Production and purification of high-titer foamy virus vector for the treatment of leukocyte adhesion deficiency

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INTRODUCTION

Foamy viruses (FVs), also known as spumaretroviruses, derive their name from the vacuolating foamy-like cytoplasm of productively infected cells and multinucleated syncytia. They are endemic in a number of mammals, including cats, cows, and captive non-human primates, but not found in humans. Despite their highly cytopathic nature in cell culture, they are not associated with any detectable disease in infected hosts. The development of leukemia in X-linked severe combined immunodeficiency patients and occurrence of myelodysplastic syndrome in chronic granulomatous disease patients caused by gamma-retrovirus vector-mediated insertional mutagenesis after ex vivo stem cell gene therapy has stimulated the development of vectors with improved safety profiles for clinical application. FVs have several distinct advantages over other integrating viral vectors such as gamma-retroviruses and lentiviruses as a gene transfer tool. These include a large packaging capacity (up to 12 Kb) and a broad host and cell-type tropism. Furthermore, FVs can efficiently transduce quiescent cells, since the FV genome can persist in a stable form as cDNA in growth-arrested cells/quiescent cells and can integrate into the host genome when the cells exit the G0 phase of the cell cycle. In addition, as compared to gamma-retrovirus or lentivirus, FV has a safer integration profile with lower risk of insertional mutagenesis. Foamy virus vectors have been used to correct genetic disorders of hematopoietic stem cells in several animal models, including leukocyte adhesion deficiency (LAD) in dogs, and Wiskott–Aldrich syndrome, Fanconi anemia, and X-linked chronic granulomatous disease in mice.

Patients with LAD type 1 (LAD-1) and dogs with canine LAD suffer from recurrent and life-threatening bacterial infections. Both diseases are caused by mutations in the leukocyte integrin CD18 subunit that prevent the formation and surface expression of CD11/CD18 heterodimeric adhesion molecules resulting in an inability of leukocytes to adhere to the endothelium and migrate toward the sites of infection.

Successful gene therapy of canine LAD was demonstrated in four dogs transplanted with autologous CD34+ cells transduced by FV vectors expressing canine CD18. However, the low titers typically obtained with FV vectors have precluded their use for clinical application in LAD-1 patients. In addition, processes used previously were not scalable and not compatible with the large-scale manufacturing needed for clinical application. Major obstacles for scale-up of FV vector production and purification include: (i) the low titer of
calcium phosphate-mediated transfection commonly used in gene transfer vector production, (ii) the limited stability of FV vectors in ambient or high temperature, acidic, or basic pH, and high salt concentrations, (iii) their sensitivity to shear forces, and (iv) the necessity to freeze FV vectors in 5% dimethyl sulfoxide (DMSO) and consequently to significantly dilute the vector to minimize toxicity to stem and progenitor cells during transduction.

In this study, we have successfully addressed each obstacle for large-scale manufacturing of FV vectors compatible with current good manufacturing practices. We first improved vector production by optimizing transfection with the use of polyethylenimine (PEI) and by varying parameters of producer cell culture, plasmid concentration, and harvest time. We next improved vector purification with the use of heparin affinity chromatography since heparan sulfate was identified as a receptor for FV, and chromatography-based purification methods are scalable and can be performed in a closed system compatible with production of clinical-grade vectors. Finally, we used tangential flow filtration (TFF) and ultra-centrifugation for the final step of vector concentration. This optimized process resulted in highly concentrated FV vectors carrying the human CD18 cDNA (FV-hCD18) that can now be scaled up for clinical application.

