THE JOURNAL OF GENE MEDICINE
J Gene Med 2003; 5: 929–940.
Published online 16 June 2003 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jgm.430

RESEARCH ARTICLE

A soluble CAR-SCF fusion protein improves adenoviral vector-mediated gene transfer to c-Kit-positive hematopoietic cells

Akira Itoh[1,3]
Takashi Okada[1]
Hiroyuki Mizuguchi[4]
Takao Hayakawa[4]
Hiroaki Mizukami[1]
Akihiro Kume[1]
Masaaki Takatoku[2]
Norio Komatsu[2]
Yutaka Hanazono[1]
Keiya Ozawa[1,2]*

[1]*Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Japan*

[2]*Division of Hematology, Department of Medicine, Jichi Medical School, Japan*

[3]*The Second Research Department, Central Technology Laboratory, Asahi Kasei Corporation, Japan*

[4]*Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Japan*

*Correspondence to:
Professor Keiya Ozawa, Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Tochigi 329-0498, Japan. E-mail: kozawa@jichi.ac.jp

Received: 30 October 2002
Revised: 24 March 2003
Accepted: 14 April 2003

Abstract

Background Although adenoviral vectors primarily derived from the adenovirus serotype 5 (Ad5) are widely used for many gene transfer applications, they cannot efficiently infect hematopoietic cells, since these cells do not express the coxsackie-adenoviral receptor (CAR).

Methods We have developed a soluble fusion protein that bridges adenoviral fibers and the c-Kit receptor to alter Ad5 tropism to immature hematopoietic cells. The CAR-SCF fusion protein consists of the extracellular domains of CAR and stem cell factor (SCF). The human megakaryoblastic leukemia cell lines UT-7 and M07e, human chronic myelogenous leukemia cell line K-562, and erythroleukemia cell line TF-1 were used to assess CAR-SCF-assisted Ad5-mediated gene transfer. Hematopoietic cell lines were infected with an Ad5 vector (Ad5-eGFP) or a fiber-mutant Ad5/F35 (Ad5/F35-eGFP) expressing the enhanced green fluorescent protein gene in the presence or absence of CAR-SCF.

Results Twenty-four hours after infection, more than 80% of M07e cells infected in the presence of CAR-SCF were eGFP-positive, compared with very few eGFP-positive cells following Ad5-eGFP infection in the absence of CAR-SCF. The enhancement of Ad5-eGFP infection by CAR-SCF was greater than that caused by Ad5/F35-eGFP (50%). The ability of CAR-SCF to enhance Ad5-eGFP infectivity was highly dependent on cellular c-Kit expression levels. Furthermore, CAR-SCF also enhanced Ad5-mediated gene transfer into human primary $CD34^+$ cells.

Conclusions The CAR-SCF fusion protein assists Ad5-mediated transduction to $c\text{-}Kit^+$ CAR^- hematopoietic cells. The use of this fusion protein would enhance a utility of Ad5-mediated hematopoietic cell transduction strategies.

Keywords adapter; adenovirus; hematopoietic cell; c-Kit

Introduction

Hematopoietic stem cells (HSCs) are often used as targets for gene therapy because of their self-renewal and multilineage differentiation capabilities. Retroviral vectors derived from murine leukemia viruses are most frequently used to infect HSCs, since they are well characterized and can integrate into the target cell genome, which allows transmission of the vector to daughter cells *in vivo*. However, when temporary gene expression in HSCs is required,

as is the case when using the Cre recombinase system, non-integrating, non-replicating adenovirus vectors would be ideal. Although adenovirus vectors derived from adenovirus serotype 5 (Ad5) are widely used for many gene transfer applications, they cannot efficiently infect hematopoietic cells [1], primarily because the Ad5 receptor, the coxsackievirus and adenovirus receptor (CAR) protein, is only very weakly expressed on the surface of hematopoietic cells [2]. It is believed that Ad5 infection of cells begins with the attachment of Ad5 to the cell surface by binding of the viral fiber to cellular CAR [3], followed by internalization of the virus via association with viral capsid penton proteins to the cellular αv integrins ($\alpha v\beta 3$ and $\alpha v\beta 5$) [4]. Recently, CAR was identified as a component of the tight junction that functions as a barrier to paracellular solute movement [5], which may explain why it is not expressed in non-adherent hematopoietic cells.

To improve adenoviral gene transfer to immature hematopoietic cells, a few strategies have been reported to provide altered and expanded tropism of Ad5 in a CAR-independent manner. One of these approaches uses a chimeric Ad5 vector in which the fiber protein from the subgroup B adenoviruses was substituted for the original fiber. These chimeric Ad5 vectors can infect hematopoietic cells in a CAR-independent manner [6–9], since subgroup B adenoviruses do not associate with CAR [10]. Chimeric Ad5 vector can also infect many primary cells, including synoviocytes and bone marrow stromal cells [11]. The second strategy involves ligand-specific targeting using a fiber-mutant Ad5 vector in which the fiber knob of Ad5 is genetically modified to bind to specific target molecule(s) such as heparin/heparan sulfate [12] and receptors for the RGD sequence [13,14]. Such fiber-mutant Ad5 vectors can target specific cells of interest. The third approach employs a chemically modified Ad5 vector that is conjugated to a ligand recognized by receptors expressed on specific hematopoietic cells [15–17]. All of these strategies, however, require the reconstruction or modification of current Ad5 vectors. Furthermore, the third approach requires ligand purification in addition to chemical modification of the Ad5 vector.

In contrast, a soluble adapter protein that links the Ad5 fiber to specific cell-surface molecules provides a simpler system to increase Ad5 infectivity of certain cell types. By adding the adapter protein to the culture medium, Ad5 tropism to target cells can be changed without requiring any reconstruction or modification of Ad vectors. For example, a fusion protein linking the extracellular domain of CAR to epidermal growth factor (EGF) could retarget Ad5 vectors to EGF receptor-positive cancer cell lines [18]. Another fusion protein linking CAR and the Fc region of human immunoglobulin could potentially retarget Ad5 vectors to Fcγ receptor I-positive cells [19]. This method should allow us to manipulate numerous Ad5 vector stocks to infect cells of interest; however, a protein that retargets Ad5 to immature hematopoietic cells has not yet been reported. In this study, we have developed a novel soluble fusion protein linking CAR and stem cell factor (SCF), a ligand of c-Kit, to achieve altered and expanded Ad5 tropism for efficient transduction to undifferentiated hematopoietic cells, including HSCs. Here we report that the CAR-SCF fusion protein facilitates retargeting of adenoviral vectors to human c-Kit$^+$ immature hematopoietic cells, including subsets with potential stem cell capacity.

Materials and methods

Cell culture

Human megakaryoblastic leukemia cell lines UT-7 [20] and M07e [21], human chronic myelogenous leukemia cell line K-562, and erythroleukemia cell line TF-1 [22] were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ, USA), 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified incubator under a 5% CO_2 atmosphere. Human cord blood CD34$^+$ cells were either isolated from placental and umbilical blood mononuclear cells using a stem cell concentration system (CellPro Systems; CellPro Inc., Bothell, WA, USA) or purchased from Bio Whittaker Inc. (Walkersville, MD, USA). Human cord blood was obtained and treated after informed consent was received following our hospital's guidelines. Purified cord blood CD34$^+$ cells were cultured in IMDM with 10% FBS and 100 ng/ml of human stem cell factor (SCF; Peprotech), 100 ng/ml of human Flt3 ligand (FL; Peprotech), human thrombopoietin (TPO; Peprotech), and 100 units/ml penicillin + 100 µg/ml streptomycin for 2 days. A human embryonic kidney cell line, 293, and 293T, a derivative of the 293 cell line that expresses SV40 large T antigen [23], were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Plasmid construction

We constructed plasmids expressing two fusion proteins, which each consist of two domains: (i) the extracellular domain of the human CAR (CARex), and (ii) the extracellular domain of the mouse or human SCF (mSCFex or hSCFex). These two domains were linked via a six-histidine (His^6)-tag and a six-amino-acid (Ser Ala Ser Ala Ser Ala) linker to generate the fusion proteins, which were called CARex-mSCFex (CmS) and CARex-hSCFex (ChS; Figure 1A). A cDNA encoding CARex with a natural signal peptide was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) from an mRNA pool of the human glioblastoma cell line U251MG and subsequently cloned. Briefly, mRNA was isolated from the cell culture using an mRNA isolation kit (Roche Diagnostics, Basel, Switzerland), and

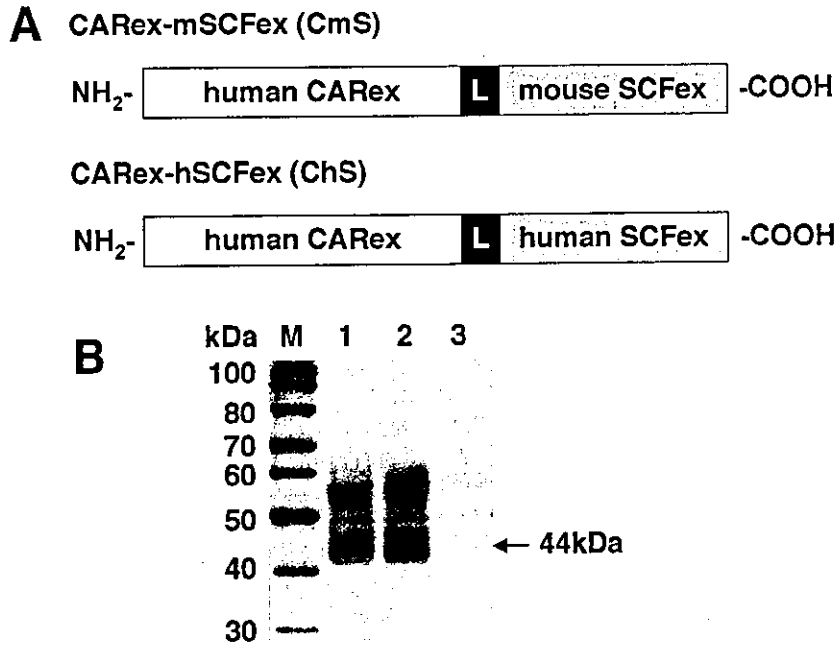

Figure 1. Construction of soluble CAR-SCF fusion proteins. (A) The extracellular domains of human CAR (CARex) and mouse or human SCF (SCFex) were fused via a linker (L) consisting of a six-histidine (His^6)-tag and six amino acids (SASASA) to generate the fusion proteins CARex-mSCFex (CmS) and CARex-hSCFex (ChS). (B) Western blot analysis of supernatants of 293T cells transfected with the CAR-SCF expression vector; lane M, His^6-tagged size marker; lane 1, CmS; lane 2, ChS; lane 3, mock transfected. An arrow indicates the theoretical molecular mass of both fusion proteins (44 kDa) calculated from their amino acid sequences

reverse-transcribed into a single-stranded cDNA using a first-strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ, USA). A DNA fragment of approximately 700 bp encoding CARex was amplified by PCR using the single-stranded cDNA as a template with the primers CAR1SL (5′-CACCATGGCGCTCCTGCTGTGCTTCGTGC-3′) and CAR3AL (5′-AGCTTTATTTGAAGGAGGGACAACG-TTTAG-3′). PCR was used to add a *Hind* III site to the 5′ end of the CARex cDNA and a DNA template encoding the His^6-tag with an *Aor*51H I recognition site to the 3′ end using the primers CAR1SHind (5′-CCAAGCTTCCACCATGGCGCTCCTGCTG-3′) and CAR3A-HisAor (5′-AGCGCTATGGTGATGGTGATGATGAGCTTTA-TTTGAAGGAGGGA-3′). cDNAs encoding mSCFex and hSCFex were identified in IMAGE [24] as clones #3662792 and #4247754, respectively, and were purchased from ResGen Corp. (Carlsbad, CA, USA). The cDNAs encoding mSCFex and hSCFex were both modified by the addition of an *Aor*51H I site and a DNA fragment encoding the six-amino-acid linker to the 5′ end and a *Xho* I site to the 3′ end by a PCR technique using the primers MSCF1LINK for mSCFex (5′-AGCGCTTCCGCCTCTGCCAAGGAGATCTGCGGGAATCC-3′) or HSCF1LINK for hSCFex (5′-AGCGCTTCCGCCTCTG-CCGAAGGGATCTGCAGGAATCG-3′) and HSCF2ASTOP for both mSCFex and hSCFex (5′-CTCGAGCTATGCAACA-GGGGGTAACATAA-3′). These two cDNA fragments encoding modified CAR and modified SCF were ligated to a single cDNA encoding CARex-mSCFex or CARex-hSCFex, and were then inserted into *Hind* III/*Xho* I-digested pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA), to obtain pcDNA/CmS and pcDNA/ChS, respectively.

