

Multilineage Cells from Adipose Tissue as Gene Delivery Vehicles

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ABSTRACT

We have characterized a population of mesenchymal progenitor cells from adipose tissue, termed processed lipoaspirate (PLA) cells, which have multilineage potential similar to bone marrow-derived mesenchymal stem cells and are also easily expanded in culture. The primary benefit of using adipose tissue as a source of multilineage progenitor cells is its relative abundance and ease of procurement. We examined the infection of PLA cells with adenoviral, oncoretroviral, and lentiviral vectors. We demonstrate that PLA cells can be transduced with lentiviral vectors at high efficiency. PLA cells maintain transgene expression after differentiation into adipogenic and osteogenic lineages after lentiviral transduction. Therefore, PLA cells and lentiviral vectors may be an efficient combination for use as a therapeutic gene delivery vehicle.

OVERVIEW SUMMARY

Mesenchymal progenitor cells from adipose tissue, termed processed lipoaspirate (PLA) cells, have multilineage potential similar to bone marrow-derived mesenchymal stem cells and are also easily expanded in culture. We examined the infection of PLA cells with adenoviral, oncoretroviral, and lentiviral vectors. We demonstrate that PLA cells can be transduced with lentiviral vectors at high efficiency and maintain transgene expression after differentiation into adipogenic and osteogenic lineages after lentiviral transduction. Therefore, PLA cells and lentiviral vectors may be an efficient combination for use as a therapeutic gene delivery vehicle.

INTRODUCTION

GENE THERAPY involves the introduction of functional genes into the body. One approach for introduction of a functional gene involves cells as gene delivery vehicles. The ideal cell for gene delivery is autologous and immunoprivileged, easily expanded in culture, and capable of long-term transgene ex-

pression. Human mesenchymal stem cells (MSCs) derived from bone marrow have been described as an attractive cellular vehicle for gene delivery applications because of their capacity for multilineage differentiation and *ex vivo* culture expansion (Friedenstein, 1990; Prockop, 1997; Pittenger *et al.*, 1999; Caplan, 2000; Caplan and Bruder, 2001). We have characterized an alternative source of mesenchymal stem cells from adipose tissue that we term processed lipoaspirate (PLA) cells. Less is known regarding the properties of these cells than about bone marrow-derived MSCs, but they share the capacity to expand in culture and differentiate along the adipogenic, osteogenic, chondrogenic, and myogenic lineages (Zuk *et al.*, 2001; Erickson *et al.*, 2002; Mizuno *et al.*, 2002). These cells can be obtained in relatively large numbers through a standard clinical procedure. In addition, the progenitor cells in adipose tissue appear to be present in the PLA population at higher frequency than in bone marrow. Investigation of gene transfer to these cells is an important first step to assess the potential of these cells as a gene delivery vehicle.

Oncoretrovirus and lentiviral vectors are commonly used for long-term gene expression because they can integrate their genes into the host genome efficiently. The oncoretroviral vector has been proposed as a tool for gene transfer in MSCs de-

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rived from bone marrow (Gordon *et al.*, 1997; Hurwitz *et al.*, 1997; Cherington *et al.*, 1998; Marx *et al.*, 1999; Mason *et al.*, 2000; Mosca *et al.*, 2000; Bartholomew *et al.*, 2001; Lee *et al.*, 2001; Jiang *et al.*, 2002), but it has the disadvantage that it requires cell division for efficient transduction (Roe *et al.*, 1993; Case *et al.*, 1999). In contrast, the lentiviral vector does not require cell division for transduction, and it has been demonstrated to have higher transduction efficiencies in many tissues and cells *in vivo* and *in vitro* (Naldini *et al.*, 1996; Miyoshi *et al.*, 1997). In addition, adenoviral vectors can transduce a variety of cells and tissues and high multiplicity of infection (MOI) with adenoviral vectors was reported to result in integration and long-term gene expression (Harui *et al.*, 1999). We sought to investigate the efficiency of gene transduction in PLA cells with various vectors. We achieved long-term transgene expression and stable transgene expression after differentiation when using lentiviral vectors.

