expression persists without transcriptional silencing. This highly efficient gene transfer method allows for safe and faithful gene delivery to primate ES cells and HSCs to test potential research and therapeutic applications. (Trends Cardiovasc Med 2003;13:106–110) © *2003, Elsevier Science Inc.*

Yutaka Hanazono, Takayuki Asano, and Keiya Ozawa are at the Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan. Yasuji Ueda is at the DNAVEC Research Inc., Ibaraki, Japan.

*Address correspondence to: Yutaka Hanazono, MD, PhD, Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi, Kawachi, Tochigi 329-0498, Japan. Tel.: (+81) 285-58-7402; fax: (+81) 285-44-8675; e-mail: hanazono@jichi.ac.jp.

• Stem Cell Therapy and Animal Models

With the establishment of embryonic stem (ES) and embryonic germ (EG) cell lines (Reubinoff et al. 2000, Shamblott et al. 1998, Thomson et al. 1998), great attention has been given to the therapeutic potential of these cells from both a biologic and medical perspective. This potential is based on their remarkable ability to differentiate into most of the specialized cells of the three EG layers and their ability

to divide for indefinite periods in culture. These extraordinary features have stimulated many researchers to consider using human pluripotent stem cells as cellular therapy for a variety of diseases and injured states. Human ES cells have been reported to differentiate into neurons, cardiac myocytes, and blood cells (Kaufman et al. 2001, Kehat et al. 2001, Reubinoff et al. 2000). Of particular note, ES cell-derived cardiomyocytes have been engrafted following implantation in mice (Klug et al. 1996), suggesting that ES cells can be used as a source of donor cardiomyocytes.

In animal studies, primarily using murine models, researchers also have recently observed that hematopoietic cells have the capacity to form other kinds of cells, such as liver, muscle, and blood vessel cells (Graf 2002); although cell fusions may, in part, account for such a change of phenotype (Terada et al. 2002, Ying et al. 2002). Several articles have suggested the possible use of bone marrow cells for the treatment of cardiac ischemia (Jackson et al. 2001, Kocher et al. 2001, Orlic et al. 2001), and bone marrow cells have been successfully used for the treatment of patients with ischemic limbs (Tateishi-Yuyama et al. 2002).

Clearly, animal models will prove to be important in the study of ES cells and hematopoietic stem cells (HSCs). Although mouse models have been used widely, they may not predict reliably the biology of ES cells and HSCs in humans. Nonhuman primate ES cells and HSCs may reflect human ES cell and HSC characteristics and behavior more closely, due to their close phylogenetic relationship (Wagner and Storb 1996). We have used cynomolgus ES cells and HSCs for the study of genetic manipulation and transplantation, with the future goal of transplantation-based applications. This article focuses on genetic manipulation of primate ES cells and HSCs using simian lentivirus vectors.

• Primate Lentivirus Vectors

Genetic manipulation of human ES cells and HSCs likely will be required for their future clinical application, at least in some disorders. Until now, murine oncoretroviral vectors (henceforth referred to as retroviral vectors) have been used predominantly for the purpose of stable expression of transgenes in murine ES cells and HSCs (Cherry et al. 2000). In an attempt to improve transgene expression in either ES cells or HSCs, retroviral vectors derived from the mouse stem cell virus (MSCV) have been developed (Grez et al. 1990, Hawley et al. 1994). These vectors differ from the standard Moloney mouse leukemia virus vectors in the long terminal repeat (LTR) promoter-enhancer region and the primer-binding site. However, even with MSCV-based vectors, transgene expression has remained low and expression is repressed over time (Cherry et al. 2000).

