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Efficient Lentiviral Transduction and Improved Engraftment of Human Bone Marrow Mesenchymal Cells

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Key Words. Mesenchymal cell • Lentiviral • Bone marrow

ABSTRACT

Human bone marrow (BM) mesenchymal stem/progenitor cells are potentially attractive targets for ex vivo gene therapy. The potential of lentiviral vectors for transducing BM mesenchymal cells was examined using a self-inactivating vector that expressed the green fluorescent protein (GFP) from an internal cytomegalovirus (CMV) promoter. This vector was compared with oncoretroviral vectors expressing GFP from the CMV promoter or a modified long-terminal repeat that had been optimized for long-term expression in stem cells. The percentage of GFP-positive cells was consistently higher following lentiviral versus oncoretroviral transduction, consistent with increased GFP mRNA levels and increased gene transfer efficiency measured by polymerase chain reaction and Southern blot analysis. In vitro GFP and FVIII expression lasted for several months post-transduction, although expression slowly declined. The transduced cells retained their stem/progenitor cell properties since they were still capable of differentiating along adipogenic and osteogenic lineages in vitro while maintaining high GFP and FVIII expression levels. Implantation of lentivirally transduced human BM mesenchymal cells using collagen scaffolds into immunodeficient mice resulted in efficient engraftment of gene-engineered cells and long-term transgene expression in vivo. These biocompatible BM mesenchymal implants represent a reversible, safe, and versatile protein delivery approach because they can be retrieved in the event of an unexpected adverse reaction or when expression of the protein of interest is no longer required. In conclusion, efficient gene delivery with lentiviral vectors in conjunction with the use of bioengineered reversible scaffolds improves the therapeutic prospects of this novel approach for gene therapy, protein delivery, or tissue engineering. STEM CELLS *2006;24: 896–907*

INTRODUCTION

Human bone marrow (BM) mesenchymal cells are attractive target cells for ex vivo gene therapy for a broad range of diseases [1]. They are derived from the nonhematopoietic BM compartment and contain, apart from differentiated mesenchymal cells, multipotent primitive stem cells such as the mesenchymal stem cells (MSCs) and the multipotent adult progenitor cells (MAPCs). These MSCs and MAPCs can give rise to distinct mesenchymal lineages and can self-renew by proliferation without differentiation and thereby maintain their stem-cell phenotype [2–4]. In addition, in vitro engineered BM mesenchymal cells can engraft long term in vivo without myeloablative conditioning [5, 6] and can lead to sustained transgene expression [7, 8].

The development of efficient gene delivery systems is warranted to achieve high expression levels of potentially therapeutic transgenes in BM mesenchymal cells. This would obviate the need for selective enrichment and long-term culture that may contribute to senescence or compromise their long-term engraftment efficiency and/or multipotency [9]. In addition, by increasing gene transfer efficiency, fewer cells may be required to achieve a therapeutic effect. This justifies the use of lentiviral vectors for transducing BM mesenchymal cells, by virtue of their ability to transduce both dividing and nondividing cells.

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Furthermore, lentiviral vectors might be less prone to transcriptional silencing than oncoretroviral vectors, particularly in hematopoietic stem cells and embryonic stem cells [10, 11]. Lentiviral vectors integrate stably into the target cell genome, which guarantees that the transgene is transmitted to the progeny of a transduced cell in an expanding BM mesenchymal cell population. Transduction with adenoviral vectors is generally less efficient and requires very high vector doses [12].

The present study aimed at determining whether transduction efficiency of human BM mesenchymal cells could be improved when HIV-1 derived lentiviral vectors are used compared with oncoretroviral vectors, and if so, whether this would improve transgene expression levels and/or the duration of gene expression. Because the BM mesenchymal cell fraction contains mesenchymal stem/progenitor cells, transduction with lentiviral vectors could give rise to prolonged transgene expression in differentiated progeny cells belonging to distinct lineages. The differentiation potential and transgene expression of lentivirally transduced BM mesenchymal cells was therefore analyzed. Because engraftment of engineered BM mesenchymal cells typically results in rapid clearance and limited and/or short-term engraftment [5], we also investigated the potential of using retrievable, collagen-based scaffolds to improve the long-term engraftment of lentivirally transduced BM mesenchymal cells.

Materials and Methods

Isolation and In Vitro Culture of Human BM Mesenchymal Cells

Human BM was isolated and purified as described previously [5]. Cells were subsequently cultured in Dulbecco's modified Eagle's medium (DMEM)-low glucose (1,000 mg/l glucose) or Iscove's modified Dulbecco's medium (Invitrogen, Merelbeke, Belgium, http://www.invitrogen.com) supplemented with hydrocortisone (10^{-6} M), 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. Human BM mesenchymal cells were either seeded at a density of $0.8–1.5 \times 10^5$ cells per well in six-well plates containing 2 ml of medium per well or in cell trays (Nunc, Invitrogen) at a density of $10–12 \times 10^6$ cells in 100 ml medium per cell tray.

Viral Vector Production

The MND-GFP-SN vector, which expresses the GFP from the modified MND long-terminal repeat (LTR) and contains a simian virus-40 neo^R cassette, was a kind gift of Dr. D. Kohn (Children's Hospital of Los Angeles, Los Angeles, CA) (Fig. 1A) [13]. The oncoretroviral vector expressing the B-domain deleted FVIII cDNA from the murine leukemia virus (MLV) LTR was described previously [14]. The MLV-$_{SIN}$-cytomegalovirus (CMV)-GFP vector was generated by cloning a 1.3-kb BamHI-Asp718I (Klenow blunt ended) fragment from the pHIV$_{SIN}$-CMV-GFP plasmid [15, 16] (see below), which contains the CMV-GFP-woodchuck hepatitis virus (WPRE) expression cassette, into the self-inactivating pQCXIX vector backbone (Clontech, Palo Alto, CA, http://www.clontech.com; Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) (Fig. 1B). The 293T cell line was cultured in D10 medium. Cells were seeded in cell factories (Nunc, Invitrogen) and transiently transfected at 90%–95% confluency using a modified calcium-phosphate

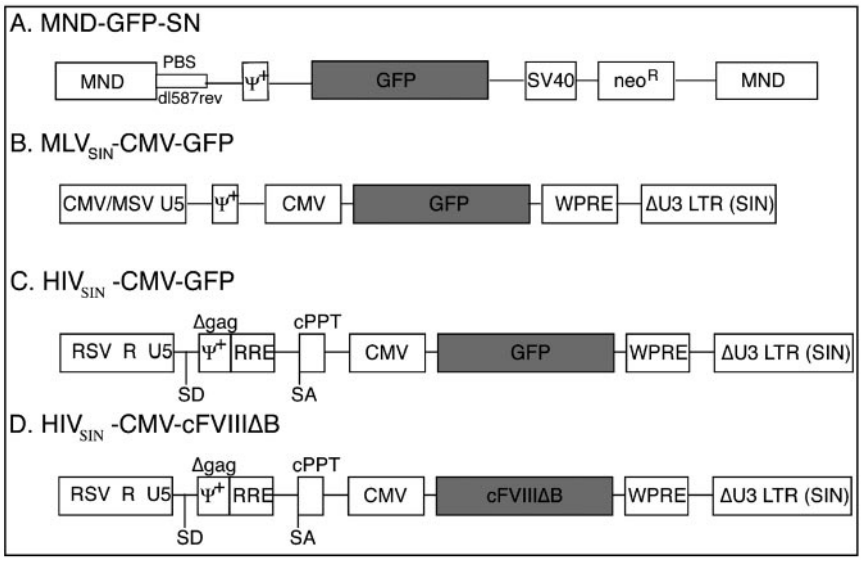


Figure 1. Schematic diagram depicting the oncoretroviral and lentiviral vector design. The MND-GFP-SN vector expresses the *GFP* from the modified *MND* long-terminal repeat (LTR) and contains a *SV40-neo^R* cassette **(A)**. In the lentiviral vector backbone *HIV$_{SIN}$-CMV-GFP* **(C)**, the internal *CMV* promoter drives the *GFP* gene or the canine B-domain deleted *FVIII cDNA (cFVIIIΔB).* The *WPRE* and *cPPT* or DNA flap have been incorporated in the vector to improve vector performance. The Rev-responsive element (*RRE*), splice donor and acceptor sites (*SD, SA*), and packaging signal (Ψ) are shown. The lentiviral vector is self-inactivating by virtue of the deletion in the *3′ LTR (ΔU3).* The full-length genomic RNA is expressed from the *CMV-RU5* chimeric promoter in the packaging cells. The oncoretroviral vector *MLV$_{SIN}$-CMV-GFP* **(B)** drives the *GFP* gene from the *CMV* promoter and contains the WPRE. The oncoretroviral vector is, similar to the lentiviral vector, self-inactivating. Abbreviations: CMV, cytomegalovirus; cPPT, central polypurine tract; GFP, green fluorescent protein; MLV, murine leukemia virus; MND, myeloproliferative sarcoma virus enhancer, negative control region-deleted, dl587rev primer-binding site-substituted; PBS, phosphate buffered saline; RSV, Rous sarcoma virus; SIN, self-inactivating; SN, simian virus-40 (SV40) neo^R cassette; WPRE, woodchuck post-transcriptional regulatory element.