MATERIALS AND METHODS

Isolation of PLA cells from adipose tissue

Adipose tissue was collected from three healthy human donors under an institutional review board-approved study (HSPC 98-09-007-3). PLA cells were isolated by a technique previously described (Zuk *et al.*, 2001). Briefly, adipose tissue was washed with phosphate-buffered saline (PBS) and enzymatically digested at 37°C for 30 min with 0.075% type IA collagenase (C-2674; Sigma, St. Louis, MO) in PBS. The digested adipose tissue was centrifuged at 1200 × *g* for 5 min to obtain a cell pellet. The pellet was resuspended and passed through a 100- μ m pore size filter to remove debris. The concentration of multinucleated cells was determined with a Coulter Counter (Beckman Coulter, Fullerton, CA). Cells were plated at 5 × 10⁶ nucleated cells/60 cm² onto standard 100-mm tissue culture dishes. PLA cells were expanded in medium consisting of Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 mg/liter) and L-Glutamine (Cellgro; Mediatech, Herndon, VA), 10% fetal bovine serum (HyClone, Logan, UT), and 1% antibiotic/antimycotic (Cellgro; Mediatech).

Vector production

Lentiviral and retroviral vectors were generated by calcium phosphate-mediated cotransfection of 293T cells as described previously (Sambrook *et al.*, 1998). For the generation of lentiviral vector, 293T cells (2 × 10⁷) were transfected with 5 μ g of pHCMVG, 12.5 μ g of pCMVdR8.2DVPR, and 12.5 μ g of a lentivirus reporter vector, pSIN18-Rh-MLV-E or pRRL-PGK-EGFP-SIN18 (Zufferey *et al.*, 1998; Kung *et al.*, 2000). For the generation of oncoretroviral vector, 5 μ g of pHCMVG, 12.5 μ g of pSV ψ -env-MLV, and 12.5 μ g of pSR α L-EGFP were used (An *et al.*, 1997). 293T cells were cultured in DMEM with 10% calf serum (GIBCO-BRL, Gaithersburg, MD), penicillin (100 U/ml), and streptomycin (100 μ g/ml). Supernatants were collected on days 2 and 3 post-transfection and filtered through 0.22- μ m pore size filters. The virus was concentrated 100-fold by ultracentrifugation. Stocks were maintained at -70°C.

Enhanced green fluorescent protein (EGFP) transduction units of all viral vectors were titrated on 293T cells. Briefly, 293T cells were infected with serial dilutions of each viral vector for 2 hr at 37°C. The infection with lentivirus or oncoretrovirus was done in the presence of Polybrene (8 μ g/ml). After incubation with the viral vector, the virus was removed and fresh medium was added. EGFP expression was analyzed 3 days after infection by flow cytometry. The titer was calculated at viral dilutions showing EGFP positivity of less than 10% to avoid multiple infection of cells. The titer (expressed as EGFP transduction units per milliliter) of each virus is as follows: SIN18-Rh-MLV-E (VSV-G), 1.8 × 10⁸; RRL-PGK-EGFP-SIN18 (VSV-G), 1.7 × 10⁸; SR α L-EGFP (VSV-G), 1.7 × 10⁶.

Adenoviral vector pAdCMVVEGFP is a first-generation, E1-deleted, replication-deficient adenoviral type 5 vector. The adenoviral vector was prepared as previously reported and kindly provided by L. Wu (Wu *et al.*, 2001).