Lentiviral vectors are another group of integrating vectors. Unlike retroviral vectors, lentiviral vectors can transduce nondividing cells (Naldini et al. 1996). They have been developed by modifying human immunodeficiency virus 1 (HIV-1) (Nadini et al. 1996), feline immunodeficiency virus (Poeschla et al. 1998), equine infectious anemia virus (Olsen 1998), simian immunodeficiency virus (SIV) (Nakajima et al. 2000, Schnell et al. 2000), or bovine immunodeficiency virus (Berkowitz et al. 2001). Although the HIV-1-based vectors are the most widely used among them, our lentiviral vector is based on an SIV derived from an African green monkey (SIVagm) (Nakajima et al. 2000). The advantage of SIVagm vectors over HIV-1 vectors is in safety. Replicative HIV-1 is severely pathogenic in humans, and thus HIV-1 vectors have potential safety concerns. A series of safety modifications have been made in HIV-1 vectors to decrease the chance of recombination events resulting in replication-competent viruses. Increasingly more HIV-1 sequences have been removed from the vectors, but the ability to produce high-titer vectors that can transduce nondividing cells and integrate efficiently is dependent on the inclusion of at least some residual HIV-1 elements (such as *gag*, *pol*, and *rev*) in addition to the LTRs (Dull et al. 1998). Thus, some safety concerns remain. In contrast, SIVagm has no pathogenicity in its immunocompetent natural host and in experimentally inoculated macaque monkeys (Honjo et al. 1990). In addition, the sequence homology between HIV-1 and SIVagm is relatively low (about 50%) (Figure 1). The generation of replication-competent virus by recombination between SIVagm vectors and HIV-1 in human subjects is therefore highly unlikely. This provides an advantage in safety, especially if target cells are already infected with HIV or are permissive to HIV infection.

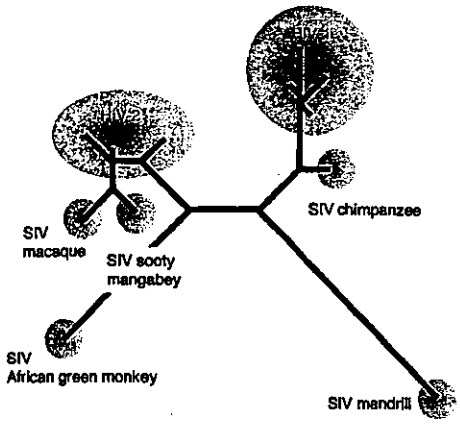


Figure 1. Primate lentiviruses. Primate lentiviruses are divided into several subgroups. The simian immunodeficiency virus (SIV) African green monkey (SIVagm) is phylogenetically most distant to the human immunodeficiency virus 1 (HIV-1), and the homology between the two viruses is rather low (around 50%). The generation of replication-competent virus by recombination between SIVagm vectors and HIV-1 in vitro or in human subjects is therefore unlikely. Thus, SIVagm vectors should offer a safety advantage over HIV-1 vectors. This tree was constructed from the nucleotide sequences of the *rev* gene by the Neighbor-Joining Method in Genetyx-Mac (Software Development Co., Ltd., Tokyo, Japan). Twelve sequences of the virus samples (4 HIV-1, 3 HIV-2, and 5 SIV strains) were analyzed.

• Gene Transfer into Primate ES Cells

Successful therapeutic use of human ES cell-derived donor cells would require the generation of essentially pure differentiated cell cultures, because the engraftment of undifferentiated pluripotent ES cells could result in teratoma formation. In addition, evasion of host immune responses to allogeneic ES cell-derived donor cells would be required to maintain prolonged engraftment. Nonhuman primate ES cell lines (rhesus, common marmoset, and cynomolgus ES cell lines) have been established (Suemori et al. 2001, Thomson et al. 1995 and 1996) and are a useful model system to study these issues. These cells were derived from blastocysts and show prolonged undifferentiated proliferation while maintaining a normal karyotype and developmental potential to differentiate into all three EG layer cells. Nonhuman primate ES cells differ from mouse ES cells, but

are remarkably similar to human ES cells in morphology, surface marker expression, and lack of leukemia inhibitory factor dependency.

We have utilized cynomolgus ES cells and explored the potential of SIV vectors to introduce new genetic material into the cells (Asano et al. 2002). When cynomolgus ES cells were transduced once with an SIV vector carrying the enhanced green fluorescent protein (EGFP or GFP) gene, most cells fluoresced after a single transduction (Figure 2). The GFP expression persisted for months without selection procedures and was not repressed during embryoid body formation. On the other hand, retroviral GFP expression in cynomolgus ES cells was low (Asano et al. 2002).

Taking advantage of the fact that the 3'LTR duplicates and becomes the 5'LTR in vivo after reverse transcription and integration, the SIV vector was constructed with a deletion of the U3 region in the 3'LTR, allowing for production of a self-inactivating (SIN) vector. The SIN SIV vector loses the transcriptional capacity of the LTR once it is integrated into the target genome. One possible explanation for the sustained lentiviral transgene expression is that the transcriptionally inactive state of the lentiviral LTR might allow the provirus to escape epigenetic mechanisms of silencing (Yoder et al. 1997). Stable transgene expression, however, also was observed when a normal SIV vector with an intact LTR was utilized (Asano et al. 2002). Lentiviral vectors might have less intrinsic susceptibility to silencing than do retroviral vectors.

