THE JOURNAL OF GENE MEDICINE
J Gene Med 2010; **12**: 137–146.
Published online 5 January 2010 in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/jgm.1429

RESEARCH ARTICLE

The influence of semen-derived enhancer of virus infection on the efficiency of retroviral gene transfer

Melanie Wurm[1]
Axel Schambach[2]
Dirk Lindemann[3]
Helmut Hanenberg[4,5]
Ludger Ständker[6]
Wolf-Georg Forssmann[6]
Rainer Blasczyk[1]
Peter A. Horn[7]*

[1]*Institute for Transfusion Medicine, Hannover Medical School, Hannover, Germany*

[2]*Department of Experimental Hematology, Hannover Medical School, Hannover, Germany*

[3]*Institute of Virology, Technische Universität Dresden, Dresden, Germany*

[4]*Department of Pediatric Oncology, Hematology and Immunology, Heinrich Heine University, Duesseldorf, Germany*

[5]*Department of Pediatrics, Herman B Wells Center for Pediatric Research, James Whitcomb Riley Hospital for Children, Indiana University School of Medicine, IN, USA*

[6]*Clinic for Immunology and Rheumatology, Hannover Medical School, Hannover, Germany*

[7]*Institute for Transfusion Medicine, University Hospital Essen, Essen, Germany*

*Correspondence to: Peter A. Horn, Institute for Transfusion Medicine, University Hospital Essen, Virchowstrasse 179, 45147 Essen, Germany. E-mail: peter.horn@uk-essen.de

Received: 3 June 2009
Revised: 10 November 2009
Accepted: 29 November 2009

Abstract

Background An improvement of retroviral infection has been postulated using a naturally occurring fragment of the abundant semen marker prostatic acidic phosphatase. This peptide, termed semen-derived enhancer of virus infection (SEVI), promotes HIV attachment to the target cells.

Methods In the present study, we examined whether SEVI would also enhance the infectivity of other viruses with different envelope proteins. We focused on retroviruses pseudotyped with envelopes that are commonly used for the genetic modification of cells, in particular, T cells and hematopoietic progenitor cells. Because the effect of SEVI is considered to be a result of its cationic properties, we compared SEVI with other cationic agents such as protamine sulfate and Polybrene.

Results We found that SEVI increases the efficiency of gene transfer for lentiviral and gammaretroviral vector constructs pseudotyped with VSV-G, GALV, RD114 or foamy viral envelopes on hematopoietic and nonhematopoietic cell lines. On T cells, the transduction efficiency of GALV and RD114 pseudotyped vectors was significantly increased by SEVI. A significant increase of the gene transfer rate was also detected for foamy virally pseudotyped lentivirus on murine hematopoietic progenitor cells. No toxic effect of SEVI treatment was detected on any cell type tested, including human and murine hematopoietic stem/progenitor cells. When directly comparing the effect of SEVI with Polybrene or protamine sulfate, we show that the semen-derived protein is more efficient in increasing the gene transfer rate.

Conclusions SEVI is a promising agent for promoting and improving gene transfer and may also be useful for clinical gene therapy studies. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords gene therapy; retroviral gene transfer; SEVI; transduction efficiency

Introduction

In near future, hematopoietic stem cell transplantation and, moreover, the genetic modification of these cells may play an important role in the therapy of malignancies, bone marrow failures and in the therapy of numerous genetic disorders, including immunological or metabolic deficiencies.

Until now, most clinical gene therapy trials and various experiments in large animal models have been based on gammaretroviral and lentiviral gene transfer systems [1–4]. Currently, numerous different envelopes are in use for the packaging of gene transfer vectors. Four of them, which are also in the

focus of the present study, have already been tested in large animal models or in clinical gene therapy trials [5,6]: the glycoprotein of the vesicular stomatitis virus (VSV-G), the envelope of the feline leukemia virus (RD114), the envelope of the gibbon ape leukemia virus (GALV) and the foamy virus (FV) envelope.

A high efficiency of gene transfer into primary cells and the ability to produce sufficient virus particles for the large-scale transduction of patient cells are prerequisites to using a vector system in clinical trials. Packaging cell lines for the large-scale production of lentiviral vectors, which were used in the present study, only exist for GALV and RD114 envelopes; however, both packaging cell lines do not produce high titer virus for efficient gene transfer. By contrast, VSV-G and FV envelopes are very efficient, although, for these envelopes, there is a lack of producer cells. Thus, the large-scale production of these viruses is extremely complex and expensive. Finally, every improvement of lentiviral transduction leads to a reduction in the need for virus production and therefore helps to reduce the cost of clinical trials.

Increasing the efficiency of gene transfer can be achieved by different strategies. The concentration of the virus supernatant by ultracentrifugation before its use in transduction is one possibile way to improve the efficiency of gene transfer [7,8].

Coating plates with the recombinant retronectin fragment CH296 was reported to enhance the viral gene transfer. Cells bind to the VLA-4 and VLA-5 binding domains and the virus particles bind to a heparin binding domain. Acting in this way, viruses and cells become closer to each other [9,10].

Another frequently used strategy to enhance the gene transfer rate is the supplementation of different agents such as polycations, cationic liposomes and calcium phosphate. For polycations such as Polybrene (Sigma-Aldrich, St. Louis, MO, USA) or protamine sulfate [11,12], it has been proposed that these agents reverse the negative electrostatic charge of the cells and the virus membranes, leading to a lower repulsion of both components [13]. Cationic liposomes such as Lipofectamine (Invitrogen, Carlsbad, CA, USA) [14] enhance retroviral gene transfer as a result of the formation of a stable virion-liposome complex [15]. Finally, the efficiency of transduction can be improved by the enhancement of cell–virus fusion using calcium phosphate [16]. However, most of these adjuvants and treatments are toxic for the cells, limiting their use with sensitive cells such as hematopoietic stem cells.

Recently, an enhancement of infection with HIV has been demonstrated using a natural occurring fragment of the semen marker prostatic acidic phosphatase (PAP) [17]. Most new infections of HIV worldwide occur through genital exposure to semen fluid [18]. It was hypothesized that these peptides, present in semen, form amyloid fibrils and are able to capture HIV virions. It was observed that these fibrils promote the attachment of the virus to the target cells. Consequently, the fibrils were termed semen-derived enhancer of virus infection (SEVI). The virus binds directly to SEVI and the dependency of binding to basic residues of the protein was also demonstrated [19].

Thus, we hypothesized that the cationic properties of SEVI may also facilitate and improve gene transfer with retroviruses pseudotypes with other commonly used envelope proteins. The effect of the SEVI protein was examined on different cell lines and hematopoietic progenitor cells were examined.

Materials and methods

Cell lines and primary cells

HeLa and NIH-3T3 (DSMZ, Braunschweig, Germany) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Cologne, Germany). K562 cells were cultured in RPMI. Both media additionally contained 10% fetal calf serum (FCS) (PAA, Coelbe, Germany) and 1% penicillin/streptomycin (cc pro GmbH, Oberdorla, Germany).