Expression and detection of CAR-SCF fusion proteins

To produce the fusion proteins, 293T cells were transiently transfected with the plasmid pcDNA/CmS or pcDNA/ChS using a lipofection reagent (LipofectAmine Plus; Invitrogen). The supernatants containing the fusion proteins were concentrated approximately 100-fold and the culture medium was exchanged into phosphate-buffered saline (PBS) using a centrifugal ultrafilter device (Ultrafree-15; Millipore Corp., Bedford, MA, USA). The concentrated fusion proteins were analyzed by Western blotting against the His^6-tag. Briefly, aliquots of the concentrated supernatant were electrophoresed on a 4–12% polyacrylamide gradient gel (NuPAGE; Invitrogen), electrotransferred onto a nitrocellulose membrane, and immunostained with an anti-penta-histidine antibody conjugated to horseradish peroxidase (Qiagen GmbH, Hilden, Germany). The fusion proteins were visualized by chemiluminescence (ECL; Amersham Biosciences Corp.). The concentrations of fusion proteins in samples were estimated by a colorimetric ELISA for human or mouse SCF (R&D Systems Inc., Minneapolis, MN, USA).

Adenovirus vectors

We examined the gene transfer activity of the adenovirus vector by using the Ad5-based vector as well as that containing the Ad5/35 chimeric fiber protein (Ad5/F35), which comprises Ad35-derived fiber knob and fiber

shaft, and Ad5-derived fiber tail and penton base [25]. Enhanced green fluorescent protein (eGFP)-expression adenovirus vectors, Ad5-eGFP and Ad5/F35-eGFP, were constructed by an *in vitro* ligation method as described previously [26]. Briefly, pCMVeGFP was constructed by inserting the eGFP gene, derived from pEGFP-N1 (Clontech Laboratories, Palo Alto, CA), into the shuttle plasmid pHMCMV5, which contains a multi-cloning site between the I-*Ceu*I and PI-*Sce*I sites. pCMVeGFP was digested with I-*Ceu*I and PI-*Sce*I and was ligated to I-*Ceu*I/PI-*Sce*I-digested pAdHM4 or pAdHM34 [25], resulting in pAdHM4-eGFP or pAdHM34-eGFP, respectively. To generate the viruses, *Pac*I-digested pAdHM4-eGFP or pAdHM34-eGFP was introduced into 293 cells by calcium phosphate transfection to obtain the adenovirus vectors Ad5-eGFP or Ad5/F35-eGFP, respectively. These adenovirus vectors were propagated with 293 cells and purified as described previously [27]. Virus particle titers and infectious titers (plaque forming units, PFU) were determined as described previously [28,29]. The PFU/particle ratio was 1:7 for Ad5-eGFP and 1:17 for Ad5/F35-eGFP.

Ad-mediated gene transfer

The culture medium of the hematopoietic cell lines was exchanged for fresh medium, and a 100-μl aliquot of the cell suspension was placed in each well of 48-well plates at a density of 1×10^5 cells per well. Solution containing the fusion protein and the Ad vector at an multiplicity of infection (MOI; PFU/cell) of 25–100 was individually added to each well, and the cells were then incubated for 2 h. After 400 μl of fresh medium had been added to each well, the cultures were incubated for an additional 22 h. Pre-cultured human cord blood $CD34^+$ cells were treated similarly, except they were incubated for 48 h following infection.

Flow cytometric analysis

Flow cytometric analysis was performed using a FACS LSR (Becton Dickinson, San Jose, CA, USA). The mouse monoclonal antibody RmcB against human CAR was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Phycoerythrin (PE)-labeled antibodies to human CD34 and CD117 (c-Kit) were purchased from Pharmingen (San Diego, CA, USA). PE-labeled monoclonal antibody (13C2) to human integrin αv chain (CD51) was purchased from Cymbus Biotechnology Ltd. (Chandlers Ford, UK). 7-Aminoactinomycin-D (Via-Probe; Pharmingen) was used for determination of dead cells. Cells were washed with FACS buffer (PBS supplemented with 5% FBS and 0.05% sodium azide) and incubated with antigen-specific antibodies for 30 min on ice. When unlabeled RmcB antibody was used, the cells were washed with FACS buffer and further incubated with a secondary antibody, PE-labeled anti-mouse IgG_1 (Dako, Glostrup, Denmark). Fluorescence of eGFP protein, PE, and Via-Probe assessment of the cells was detected by FL1, FL2, and FL3, respectively. The Via-Probe-negative cell fraction reflecting viable cells were used for detection of eGFP- and/or PE-positive cells.

Results

Production of CAR-SCF fusion proteins

Plasmids expressing either CARex-mSCFex (CmS) or CARex-hSCFex (ChS; Figure 1A) were transiently transfected into 293T cells. The fusion proteins were then concentrated approximately 100-fold from the culture medium, which was exchanged for PBS using an ultrafilter device. ELISA for human or mouse SCF revealed that the final concentration was equivalent to approximately 10 μg/ml free SCF. Western blotting with an antibody against the His^6-tag revealed a major 44-kDa band as well as smear-like bands between 44 and 57 kDa for both CmS and ChS (Figure 1B). The theoretical molecular weight of both CmS and ChS calculated from their amino acid sequences is 44 kDa. The smear-like bands may represent heterogeneously glycosylated forms of the proteins.

Ad receptor and c-Kit expression in hematopoietic cell lines

We examined the expression of the major Ad receptors, CAR and integrin αv chain (CD51), on the surface of hematopoietic cells using flow cytometry (Figure 2). CAR was very weakly expressed in the human megakaryoblastic leukemia cell line M07e (1.1%) and in the human erythroleukemia cell line TF-1 (2.2%), faintly expressed in the human megakaryoblastic leukemia cell line UT-7 (19%), and moderately expressed in the human chronic myelogenous leukemia cell line K-562 (66%). In contrast, CD51 was abundantly expressed in all of these cell lines. We also analyzed c-Kit expression, and found that it was expressed in M07e (100%), UT-7 (80%), and TF-1 (60%) cells, respectively, but not in K-562 (<1%) cells. Since M07e cells weakly express CAR but highly express c-Kit, we used these cells to assess the ability of the CAR-SCF fusion proteins to enhance the efficiency of Ad5-mediated gene transfer.

Ad-mediated gene transfer to M07e cells

While M07e cells did not fluoresce following infection with Ad5-eGFP alone, addition of either CmS or ChS to the culture medium resulted in remarkable fluorescence (Figure 3A). The cells also fluoresced to a lesser extent when infected with Ad5/F35-eGFP, a same construct to Ad5-eGFP except for having the Ad5/35 chimeric fiber.

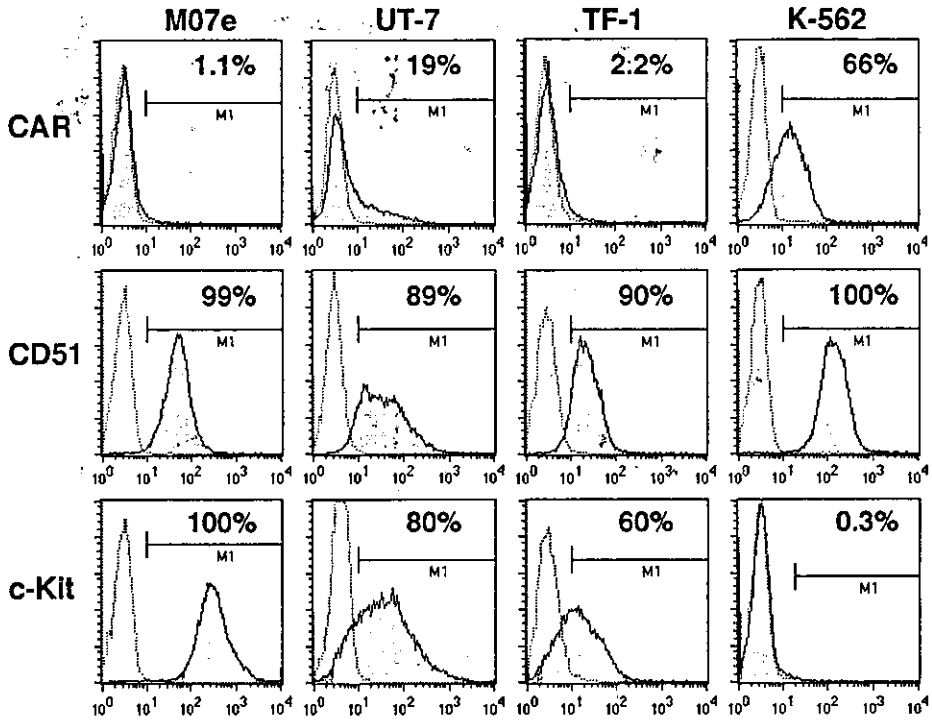

Figure 2. Expression of adenoviral receptors and c-Kit in hematopoietic cell lines. Cells were stained with monoclonal antibodies to human CAR (clone RmcB), CD51 (integrin αv chain, clone 13C2), or c-Kit (CD117, clone YB5.B8) and analyzed for their expression by flow cytometry. Dotted histograms show background staining obtained with isotype control antibodies

The expression of eGFP was quantitatively analyzed by flow cytometry (Figure 3B). The percentage of eGFP-positive M07e cells infected with Ad5-eGFP was comparable to the negative control (0.05%), even at an MOI of 100. In contrast, when 10 ng/ml of CmS were added along with Ad5-eGFP, more than 80% of the cells were eGFP-positive. Since ChS enhanced Ad5-mediated eGFP expression to a slightly weaker extent, we used CmS in later experiments. M07e cells infected with Ad5/F35-eGFP fluoresced at lower levels than those given by the combination of Ad5-eGFP and the CAR-SCF fusion proteins. In addition, the mean fluorescence intensity of eGFP-positive cells infected with Ad5-eGFP in the presence of the CAR-SCF fusion proteins was much higher than that of cells infected with Ad5/F35-eGFP.