Recently, it has been reported that human ES cells can be transduced efficiently by HIV-1-based lentiviral vectors (Pfeifer et al. 2002). The lentiviral gene transfer method should allow for efficient introduction of a variety of genes of interest into primate ES cells and for their sustained expression.

• Gene Transfer into Primate CD34$^+$ Cells

If plasticity of adult HSCs can be applied to human cells, it eventually may be possible to use HSCs to repair a wide array of cells and tissues. We have utilized cynomolgus monkeys (*Macaca fascicularis*) as predictive preclinical HSC transplantation models (Ageyama et al. 2002). We also have developed a cynomolgus model of acute cardiac infarction. This model will provide an important framework for future clinical studies using hematopoietic cells aimed at regeneration of ischemic cardiac muscle.

CD34 is a cell-surface marker of undifferentiated HSCs. Although all HSCs may not express CD34 (Osawa et al. 1996), clinical CD34$^+$ cell transplantation has been conducted successfully in HSC gene therapy studies with retroviral vectors (Aiuti et al. 2002, Cavazzana-Calvo et al. 2000). CD34$^+$ cells can be isolated from bone marrow, peripheral blood, or cord blood. For genetic manipulation, the cells usually are cultured ex vivo for several days prior to reinfusion to allow for cell cycling, because retroviral transduction requires cell division. We have utilized the standard culture conditions that include several cytokines (stem cell factor, Flt-3 ligand, and thrombopoietin) for retroviral transduction of primate CD34$^+$ cells (Hanazono et al. 2002). Prolonged cytokine treatment of HSCs, however, may result in the loss of multilineage differentiation and engraftment abilities (Dunbar et al. 2001). On the other hand, lentiviral vectors can transduce nondividing cells, and thus cytokine treatment is not necessarily required for lentiviral transduction. This is the great advantage of using lentiviral vectors over retroviral vectors for transduction of HSCs.

In our laboratory, human CD34$^+$ cells were transduced with SIV vectors carrying the GFP gene. Twenty percent to forty percent of cells fluoresced after a single infection with the vector (Figure 3A). The transduced CD34$^+$ cells were transplanted into sublethally irradiated nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. This model takes advantage of the immunologically naive state of the mice. Because the NOD/SCID mice are severely immuno-

