

Comparison of Multi-Lineage Cells from Human Adipose Tissue and Bone Marrow

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Key Words

Mesenchymal stem cell · Lipoaspirate · Processed lipoaspirate cells · Adipose tissue

Abstract

Our laboratory has recently characterized a population of cells from adipose tissue, termed processed lipoaspirate (PLA) cells, which have multi-lineage potential similar to bone-marrow-derived mesenchymal stem cells (MSCs).

This study is the first comparison of PLA cells and MSCs isolated from the same patient. No significant differences were observed for yield of adherent stromal cells, growth kinetics, cell senescence, multi-lineage differentiation capacity, and gene transduction efficiency. Adipose tissue is an abundant and easily procured source of PLA cells, which have a potential like MSCs for use in tissue-engineering applications and as gene delivery vehicles.

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Abbreviations used in this paper

ABAM	antibiotic-antimycotic
AP	alkaline phosphatase
CM	control medium
DMEM	Dulbecco's modified media
FBS	fetal bovine serum
GPDH	glycerophosphate dehydrogenase
HIV-1	human immunodeficiency virus type-1
MLV	murine leukemia virus
MSC	mesenchymal stem cells
PBS	phosphate-buffered saline
PLA	processed lipoaspirate cells
VSV-G	vesicular stomatitis virus G protein

Introduction

Mesenchymal stem cells (MSCs) derived from bone marrow have been used experimentally for gene therapy and tissue-engineering applications [Friedenstein, 1990; Prockop, 1997; Pittenger et al., 1999; Caplan, 2000; Mason et al., 2000; Mosca et al., 2000]. Currently, MSCs are obtained by aspiration of 10–40 ml of bone marrow from the iliac crest or at the time of bone marrow biopsy and isolated by their adherence properties. Ex vivo culture expansion results in hundreds of millions of cells within a few passages [Pittenger et al., 1999; Caplan, 2000]. Moreover, the apparent pluripotent nature of MSCs makes

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them excellent candidates for tissue engineering. However, MSCs have been reported to require selective sera lots and growth factor supplements for culture expansion [Lennon et al., 1996]. Furthermore, traditional bone marrow procurements, particularly in volumes larger than a few milliliters may be painful, frequently requiring general or spinal anesthesia [Auquier et al., 1995; Nishimori and Yamada, 2002], and may yield low numbers of MSCs upon processing [Pittenger et al., 1999]. As an alternative to MSCs, our laboratory has identified a population of cells isolated from human liposuctioned adipose tissue termed processed lipoaspirate cells (PLA cells), which appear to have similar multi-lineage mesodermal potential as MSCs [Zuk et al., 2001; Erickson et al., 2002; Mizuno et al., 2002; Safford et al., 2002]. The advantage of adipose tissue as a source of multi-lineage cells is its relative abundance and ease of procurement by local excision or suction-assisted liposuction. These factors may make PLA cells a viable clinical alternative to MSCs. However, there is a need to more accurately compare these two populations. Therefore, in this study, we sought to compare PLA cells and MSCs based on several parameters. Given donor variability and to make the comparison internally consistent, we compared PLA cells and MSCs obtained from the same patient for the following: (1) cell yield, (2) growth kinetics, (3) cell senescence, (4) multi-lineage differentiation capacity, and (5) gene transduction efficiency.

Materials and Methods

Materials

All materials were from Sigma unless otherwise stated. All tissue culture plasticware was from Fisher. Fetal bovine serum (FBS) was purchased from Hyclone (Logan, Utah, USA). Dulbecco's modified media (DMEM), trypsin/EDTA, and antibiotic-antimycotic (ABAM) were purchased from Cellgro (Herndon, Va., USA).

Harvest of Adipose Tissue and Bone Marrow

A bone marrow biopsy and a small piece of adipose tissue were obtained from 5 patients undergoing elective hip surgery after informed consent using an IRB-approved protocol. The patients included 3 males and 2 females ranging in age from 7 to 71 (mean 50). The average age of the patients was 50 years (range 7–71). Tissues were processed within 3 h of harvesting.

