

# Multilineage Cells from Human Adipose Tissue: Implications for Cell-Based Therapies

PATRICIA A. ZUK, Ph.D.,<sup>1,2</sup> MIN ZHU, M.D.,<sup>1,2</sup> HIROSHI MIZUNO, M.D.,<sup>2</sup>  
JERRY HUANG, B.S.,<sup>2</sup> J. WILLIAM FUTRELL, M.D.,<sup>3</sup> ADAM J. KATZ, M.D.,<sup>3</sup>  
PROSPER BENHAIM, M.D.,<sup>2</sup> H. PETER LORENZ, M.D.,<sup>2</sup>  
and MARC H. HEDRICK, M.D.<sup>2</sup>

## ABSTRACT

Future cell-based therapies such as tissue engineering will benefit from a source of autologous pluripotent stem cells. For mesodermal tissue engineering, one such source of cells is the bone marrow stroma. The bone marrow compartment contains several cell populations, including mesenchymal stem cells (MSCs) that are capable of differentiating into adipogenic, osteogenic, chondrogenic, and myogenic cells. However, autologous bone marrow procurement has potential limitations. An alternate source of autologous adult stem cells that is obtainable in large quantities, under local anesthesia, with minimal discomfort would be advantageous. In this study, we determined if a population of stem cells could be isolated from human adipose tissue. Human adipose tissue, obtained by suction-assisted lipectomy (*i.e.*, liposuction), was processed to obtain a fibroblast-like population of cells or a processed lipoaspirate (PLA). These PLA cells can be maintained *in vitro* for extended periods with stable population doubling and low levels of senescence. Immunofluorescence and flow cytometry show that the majority of PLA cells are of mesodermal or mesenchymal origin with low levels of contaminating pericytes, endothelial cells, and smooth muscle cells. Finally, PLA cells differentiate *in vitro* into adipogenic, chondrogenic, myogenic, and osteogenic cells in the presence of lineage-specific induction factors. In conclusion, the data support the hypothesis that a human lipoaspirate contains multipotent cells and may represent an alternative stem cell source to bone marrow-derived MSCs.

## INTRODUCTION

**T**HE THERAPEUTIC POTENTIAL of multilineage stem cells for applications such as tissue engineering and gene therapy is enormous. Conceptually, there are two general types of stem cells potentially useful for these applications: embryonic stem cells (ESCs) and autologous stem cells. Although theoretically appealing because of their pluripotentiality, the practical use of ESCs is limited due to potential problems of

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<sup>1</sup>Dr. Zuk and Dr. Zhu are co-first authors.

<sup>2</sup>Laboratory for Regenerative Bioengineering and Repair, Departments of Surgery and Orthopaedic Surgery, UCLA School of Medicine, Los Angeles, California.

<sup>3</sup>Division of Plastic and Reconstructive Surgery, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

cell regulation and ethical considerations. In contrast, autologous stem cells, by their nature, are immunocompatible and have no ethical issues related to their use. For the engineering of mesodermally derived tissues, autologous stem cells obtained from bone marrow have proven experimentally promising. Human bone marrow is derived from the embryonic mesoderm and is comprised of a population of hematopoietic stem cells (HSCs), supported by a mesenchymal stroma.<sup>1-5</sup> Although the proliferation and differentiation of HSCs have been well documented, less is known about the stromal component. The bone marrow stroma, in both animals and humans, is heterogenous in composition, containing several cell populations, including a stem cell population termed mesenchymal stem cells or MSCs.<sup>6</sup> Studies on MSCs have demonstrated their differentiation into adipocytes,<sup>7,8</sup> chondrocytes,<sup>6,8-11</sup> myoblasts,<sup>12,13</sup> and osteoblasts.<sup>6,8,14-19</sup> These cells represent a promising option for future tissue engineering strategies. However, traditional bone marrow procurement procedures may be painful, frequently requiring general or spinal anesthesia and may yield low numbers of MSCs upon processing (approximately 1 MSC per 10<sup>5</sup> adherent stromal cells<sup>8,17,20</sup>). From a practical standpoint, low stem cell numbers necessitate an *ex vivo* expansion step to obtain clinically significant cell numbers. Such a step is time consuming, expensive, and risks cell contamination and loss. An ideal source of autologous stem cells would, therefore, be both easy to obtain, result in minimal patient discomfort, yet be capable of yielding cell numbers substantial enough to obviate extensive expansion in culture.

Adipose tissue may represent such a source. Although it is known that many tissues contain lineage-committed progenitor cells for tissue maintenance and repair, several studies have demonstrated the presence of uncommitted MSCs within the connective tissue matrices of several organs in birds, mice, rats, and rabbits.<sup>21-26</sup> Furthermore, adipose tissue, like bone marrow, is derived from the embryonic mesoderm and contains a heterogenous stromal cell population.<sup>27-31</sup> These similarities, together with the identification of MSCs in several tissues, make plausible the concept that a stem cell population can be isolated from human adipose tissue. Therefore, in this study, we sought to determine if a population of multipotential stem cells could be isolated from human adipose tissue.

## MATERIALS AND METHODS

### *Materials*

All materials were purchased from Sigma (St. Louis, MO) unless otherwise stated. All tissue culture reagents were purchased from Life Technologies (New York, NY). Fetal bovine serum (FBS) and horse serum (HS) were purchased from Hyclone (Logan, UT) and Life Technologies, respectively.

### *Cell lines*

Normal human osteoblasts (NHOst), human skeletal muscle (SkM) cells, and population of MSCs derived from bone marrow were purchased from Clonetics (Walkersville, MD). The murine 3T3-L1 preadipocyte cell line<sup>32</sup> was obtained from ATCC (Rockville, MD). Human foreskin fibroblasts (HFFs) were obtained from Cascade Biologics (Portland, OR).

### *Isolation and culture of stem cells—PLA and MSCs*

Human adipose tissue was obtained from elective liposuction procedures under local anesthesia (HSPC #98-08 011-02). In this procedure, a hollow blunt-tipped cannula was introduced into the subcutaneous space through small (~1 cm) incisions. The cannula was attached to gentle suction and moved through the adipose compartment, mechanically disrupting the fat tissue. A solution of saline and the vasoconstrictor epinephrine was infused into the adipose compartment to minimize blood loss and contamination of the tissue by peripheral blood cells. The raw lipoaspirate (~300 cc) was processed according to established methodologies to obtain a stromal vascular fraction (SVF).<sup>33,34</sup> To isolate the SVF, lipoaspirates were washed extensively with equal volumes of phosphate-buffered saline (PBS), and the ECM was digested at 37°C for 30 min with 0.075% collagenase. Enzyme activity was neutralized with Dulbecco's modified Eagle's medium (DMEM), containing 10% FBS and centrifuged at 1200 × *g* for 10 min to obtain a high-density

SVF pellet. The pellet was resuspended in 160 mM  $\text{NH}_4\text{Cl}$  and incubated at room temperature for 10 min to lyse contaminating red blood cells. The SVF was collected by centrifugation, as detailed above, filtered through a 100- $\mu\text{m}$  nylon mesh to remove cellular debris and incubated overnight at 37°C/5%  $\text{CO}_2$  in control medium (DMEM, 10% FBS, 1% antibiotic/antimycotic solution). Following incubation, the plates were washed extensively with PBS to remove residual nonadherent red blood cells. The resulting cell population was termed a processed lipoaspirate (PLA), to distinguish it from the SVF obtained from excised adipose tissue. PLA cells were maintained at 37°C/5%  $\text{CO}_2$  in noninductive control medium. Cells did not require specific FBS sera lots for expansion and differentiation (data not shown). For immunofluorescence studies, a population of MSCs was obtained from human bone marrow aspirates according to the protocol of Rickard *et al.*<sup>17</sup> and maintained in control medium. To prevent spontaneous differentiation, cells were maintained at subconfluent levels.