Gene transduction of processed lipoaspirate cells and mouse embryonic stem cells

Murine embryonic stem (ES) cells were obtained from S.J. Elledge (Baylor College of Medicine, Houston, TX) (Liu *et al.*, 2000). The murine ES cells were cultured in KO-DMEM (GIBCO-BRL) with 10% fetal calf serum (GIBCO-BRL), penicillin (100 U/ml), streptomycin (100 μ g/ml), and 50 μ M 2-mercaptoethanol. The culture flask was coated with 0.2% gelatin (Sigma). Irradiated mouse fibroblasts were used as feeder cells. Murine ES cells were trypsinized on the day of infection. Feeder cells were removed by adherence to culture flasks (Falcon; BD Biosciences Discovery Labware, Bedford, MA) at 37°C for 2 hr. Murine ES cells (5 × 10⁴) were incubated with virus for 2 hr at 37°C. The virus was removed and cells were washed once with fresh medium. The cells were cultured for 3 days and EGFP expression was analyzed by flow cytometry after feeder cells were removed. Forward and side scatter of flow cytometry analysis clearly distinguished the feeder cell population from the murine ES cell population. PLA cells (1 × 10⁴) were seeded in each well of a 24-well plate the day before infection. The PLA cells were infected with oncoretrovirus or lentivirus at an MOI of 1.4 or 14. The PLA cells were also infected with adenoviral vector at MOIs of 1, 10, or 100. The MOI is calculated by dividing the EGFP transduction units of each vector on 293T cells by the number of infected PLA cells. EGFP expression of the infected cells was analyzed by flow cytometry 3 days after infection.

Differentiation of processed lipoaspirate cells

Adipogenic differentiation was induced by culturing cells in adipogenic medium (AM) consisting of complete medium (CM) supplemented with 0.5 mM isobutylmethylxanthine (IBMX), 1 μ M dexamethasone, 10 μ M insulin, and 200 μ M indomethacin (Green and Meuth, 1974; Hauner *et al.*, 1987; Zuk *et al.*, 2001) for 3 weeks. Differentiation was confirmed histologically by using oil red O stain as an indicator of intracellular lipid accumulation (Preece, 1972; Zuk *et al.*, 2001). Briefly, the cells were fixed for 60 min at room temperature in 4% formaldehyde-1% calcium and washed with 70% ethanol. The cells were then in-