RESULTS
Optimization of transfection conditions to maximize FV titers
FV vectors were previously produced by calcium phosphate-mediated transient transfection of HEK293T cells with helper (gag, pol, and env) and gene transfer vector plasmids. Unconcentrated titers of FV-GFP were $1.2 \pm 0.2 \times 10^5$ infectious units (IU)/ml as determined on HT1080 cells and those of FV-hCD18 were $1.7 \pm 0.1 \times 10^3$ IU/ml as determined on RAW264.7 cells. We have recently published that PEI-mediated transfection resulted in up to a 50-fold increase in FV vector titers over calcium phosphate transfection. In this study, PEI-mediated transfection was further optimized to maximize FV-GFP and FV-hCD18 vector titers. For both FV-GFP (Figure 1a) and FV-hCD18 (Figure 1b) vectors, titers improved with increasing concentrations of PEI, with a peak titer at 70–80 μg PEI per T75 flask. Further increases in PEI led to reduced titers (Supplementary Figure S1). After optimization, 70 μg of PEI per T75 flask was used during FV vector production in all experiments.

We also evaluated the effect of poly-L-lysine coating of culture plastic on FV-GFP vector production. Coating culture plastic with 0.1% of poly-L-lysine prior to seeding HEK293T cells significantly increased FV vector titers (Figure 1c). Although it has been suggested that a 15 minutes PEI-DNA precipitation time is optimal for high-titer FV vector production, our current data showed that a 10 minutes precipitation time yielded the highest titers (Figure 1d). Calcium phosphate-mediated transfection requires a medium change the next day to limit cellular toxicity and increase FV vector titers. Similarly, we tested whether a change in medium after PEI-mediated transfection would also increase FV vector titers. Unexpectedly, this actually decreased FV vector titers by twofold to fivefold (Figure 1e). It is not clear whether this is due to a physiological response of the cells or related to a prolonged exposure to PEI and plasmid. Irrespective, we adopted a protocol in which the transfection medium containing PEI was not removed posttransfection but left with the cells until harvesting the vector. In addition, we optimized the harvest time for FV vectors after transfection of the producer cells. FV-hCD18 vectors were sampled from 24 to 93 hours posttransfection without medium change and titered. In our hands, harvesting of FV vectors around 66 hours posttransfection yielded the highest titers (Figure 1f).

We next compared pCiGSΔΨ (original gag) and pCiGAopt (codon optimized gag) plasmids for FV-hCD18 vector production (Figure 2a,b). We previously observed that transfection of HEK293T cells with 10.4 μg of pCiGSΔΨ per T75 flask resulted in optimal FV-hCD18 vector titers (data not shown). However, significant toxicity to HEK293T was observed when the same amount of pCiGAopt was transfected, resulting in a 10-fold reduction in FV-hCD18 titers (Figure 2a). When amounts of pCiGAopt were reduced from 10.4 to 1.3 μg per T75 flask in transfection, the titers of FV-hCD18 vectors increased proportionally (Figure 2a). In a follow-up study, the highest FV titer was obtained with 0.65 μg of pCiGAopt plasmid (Figure 2b). Thus, the use of codon optimized gag resulted in doubling of the FV-hCD18 vector titers while using 16-fold less plasmid as compared to the previously optimized amount of pCiGSΔΨ.

Benzonase treatment of cultures posttransfection to reduce residual plasmid
Benzonase endonuclease is commonly used to reduce the amount of residual plasmid and cellular genomic DNA and RNA in the vector product. Treatment of FV vectors for 16 hours with increasing concentrations of Benzonase had only minimal impact on vector titers (Supplementary Figure S2a). Longer exposure (40 hours) with 50 U/ml Benzonase further reduced FV vector titers minimally (Supplementary Figure S2b). While differences were not statistically significant, we chose a 16-hour exposure of Benzonase at 50 U/ml to limit the potential impact of Benzonase on FV titers. Overall, when all optimized conditions are combined, nonpurified, and unconcentrated FV-hCD18 titers of $\sim 1 \times 10^7$ IU/ml were consistently obtained, a 50-fold increase compared to titers obtained with the nonoptimized protocol.

