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Original Research Report

Phenotypical and Functional Characterization of Freshly Isolated Adipose Tissue-Derived Stem Cells

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ABSTRACT

Adipose tissue contains a stromal vascular fraction (SVF) that is a rich source of adipose tissue-derived stem cells (ASCs). ASCs are multipotent and in vitro-expanded ASCs have the capacity to differentiate, into amongst others, adipocytes, chondrocytes, osteoblasts, and myocytes. For tissue engineering purposes, however, it would be advantageous to use the whole SVF, which can be transplanted without further in vitro selection or expansion steps. Because little is known about the freshly isolated ASCs in the SVF, we phenotypically characterized human freshly isolated ASCs, using flow cytometry. In addition, we investigated whether freshly isolated ASCs have functional properties comparable to cultured ASCs. For this, the differentiation potential of both freshly isolated ASCs and cultured ASCs into the osteogenic pathway was analyzed. Freshly isolated ASCs slightly differed in immunophenotype from cultured ASCs. Contrary to cultured ASCs, freshly isolated ASCs were shown to be highly positive for CD34, and positive for CD117 and HLA-DR. On the other hand, expression of CD105 and especially CD166 on the freshly isolated ASCs was relatively low. After osteogenic stimulation of freshly isolated ASCs, both *Runx-2* and *Col1aI* gene expression were significantly increased ($p < 0.05$). However, there was a difference in the kinetics of gene expression between freshly isolated and cultured ASCs and also between the different SVF isolates tested. There was no difference in alkaline phosphatase activity between freshly isolated ASCs and cultured ASCs. In addition, freshly isolated ASCs stained positive for osteonectin and showed matrix mineralization. We conclude that although there are minor differences in phenotype and kinetics of differentiation between freshly isolated ASCs and cultured ASCs, the use of freshly isolated ASCs for tissue engineering purposes involving bone repair is potentially applicable.

INTRODUCTION

AUTOLOGOUS STEM CELL THERAPY aiming at tissue regeneration is a fast-growing field of research that involves transplantation of stem cells into a patient, either directly into the defect site or by systemic infusion. Autologous grafts provide the advantage of low risk of immunogenic response and the absence of external pathogen transfer. The most potent stem cells, still capable of differentiating into cell types of all three embryonic germ

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Part of this study was presented at the International Fat Applied Technology Society (IFATS), September 10–13, 2005, Charlottesville, VA. The Abstract, "Adipose tissue-derived mesenchymal stem cells do express the stem cell markers CD34 and CD117, but expression is lost upon in vitro propagation," by van Milligen, FJ, Oedayrajsingh Varma MJ, Helder MN, Klein-Nulend J, Bontkes HJ, Schuurhuis GJ, van Ham SM and Meijer CJLM, was originally published in *Adipocytes* 1:177 (2005), and is reprinted with permission.

layers, are embryonic stem cells (1). However, the ongoing intense ethical and political debate restricts their use. An alternative source is provided by adult, bone marrow-derived mesenchymal stem cells (MSCs), present within the bone marrow stroma. These bone marrow-derived stromal cells have already demonstrated efficacy in multiple examples of cellular therapies (2–7). The retrieval of stem cells from the bone marrow, however, is highly invasive and provides only low numbers of stem cells. Therefore, alternative sources for MSCs have been investigated.

Only recently, adipose tissue was defined as a new source of MSCs. Like the bone marrow, adipose tissue contains a stromal fraction, further referred to as stromal vascular fraction or SVF, which contains a population of adipose tissue-derived stem cells (ASCs) (8). An advantage of using adipose tissue as the source of stem cells is that the harvesting of adipose tissue is minimally invasive and can be done under local anesthesia, thus resulting in minimal patient discomfort and low patient risk. Furthermore, adipose tissue provides large numbers of stem cells as compared to bone marrow. A bone marrow transplant of 100 ml contains approximately 6×10^8 nucleated cells (9), of which only 0.001–0.01% ($0.006–0.06 \times 10^6$ cells) is a stem cell (10). In comparison, the number of viable SVF cells that can be retrieved from subcutaneous fat is approximately $0.5–2.0 \times 10^6$ cells/gram of adipose tissue (8,9,11,12), whereby the percentages of stem cells within the SVF range from 1 to 10% (12–14), most likely depending on donor and tissue harvesting site. This implicates that approximately between 0.5×10^6 and 20×10^6 stem cells can be retrieved from 100 grams of fat, which is an amount that can easily be obtained from a patient. The ASCs have shown to be multipotent and have the capacity to differentiate into adipocytes, chondrocytes and osteoblasts (15), myocytes (16,17), neuronal cells (15,18,19), cardiomyocytes (20), and hepatocytes (21) in vitro. Therefore, ASCs may have the potential to be used for various clinical applications, including bone, cartilage, and cardiac repair. The stromal fraction of adipose tissue is a heterogeneous mixture of cells, including endothelial cells, smooth muscle cells, pericytes, fibroblasts, mast cells, and preadipocytes (8,22). The capacity of the ASCs to adhere to plastic and expand in vitro is generally used to select and expand the stem cells. The current practice of culturing cells is time consuming, expensive, and inefficient because it requires the incorporation of current Good Manufacturing Practice (cGMP) to allow reinfusion into patients. Perhaps more importantly, culturing may affect the functional characteristics of the ASCs, such as their potential to home to the site of tissue damage (23) due to a change in the expression of adhesion molecules during culture. Such culture-expanded cell isolates, enriched for ASCs, may therefore become unsuitable for systemic infusion purposes. Culturing may also have an effect on the differentiation potential of the ASCs; however, this has not been investigated yet.

For clinical practice, it would be advantageous to use the SVF cells, because they can be harvested during the operative procedure itself and given back to the patient without the need for further in vitro expansion. Because adipose tissue is such an abundant source of stem cells, such an approach may be feasible. Most of our knowledge of ASCs, however, is based on phenotypical and functional characterization of cultured ASCs (14–22, 24–31). In this study, we investigated the phenotypical and functional characteristics of freshly isolated ASCs in the SVF of adipose tissue, as compared to cultured ASCs. Whereas culture-expanded ASCs represent a rather homogeneous cell population (8,13,32–34), the SVF is a heterogeneous mixture of cells. The immunophenotype of the whole SVF fraction has been previously investigated (13,33). However, to our knowledge, the ASCs in the SVF have not been characterized yet. For analyzing the phenotypical marker profile of freshly isolated ASCs, the ASCs were identified in the SVF on the basis of their high expression of CD34 and lack of expression of platelet endothelial cell adhesion molecule (PECAM or CD31) (24). Subsequently, the expression of stem cell markers CD117, Flt3, and CD133, several cell adhesion molecules, CD105 (endoglyn), HLA-ABC and HLA-DR, common leukocyte antigen CD45, and melanoma cell adhesion molecule CD146 on this cell population was investigated using three-color staining and flow cytometry. The expression of these surface markers on freshly isolated ASCs was compared to the expression on cultured ASCs. Next, we investigated whether freshly isolated ASCs in the SVF have functional properties comparable to cultured ASCs. In this part of the study, we selected for the ASCs in the SVF by seeding SVF cells in plastic culture wells. We demonstrated by confocal microscopy that more than 85% of the SVF cells that initially adhered to the culture wells had a $CD34^+CD31^-CD45^-CD146^-$ ASC-like phenotype. Differentiation of the freshly isolated ASCs into the osteogenic pathway was investigated and compared with that of cultured ASCs.