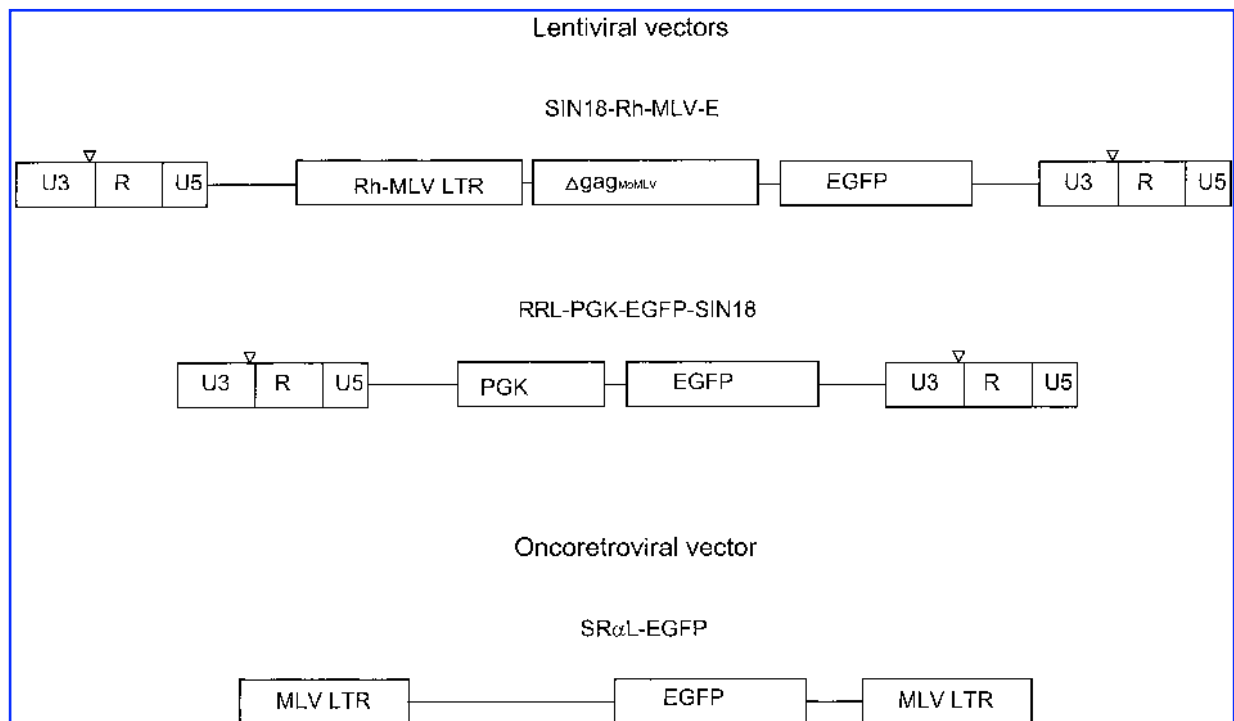


FIG. 1. Schematic diagram of lentiviral and oncoretroviral constructions. Both lentiviral vectors have a 400-bp deletion (∇) in the U3 region of the 3' LTR (Gill *et al.*, 2001). SIN18-Rh-MLV-E has an internal LTR derived from the amphi-mink cell focus-forming retrovirus in the serum of one rhesus macaque monkey that developed T cell lymphoma after autologous transplantation of enriched bone marrow stem cells transduced with a retrovirus preparation containing replication-competent virus (Kung *et al.*, 2000). RRL-PGK-EGFP-SIN18 has a housekeeping PGK promoter as an internal promoter (Gill *et al.*, 2001). SR α L-EGFP uses the MuLV LTR as promoter. SIN18-Rh-MLV-E and SR α L-EGFP have a partial MuLV gag sequence that is untranslated (Kung *et al.*, 2000). All vectors have EGFP as reporter gene. The vectors are drawn to scale.

cubated in 2% (w/v) oil red O reagent for 5 min at room temperature. Excess stain was removed by washing with 70% ethanol, followed by several changes of distilled water. The cells were counterstained for 2 min with hematoxylin.

Osteogenic differentiation was induced after culturing cells

in osteogenic medium (OM) consisting of CM supplemented with 0.1 μ M dexamethasone, 50 μ M ascorbate 2-phosphate, and 10 mM β -glycerophosphate. Differentiation was confirmed by alkaline phosphatase (AP) activity that is upregulated in osteogenic tissues (Jaiswal *et al.*, 1997; Pittenger *et al.*, 1999; Zuk

TABLE 1. EGFP EXPRESSION OF PROCESSED LIPOASPIRATE CELLS AND MURINE EMBRYONIC STEM CELLS TRANSDUCED BY LENTIVIRAL AND ONCORETROVIRAL VECTORS

Cell	Oncoretrovirus		Lentivirus	
	MOI ^a	(SR α L-EGFP)	SIN18-Rh-MLV-E	RRL-PGK-EGFP-SIN18
PLA donor A	14	13.8 ^b	92.6	84.7
	1.4	2.4	20.1	26.2
PLA donor B	14	18.5	93.8	87.0
PLA donor C	14	21.0	89.0	73.0
Mouse ES		—	<0.01 ^c	11.8 ^c

^aMOI was calculated by dividing the number of EGFP transduction units on 293T cells by the number of PLA cells infected.

^bPercentage of EGFP⁺ cells determined by flow cytometry.

^cMOI of SIN18-Rh-MLV-E was 0.5. MOI of RRL-PGK-EGFP-SIN18 was 0.6. A representative experiment is shown.

et al., 2001). To detect AP activity, cells were rinsed with PBS and stained with a 1% AP solution (1% naphthol-ASBI phosphate, fast red TR [1 mg/ml]) at 37°C for 30 min.

The EGFP expression of differentiated cells was confirmed by fluorescence microscopy.

RESULTS AND DISCUSSION

We examined different vectors for efficiency of transduction of PLA cells. The oncoretroviral vector utilized was a vesicular stomatitis virus G envelope (VSV-G)-pseudotyped murine leukemia virus (MuLV) vector, SR α L-EGFP (VSV-G); the lentiviral vector we used was the VSV-G-pseudotyped human immunodeficiency virus type 1 (HIV-1) vector SIN18-Rh-MLV-E (VSV-G) (Fig. 1). EGFP was used as the reporter gene for both vectors. To compare the transduction efficiency of each vector simply by EGFP expression, we used lentiviral and oncoretroviral vectors, which have similar transcriptional activity in transduced cells. We used SIN18-Rh-MLV-E as a lentiviral vector because the pro-