Isolation of PLA Cells from Adipose Tissue

PLA cells were isolated using a modification of a technique described previously by our laboratory [Zuk et al., 2001]. Adipose tissue was weighed, extensively washed with phosphate-buffered saline (PBS), minced for 10 min with fine scissors, and enzymatically digested at 37°C for 30 min with 0.075% type IA collagenase (C-2674; Sigma®, St. Louis, Mo., USA) in PBS. The digested adipose

tissue was centrifuged at 1,200 g for 5 min to obtain a cell pellet. The pellet was resuspended and passed through a 100- μ m filter to remove debris. The concentration of nucleated cells was determined using a Coulter Counter. Cells were plated in medium at a density of 5×10^6 nucleated cells/100-mm tissue culture dish. The plating density used was based on previous experience [Zuk et al., 2001] and designed to result in subconfluent plates within 2 weeks of initial plating. The number of adherent cells was estimated by multiplying the number of plates isolated by the cell density. The yield was determined by calculating the number of adherent cells per gram of tissue harvested.

Isolation of MSCs from Bone Marrow

MSCs were isolated using a modification of a technique previously described [Haynesworth et al., 1992a, b]. The bone marrow sample was weighed, washed with 15 ml of control medium (CM) and vortexed. The cell suspension was passed through a 100- μ m nylon cell strainer (BD®; Franklin Lakes, N.J., USA). The residual bone marrow was washed, vortexed, and filtered twice. The filtered cell suspension was centrifuged at 1,200 g. The cell pellet was resuspended in CM and the cell concentration determined using a Coulter Counter. MSCs were plated at a concentration of 5×10^7 to 1×10^8 nucleated cells/100-mm tissue culture dish. This plating density was based on established isolation methods [Haynesworth et al., 1992a, b] and resulted in subconfluent plates within 2 weeks of plating. The number of adherent cells and yield were estimated as described for PLA cells.

Culture and Expansion

Following plating, both MSCs and PLA cells were maintained in a humidified incubator at 37°C/5% CO₂ in CM consisting of DMEM (Cellgro), 10% FBS (Hyclone®), and 1% ABAM (Cellgro). The medium was changed after 3–5 days to remove nonadherent cells and twice weekly thereafter. Cells were passaged with 0.25% trypsin/0.1% EDTA (Cellgro) upon reaching 90% confluency and expanded until passage 4, whereupon they were analyzed.

Growth Kinetics

Analysis of growth kinetics was performed using modified techniques previously described [Bruder et al., 1997; DiGirolamo et al., 1999; Zuk et al., 2001]. Passage 4 cells were plated into 6-well dishes at a high seeding density (1.85×10^3 cells/cm²). Cells from three wells were trypsinized every other day from day 1 to day 11 and viable cells were counted using a hemacytometer. The mean number of cells was calculated and plotted against culture time to generate a growth curve. The logarithmic phase of growth occurred between days 3 and 9. The number of population doublings was calculated using the formula $\log N_3 / \log N_9$, where N_3 is the number of cells on day 3 and N_9 is the number of cells on day 9. Doubling time was determined by dividing the total number of hours in culture (i.e. 144 h) by the number of doublings.

Cell Senescence Assay

Cell senescence was assessed using a β -gal staining assay, in which β -galactosidase activity is detected in senescent cells at pH 6.0 [Dimiri et al., 1995]. Passage 4 cells were plated into 6 wells at a seeding density of 1.85×10^3 cells/cm² and cultured for 2 weeks in CM. Cells were fixed for 5 min in 2% formaldehyde-glutaraldehyde, and incubated in a β -gal reaction buffer [1 mg/ml X-gal, 40 mM citric acid/sodium phosphate buffer (pH 6.0), 5 mM each of potassium ferro-

cyanide and potassium ferricyanide, 150 mM NaCl and 2 mM MgCl₂] for 2 h. The percentage of senescent cells (number of blue cells/total number of cells counted) was calculated for three randomly selected visual fields at 400 × magnification.

Confirmation of in vitro Multi-Lineage Differentiation – Histology and Spectrophotometric Assays

Cells were analyzed for their capacity to differentiate along the adipogenic, osteogenic, chondrogenic, and neuron-like lineage using the methods described below. Cells maintained in CM were used as negative controls.

Adipogenic Differentiation. Cells were cultured in CM for 1 week. Adipogenic differentiation was induced by culturing cells for 3 weeks in adipogenic medium consisting of CM supplemented with 0.5 mM isobutyl-methylxanthine, 1 μM dexamethasone, 10 μM insulin, and 200 μM indomethacin [Green and Meuth, 1974; Hauner et al., 1987]. Differentiation was confirmed histologically using the oil red O stain as an indicator of intracellular lipid accumulation [Preece, 1972]. 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 incubated 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. Adipogenic differentiation was quantified by counting cells in three randomly selected visual fields at low magnification (100 ×) and calculating the percentage of adipogenic cells (number of cells with oil red O-positive intracellular vesicles/total number of cells × 100). To quantify adipogenesis biochemically, the lipogenic enzyme glycerol-3-phosphate dehydrogenase (GPDH) was measured spectrophotometrically. The assay was performed as previously described [Wise and Green, 1979]. Triplicate samples of cells were washed with and harvested in PBS using a cell scraper. One unit of GPDH was defined as the oxidation of 1 nmol of NADH/min. GPDH activity was expressed as units GPDH/μg protein. Values were expressed as the mean ± SD.