MATERIALS AND METHODS

Tissue sampling

Human subcutaneous adipose tissue samples were obtained as waste material after elective surgery and donated upon informed consent of the patients by the department of Plastic Surgery from the VU University Medical Center. Adipose tissue was harvested from the abdomen or hip and thigh region of healthy females, using either resection or tumescent liposuction. Eight fe-

male donors (mean age 42; range 28–62 years) were included in this study. The mean body mass index was 26.3 ± 2.4 (mean ± SD).

Isolation and culture of the SVF of adipose tissue

Adipose tissue was weighed and extensively washed with phosphate-buffered saline (PBS). Resected material was first cut into small pieces using a surgical scalpel. Tissue was digested enzymatically with 0.1% Collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) in PBS and 1% bovine serum albumin (BSA), for 45 min at 37°C with intermittent shaking. Enzyme activity was then neutralized by adding Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose (BioWhittaker, Cambrex, Verviers, Belgium) and supplemented with 10% fetal bovine serum (FBS). The digested adipose tissue (AT) was centrifuged for 10 min at 600 × *g*. The cell-containing pellet was resuspended in PBS and passed through a 200-μm nylon mesh (Braun/Beldico s.a-n.v, Marche-en Famenne, Belgium) to remove debris. Subsequently, cells were subjected to a Ficoll density centrifugation step (ρ = 1,077 g/ml, osmolarity 290 ± 15 mOsm; Axis-Shield, Oslo, Norway) to remove erythrocytes. The cell-containing interface was harvested and washed with DMEM and 10% FBS. Viability of the cells was assessed using the Trypan Blue exclusion assay. Viable, large cells with an irregular morphology were counted, using light microscopy. A total of 5×10^6 cells were resuspended in a mixture (1:1) of DMEM and 10% FBS and cryoprotective medium (Freezing Medium, BioWhittaker, Cambrex, Verviers, Belgium), frozen under "controlled rate" conditions in a Kryosave (HCI Cryogenics BV., Hedel, The Netherlands), and stored in the vapor phase of liquid nitrogen according to standard practice in the Department of Pathology of the VU University Medical Center, and following the guidelines of cGMP.

Single-cell suspensions of SVF cells were seeded at 1×10^5 cells/cm^2 in normal culture medium, consisting of DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all from Gibco, Invitrogen, CA). The cultures were maintained in a 5% CO_2 incubator at 37°C in a humidified atmosphere. The medium was changed twice a week. When reaching 80–90% confluency, cells were detached with 0.5 mM EDTA/0.05% trypsin (Gibco, Invitrogen, CA) for 5 min at 37°C and then replated. Cell viability was assessed using the Trypan Blue exclusion assay.

Flow cytometry

Single-cell suspensions of cryopreserved, freshly isolated adipose tissue-derived SVF cells and cultured passage-4 ASCs were phenotypically characterized using fluorescence-activated cell sorting (FACS; FACSCalibur, Becton Dickinson, USA). Cells were washed in FACS buffer (PBS containing 1% BSA and 0.05% sodium azide) and 1×10^5 per sample cells were incubated on ice with the optimal dilution of conjugated monoclonal antibody (mAb) in FACS buffer. All mAbs were of the immunoglobulin G_1 (IgG_1) isotype. We used allophycocyanin (APC)-conjugated antibodies against CD34 (clone 8612) and CD45, both from BD Biosciences (San José, CA). Fluorescein isothiocyanate (FITC)-conjugated mAbs against CD2, CD3 and CD14 (all from BD), CD19, CD11b (PharMingen, San Diego, Cal), activated leukocyte cell adhesion molecule (ALCAM or CD166, clone 3A6, RDI Research Diagnostics, Flanders, NJ), and PECAM (or CD31, BD) were used. Phycoerythrin (PE)-conjugated mAbs against c-kit (CD117, PharMingen, San Diego, CA), CD133 (clone 293C3, Miltenyi Biotec GmbH, Germany), Flt3 (Immunotech), endoglyn (CD105, clone SN6, Caltag Laboratories, Burlingame, CA), melanoma cell adhesion molecule (MELCAM or CD146, mAb 16985H, Chemicon Temecula, CA), Thy-1 (CD90, clone 5E10, BD), HLA-DR, HLA-ABC, vascular cell adhesion molecule-1 (VCAM-1 or CD106), and intercellular adhesion molecule-1 (ICAM-1 or CD54) (all from BD) were used. After a 30-min incubation, cells were washed twice with FACS buffer. Nonspecific fluorescence was determined by incubating cells with conjugated mAb anti-human IgG_1 (DAKO Cytomation, Glostrup, Denmark). Flow cytometry data were analyzed using CELLQUEST software (Becton Dickinson). Of three donors, cell populations were sorted based on the levels of CD34 and CD31 expression, using FACSvantage™ (Becton Dickinson). Sorted cells were seeded at a density of 10^4 cells/cm^2 and expanded in vitro.

Phenotypical characterization of SVF cells adhering to plastic culture wells

A total of 40,000 SVF cells were seeded (n = 3) per Lab Tek® Chamber Slide of 1.9 cm^2 (Nalge Nunc Int Corp, Naperville, IL). After 2 and 6 days of culture, cells were washed with PBS and fixed in 4% paraformaldehyde for 30 min. Cells were then washed two times with PBS and once with PBS and 1% BSA. Cells were incubated for 1 h with a 1:50 dilution of mAbs against CD34, CD31, CD45 (all from BD Biosciences, San José, CA), and CD146 (Chemicon Temecula, CA) in PBS and 1% BSA. Cells were then washed three times with PBS and 1% BSA and incubated with a 1:500 dilution of biotin-conjugated rabbit antibodies against mouse immunoglobulins RaMBioF(ab′)$_2$ (DAKO Cytomation, Glostrup, Denmark) in PBS and 1% BSA for 1 h. After three washings with PBS, a 1:500 dilution of Streptavidin Alexa Fluor™ 488 conjugate (Molecular Probes, Eugene, OR) was added for 1 h. Cells were washed three times

with PBS and nuclei were stained with 7.5 μM propidium iodide (PI) during 15 min. After washing three times with PBS, cells were covered with Vectashield mounting medium (Vector Laboratories, Inc. Burlingame, CA). Antibody staining of the cells was analyzed by confocal microscopy, using a Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany). The percentage of adhering cells that stained positive for CD34, CD31, CD146, and CD45, respectively, was quantified by previously developed image analysis software (35).