motor of SIN18-Rh-MLV-E was derived from an oncoretroviral vector and its transcriptional activity is similar to that of SR α L-EGFP (Kung *et al.*, 2000). The viruses were generated by calcium phosphate-mediated cotransfection of 293T cells and titrated on 293T cells as described previously. We infected PLA cells derived from three donors with both vectors, with the same number of EGFP transduction units. EGFP expression was analyzed by flow cytometry 3 days after infection. The efficiency of expression of lentivirus on PLA cells was 4- to 10-fold greater with lentivirus than with oncoretrovirus at the same MOI (Table 1 and Fig. 2). The percentage of EGFP-positive cells at 1 week was not different from the percentage of EGFP-positive cells determined 3 days after infection (data not shown). Because the same envelope protein was used for both vectors and titrations were performed on the same cell line, the efficiency of entry of both vectors would be expected to be similar. The requirement of the oncoretroviral vector for cell division could be the reason for the apparently lower infectivity of the oncoretroviral vector. Nuclear localization of the oncoretrovirus preintegration complex in PLA cells would not be expected to be efficient because of the slower

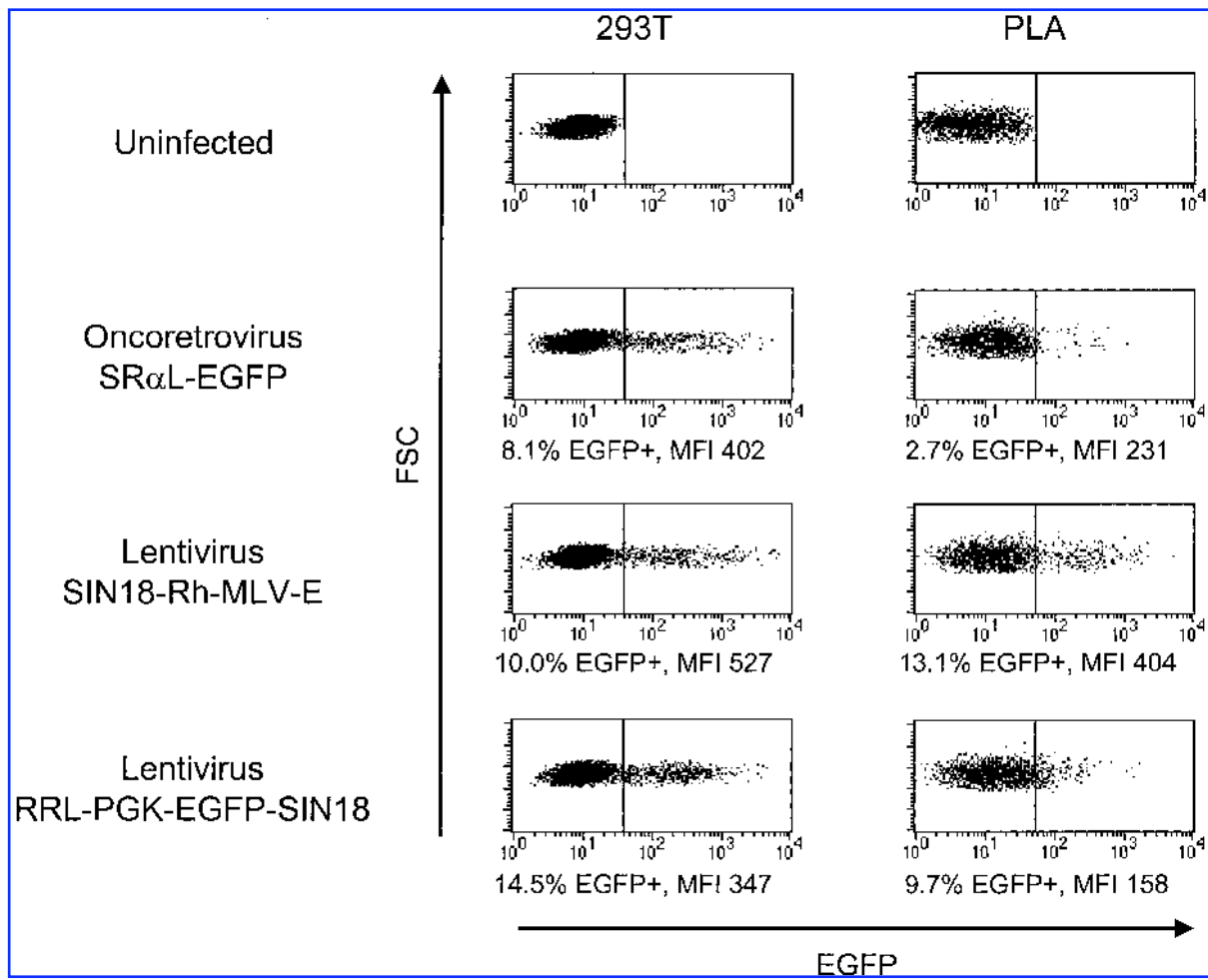


FIG. 2. EGFP expression in 293T cells and PLA cells transduced by oncoretroviral vector or lentiviral vector. 293T cells (2×10^5 cells) and PLA cells (4×10^4 cells) were infected with the same amount of lentiviral vector or oncoretroviral vector. Control cells were treated by addition to the medium of 8 μ g/ml of Polybrene. The percentage and mean fluorescence intensity (MFI) of GFP-positive cells 72 hr after transduction are shown.