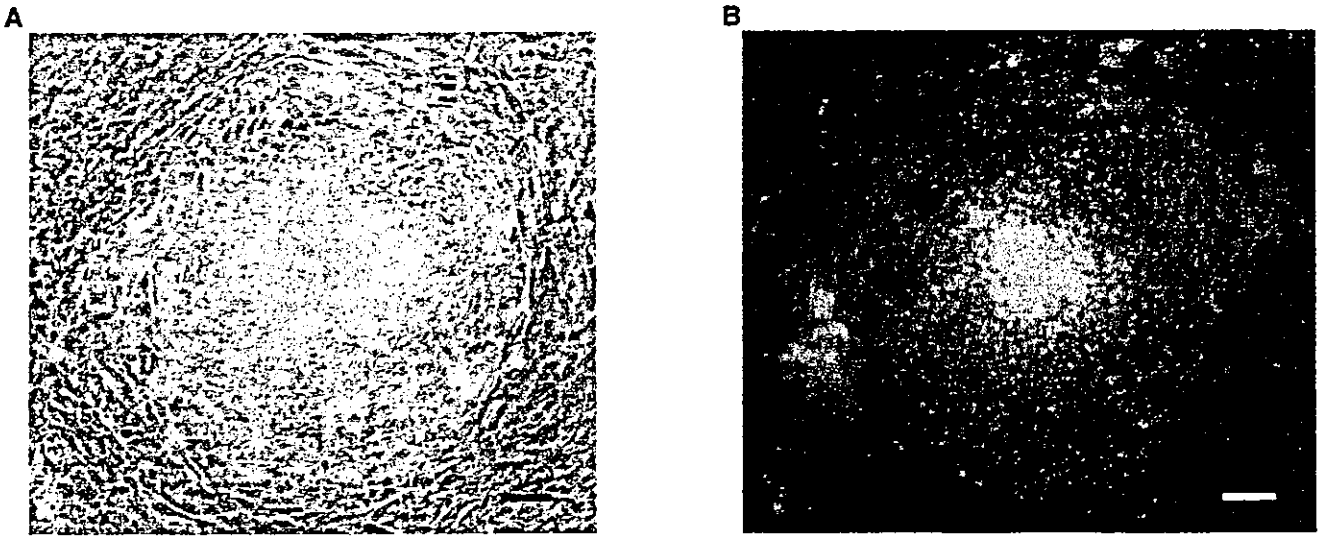


Figure 2. Gene transfer into cynomolgus embryonic stem (ES) cells by simian immunodeficiency virus (SIV) vector. Cynomolgus ES cells (CMK6) were transduced once with the SIV vector expressing the green fluorescent protein (GFP) gene at 60 transducing units per target cell. The transduced cells were observed at day 6 after transduction in a bright field **(A)** and in a dark field **(B)** under a fluorescence microscope. Cells that form colonies are cynomolgus ES cells, and background cells are mouse embryonic fibroblast feeder cells. Fluorescence was observed in many ES cells. Scale bar = 100 μm.

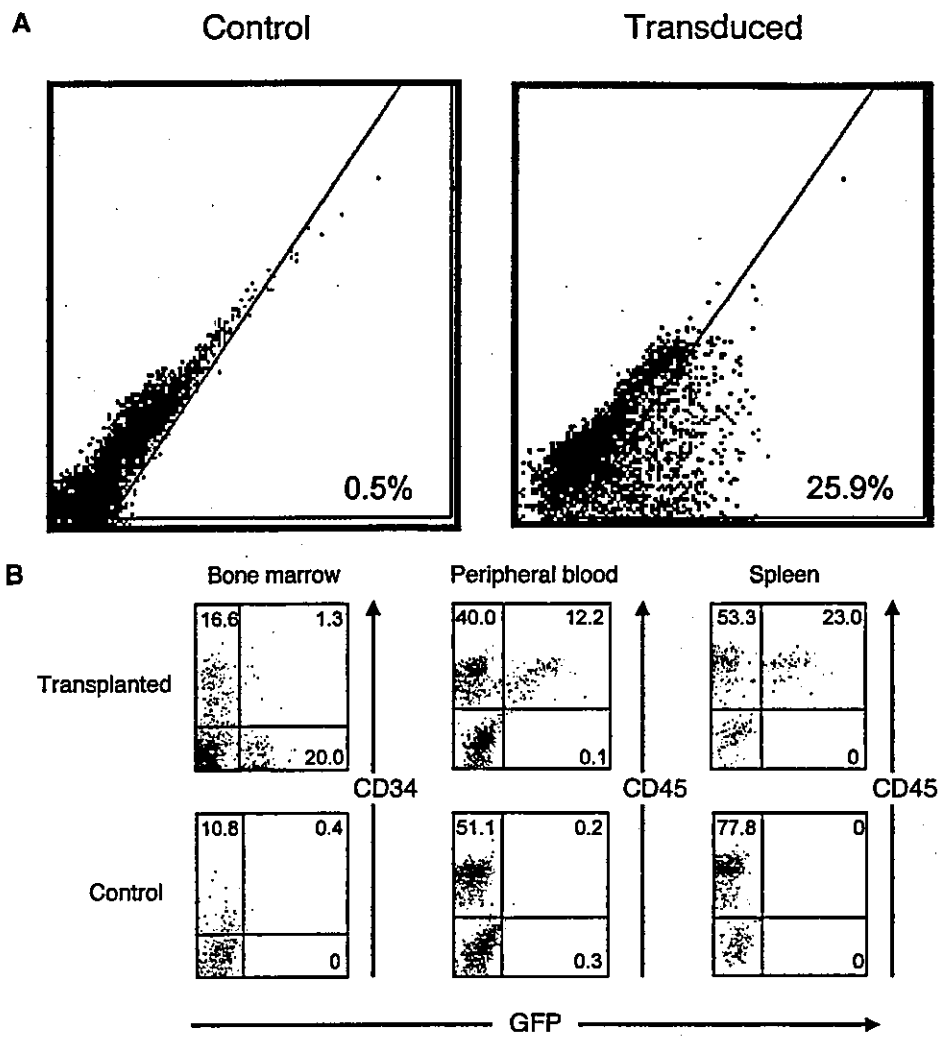


Figure 3. Gene transfer into human CD34$^+$ cells by simian immunodeficiency virus (SIV) vector and transplantation into sublethally irradiated nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. **(A)** Human CD34$^+$ cells were transduced with a green fluorescent protein (GFP)-expressing SIV vector and 25.9% of the cells fluoresced. After transduction, the CD34$^+$ cells were transplanted into sublethally irradiated NOD/SCID mice. **(B)** Six weeks after transplantation, bone marrow, peripheral blood, and spleen cells were harvested and examined for human CD34 or CD45 expression (*vertical axes*) and GFP expression (*horizontal axes*). Fluorescent human cells were detected (*right upper quadrants*), clearly indicating that the progeny of engrafted human cells expressed the GFP gene.