#### *Indirect immunofluorescence of PLA cells*

PLA cells and MSCs obtained from human bone marrow aspirates were plated onto glass chamber slides and fixed for 15 min in 4% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.0). The cells were washed for 10 min in 100 mM glycine in PBS (PBS/glycine) and blocked for 1 h in immunofluorescent blocking buffer (IBB) containing 5% bovine serum albumin (BSA), 10% FBS, 1  $\times$  PBS, 0.1% Triton X-100). The cells were subsequently incubated for 1 h in IBB containing the following cell-specific monoclonal antibodies: (1) anti-smooth muscle actin (anti-SMA; Cedarlane Inc., Hornby, Ontario), to identify smooth muscle cells and pericytes<sup>35-38</sup>; (2) anti-Factor VIII (anti-FVIII; Calbiochem, San Diego, CA), to identify endothelial cells<sup>39,40</sup>; and (3) ASO2 (dianova, Hamburg, Germany), to identify fibroblasts and cells of mesenchymal origin.<sup>41,42</sup> The cells were washed extensively with PBS/glycine and incubated for 1 h in IBB containing a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. The cells were washed with PBS/glycine and mounted with a solution containing DAPI to detect nuclei (VectaShield, Vector Labs, Burlingame, CA).

#### *Flow cytometry*

PLA samples from 5 donors were cultured in control medium for 72 h prior to analysis. Flow cytometry was performed on a FACScan argon laser cytometer (Becton Dickson, San Jose, CA). Cells were harvested in 0.25% trypsin/EDTA and fixed for 30 min in ice-cold 2% formaldehyde. Following fixation, cells were washed in flow cytometry buffer (FCB; 1  $\times$  PBS, 2% FBS, 0.2% Tween-20). Cell aliquots (1  $\times$  10<sup>6</sup> cells) were incubated in FCB containing monoclonal antibodies to FVIII, smooth muscle actin, or ASO2. In addition, cells were also incubated with FCB containing a monoclonal antibody to vimentin (anti-VIM; Biogenesis, Brentwood, NH), to identify mesenchymal cells.<sup>43,44</sup> To assess viability, duplicate samples were harvested, fixed for 30 min with ice-cold 1% paraformaldehyde, permeabilized with 0.05% Nonidet-40, and incubated with propidium iodide (PI) at a concentration of 25  $\mu\text{g}/\text{mL}$ . Debris and dead cells were excluded by eliminating PI-positive events. All subsequent PLA samples were corrected accordingly.

#### *Cumulative population doubling*

PLA cells were maintained in control medium until 80% confluent. Cells were harvested at confluence and population doubling calculated using the formula  $\log N_1/\log N_2$ , where  $N_1$  is the number of cells at confluence prior to passaging and  $N_2$  is the number of cells seeded after passaging. Cumulative population doubling was determined in cultures maintained until passage 13 (approximately 165 days). The mean cumulative population doubling obtained from 3 donors was expressed as a function of passage number.

#### *Cell senescence assay*

Senescence was assessed using a  $\beta$ -galactosidase ( $\beta$ -Gal) staining assay, in which  $\beta$ -Gal activity is detected in senescent cells at pH 6.0 but is absent in proliferating cells.<sup>45</sup> Cells from each culture passage (passage 1 to passage 15) were fixed for 5 min in 2% formaldehyde/glutaraldehyde and incubated in a  $\beta$ -Gal reaction buffer (containing 1 mg/ml X-Gal, 40 mM citric acid/sodium phosphate buffer (pH 6.0), 5 mM

**TABLE 1. LINEAGE-SPECIFIC DIFFERENTIATION INDUCED BY MEDIA SUPPLEMENTATION**

<i>Medium</i>	<i>Media</i>	<i>Serum</i>	<i>Supplementation</i>
Control	DMEM	10% FBS	none
Adipogenic (AM)	DMEM	10% FBS	0.5 mM isobutyl-methylxanthine (IBMX), 1 $\mu$ M dexamethasone, 10 $\mu$ M insulin, 200 $\mu$ M indomethacin, 1% antibiotic/antimycotic
Osteogenic (OM)	DMDM	10% FBS	0.1 $\mu$ M dexamethasone, 50 $\mu$ M ascorbate-2-phosphate, 10 mM $\beta$ -glycerophosphate, 1% antibiotic/antimycotic
Chondrogenic (CM)	DMEM	1% FBS	6.25 $\mu$ g/ml insulin, 10 ng/ml TGF- $\beta$ 1, 50 nM ascorbate-2-phosphate, 1% antibiotic/antimycotic
Myogenic (MM)	DMEM	10% FBS, 5% HS	0.1 $\mu$ M dexamethasone, 50 $\mu$ M hydrocortisone, 1% antibiotic/antimycotic

each of potassium ferrocyanide and potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl<sub>2</sub>). Senescent cells (blue) were identified by light microscopy.

#### *Confirmation of multilineage differentiation of PLA cells*

PLA cells at passage 1 were analyzed for their capacity to differentiate toward the adipogenic, osteogenic, chondrogenic, and myogenic lineages. To induce differentiation, PLA cells were cultured with specific induction media, as detailed in Table 1. Each medium has been previously described and shown to induce multilineage differentiation of MSCs.<sup>8,14,15,31,33</sup> Differentiation was confirmed using the histological and immunohistological assays outlined in Table 2. A commercial source of bone marrow-derived MSCs and lineage-specific precursors were examined as positive controls. PLA cells maintained in control medium and HFFs were analyzed as negative controls.

**Adipogenesis:** Adipogenic differentiation was induced by culturing PLA cells for 2 weeks in adipogenic medium (AM) and assessed using an Oil Red O stain as an indicator of intracellular lipid accumulation.<sup>46</sup> Prior to staining, the cells were fixed for 60 min at room temperature in 4% formaldehyde/1% calcium and washed with 70% ethanol. The cells were incubated in 2% (wt/vol) 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.