Purification of FV vectors using heparin affinity chromatography
Since membrane-associated heparan sulfate, a heparin-related molecule, is a receptor for FV in cells, we hypothesized that FV vector particles could be purified by heparin affinity chromatography. We evaluated the binding, washing, and elution conditions needed for effective purification of FV vector. Prior to chromatography, nuclease-treated FV vector supernatants were filtered through a 0.45 μm filter to remove any coarse cellular debris. Vector supernatants were subsequently loaded onto a 7.9 ml bed volume POROS-OH 50 μm heparin affinity chromatography column at a linear flow rate of 267 cm/hour and a residence time of 2.3 minutes. Faster flow rates and shorter residence time resulted in FV vector into the flow-through fraction (data not shown). After loading, the heparin column was washed with sodium phosphate or Tris-HCl buffer containing 150 mmol/l sodium chloride (pH 7.0). The washing step was continued until the ultraviolet absorbance curve (280 nm) returned to baseline and became stabilized. To evaluate elution conditions, bound virus particles were eluted using a salt gradient from 100 mmol/l to 1.0 mol/l NaCl (pH 7.0). The optimal NaCl concentration for elution was determined based on the presence of infectious FV-GFP particles in individual chromatography fractions as measured by ELISA on HT1080 cells and sample conductivity which correlated to NaCl concentration (Supplementary Figure S3). We found that most of the FV-hCD18 was eluted at 600 mmol/l of NaCl (Figure 3). In addition, we did not observe any significant loss of FV particles in the flow-through during loading and washing. The average recovery of FV vector in the elution fraction was 69 ± 6% (n = 5) as shown in Table 1.
Concentration of FV vectors
TFF is a rapid, efficient, and scalable method for concentration of small and large volumes of biological samples. Here, we used TFF as a method to concentrate heparin affinity chromatography purified FV vector. Ultrafiltration was performed by recirculating the sample at 280 ml per minute through a TFF cartridge with a 750 KDa nominal cut off using a trans-membrane pressure between 5 and 6 psi. Vector particles were retained within the membrane, whereas proteins smaller than 750 kDa were removed resulting in concentration and further purification of the vector. Vector was subsequently diafiltered using 100 ml of 150 mmol/l NaCl, 25 mmol/l Tris–HCl (pH 7.4) buffer. This step changed the concentration of salt to a physiological level. Using TFF, vectors were concentrated 20- to 30-fold with an average recovery of 89 ± 13% (n = 5) as shown in Table 2. The material was subsequently concentrated by ultracentrifugation at 50,000 g for 2 hours. Pellets were resuspended in final formulation buffer consisting of X-VIVO 10, 1% human serum albumin, and 5% DMSO. This last step concentrated the vector an additional 60-fold with 48 ± 14% (n = 5) recovery (Table 2). Overall, using the optimized conditions established for heparin affinity chromatography, TFF, and ultracentrifugation, the FV vectors were concentrated ~5,000-fold with a net recovery of 19 ± 3.1% (n = 5).

FV-hCD18 vector transduction
We next tested the ability of purified FV-hCD18 vectors to transduce granulocyte-colony stimulating factor (G-CSF)–mobilized peripheral blood CD34+ cells obtained from two subjects diagnosed with LAD-1, using two independent FV-hCD18 vector pilot batches.
cryopreservation, the highly concentrated FV-hCD18 vector was since DMSO must be added for optimal recovery of FV vectors after allowing maximal detection of CD18 expression by flow cytometry. Cells were cultured in the presence of cytokines with 600 mmol/l NaCl. Infectious unit (IU) of FV vectors was estimated with gray squares showing the volume of FV sample loaded; the line with dark diamonds shows the total infectious units of FV-hCD18 vector in each fraction (43.5 µg of pCiGSΔΨ). (Figure 4). CD34+ cells were cultured in the presence of cytokines on Retronectin-coated plates and transduced for 16 hours with concentrated and purified FV-hCD18 vector at various dilutions. Cells were washed and continued in culture for an additional 3 days to allow maximal detection of CD18 expression by flow cytometry. Since DMSO must be added for optimal recovery of FV vectors after cryopreservation, the highly concentrated FV-hCD18 vector was diluted to reduce DMSO concentration to ≤0.1% to limit the toxicity to CD34+ cells during transduction. Increasing doses of DMSO, especially with a prolonged exposure are well known to be toxic to murine and human hematopoietic cells and other types of stem cells, including human embryonic stem cells. We confirmed these results and observed reduced viable CD34+ cells when DMSO concentrations exceeded 0.1% (Supplementary Figure S4). For both subjects, percentages of transduction in bulk CD34+ cells increased proportionally with increasing volumes of FV vector. Subject 1 has a moderate clinical phenotype and 18.7% of CD34+ cells expressed CD18 at baseline; CD18+ cells increased to 77.4% (i.e., 59% over baseline CD18 expression) after transduction at the highest MOI of FV vector tested. This level was similar to baseline CD18+ cells after transduction at the highest MOI of FV vector tested. This level was similar to baseline CD18 expression) after transduction at the highest MOI (87.3%) measured in mobilized peripheral blood CD34+ cells from a healthy subject (Figure 4, upper panel). In subject 2 with a severe clinical phenotype and 21.2% of LAD-1 CD34+ cells expressed CD18 after transduction, compared to untransduced LAD-1 CD34+ cells. Overall, these experiments provide proof of principle that clinical-grade high-titer FV vectors can be produced and purified.