deficient, human HSCs can engraft and generate their progeny in these animals (Bhatia et al. 1998). As shown in Figure 3, the progeny of engrafted cells also fluoresced, suggesting that transcriptional silencing did not occur.

• Discussion

We have shown that primate ES cells and HSCs can be transduced efficiently with SIV vectors and that the transgene expression persists without transcriptional silencing. Recently, two groups (Lois et al. 2002. Pfeifer et al. 2002) revealed that trangenes delivered into mouse ES cells by HIV-1-based lentiviral vectors, in contrast to retroviral vectors, were expressed without transcriptional silencing during in vivo embryogenesis, resulting in the generation of transgenic mice. Retroviral and lentiviral vectors might have very different intrinsic susceptibilities to silencing, presumably as a consequence of their contrasting lifestyles. Whereas retroviruses rely on germline transmission as one form of spreading, lentiviruses rely on horizontal and nongermline vertical transfer. Thus, organisms might have evolved mechanisms to suppress the activity of endogenous retroviruses that would otherwise lead to their parasitic expansion in the genome (Yoder et al. 1997). In contrast, such mechanisms might not target lentiviral sequences, because endogenous lentiviruses have not been found in any mammalian genome. The highly efficient gene transfer method using lentiviral vectors without transgene silencing allows for faithful gene delivery to primate ES cells and HSCs with the potential for research and therapeutic application.

• Acknowledgments

The authors thank Dr. Norio Nakatsuji (Kyoto University, Kyoto, Japan) and Tanabe Seiyaku Co. Ltd. (Osaka, Japan) for providing the cynomolgus ES cells.

References

Ageyama N, Hanazono Y, Shibata H, et al.: 2002. Safe and efficient methods of autologous hematopoietic stem cell transplantation for biomedical research in cynomolgus monkeys. Comp Med 52:445–451.

Aiuti A, Slavin S, Aker M, et al.: 2002. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Science 296:2410–2413.

Asano T, Hanazono Y, Ueda Y, et al.: 2002. Highly efficient gene transfer into primate embryonic stem cells with a simian lentivirus vector. Mol Ther 6:162–168.

Berkowitz R, Ilves H, Lin WY, et al.: 2001. Construction and molecular analysis of gene transfer systems derived from bovine immunodeficiency virus. J Virol 75:3371–3382.

Bhatia M, Bonnet D, Murdoch B, et al.: 1998. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat Med 4:1038–1045.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, et al.: 2000. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 288:669–672.

Cherry SR, Biniszkiewics D, van Parijs L, et al.: 2000. Retroviral expression in embryonic stem cells and hematopoietic stem cells. Mol Cell Biol 20:7419–7426.

Dull T, Zufferey R, Kelly M, et al.: 1998. A third-generation lentivirus vector with a conditional packaging system. J Virol 72: 8463–8471.

Dunbar CE, Takatoku M, Donahue RE: 2001. The impact of ex vivo cytokine stimulation on engraftment of primitive hematopoietic cells in a non-human primate model. Ann NY Acad Sci 938:236–245.

Graf T: 2002. Differentiation plasticity of hematopoietic cells. Blood 99:3089–3101.

Grez M, Akgün E, Hilberg F, Ostertag, W: 1990. Embryonic stem cell virus, a recombinant murine retrovirus with expression in embryonic stem cells. Proc Natl Acad Sci USA 87:9202–9206.

Hanazono Y, Nagashima T, Takatoku M, et al.: 2002. In vivo selective expansion of gene-modified hematopoietic cells in a non-human primate model. Gene Ther 9:1055–1064.

Hawley RG, Lieu FH, Fong AZ, Hawley TS: 1994. Versatile retroviral vectors for potential use in gene therapy. Gene Ther 1:136–138.