transfection protocol (Invitrogen). The following amount of DNA was used per cell factory: 4 mg of $pMLV_{SIN}$-CMV-GFP or pMND-GFP-SN, 1.5 mg of pMD-GP (gag-pol) and 1.5 mg of pCI-VSV-G (VSV-G envelope). The pMD-GP and pCI-VSV-G plasmids were kindly provided by Dr. D. Ory (University of Washington, St. Louis). Twenty-four hours after transfection, cells were washed with PBS, fresh medium was added, and viral vector-containing supernatant was collected at 24-hour intervals and snap-frozen for later use. Vector titers were determined by RNA dot blot analysis.

The lentiviral *HIV_{SIN}-CMV-GFP* vector has been described previously and was similar to a self-inactivating second-generation construct [17], except that an additional 118-bp sequence containing the central polypurine tract (*cPPT*) and central termination sequences and a post-transcriptional regulatory element from WPRE had been introduced in the vector backbone to augment transgene expression and/or viral vector titer [15, 16] (Fig. 1C) (kindly provided by Dr. Luigi Naldini, San Rafaelle Institute, Milano, Italy). The *HIV_{SIN}-CMV-cFVIIIΔB* vector was constructed by first introducing an *Xma I* site downstream of the *GFP* reporter gene into the *$pHIV_{SIN}$-CMV-GFP* plasmid vector and subsequently replacing the *Xba I–Xma I* fragment containing the *GFP* gene with a 4.4-kb *Xba I–Xma I* fragment containing the B-domain–deleted canine *FVIII* cDNA from plasmid *pBK-CMV-mun 2bdd1–6* [18]. For lentiviral vector production, 293T cells were transiently transfected as described above. The following amount of DNA was used per cell factory: 3 mg of *$pHIV_{SIN}$-CMV-GFP* or *$pHIV_{SIN}$-CMV-cFVIIIΔB* vector plasmid, 1.5 mg of *pMDL gag/pol RRE* helper plasmid, 1.5 mg of *Rev*-expressing plasmid, and 1.5 mg *pCI-VSV-G* envelope-encoding plasmid, which were previously described [19, 20]. During lentiviral production, D10 medium was supplemented with 1.1 mg/ml Na-butyrate (Sigma, Bornem, Belgium, http://www.sigmaaldrich.com). Functional titer of the HIV_{SIN}-CMV-GFP vector was determined by transducing NIH-3T3 fibroblasts seeded in a six-well plate (7.5×10^4 cells per well) with serially diluted vector-containing supernatant supplemented with 8 μg/ml polybrene. Since GFP was used as a marker, the number of fluorescent cells per microscopic field (five fields) was counted 48 hours post-transfection, on which basis the total number of fluorescent cells per ml of viral vector-containing supernatant could be calculated (= titer in TU/ml, transducing units per ml). Alternatively, lentiviral vectors were titered using RNA dot blot analysis and/or HIV-1 p24 core profile enzyme-linked immunosorbent assay (ELISA) (PerkinElmer Life and Analytical Sciences, Boston, http://www.perkinelmer.com). HIV_{SIN}-CMV-GFP vectors with known functional titer (see above) from the same batch were used as controls.

Transduction of BM Mesenchymal Cells

For comparison of transduction efficiency of oncoretroviral and lentiviral vectors, supernatant containing the MND-GFP-SN vector, MLV_{SIN}-CMV-GFP vector, or HIV_{SIN}-CMV-GFP vector supplemented with protamine-sulphate (4 μg/ml) and hydrocortisone (10^{-6} M) was added to the cells at different multiplicities of infection (MOI). Where indicated, cells were subsequently centrifuged at 1,400*g* for 1 hour at 32°C and incubated at 37°C for 24 hours, or incubated overnight without centrifugation. Transductions were performed either once or successively, as indicated.

Polymerase Chain Reaction and Southern Blot Analysis

Genomic DNA was isolated from the transduced BM mesenchymal cells by phenol-chloroform extraction and quantified spectrophotometrically. Polymerase chain reaction (PCR) was performed with primers specific for the *GFP* gene (5′-TCGCCACCATGGTGAGCAAGG-3′ and 5′-GGCCGCTTTACTTGTACAGCTCG-3′). *β-actin*–specific primers (5′-CATTGTGATGGACTCCGGAGACGG-3′ and 5′-CATCTCCTGCTCGAAGTCTAGAGC-3′) were used as controls for normalization. PCR was performed with Platinum Taq (Invitrogen) by denaturing for 2 minutes at 94°C, followed by 32 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 1 minute at 72°C, and a final extension at 72°C for 2 minutes for *GFP* amplification, yielding a 0.7-kb *GFP*-specific PCR product. Amplification of *β-actin* was performed with AmpliTaq Gold (PerkinElmer) by denaturing for 10 minutes at 95°C, followed by 28–30 cycles of 1 minute at 95°C, 1 min at 59°C, 2 minutes at 72°C, and a final extension for 5 minutes at 72°C, yielding a 0.2-kb *β-actin*–specific product. The intensity of the PCR products relative to the standard was quantified with a Stratagene (La Jolla, CA, http://www.stratagene.com) Eagle Eye II and NIH Image 1.62 software after background subtraction.

For reverse transcription (RT)-PCR, total RNA was extracted from the transduced cells with Trizol (Invitrogen). PCR conditions and primers for *β-actin* amplification were identical to those mentioned above except that 26 cycles were performed. Amplification of *GFP* on the cDNA was carried out with Platinum Taq by denaturing for 2 minutes at 94°C, followed by 32 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 1 minute at 72°C, and a final extension for 2 minutes at 72°C, yielding the 0.7-kb *GFP*-specific PCR product. Serially diluted *MND-GFP-SN* or *MLV_{SIN}-CMV-GFP* plasmid or *β-actin* PCR products of known concentrations were used as standards to validate the linearity of the *GFP* and *β-actin* RT-PCR respectively. Integrated vector copies were analyzed by Southern blot analysis [21] using the 599-bp *Xba I–Cla I* fragment of *HIV_{SIN}-CMV-GFP* containing the *CMV* promoter as a probe. Serially diluted *HIV_{SIN}-CMV-GFP* plasmid spiked into genomic DNA was used as a reference. After overnight hybridization, blots were washed twice with 2× standard saline citrate (SSC) and once with 0.5× SSC and 0.1% SDS.

Differentiation Studies

In vitro osteogenesis was performed as described elsewhere [22]. Human BM mesenchymal cells (untransduced controls or cells transduced with HIV_{SIN}-CMV-GFP or HIV_{SIN}-CMV-cFVIIIΔB) were plated at a density of 8×10^4 cells per well in 12-well tissue culture plates in DMEM supplemented with 2% FBS and antibiotics. The following day, fresh medium (DMEM containing 10% FBS and 100 IU/ml penicillin, 100 mg/ml streptomycin, and 250 ng/ml amphotericin B) was added. One week later, osteogenesis was induced by culturing the cells in the presence of 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid (Sigma), which was changed twice a week for 10 days. Controls for osteogenic differentiation were cultured in medium containing vehicle solutions of the compounds of the osteogenic medium. Osteogenic differentiation was confirmed by assessing calcium deposition, alizarin red

staining and alkaline phosphatase (ALP) activity [22]. In a separate experiment, osteogenic differentiation was assessed 3 or 20 days post-transduction using calcium quantification and alizarin red staining, as described elsewhere [22].