The OG2 mouse embryonic fibroblasts (MEF) that were used for the cytotoxicity assay were obtained from d13.5 mouse embryos of the OG2 strain (strain 004 654; Jackson Laboratory, Bar Harbor, ME, USA). Cells were cultured in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin and 1% non-essential amino acids (Gibco, Invitrogen, Karslruhe, Germany).

The human T cells were extracted from peripheral blood samples. Mononuclear cells were separated from other blood components using Lymphosep (cc pro GmbH) and the T cells were enriched in a MACS™ cell separation system and a Pan T-cell Isolation Kit II, MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Verification of the cells was performed by adding a CD3-phycoerythrin (PE) antibody (Clone A07747; BD Biosciences, Heidelberg, Germany) and subsequent analysis of the cell solution by flow cytometry. Separated human T cells were cultivated in IMDM (Lonza) additionally containing 10% FCS and 1% penicillin/streptomycin. Medium was supplemented with interleukin (IL)-2 (100 IU/ml) (Peprotech, Hamburg, Germany) and αCD3/CD28 Expander Beads (Dynal Biotech, Karlsruhe, Germany). Finally, T cells were isolated and activated 2 days before transduction.

Human $CD34^+$ cells were selected from hematopoietic stem cell apheresis. Donor $CD34^+$ cells were mobilized with granulocyte-colony stimulating factor (G-CSF) (Lenograstim, Granocyte®; Chugai, Frankfurt, Germany), at 10 μg/kg body weight. G-CSF was given on four consecutive days and, on day 5, apheresis was performed. Mononuclear cells were purified in a similar way as described for T cells. The cells were also enriched using the MACS™ cell separation system combined with the CD34 Micro Bead Kit (Miltenyi Biotec). The $CD34^+$ cells were labeled with a CD34-PE antibody (clone 8G12; BD Biosciences) and detection of positive cells was performed by flow cytometry. The human $CD34^+$ cells were cultivated in IMDM supplemented with 10% FCS (PAA) and 1% penicillin/streptomycin (cc pro GmbH).

DOI: 10.1002/jgm

The human cytokines thrombopoietin, stem cell factor, IL-6 and Flt-3 ligand (Peprotech), each at 100 ng/ml, were added to the medium.

Bone marrow was collected from C57BL/6 mice (Charles River Laboratories, Kisslegg, Germany). Hematopoietic progenitor cells, Lin^- cells, were separated using a MACS™ cell separation system and a Lineage Cell Depletion Kit (Miltenyi Biotec). Lin^- cells were cultured in Stemspan media (StemCell Technologie Inc., Cologne, Germany) supplemented with 1% glutamine and 2% penicillin/streptomycin (cc pro GmbH). The cytokines human Flt-3 ligand (100 ng/ml) and IL-11 (100 ng/ml) and the mouse cytokines stem cell factor (50 ng/ml) and IL-3 (20 ng/ml) (all from Peprotech) were added to the medium. Lin^- cells were pre-stimulated for 2 days before transduction.

Cytotoxicity assay

For adherent cell lines (HeLa and MEF), 5×10^3 cells were seeded in a 96-well dish, 1 day before starting the SEVI treatment. All blood cells were cultured in suspension in a 96-well dish with an initial seeding density of 1×10^5 cells per well together at various SEVI concentrations. T cells were plated after an activation of 2 days. All cells were cultured for 3 days in a total volume of 100 µl before MTT assays (Biochain, Hayward, CA, USA) were performed according to the manufacturer's instructions. The experiment was performed in triplicate for the primary blood cells and with six technical replicas for the K562, HeLa and MEF cells. The metabolic activity is presented as the mean ± SD after normalization to untreated control cells (set to 100%).

Constructs and transfection

In the present study, three different retroviral vectors, all coding for a green fluorescent protein (GFP), were used in different gene transfer protocols. The gammaretroviral vector SRS11.SF.GFPpre [20] is a gammaretroviral SIN vector based on murine leukemia virus (MLV), the lentiviral vector pCL7.SFFV from HIV-1 and the FV vector puc2MD9 was generated from a primate FV. Viral vectors were produced by co-transfection of MLV g/p for the gamma retroviral vector, psPax2 (from Professor Didier Trono, NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) for the lentiviral vector and pcziGag together with pcziPol for the FV system. Viruses were pseudotyped with VSV-G (pMD2G, from Prof. Didier Trono), RD114-TR [21], paLF GALV -TM [22] or a modified FV envelope (Lindemann *et al.* manuscript in preparation). All vectors were produced by transfection of 293T cells applying Polyethylenimine (Sigma) as a transfection reagent.

The virus supernatants were diluted before transduction of the cell lines. This was performed to achieve a modest level of gene transfer so that both increases and decreases in gene transfer could be easily quantified, allowing the detection of both positive and negative influences. All viral supernatants were diluted with DMEM, which was also used for virus production. The GALV and RD114 pseudotyped constructs were diluted 1 : 2, FV was diluted 1 : 10 and VSV-G constructs were diluted 1 : 20. After dilution, the titer of the GALV and RD114 pseudotyped virus constructs was in the range $2–7 \times 10^3$ transducing units/ml; for FV, it was in the range $0.8–1 \times 10^5$ transducing units/ml; and, for VSV-G, it was in the range $5–7 \times 10^4$ transducing units/ml media. This resulted in gene transfer rates typically in the range 0.5–50%. The fold change in transduction was selected to compare the gene transfer efficiencies of different treatments.

The virus supernatant for the transduction of the primary cells was concentrated by centrifugation for 2 h at 16 000 ***g*** at 37 °C. Subsequently, the old medium from the producer cells was discarded and replaced by fresh medium. For the transduction of the primary cells, the virus titers were $2–3 \times 10^4$ transfecting units/ml for GALV and RD114; $2–3 \times 10^6$ transfecting units/ml for FV; and 1×10^6 transducing units/ml for VSV-G. All virus containing supernatants were titrated on HT1080 cells.

SEVI activation

SEVI peptides were prepared by 9*H*-fluorenylmethoxycarbonyl solid-phase peptide synthesis, as described previously [23], and subsequently purified by reverse-phase chromatography. The identity and homogeneity of the products were checked by analytical high-performance liquid chromatography and electrospray ionization- and matrix-assisted laser desorption ionization-mass spectrometry.

The PAP 248–286 (referred to below as the 'SEVI protein') and different PAP variants were tested in the present study. Indeed, PAP 247–282, PAP 247–286, PAP 253–285 and PAP 248–286, in which eight different amino acids were changed to alanine (8× Ala), were examined. Lyophilized peptides were dissolved in phosphate-buffered saline (PBS). Only the 8× Ala PAP 248–286 was dissolved in dimethyl sulfoxide (Sigma-Aldrich, Seelze, Germany) and diluted twice with PBS. The concentration was adjusted to 5 mg/ml for all PAP variants. Fibril formation was activated by 24 h of incubation at 37 °C and under constant agitation using an Eppendorf Thermomixer (Eppendorf AG, Hamburg, Germany).