Osteogenic differentiation of SVF cells and cultured ASCs

For osteogenic differentiation, SVF cells were seeded at 50,000 cells/cm^2 and cultured ASCs (passage 4) at 5,000 cells/cm^2. Because the SVF isolates are heterogeneous mixtures of cells, the contribution of stem cells in these isolates is relatively low and ranges between approximately 1 and 10% (12–14). In contrast, after four passages in culture, the ASCs form a homogeneous population of cells regarding their surface marker expression and probably do contain almost exclusively stem cells (9,11,27). To add similar numbers of stem cells to the culture wells, SVF cells were plated at 10-fold higher densities than cultured ASCs. Cells were cultured in monolayer in osteogenic medium, consisting of normal culture medium supplemented with 10 mM β-glycerol phosphate, 50 μg/ml ascorbate-2-phosphate, and 100 ng/ml bone morphogenetic protein-2 (BMP-2; Peprotech EC LTD, London, UK). Control cells were cultured in normal culture medium. Osteogenic and control media were changed twice a week. BMP-2 has been shown to induce osteogenic differentiation both in cultured bone marrow-derived MSCs and cultured ASCs, in vivo and in vitro (36–39).

Reverse transcription PCR

Osteogenic gene expression by SVF cells ($n = 4$) was analyzed by measuring Runx-2, osteopontin, and Collagen type I mRNA (40–42) after 7, 10, and 12 days of osteogenic stimulation using RT-PCR. Total RNA from the cells was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA). cDNA synthesis (GeneAmp® PCR System9700, PE Applied Biosystems, CA) was performed using 0.5–1 μg of total RNA in a 20-μl reaction mix containing 5 Units of Transcriptor Reverse Transcriptase (Roche Diagnostics), 0.08 A_{260} units random primers (Roche Diagnostics), and 1 mM of each dNTP (Invitrogen). The cDNA was stored at −80°C prior to real-time PCR.

Real-time PCR reactions were performed using the SYBRGreen reaction kit according to the manufacturer's instructions (Roche Diagnostics) in a LightCycler (Roche Diagnostics). Primers (Invitrogen) used for real-time PCR are listed in Table 1. For real-time PCR, the values of relative target gene expression were normalized for relative 18*S* housekeeping gene expression.

Alkaline phosphatase activity

To assess the osteoblastic phenotype of the osteogenic stimulated SVF cells ($n = 4$), alkaline phosphatase (ALP) activity was measured after 14 days of culture in osteogenic medium or control medium. ALP activity and protein content were determined in the cell lysate. As substrate, *p*-nitrophenyl phosphate (Merck) at pH 10.3 was used to determine ALP activity. The absorbance was read at 410 nm. ALP activity data were expressed as millimole per microgram of protein in the cell layer. The amount of protein was determined using a BCA Protein Assay Reagent kit (Pierce, Rockford, IL), and the absorbance was read at 570 nm with a microplate reader (BioRad Laboratories Inc., Hercules, CA).

Von Kossa staining

To assess matrix mineralization in cultures of freshly isolated ASCs, SVF cells ($n = 3$) were cultured for 21 days in either osteogenic medium or control medium. Then wells were rinsed with PBS and fixed in 4% paraformaldehyde for 1 h. Cells were then rinsed with dis-

Table 1. Primers Used for Real-Time PCR

Target gene	*Primer sets*	*Expected product size*
18*S*	Forward, 5′-gtaacccgttgaaccccatt-3′	
	Reverse, 5′-ccatccaatcggtagtagccg-3′	151 bp
Runx-2	Forward, 5′-atgcttcattcgcctcac-3′	
	Reverse, 5′-actgcttgcagccttaaat-3′	156 bp
OPN	Forward, 5′-ttccaagtaagtccaacgaaag-3′	
	Reverse, 5′-gtgaccagttcatcagattcat-3′	181 bp
Col1αI	Forward, 5′-aagccgaattcctggtct-3′	
	Reverse, 5′-tccaacgagatcgagatcc-3′	195 bp

tilled water and overlaid with a 5% silver nitrate solution in the dark for 30 min, followed by rinsing with destilled water. After exposure to ultraviolet light for 1 h, cells were treated with sodium thiosulfate for 5 min and rinsed with distilled water. Finally, cells were counterstained using Fast Green. Calcified extracellular matrix was detected as black spots.

Immunohistochemistry

SVF cells ($n = 4$) were seeded at 50,000 cells/cm^2 in Lab Tek Chamber slides, and cultured in osteogenic medium. After 7 and 14 days of culture, cells were washed with PBS and fixed in 4% paraformaldehyde for 30 min. Cells were then washed two times with PBS and once with PBS, 1% BSA, and 0.1% saponin. Cells were incubated for 2 h with a 1:50 dilution of rabbit antibodies against osteonectin (EMD Biosciences, Inc., La Jolla, CA) in PBS, 1% BSA, and 0.1% saponin. Cells were then washed three times with PBS, 0.1% BSA, and 0.1% saponin and incubated with a 1:200 dilution of biotin-conjugated goat antibodies against rabbit immunoglobulins (BD Biosciences Pharmingen, San Diego, CA), in PBS, 1% BSA, and 0.1% saponin for 1 h. After three