Transduced and nontransduced BM mesenchymal cells were plated as described above and grown to confluency in complete medium. Adipogenic induction medium was then added, consisting of high-glucose DMEM supplemented with 10% FBS and antibiotics, 1 μM dexamethasone, 0.5 mM methyl-isobutylxanthine, 10 μg/ml insulin, and 100 mM indomethacin (Sigma). After 72 hours, the medium was changed to adipogenic maintenance medium (10 μg/ml insulin in high-glucose DMEM supplemented with 10% FBS and antibiotics) for 24 hours. This cycle was repeated four times. Controls for adipogenic differentiation were cultured in medium containing vehicle solutions of the compounds of the osteogenic medium. Adipogenesis was assessed by oil red-O staining as described elsewhere [22]. In a separate experiment, adipogenic differentiation was assessed 4 or 20 days post-transduction.

Analysis of Transgene Expression

In vitro FVIII activity in the transduced BM mesenchymal cells was quantified by measuring the FVIII-dependent generation of factor Xa from factor X using a chromogenic assay (Coatest FVIII, Chromogenix, Molndal, Sweden, http://www.chromogenix.com). Human plasma purified FVIII (Octapharma, Langenfeld, Germany, http://www.octapharma.com) of known activity was used as a standard, and 1,000 mU was defined as 200 ng FVIII/ml. BM mesenchymal cell populations transduced with *GFP* vectors were analyzed by confocal microscopy, (Zeiss Axiovert; Carl Zeiss, Oberokochen, Germany, http://www.zeiss.com) and by cytofluorimetric analysis (FACSCalibur, equipped with CELLquest software; Becton Dickinson) to quantify the percentage GFP-positive cells.

Bioengineering of BM Mesenchymal Cells into Collagen Implants

BM mesenchymal cells stably transduced with HIV_{SIN}-CMV-GFP were suspended using trypsin/EDTA, pelleted, and resuspended in a chilled DMEM-based culture medium containing freshly pre-made collagen mix (1–2 mg/ml of Type I bovine collagen, Zyderm; Inamed, Irvine, CA, http://www.inamed.com), and neutralized with NaOH as previously described [23]. The cell–gel mixture (2×10^6 cells/ml) was cast into 25-mm long silicone rubber molds with end attachment sites, placed in a humidified 37°C, 5% CO_2 incubator for 2–6 hours, and then flooded with 10 ml of DMEM-based culture medium and left in the incubator for an additional 2 days before implantation. Within 24 hours after casting, the cell–gel mix contracted, detaching from the molds and held in place only at the two end attachment sites. Two days later, the bioengineered BM mesenchymal implants were removed from their molds and formed 20- to 25-mm long, 1- to 3-mm diameter cylindrical shaped structures. Six bioengineered implants with each containing 2×10^6 cells were subsequently implanted s.c. into adult immunodeficient NOD-SCID mice. All animal procedures were approved by the Animal Ethical Commission of the University of Leuven.

Statistical Analysis

Statistical analyses were performed using the two-tailed Student's *t*-test. Results are shown as mean $\pm$ standard deviation. Fluorescence-activated cell sorting (FACS) data were analyzed by Kolmogorov-Smirnov statistics.

RESULTS

GFP Expression in Transduced BM Mesenchymal Cells

To determine whether BM mesenchymal cells could be transduced efficiently with lentiviral vectors, a subconfluent BM mesenchymal cell culture was exposed to the VSV-G pseudotyped HIV_{SIN}-CMV-GFP vector particles. Typically, vector titers ranged between 10^6 and 5×10^6 TU/ml as determined by transducing NIH-3T3 fibroblasts. For comparison, a VSV-G pseudotyped oncoretroviral vector was used (MLV_{SIN}-CMV-GFP vector), which expressed *GFP* from the *CMV* promoter in the context of the same expression cassette as in the HIV_{SIN}-CMV-GFP vector (Fig. 1B). Confocal microscopical analysis and flow cytometry confirmed that transduction of the human BM mesenchymal cells was significantly more efficient with the HIV_{SIN}-CMV-GFP lentiviral vector than with the MLV_{SIN}-CMV-GFP oncoretroviral vector. A single exposure of human BM mesenchymal cells to a high dose of HIV_{SIN}-CMV-GFP (MOI of 140) resulted in nearly 70% (Fig. 2D; Table 1) of the target cell population expressing *GFP* (background 0.02%). In contrast, when the same batch of human BM mesenchymal cells was transduced in parallel with the same dose of the MLV_{SIN}-CMV-GFP vector instead, only 5.2% (Fig. 2A; Table 1) GFP-positive cells were detected by FACS analysis. This constituted a 13-fold greater efficiency in lentiviral versus oncoretroviral transduction. Similarly, at lower vector doses, lentiviral transduction of human BM mesenchymal cells was more efficient than oncoretroviral gene transfer. At an MOI of 14, 14% of the BM mesenchymal cell population expressed *GFP* following lentiviral gene transfer, compared with only 1.4% following oncoretroviral gene transfer, which represents a 10-fold difference in transduction efficiency (Table 1). At the lowest vector dose (MOI of 1.4), 5.7% of the BM mesenchymal cell population expressed GFP following lentiviral gene transfer, compared with 0.2% following oncoretroviral gene transfer (Table 1). Kolmogorov-Smirnov statistics confirmed that the difference in GFP expression was statistically significant ($p < .001$) between HIV_{SIN}-CMV-GFP and MLV_{SIN}-CMV-GFP. GFP expression levels could be augmented further by repeated vector exposure (Fig. 2; Table 1). Following three transductions with the HIV_{SIN}-CMV-GFP lentiviral vector, a maximum of 92% of BM mesenchymal cells that expressed GFP was achieved (Fig. 2F; Table 1), compared with only 17.2% (Fig. 2C; Table 1) with the MLV_{SIN}-CMV-GFP oncoretroviral vector. As shown in Figures 2 and 3, not only the percentage of GFP-positive cells was higher in the lentivirally transduced cells, but the mean fluorescence intensity (MFI) was also higher, indicating that the average vector copy number per cell was higher than with oncoretroviral transduction, which was subsequently confirmed by Southern blot analysis (see below; Fig. 3D, 3E). Lentiviral transduction was twofold greater in the presence of protamine sulphate (data not shown), and this was therefore routinely added to the viral vector during transduction,