Transduction

HeLa cells were plated in six-well plates at 100 000 cells per well 16–24 h prior transduction. NIH-3T3 cells were plated at 50 000 cells per well. Non-adherent cells, K562 and human T cells were seeded at 100 000 cells per well at the day of transduction. Human $CD34^+$ cells were transduced on the same day they were isolated. They

DOI: 10.1002/jgm

were plated at 100 000 $CD34^+$ cells per well, which were coated with Retronectin 4 μg/cm^2 (TaKara Bio Inc., Shiga, Japan) prior to cell seeding. Murine Lin^- cells were plated at 180 000 cells per well.

Virus supernatant was added to the cells and SEVI or one of the other PAP variants were supplemented at a final concentration of 50 μg/ml, as described previously [17]. Polybrene and protamine sulfate (Sigma-Aldrich) were used at 8 μg/ml final concentrations.

Progenitor assays were set up from human $CD34^+$ cells the day after transduction in Methocult (StemCell Technologies Inc., Vancouver, BC, Canada) at 250 or 500 cells/ml. Colonies were counted in a fluorescence microscope 14 days after transduction.

The efficiency of the transduction measured as the efficiency of gene transfer was examined 4 days after the transduction by flow cytometry. Accordingly, T cells were labeled with a CD3-PE and $CD34^+$ cells with a CD34-PE antibody as previously described.

Statistical analysis

Differences in the efficiency of gene transfer were described as the fold difference compared to untreated cells. All data are shown as the mean ± SD of at least three independently repeated experiments. The statistic analysis of the data was calculated with using SAS software, version 9.1 (SAS Institute, Cary, NC, USA). Data were examined for normality using a *t*-test (procedure TTEST, SAS). $p \leq 0.05$ was considered statistically significant.

Results

Cytotoxicity of SEVI on adherent and suspension cells

To evaluate the toxic effects of SEVI protein on different cell lines, we analysed the metabolic activity of these cells 3 days after treatment with a SEVI concentration in the range 0.1–100 μg/ml. For adherent cell lines, we chose HeLa cells and OG2 MEF and, for hematopoietic suspension culture cells, we evaluated K562 cells, human T cells and human $CD34^+$ cells. The influence of various SEVI concentrations on the cell viability was evaluated using MTT tests. On adherent cells, SEVI has a mild negative effect on the metabolic activity, resulting in metabolic activity levels of 58–71% when applying the highest SEVI concentration (Figure 1, gray samples). Importantly for hematopoietic cells, no negative effect of SEVI was detected (Figure 1, black samples) and even a slightly increased metabolic activity of 126% and 111% could be detected for T cells and $CD34^+$ cells, respectively.

In another experiment, $CD34^+$ cells were plated in methylcellulose and the number of colonies was used to evaluate the effect of SEVI on the colony formation capabilities of hematopoietic stem cells. On average, 26.4 colonies per 100 plated $CD34^+$ cells grew in the absence and 29.7 colonies grew in the presence of 50 μg/ml SEVI. In addition, the ratio between myeloid and lymphoid colonies was not significantly affected by the SEVI treatment (data not shown). This result suggests that the SEVI protein is not toxic for $CD34^+$ cells and that a dose of 50 μg/ml does not influence their differentiation potential.

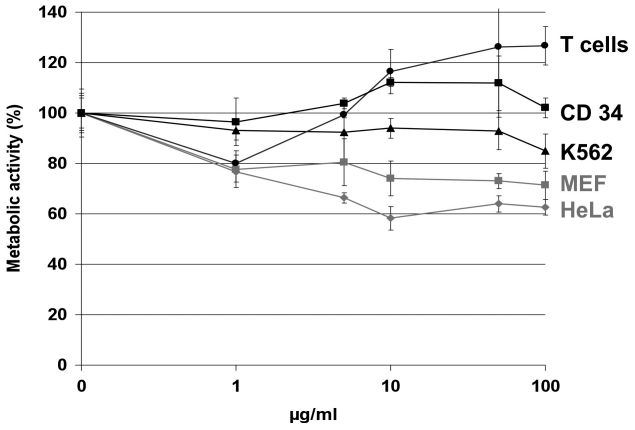

Figure 1. Cytotoxicity assays of SEVI protein effects on different cell types. Cells were cultivated with various SEVI concentrations. After 3 days of cultivation, metabolic activity was evaluated at MTT tests. The levels of the metabolic activity of untreated cells were normalized to 100%

Influence of SEVI in combination with different vectors and envelopes on the efficiency of gene transfer

The study also aimed to evaluate whether it is possible to enhance the gene transfer rate using a SEVI protein for viruses other than HIV. Furthermore, whether the packed vector has any influence on the interaction between virus particles and SEVI was examined.

The influence of SEVI on the efficiency of gene transfer was evaluated in two different human cell lines. Lentiviral and gammaretroviral vectors each pseudotyped with four different envelopes were used in the presence or absence of SEVI. Cells transduced without SEVI were designated as 'untreated' cells and were used to calculate the fold increase caused by the SEVI treatment.

When viral constructs were pseudotyped with the VSV-G or the FV envelope, supplementation of SEVI did not lead to a significant increase of the efficiency of gene transfer in any tested cell line, although the efficiency of gene transfer was even reduced in most of the cases in the presence of SEVI (Figure 2A). However, using SEVI and gammaretroviral constructs, which were pseudotyped with GALV or RD114, a marked increase of the efficiency of gene transfer was observed in HeLa cells (Figure 2B). The lentiviral vector pseudotyped with the GALV or RD114 envelope showed a significant increase as a result of SEVI treatment on K562 cells; the increase was up to 2.5-fold for RD114 ($p = 0.02$)