Osteogenic Differentiation. Cells were cultured in CM for 1 week. Osteogenic differentiation was induced after culturing cells for 3 weeks in osteogenic medium consisting of CM supplemented with 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, and 10 mM β-glycerophosphate [Pittenger et al., 1999]. Spectrophotometric assays were performed to detect alkaline phosphatase (AP) activity [Beresford et al., 1986] and calcium content (Sigma; per package insert). For AP activity, triplicate samples of cells were washed with PBS and harvested into PBS/0.1% Triton X-100. AP enzyme activity was assayed using a commercial AP enzyme kit (Sigma) according to the manufacturer. AP activity was expressed as nmol *p*-nitrophenol produced/min/μg protein. For the calcium assay, triplicate samples of cells were harvested in PBS and centrifuged. The cell pellet was extracted in 0.1 N HCl in 4 °C for a minimum of 4 h. The extracts were centrifuged for 5 min at 10,000 *g* and total calcium in the supernatant was determined using a commercial kit (Sigma #587). Levels were expressed as mM Ca/μg protein. Values were expressed as the mean ± SD.

Chondrogenic Differentiation. Chondrogenic differentiation was induced using a modified micromass culture technique [Denker et al., 1995; Mackay et al., 1998; Pittenger et al., 1999; Zuk et al., 2001]. Briefly, 5 μl of a concentrated cell suspension (1 × 10⁷ cells/ml) was pipetted into the center of each well and allowed to attach at 37 °C for 2 h. Chondrogenic medium consisting of DMEM supplemented with 1% FBS, 6.25 μg/ml insulin, 10 ng/ml TGFβ1, 50 nM

ascorbate-2-phosphate, and 1% ABAM was gently overlaid so as not to detach the cell nodules. Cultures were maintained in chondrogenic medium for 2 weeks prior to analysis. To confirm differentiation, cell nodules were fixed in 4% paraformaldehyde for 15 min at room temperature, washed extensively with PBS, incubated for 30 min in 1% (w/v) Alcian blue (Sigma A-3157) in 0.1 N HCl (pH 1.0), and washed with 0.1 N HCl for 5 min to remove excess stain [Lev and Spicer, 1964].

Neuron-Like Differentiation. Cells were cultured in complete medium for 1 week. Neuron-like differentiation was induced after culturing cells for 2 weeks in a neurogenic medium modified from that described previously [Woodbury et al., 2000]. Neurogenic medium consisted of DMEM supplemented only with 5–10 mM β-mercaptoethanol. Neuronal morphology was defined as the presence of a refractile cell body and a minimum of two filopodial projections. The percentage of cells with neuron-like morphology was calculated (number of cells with neuronal morphology/total number of cells × 100).

Gene Transduction

Gene transduction efficiency of PLA cells and MSCs was examined using a first generation adenovirus vector, a human immunodeficiency virus type-1 (HIV-1)-based lentivirus vector, and a murine leukemia virus (MLV)-based oncoretrovirus vector. The adenovirus vector was an E1-deleted, replication-deficient adenoviral type 5 vector with EGFP reporter gene driven by a CMV promoter (pACCMVGFP) being used as the adenovirus vector [Tan et al., 1999; Miller et al., 2000]. The lentivirus vector was a vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1-based vector, SIN18-Rh-MLV-E (VSV-G). The oncoretrovirus was a VSV-G-pseudotyped MLV vector, SRαL-EGFP (VSV-G) [Kung et al., 2000]. EGFP was used as reporter gene for all vectors. The lentiviral and retroviral viruses were generated by calcium phosphate-mediated cotransfection of 293T cells [Kung et al., 2000]. Cells were infected with all viral vectors for 2 h at 37 °C. The infection by lentivirus and oncoretrovirus vector was done in the presence of 8 μg/ml polybrene. EGFP expression was analyzed 3 days after infection by flow cytometry. EGFP transduction units of all virus vectors were titrated on HeLa cell. PLA cell- and bone marrow-derived MSC were infected by the vector at the same multiplicity of infection. The multiplicity of infection of adenovirus, lentivirus, and oncoretrovirus vector were 5, 14, and 14, respectively. Of note, equal transduction units were used for lentivirus and retrovirus. In order to quantify GFP expression for transduced cells, the mean fluorescence intensity (FL1+ gated population) was also recorded.