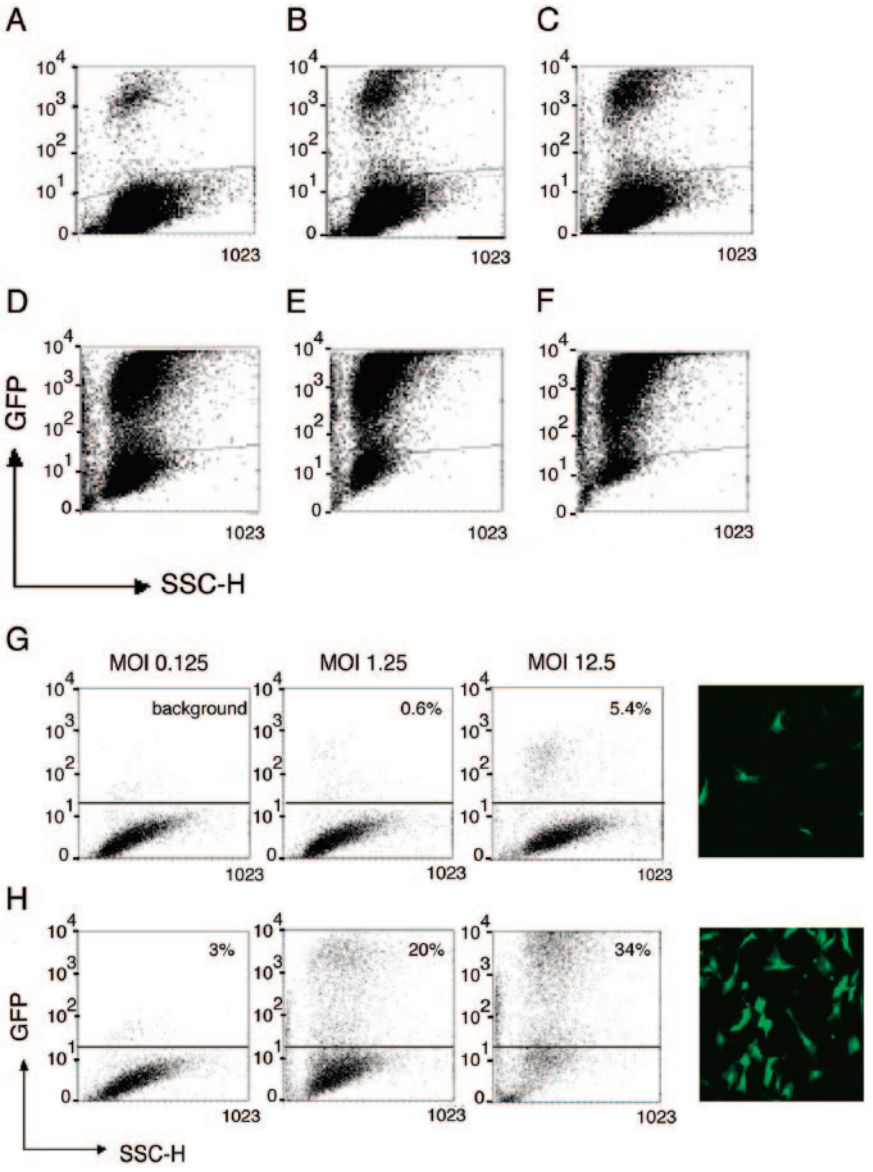


Figure 2. GFP expression in transduced human BM mesenchymal cells measured by flow cytometry. Cells were exposed overnight to MLV_{SIN}-CMV-GFP **(A–C)** and HIV_{SIN}-CMV-GFP **(D–F)** at an MOI of 140. Transductions were performed one **(A, D)**, two **(B, E)**, or three **(C, F)** times. BM mesenchymal cells were transduced with MND-GFP-SN oncoretroviral **(G)** and HIV_{SIN}-CMV-GFP lentiviral **(H)** vectors at doses of 10^4 TU/ml (MOI, 0.125), 10^5 TU/ml (MOI, 1.25), and 10^6 TU/ml (MOI, 12.5). Values given correspond to the percentage GFP-positive cells after background subtraction. Also shown are confocal microscopical images of BM mesenchymal cells transduced with MND-GFP-SN oncoretroviral **(G**, right) and HIV_{SIN}-CMV-GFP lentiviral vectors **(H**, right) at a dose of 10^6 TU/ml (MOI, 12.5). Abbreviations: BM, bone marrow; CMV, cytomegalovirus; GFP, green fluorescent protein; MLV, murine leukemia virus; MND, myeloproliferative sarcoma virus enhancer, negative control region-deleted, dl587rev primer-binding site-substituted; MOI, multiplicity of infection; SIN, self-inactivating; SN, simian virus-40 (SV40) neo^R cassette; SSC-H, side scatter; TU, transducing units.

just like with oncoretroviral vectors. Following seven transductions with the HIV_{SIN}-CMV-GFP vector, BM mesenchymal cells expressed *GFP* in 99.9% of cells, as determined by FACS analysis. After 4 months of continuous in vitro culture, GFP expression was still detectable in 98.3% of the transduced cells. However, the MFI of GFP expression in these samples decreased from 3,423 to 1,974 arbitrary units (negative control 4.0 arbitrary units) during this interval, indicating that GFP expression gradually declined.

In a separate experiment, an oncoretroviral vector was used (MND-GFP-SN), which expressed *GFP* from a modified *LTR* that confers long-term expression in various stem cells (Fig. 1A) [13]. Confocal microscopical analysis and flow cytometry indicated that transduction of the human BM mesenchymal cells was significantly more efficient with the lentiviral HIV_{SIN}-CMV-GFP vector (Fig. 2H) than with the MND-GFP-SN oncoretroviral vector (Fig. 2G). A single exposure of human BM mesenchymal cells to a vector dose of 10^6 TU/ml HIV_{SIN}-CMV-GFP (MOI, 12.5) resulted in 34% of the target cell population expressing *GFP* (Fig. 2H). In contrast, when the same batch of human BM mesenchymal cells was transduced in parallel with the same dose of MND-GFP-SN vector instead, only 5.4% GFP-positive cells were detected by FACS analysis (Fig. 2G). This constituted a sixfold greater efficiency in lentiviral versus oncoretroviral transduction. Similarly, at lower vector doses, lentiviral transduction of human BM mesenchymal cells was more efficient than oncoretroviral gene transfer. At a vector dose of 10^5 TU/ml (MOI, 1.25), 20% of the BM mesenchymal cell population expressed *GFP* following HIV_{SIN}-CMV-GFP lentiviral gene transfer, compared with only 0.6% following MND-GFP-SN gene transfer, which represents a 32-fold difference in transduction efficiency. At the lowest vector dose of only 10^4 TU/ml (MOI, 0.125), 3% of the BM mesenchymal cell population expressed *GFP* following lentiviral gene transfer, whereas *GFP* expression was not significantly

Table 1. Transduction efficiency of human BM mesenchymal cells transduced with MLV_{SIN}-*CMV-GFP* and HIV_{SIN}-*CMV-GFP*

MOI	Transduction rounds	*MLV_{SIN}-CMV-GFP*	*HIV_{SIN}-CMV-GFP*
140	1	5.2	66.6
	2	13.7	89.4
	3	17.2	91.9
14	1	1.4	13.9
	2	4.5	34.6
	3	4.6	46.4
1.4	1	.2	5.7
	2	.8	10.7
	3	.9	14.6

Cells were subjected to subsequent transductions at different MOIs (140, 14, and 1.4). The value given is the percentage of GFP-positive cells as measured by FACS analysis.

Abbreviations: BM, bone marrow; MOI, multiplicity of infection; CMV, cytomegalovirus; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; SIN, self-inactivating.

different from background levels following oncoretroviral gene transfer. Kolmogorov-Smirnov statistics confirmed that the difference in GFP expression was significant ($p < .001$) when lentiviral vectors were employed (HIV_{SIN}-CMV-GFP) compared with the MND-GFP-SN oncoretroviral vector.

Gene Transfer Efficiency and GFP mRNA Expression in Transduced BM Mesenchymal Cells

To ascertain that the greater percentage of GFP-positive BM mesenchymal cells following HIV_{SIN}-CMV-GFP lentiviral gene transfer versus MLV_{SIN}-CMV-GFP gene transfer, PCR analysis was performed using *GFP*-specific primers. Figure 3A and 3C show that HIV_{SIN}-CMV-GFP lentiviral gene transfer using high vector doses (MOI, 14; MOI, 140) resulted in a significantly greater *GFP* gene transfer than when the MLV_{SIN}-CMV-GFP oncoretroviral vector was employed. Similarly, when the relative *GFP* mRNA expression levels were determined by RT-PCR analysis (Fig. 3B), significantly higher *GFP* mRNA levels were apparent following HIV_{SIN}-CMV-GFP lentiviral gene transfer than following transduction with the MLV_{SIN}-CMV-GFP vector. This is consistent with the differences in gene transfer efficiency (Fig. 3A) and with the differences in GFP protein expression as determined by confocal and FACS analysis (Fig. 2). PCR and RT-PCR analysis using *β-actin* as a control revealed no significant difference between oncoretrovirally and lentivirally transduced BM mesenchymal cells.