**Osteogenesis:** Osteogenic differentiation was induced by culturing PLA cells for a minimum of 2 weeks in osteogenic medium (OM) and examined for alkaline phosphatase (AP) activity and ECM calcification by von Kossa staining. To detect AP activity, cells were incubated in OM for 2 weeks, rinsed with PBS, and stained with a 1% AP solution (1% naphthol ABSI phosphate, 1 mg/mL Fast Red TR) at 37°C for 30 min. For von Kossa staining, the cells were incubated in OM for 4 weeks and fixed with 4% paraformaldehyde.

**TABLE 2. DIFFERENTIATION MARKERS AND ASSAYS OF LINEAGE-SPECIFIC DIFFERENTIATION**

<i>Lineage</i>	<i>Lineage-specific determinant</i>	<i>Histologic/immunohistochemical assay</i>
Adipogenic	Lipid accumulation	Oil Red O stain
Osteogenic	1. AP activity 2. Calcified matrix production	1. AP stain 2. Von Kossa stain
Chondrogenic	1. Sulfated proteoglycan-rich matrix 2. Collagen II synthesis	1. Alcian Blue (pH 1.0) stain 2. Collagen II-specific monoclonal antibody
Myogenic	1. Multinucleation 2. Skeletal muscle myosin heavy-chain and MyoD1 expression	1. Phase contrast microscopy 2. Myosin- and MyoD1-specific monoclonal antibodies

hyde for 60 min at room temperature. The cells were rinsed with distilled water and then overlaid with a 1% (wt/vol) silver nitrate solution in the absence of light for 30 min. The cells were washed several times with distilled water and developed under UV light for 60 min. Finally, the cells were counter-stained with 0.1% eosin in ethanol.

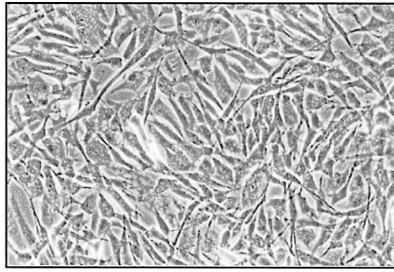
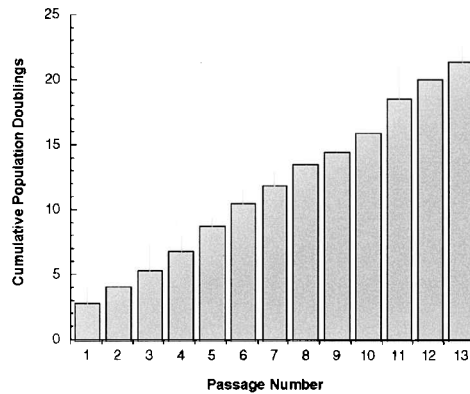
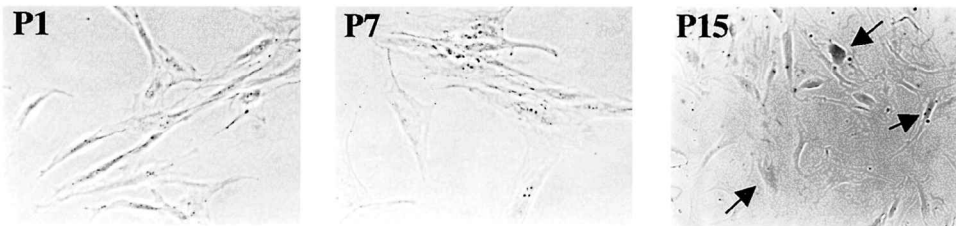
**Chondrogenesis:** Chondrogenic differentiation was induced using the micromass culture technique.<sup>47–49</sup> Briefly, 10  $\mu$ L of a concentrated PLA cell suspension ( $8 \times 10^6$  cells/mL) was plated into the center of each well and allowed to attach at 37°C for 2 h. Chondrogenic medium (CM) was gently overlaid so as not to detach the cell nodules, and cultures were maintained in CM for 2 weeks prior to analysis. Chondrogenesis was confirmed using the histologic stain Alcian Blue at acidic pH. PLA cell nodules were fixed with 4% paraformaldehyde for 15 min at room temperature and washed with several changes of PBS.

Studies have shown specific staining of sulfated proteoglycans, present in cartilagenous matrices, at pH levels of 1 and below.<sup>50</sup> In light of this, the cells were incubated for 30 min with 1% (wt/vol) 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. In addition to Alcian Blue staining, expression of the cartilage-specific collagen type II isoform was also determined. PLA cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Cells were incubated in 0.2 U/mL chondroitinase ABC for 40 min at 37°C to facilitate antibody access to collagen II. The cells were rinsed in PBS and endogenous peroxidase activity quenched by incubating for 10 min in 3% hydrogen peroxide in methanol. Following a wash in PBS, nonspecific sites were blocked by incubating cells for 1 h in blocking buffer (PBS, containing 10% horse serum). The cells were subsequently incubated for 1 h in blocking buffer containing a monoclonal antibody specific to human collagen type II (ICN Biomedical, Costa Mesa, CA). The cells were washed extensively in blocking buffer, and collagen type II was shown using a commercially available kit for the detection of monoclonal antibodies according to the manufacturer (VectaStain ABC kit, Vector Labs Inc., Burlingame, CA).

**Myogenesis:** Myogenic differentiation was induced by culturing PLA cells myogenic medium (MM) for 6 weeks and confirmed by immunohistochemical staining for the muscle-specific transcription factor MyoD1 and the myosin heavy chain. Cells were rinsed twice with PBS, fixed for 20 min with 4% paraformaldehyde, and washed several times with PBS. The cells were incubated with 3% hydrogen peroxide in PBS for 10 min to quench endogenous peroxidase enzyme activity, and nonspecific sites were blocked by incubation in blocking buffer (PBS, 10% HS, 0.1% Triton X-100) for an additional 60 min. The cells were washed three times for 5 min each in blocking buffer and incubated for 1 h in blocking buffer containing either a monoclonal antibody specific to skeletal muscle myosin heavy chain (Biomed, Foster City, CA) or to MyoD1 (Dako Corp, Carpinteria, CA). The cells were washed extensively in blocking buffer and the monoclonal antibodies detected using the VectaStain ABC kit according to manufacturer's specifications. The cells were counterstained with hematoxylin for 3 min.

## RESULTS

In this study, we examined the constituent cell types and multilineage potential of a putative mesodermal stem cell population obtained from human adipose tissue. Human adipose tissue was obtained by suction-assisted lipectomy (*i.e.*, liposuction), and the lipoaspirates were processed based on adapted methodologies,<sup>34</sup> to obtain a PLA cell population containing the putative stem cell fraction. Processing of 300 cc of liposuctioned tissue routinely yielded PLA samples of  $2\text{--}6 \times 10^8$  cells. PLA cultures were maintained in DMEM supplemented with 10% FBS. Supplementation with FBS has been shown to be important for human and animal MSC attachment and proliferation *in vitro*.<sup>16,51,52</sup> However, studies suggest that proliferation and differentiation of human MSCs may be dependent upon FBS source and quality, making sera screening critical.<sup>51,52</sup> PLA cells expanded easily *in vitro* and exhibited a fibroblast-like morphology, consistent with that of MSCs obtained from bone marrow and a commercial source (Fig. 1A). PLA cells did not appear to require specific sera lots for expansion and multilineage differentiation. Ten FBS lots from three manufacturers were tested and did not appear to alter PLA cell morphology, proliferation rate, or their differentiative capacity *in vitro* (data not shown).