DOI: 10.1002/jgm

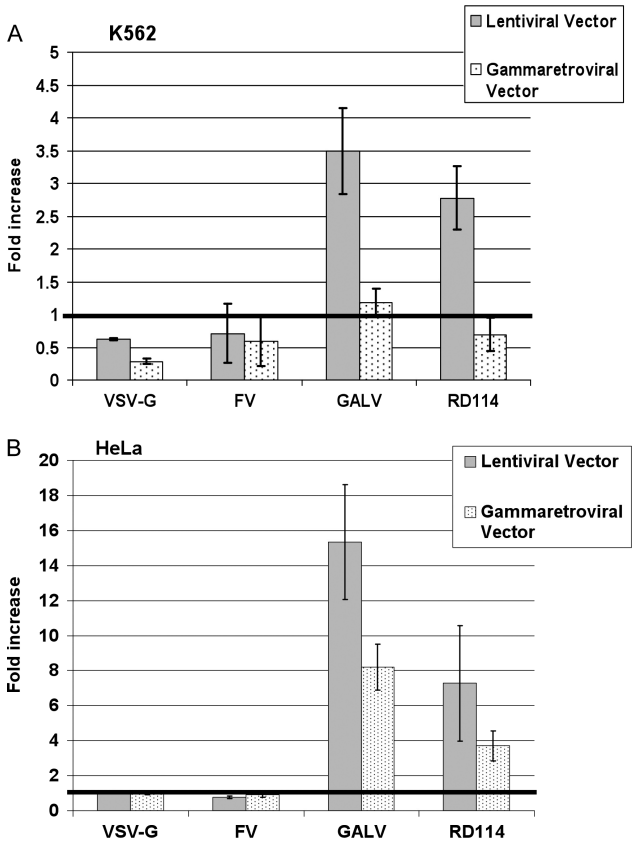

Figure 2. Influence of the SEVI treatment on the gene transfer rate for GALV and RD114 pseudotyped retroviruses on two different human cell lines. Gammaretroviral and lentiviral vectors were pseudotyped with VSV-G, FV envelope, GALV and RD114, respectively. The transduction was performed with SEVI or without SEVI. Differences in the gene transfer rate are described as the fold increase compared to untreated cells ($n = 3$). (A) The increase of the gene transfer rate by the SEVI treatment is significant for lentiviral vectors pseudotyped with GALV and RD114 ($p = 0.0011$ and $p = 0.02$) on K562 cells. (B) On HeLa cells, the increase was significant not only for GALV and RD114 pseudotyped lentiviruses but also for gammaretroviruses (lentiviral vector: $p = 0.0002$ and $p = 0.001$; gammretroviral vector: $p = 0.0005$ and $p = 0.0003$)

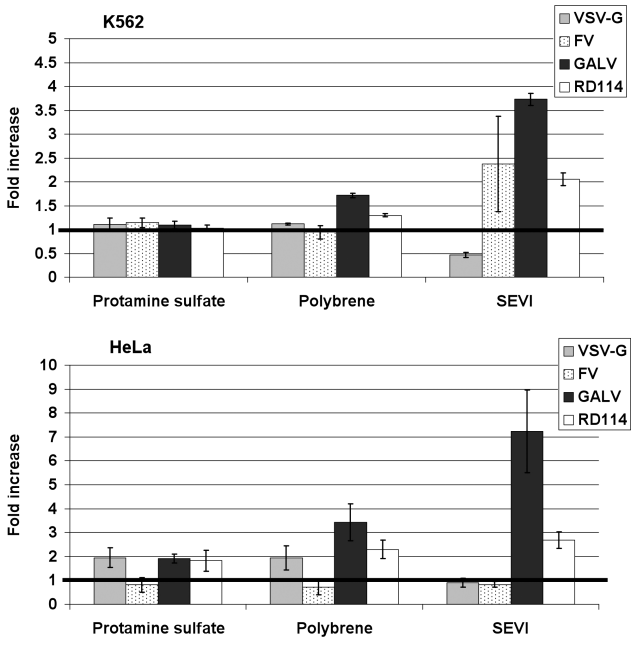

Figure 3. The influence on the efficiency of gene transfer by SEVI protein treatment is compared with treatments using protamine sulfate or Polybrene. A lentiviral vector was pseudotyped with VSV-G, FV envelope, GALV and RD114, respectively. The transductions were performed with protamine sulfate or Polybrene, with an 8 μg/ml concentration each time, and the SEVI protein was used at a concentration of 50 μg/ml. Differences in the efficiency of gene transfer are described as the fold increase compared to untreated cells ($n = 3$). A significant increase of the gene transfer rate was induced by the SEVI treatment for the GALV and RD114 pseudotyped viruses on K562 cells (A) ($p = 0.005$ and $p = 0.0001$) and also on HeLa cells (B) ($p = 0.05$ and $p = 0.04$). (B) The gene transfer rate of the GALV pseudotyped virus was also significantly increased by Polybrene treatment ($p = 0.05$)

and 3.5-fold for GALV ($p = 0.0011$). The highest increase of the efficiency of gene transfer was reached on HeLa cells with the lentiviral vector and GALV envelope (Figure 2B). Here, the efficiency of gene transfer was significantly elevated up to 15-fold compared to cells without SEVI ($p = 0.0002$). The efficiency of gene transfer of the same lentiviral vector pseudotyped with RD114 increased seven-fold compared to cells transduced in the absence of SEVI ($p = 0.001$; Figure 2B). Furthermore, the gammaretroviral vector systems pseudotyped with GALV or RD114 showed a significant increase of the efficiency of gene transfer compared to the SEVI untreated cells ($p = 0.005$ and $p = 0.0003$).

Taken together, SEVI is able to enhance the efficiency of gene transfer for other virus constructs than HIV and this enhancement is independent of the vector packaging system.

The efficiency of SEVI in comparison with cationic agents to increase the efficiency of gene transfer

The study also aimed to determine the enhancement of the gene transfer rate by SEVI in comparison with cationic agents such as protamine sulfate and Polybrene, which are widely used to enhance gene transfer.

The experiments were also performed in the human cell lines K562 and HeLa. The lentiviral vector system was pseudotyped with all four envelopes. The increase of the efficiency of gene transfer was normalized to the value reached by 'untreated' cells, which were transduced in the absence of SEVI or cationic agents.

In K562 cells, Polybrene and protamine sulfate enhanced the gene transfer rate only for RD114 pseudotyped virus (Figure 3A). The efficiency of gene transfer of GALV or RD114 pseudotyped viruses was increased by the cationic agents in HeLa cells (Figure 3B). The highest increase was detected when Polybrene and GALV pseudotyped vectors were combined ($p = 0.05$).

However, SEVI significantly increased the efficiency of gene transfer for retroviruses pseudotyped with GALV or RD114 envelopes in each cell line tested (K562:

DOI: 10.1002/jgm

$p = 0.0045$ and $p = 0.0001$; HeLa: $p = 0.05$ and $p = 0.036$; Figure 3). The highest increase of the transduction efficiency was 9.5-fold, as detected in HeLa cells with the GALV vector system. Neither the VSV-G nor the FV pseudotyped vector systems showed a significant increase of the efficiency of gene transfer as a result of treatment with cationic agents or SEVI.

SEVI in mouse fibroblasts

Phagocytosis of SEVI fibrils by different target cells was previously shown by electron microscopy in the supplementary data to a study by Münch *et al*. [17]. If SEVI were able to bind to a virus particle and enter the cell together with the attached virus, this would result in a gene transfer without the necessity of the virus to bind to a cellular receptor, allowing removal of an envelope's tropism restriction. We therefore tested the possibility of overcoming the species barrier by the use of SEVI.

Mouse cells have no receptor for GALV and RD114, resulting in a virus pseudotyped with GALV and RD114 envelopes not being able to enter mouse cells. By contrast, VSV-G and FV envelope show no tissue tropism because the need for any specific receptor is missing.