**Table 1** Recovery of FV-hCD18 vector after each step of heparin affinity chromatography run

| Step | % of recovery (average ± SEM)** | n  |
|------|-------------------------------|----|
| Pre-load | 100 ± 0 | 5  |
| Loading | 4 ± 1.8 | 5  |
| Washing | 0 ± 0 | 5  |
| Elution | 69 ± 2.7 | 5  |

*Data represent mean and SEM of five independent experiments.

**Table 2** Estimated recovery from 1 l of FV-hCD18 vector after each step of purification

| Step                | Volume of vector (ml) | Processing time (average ± SEM)* | % of step recovery | n  |
|---------------------|-----------------------|---------------------------------|--------------------|----|
| Heparin column      | 333.3                 | 5 hours                         | 69 ± 2.7           | 5  |
| TFF                 | 11.9                  | 45 minutes                      | 89 ± 5.8           | 5  |
| 0.2 m filter        | 11.9                  | 15 minutes                      | 84 ± 4.5           | 5  |
| Ultracentrifugation | 0.2                   | 2 hours                         | 48 ± 6.3           | 5  |
| Net recovery        | 0.2                   | 8 hours                         | 19 ± 3.1           | 5  |

*Data represent mean and standard error of mean (SEM) of five independent experiments. *Total processing time.
for efficient transduction of LAD CD34+ cells with minimal DMSO-related toxicities.

**DISCUSSION**

FV vectors represent a potentially safer alternative to currently used integrating viral vectors for gene therapy application. However, approaches customarily used to manufacture large-scale lentiviral vector for clinical application have resulted in low titers for FV vectors,6,7 hampering their clinical development. In this study, we have presented process development with a step-by-step optimization of FV vector production and purification (Figure 5).

PEI-mediated transfection of FV plasmids into HEK293T cells significantly increases the titers over those achieved with calcium phosphate.11 PEI has the ability to avoid trafficking to degradative lysosomes and its buffering capacity leads to osmotic swelling and rupture of endosomes, resulting in release of the vector particles into the cytoplasm and subsequently to the culture medium.29 PEI has a high cationic charge density at physiological pH due to partial protonation of the amino groups in every third position. These amino groups form noncovalent complexes with negatively charged DNA, which leads to condensation and shielding of the negative charges, thereby allowing endocytosis into the cells, resulting in efficient transfection of vector producer cells.8,9

Substantial plasmid DNA contamination is carried over in vector supernatants produced by transient transfection.11 Plasmid DNA present in vector supernatants artificially increases the PCR-based titer of vectors and may be toxic to primary cells such as hematopoietic stem and progenitor cells exposed to the concentrated vectors. Nucleic acids also result in increased supernatant viscosity which interferes with purification steps and reduces vector titers. Addition of benzonase endonuclease during FV vector production allowed complete digestion of all forms of DNA and RNA to 5'-monophosphate terminated oligonucleotides 2 to 5 bases in length.32 It is effective over a wide range of temperature and pH and has no proteolytic activity, providing a simple approach to enhance FV vector production. Our data supports that Benzonase endonuclease can be safely used in the manufacture of FV vector without significant loss of infectious titer.