Honjo S, Narita T, Kobayashi R, et al.: 1990. Experimental infection of African green monkeys and cynomolgus monkeys with an SIVagm strain isolated from a healthy African green monkey. J Med Primatol 19:9–20.

Jackson KA, Majka SM, Wang H, et al.: 2001. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 107:1395–1402.

Kaufman DS, Hanson ET, Lewis RL, et al.: 2001. Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci USA 98:10,716–10,721.

Kehat I, Kenyagin-Karsenti D, Snir M, et al.: 2001. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 108:407–414.

Klug MG, Soonpaa MH, Koh GY, Field LJ: 1996. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. J Clin Invest 98:216–224.

Kocher AA, Schuster MD, Szabolcs MJ, et al.: 2001. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 7:430–436.

Lois C, Hong EJ, Pease S, et al.: 2002. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science 295:868–872.

Nakajima T, Nakamaru K, Ido E, et al.: 2000. Development of novel simian immunodeficiency virus vectors carrying a dual gene expression system. Hum Gene Ther 11:1863–1874.

Naldini L, Blömer U, Gallay P, et al.: 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272:263–267.

Olsen JC: 1998. Gene transfer vectors derived from equine infectious anemia virus. Gene Ther 5:1481–1487.

Orlic D, Kajstura J, Chimenti S, et al.: 2001. Bone marrow cells regenerate infarcted myocardium. Nature 410:701–705.

Osawa M, Hanada K, Hamada H, Nakauchi H: 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273:242–245.

Pfeifer A, Ikawa M, Dayn Y, Verma IM: 2002. Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc Natl Acad Sci USA 99:2140–2145.

Poeschla EM, Wong-Staal F, Looney DJ: 1998. Efficient transduction of nondividing human cells by feline immunodeficiency lentiviral vectors. Nat Med 4:354–357.

Reubinoff BE, Pera MF, Fong C-Y, et al.: 2000. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotech 18:399–404.

Schnell T, Foley P, Wirth M, et al.: 2000. Development of a self-inactivating, minimal lentivirus vector based on simian immunodeficiency virus. Hum Gene Ther 11:439–447.

Shamblott MJ, Axelman J, Wang S, et al.: 1998. Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci USA 95:13,726–13,731.

Suemori H, Tada T, Torii R, et al.: 2001. Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. Dev Dyn 222:273–279.

Tateishi-Yuyama E, Matsubara H, Murohara T, et al.: 2002. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet 360:427–435.

Terada N, Hamazaki T, Oka M, et al.: 2002. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:542–545.

Thomson JA, Kalishman J, Golos TG, et al.: 1995. Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci USA 92:7844–7848.

Thomson JA, Kalishman J, Golos TG, et al.: 1996. Pluripotent cell lines derived from common marmoset (*Callithrix jacchus*) blastocysts. Biol Reprod 55:254–259.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al.: 1998. Embryonic stem cell lines derived from human blastocyts. Science 282:1145–1147.

Wagner JL, Storb R: 1996. Preclinical large animal models for hematopoietic stem cell transplantation. Curr Opin Hematol 3:410–415.

Ying QL, Nichols J, Evans EP, Smith AG: 2002. Changing potency by spontaneous fusion. Nature 416:545–548.

Yoder JA, Walsh CP, Bestor TH: 1997. Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13:335–340.

PII S1050-1738(02)00253-0 **TCM**

ARTICLE

doi:10.1006/mthe.2002.0655, available online at http://www.idealibrary.com on IDEAL

Highly Efficient Gene Transfer into Primate Embryonic Stem Cells with a Simian Lentivirus Vector

Takayuki Asano,[1,6] Yutaka Hanazono,[1,*] Yasuji Ueda,[3] Shin-ichi Muramatsu,[2] Akihiro Kume,[1] Hirofumi Suemori,[4] Yutaka Suzuki,[5] Yasushi Kondo,[5] Kiyonori Harii,[6] Mamoru Hasegawa,[3] Norio Nakatsuji,[4] and Keiya Ozawa[1]

[1]*Division of Genetic Therapeutics and* [2]*Department of Neurology, Jichi Medical School, Tochigi 329-0498, Japan*
[3]*DNAVEC Research Inc., Ibaraki 305-0856, Japan*
[4]*Department of Development and Differentiation, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan*
[5]*Tanabe Seiyaku Co., Ltd., Osaka 532-8505, Japan*
[6]*Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan*