Statistics

Values for bone marrow and fat cells were compared using Student's *t* test for paired samples assuming a two-tailed distribution.

Results

Tissue Harvest and Cell Isolation

The mean mass of collected tissue was 7 g (range 0.5–11 g) for bone marrow and 17 g (range 3–26 g) for adipose tissue. The mean number of nucleated cells isolated per gram of bone marrow was significantly higher than of adi-

Table 1. Cell yield of PLA cells and MSCs

Patient data			Adipose				Bone marrow			
number	age	scatter plot symbol	mass of harvested tissue, g	total nucleated cells	number of adherent cells	yield adherent cells/g	amount of tissue harvested, g	total nucleated cells	number of adherent cells	yield adherent cells/g
1	54	+	15	2×10^7	4×10^6	3×10^5	6	4×10^8	2×10^6	3×10^5
2	71	□	19	5×10^6	1×10^6	0.5×10^5	11	5×10^7	0.5×10^6	0.5×10^5
3	61	△	26	3×10^7	6×10^6	2×10^5	10	8×10^8	4×10^6	4×10^5
4	7	◇	3	5×10^6	1×10^6	3×10^5	0.5	2×10^7	0.08×10^6	2×10^5
5	55	×	22	1.5×10^7	3×10^6	1×10^5	6	6×10^8	3×10^6	5×10^5
Average	50	–	17	1.5×10^7	3×10^6	2×10^5	7	4×10^8	2×10^6	3×10^5

The amount of tissue harvested, total nucleated cells isolated, number of adherent cells, and cell yield from the adipose and bone marrow of 5 patients are summarized. The yield was calculated by dividing the number of adherent cells by tissue mass (adherent cells/g).

pose tissue (6×10^7 vs. 1×10^6 ; $p < 0.03$). Because of the higher number of contaminating hematopoietic cells, bone marrow isolates were plated at 10- to 20-fold higher densities [Haynesworth et al., 1992 a, b]. Both bone marrow and adipose isolates grew to subconfluence within 2 weeks. Table 1 provides a patient summary for the mass of tissue collected, number of nucleated cells, number of adherent cells, and yield of adherent cells per gram of tissue. There was no significant difference in yield between bone marrow and adipose tissue.

Growth Kinetics and Cell Senescence

At a plating density of 1.85×10^3 cells/cm², passage for PLA cells and MSCs reached confluence by day 13. Mean population doubling times during the logarithmic phase of growth from days 3 to 9 were not statistically significant (78 ± 26 h for PLA cells vs. 86 ± 23 h for MSCs; fig. 1). No significant differences were observed for cell senescence based on β -galactosidase activity (fig. 2).

In vitro Differentiation

No significant differences were observed between PLA cells and MSCs for markers of adipogenesis. The percentage of cells staining positive for oil red O was $49 \pm 14\%$ of PLA cells and $55 \pm 15\%$ of MSCs (fig. 3a). Activity of the lipogenic enzyme GPDH was 0.05 ± 0.04 units/ μ g in PLA cells and 0.1 ± 0.2 units GPDH/ μ g protein in MSC (fig. 3b).

As with adipogenesis, no significant difference was observed between PLA cells and MSCs for the osteogenic markers AP (0.10 ± 0.12 vs. 0.08 ± 0.07 nmol *p*-nitro-

phenol produced/min/ μ g protein; fig. 4a) or total calcium (33 ± 38 vs. 42 ± 55 mM Ca/ μ g protein; fig. 4b).

PLA cells could be successfully used to make micro-mass nodules which stained positive for Alcian blue (data not shown). By contrast, we were unable to form Alcian-blue-positive micromass nodules with the MSCs under these conditions.

Finally, no significant difference was observed between PLA cells and MSCs for the percentage of cells that developed a neuron-like morphology (5 ± 5 vs. $3 \pm 3\%$; fig. 5).