Commonly used purification methods such as ultracentrifugation can precipitate FV particles along with cellular debris and serum
ceptibility of retroviruses to osmotic shock36 and limited stability of
stability requires relatively low salt concentrations for dissocia-
reversibly, requiring relatively low salt concentrations for dissocia-
is also reduced. Diffusion is no longer limiting and flow rates can be
Carrying sample molecules to short "diffusive" pores inside. By
convection flow to occur through the particles themselves, quickly
have two discreet classes of pores. Large "through pores" allow
medium, POROS perfusion chromatography medium is engineered
with PEI. Cultures were treated with Benzonase for 16 hours prior to
supernate was filtered, purified with heparin affinity chromatography and filtered, concentrated, and diafiltered with TFF, sterile filtered, and concentrated aseptically using ultra centrifugation. FV vector supernatants were stored at –80°C in the presence of 5% DMSO.
proteins33 which can be toxic to the target cells. Heparin affinity
medium strongly binds only those particles that have affinity for
hemiparin molecules.34,35 Unbound and loosely bound material pres-
ent in FV supernatant, including cellular debris and serum proteins, elute in the flow-through during sample loading and washing with
low salt containing buffer. The FV-heparin interaction is stable but
reversible, requiring relatively low salt concentrations for dissocia-
as demonstrated here. This is important in considering the sus-
cceptibility of retroviruses to osmotic shock36 and limited stability of
FV vectors in high salt (data not shown).
In contrast to conventional heparin affinity chromatography
medium, POROS perfusion chromatography medium is engineered
to have two discreet classes of pores. Large "through pores" allow
convection flow to occur through the particles themselves, quickly
carrying sample molecules to short "diffusive" pores inside. By
reducing the distance over which diffusion needs to occur, the time
required for sample molecules to interact with interior binding sites
is also reduced. Diffusion is no longer limiting and flow rates can be
dramatically increased without compromising resolution or capac-
ity. Separation can be achieved at speeds up to 100-fold faster as
compared to conventional heparin medium.37 We have carefully
optimized the binding conditions and found POROS-Heparin to be
superior in its ability to effectively capture FV particles as compared
to Heparin-Sepharose medium such as Hi-Trap Heparin (data not
shown).
The stability of vectors is strongly dependent on ultrafiltration
parameters such as trans-membrane pressure, shear, and process
run duration.38 These parameters were optimized to maximize the
concentration and recovery of FV vector. Although higher shear
forces were helpful in reducing membrane fouling, these reduced
vector titer (data not shown). Shear values between 2,000 and
3,000 s−1 resulted in an 89% recovery of infectious virus particles in
our study. Membrane fouling was not an issue since most of the pro-
teins were removed during the chromatography run. Since TFF is a
closed system and ultracentrifugation tubes are sealed prior to the
centrifugation step, both are compatible with clinical grade vector
production.21,22
After optimization of the process, two pilot batches of FV vectors
produced showed 21–59% transduction efficiencies in G-CSF mobi-
ilized CD34+ cells derived from two LAD-1 subjects. In a preclinical
gene therapy study of canine LAD, clinical benefit was observed
with CD18 gene marking of 14–25% in bulk canine hematopoietic stem
and progenitor cells after transduction,9,39 suggesting clinically relevant transduction efficiencies were achieved. FV vec-
tor cryopreservation necessitates 5% DMSO[1]30 and, therefore,
the time required for cryopreservation of CD34+ cells was not feasible due to DMSO-induced toxicity on target CD34+ cells
( Supplementary Figure S4). Despite nearly identical vector copy number between subjects 1 and 2, expression of CD18 was quite
different. The timing of flow cytometry for optimal CD18 gene
expression in bulk CD34+ cells after transduction may vary between
patients. For consistency, we have chosen a period of 72 hours for
both subjects but this may not be optimal for subject 2. Given the
scarcity of LAD CD34+ cells, kinetic expression studies are impracti-
cal. Other explanations related to molecular differences (different
mutations), phenotypic differences (subject 1: moderate; subject 2:
severe), age differences (subject 1:19YO; subject 2:33 YO), or tech-
nical differences (widely different duration of cryopreservation of
CD34+ cells, 4 years versus 1 month) between subjects 1 and 2 can-
not be entirely ruled out. Given that transduction differed between
the two patients tested here, it may be helpful to examine trans-
duction efficiencies of patients CD34+ cells prior to gene therapy to
optimize clinical transduction and even attempt correlating with
heparan sulfate expression. If differences in transduction correlate
with heparan sulfate levels, heparan sulfate expression may be used
as a marker to predict transducibility. Based on the average FV titers
using this methodology and the data in Figure 4, where 450,000
cells transduced at 21.2% with 6 µl FV vector, transduction of 250
million cells (to treat a 50 kg individual with 5×10^6 transduced cells/
kg) will require approximately 3 ml of 5,000-fold concentrated vec-
tor. This represents the equivalent of approximately 15 l of initial cul-
ture volume per patient, which is feasible from the manufacturing
standpoint. In addition, canine data and some of our unpublished
results show that transduction efficiencies of ~20% are sufficient for
long-term correction of LAD. Therefore, the FV vector production
process described in this study paves the way to scale-up FV pro-
duction for clinical manufacturing of FV-hCD18 vectors for a clinical
trial in LAD-1 patients.