Experiments similar to those performed with the human cell lines were also performed on NIH-3T3 mouse fibroblast cells. The lentiviral vector was pseudotyped with each of the four different envelopes. The evaluation of the increase of the efficiency of gene transfer was compared with 'untreated' NIH-3T3 cells, which were transduced in the absence of SEVI.

GALV and RD114 pseudotyped vectors were unable to infect the cells, even in the presence of SEVI (Figure 4). The virus with the FV envelope showed no significant influence on the efficiency of gene transfer by the SEVI treatment. The efficiency of gene transfer of VSV-G pseudotyped viruses was increased five-fold when SEVI was added and this effect was highly significant ($p = 0.025$; Figure 4).

In conclusion, the use of SEVI does not enable the species barrier to be overcome.

Analysis of different PAP variants

The analysis of peptide libraries from human sperm revealed different PAP variants, and it was shown that some of these variants are able to increase the infection rate of HIV. In addition, some of these peptides were artificially modified to enhance the SEVI effect.

The hypothesis of these experiments was that not only is SEVI itself able to enhance the efficiency of gene transfer rates, but also other PAP variants. A further study aim was to examine whether these PAP variants work as efficiently as SEVI with different envelopes.

Overall, five PAP variants were examined in the present study with the lentiviral vector system pseudotyped with VSV-G, FV envelope, GALV or RD114. The increase of the transduction efficiency was calculated to the value obtained by 'untreated' cells, which were transduced with the virus supernatant without any kind of PAP.

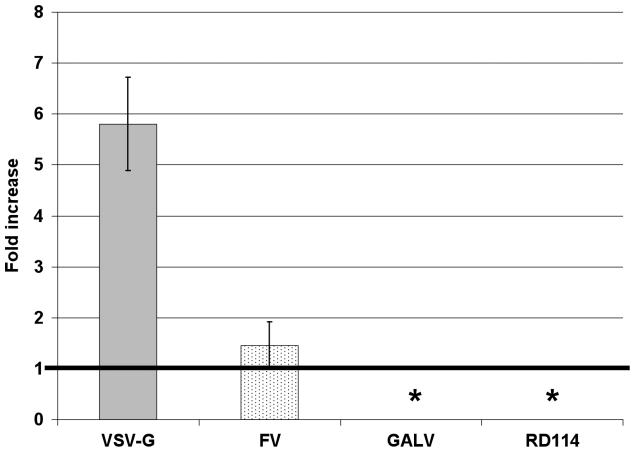

Figure 4. A comparison of the influence of the SEVI protein on the gene transfer rate in NHI-3T3 cells, a mouse cell line. Cells were transduced with a lentiviral vector, pseudotyped with VSV-G, FV, GALV and RD114 envelopes, respectively. Differences in the gene transfer rate between the SEVI-treated and the untreated cells are described as the fold increase compared to untreated cells ($n = 3$). In the marked field (*), no gene transfer was detectable in untreated or SEVI-treated cells. The increase of the VSV-G pseudotyped construct was highly significant ($p = 0.025$)

SEVI, the variant PAP 253–285 and the variant 247–286 enhanced the gene transfer rate of GALV and RD114 pseudotyped viruses on K562 and HeLa cells (Figure 5). They enhanced the transduction rate between two- and seven-fold. For the PAP variants 247–282 and the 8x Ala variant, a significant increase of the gene transfer rate was not seen. Moreover, for the VSV-G and the FV envelope, no increase of the gene transfer rate by any of the tested PAP variants was observed. The opposite was detected for the FV envelope, where, in some cases, the gene transfer rate was reduced by the protein treatment.

SEVI increases the efficiency of gene transfer on primary hematopoietic cells

Next, we wanted to test the possibility of enhancing the gene transfer rate in primary hematopoietic cells such as T cells (Figures 6 and 7) and hematopoietic progenitor cells from human and mice (Figures 8 and 9) and to rule out any negative influence of SEVI treatment on these sensitive primary cells, which are often the target of gene therapy.

Lentiviral constructs pseudotyped with each of the four different envelopes were used. To evaluate the efficiency of gene transfer, the value of SEVI-treated cells was compared with that of untreated cells, which had been prestimulated and transduced in the same way but without SEVI.

DOI: 10.1002/jgm

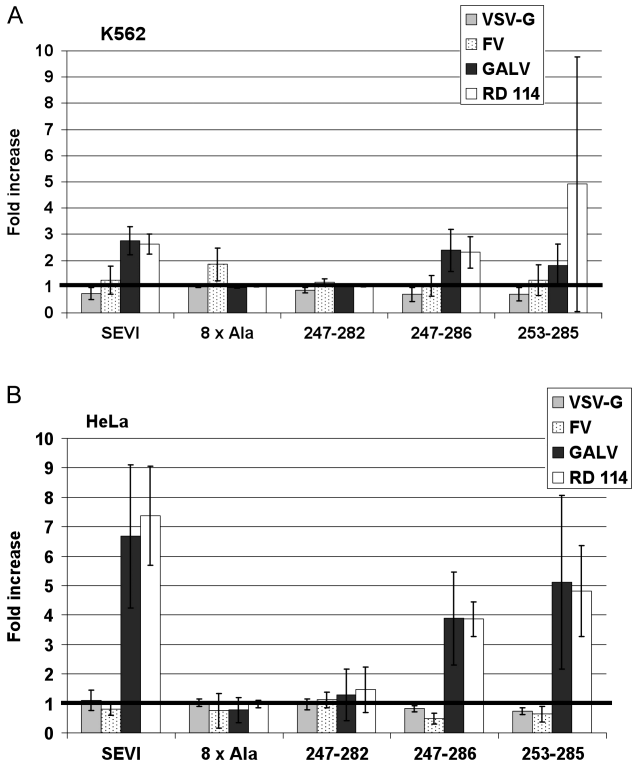

Figure 5. A comparison of the efficiency of gene transfer between SEVI-treated cells and cells treated with other PAP variants. All PAP variants were used at a concentration of 50 μg/ml. Cells were transduced with a lentiviral vector, pseudotyped with VSV-G, FV, GALV and RD114 envelopes, respectively. Differences in the efficiency of gene transfer are described as the fold increase compared to untreated cells ($n = 3$). The effect of the PAP treatment was tested on the human cell lines K562 (A) and on HeLa cells (B)