Gene Transduction

Both PLA cells and MSCs were successfully transduced with adenovirus, lentivirus, and retrovirus (fig. 6) with no observed cytotoxicity at the multiplicities of infection used. While adenoviral transduction efficiency was greater for MSCs than PLA cells (85 ± 18 vs. $63 \pm 15\%$; $p < 0.05$), no significant difference was observed for lentivirus (92 ± 2.3 vs. $90 \pm 13\%$) and retrovirus (15 ± 6.2 vs. $9 \pm 8.5\%$). Transduction efficiencies were significantly higher for lentivirus than that for retrovirus for both PLA cells and MSCs ($p < 0.001$). No significant difference was noted for the expression level (mean linear fluorescence intensity) between PLA and MSCs for all vectors tested (data not shown).

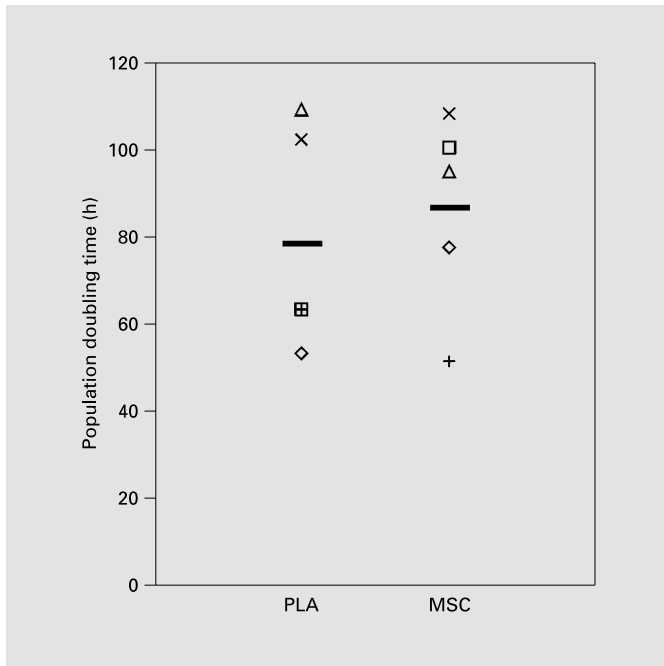


Fig. 1. Populating doubling time of PLA cells and MSCs. Cells were seeded at identical plating densities and counted every other day. Doubling time was not significantly different between PLA cells and MSCs. Mean values are represented by a horizontal bar.

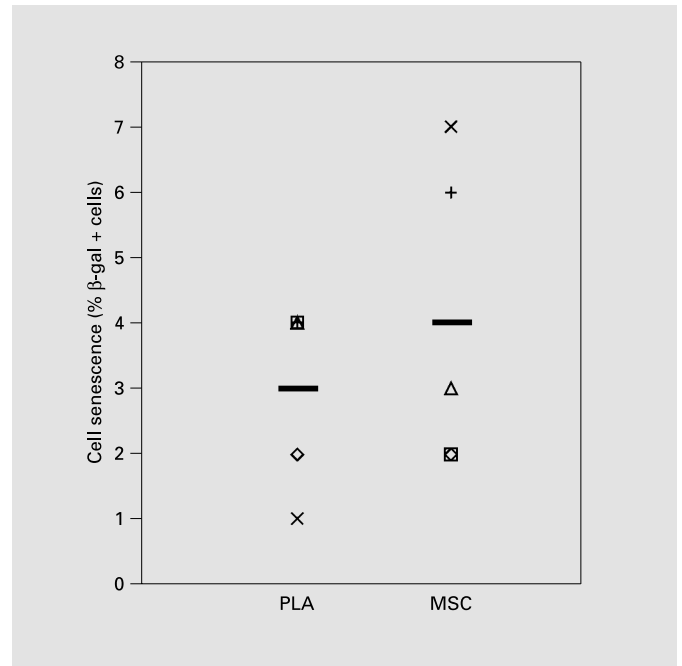


Fig. 2. Cell senescence of PLA cells and MSCs. β -gal staining was used to ascertain the number of senescent cells. The percentage of cells staining blue is graphically summarized. Mean values are represented by a horizontal bar.