Dose effect of CAR-SCF on Ad5-mediated gene expression

We investigated Ad5-mediated gene expression in the presence of various amounts of CmS. A CmS stock solution of approximately 10 μg/ml was serially diluted and added to each Ad5-eGFP infection culture of M07e, UT-7, TF-1, and K-562 cells. The percentage of eGFP-positive cells increased in proportion to the CmS concentration in c-Kit positive cell lines, M07e and UT-7 infected with Ad-5-eGFP at an MOI of 50. However, addition of a larger amount of CmS (100 ng/ml) reduced Ad-5-eGFP-mediated transduction of these cells (Figure 4A). These results suggest that CAR-SCF enhances Ad5-mediated gene expression in accordance with the level of c-Kit expression on the target cells, although an excess amount of CAR-SCF may act as a competitor which blocks the interaction between Ad5 and cells. In contrast, the percentage of eGFP-positive cells decreased as CmS concentration was increased in TF-1 and K-562 cell culture when these were infected with Ad-5-eGFP at an MOI of 50 (Figure 4B). We further investigated the effect of CmS concentration on Ad5-mediated transduction of M07e cells. Addition of 10–30 ng/ml of CmS was optimal for Ad5-eGFP infection of M07e cells at all MOIs assessed (Figure 4C). The highest fluorescence intensity of the eGFP-positive cells was observed with 30 ng/ml of CmS (Figure 4D). Therefore, the optimal dose of CmS may be dependent on the amount of the c-Kit expression on the target cells rather than the amount of the Ad5.

Comparison of CAR-SCF-assisted Ad5-mediated transduction and Ad5/F35-mediated transduction for gene transfer to hematopoietic cell lines

We compared CAR-SCF-assisted Ad5-mediated gene transfer to hematopoietic cell lines with that of Ad5/F35. Infection of UT-7 cells, which express CAR weakly (19%) and c-Kit moderately (80%; Figure 2), with Ad5-eGFP alone resulted in 30, 33, and 39% eGFP-positive cells at MOIs of 25, 50, and 100, respectively (Figure 5).

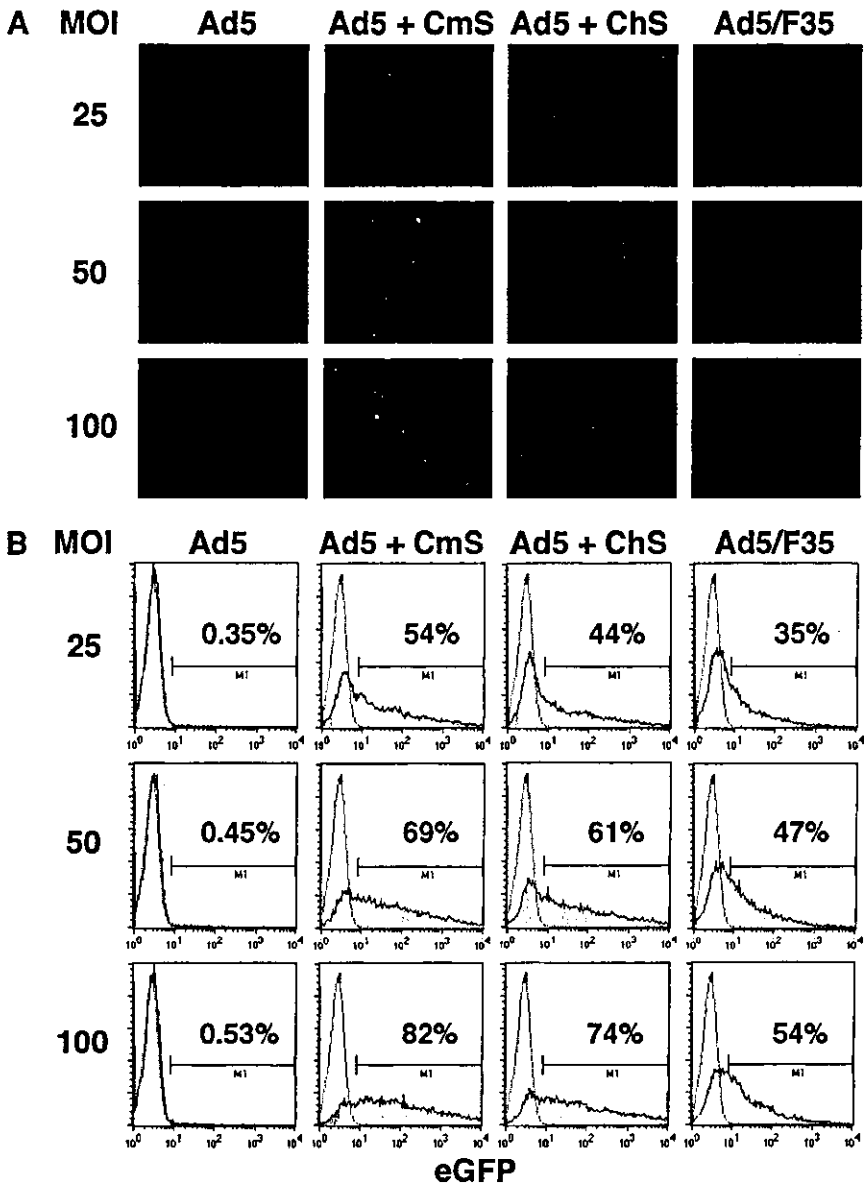

Figure 3. Ad-mediated eGFP gene transfer to M07e cells. CmS or ChS condensed solution (10 μg/ml, final 10 ng/ml) was added to M07e cell culture. The culture was left for 10 min at room temperature, and concentrated Ad5-eGFP or Ad5/F35-eGFP vector was added at MOIs of 25, 50, and 100. Twenty-four hours after the incubation, eGFP-positive cells were examined under a fluorescent microscope (A) and analyzed for their eGFP expression by flow cytometry (B)

Addition of CmS to the culture medium increased the percentage of eGFP-positive cells to 59, 65, and 70%, respectively. Infection of UT-7 cells with Ad5/F35-eGFP resulted in larger fractions of eGFP-positive cells: 82, 93, and 96% at MOIs of 25, 50, and 100, respectively. Interestingly, the mean fluorescence intensity of eGFP-positive UT-7 cells infected with Ad5-eGFP in the presence of CmS was much higher than that of cells infected with either Ad5-eGFP alone or with Ad5/F35-eGFP. The mean fluorescence intensity of eGFP-positive UT-7 cells infected with Ad5/F35-eGFP was comparable to that of UT-7 cells infected with Ad5-eGFP alone.

TF-1 cells very weakly express CAR (2.2%) and moderately express c-Kit (60%; Figure 2). Ad5-eGFP infection of these cells resulted in 24, 38, and 50% eGFP-positive cells at MOIs of 25, 50, and 100, respectively. Addition of CmS to the culture medium increased these percentages to 42, 52, and 65%, respectively. Ad5/F35-eGFP was more efficient at infecting TF-1 cells, with 96, 99, and 99% eGFP-positive cells at MOIs of 25, 50, and 100, respectively. The highest mean fluorescence intensity of eGFP-positive TF-1 cells occurred with Ad5/F35-eGFP infection.

K-562 cells express CAR moderately (66%) but very weakly express c-Kit (0.3%; Figure 2). Ad5-eGFP infection of these cells resulted in 54, 70, and 82% eGFP-positive cells at MOIs of 25, 50, and 100, respectively. Addition of CmS did not increase the efficiency of Ad5-eGFP infection.

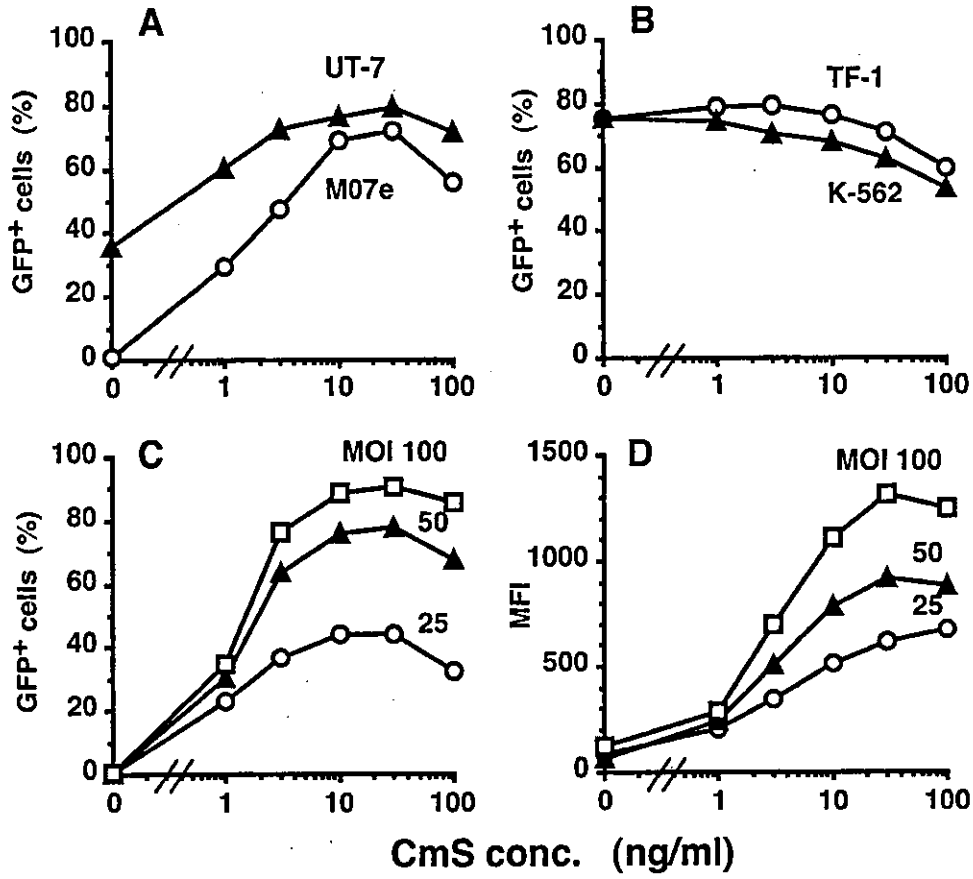

Figure 4. Dependency on CAR-SCF dose for Ad5-mediated gene transfer to hematopoietic cell lines. CmS (0, 1, 3, 10, 30 or 100 ng/ml) was added in M07e, UT-7, TF-1, or K-562 cell culture before infection with Ad5-eGFP at an MOI of 50 (A, B). Twenty-four hours after the treatment, cells were analyzed for eGFP expression by flow cytometry. Percentage of eGFP-positive cells in c-Kit-positive cell lines, M07e and UT-7 (A); c-Kit weak positive cell line TF-1 and c-Kit-negative cell line K-562 (B). M07e cells were transfected by Ad5-eGFP at MOIs of 25, 50 and 100 in the presence of various concentration of CmS (C, D). Percentage of eGFP-positive cells (C) and mean fluorescence intensity (MFI) of eGFP-positive cells (D)

Infection with Ad5/F35-eGFP resulted in 92, 96, and 98% eGFP-positive K-562 cells, respectively.