**A.****B.****C.**

**FIG. 1.** Morphology, growth kinetics and senescence of PLA cells over long-term culture. **(A)** The morphology of a processed lipoaspirate or PLA obtained from liposuctioned adipose tissue is shown. **(B)** PLA cells, obtained from 3 donors, were cultured for an extended period and cumulative population doubling was measured and expressed as a function of passage number. **(C)** Senescence in PLA cultures was detected by staining cells at passages 1, 7, and 15 (P1, P7, and P15, respectively) for  $\beta$ -Gal expression at pH 6.0. Representative senescent cells are shown (arrows).

#### *Growth kinetics and composition of the PLA*

PLA cells, obtained from 20 donors and cultured under standard conditions (*i.e.*, 10% FBS), exhibited an average population doubling time of 60 h using several sera sources and lots (data not shown). Following isolation, an initial lag time of 5–7 days was observed in PLA cultures (data not shown). Cells then entered a proliferative phase, reaching confluence within 48 h. To examine long-term growth kinetics of PLA cultures, we measured cumulative population doublings with respect to passage number in multiple donors. Consistent with the observed lag time upon initial culture, PLA cells underwent an average of three population doublings prior to the first passage (Fig. 1B). An average of 1.5 population doublings was observed upon subsequent passages. A linear relationship between cumulative population doubling and passage number was observed, indicating a relatively constant population doubling rate over the range studied. Fur-

thermore, no appreciable decrease in cumulative population doublings was observed at later passages (P13 = 165 days in culture), suggesting that PLA cultures maintain their proliferative potential during extended culture periods.

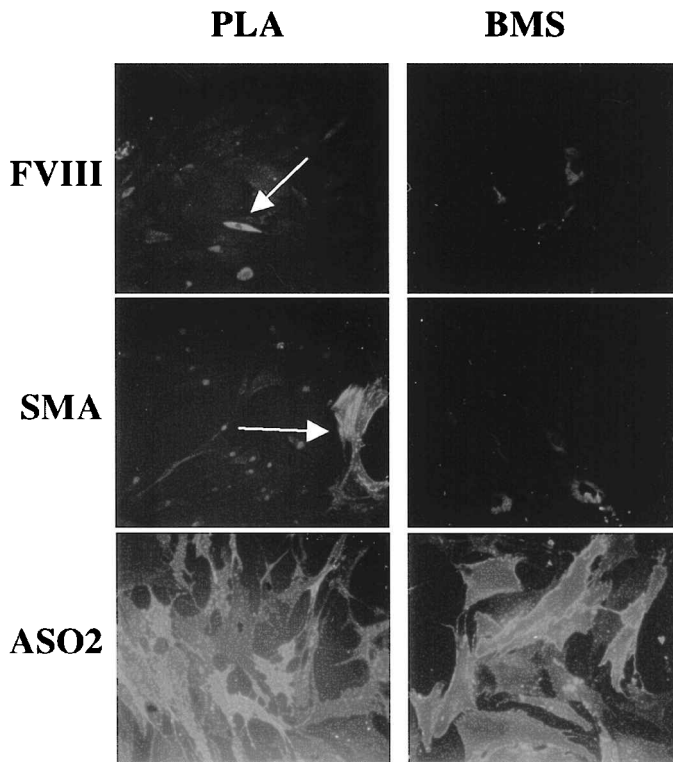
In addition to cumulative population doubling, we also examined cell senescence in long-term PLA cultures using a  $\beta$ -Gal staining protocol, in which  $\beta$ -Gal expression is absent in proliferating cells but can be detected in senescent cells at a pH of 6.0.<sup>45</sup> Using this assay, PLA cultures were examined for senescence at each passage. PLA cultures at passage 1 exhibited no appreciable  $\beta$ -Gal staining (Fig. 1C, P1). An increase in  $\beta$ -Gal staining was observed at later passages (P7 and P15); however, the percentage of senescent cells remained below 5% through 10 passages and increased to 15% at passage 15 (data not shown). Taken together, the data indicate that PLA samples are relatively stable over long-term culture, maintaining a consistent population doubling rate and exhibiting low levels of senescence.

The SVF processed from excised adipose tissue is a heterogenous population including mast cells, endothelial cells, pericytes, fibroblasts, and lineage-committed progenitor cells, or preadipocytes.<sup>29,33</sup> These components may also be present, together with the putative stem cell fraction, in the PLA obtained from liposuctioned adipose tissue. However, no literature regarding this has been published. To characterize the PLA phenotypically, samples from several donors were examined by indirect immunofluorescence using antibodies specific to established cell-surface markers. A bone marrow stromal fraction obtained from human marrow aspirates was also examined as a control. To identify endothelial cells, PLA cells were incubated with a monoclonal antibody to FVIII.<sup>39,40</sup> Smooth muscle cells were identified using a monoclonal antibody to smooth muscle actin.<sup>43,44</sup> This antibody has also been shown to react with transitional pericytes (*i.e.*, pericytes of pre- and post-capillaries) and the contractile apparatus of pericytes committed to the smooth muscle lineage.<sup>37,53</sup> Low levels of endothelial cells, smooth muscle cells and pericytes were observed in the PLA (Fig. 2). In comparison, no staining for these markers was observed in processed bone marrow stromal samples. In addition to FVIII and smooth muscle actin, cells were also incubated with a monoclonal antibody (ASO2) specific to fibroblasts and mesenchymal cells.<sup>41,42</sup> The majority of the PLA and bone marrow stromal cells stained positively with ASO2, suggesting a mesenchymal origin (Fig. 1, ASO2 panels).

To determine PLA composition quantitatively, samples were analyzed by flow cytometry using the cell-surface markers described above. PLA samples were obtained and cultured for 72 h in control medium. Cell size and granularity were measured using forward- and side-scatter settings (Fig. 3A). The majority of the PLA sample was comprised of small, agranular cells. In addition, PLA cells were incubated with monoclonal antibodies to FVIII, smooth muscle actin, and ASO2 and a monoclonal antibody to vimentin, an intermediate filament protein found predominantly in cells of mesenchymal origin.<sup>43,44</sup> Viability was assessed using propidium iodide and samples were corrected for viability, nonspecific fluorescence, and autofluorescence. Data are shown from a representative patient (Fig. 3B). Cytometry data was collected from 5 donors, and the number of positive events for each cell-specific marker was expressed as a percentage of the total PLA cell number. Consistent with the immunofluorescent data, a fraction of the PLA cells expressed FVIII (FVIII-positive cells =  $24.9\% \pm 8.2$  of total PLA cell number) and SMA (SMA-positive cells =  $29.2\% \pm 2.1$  of total PLA cell number) (Fig. 3C), indicating that the PLA contains endothelial cells, smooth muscle cells, and, possibly, pericytes. Furthermore, the majority of the PLA cells stained positively for ASO2 (ASO2-positive cells =  $85.0\% \pm 12.8$  of total PLA cell number) and vimentin (VIM-positive cells =  $63.2\% \pm 5.6$  of total cell number), indicative of cells of mesenchymal origin. Taken together, the results suggest that the PLA is a relatively homogenous population of mesodermal or mesenchymal cells with low contamination by endothelial cells, pericytes, and smooth muscle cells.