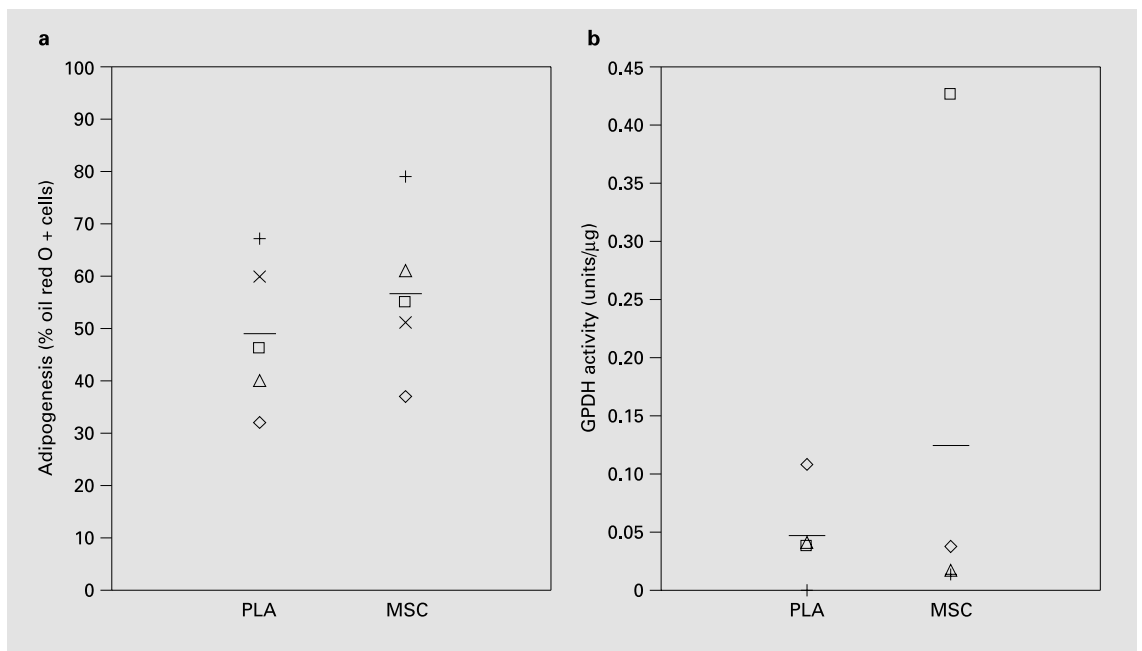


Fig. 3. Adipogenic differentiation. PLA cells and MSCs were incubated in adipogenic media. Cells were stained with oil red O and the number of cells staining positive were counted and expressed as a percentage of the total number of cells **(a)**. **(b)** Levels of the lipogenic enzyme GPDH were quantified using a spectrophotometric assay. Mean values are represented by a horizontal bar.

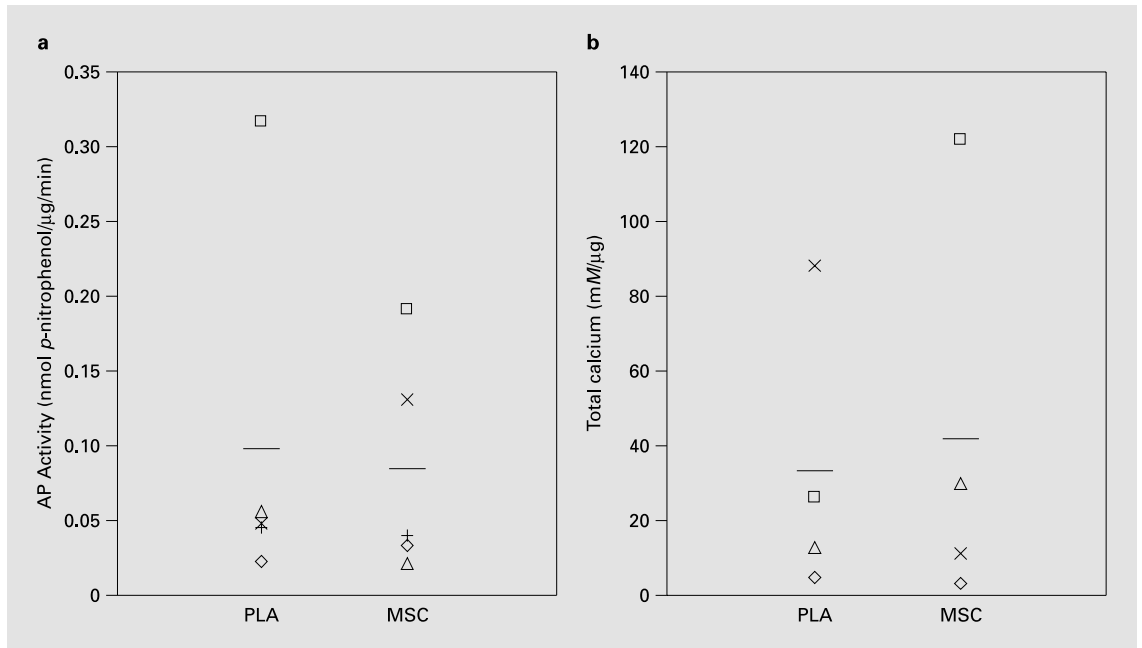


Fig. 4. Osteogenic differentiation. PLA cells and MSCs were incubated in osteogenic medium. The AP activity (**a**) and calcium content (**b**) were determined using spectrophotometric assays. Mean values are represented by a horizontal bar.