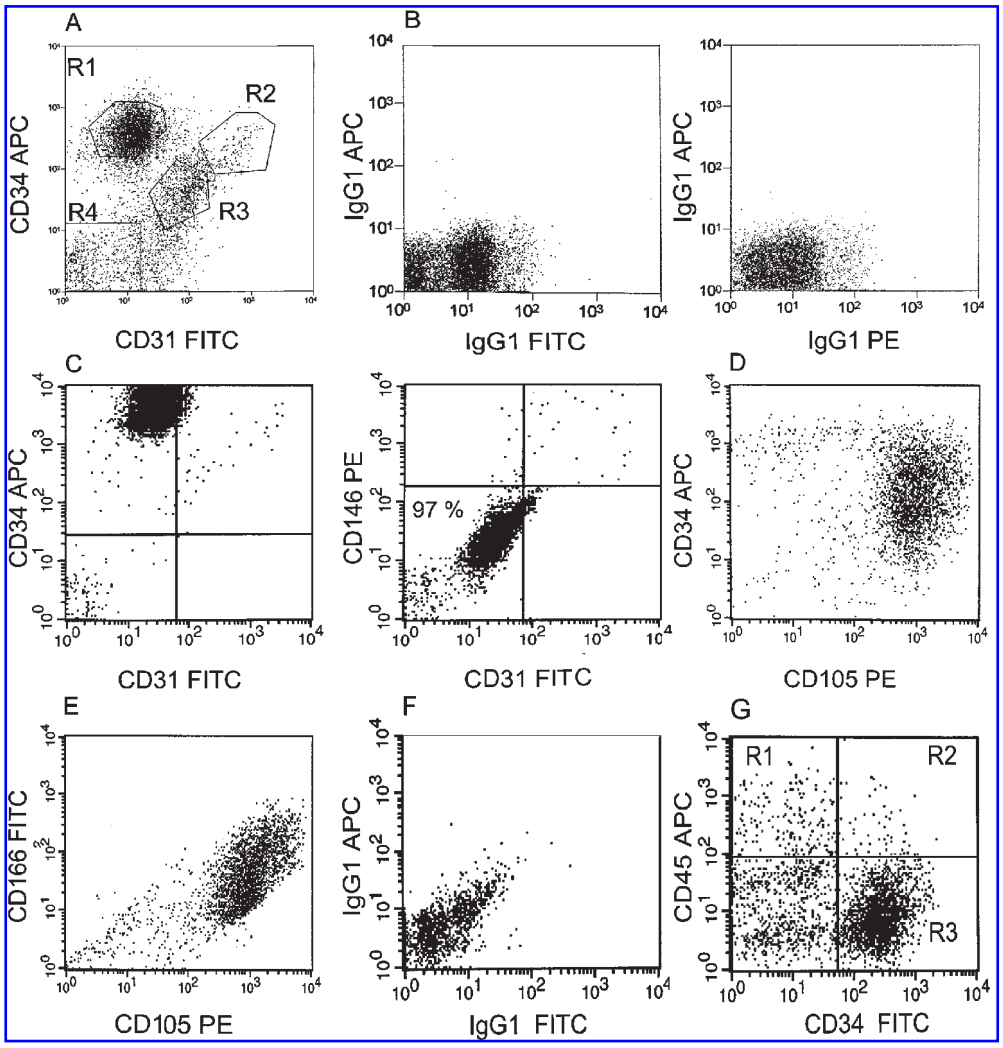


FIG. 1. Identification of ASC population in the SVF of adipose tissue. Freshly isolated SVF cells were stained with APC-labeled mAb anti-CD34, FITC-labeled mAb anti-CD31, and PE-labeled mAb anti-CD146. (**A**) Populations of cells defined by gates R1, R2, R3, and R4 were isolated by FACS. (**B**) Staining of isotype-matched control mAbs. (**C**) Sorted cells of gate R1 were analyzed for CD34, CD31, and CD146 expression. Data are from one isolate and representative of analyses of 3 donors. (**D,E**) CD34, CD105, and CD166 expression of R1 cells (defined in Fig. 1A) after 1 week of culture (passage 1). (**F**) Staining of isotype-matched control mAbs. (**G**) Double staining of SVF cells with FITC-labeled mAb against CD34 and APC-labeled mAb against CD45. Data are from one isolate and representative of analyses of 3 donors. Abbreviations: APC, allophycocyanin; ASC, adipose tissue-derived stem cell; PE, phycoerythrin; SVF, stromal vascular fraction.

washings with PBS, a 1:500 dilution of Streptavidin Alexa Fluor™ 488 conjugate was added for 1 h. Cells were washed three times with PBS, and nuclei were stained with 7.5 μM PI for 15 min. After washing three times with PBS, cells were covered with Vectashield mounting medium. Antibody staining of the cells was analyzed by confocal microscopy, as described above.

Statistics

Increased gene expression and ALP activity were analyzed using a one-tailed, paired Student's *t*-test.

RESULTS

ASCs are present in the CD34^{bright} CD31^{-} cell fraction of the SVF of adipose tissue

First we verified that the CD34^{+}CD31^{-} fraction of SVF cells contained the ASCs, as previously demonstrated by Boquest et al. (18). For this, three-color stainings of freshly isolated SVF cells were performed using CD34, PECAM (or CD31), and MELCAM (or CD146). Cell populations were FACS sorted on the basis of CD31 and CD34 expression. In each donor, CD34^{bright}CD31^{-} cells (Fig. 1A, gate R1), CD34^{bright}CD31^{+} cells (gate R2), CD34^{dim}CD31^{dim} cells (gate R3), and CD34^{-} CD31^{-} cells (gate R4) were discriminated. The isolated cells that were defined by gates R1, R2, R3, and R4, were expanded in vitro in normal culture medium. Only the sorted cells within gate R1 adhered to the plastic culture flask and showed proliferative activity. R1 cells (97%, Fig. 1C) were highly positive for CD34 and negative for CD31 and CD146. After 1 week, cells had reached 80–90% confluency. Flow cytometry demonstrated that after 1 week of culture more than 95% of the cells were still CD34 positive, but that the level of expression had decreased dramatically [Fig. 1D; mean fluorescence intensity (MF) = 200, versus Fig. 2C, MF = 4,000]. This demonstrates that the loss in CD34 expression on these cells during culture is not due to a selective outgrowth of