### *PLA cells exhibit multilineage potential*

To study the multilineage capacity of PLA cells, cells were differentiated toward the adipogenic, osteogenic, chondrogenic, and myogenic lineages using lineage-specific induction factors (Table 1). Human and animal bone marrow-derived MSCs have been shown to differentiate toward the adipogenic, osteogenic, and chondrogenic lineages with appropriate medium supplementation.<sup>8,14,15,31,33</sup> Following induction, differentiation was assessed using histology and immunohistochemistry (Table 2). Commercially available



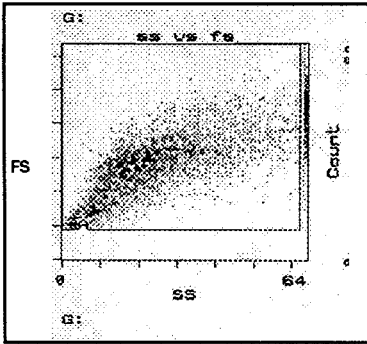
**FIG. 2.** Composition of the PLA: indirect immunofluorescence. PLA cells (PLA), in addition to bone marrow stromal cells (BMS), were processed for immunofluorescence using the following antibodies to cell type-specific markers: (1) anti-Factor VIII (FVIII); (2) anti-smooth muscle actin (SMA); and (3) ASO2 (ASO2). Factor VIII- and smooth muscle actin-expressing cells are shown (arrows).

MSCs and lineage-committed progenitor cells served as positive controls whereas PLA cells maintained in control medium and HFF cells were examined as negative controls.

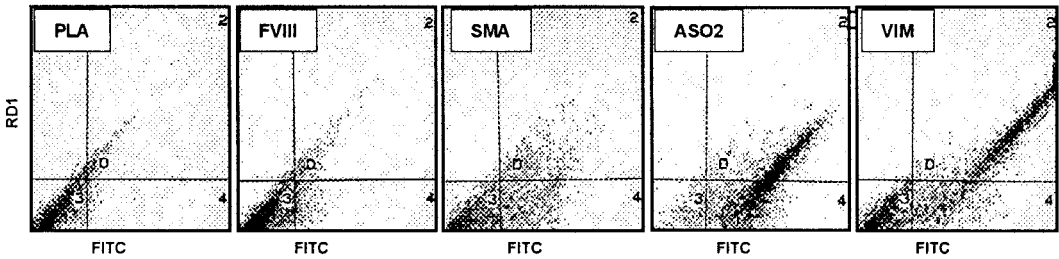
Preadipocytes and MSCs treated with adipogenic induction medium, containing cAMP agonists and induction agents such as isobutyl-methylxanthine (IBMX), indomethacin, insulin, and dexamethasone, develop lipid-containing droplets that accumulate the lipid dye Oil Red-O.<sup>8,54,55</sup> To determine if PLA cells undergo adipogenesis, cells were cultured in medium containing these agents (adipogenic medium, AM) and stained with Oil Red-O. PLA cells cultured in AM were reproducibly induced toward the adipogenic lineage as early as 2 weeks post-induction (Fig. 4). A significant fraction of the cells contained multiple, intracellular lipid-filled droplets that accumulated Oil Red-O. The Oil Red O-containing PLA cells exhibited an expanded morphology with the majority of the intracellular volume (90–98%) occupied by lipid droplets, consistent with the phenotype of mature adipocytes. The mean level of adipogenic differentiation measured in 6 donors under 35 years of age was  $42.4\% \pm 10.6\%$  (% Oil Red O-positive cells/total PLA cell number; data not shown). Prolonged culture times (*i.e.*, 4 weeks) resulted in the detachment of differentiating cells from the culture plate and flotation to the surface (data not shown). The observed morphology and lipid accumulation of differentiated PLA cells were similar to that observed upon treatment of bone marrow-derived MSCs and the preadipocyte cell line 3T3-L1 in AM. No lipid droplets were observed in undifferentiated PLA cells or in HFF negative controls (data not shown). In contrast to MSCs, in which adipogenic differentiation dramatically decreases beyond the third culture passage,<sup>56</sup> the adipogenic potential of PLA cells was maintained over long-term culture (*i.e.*, passage 15 = 175 days culture) (data not shown). Taken together, the results indicate that PLA cells undergo adipogenic differentiation.