To further confirm that lentiviral vectors transduced BM mesenchymal cells more efficiently than oncoretroviral vectors, an independent experiment was conducted in which integrated vector copy numbers of cells transduced with HIV_{SIN}-CMV-GFP or MLV_{SIN}-CMV-GFP were assessed by Southern blot. Southern blot analysis confirmed that after three successive transduction rounds, HIV_{SIN}-CMV-GFP resulted in significantly greater (4.8-fold; $p < .05$, one-tailed *t*-test) stably integrated vector copies than when MLV_{SIN}-CMV-GFP was used. Comparison of HIV_{SIN}-CMV-GFP with MLV_{SIN}-CMV-GFP showed a highly significant difference ($p < .0001$) in MFI in transduced BM mesenchymal cells, as determined by FACS (three rounds of transduction) (Fig. 3E). Successive rounds of lentiviral transduction resulted in a significantly (1.9-fold) greater MFI ($p < .005$). The greater MFI when using a lentiviral versus oncoretroviral vector, or following successive lentiviral transduction rounds, correlated strongly with the significantly greater vector copy number (correlation coefficient $R^2 = 0.94$). These Southern blot data confirm the superiority of lentiviral over oncoretroviral vectors to transduce BM mesenchymal cells and are therefore consistent with the GFP expression data provided by confocal microscopy and FACS (Figs. 2, 3E) and semiquantitative PCR analysis on both RNA and DNA (Fig. 3).

Similarly, Figure 4A and 4C show that HIV_{SIN}-CMV-GFP lentiviral gene transfer using different vector doses consistently resulted in significantly greater *GFP* gene transfer than when the MND-GFP-SN oncoretroviral vector was employed. When the relative GFP mRNA expression levels were determined by semiquantitative RT-PCR analysis (Fig. 4B), significantly higher GFP mRNA levels were apparent following HIV_{SIN}-CMV-GFP lentiviral gene transfer than following transduction with the MND-GFP-SN vector. This is consistent with the differences in gene transfer efficiency (Fig. 4A) and with the differences in GFP protein expression as determined by confocal and FACS analysis (Fig. 2G, 2H). PCR and RT-PCR analysis using *β-actin* as a control revealed no significant difference between oncoretrovirally and lentivirally transduced BM mesenchymal cells.

FVIII Expression in Lentivirally Transduced BM Mesenchymal Cells

Following successive transductions of BM mesenchymal cells with the HIV_{SIN}-CMV-cFVIIIΔB vector that encodes canine B-domain deleted FVIII (Fig. 1C), in vitro FVIII levels varied from 800 to 3,600 mU of cFVIII/10^6 cells per 24 hours, depending on the BM donor. The in vitro FVIII production from the same transduced population of BM mesenchymal cells was monitored over a 5-month period. After 3 weeks in culture, expression started to decline gradually over time but remained detectable in the culture for at least 15 weeks (FVIII >130 mU/10^6 cells per 24 hours) (Fig. 5A). An unpaired *t*-test comparing peak expression at week 2 with the last data point at week 15 shows that expression declined significantly ($p < .0001$) during this period. Similar expression kinetics were observed when BM mesenchymal cells were transduced with an oncoretroviral vector (supplemental online Fig. 2). These particular kinetics further justify the importance of using

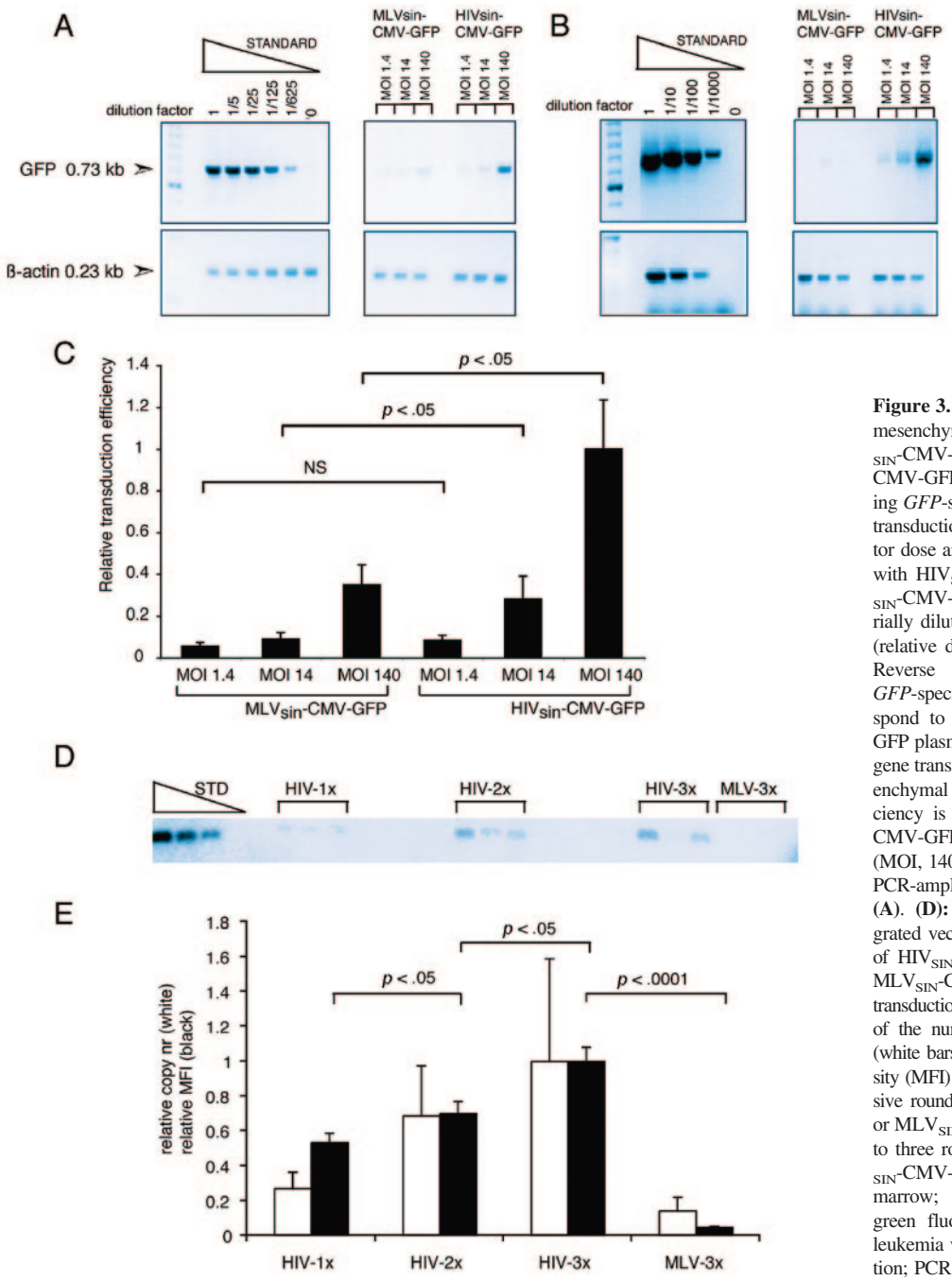


Figure 3. Gene transfer efficiency to BM mesenchymal cells transduced with HIV_{SIN}-CMV-GFP in parallel with MLV_{SIN}-CMV-GFP. **(A):** Semiquantitative PCR using *GFP*-specific primers shows increasing transduction efficiency with increasing vector dose and higher transduction efficiency with HIV_{SIN}-CMV-GFP than with MLV_{SIN}-CMV-GFP. Standards correspond to serially diluted MLV_{SIN}-CMV-GFP plasmid (relative dilution factor is indicated). **(B):** Reverse transcription (RT)-PCR using *GFP*-specific primers. Standards correspond to serially diluted MLV_{SIN}-CMV-GFP plasmid. **(C):** Quantitative analysis of gene transfer efficiency in human BM mesenchymal cells. The gene transfer efficiency is shown, relative to the HIV_{SIN}-CMV-GFP transduced cell population (MOI, 140), based on the intensities of the PCR-amplified *GFP* fragment shown in **(A)**. **(D):** Southern blot comparing integrated vector copies after successive rounds of HIV_{SIN}-CMV-GFP transduction or with MLV_{SIN}-CMV-GFP after three rounds of transduction. **(E):** Quantitative representation of the number of integrated vector copies (white bars) or the mean fluorescence intensity (MFI) of cells transduced during successive rounds with either HIV_{SIN}-CMV-GFP or MLV_{SIN}-CMV-GFP. Values are relative to three rounds of transduction with HIV_{SIN}-CMV-GFP. Abbreviations: BM, bone marrow; CMV, cytomegalovirus; GFP, green fluorescent protein; MLV, murine leukemia virus; MOI, multiplicity of infection; PCR, polymerase chain reaction.

efficient gene delivery methods, like lentiviral vectors, obviating extensive and prolonged in vitro culture. Untransduced BM mesenchymal cells did not produce any detectable FVIII in culture (data not shown).