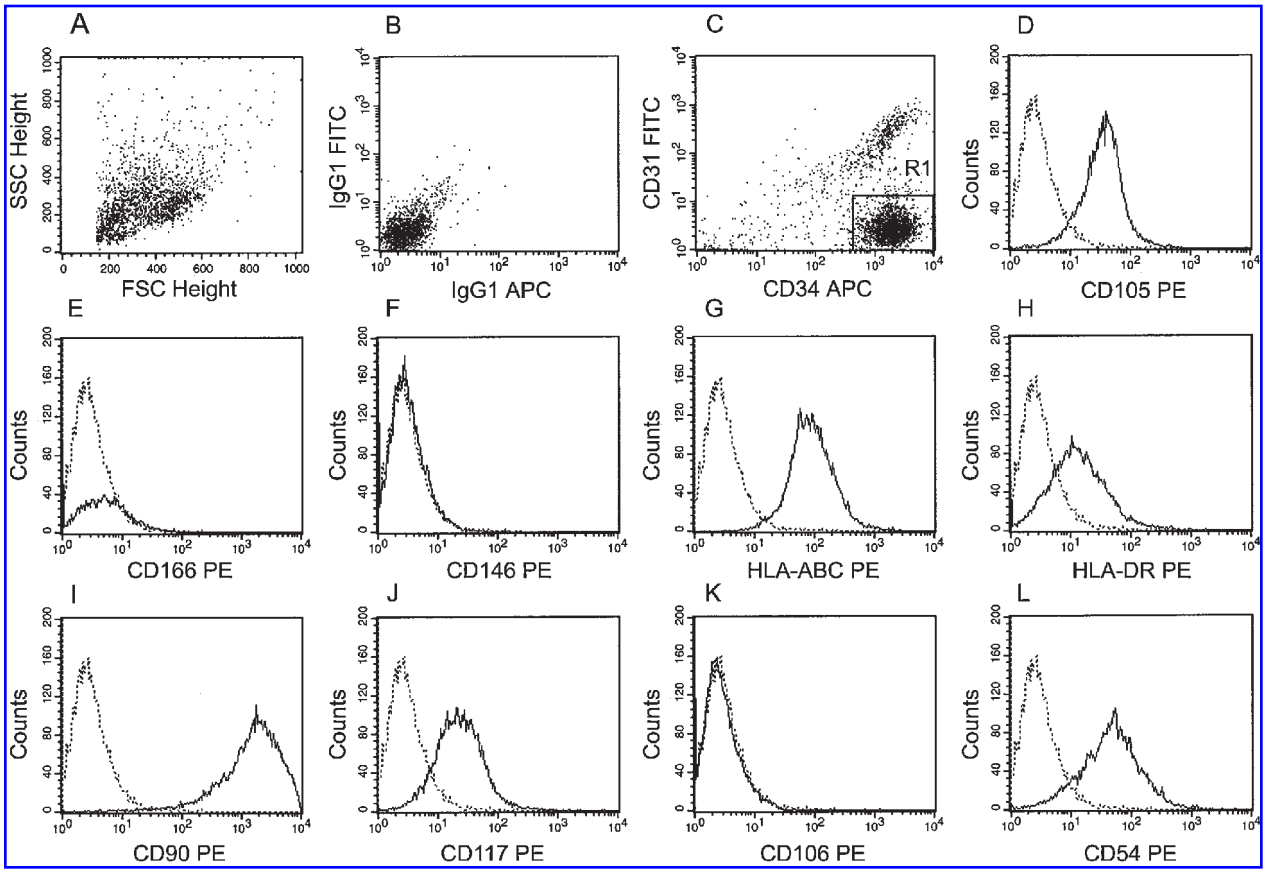


FIG. 2. Phenotypical characterization of ASC-containing cell fraction in the SVF of adipose tissue using flow cytometry. (**A**) FSC/SSC distribution of all cells. (**B**) Staining of isotype-matched control mAbs. (**C**) Triple stainings of SVF cells with APC-labeled mAb anti-CD34, FITC-labeled mAb anti-CD31, and each of the indicated mAbs, labeled with PE. The CD34^{bright}CD31^{-} cell fraction was gated (gate R1) and the histogram plots of the cells within R1 are shown in **D–L.** Data are from one isolate and representative of analyses of 5 individuals. Each histogram plot contains an isotype-matched mAb control, which is indicated as a dashed line. Abbreviations: APC, allophycocyanin; ASC, adipose tissue-derived stem cell; SVF, stromal vascular fraction.

$CD34^-$ cells, but is caused by a decrease in CD34 expression of the ASCs. Cultured cells were also positive for CD105 and CD166 (Fig. 1E).

Phenotypical characterization of freshly isolated ASCs

The $CD34^{bright}CD31^-$ cells in the SVF of adipose tissue were characterized further phenotypically in 5 donors (Figs. 2 and 3). To this end, three-color stainings of freshly isolated SVF cells were performed, whereby the $CD34^{bright}CD31^-$ cells were gated (Fig. 2C, gate R1), and examined for the expression of CD105, CD166, CD146, HLA-ABC, HLA-DR, CD90, CD117, CD106, CD54, CD133, and Flt3, respectively. In Fig. 2, D–L, the flow cytometric histograms of the $CD34^{bright}CD31^-$ cell fraction from a representative donor are shown. In Fig. 3, staining results of four additional donors are presented as the MF intensity. The $CD34^{bright}CD31^-$ cells demonstrated a homogeneous expression of the different surface markers. Cells were positive for CD105, HLA-ABC, HLA-DR, CD90, CD117, and CD54. Surprisingly, expression of CD166, a cell adhesion molecule that is generally accepted to be present on cultured MSCs, was very low. The $CD34^{bright}CD31^-$ cells were negative for CD146 and CD106, and for CD45, CD133, and Flt3 (not shown).

Differences in surface marker expression between freshly isolated ASCs and cultured ASCs

To compare the surface marker expression of freshly isolated ASCs with that of cultured ASCs, cultured ASCs from 3 donors (passage 4) were characterized phenotypically using flow cytometry (Table 2). Cultured ASCs demonstrated to be a homogeneous population of cells in surface marker expression showed an increased expression of CD105 and CD166, as compared to the ASCs in fresh cell isolates. On the other hand, CD34 expression on the cultured ASCs was diminished dramatically and HLA-DR and CD117 expression was lost.

Identification of other cell populations within the SVF of adipose tissue

The $CD34^{bright}CD31^-$ cells (Fig. 1A, gate R1) represented on average 34.6% ± 17.8% (mean ± SD, $n = 5$) of the SVF cells. The $CD34^{bright}CD31^+$ endothelial cells (Fig. 1A, gate R2) were also $CD146^+$ (not shown) and represented on average 12.2% ± 9.5% of the SVF cells. The $CD34^{dim}$ cells (gate R3) represented on average 16.4% ± 9.1% of the SVF cells. CD31 and CD146 expression on these $CD34^{dim}$ cells differed per donor, also with a population of cells that did express CD146, but no CD31. The frequency of these $CD34^{dim}CD146^+CD31^-$