Taken together, these results suggest that enhancement of Ad5-mediated gene transfer and transgene expression by CmS would be expected to depend on the relative levels of CAR and c-Kit on the target cells. In contrast, infection with Ad5/F35 may be independent of CAR and c-Kit expression of cells.

Commitment of CAR-SCF-assisted Ad5 infection of M07e cells to c-Kit

To confirm that CAR-SCF mediates adenoviral infection by binding to c-Kit, we used soluble mouse SCF which competes with CAR-SCF for binding to cellular c-Kit or a monoclonal antibody RmcB specific for human CAR to block the interaction between CAR and Ad5. Before infection of M07e cells with Ad5-eGFP at an MOI of 100, various concentrations of mouse SCF or RmcB were added to the M07e culture in the presence of 3 or 30 ng/ml of CmS. Percentages of eGFP-positive cells decreased in response to the increasing amount of the mouse SCF (Figure 6A) or RmcB (Figure 6B). These results suggest that both affinity domains of CAR-SCF participate in linking Ad5 fiber knob and c-Kit protein during Ad5 infection.

Effect of SCF on CAR-SCF-assisted Ad-mediated gene transfer

The CAR-SCF fusion proteins theoretically mediate the interaction between the Ad5 fiber and cellular c-Kit. However, the SCF portion of the fusion proteins alone may also increase transgene expression via activation of the c-Kit signaling pathway. To support this explanation, 30 ng/ml of mouse SCF instead of CmS were added to M07e cell culture before infection of Ad5-eGFP or Ad5/F35-eGFP at MOIs of 25, 50, or 100. As described above, addition of CmS to the culture medium of M07e cells with Ad5-eGFP significantly increased both the percentage of eGFP-positive cells and the mean fluorescence intensity. However, addition of the same molar concentration of mouse SCF alone also slightly increased the eGFP-positive fraction ($p < 0.001$; Figure 7A, left). Although M07e cells infected with Ad5/F35-eGFP fluoresced at lower levels than those infected by a combination of Ad5-eGFP and the CAR-SCF

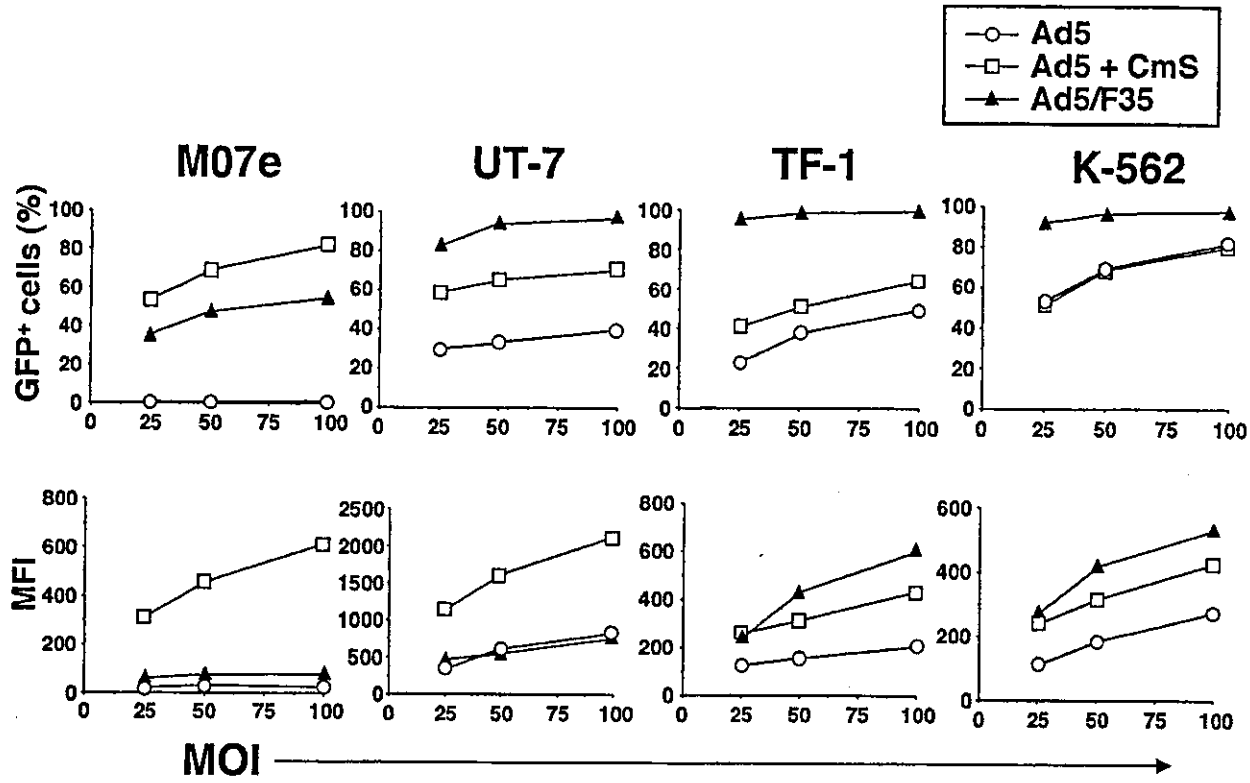

Figure 5. Comparison of CAR-SCF-assisted Ad5 with Ad5/F35 for gene transfer to hematopoietic cell lines. M07e, UT-7, TF-1, and K-562 cell cultures were transfected by Ad5-eGFP, Ad5-eGFP plus 10 ng/ml of CmS, or Ad5/F35-eGFP, at MOIs of 25, 50 or 100. Twenty-four hours after the infection, the eGFP-positive cells were analyzed by flow cytometry. Percentage of eGFP-positive cells (upper) and mean fluorescence intensity (MFI) of the eGFP-positive cells (lower) are shown

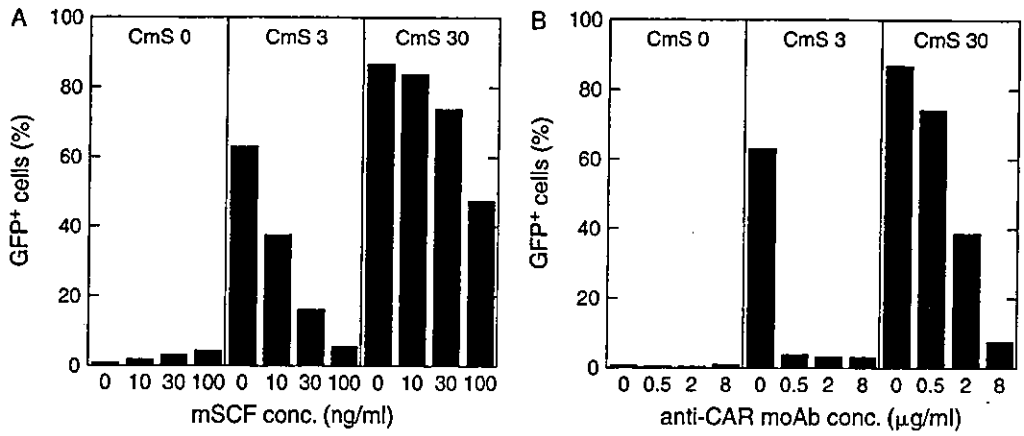

Figure 6. Blocking examination of CAR-SCF-assisted Ad5 gene transfer to M07e cells. As inhibitors for CAR-SCF fusion protein, soluble mouse SCF (A) or an anti-CAR monoclonal antibody RmcB (B) was added to M07e cell culture in the absence or presence of CmS (3 or 30 ng/ml) before infection with Ad5-eGFP at an MOI of 100. Twenty-four hours after the infection, the eGFP-positive cells were analyzed by flow cytometry

fusion proteins, treatment with CmS or mouse SCF alone significantly enhanced the gene transduction efficiency of Ad5/F35-eGFP (Figure 7A, right). Similar results were obtained in the study with UT-7 cells (Figure 7B). Taken together, these results suggest that CAR-SCF assists Ad5 gene transfer primarily by linking the Ad5 fiber and cellular c-Kit and also by other mechanisms such as activating the c-Kit intracellular signaling pathway.

Ad-mediated gene transfer to human primary CD34$^+$ cells

To test whether the CAR-SCF fusion protein improves Ad-mediated gene transfer to primary immature hematopoietic cells, human CD34$^+$ cells isolated from cord blood (n = 3) and bone marrow (n = 1) were used as targets. The cells were pre-cultured in the presence of multiple