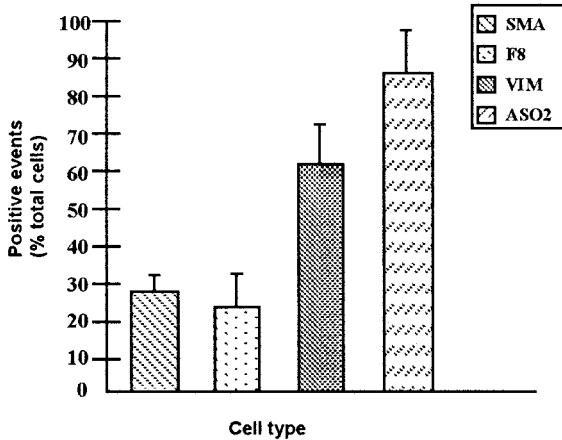
A. FS vs. SS



B. Cell-specific markers

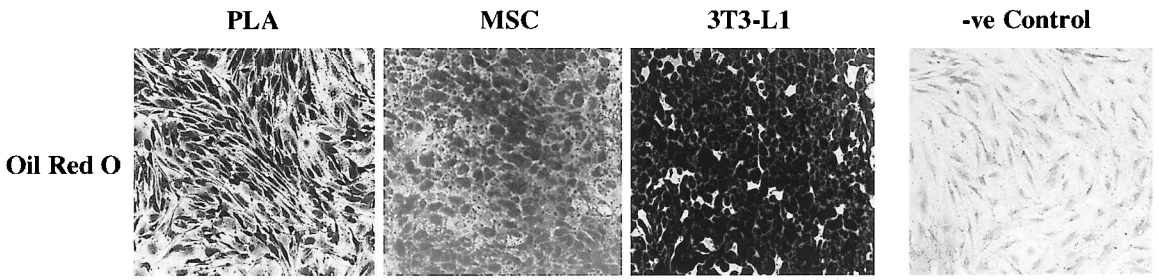


C. PLA composition



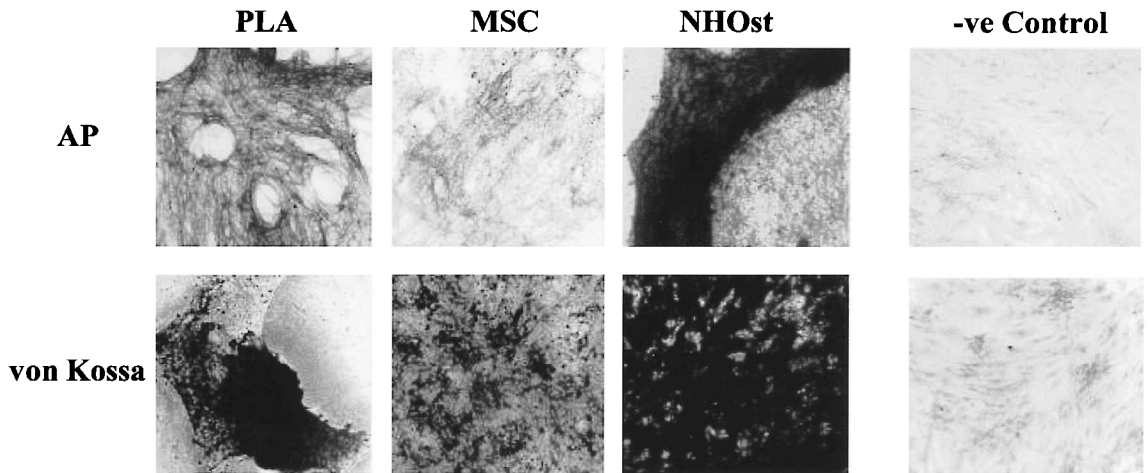
**FIG. 3.** Composition of the PLA: flow cytometry. (A) PLA samples were examined by flow cytometry using forward and side scatter (FS and SS, respectively). A representative PLA sample is shown. (B) The cell composition of a representative PLA sample from one donor (PLA) was determined by incubating the sample with the following monoclonal antibodies: anti-Factor VIII (FVIII), anti-smooth muscle actin (SMA), ASO2, and a monoclonal antibody to vimentin (VIM), an additional marker for cells of mesenchymal origin. (C) Flow cytometry data from 5 donors was collected and the mean number of positive events for each cell-specific marker is expressed as a percentage of total PLA cell number.

Differentiation of osteoprogenitor cells and marrow-derived MSCs into osteoblasts is induced *in vitro* by treating cells with low concentrations of ascorbic acid,  $\beta$ -glycerophosphate, and dexamethasone.<sup>8,15,56</sup> Early differentiation of these cells into immature osteoblasts is characterized by AP enzyme activity with human MSCs expressing AP as early as 4 days and maximum levels observed at 12 days post-induction.<sup>57</sup> To confirm their osteogenic capacity, PLA cells were treated with OM for 14 days, and the expression of AP was



**FIG. 4.** PLA cells accumulate lipid-filled droplets upon treatment with adipogenic medium (AM). PLA cells (PLA), bone marrow-derived MSCs (MSC), and 3T3-L1 preadipocyte cells (3T3-L1) were cultured for 2 weeks in AM and stained with Oil Red O to identify lipid-filled intracellular vacuoles. Undifferentiated PLA cells maintained in control medium (–ve Control) were stained as a negative control.

examined. PLA cells cultured in OM formed an extensive network of dense, multilayered nodules that stained positively for AP (Fig. 5). The mean number of AP-positive staining cells measured in 6 donors was  $50.2\% \pm 10.8\%$  of total PLA cell number (data not shown). Expression of AP was also observed in both MSCs and NHOst-positive controls maintained in OM. In contrast, undifferentiated PLA cells and HFF negative controls (data not shown) did not show evidence of AP expressions. Although AP expression is dramatically upregulated in osteogenic tissues, its expression has been observed in several non-osteogenic cell types and tissues such as cartilage, liver, and kidney.<sup>58–60</sup> Therefore, AP expression is frequently used, in conjunction with other osteogenic-specific markers, as an indicator of osteogenesis. One such indicator is the formation of a calcified ECM. Mature osteoblasts secrete a collagen I-rich ECM that becomes calcified during the later stages of differentiation.<sup>61</sup> Therefore, to confirm osteogenic differentiation, calcification of the ECM matrix was assessed in PLA cells using a von Kossa stain. Calcification appears as black regions within the cell monolayer. Consistent with osteogenesis, several black regions, indicative of a calcified ECM, were observed in PLA cells treated for 4 weeks in OM. Calcification was also identified in MSC and NHOst-positive controls, whereas no calcification was observed in undifferentiated PLA cells or HFF cells (data not shown). The osteogenic potential of PLA cells was maintained over long-

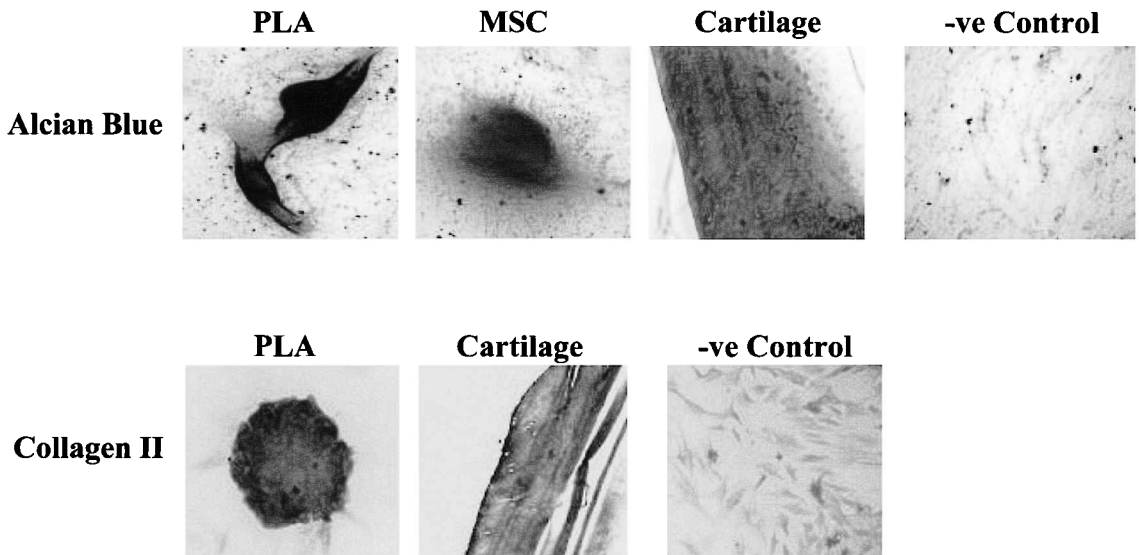


**FIG. 5.** PLA cells induced with osteogenic medium (OM) express AP and are associated with a calcified ECM. PLA cells (PLA), MSCs (MSC), and a human osteoblast cell line (NHOst) were cultured in OM to induce osteogenesis. Cells were stained at 2 weeks for AP activity (AP; red). The presence of a calcified extracellular matrix (black regions) was examined at 4 weeks (von Kossa). Undifferentiated PLA cells maintained in control medium were examined for AP expression and matrix calcification as a negative control (–ve Control).