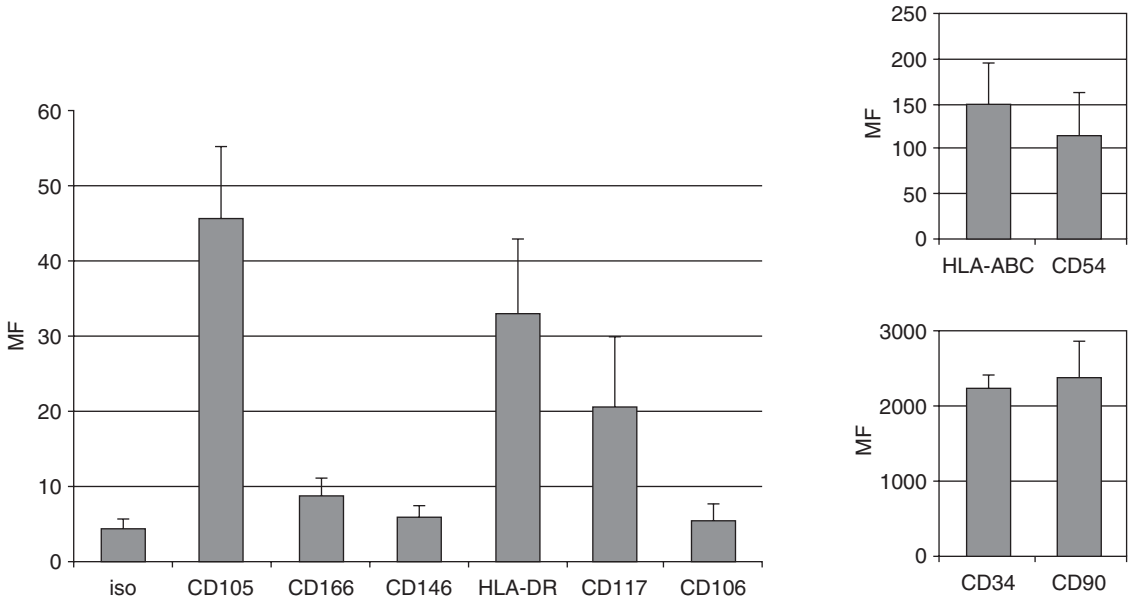


FIG. 3. Phenotypical characterization of the ASC-containing cell fraction in the stromal vascular fraction of adipose tissue, using flow cytometry. Freshly isolated SVF cells of 4 donors were analyzed in three-color stainings using APC-labeled mAb anti-CD34, FITC-labeled mAb anti-CD31, and each of the indicated mAbs, labeled with PE. The $CD34^{bright}CD31^-$ cell fraction was gated (as in Fig. 2C, gate R1) and staining results of the PE-labeled mAbs are expressed as the mean fluorescence (+SD). Note the different *y* axis scales. Abbreviations: APC, allophycocyanin; ASC, adipose tissue-derived stem cell; MF, mean fluorescence intensity; SVF, stromal vascular fraction.

Table 2. Surface Marker Expression on Freshly Isolated and Cultured ASCs

Cell-surface marker	*Freshly isolated ASCs* (n = *5*)	*Cultured ASCs passage 4* (n = *3*)
CD34	+++	+
CD166	+/−	+
CD105	+	++
CD90	+++	+++
HLA-ABC	++	++
HLA-DR	+	−
CD45	−	−
CD117	+	−
Flt3	−	−
CD133	−	−
CD106	−	−
CD54	++	++

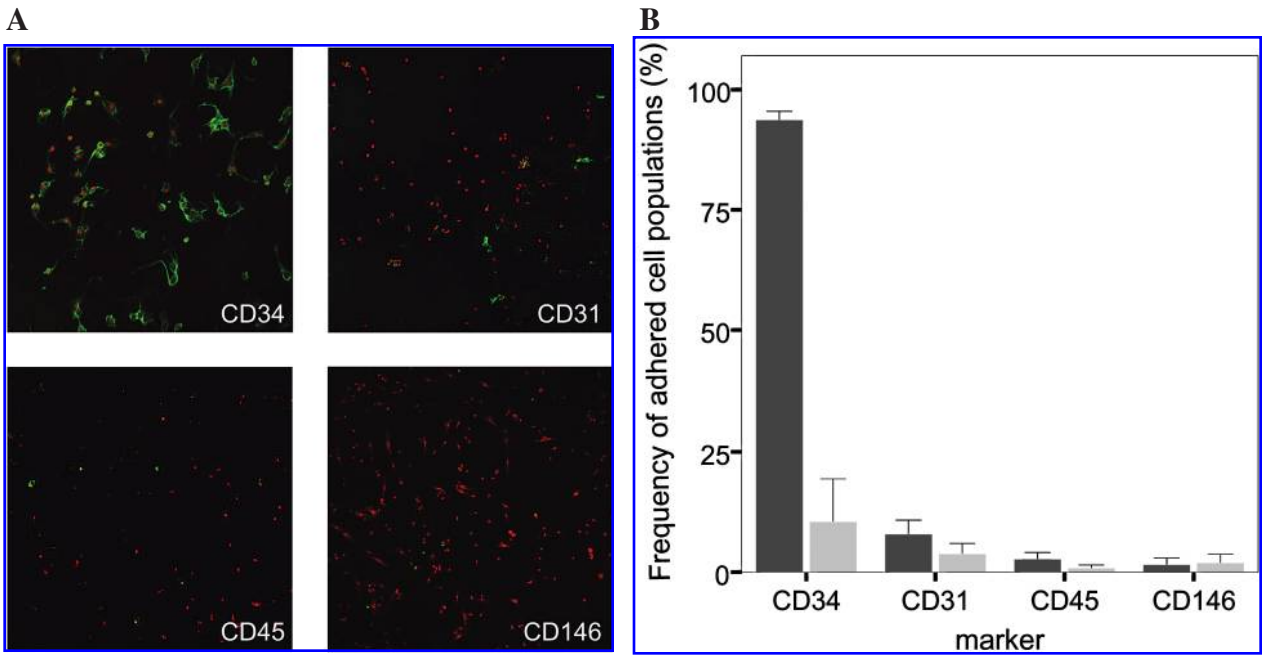
MF isotype control < 4.25
−, MF < 8.50; +/−, $8.5 <$ MF < 9; +, $10 <$ MF < 100; ++, $100 <$ MF $< 1{,}000$; +++, MF $> 1{,}000$.
Abbreviations: Mean fluorescence intensity, MF.