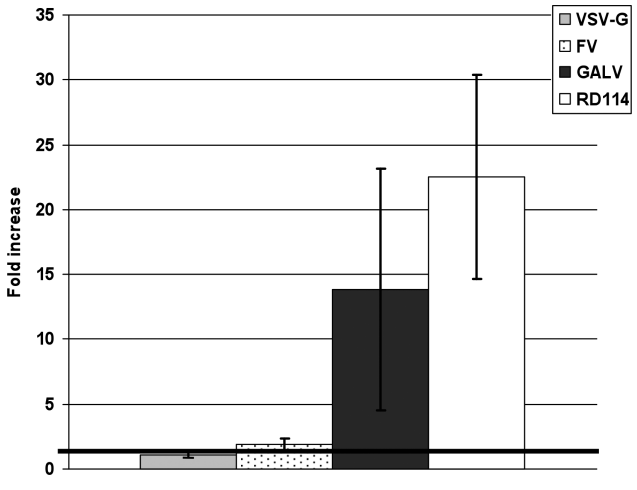

Figure 6. Influence on the efficiency of gene transfer by a SEVI protein treatment in human T cells. Two days before transduction, T cells were pre-stimulated with IL-2 plus a αCD3/CD28 bead. T cells were transduced with a lentiviral vector, pseudotyped with VSV-G, FV, GALV and RD114 envelopes, respectively. The efficiency of SEVI treatment was compared to an untreated transduction. Differences in the efficiency of gene transfer are described as the fold increase compared to untreated cells ($n = 3$)

At first, the effect of SEVI on the transduction rate of primary human T cells and human $CD34^+$ progenitors was examined.

The efficiency of gene transfer for T cells were increased up to 22-fold for the combination of SEVI protein treatment and RD114 pseudotyped virus ($p = 0.04$; Figure 6). The transduction efficiency of the GALV pseudotyped vector was increased up to 13-fold, when SEVI was applied (Figure 6). The dot plots obtained by flow cytometry from T cells of one of the T cell donors are shown (Figure 7).

On human $CD34^+$ cells, which were cultivated in liquid media, SEVI increased the efficiency of gene transfer of all four different envelopes. A maximum increase of almost ten-fold was detected for the RD114 envelope (Figure 8). The transduction efficiency of the FV envelope improved up to 7.8-fold. Furthermore, the efficiency of gene transfer of the VSV-G and the GALV pseudotyped viruses increased by 3.5- and three-fold, respectively.

In addition, the effect of SEVI on the transduction of murine progenitor cells was tested. Both VSV-G and the FV envelope were tested on murine Lin^- cells. A significant six-fold increase on the efficiency of gene transfer as a result of SEVI treatment was observed in combination with the FV envelope ($p = 0.0001$; Figure 9). Again, no effect on the growth rate of these murine hematopoietic stem and progenitor cells as a result of SEVI treatment was observed.

Discussion

In the present study, we demonstrated that SEVI enhances the gene transfer rate not only for HIV, but also for other retroviruses packaged with different envelope proteins. The magnitude of enhancement varies with different envelopes and cell types. Importantly, with all the tested cell types, in particular with hematopoietic progenitor cells, no major toxic effect of the protein was detected.

The first step was to evaluate the effect on the cell viability by measuring the metabolic activity of cells after treatment with different SEVI protein concentrations. As depicted in Figure 1, we did not observe a decrease of metabolic activity in K562, human T cells and human $CD34^+$ and only observed a mild reduction of metabolic activity for the adherent cells (HeLa and MEF). These data are consistent with the results obtained by Münch *et al.* [17] who demonstrated in their supplementary data that SEVI does not have serious effects one cell viabilities of various cells. Taken together, these results indicate that SEVI is safely applicable to various cell lines, particularly to blood cells.

SEVI highly significantly increased gene transfer efficiency for different experimental set-ups. The results obtained strongly depend on the envelope and the target cell line used for transduction. The envelopes GALV and RD114 showed a significant increase of the transduction efficiency by the use of SEVI in all human cell lines examined compared to those cells transduced without

DOI: 10.1002/jgm

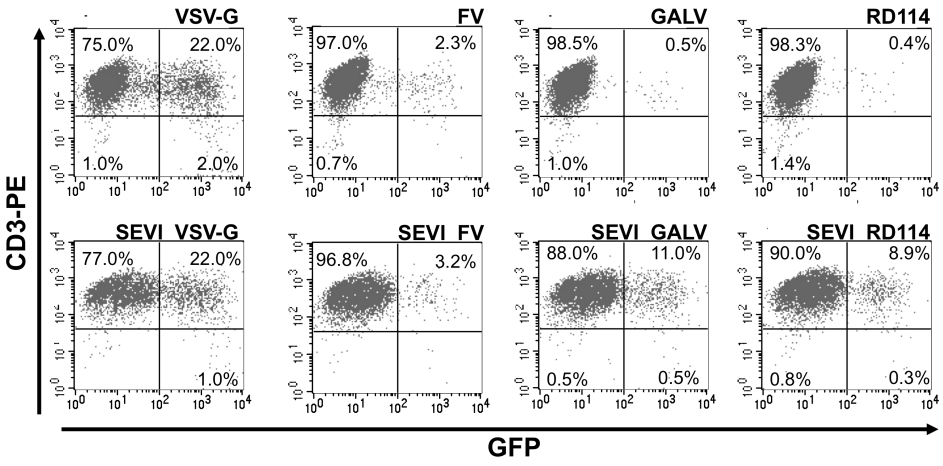

Figure 7. Flow cytometry plots from a reprensentative human T cell experiment are shown. T cells were pre-stimulated 2 days before transduction with IL-2 and αCD3/CD28 Beads. The effect of SEVI protein treatment is compared with an untreated transduction. T cells were transduced with a lentiviral vector, pseudotyped with VSV-G, FV, GALV and RD114 envelopes, respectively. For the flow cytometry analyses, cells were labeled with a CD3-PE antibody. The GFP positive parts of the whole CD3 positive cell fraction are shown

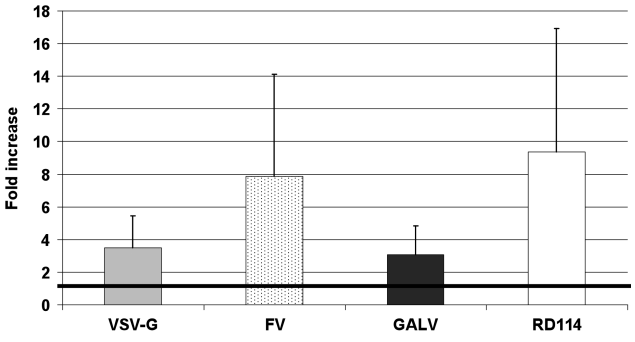

Figure 8. SEVI enhances transduction of human CD34$^+$ cells. CD34$^+$ cells from three patients were transduced with the indicated vectors. The effect of the SEVI treatment is compared with an untreated transduction. The average fold increase in transduction efficiency as determined by enhanced GFP expression 4 days after vector exposure is indicated. For all vectors, SEVI increased the transduction efficiency. The standard error of the mean is shown for each vector