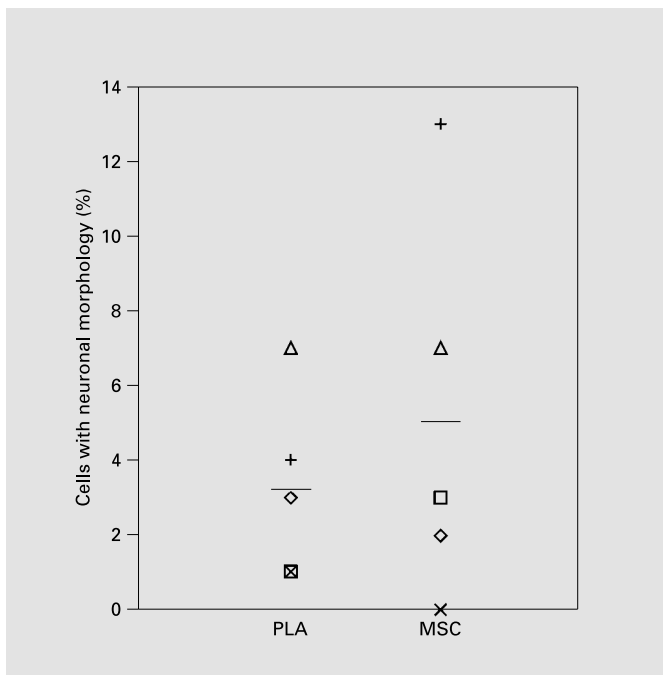


Fig. 5. Neuron-like differentiation. Cells were incubated in neurogenic medium. The percentage of PLA cells and MSCs with neuronal morphology (defined as a refractile cell body and a minimum of two filopodial projections) was compared. Mean values are represented by a horizontal bar.

Discussion

MSCs can be isolated by their adherence properties to plastic in tissue culture. The presence of a similar population of cells derived from adipose tissue (PLA cells) provides the opportunity to obtain stromal cells with multilineage potential from a much more readily available and abundant source. Therefore, a direct comparison of PLA cells and MSCs isolated from the same patients was performed. We observed that the yield of adherent stromal cells per gram of tissue was similar between bone marrow and adipose tissue. However, experience with large volume bone marrow harvests in the context of bone marrow transplantation has demonstrated that scaling from small to large harvest volumes is compromised by a substantially increased contamination with whole blood [Batinic et al., 1990; Bacigalupo et al., 1992] such that a 1,000-ml harvest contains significantly less than 100 times the stem cell content of a 10-ml harvest. Adipose tissue harvested by liposuction is not subject to such dilution effects. Indeed, we have previously described essentially the same cell yields from considerably larger amounts of adipose tissue [Zuk et al., 2001]. As a result, our data demonstrate that a typical lipoaspirate of 1,000 ml will yield 1.5×10^{10} PLA cells after two passages. Furthermore, PLA cells had