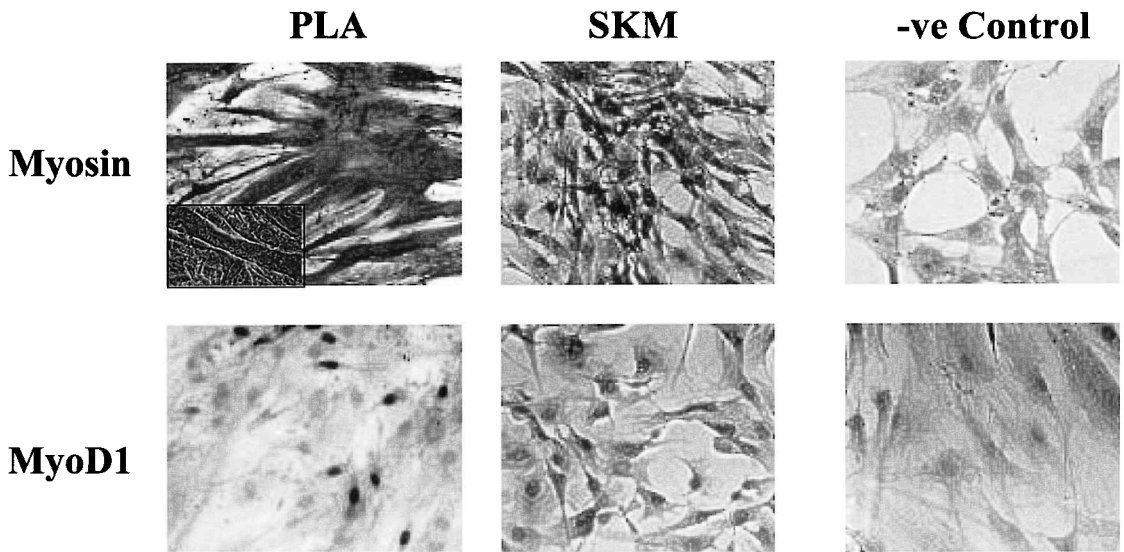
term culture, with cells expressing AP as late as 175 days of culture (data not shown). Taken together, the expression of AP by PLA cells and the production of a calcified ECM strongly suggest that these adipose-derived cells can be induced toward the osteogenic lineage.

Chondrogenic differentiation can be induced *in vitro* using a micromass culture technique, in which cellular condensation (a critical first event of chondrogenesis) is duplicated.<sup>47–49,62</sup> Enhanced differentiation can be obtained by treating cells with dexamethasone and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1).<sup>63</sup> Marrow-derived MSCs, cultured with these agents under micromass conditions, form cell nodules associated with a well-organized ECM rich in collagen II and sulfated proteoglycans.<sup>8,64</sup> These sulfated proteoglycans can be specifically detected using the stain Alcian Blue under acidic conditions.<sup>50</sup> To assess the chondrogenic capacity of PLA cells, cells were cultured via micromass in CM, containing dexamethasone and TGF- $\beta$ 1. Micromass culture of PLA cells resulted in the formation of dense nodules consistent with chondrogenic differentiation. The PLA nodules were associated with an Alcian Blue-positive ECM, indicative of the presence of sulfated proteoglycans within the matrix (Fig. 6). Cartilaginous nodules were also observed upon micromass culture of MSC controls. To confirm the specificity of Alcian Blue for cartilaginous matrices, human cartilage and bone sections were stained with Alcian Blue under acidic conditions. As expected, human cartilage sections stained positively with Alcian Blue, whereas no staining was observed in bone sections (data not shown). In addition to the presence of sulfated proteoglycans within the ECM, both PLA cells and human cartilage sections expressed the collagen type II isoform, but no staining was observed in undifferentiated PLA cells. Consistent with adipogenic and osteogenic differentiation, PLA cells retained their chondrogenic differentiation potential after extended culture periods (*i.e.*, up to 175 days; data not shown). The above results suggest that PLA cells possess the capacity to differentiate toward the chondrogenic lineage.

Myogenesis is characterized by a period of myoblast proliferation, followed by the expression of muscle-specific proteins and fusion to form multinucleated myotubules. Early myogenic differentiation is char-



**FIG. 6.** PLA cells treated with chondrogenic medium (CM) are associated with a proteoglycan-rich matrix and express collagen type II. PLA cells (PLA) and MSCs (MSC) were cultured for 2 weeks in CM using the micromass technique to induce chondrogenesis. The cells were fixed and processed for the presence of sulfated proteoglycans with Alcian Blue under acidic conditions (Alcian Blue). Paraffin sections of human cartilage were used as a positive control (Cartilage) while undifferentiated PLAs maintained in control medium were processed as a negative control (-ve Control). In addition, the expression of collagen type II (Collagen II) was examined in PLA cells and human cartilage sections. PLA cells cultured in control medium (-ve Control) were stained with Alcian Blue and for collagen II expression as a negative control.



**FIG. 7.** PLA cells cultured in myogenic medium (MM) express the myosin heavy chain and MyoD1. PLA cells (PLA) were treated with MM and stained with antibodies specific to skeletal muscle myosin heavy chain (Myosin) or MyoD1 (MyoD1). A human skeletal muscle cell line (SKM) was examined as a positive control. In addition, the presence of multinucleated cells in PLA cultures is shown (PLA, inset box). Myosin and MyoD1 expression were also assessed in undifferentiated PLA cells (-ve Control) as a negative control.

acterized by the expression of several myogenic regulatory factors including myogenic determination factor 1 (MyoD1).<sup>65-67</sup> Terminally differentiated myoblasts can be characterized by the expression of myosin and the presence of multiple nuclei.<sup>68</sup> Proliferation and myogenic differentiation of muscle precursors and bone marrow-derived stem cells can be induced by dexamethasone and results in the expression of muscle-specific proteins.<sup>14,69,70</sup> Furthermore, addition of hydrocortisone is known to stimulate human myoblast proliferation, prior to their transition into differentiated myotubules.<sup>71</sup> To examine if PLA cells undergo myogenesis, cells were cultured for 6 weeks in the presence of dexamethasone and hydrocortisone, and incubated with antibodies specific to MyoD1 and myosin (heavy chain). Consistent with early myogenic differentiation, treatment of PLA cells with MM for 1 week induced the expression of MyoD1 (Fig. 7). PLA cells treated for longer time periods (6 weeks) stained positively for myosin. In addition to myosin expression, the presence of discrete 'patches' of large, elongated cells with multiple nuclei were also observed, suggesting that PLA cells underwent myoblast fusion (PLA panel, inset). Like PLA cells, MyoD1 and myosin heavy-chain expression were also detected in human skeletal muscle-positive control cells. Using myogenic medium, myogenic differentiation was not observed in MSC controls even at 6 weeks of induction (data not shown). These cells may be adversely affected by hydrocortisone and may require alternate conditions to induce differentiation. Myogenic differentiation levels in PLA cells averaged 12% (data not shown). Multinucleation, myosin heavy-chain, and MyoD1 expression were not observed in undifferentiated PLA cells nor in HFF negative controls (data not shown). The presence of multinucleated cells and the expression of both MyoD1 and myosin heavy chain suggest that PLA cells have the capacity to undergo myogenic differentiation.