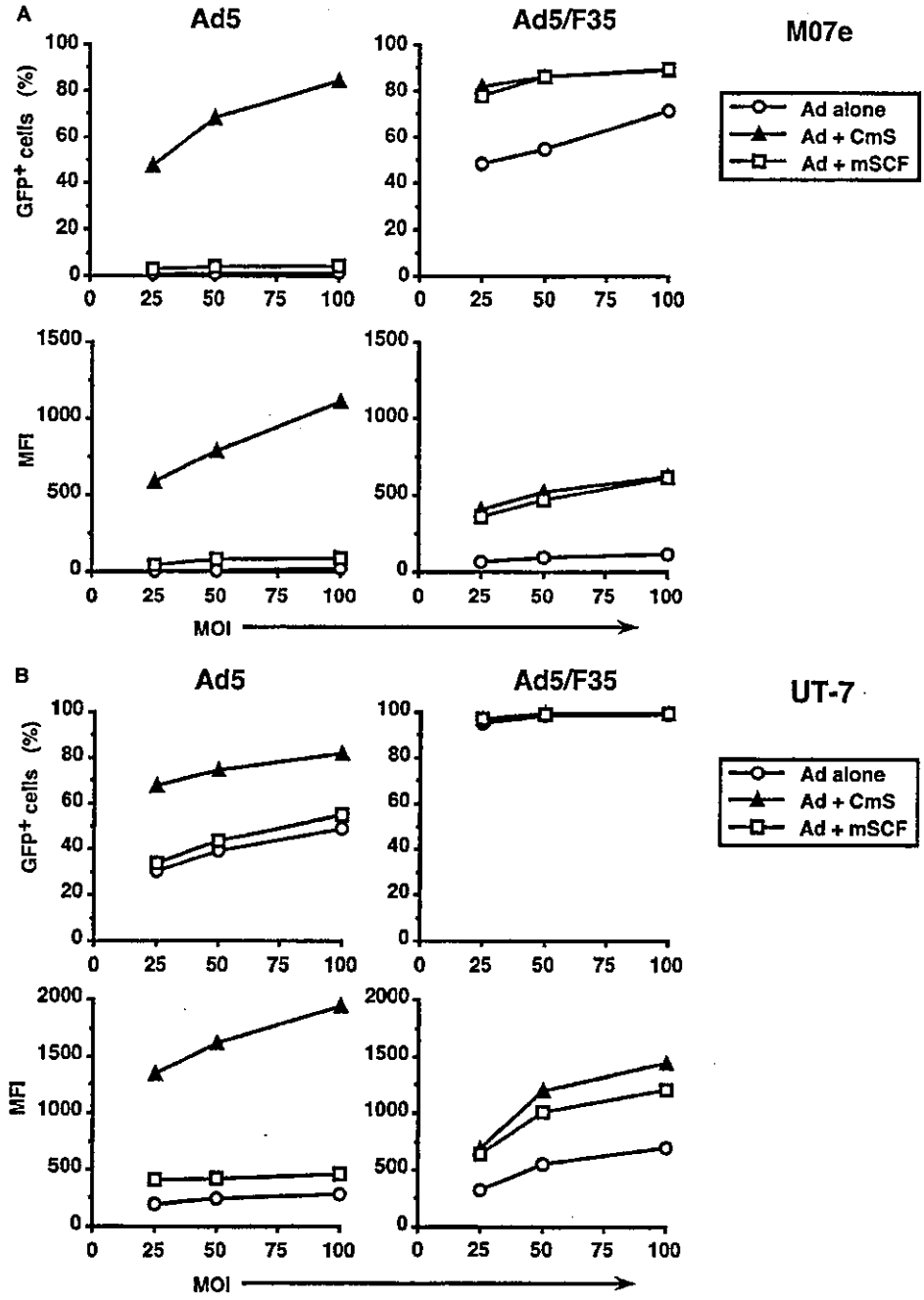

Figure 7. The effect of SCF on Ad-mediated gene transfer to c-Kit-positive cells. Soluble mouse SCF or CmS at the same molar concentration (final 30 ng/ml of mouse SCF equivalent) was added to M07e (A) or UT-7 (B) cell culture before infection with Ad5-eGFP or Ad5/F35-eGFP at MOIs of 25, 50 and 100. Twenty-four hours after the infection, eGFP-positive cells were analyzed by flow cytometry. Percentages of the eGFP-positive cells (upper) and the mean fluorescence intensity (MFI) of eGFP-positive cells (lower) are shown. Experiments at an MOI of 100 were performed in triplicate and the values represent the means. As the standard deviations were very small, the error bars disappeared under the symbols. Statistical analysis with Student's *t*-test indicated that *p* values of Ad alone vs. Ad plus CmS and Ad alone vs. Ad plus mSCF were less than 0.001 in both cell lines

cytokines (Flt-3 ligand/thrombopoietin or SCF/Flt-3 ligand/thrombopoietin) for 2 or 3 days and incubated with Ad5-eGFP or Ad5/F35-eGFP at MOIs of 50 to 100 in the presence or absence of 30 ng/ml CmS for 2 days. Representative data are shown in Figure 8. While Ad5-eGFP alone weakly infected the $CD34^+$ cells, addition of CAR-SCF doubled the infection efficiency. The Ad5/F35 vector was shown to have similar infectivity to Ad5-eGFP vector with CmS. Staining the cells with anti-CD51 antibody indicated that human primary $CD34^+$ cells expressed αv integrins at lower level (approximately 30%) than leukemic cell lines. The $CD51^+$ cells were infected with both Ad5 and Ad5/F35 vectors (Figure 8, lower panels). When we took into account varying conditions, such as different combinations of cytokines, pre-incubation time, cord blood lot, and MOI, we observed that CAR-SCF

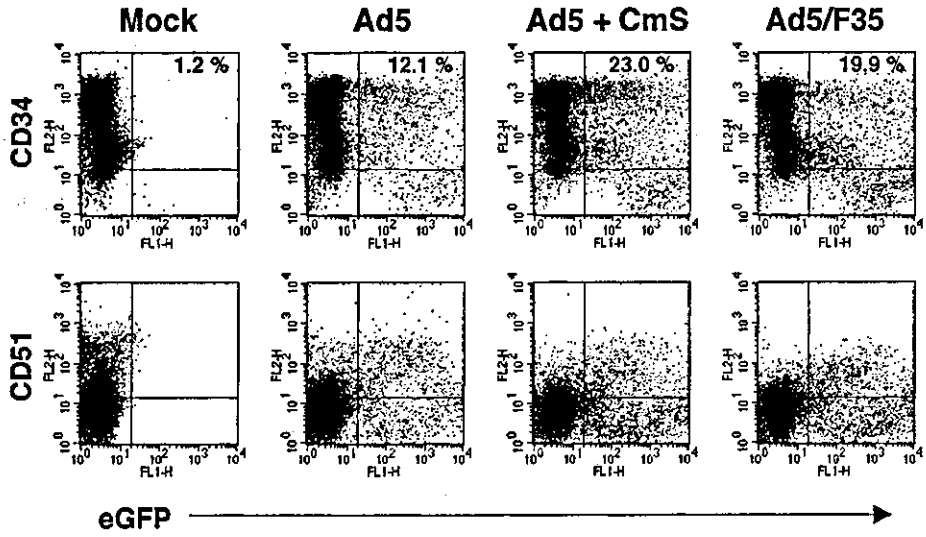

Figure 8. Adenoviral gene transfer to human primary CD34$^+$ cells. Cryopreserved human bone marrow CD34$^+$ cells were pre-stimulated with Flt-3 ligand (FL) and thrombopoietin (TPO, 100 ng/ml each) for 2 days, and then infected with Ad5-eGFP or Ad5/F35-eGFP at an MOI of 100 in the presence of SCF/FL/TPO (Ad5 alone), CmS/FL/TPO (Ad5 plus CmS), or SCF/FL/TPO (Ad5/F35) for 2 days. Cells were stained with a phycoerythrin-labeled monoclonal antibody against human CD34 or CD51, and then expression profile of CD34, CD51 and eGFP of the cells were analyzed by flow cytometry

Table 1. Adenovirus-mediated gene transfer to human primary CD34$^+$ cells

Fraction	Parameter	Mock	Ad5	Ad5 + CmS	Ad5/F35
Whole cells	% GFP$^+$	1.19 ± 0.14	10.8 ± 3.7[a]	19.1 ± 3.5[a]	18.5 ± 9.4
	MFI		660	1459	1307
CD34$^+$ cells	% GFP$^+$	1.19 ± 0.26	6.45 ± 2.93	13.2 ± 5.6	10.4 ± 4.8
	MFI		666	1937	2629

Fresh isolated or cryopreserved human CD34$^+$ cells were preincubated with combination of cytokines, SCF, Flt-3 ligand, and thrombopoietin or Flt-3 ligand and thrombopoietin for 2 or 3 days. The cells were infected by Ad5-eGFP or Ad5/F35-eGFP at an MOI of 50 or 100 either alone or 30 ng/ml of CmS for 2 days. MFI: representative value of mean fluorescence intensity. Data were indicated as mean ± SD ($n = 4$), and analyzed with Student's *t*-test.
[a] $p < 0.05$.

reproducibly enhanced Ad5-mediated gene transfer to CD34$^+$ cells (Table 1).

Discussion

In this study, we developed a CAR-SCF fusion protein that allows an Ad5 vector to infect immature hematopoietic cells expressing c-Kit. We found that CAR-SCF remarkably enhanced Ad5-mediated gene transfer to c-Kit$^+$ CAR$^-$ CD51$^+$ M07e cells, and that this enhancement was dependent on the relative expression levels of CAR and c-Kit on the target cells. The CAR-SCF fusion protein also assisted Ad5-mediated gene transfer to human primary CD34$^+$ cells.

Previous studies have shown that avidin/biotin-mediated SCF conjugation to Ad5 vectors can efficiently improve gene transfer to M07e cells [15–17]. Our data demonstrate that a soluble form of the adapter can also sufficiently improve gene transfer, indicating that the affinity of the Ad5 fiber for CAR is stable and potent enough for CAR fusion proteins to link Ad5 with cells.

Other studies have shown that a bispecific single-chain antibody (scFv) with affinity for both Ad5 and a cell-specific antigen can be used to link Ad5 to target cells [30–32]. A fusion protein consisting of CAR and scFv to c-erbB-2 and another fusion protein of CAR and scFv to CD40 have also been reported to retarget Ad to tumor cells and dendritic cells, respectively [33,34]. Furthermore, trimerization of a CAR fusion protein can strengthen the affinity of the Ad5 fiber for the target cell [35]. As an alternative to CAR fusion proteins, which target Ad vectors to undifferentiated hematopoietic cells, other molecules such as CD34 and AC133 that are expressed on specific cell types are potential candidates for fusion proteins. Even if the targeting molecule ligand is unknown, scFv can be used for target binding. However, the CAR-SCF fusion protein has an advantage over CAR-scFv in that it activates the c-Kit signaling pathway, which is necessary for maintenance and growth of immature hematopoietic cells *in vitro*.

We observed that while M07e and TF-1 cells have a similar expression profile of the adenoviral receptors CAR and αv integrins, their susceptibility to Ad5 infection

was considerably different. This may be due to different expression levels of other coreceptors such as heparan sulfate glycosaminoglycans [36] and dynamin [37].

The CAR-SCF fusion protein also improved Ad5-mediated gene transfer to human primary CD34$^+$ cells. Whereas more than 80% of CD34$^+$ cells expressed c-Kit (data not shown), approximately 20% of the cells could be infected with the Ad5-eGFP vector in the presence of CAR-SCF. In addition to reduced CAR expression, we found that CD34$^+$ cells express αv integrins at weak levels (6–49% positive cells), suggesting that αv integrin dependent Ad internalization following attachment to the cell may be rate-limiting in CD34$^+$ cells. Ad5/F35-eGFP vector also infected CD34$^+$ cells at a similar extent to that of the Ad5-eGFP vector in the presence of CAR-SCF. As the Ad5/F35 vector has Ad5-derived penton bases, Ad5/F35 is also supposed to utilize αv integrins for internalization. The addition of certain reagents such as histone deacetylase inhibitor [38] and a mixture of cytokines [39] can upregulate the expression of αv integrins. Combination of such reagents and the CAR-SCF fusion proteins may further improve Ad5-mediated gene transfer to CD34$^+$ cells.

CAR has two immunoglobulin-like domains, D1 and D2, in the extracellular domain, and D1 alone can bind to the adenoviral fiber [40]. Using just D1 instead of the full-length extracellular domain in the fusion protein should compact the protein for more efficient protein production.

In conclusion, the CAR-SCF fusion protein facilitates retargeting of adenoviral vectors to c-Kit$^+$ human immature hematopoietic cells, including subsets with potential stem cell capacity. The simplicity and utility of this type of fusion protein allows not only easy transduction of Ad5-mediated gene transduction, but also retargeting of the vector to hematopoietic cells without any modification of the huge Ad5-based vector stocks. These findings should prove valuable for the development of efficient gene delivery to hematopoietic cells.

Acknowledgements

This study was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labor and Welfare of Japan.