Adipogenic and Osteogenic Differentiation of Transduced BM Mesenchymal Cells

Although FVIII expression was detectable for several months in vitro following lentiviral transduction of human BM mesenchymal cells, expression levels were not stable and gradually declined (Fig. 5A). Since differentiated cells (adipocytes, osteocytes) were present in the BM mesenchymal cultures (Fig. 5B), it was important to determine whether differentiation of BM mesenchymal cells contributed to this decline. Human BM mesenchymal cells transduced with the HIV_{SIN}-CMV-GFP vector and containing more than 99% GFP-expressing cells, were therefore induced to differentiate along adipogenic and osteogenic lineages. Untransduced human BM mesenchymal cells were induced to differentiate in parallel as a control. Adipogenesis was measured by determining the percentage of adipocytes originating in the culture by oil red-O staining (supplemental online Fig. 1D–1F). Most of these adipocytes (Fig. 5B, 5C) expressed GFP, indicating that lentiviral expression of GFP is retained following adipogenic differentiation. Similarly, lentivi-

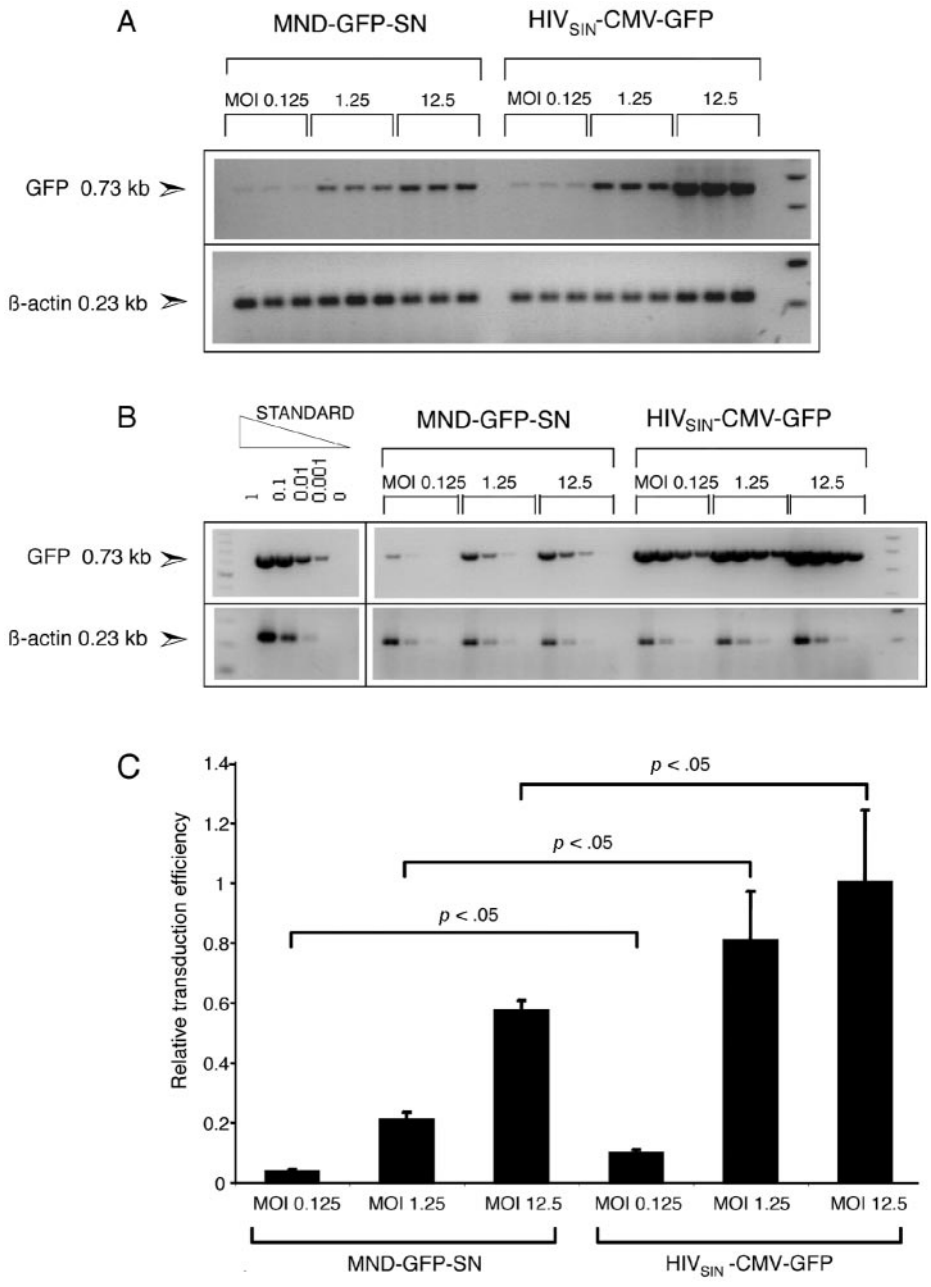


Figure 4. Gene transfer efficiency to BM mesenchymal cells transduced with MND-GFP-SN or HIVSIN-CMV-GFP. **(A):** Analysis of gene transfer efficiency in human BM mesenchymal cells by semiquantitative PCR. A *GFP*-specific primer pair was used yielding a 0.7-kb PCR product. *β-actin*–specific primers were used as controls for normalization yielding a 0.2-kb PCR product. Samples are represented in triplicate. **(B):** Analysis of *GFP* mRNA expression in human BM mesenchymal cells by semiquantitative reverse transcription (RT)-PCR. A *GFP*-specific primer pair was used yielding a 0.7-kb PCR product. *β-actin*–specific primers were used as controls for normalization yielding a 0.2-kb PCR product. Samples were serially diluted to the linear range of the assay. Standards correspond to serially diluted pMND-GFP-SN plasmids and serially diluted *β-actin* PCR product. The markers shown are 100-bp molecular weight markers. **(C):** Quantitative analysis of gene transfer efficiency in human BM mesenchymal cells. The gene transfer efficiency is shown, relative to the HIV$_{SIN}$-CMV-GFP–transduced cell population (MOI, 12.5), based on the intensities of the PCR-amplified *GFP*-fragment shown in **(A)**. Abbreviations: BM, bone marrow; CMV, cytomegalovirus; GFP, green fluorescent protein; MND, myeloproliferative sarcoma virus enhancer, negative control region-deleted, dl587rev primer-binding site-substituted; MOI, multiplicity of infection; PCR; polymerase chain reaction; SIN, self-inactivating; SN, simian virus-40 (SV40) neo^R cassette.