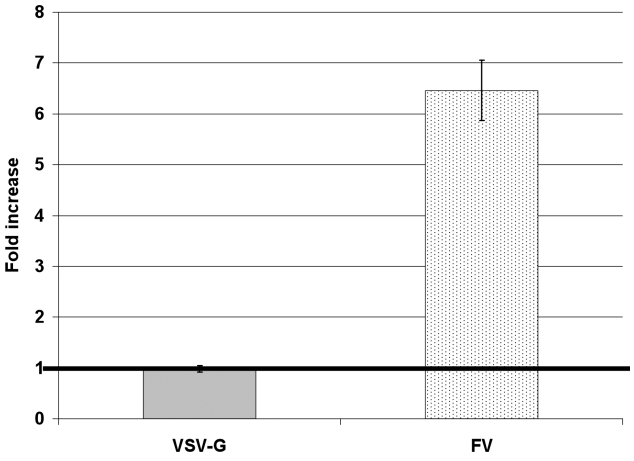

Figure 9. A comparison of the efficiency of gene transfer in SEVI-treated murine Lin$^-$ cells and untreated Lin$^-$ cells. Lin$^-$ cells were cultivated in Stem span media with the cytokines human Flt-3 ligand and IL-11 and the mouse cytokines stem cell factor and IL-3. Cells were expanded for 2 days before transduction. A lentiviral vector pseudotyped with either VSV-G or FV envelope was used. Differences in the efficiency of gene transfer between SEVI-treated and untreated cells are described as the fold increase compared to untreated cells ($n = 3$). The increase of the gene transfer rate was highly significant ($p = 0.0001$) for the FV pseudotyped virus construct

SEVI. These two envelopes are both characterized by binding to a specific receptor of the target cell [24,25]. These findings are in line with the results obtained by Münch *et al.* [17], who observed a high enhancement of the HIV-1 infectivity by SEVI treatment. Notably, HIV-1 also uses a specific receptor, the CD4 receptor, for viral entry into the target cell. By contrast, our results from the FV and VSV-G envelope, for which the receptor interaction partners are less well understood, did not show an increased gene transfer efficiency. Currently, the mechanism of SEVI peptide leading to an increased infection rate of HIV is considered to be based on binding of the SEVI fibrils to the virus directly with basic residues of the protein [19]. It is noteworthy that SEVI proteins enter the cell membrane when the virus is attached to the protein, as demonstrated by electron microscopy [17]. A specificity of this physical conjunction may be responsible for the contrasting results obtained when using different envelopes. It was also suggested that only the envelope and not the vector system is mainly responsible for the SEVI effect. These results are consistent with those obtained in other studies, which only focused on the infectivity of the vector systems and also concluded that the vector system does not influence infectivity [26]. However, by comparing lenti- and gammaretroviral vector systems pseudotyped with the same envelope proteins, we provide consistent evidence indicating that the vector itself also has an influence on the SEVI-mediated increased gene transfer rates. In our hands, the enhancement of the gene transfer rate is lower for the gammaretroviral vector than for the lentiviral vector, pseudotyped with the same

DOI: 10.1002/jgm

envelope. One reason for this observation might be that not only virus entry, but also viral gene expression can be enhanced by SEVI to various extents. This issue has previously been discussed by Münch *et al*. [17], who noted that SEVI enhance the gene expression of the lentiviral genome. However, the underlying mechanism remains unknown and, at present, we can only assume that it is less efficiently (if at all) working for the gammaretroviral vector.

The hypothesis that virus particles bind to the SEVI and subsequently enter the cell membrane independently of cellular receptors had to be negated. It was shown that GALV and RD114 envelopes failed to result in any appreciable gene transfer because both envelopes require a receptor for cell entry, which is missing on the surface of mouse cells, even in the presence of SEVI.

In addition, the effect of SEVI protein on the efficiency of gene transfer was compared with protamine sulfate and Polybrene. In our hands, Polybrene increased the efficiency of gene transfer for the RD114 and GALV-pseudotyped virus by 3.4- and 2.2-fold and protamine sulfate demonstrated an increase for the VSV-G, the GALV and the RD114 envelope of 3.2-, 1.9- and 1.8-fold, respectively. In other studies, the transduction efficiency in tumour cells was enhanced up to eight- to ten-fold by using either protamine sulfate or Polybrene [27]. VSV-G-pseudotyped vectors were tested on human neuronal cells and the maximum increase was 1.4-fold for Polybrene and 1.2-fold for protamine sulfate [28]. However, those experiments differed regarding cell type, vector systems, envelope, marker gene, experimental settings and other components. Thus, it is difficult to directly compare the effects of Polybrene and protamine sulfate from these studies with respect to transduction efficiency against the results obtained in the present study. Supplementation of Polybrene or protamine sulfate became a standard procedure in lentiviral gene transfer, leading to an increase of the efficiency of gene transfer. In the present study, we demonstrate that supplementation of SEVI also leads to a consistent and, importantly, a higher increase of the efficiency of gene transfer in a side-by-side comparison with Polybrene and protamine sulfate.

The effects of five different variants of PAPs, either isolated from human sperm or artificially modified, were evaluated in the present study. Taken together, a statistically significant increase of transduction efficiency was detected only for SEVI. Depending on the cell type and envelope employed, a smaller increase was also seen for the PAP variants 247–286 and 253–285. Most variants of PAP differ only by a few amino acids, although this could be a reason for the significant differences by which the protein influences the infectivity of virus particles. It is possible that PAP variants such as 247–282, which do not facilitate the virus infection, could partially protect against HIV infection in a natural way by competing with SEVI in virus binding.

Hematopoietic cells, including T cells and hematopoietic stem and progenitor cells, are frequently targeted for gene therapy studies. Polycations such as Polybrene and protamine sulfate are agents frequently used to enhance gene transfer but they are often toxic for primary cells [11]. Polybrene also inhibits proliferation of human hematopoietic progenitor cells [29,30]. A significant increase of the efficiency of gene transfer by SEVI was detected on human T cells.

The efficiency of gene transfer on human $CD34^+$ cells in liquid culture was increased for all viruses tested as a result of SEVI treatment. No toxicity of the SEVI protein was observed in sensitive cells such as human $CD34^+$ cells. Both in liquid culture and in colony assays, the cells showed a normal growth and differentiation rate. Furthermore, no toxicity of SEVI was detected in murine Lin^- cells.

In conclusion, in the present study, we have shown that the SEVI protein is a feasible and effective method for enhancing the efficiency of gene transfer. The SEVI protein is not toxic, even for sensitive cells such as hematopoietic stem cells. Especially, the GALV and the RD114 envelope show a clear improvement of transduction efficiency. GALV has already been used in several gene therapy trials [4,31] and RD114 is also a very promising alternative, because this envelope is resistant to human complement [21]. An improvement of lentiviral transduction facilitates a reduction of virus production in large-scale production, which is extremely complex and expensive. Moreover, this artificially constructed protein can be produced relatively simple according to good manufacturing practice and would thus be available for clinical purposes.