**MATERIALS AND METHODS**

**Plasmids**
Self-inactivating FV gene-transfer vector plasmids pΔΦ-MSCV-green fluores-
rescent protein (GFP) and pΔΦ-MSCV-huCD18, as well as packaging gene
plasmids pCiGSS2W (gag), pCiGAGopt (codon optimized gag), pCiPS (pol),
and pCIES (env) (Supplementary Figure S5) were constructed by Dr David
Russell.7 FV gene transfer, gag, pol, and env vector plasmids were used at a
ratio of 14:14:2.1. When gag plasmid pCiGAGopt was used instead of
pCiGSS2W, a 16-fold lower concentration of the plasmid was used for optimal
titer. Plasmids were manufactured by Puresyn (Malvern, PA).

**Cell culture**
Human embryonic kidney cell line HEK293T, mouse macrophage cell line RAW
264.7, and human fibrosarcoma cell line HT1080 were grown in Dulbecco's
modified Eagle's medium, high glucose, (DMEM; Invitrogen, San Diego, CA)
supplemented with 10% fetal bovine serum, 1 mmol/l l-glutamax, 1 mmol/l
sodium pyruvate, and 1 mmol/l nonessential amino acids (Invitrogen, San
Diego, CA). Human CD34+ cells from two LAD-1 patients were cultured in

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**Figure 5** Flow diagram of FV vector production and purification. HEK293T producer cells were seeded in cell culture vessels treated with
d poly-L-Lysine and FV vector plasmids were transfected into the cells with
pepT1. Cultures were treated with Benzonase for 16 hours prior to
vector harvest. FV supernate was filtered, purified with heparin affinity
chromatography and filtered, concentrated, and diafiltered with TFF, sterile filtered, and concentrated aseptically using ultra centrifugation. FV
vector supernatants were stored at –80°C in the presence of 5% DMSO.

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vector harvest. FV supernate was filtered, purified with heparin affinity
chromatography and filtered, concentrated, and diafiltered with TFF, sterile filtered, and concentrated aseptically using ultra centrifugation. FV
vector supernatants were stored at –80°C in the presence of 5% DMSO.
for GFP expression or stained with mouse anti-human CD18-APC (Clone 6.7, BD Biosciences, San Diego, CA) diluted in 1% bovine serum albumin (BSA) in PBS. Flow cytometry analysis was performed on a flow cytometer (LSR-Fortessa, BD Biosciences). Titers (IU per ml) were calculated based on the number of cells at the time of infection, the dilution factor, and percentage of GFP+ or CD18+ cells.