Table 3. Frequency of Cell Populations in the Stromal Vascular Fraction of Adipose Tissue

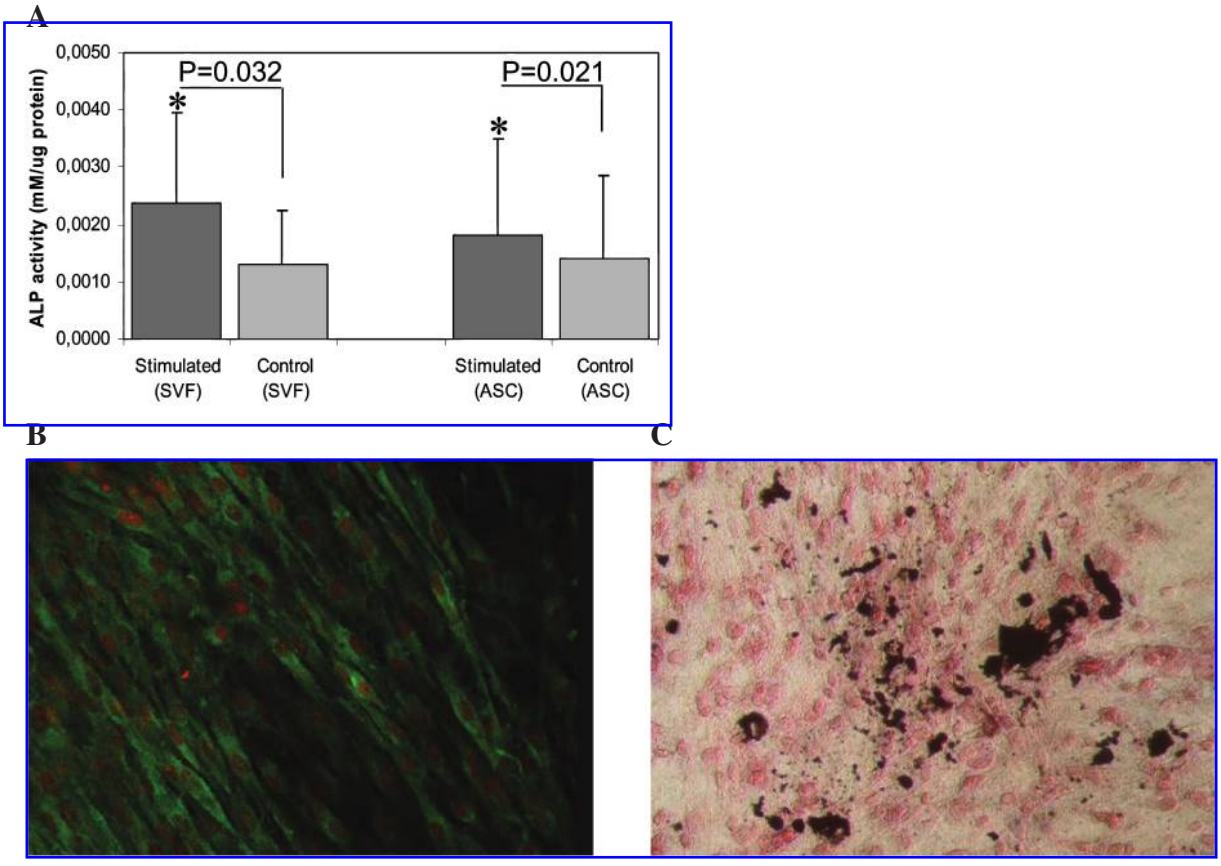
Phenotype	*Frequency (%)* (n = *5*)	*Cell type*
$CD34^{bright}CD31^{-}CD146^{-}$	34.6 ± 17.8	ASC-like
$CD34^{bright}CD31^{+}CD146^{+}$	12.2 ± 9.5	Endothelial cell
$CD34^{dim}CD31^{-}CD146^{+}$	10.3 ± 9.9	Vascular smooth muscle cell/pericyte
$CD45^{+}CD34^{-}$	14.2 ± 2.3	Leukocyte
$CD45^{+}CD34^{+}$	5.2 ± 1.3	HSC-like
$CD45^{+}CD34^{+}CD117^{+}$	0.75 ± 0.1	HSC-like
$CD45^{+}CD34^{+}CD133^{+}$	0.02 ± 0.03	HSC-like
$CD45^{+}CD34^{-}CD3^{+}$	4.8 ± 1.9	T cell
$CD45^{+}CD34^{-}CD19^{+}$	0.2 ± 0.3	B cell
$CD45^{+}CD34^{-}CD117^{+}$	0.56 ± 0.2	Mast cell
$CD45^{+}CD11b^{+}$	6.1 ± 3.2	Granulocyte
$CD45^{+}CD14^{+}$	5.2 ± 4.3	Monocyte

Cell populations in the stromal vascular fraction of 5 donors were phenotypically characterized in triple stainings, using flow cytometry. Results are expressed as mean ± SD.
Abbreviations: HSC, hematopoietic stem cell; ASC, adipose tissue-derived stem cell.

COLOR PLATE 2. Osteogenic differentiation of freshly isolated ASCs and cultured ASCs. SVF cells and passage-4 ASCs were cultured in osteogenic medium (containing 10 mM β-glycerol phosphate, 50 μg/ml ascorbic acid, and 100 ng/ml BMP-2), and in normal culture medium, respectively. (**A**) After 14 days of culture, ALP activity and protein content were determined in the cell lysates of SVF cultures ($n = 4$) and ASC cultures ($n = 8$). (**B**) After 14 days of culture, cells were fixed and stained with rabbit antibodies against human osteonectin. mAb binding was detected by subsequent stainings with biotin-conjugated goat antibodies against rabbit immunoglobulins and Streptavidin Alexa Fluor™ 488 conjugate, followed by confocal microscopy (magnification, 400×). Positive staining was not detected when PBS was used instead of the primary antibody (not shown). (**C**) Matrix mineralization was detected after 21 days in von Kossa staining. Positive staining was not detected when SVF cells were cultured in normal culture medium (not shown). Abbreviations: ASC, adipose tissue-derived stem cell; BMP-2, bone morphogenetic protein 2; SVF, stromal vascular fraction.



COLOR PLATE 1. Phenotypical characterization of cell populations in the SVF adhering to plastic culture wells, using confocal microscopy. A total of 40,000 SVF cells were seeded per chamber slide (1.9 cm^2), and after 2 and 6 days of culture cells were fixed and stained with mAbs against CD34, CD31, CD45, or CD146. mAb binding was detected by subsequent incubations with biotin-conjugated rabbit antibodies against mouse immunoglobulins RaMBioF(ab′)$_2$ and Streptavidin Alexa Fluor™ 488 conjugate. Nuclei were stained with PI. Cells are from 1 SVF isolate and representative of analyses of 3 donors. (**B**) Percentages of adhering cells that stained positive for CD34, CD31, CD45, and CD146 were quantified by previously developed image analysis software (35). Abbreviations: SVF, stromal vascular fraction.



COLOR PLATE 2.