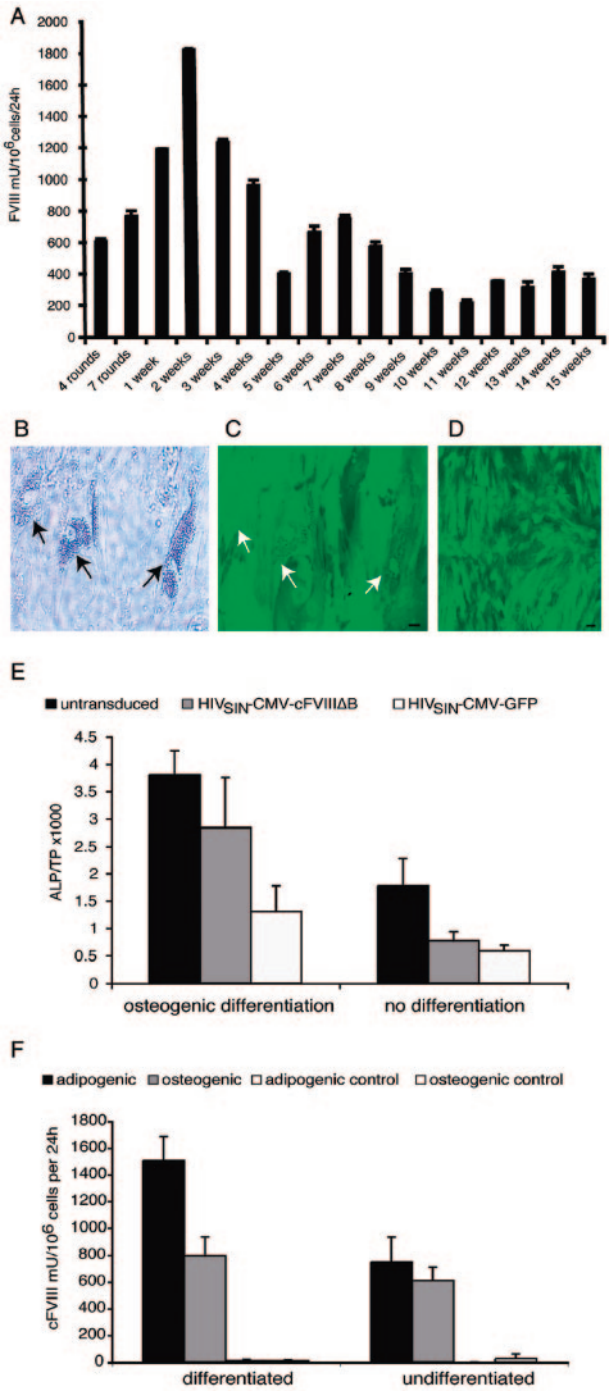
ral GFP expression is retained following differentiation of BM mesenchymal stem/progenitor cells along the osteogenic lineage (Fig. 5D). Osteogenic differentiation was confirmed by the significant increase in normalized ALP activity (Fig. 5E; $n = 4$, $p = .03$), by calcium deposition and by alizarin red staining (supplemental online Fig. 1A–1C).

We subsequently tested whether differentiation was affected by the culture period of the BM mesenchymal cells post-transduction. A more extensive differentiation experiment was therefore performed using BM mesenchymal cells that were cultured for only 4 days post-transduction (i.e., "early" culture) compared with the differentiation potential of transduced BM mesenchymal cells that had been cultured over 20 days (i.e., "late" culture). In vitro osteogenesis and adipogenesis were assessed in the early and late BM mesenchymal cells (supplemental online Fig. 1). The differentiation data presented in supplemental online Figure 1F indicate that no significant difference was observed in adipogenic differentiation when comparing BM mesenchymal cells grown over a short versus a longer period after transduction. Indeed, 35% of the transduced BM mesenchymal cells differentiated into adipocytes in both cell populations, regardless of the duration of the culture post-transduction. The adipogenic differentiation potential was also similar in nontransduced cells grown over 4 versus 20 days in culture. Moreover, the results shown in supplemental online Figure 1F indicate that lentiviral transduction did not affect adipogenesis.

The osteogenic differentiation potential was more pronounced in transduced BM mesenchymal cells grown over 20 days than in

those grown over 4 days for both transduced and nontransduced cells (supplemental online Fig. 1C). Hence, BM mesenchymal cells showed greater osteogenic differentiation following extended in vitro culture for at least 20 days. Interestingly, transduction with HIV_{SIN}-CMV-GFP inhibited osteogenic differentiation (Fig. 5E; supplemental online Fig. 1C), based on significantly lower normalized ALP activity and calcium deposition. However, transduction with HIV_{SIN}-CMV-cFVIIIΔB did not appear to inhibit osteogenic differentiation (Fig. 5E).

To determine whether differentiation of BM mesenchymal stem/progenitor cells accounted for the decline in FVIII expression in vitro, cells were transduced with the HIV_{SIN}-CMV-cFVIIIΔB vector and induced to differentiate along the adipogenic and osteogenic lineage. Untransduced human BM mesenchymal cells were induced in parallel as a control. Functional cFVIII expression did not decline upon induction of adipogenic or osteogenic differentiation (Fig. 5F). In fact, adipogenesis appears to enhance FVIII production. These results indicate that differentiation of transduced cells along the adipogenic or osteogenic lineage does not contribute to FVIII downregulation.



In Vivo Engraftment of Bioengineered Implants Based on BM Mesenchymal Cells

Typically, systemic administration of gene-engineered BM mesenchymal cells results in rapid clearance and limited or transient engraftment [1, 5, 6]. To overcome these limitations, BM mesenchymal cells transduced with the HIV_{SIN}-CMV-GFP vector were seeded onto biocompatible U.S. Food and Drug Administration–approved collagen scaffolds. These bioengineered human BM mesenchymal cell implants were then implanted s.c. into immunodeficient NOD-SCID mice. Efficient engraftment of the engineered cells in vivo was apparent, because GFP fluorescence could readily be detected following whole-body transdermal imaging in living animals for at least 2 months postimplantation (duration of the experiment) (Fig. 6).

DISCUSSION

BM mesenchymal cells are attractive target cells for the treatment of a variety of diseases such as hemophilia, bone disorders, and cancer. Previously, we have shown that they could be transduced with oncoretroviral vectors [24] using complex optimized transduction protocols. In the present study, we compared the transduction efficiency of the lentiviral HIV_{SIN}-CMV-GFP vector with those of the MLV_{SIN}-CMV-GFP and MND-GFP-SN oncoretroviral vectors at varying MOI by cytofluorimetric analysis. Transduction was invariably more efficient with the lentiviral vector than with the oncoretroviral vectors, resulting in nearly 100% of the cells expressing GFP after successive transduction rounds. The greater percentage of GFP-positive BM mesenchymal cells following lentiviral versus oncoretroviral transduction was consistent with a concomitantly greater gene transfer efficiency and greater GFP mRNA expression by semiquan-

Figure 5. Effect of transduction on differentiation of BM mesenchymal cells. **(A):** Kinetic analysis of FVIII expression in cultures transduced with HIV_{SIN}-CMV-cFVIIIΔB. **(B, C):** Persistent GFP expression following adipogenic and osteogenic differentiation in human BM mesenchymal cells transduced with HIV_{SIN}-CMV-GFP. **(B)** and **(C)** depict the same area, showing that cells with the adipogenic phenotype express GFP (arrows) (magnification ×710). **(D):** GFP expression in cells with the osteogenic phenotype (bars represent 100 μm). **(E):** Alkaline phosphatase (ALP) activity normalized over total protein (TP) content of undifferentiated and differentiated human BM mesenchymal cell cultures following osteogenic lineage induction (enzyme-linked immunosorbent assay) in untransduced (black), HIV_{SIN}-CMV-cFVIIIΔB transduced (light gray), and HIV_{SIN}-CMV-GFP transduced (white) cell populations. **(F):** cFVIII expression in adipogenic (black) and osteogenic (gray) mesenchymal cell cultures and untransduced controls (white). Abbreviations: BM, bone marrow; CMV, cytomegalovirus; GFP, green fluorescent protein; SIN, self-inactivating.

titative PCR and Southern blot and RT-PCR analysis, respectively. Previous studies failed to prove the superiority of lentiviral vectors over oncoretroviral vectors for transducing BM mesenchymal cells [25] because of a lack of normalized vector design and the absence of PCR or Southern blot analysis, in contrast to the present study. The greater stable gene transfer efficiency is likely a result of the ability of lentiviral vectors to transduce both cycling and noncycling BM mesenchymal cells; whereas stable oncoretroviral gene transfer is restricted to those cells that are cycling at the time of transduction. In addition, since the *cPPT* element in the lentiviral vector facilitates intranuclear transport of the lentiviral preintegration complex, it could have further contributed to the greater gene transfer efficiency [15, 16, 26, 27].