Acknowledgements

These studies were supported in part by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) for the Cluster of Excellence REBIRTH (From Regenerative Biology to Reconstructive Therapy) and by the Else Kröner Foundation (A.S.). The work of H.H. was supported by the DFG spp1230 (HA2322/2 – 1). D.L. is supported by the DFG (LI 621/3-3, LI 621/4-1) and the German Ministry of Science (BMBF) (BMBF-NBL3 Az. 01ZZ0102). P.A.H. is supported by the DFG (SE1093/2-1) and German Ministry of Science (BMBF) (BMBF-01GN0814). We would also like to thank Professor Christopher Baum from the Department of Experimental Hematology, Hannover Medical School, for providing viral vectors and support. Furthermore, we would like to thank Kirsten Elger and Andreas Zgraja for their excellent technical assistance.

References

1. Aiuti A, Cattaneo F, Galimberti S, *et al*. Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med* 2009; **360**: 447–458.
2. Beard BC, Keyser KA, Trobridge GD, *et al*. Unique integration profiles in a canine model of long-term repopulating cells transduced with gammaretrovirus, lentivirus, or foamy virus. *Hum Gene Ther* 2007; **18**: 423–434.
3. Cavazzana-Calvo M, Hacein-Bey-Abina S, Fischer A. Gene therapy of X-linked severe combined immunodeficiency. *Curr Opin Allergy Clin Immunol* 2002; **2**: 507–509.
4. Hacein-Bey-Abina S, Le Deist F, Carlier F, *et al*. Sustained correction of X-linked severe combined immunodeficiency

by ex vivo gene therapy. *N Engl J Med* 2002; **346**: 1185–1193.
5. Relander T, Johansson M, Olsson K, *et al*. Gene transfer to repopulating human CD34+ cells using amphotropic-, GALV–, or RD114-pseudotyped HIV-1-based vectors from stable producer cells. *Mol Ther* 2005; **11**: 452–459.
6. Kiem HP, Allen J, Trobridge G, *et al*. Foamy-virus-mediated gene transfer to canine repopulating cells. *Blood* 2007; **109**: 65–70.
7. Burns JC, Friedmann T, Driever W, *et al*. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc Natl Acad Sci USA* 1993; **90**: 8033–8037.
8. Yang J, Friedman MS, Bian H, *et al*. Highly efficient genetic transduction of primary human synoviocytes with concentrated retroviral supernatant. *Arthritis Res* 2002; **4**: 215–219.
9. Hanenberg H, Xiao XL, Dilloo D, *et al*. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat Med* 1996; **2**: 876–882.
10. Hanenberg H, Hashino K, Konishi H, *et al*. Optimization of fibronectin-assisted retroviral gene transfer into human CD34+ hematopoietic cells. *Hum Gene Ther* 1997; **8**: 2193–2206.
11. Cornetta K, Anderson WF. Protamine sulfate as an effective alternative to polybrene in retroviral-mediated gene-transfer: implications for human gene therapy. *J Virol Methods* 1989; **23**: 187–194.
12. Seitz B, Moreira L, Baktanian E, *et al*. Retroviral vector-mediated gene transfer into keratocytes in vitro and in vivo. *Am J Ophthalmol* 1998; **126**: 630–639.
13. Coelen R, Jose D, May J. The effect of hexadimethrine bromide (polybrene) on the infection of primate retroviruses SSV1/SSAV1 and BaEV. *Arch Virol* 1983; **75**: 307–311.
14. Swaney WP, Sorgi FL, Bahnson AB, *et al*. The effect of cationic liposome pretreatment and centrifugation on retrovirus-mediated gene transfer. *Gene Ther* 1997; **4**: 1379–1386.
15. Porter CD, Lukacs KV, Box G, *et al*. Cationic liposomes enhance the rate of transduction by a recombinant retroviral vector in vitro and in vivo. *J Virol* 1998; **72**: 4832–4840.
16. Sakoda T, Kasahara N, Kedes L, *et al*. Calcium phosphate coprecipitation greatly enhances transduction of cardiac myocytes and vascular smooth muscle cells by lentivirus vectors. *Exp Clin Cardiol* 2007; **12**: 133–138.
17. Munch J, Rucker E, Standker L, *et al*. Semen-derived amyloid fibrils drastically enhance HIV infection. *Cell* 2007; **131**: 1059–1071.
18. Royce RA, Sena A, Cates W Jr, *et al*. Sexual transmission of HIV. *N Engl J Med* 1997; **336**: 1072–1078.
19. Roan NR, Munch J, Arhel N, *et al*. The cationic properties of SEVI underlie its ability to markedly enhance HIV infection. *J Virol* 2009; **83**: 73–80.
20. Schambach A, Mueller D, Galla M, *et al*. Overcoming promoter competition in packaging cells improves production of self-inactivating retroviral vectors. *Gene Ther* 2006; **13**: 1524–1533.
21. Cosset FL, Takeuchi Y, Battini JL, *et al*. High-titer packaging cells producing recombinant retroviruses resistant to human serum. *J Virol* 1995; **69**: 7430–7436.
22. Stitz J, Buchholz CJ, Engelstadter M, *et al*. Lentiviral vectors pseudotyped with envelope glycoproteins derived from gibbon ape leukemia virus and murine leukemia virus 10A1. *Virology* 2000; **273**: 16–20.
23. Fields GB, Noble RL. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int J Pept Protein Res* 1990; **35**: 161–214.
24. Miller AD, Chen F. Retrovirus packaging cells based on 10A1 murine leukemia virus for production of vectors that use multiple receptors for cell entry. *J Virol* 1996; **70**: 5564–5571.
25. Rasko JE, Battini JL, Gottschalk RJ, *et al*. The RD114/simian type D retrovirus receptor is a neutral amino acid transporter. *Proc Natl Acad Sci USA* 1999; **96**: 2129–2134.
26. Logan AC, Nightingale SJ, Haas DL, *et al*. Factors influencing the titer and infectivity of lentiviral vectors. *Hum Gene Ther* 2004; **15**: 976–988.
27. Clark PR, Stopeck AT, Brailey JL, *et al*. Polycations and cationic lipids enhance adenovirus transduction and transgene expression in tumor cells. *Cancer Gene Ther* 1999; **6**: 437–446.
28. Lee H, Song JJ, Kim E, *et al*. Efficient gene transfer of VSV-G pseudotyped retroviral vector to human brain tumor. *Gene Ther* 2001; **8**: 268–273.
29. Dybing J, Lynch CM, Hara P, *et al*. GaLV pseudotyped vectors and cationic lipids transduce human CD34+ cells. *Hum Gene Ther* 1997; **8**: 1685–1694.
30. Flasshove M, Banerjee D, Mineishi S, *et al*. Ex vivo expansion and selection of human CD34+ peripheral blood progenitor cells after introduction of a mutated dihydrofolate reductase cDNA via retroviral gene transfer. *Blood* 1995; **85**: 566–574.
31. Gaspar HB, Parsley KL, Howe S, *et al*. Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* 2004; **364**: 2181–2187.

DOI: 10.1002/jgm