growth rate of PLA cells (population doubling time is 60 hr) (Zuk *et al.*, 2001) as compared with 293T cells used for the titration of the vectors (doubling time is 24 hr). Another lentiviral vector, utilizing an internal phosphoglyceratekinase (PGK) promoter (RRL-PGK-EGFP-SIN18), gave similar levels of EGFP-positive PLA cells (Table 1). The percentage of transduced cells remained stable over at least 10 cell generations (100 days) (data not shown).

Adenoviral vectors can transduce a variety of tissues and cell types. They can also infect nondividing cells (Barnett *et al.*, 2002). Adenoviral vectors applied at high MOIs can result in integration and long-term gene expression (Harui *et al.*, 1999). We transduced PLA cells with a first-generation, E1-deleted, replication-deficient adenoviral type 5 vector (pAdCMVGFP) prepared as previously reported (Tan *et al.*, 1999; Miller *et al.*, 2000). At MOIs of 10 and 100 we achieved transduction effi-

ciencies of 51 and >99%, respectively. However, we could not perform studies subsequent to the differentiation experiment because transduction of PLA cells with the adenoviral vector at these high MOIs was cytotoxic (almost all transduced cells died), as has been reported for hematopoietic stem cells and other cell types (Teramoto *et al.*, 1995; Zheng *et al.*, 2000; Stecher *et al.*, 2001). We also transduced cells with a lentiviral vector using the same promoter as this adenoviral vector (the cytomegalovirus [CMV] promoter). We observed more than 90% EGFP positivity at an MOI of 14 without apparent toxicity (data not shown). Because the lentiviral vector was able to achieve high transduction efficiencies without any apparent cytotoxicity, we elected to use the lentiviral vector in all subsequent experiments.