References

1. Marini FC, Yu Q, Wickham T, *et al.* Adenovirus as a gene therapy vector for hematopoietic cells. *Cancer Gene Ther* 2000; **7**: 816–825.
2. Rebel VI, Hartnett S, Denham J, *et al.* Maturation and lineage-specific expression of the Coxsackie and adenovirus receptor in hematopoietic cells. *Stem Cells* 2000; **18**: 176–182.
3. Bergelson JM, Cunningham JA, Droguett G, *et al.* Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 1997; **275**: 1320–1323.
4. Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ promote adenovirus internalization but not virus attachment. *Cell* 1993; **73**: 309–319.
5. Cohen CJ, Shieh JTC, Pickles RJ, *et al.* The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc Natl Acad Sci U S A* 2001; **98**: 15 191–15 196.
6. Shayakhmetov DM, Papayannopoulou T, Stamatoyannopoulos G, Lieber A. Efficient gene transfer into human CD34$^+$ cells by a retargeted adenovirus vector. *J Virol* 2000; **74**: 2567–2583.
7. Stecher H, Shayakhmetov DM, Stamatoyannopoulos G, Lieber A. A capsid-modified adenovirus vector devoid of all viral genes: assessment of transduction and toxicity in human hematopoietic cells. *Mol Ther* 2001; **4**: 36–44.
8. Knaän-Shanzer S, van der Velde I, Havenga MJE, *et al.* Highly efficient targeted transduction of undifferentiated human hematopoietic cells by adenoviral vectors displaying fiber knobs of subgroup B. *Hum Gene Ther* 2001; **12**: 1989–2005.
9. Yotnda P, Onishi H, Heslop HE, *et al.* Efficient infection of primitive hematopoietic stem cells by modified adenovirus. *Gene Ther* 2001; **8**: 930–937.
10. Roelvink PW, Lizonova A, Lee JG, *et al.* The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J Virol* 1998; **72**: 7909–7915.
11. Havenga MJ, Lemckert AA, Ophorst OJ, *et al.* Exploiting the natural diversity in adenovirus tropism for therapy and prevention of disease. *J Virol* 2002; **76**: 4612–4620.
12. Gonzalez R, Vereecque R, Wickham TJ, *et al.* Increased gene transfer in acute myeloid leukemic cells by an adenovirus vector containing a modified fiber protein. *Gene Ther* 1999; **6**: 314–320.
13. Dmitriev I, Krasnykh V, Miller CR, *et al.* An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol* 1998; **72**: 9706–9713.
14. Garcia-Castro J, Segovia JC, Garcia-Sanchez F, *et al.* Selective transduction of murine myelomonocytic leukemia cells (WEHI-3B) with regular and RGD-adenoviral vectors. *Mol Ther* 2001; **3**: 70–77.
15. Schwarzenberger P, Spence SE, Gooya JM, *et al.* Targeted gene transfer to human hematopoietic progenitor cell lines through the c-kit receptor. *Blood* 1996; **87**: 472–478.
16. Smith JS, Keller JR, Lohrey NC, *et al.* Redirected infection of directly biotinylated recombinant adenovirus vectors through cell surface receptors and antigens. *Proc Natl Acad Sci U S A* 1999; **96**: 8855–8860.
17. Zhong Q, Oliver P, Huang W, *et al.* Efficient c-kit receptor-targeted gene transfer to primary human CD34-selected hematopoietic stem cells. *J Virol* 2001; **75**: 10 393–10 400.
18. Dmitriev I, Kashentseva E, Rogers BE, *et al.* Ectodomain of coxsackievirus and adenovirus receptor genetically fused to epidermal growth factor mediates adenovirus targeting to epidermal growth factor receptor-positive cells. *J Virol* 2000; **74**: 6875–6884.
19. Ebbinghaus C, Al-Jaibaji A, Operschall E, *et al.* Functional and selective targeting of adenovirus to high-affinity Fcγ receptor I-positive cells by using a bispecific hybrid adapter. *J Virol* 2001; **75**: 480–489.
20. Komatsu N, Nakauchi H, Miwa A, *et al.* Establishment and characterization of a human leukemic cell line with megakaryocytic features: dependency on granulocyte-macrophage colony-stimulating factor, interleukin 3, or erythropoietin for growth and survival. *Cancer Res* 1991; **51**: 341–348.
21. Brizzi MF, Avanzi GC, Veglia F, *et al.* Expression and modulation of IL-3 and GM-CSF receptors in human growth factor dependent leukaemic cells. *Br J Haematol* 1990; **76**: 203–239.
22. Kitamura T, Tojo A, Kuwaki T, *et al.* Identification and analysis of human erythropoietin receptors on a factor-dependent cell line, TF-1. *Blood* 1989; **73**: 375–380.
23. Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci U S A* 1993; **90**: 8392–8396.
24. IMAGE. Available: http://image.llnl.gov/.
25. Mizuguchi H, Hayakawa T. Adenovirus vectors containing chimeric type 5 and type 35 fiber proteins exhibit altered and expanded tropism and increase the size limit of foreign genes. *Gene* 2002; **285**: 69–77.
26. Mizuguchi H, Kay MA. A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum Gene Ther* 1999; **10**: 2013–2017.
27. Okada T, Ramsey WJ, Munir J, *et al.* Efficient directional cloning of recombinant adenovirus vectors using DNA-protein complex. *Nucleic Acids Res* 1998; **26**: 1947–1950.

28. Maizel JV, White DO, Scharff MD. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 1968; 36: 115–125.
29. Kanegae Y, Makimura M, Saito I. A simple and efficient method for purification of infectious recombinant adenovirus. *Jpn J Med Sci Biol* 1994; 47: 157–166.
30. Haisma HJ, Grill J, Curiel DT, *et al.* Targeting of adenoviral vectors through a bispecific single-chain antibody. *Cancer Gene Ther* 2000; 7: 901–904.
31. van Beusechem VW, Grill J, Mastenbroek DC, *et al.* Efficient and selective gene transfer into primary human brain tumors by using single-chain antibody-targeted adenoviral vectors with native tropism abolished. *J Virol* 2002; 76: 2753–2762.
32. Nettelbeck DM, Miller DW, Jerome V, *et al.* Targeting of adenovirus to endothelial cells by a bispecific single-chain diabody directed against the adenovirus fiber knob domain and human endoglin (CD105). *Mol Ther* 2001; 3: 882–891.
33. Kashentseva EA, Seki T, Curiel DT, Dmitriev IP. Adenovirus targeting to c-erbB-2 oncoprotein by single-chain antibody fused to trimeric form of adenovirus receptor ectodomain. *Cancer Res* 2002; 62: 609–616.
34. Pereboev AV, Asiedu CK, Kawakami Y, *et al.* Coxsackievirus-adenovirus receptor genetically fused to anti-human CD40 scFv enhances adenoviral transduction of dendritic cells. *Gene Ther* 2002; 9: 1189–1193.
35. Kim J, Smith T, Idamakanti N, *et al.* Targeting adenoviral vectors by using the extracellular domain of the coxsackie-adenovirus receptor: improved potency via trimerization. *J Virol* 2002; 76: 1892–1903.
36. Dechecchi MC, Melotti P, Bonizzato A, *et al.* Heparan sulfate glycosaminoglycans are receptors sufficient to mediate the initial binding of adenovirus types 2 and 5. *J Virol* 2001; 75: 8772–8780.
37. Wang K, Huang S, Kapoor-Munshi A, Nemerow G. Adenovirus internalization and infection require dynamin. *J Virol* 1998; 72: 3455–3458.
38. Kitazono M, Rao VK, Robey R, *et al.* Histone deacetylase inhibitor FR901228 enhances adenovirus infection of hematopoietic cells. *Blood* 2002; 99: 2248–2251.
39. Takahashi T, Yamada K, Tanaka T, *et al.* A potential molecular approach to ex vivo hematopoietic expansion with recombinant epidermal growth factor receptor-expressing adenovirus vector. *Blood* 1998; 91: 4509–4515.
40. Freimuth P, Springer K, Berard C, *et al.* Coxsackievirus and adenovirus receptor amino-terminal immunoglobulin V-related domain binds adenovirus type 2 and fiber knob from adenovirus type 12. *J Virol* 1999; 73: 1392–1398.

THE JOURNAL OF GENE MEDICINE
J Gene Med 2003; 5: 921–928.
Published online 20 June 2003 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jgm.431

RESEARCH ARTICLE

Efficient gene transfer of a simian immunodeficiency viral vector into cardiomyocytes derived from primate embryonic stem cells

Mihoko Nagata,[1] Masafumi Takahashi,[2,4]* Shin-ichi Muramatsu,[1]* Yasuji Ueda,[7] Yutaka Hanazono,[5] Koichi Takeuchi,[6] Koji Okada,[3] Yutaka Suzuki,[8] Yasushi Kondo,[8] Masafumi Suemori,[9] Uichi Ikeda,[2] Imaharu Nakano,[1] Eiji Kobayashi,[4] Mamoru Hasegawa,[7] Keiya Ozawa,[5] Norio Nakatsuji[9,10] and Kazuyuki Shimada[2]

[1]*Division of Neurology, Department of Medicine, Jichi Medical School, Tochigi 329-0498, Japan;* [2]*Division of Cardiology, Department of Medicine, Jichi Medical School, Tochigi 329-0498, Japan;* [3]*Division of Endocrinology, Department of Medicine, Jichi Medical School, Tochigi 329-0498, Japan;* [4]*Division of Organ Replacement Research, Center for Molecular Medicine, Jichi Medical School, Tochigi 329-0498, Japan;* [5]*Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi 329-0498, Japan;* [6]*Department of Anatomy, Jichi Medical School, Tochigi 329-0498, Japan;* [7]*DNAVEC Research, Inc., Ibaraki 305-0856, Japan;* [8]*Tanabe Seiyaku Co., Ltd., Osaka 532-8505, Japan;* [9]*Department of Development and Differentiation, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan;* [10]*Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan*

*Correspondence to: Masafumi Takahashi or Shin-ichi Muramatsu, Division of Organ Replacement Research or Division of Neurology, Jichi Medical School, Minamikawachi-machi, Tochigi 329-0498, Japan. E-mail: masafumi@jichi.ac.jp or muramats@jichi.ac.jp

Received: 18 December 2002
Revised: 7 April 2003
Accepted: 5 May 2003

Abstract

Background Embryonic stem (ES) cells continually proliferate and can generate large numbers of differentiated cells. Genetic manipulation of transplantable cells derived from primate ES cells offers considerable potential for development research and regenerative cell therapy. However, protocols for efficient gene transfer into primate ES-cell-derived cells have not yet been established.

Methods Spontaneously contracting areas were derived from cynomolgus monkey ES cells. Features of cardiomyocytes in the area were analyzed according to gene expression (RT-PCR), morphology (immunostaining and electron microscopy), and function (intracellular calcium transience). Beating cells were transduced using a simian immunodeficiency virus (SIV) vector expressing enhanced green fluorescence protein (EGFP), then transplanted into ischemic rat myocardium.

Results Beating cells derived from monkey ES cells displayed gene expression, ultrastructural and functional properties of early-stage cardiomyocytes. Highly efficient (97% cardiac phenotype) and stable transduction of these ES-cell-derived cardiomyocytes was achieved using SIV vector without altering contractile function. In addition, transduced cardiomyocytes survived in the myocardium of a rat myocardial infarction model.

Conclusions A lentiviral vector system based on SIV represents a useful vehicle for genetic modification of cardiomyocytes derived from primate ES cells, and can extend the application of primate ES cells to gene therapy.

Keywords embryonic stem cell; cardiomyocyte; simian immunodeficiency virus; cell transplantation; myocardial infarction

Introduction

The generation of various differentiated cells from pluripotent embryonic stem (ES) cells provides a renewable resource not only for studying the mechanism of early development *in vitro*, but also for cell transplantation therapy. Among many specialized cells in adults, cardiomyocytes are terminally differentiated and have no or only limited regenerative capacity after injury such as myocardial infarction [1]. Thus, the transplantation of functional cardiomyocytes into the damaged myocardium would have therapeutic potential. Recent studies have demonstrated that human ES cells can differentiate into cardiomyocytes with structural and functional properties *in vitro* [2–5]. Although human ES cells hold promise for clinical applications, an alternative model system based on ES cells derived from

experimental animals may be necessary for pre-clinical studies, including allogenic transplantation. We established cynomolgus monkey (*Macaca fascicularis*) ES cell lines [6] that are similar to human ES cells but distinct from murine ES cells in terms of morphology, expression of surface markers, feeder- and leukemia inhibitory factor-dependence and other factors. These features indicate that cynomolgus ES cells represent a suitable pre-clinical model for cell transplantation therapy.