**To whom correspondence and reprint requests should be addressed. Fax: (81)285-44-8675. E-mail: hanazono@jichi.ac.jp.*

The ability to stably introduce genetic material into primate embryonic stem (ES) cells could allow their broader application. We previously derived ES cell lines from cynomolgus monkey blastocysts. In this study, we examined lentiviral gene transfer into cynomolgus ES cells. When cynomolgus ES cells were transduced once with a simian immunodeficiency virus (SIV)-based lentivirus vector encoding the green fluorescent protein (GFP) gene, most cells (around 90%) fluoresced, and high levels of GFP expression persisted for 5 months without selection procedures. In addition, high levels of GFP expression were observed during embryoid body formation. On the other hand, transduction of mouse ES cells with the SIV-based vector resulted in lower gene transfer rates, implying that SIV vectors can transduce primate ES cells more efficiently than mouse ES cells. The use of GFP as a reporter gene allows direct and simple detection of successfully transduced ES cells and facilitates monitoring of ES cell proliferation and differentiation both *in vitro* and potentially *in vivo*. Furthermore, this highly efficient gene transfer method allows faithful gene delivery to primate ES cells with potential for both research and therapeutic application.

Key Words: primate embryonic stem cell, gene transfer, simian immunodeficiency virus, lentivirus vector, green fluorescent protein

INTRODUCTION

Since human pluripotent stem cells, including embryonic stem (ES) and embryonic germ (EG) cell lines, were established [1–3], great attention has been drawn to these cells from both biological and medical perspectives because of their remarkable ability to differentiate into most of the specialized cells of the three embryonic germ layers. In addition, they can divide for indefinite periods in culture. These extraordinary features have stimulated many researchers to consider human pluripotent stem cells as cellular therapies for a variety of diseases and injury states, as well as for drug development and toxicity testing [4]. However, great ethical debates have also been evoked in the public forum [5,6]. Although these cells indeed hold great promise, an alternative model system, especially that of the nonhuman primate, could prove to be invaluable to validate the promises of pluripotent stem cells and to address the efficacy and safety of pluripotent stem cell-based transplantation therapies.

Nonhuman-primate (rhesus and common marmoset) ES cell lines have been established by Thomson's group [7,8]. We have recently established cynomolgus monkey (*Macaca fascicularis*) ES cell lines [9]. The cynomolgus ES cell lines were derived from blastocysts, maintain a normal karyotype, show prolonged undifferentiated proliferation, and have developmental potential along all three embryonic germ layers. Cynomolgus ES cells are remarkably similar to human ES cells in all aspects, including morphology and surface marker expression. On the other hand, primate (human, rhesus, and cynomolgus) ES cells are quite distinct from mouse ES cells, for instance, in their growth velocity, in there feeder- and leukemia inhibitory factor (LIF)-dependency, and in their morphology and surface marker expression. Therefore, experimental results using mouse ES cells may not be predictive of those in primates. These discrepancies stimulated us to develop nonhuman-primate (cynomolgus) ES cells as a predictive model to more closely reflect human ES cell characteristics and behavior.

1525-0016/02 $35.00

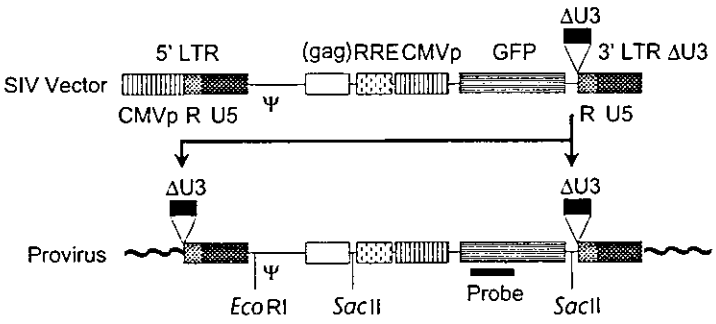


FIG. 1. Vector construction. The SIV vector contains the internal CMV promoter to drive the expression of the GFP gene. It is a SIN vector that was created by deletion of the U3 region of the 3' LTR. Once the SIN vector is transferred into target genome, most of the LTR sequences are lost. Ψ, packaging signal; CMVp, cytomegalovirus promoter; RRE, Rev-responsive element.