Our goal was to achieve stable gene expression in PLA cells

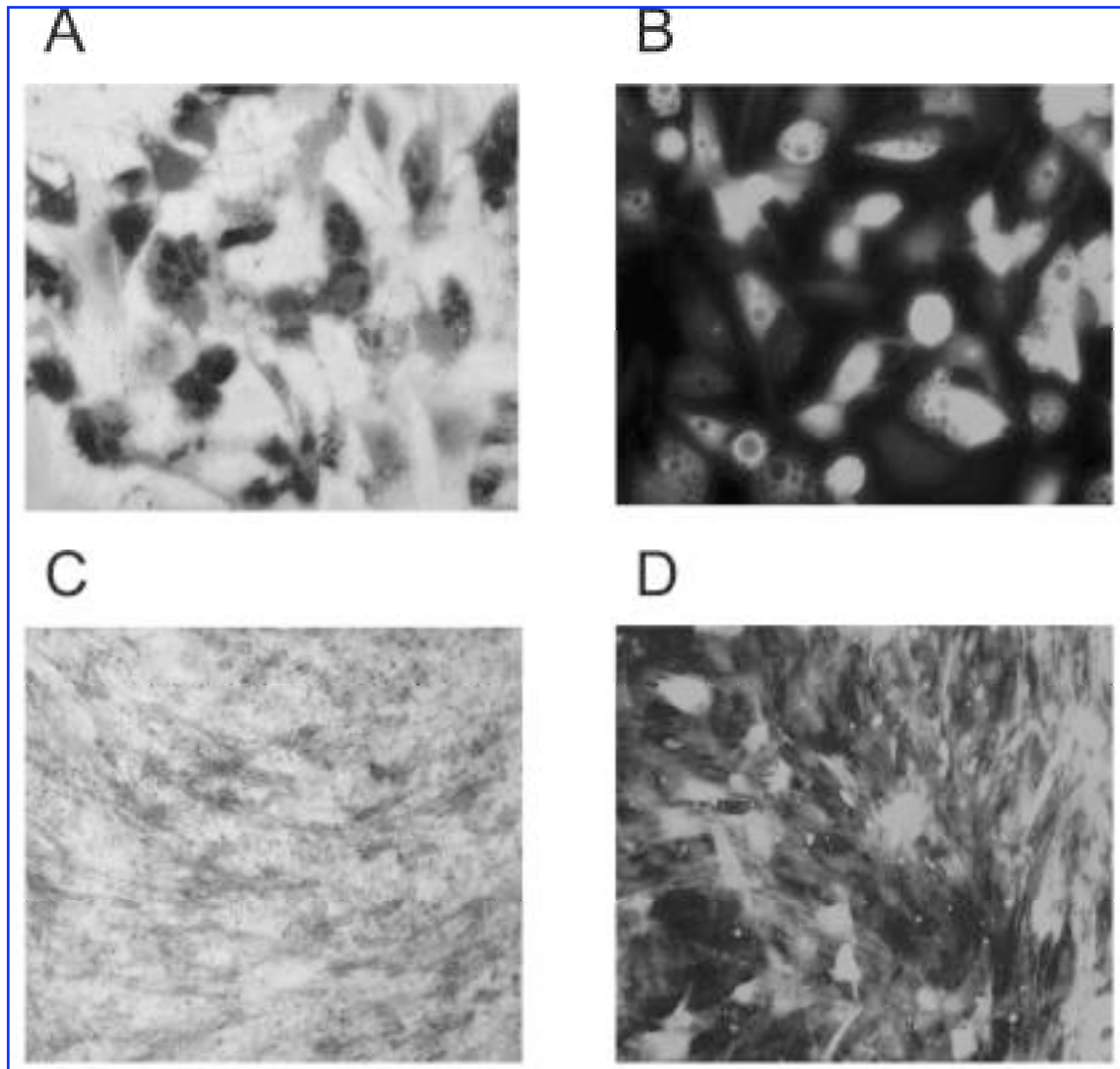


FIG. 3. (A) Adipogenic differentiation of PLA cells confirmed by the presence of intracellular lipid vesicles staining positive for oil red O. (B) EGFP expression of PLA cells after adipogenic differentiation. EGFP was visualized by fluorescence microscopy. (C) Osteogenic differentiation of PLA cells confirmed by the presence of positive alkaline phosphatase staining. Erythrocytes are positive for alkaline phosphatase activity. (D) EGFP expression of PLA cells after osteogenic differentiation. EGFP was visualized by fluorescence microscopy.