Gene transfer into transplantable cells has potential to enhance the effects of cell replacement therapy. Although murine ES cells can be transduced by electroporation or mouse stem cell virus (MSCV)-based retroviral vectors [7–9], primate ES cells are not efficiently transduced by these methods [10]. Lentiviral vectors can transduce both dividing and non-dividing cells and long-term expression of the transgene is stable in a wide range of target cells [11–13]. We have described the highly efficient transfer of a gene into cynomolgus monkey undifferentiated ES cells using a lentivirus vector based on simian immunodeficiency virus (SIV) [14].

The present study examines the differentiation of cynomolgus ES cells into functional cardiomyocytes and determines the efficiency and stability of gene transduction into these cardiomyocytes using an SIV-based lentiviral vector encoding the enhanced green fluorescence protein (EGFP) gene. We also evaluate the survival of transplanted cardiomyocytes derived from cynomolgus ES cells in the injured myocardium of a rat myocardial infarction model.

Materials and methods

Cell preparations

The cynomolgus monkey ES cell line CMK6 [6] was cultured in DMEM/F12 (Sigma, St. Louis, USA) on a mouse embryonic fibroblast feeder layer that was mitotically inactivated with mitomycin C (Kyowa, Tokyo, Japan). The medium was supplemented with 0.1 mM 2-mercaptoethanol (Sigma), 2 mM glutamine (Invitrogen, Carlsbad, USA), 1 mM sodium pyruvate (Invitrogen) and 15% fetal bovine serum (FBS, ICN Biomedicals, Inc., Ohio, USA). To induce differentiation, ES cells were dispersed into small clumps using collagenase IV (Wako, Osaka, Japan), transferred to plastic Petri dishes and suspension-cultured for 10 days. During this period, the cells aggregated to form embryoid bodies (EBs), which were then plated on plastic plates, and the appearance of spontaneous contractions was observed under a microscope. Rat neonatal cardiomyocytes were prepared from cardiac ventricles of 1-day-old Sprague-Dawley rats as described previously [15]. The cells were grown in DMEM (Sigma) supplemented with 10% FBS (ICN Biomedicals, Inc.), and 1% penicillin/streptomycin solution (Invitrogen). All experiments were carried out in full compliance with the institutional animal care and use committee of the Jichi Medical School.

RT-PCR

Total RNA from undifferentiated ES cells, contracting EBs, and heart tissue of an adult cynomolgus monkey that was killed for unrelated reasons was extracted using a RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Complementary DNA synthesized from 1 μg total RNA using SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) was amplified by PCR using the following primers selective for human cardiac genes (oligonucleotide sequences are given in brackets in the order of anti-sense, sense primer followed by the annealing temperature, cycles used for PCR and length of the amplified fragment): cardiac troponin T (cTnT, 5′-GGCAGCGGAAGAGGATGCTGAA and 5′-GAGGCACCAAGTTGGGCATGAACGA; 60 °C; 35 cycles; 150 bp), atrial myosin light chain (MLC-2A, 5′-ACAGAGTTTATTGAGGTGCCCC and 5′-AAGGTGAAGTG-TCCCAGAGG; 61 °C; 35 cycles; 381 bp), ventricular myosin light chain (MLC-2V, 5′-TATTGGAACATGGCCTC-TGGAT and 5′-GGTGCTGAAGGCTGATTACGTT; 61 °C; 35 cycles; 382 bp), α-myosin heavy chain (αMHC, 5′-GTCATTGCTGAAACCGAGAATG and 5′-GCAAAGTACTG-GATGACACGCT; 61 °C; 40 cycles; 413 bp), octamer-binding protein 4 (Oct-4, 5′-GAGAACAATGAGAACCTTC-AGGAGA and 5′-TTCTGGCGCCGGTTACAGAACCA; 55 °C; 35 cycles; 219 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 5′-ATGCCAGTGAGCTTCCCGTT and 5′-CATCACCATCTTCCAGGAGC; 58 °C; 30 cycles; 473 bp).

Electron microscopy

For transmission electron microscopy, tissues were fixed in 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, at 4 °C for 24 h, postfixed in 1% OsO_4 in PBS for 1 h, dehydrated in a graded ethanol series and embedded in Epon 812. Thin (60–90 nm) sections were stained with uranyl acetate and lead citrate, and observed using a JEM-2000EX transmission electron microscope operating at 80 kV.

Intracellular calcium transience

Intracellular calcium transience of the EBs and rat neonatal cardiomyocytes was measured as described previously [16]. Briefly, cells were loaded with fura-2-AM (Dojin Biochemicals, Kumamoto, Japan), washed and transferred to the chamber of a fluorescence spectrophotometer (CAF-100; Japan Spectroscopic Co., Tokyo, Japan). Fura-2 fluorescence was measured using a dual wavelength system. Fluorescence was monitored at 500 nm, with excitation at 340 and 380 nm in the ratio mode. After achieving a stable fluorescence signal, the cells were stimulated with 100 nM angiotensin II (Sigma) or endothelin-1 (Peptide Institute Inc., Osaka, Japan). The cells pretreated with [Sar^1,Ile^8]-angiotensin II (non-selective antagonist; Peptide Institute Inc.) or CV-11 974

(angiotensin II type 1 receptor antagonist; kind gift from Takeda Chemical Industries, Ltd., Osaka, Japan) were also stimulated with angiotensin II.

Immunohistochemical staining

Contracting areas in EBs were mechanically dissected using a sterile micropipette. The samples were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) containing 8% sucrose at 4 °C for 4 h, washed with PBS containing 10, 20, and 30% sucrose in that order, embedded in OCT compound (Miles Laboratory, IN, USA), frozen in liquid nitrogen and cut into thin (8–10 μm) sections. We incubated the sections with monoclonal antibodies against human cardiac troponin I (cTnI) and cardiac myosin (both from Biogenesis, England, UK; diluted 1 : 200) for 1 h at room temperature. These anti-human antibodies cross-react against cynomolgus monkey cardiomyocytes but not against rat cardiomyocytes. Texas red labelled anti mouse IgG (Vector, Burlingame, CA, USA) was the secondary antibody. Samples were fixed in 4% paraformaldehyde in PBS for 20 min and immersed in 4′,6-diamidino-2-phenylindole (DAPI, 500 ng/ml; Sigma) containing Tris buffer (pH 7.4) for 10 min at room temperature to stain nuclei.

Vector construction and transduction

The SIV vector expressing the EGFP gene (Clontech, CA, USA) was produced by transient transfection into 293T cells as described [17]. Briefly, the envelope plasmid (pVSV-G; Clontech) encoding the vesicular stomatitis virus G (VSV-G) protein, the packaging plasmid (pCAGGS/Sagm-gtr), and the vector plasmid (pBS/CG2-Rc/s-CMV-ΔU) expressing the EGFP gene under the control of the cytomegalovirus (CMV) promoter were transfected into 293T cells and supernatants were harvested 48 h later. The SIV vector was concentrated by centrifugation of the supernatants at 42 500 *g* for 90 min and the titer assessed by fluorescence activated cell sorting (FACS) using 293T cells as targets was 1.87×10^8 TU/ml.

Spontaneously contracting EBs were transduced with SIV vector expressing the EGFP gene at a multiplicity of infection (MOI) of 100. Cells were washed with PBS 10 h later and incubated in fresh medium for 7–14 days. Contracting EBs were micro-dissected with a sterile micropipette and prepared for immunohistological analysis. Other dissected cells were enzymatically dispersed in trypsin/EDTA solution (0.05% trypsin, 0.53 mM EDTA; Invitrogen) for 7 min at 37 °C and resuspended in DMEM/F12 for use in cell transplantation experiments. To determine the transduction efficiency with the SIV vector, some dissociated cells were centrifuged with Cytospin and stained with anti-cTnI antibody. We then calculated the ratio (%) of EGFP-positive cells among cTnI-immunoreactive cells.

Cell transplantation in a rat myocardial infarction model

Immunodeficient (F344/N *rnu/rnu*) nude rats (male, initial body weight 140–180 g) were used (n = 4) to avoid graft-versus-host disease. Left thoracotomy proceeded under general anesthesia, then the pericardium was opened and the left descending coronary artery was ligated. Cynomolgus ES-cell-derived cardiomyocytes expressing EGFP (1×10^5 cells/50 μl) were implanted in the injured myocardium 30 min after myocardial infarction induction. Two weeks later, rats were killed and hearts were extracted for immunohistological study. All experiments in this study were performed in accordance with the Jichi Medical School Guide for Laboratory Animals.

Statistical analysis

Results are presented as mean ± SD. For comparisons between multiple groups, we determined the significance of differences between group means by ANOVA using the least significant difference for multiple comparisons. Differences at values of $p < 0.05$ were considered to be statistically significant.

Results

Differentiation of cynomolgus ES cells into cardiomyocytes

Rhythmically contracting areas appeared between 3 and 10 days after plating EBs on plastic plates, and were maintained for 3–4 weeks. Figure 1A shows that the ratio of EBs containing beating areas as a function of time after plating was 8.7% (of 751 EBs) at 14 days.

We investigated the expression of cardiac-specific genes in spontaneously contracting EBs. The RT-PCR results revealed that these cells expressed cTnT, MLC-2A, MLC-2V, and αMHC (Figure 1B). In contrast, Oct-4, a marker of undifferentiated ES cells, was expressed in undifferentiated ES cells, but not in contracting EBs or cynomolgus heart tissues. Light microscopy showed that the contracting areas were composed mainly of round or rod-shaped mononuclear cells. Myofibers were detected in the high power light microscopy image stained with toluidine blue (Figure 2A). Transmission electron microscopy revealed that these cells had the mature sarcomeric organization and desmosome structure of cardiomyocytes (Figures 2B–2D). To further elucidate whether these cells have functional features as cardiomyocytes, we measured the effect of angiotensin II or endothelin-1 on intracellular calcium transience. Intracellular calcium transience was obviously stimulated with angiotensin II or endothelin-1. The angiotensin II stimulated effect was completely inhibited by pretreatment with [Sar^1,Ile^8]-angiotensin II

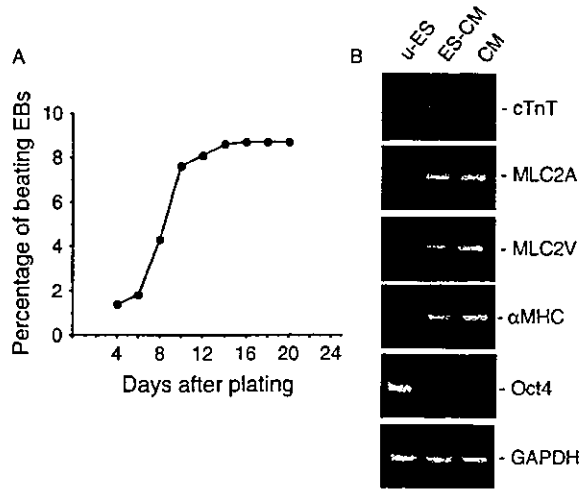

Figure 1. Differentiation of cardiomyocytes from cynomolgus ES cells. (A) Cumulative ratio of EBs derived from cynomolgus ES cells containing spontaneously contracting areas during differentiation. (B) Expression of specific cardiac markers. RNA samples from undifferentiated ES cells (u-ES), contracting 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