Genetic manipulation of human pluripotent stem cells will likely be required for their future clinical application, at least in some disorders. Although electroporation has served as the principal method for the introduction of foreign DNA into murine ES cells [10], human ES cells do not survive electroporation well [11]. Alternatively, the lipofection method has been attempted for gene transfer into human ES cells, yet resulting gene transfer efficiency rates of no more than 10% have been attained. In addition, transgene expression is transient with very few stable transfectants (about one cell per 10^5 cells) obtained [11]. Efficient gene transfer methods that result in stable transgene expression in primate ES cells are, therefore, required for their future applications. Here we report a method that results in highly efficient gene transfer along with stable long-term transgene expression in cynomolgus ES cells using a lentiviral vector derived from the simian immunodeficiency virus (SIV).

RESULTS

Vector Construction

Lentiviral vectors were developed by modifying HIV-1, feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), or SIV as described [12–16]. In this study, we constructed a lentiviral vector based on SIV derived from the African green monkey (SIVagm), which is nonpathogenic in both the natural host and other experimentally inoculated macaques [17]. Rather than relying on specific viral entry by CD4 or other coreceptors, the vesicular stomatitis virus (VSV)-G envelope, which has generally been used to pseudotype lentiviral vectors, was used in the current study [16] (Fig. 1). Taking advantage of the fact that the 3' long terminal repeat (LTR) duplicates and becomes the 5' LTR *in vivo* after reverse transcription and integration, our vector was constructed with a deletion of the U3 region in the 3' LTR, allowing production of self-inactivating (SIN) vector, which loses the transcriptional capacity of the LTR once transferred to the target genome [18,19]. The SIV vector in the current study drives expression of the green fluorescent protein (GFP) gene by an internal cytomegalovirus (CMV) promoter.

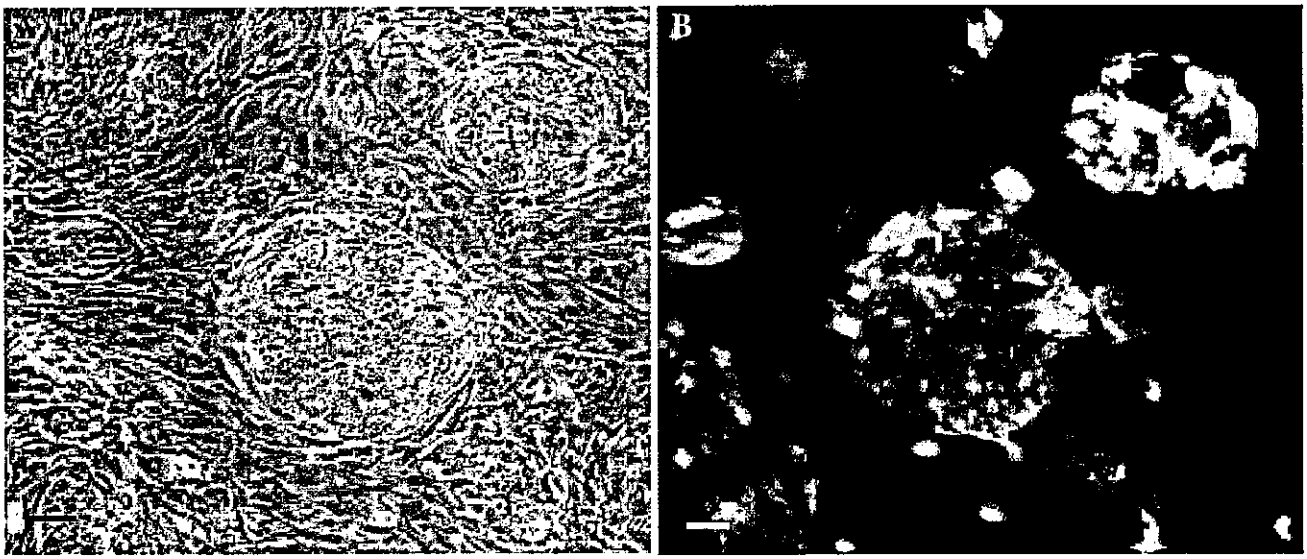


FIG. 2. Green cynomolgus ES cells. Cynomolgus ES cells were transduced once with the SIV vector expressing the GFP gene at MOI of 100 and the cells were maintained in an undifferentiated state without any selection procedures. The cells were observed on day 30 after transduction in a bright field (A) and in a dark field (B) under a fluorescent microscope. Cells that form colonies are cynomolgus ES cells and background cells are MEF cells. Bar = 100 μm.