after long-term culture and after differentiation. It has been reported that gene expression of oncoretroviruses is silenced during differentiation and development of embryonic stem cells and hematopoietic stem cells (Jaenisch *et al.*, 1975; Gautsch, 1980; Robertson *et al.*, 1986; Challita and Kohn, 1994; Cherry *et al.*, 2000; Pannell *et al.*, 2000). On the other hand, there are several reports that lentiviral vectors are resistant to this silencing effect (Hamaguchi *et al.*, 2000; Lois *et al.*, 2002; Pfeifer *et al.*, 2002). The lentiviral vector SIN18-Rh-MLV-E, consisting of a modified internal MuLV long terminal repeat (LTR) in the context of a lentiviral vector, previously shown to be highly efficient in transduction and gene expression in T cells (Kung *et al.*, 2000), also efficiently transduced PLA cells (Table 1). However, because it is a hybrid vector, with both lentiviral and oncoretroviral characteristics, its transgene expression could be downregulated during differentiation. Mouse ES cells have been used to investigate gene silencing because they shut off transgene expression efficiently (Cherry *et al.*, 2000; Hamaguchi *et al.*, 2000; Pannell *et al.*, 2000). We investigated the susceptibility of the lentiviral vector to a silencing effect by using mouse ES cells. We could not detect any expression of EGFP in mouse ES cells infected with SIN18-Rh-MLV-E (VSV-G), indicating that this lentiviral vector is also susceptible to a silencing effect (Table 1). Because other viral promoters (e.g., the CMV promoter) have also been shown to be silenced (Loser *et al.*, 1998; Gill *et al.*, 2001), we tested a different lentiviral vector, RRL-PGK-EGFP-SIN18, which utilizes an internal PGK promoter (Zufferey *et al.*, 1998). We found that RRL-PGK-EGFP-SIN18 (VSV-G) showed high EGFP expression in mouse ES cells (Table 1), indicating resistance to gene silencing, at least in ES cells. Although none of the vectors tested was silenced in PLA cells and silencing is likely to be different in different cell types, we elected to utilize RRL-PGK-EGFP-SIN18 (VSV-G) in further experiments to assess transgene expression during differentiation and long-term culture of PLA cells.

We determined the differentiation of EGFP-transduced PLA cells and EGFP expression after differentiation. We transduced PLA cells with RRL-PGK-EGFP-SIN18 (VSV-G) at a high MOI such that nearly all cells were infected. After initial transduction at an MOI of 50, 98% of PLA cells expressed EGFP. Transgene expression remained in more than 95% of cells after 100 days of *in vitro* culture. Cells were analyzed for their capacity to differentiate along multiple lineages, using methods previously described in Materials and Methods. Cells maintained in control medium were used as negative controls for each lineage of differentiation. We did not observe any differentiation of the transduced cells without induction by specific medium.

Adipogenic differentiation was induced by culturing cells in adipogenic medium for 3 weeks (Green and Meuth, 1974; Hauner *et al.*, 1987; Zuk *et al.*, 2001). Differentiation was confirmed histologically, using oil red O stain as an indicator of intracellular lipid accumulation (Preece, 1972; Zuk *et al.*, 2001) (Fig. 3A). Approximately 50% of the cells incubated in adipogenic medium contained intracellular lipid vesicles indicative of adipogenesis. No PLA cells in control medium showed intracellular lipid vesicles (data not shown). EGFP expression in differentiated cells was confirmed by fluorescence microscopy (Fig. 3B). A comparable percentage of these differ-

entiated PLA cells expressed EGFP for 2 and 3 weeks after differentiation (data not shown).

Osteogenic differentiation was induced after culturing cells in osteogenic medium for 3 weeks. Differentiation was confirmed histologically by alkaline phosphatase staining, which is upregulated in osteogenic tissues (Jaiswal *et al.*, 1997; Pittenger *et al.*, 1999; Zuk *et al.*, 2001). EGFP-transduced PLA cells showed alkaline phosphatase activity after culture in osteogenic medium (Fig. 3C) but not in control medium (data not shown). EGFP expression was maintained after osteogenic differentiation (Fig. 3D).

To our knowledge, this is the first published report demonstrating gene transfer into adipose tissue-derived mesenchymal progenitors and maintenance of transgene expression after differentiation. We used a lentiviral vector, which takes advantage of a housekeeping gene promoter that may help to prevent the silencing effect seen with other vectors in differentiating cells. We observed maintenance of transgene expression before and after lineage-specific differentiation. Furthermore, we observed transgene expression after long-term culture. These results suggest that PLA cells may represent an efficacious vehicle for gene delivery. The clinical utility of PLA cells for gene therapy is enhanced by their ease of procurement from a relatively abundant source, adipose tissue. One to 2 liters of adipose can be obtained by procedures such as liposuction, with minimal morbidity, and yield hundreds of millions of PLA cells. PLA cells can also be easily expanded by culturing. Finally, PLA cells can be obtained safely from adult donors and can be used in autologous therapies without concern about rejection and the need for immunosuppression.

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