Mobilization, apheresis, and purification of human LAD-1 CD34+ cells

Two subjects with LAD-1 received G-CSF 10 μg/kg (Amgen, Thousand Oaks, CA) for 5 days, given as a single daily s.c. injection. Large volume (15 l) leukopheresis was initiated on the morning of day 5 of G-CSF administration, using a blood cell separator (Cobe Spectra, Terumo BCT, Lakewood, CO). The mononuclear cell (MNC) concentrates were enriched in CD34+ cells using a semi-automated CliniMACS Plus instrument (Miltenyi Biotec, Auburn, CA) and cryopreserved prior to transduction. All subjects gave written informed consent on treatment protocols approved by the Institutional Review Board (IRB) of the National Heart, Lung and Blood Institute (NHLBI), National Institutes of Health (NIH), in accordance with the Declaration of Helsinki.

Transduction of human LAD-1 CD34+ cells

Human LAD-1 CD34+ cells (450,000 cells/well) were transduced with different vectors of MSCV-hCD18 in 36 cm2 cell culture plates coated with Retronectin 5 μg/cm2 (Takara, Shiga, Japan). Plates were subjected to spinoculation at 300 g for 5 minutes and incubated overnight (16–17 hours) at 37 °C. The following morning, FV vector supernatant was removed and fresh CD34+ cell culture medium was added. Three days posttransduction, cells were collected by gentle scraping, stained with anti-human CD18-FITC antibody (clone 6.7, BD Biosciences, San Jose, CA), and analyzed by flow cytometry using a LSR Fortessa instrument (BD Biosciences).

Real-time PCR for vector copy number determination

The presence of CD18 proviral sequences in genomic DNA isolated from CD34+ cells after transduction was determined using the ABI PRISM 7500 Real-Time PCR System (Life Technologies, Grand Island, NY). Briefly, primers MSCV-F (5′-AGTCTCCTCGATAGACTGC GT-3′) and CD18-R (5′-CTTCTGAGCATTCTGAGAGA-3′) amplified a vector-specific 123-bp fragment spanning the MSCV promoter and hCD18 cDNA. Amplification was detected with the MSCV-CD18 probe (5′-56FAM-CTCCTCGATAGACTGC GT-36BHQ-3′). The human albumin gene was used as an endogenous control for data normalization. Primers Hs Albumin-F (5′-GCT CTC CTG CCT GTT CTA TA-3′) and Hs Albumin-R (5′-GGATTCTGTG CAGCATTTGG-3′) amplified a 123-bp fragment spanning the intron 11-exon 12 junction of the human albumin gene. Amplification was detected with the Hs Albumin probe (5′-56FAM-CCGTCCCTGAGAGA-3′). Amplification of plasmids containing cloned target sequences of both MSCV-hCD18 or Hs Hs Albumin intron 11-exon 12 junction was used to prepare a standard curve to quantify the number of FV-hCD18 vector integrations per diploid genome. For multiplex pPCR reactions, the FV- and albumin-specific amplicon primers were used in combination with the FAM-labeled, vector-specific TaqMan probe (MSCV-CD18) described above and the following albumin gene-specific TaqMan probe: 5′-56JOE N HS/CCTGAGAGA CC/56FAM-CTCCTCGATAGACTGC GT-3′. Samples underwent denaturation at 95 °C for 10 minutes, followed by 40 cycles of amplification (15 seconds at 95°C, 1 minute at 60°C).

Statistical analysis

Statistical analysis was done using a two-tailed Student’s t-test. A P value of ≤ 0.05 was considered statistically significant.

CONFLICT OF INTEREST

We declare that there is no financial conflict of interest in our work.

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