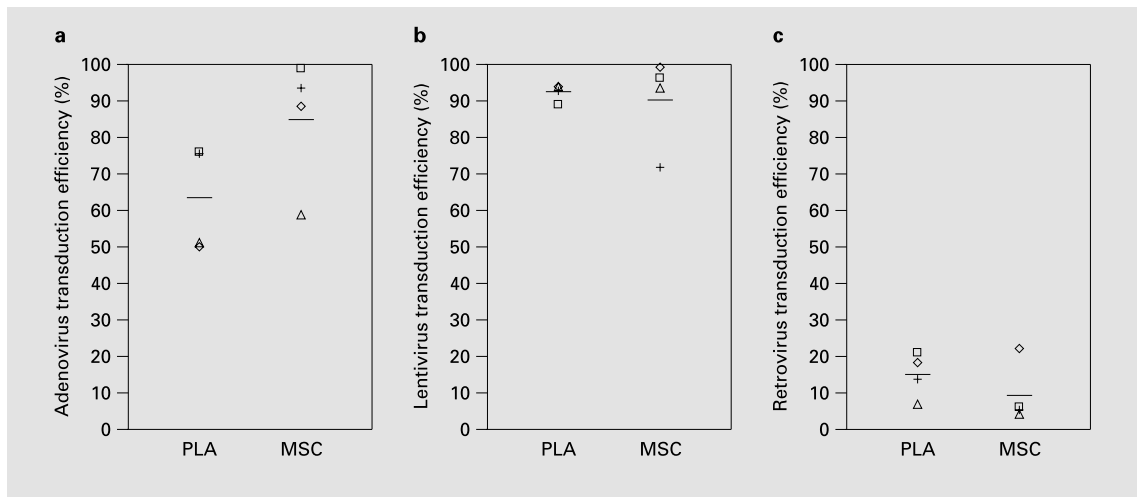


Fig. 6. Gene transduction efficiency. Cells were transduced with adenoviral (a), lentiviral (b) and retroviral (c) vectors containing a green fluorescent protein reporter gene. Transduction efficiency (GFP expression) was determined by flow cytometry. Mean values are represented by a horizontal bar.

a proliferative capacity comparable to MSCs (fig. 1) with a similar ability to undergo multi-lineage differentiation (fig. 3–5). Large-volume bone marrow harvests are associated with significant morbidity [Auquier et al., 1995; Nishimori et al., 2002]. While there is no rigorous data available on postoperative pain following liposuction the nature of this procedure is such that it is generally less invasive than a marrow harvest.

Culture expandability and self-renewal are properties intrinsic to stem cells and essential for their use in gene therapy and tissue-engineering applications. MSCs have been reported to require hydrocortisone, selected lots of fetal bovine and horse serum, and other supplements for long-term culture [Lennon et al., 1996]. In our experience, we observed no special requirements for the culture expansion of PLA cells [Zuk et al., 2001]. This may explain the relatively slow doubling times of both populations and the poor chondrogenic capacity of MSCs observed in this study compared to that reported in the literature [Johnstone et al., 1998; Yoo et al., 1998]. However, it should be noted that PLA cells did exhibit substantial chondrogenic capacity in the same culture medium. Under the conditions we describe, the population doubling time we report for nonselective media conditions was comparable for MSCs and PLA cells. Furthermore, we observed no differences between MSCs and PLA cells in cell senescence.

Our observation that PLA cells and MSCs have a similar in vitro differentiation potential suggests that PLA

cells have the potential to serve as substitutes for MSCs in tissue-engineering applications involving fat, bone, cartilage, and possibly nervous tissue. Stromal cells derived from adipose tissue have been used experimentally to regenerate adipose tissue in rats [Patrick et al., 1999], and human cells have been used to generate cartilaginous constructs [Erickson et al., 2002]. Studies are underway to investigate the potential of PLA cells to form bone. Recently, cells derived from adipose and other tissues have been demonstrated to have the potential to differentiate into cells with characteristics of neurons, oligodendrocytes, and astrocytes [Kopen et al., 1999; Brazelton et al., 2000; Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Deng et al., 2001; Toma et al., 2001]. Additional work will be required to ascertain the true neuronal potential and in vivo regenerative capacity of PLA cells.

In addition to tissue-engineering applications, PLA cells have the potential to be used as gene delivery vehicles. PLA cells and MSCs were infected successfully using adenovirus, retrovirus, and lentivirus. While retrovirus has been used in several gene therapy experiments using MSCs with promising results [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], lentivirus has been demonstrated to have higher transduction efficiencies and has been used in many tissues and cells in vitro and in vivo [Naldini et al., 1996; Blomer et al., 1997; Kafri et al., 1997; Sutton et al., 1998; Evan et al., 1999]. In order to

compare transduction efficiencies of lentivirus and retrovirus in PLA cells and MSCs, we used vectors that utilize the same promoter and have similar transcriptional activity in HeLa cells [Kung et al., 2000]. One possible explanation for the lower transduction efficiency of retrovirus is that it requires cell division for efficient transduction [Roe et al., 1993; Case et al., 1999]. The longer population doubling times of PLA cells and MSCs compared with HeLa cells (around 24 h) supports this hypothesis. We are currently investigating whether PLA cells like MSCs have the ability to maintain stable lentivirus transgene expression in vivo and following differentiation.

In summary, adipose tissue is a relatively abundant, easily procured, and promising autologous source of multi-lineage cells. The clinical utility of PLA cells is enhanced by their nonselective media requirements, ready

culture expandability, in vitro multi-lineage differentiation capacity, and amenability to gene therapy. Additional studies will be required to investigate the in vivo differentiation capacity and gene expression potential of PLA cells.

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