The inherent efficiency of lentiviral versus oncoretroviral vectors for BM mesenchymal cell transduction facilitates possible scaling-up for gene transfer studies in large animal models or ultimately for clinical trials. In addition, it obviates the need for selective enrichment of the transduced BM mesenchymal cells, which prolongs the in vitro culture and consequently may adversely influence engraftment potential [9]. However, a possible disadvantage of efficient lentiviral transduction is the higher risk for insertional oncogenesis following multiple vector integrations [28–31]. Nevertheless, the use of SIN vectors potentially diminishes the risk of activating neighboring proto-oncogenes.

Lentiviral gene transfer compares favorably with other gene transfer methods for introducing genes into BM mesenchymal cells. In particular, transduction of BM mesenchymal cells with adenoviral vectors seems relatively inefficient compared with lentiviral gene transfer, which is at least partly a result of the limited expression of the cellular coxsackie and adenovirus receptor on the BM mesenchymal cells [12, 32]. In addition, unlike lentiviral vectors, adenoviral vectors do not integrate in a typically dividing BM mesenchymal cell population. Consequently, the potentially therapeutic transgene is diluted over time, limiting the use of adenoviral vectors to gene therapy of disorders that would benefit only from short-term expression of a potentially therapeutic transgene in BM mesenchymal cells.

The relatively efficient lentiviral transduction of human BM mesenchymal cells resulted in high and long-term GFP and FVIII expression for several months in vitro. However, expression of these various transgenes gradually declined following in vitro culture. This is consistent with the gradual decline in transgene expression in oncoretrovirally transduced BM mesenchymal cells (supplemental online Fig. 2). The reason for this decline in transgene expression is currently not known. However, because the percentage of GFP-positive cells remained essentially unchanged (>98%) over at least 4 months in culture, it can be excluded that *GFP*-negative cells would have overgrown the BM mesenchymal cell culture.

It is known that lentiviral vectors can persist as episomes and can express the transgene for a prolonged period of time after vector exposure, at least in some cell types [33]. Because the transduced BM mesenchymal cells are actually dividing and because integration-defective lentiviral vectors were previously shown not to express the transgene in transduced BM mesenchymal cells [25], expression in transduced BM mesenchymal cells is by and large from the integrated proviral DNA and not from the nonintegrated episomes.

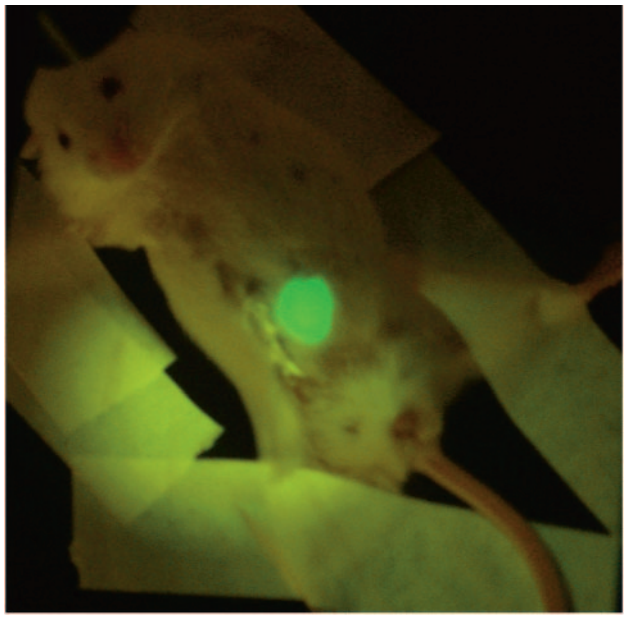


Figure 6. Detection of GFP expression by whole-body transdermal imaging in live animals. HIV_{SIN}-CMV-GFP transduced human bone marrow mesenchymal cells maintained in a collagen implant can be detected for at least up to 2 months in vivo. Abbreviations: CMV, cytomegalovirus; GFP, green fluorescent protein; SIN, self-inactivating.

Hence, the episomal vector forms likely do not account for the kinetics of FVIII expression.

It is more likely that the decline in transgene expression may have been the consequence of cell senescence, associated with telomere shortening, consistent with previous studies using BM mesenchymal cells [34]. This senescence may have led to reduced proliferation in culture, which was previously shown to diminish transgene (GFP) expression in other cell populations containing adult stem cells, such as neural stem cells [35]. This indicates that the decline in *FVIII* expression is not inherent to the use of oncoretroviral vectors but also occurs when different types of promoters (*LTR* versus *CMV*) or different vectors (oncoretroviral versus lentiviral) are employed.

Although it seemed possible that differentiation of BM mesenchymal stem/progenitor cells may have contributed to the slow decline in transgene expression, BM mesenchymal cells transduced with lentiviral vectors expressing either *GFP* or *FVIII* continue to express these transgenes strongly following adipogenic or osteogenic differentiation. Hence, differentiation of BM mesenchymal stem/progenitor cells likely does not account for the gradual decline in gene expression in vitro. In addition, lentiviral transduction of the *FVIII* gene did not influence the ability of these BM mesenchymal cell cultures to differentiate. In contrast, expression of *GFP* following lentiviral transduction seems to impair osteogenesis to some degree in vitro. These results suggest that the transgene, in this case the *GFP* marker gene, rather than the lentiviral vector itself, influences the differentiation potential of the mesenchymal cells. It is known that GFP can be toxic to certain cells, resulting in the inability of stable cell clones to express the protein [36] or in phenotypic changes in neuronal progenitor cells [37]. The impaired differentiation

of mesenchymal cells may represent a more subtle effect of GFP toxicity.

We also showed that efficient engraftment of lentivirally transduced BM mesenchymal cells could be obtained in vivo. To achieve this, BM mesenchymal cells transduced with the HIV_{SIN}-CMV-GFP vector and subsequently seeded onto collagen scaffolds were implanted into immunodeficient NOD-SCID mice. Efficient engraftment of the engineered cells in vivo was apparent by virtue of the intense and prolonged GFP fluorescence following whole-body transdermal imaging in living animals. The use of these BM mesenchymal cell implants overcomes one of the limitations using gene-engineered BM mesenchymal cells administered systemically, which typically results in rapid cell clearance if no scaffold is provided [5, 6]. One particularly attractive safety feature of this technology is that these BM mesenchymal cell implants represent a potentially reversible protein delivery approach because these implants could be retrieved in the event of an unexpected adverse reaction or when the expression of the therapeutic protein is no longer required. We have also shown that the MSC implants could be readministered (data not shown), which further underscores the versatility of this approach. The efficient gene delivery with lentiviral vectors in conjunction with the use of bioengineered reversible scaffolds improves the therapeutic prospects of this novel approach for gene therapy, protein delivery, or tissue engineering. However, efforts toward standardization in isolation and purification of these cells, and the use of purified more primitive MAPCs in particular, will be essential to exploit the full potential of this promising cell type [38].

ACKNOWLEDGMENTS

We thank Dr. D. Kohn (Children's Hospital Los Angeles) for providing the pMND-GFP-SN plasmid, Dr. D. Ory (Washington University) for the pCI-VSV-G and pMD-GP plasmids, and Dr. L. Naldini (San Rafaelle Institute) for the lentiviral vectors and helper constructs. We also thank Dr. M. Delforge (University Hospital Gasthuisberg, Leuven, Belgium) for providing the bone marrow samples. Gaby Itu, Yasushi Iwasaki, Veerle Gillijns, Veerle Vanslembrouck, Hans Zwinnen, and Els Vangoidsenhoven are acknowledged for technical assistance. This study was supported by the Flanders Fund for Scientific Research (FWO) (A.V. and T.V.), the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT-Flanders) (L.T.), a grant from the Flanders Government to the Flanders Interuniversity Institute for Biotechnology (VIB), a Concerted Research Grant (GOA) from the University of Leuven, and the Katherine Dormandy Trust. H.H.V. acknowledgessabbatical support from the Miriam Hospital. A.V.D. and L.T. contributed equally to this study.

DISCLOSURES

The authors indicate no potential conflicts of interest.

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