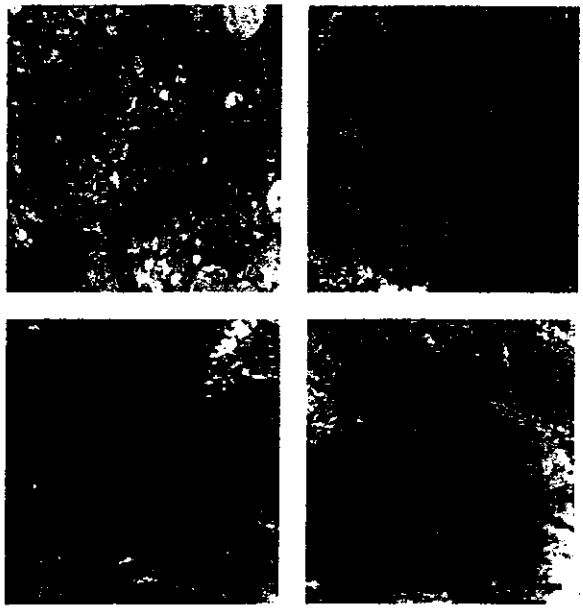

Figure 2. Morphological analysis of contracting EBs 14 days after plating. (A) High power light microscopy image stained with toluidine blue. Myofibers were observed (arrows). Magnification ×1000. (B) Low power transmission micrograph revealed sarcomeric organization (arrowheads) and desmosomes (arrows). 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

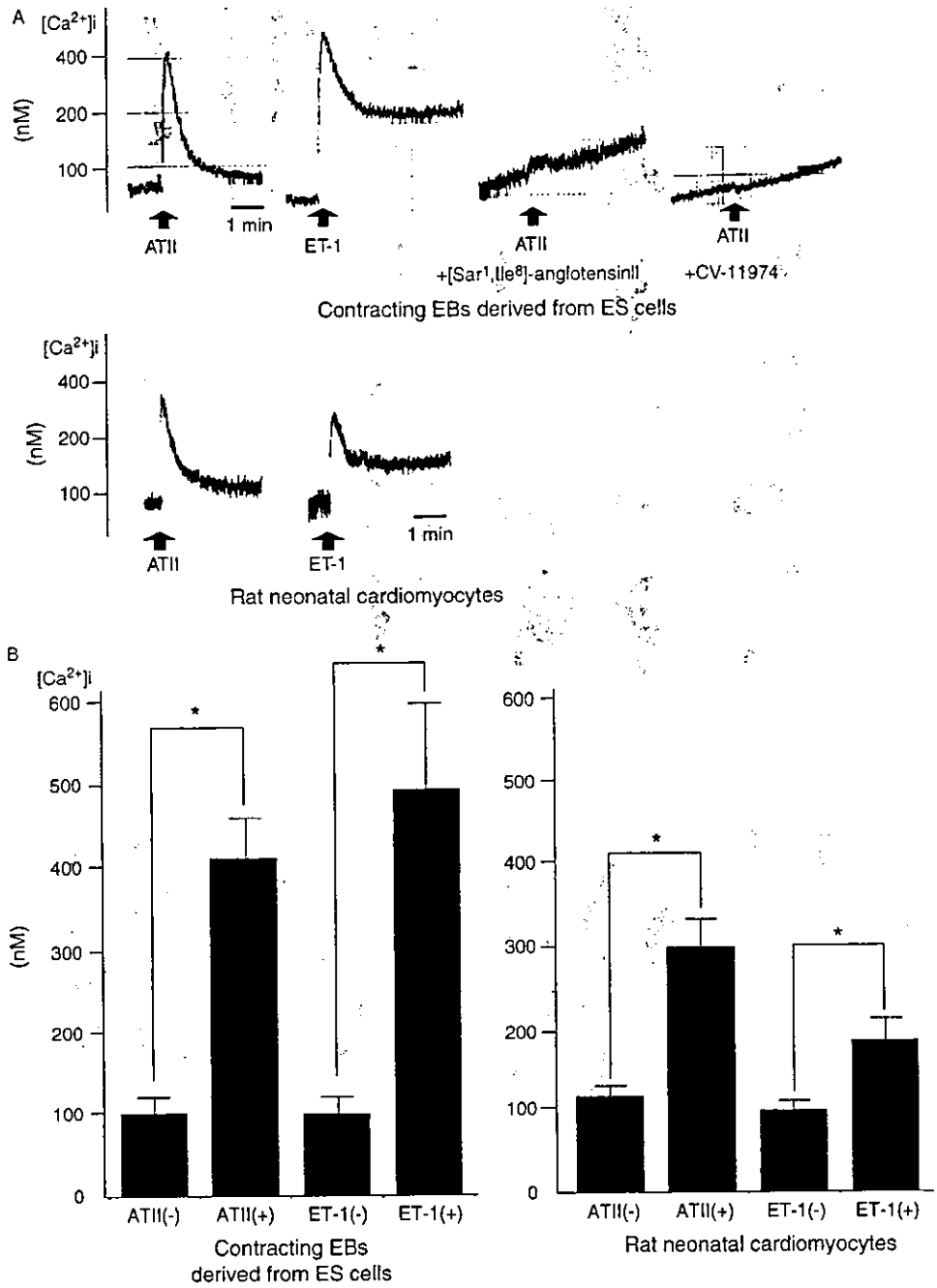

Figure 3. Functional analysis. Intracellular Ca^{2+} transience ($[Ca^{2+}]i$) of contracting EBs and rat neonatal cardiomyocytes determined by fura-2 fluorescence. (A) Typical Ca^{2+} transience appeared in response to 100 nM angiotensin II (ATII) and endothelin-1 (ET-1) in both cell types. The angiotensin-II-stimulated 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

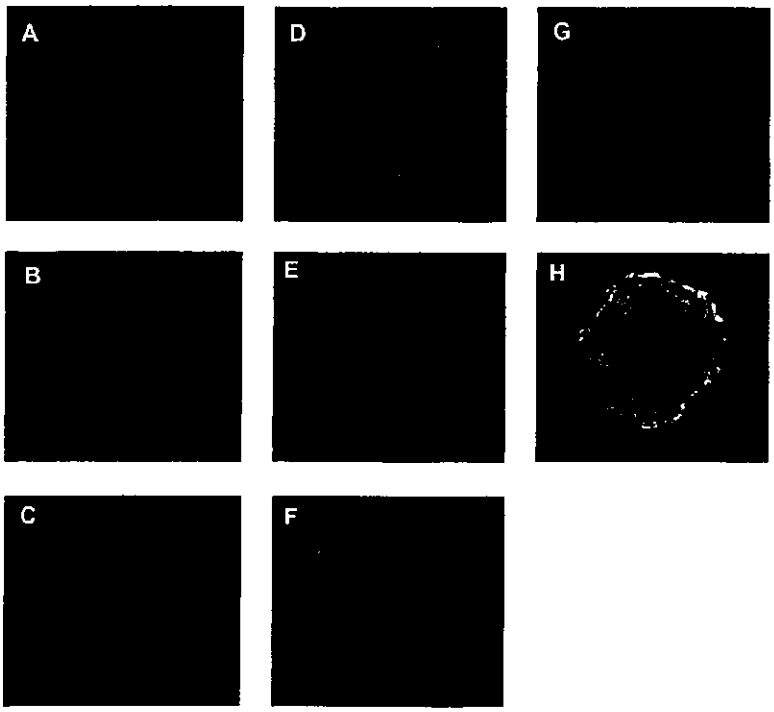

Figure 4. Immunohistochemical staining and EGFP expression. Cardiomyocytes derived from cynomolgus ES cells were transduced with EGFP-SIV vector. Contracting areas were isolated and stained with DAPI (A, D), cTnI (B) and cardiac myosin (E), then EGFP expression (C, F) was identified by fluorescent microscopy. Control staining in which mouse non-specific IgG was used as a primary antibody (G) and phase-contrast photograph (H). Magnification x100

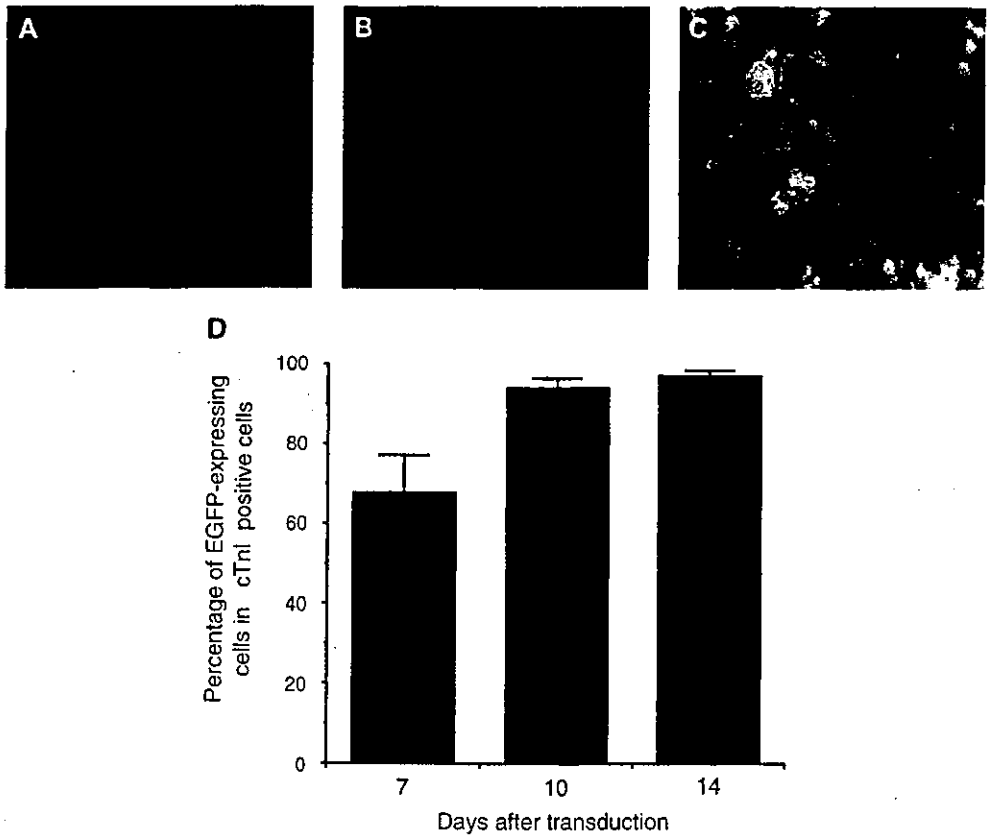

Figure 5. Transgene expression using SIV-based lentivirus vector. Cardiomyocytes derived from cynomolgus ES cells were transduced with EGFP-SIV vector. Contracting areas were isolated, trypsinized and stained with cTnI (A), then EGFP expression (B) was identified by fluorescent microscopy. (C) B merged with A. Magnification x200. (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 USA* 2001; 98: 11 450–11 455.