## DISCUSSION

In this paper, we report that a cellular fraction with multiple mesodermal lineage capabilities can be processed from human lipoaspirates. This cellular fraction, which we call a processed lipoaspirate (PLA),

is comprised of fibroblast-like cells that can be expanded easily *in vitro* without the need for specific sera lots or media supplementation. PLA samples, from several donors, maintained a linear growth rate with no appreciable senescence over extended culture periods. The PLA population was heterogeneous in nature, with the majority of the cells being mesenchymal in origin. However, contaminating endothelial, smooth muscle, and pericyte cell populations were identified. PLA cells also exhibited multilineage potential *in vitro*, differentiating toward the adipogenic, osteogenic, chondrogenic, and myogenic lineages when cultured in the presence of established lineage-specific differentiation factors. PLA differentiation results were consistent with those observed upon lineage-specific differentiation of bone marrow-derived MSCs and lineage-committed precursors.

Although the apparent multidifferentiative capacity of PLA cells suggests the presence of a stem cell population within human liposuctioned adipose tissue, it is not conclusive. Multilineage differentiation may also be due to the presence of: (1) multiple lineage-committed progenitor cells; (2) multipotent cells from other sources (*e.g.*, pericytes, marrow-derived MSCs from peripheral blood); or (3) a combination of the above.

The observed differentiation may be due to the presence of lineage-committed progenitor cells, such as preosteoblasts, premyoblasts, or preadipocytes within the PLA. Cellular fractions (*i.e.*, SVFs) obtained from excised adipose tissue are known to contain preadipocytes that differentiate into mature adipocytes.<sup>29,30</sup> It is possible that the observed adipogenic differentiation by PLA cells is simply the commitment of existing preadipocytes and not the differentiation of a multipotent cell. However, we do not believe this to be the case. As little as 0.02% of the SVF obtained from excised adipose tissue have been identified as preadipocytes capable of adipogenic differentiation.<sup>29</sup> If preadipocyte numbers within the PLA are comparable to those levels measured in the SVF from excised tissue, one would expect a relatively low level of adipogenesis. However, the degree of adipogenesis observed in the PLA is significant (~40% of the total PLA cell number) and may result from the differentiation of additional cell types (*i.e.* stem cells).

Damage to the underlying muscle during liposuction may introduce myogenic precursor cells or satellite cells into the PLA, resulting in the observed myogenic differentiation by these cells. Located between the sarcolemma and the external lamina of the muscle fiber, myogenic precursor cells in their undifferentiated state are quiescent and exhibit no distinguishing features, making their identification difficult. Several groups have attempted to identify unique markers for these precursors with limited success. Currently, the expression of the myogenic regulatory factors, MyoD1, and myogenin have been used to identify satellite cells during embryogenesis and in regenerating adult muscle in rodents.<sup>72-76</sup> In addition, MyoD1 expression has been identified in proliferating myoblasts prior to the onset of differentiation.<sup>66</sup> Although these markers have not been used to identify myogenic precursors in human subjects, MyoD1 is expressed during early myogenic differentiation in normal skeletal muscle and has been used to identify the skeletal muscle origin of rhabdosarcomas in humans.<sup>77-79</sup> The absence of MyoD1 expression in PLA cells maintained in noninductive CM (see Fig. 7), suggests that our observed myogenic differentiation is not due to the presence of myogenic precursors or proliferating myoblasts within the PLA. Consistent with this, the blunt contour of the liposuction cannula would make it extremely difficult to penetrate the fibrous fascial cavity surrounding the muscle and introduce these precursors into the adipose compartment.

Human adipose tissue is vascularized and, as such, contains potential systemic vascular 'conduits' for contamination by multipotent cells, such as pericytes and marrow-derived MSCs. Disruption of the blood supply during liposuction may result in the release of pericytes, known to possess multilineage potential both *in vivo* and *in vitro*.<sup>80-82</sup> Consistent with this, our immunofluorescent and flow cytometry data show that a small fraction of the PLA is comprised of cells that express smooth muscle actin, a component of transitional pericytes and pericytes committed to the smooth muscle lineage.<sup>37</sup> The multilineage differentiation observed in PLA may be, in part, due to the presence of pericytes. Disruption of the blood supply may also introduce MSCs into the PLA. However, the literature is conflicted as to the presence of these stem cells in the peripheral blood.<sup>83,84</sup> If the peripheral blood does indeed represent a source of MSCs, our observed multilineage differentiation by PLA cells may be due to the contamination of adipose tissue by these stem cells. However, MSCs are a small constituent of the bone marrow stroma in humans (~1 MSC per 10<sup>5</sup> adherent

stromal cells.<sup>8,17,20</sup> If these cells do exist in peripheral blood, they are likely to be in even smaller quantities than in the bone marrow and contamination levels of the PLA by these cells may be negligible.

These arguments provide support for the presence of a multipotent stem cell population within liposuctioned adipose tissue; however, definitive confirmation requires the isolation and characterization of multiple clones derived from a single cell. Preliminary data confirm that clonal PLA cell populations possess multilineage potential, capable of adipogenic, osteogenic, and chondrogenic differentiation.<sup>85</sup> Although promising, isolation and analysis of multiple PLA cell clones will be required to confirm the presence of a stem cell population within liposuctioned adipose tissue.

The future of engineering mesodermally derived tissues from stem cells is promising and the development of these strategies will likely require a readily available source of donor cells. Current research has demonstrated exciting results using bone marrow-derived MSCs. MSCs can differentiate into osteogenic and chondrogenic tissues *in vivo*,<sup>86-89</sup> and preliminary data suggest that these cells can be used to repair bony and cartilagenous defects.<sup>12,88-92</sup> We believe that PLA cells obtained from liposuctioned adipose tissue may represent another source of multilineage mesodermal stem cells. Like the bone marrow stroma, our data suggest that adipose tissue may contain a significant fraction of cells with multilineage capacity. These adipose-derived stem cells may be readily available in large quantities with minimal morbidity and discomfort associated with their harvest. The autologous nature of these stem cells, together with their putative multipotentiality and ease of procurement, may make these cells an excellent choice for many future tissue engineering strategies and cell-based therapies.

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Address reprint requests to:

*M.H. Hedrick, M.D.*

*University of California*

*Division of Plastic and Reconstructive Surgery*

*64-140 Center for Health Sciences*

*10833 LeConte Avenue*

*Los Angeles, CA 90095-1665*

*E-mail: marc2@ucla.edu*