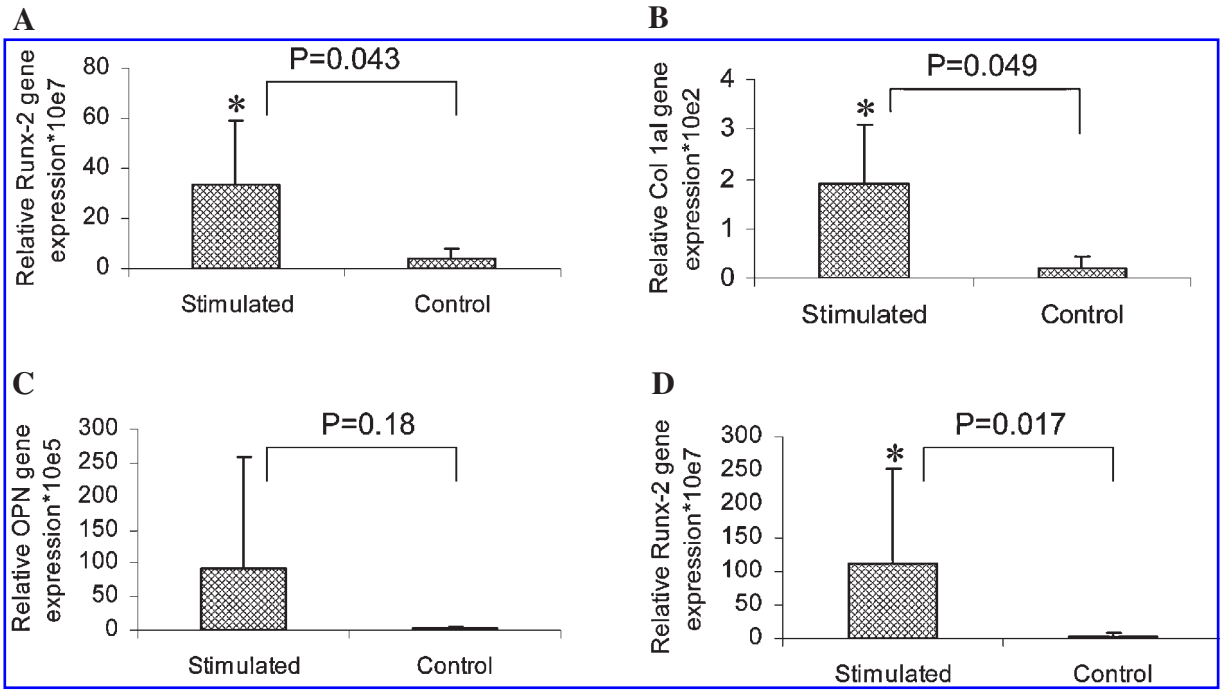


FIG. 4. Osteogenic gene expression of freshly isolated ASCs and cultured ASCs. SVF cells (**A,B,C,** $n = 4$) and passage 4 ASCs (**D,** $n = 4$) were cultured in osteogenic medium containing 10 mM β-glycerol phosphate, 50 μg/ml ascorbic acid, and 100 ng/ml BMP-2. After 7, 10, and 12 days of culture, cells were harvested and total RNA was isolated. *Runx-2*, *osteopontin*, and *Col1aI* mRNA expression was analyzed using real-time PCR (see Table 1 for primer sets used), and related to 18*S* housekeeping gene expression. Abbreviations: ASC, adipose tissue-derived stem cell; BMP-2, bone morphogenetic protein 2; OPN, osteopontin; SVF, stromal vascular fraction.

cells showed a large variation between the donors tested, ranging from 2.6% to 30% (10.3% ± 9.9%, mean ± SD, $n = 5$), and possibly represented vascular smooth muscle cells (VSMCs) and pericytes.

To identify leukocyte populations in the SVF, freshly isolated SVF cells were also stained with a combination of CD34 and CD45, the common leukocyte antigen. Of the SVF cells, 55.3% ± 5.7% stained positive for CD34 and negative for CD45 (Fig. 1G, R3). About 5.2% ± 1.3% of the cells showed expression of both CD34 and CD45, which is indicative for the presence of progenitor cells of hematopoietic origin (Fig. 1G, R2). Three-color stainings of the SVF cells for CD34, CD45, and a third surface marker specific for different types of leukocytes, identified additional populations of leucocytes. Within the $CD34^+CD45^+$ fraction also a $CD117^+$ cell population (0.75% ± 0.1%) was detected. A summary of the frequencies of the cell populations detected in the SVF of adipose tissue is listed in Table 3.

Identification of cell populations in the SVF adhering to plastic culture wells

When SVF cells are seeded in plastic culture wells, all cell populations in the SVF may potentially adhere, whereas we were only interested in the ASCs in the SVF. To study the contribution of freshly isolated ASCs and other cell populations initially binding to the culture wells, SVF cells were seeded and the adhering cell populations were identified, using confocal microscopy (Color Plate 1A). Percentages of adhering cell populations were quantified (Color Plate 1B). At day 2 after seeding 94% ± 2.4% of the adhering cells were $CD34^+$. Most $CD34^+$ cells had stretched out and showed slender protrusions (Color Plate 1A). Only low frequencies of $CD31^+$ cells, $CD45^+$ cells and $CD146^+$ cells were detected. After 6 days of culture, CD34 staining of the adhering cells had diminished dramatically in all 3 donors (Color Plate 1B). Only low frequencies of $CD31^+$ cells, $CD45^+$ cells $CD146^+$ cells were detected.

Osteogenic differentiation of freshly isolated and cultured ASCs

The capability of freshly isolated ASCs to differentiate into the osteogenic pathway was investigated by culturing SVF cells in osteogenic medium containing BMP-2. The *Runx-2*, *osteopontin*, and *Col1aI* mRNA expression increased in time in all 4 donors tested. However, the kinetics of gene expression varied between the donors with a peak expression between days 7 and 12.

To calculate the increase in gene expression, the peak expression was taken in each donor. After osteogenic stimulation, both *Runx-2* and *Col1aI* gene expression were significantly increased (Fig. 4A,B; $p < 0.05$). Osteopontin gene expression was also increased; however, this increase was not statistically significant (Fig. 4C). *Runx-2* gene expression in cultured passage-4 ASCs was significantly increased at day 7 of osteogenic stimulation (Fig. 4D; $p = 0.017$). In previous studies, we already demonstrated an increased osteopontin and *Col1aI* mRNA expression in cultured ASCs within 7 days of osteogenic stimulation (12).

Freshly isolated ASCs showed increased ALP activity after 14 days of culture in osteogenic medium, as compared to cells cultured in normal culture medium (Color Plate 2A; $p = 0.032$). There was no significant difference in ALP activity between freshly isolated and cultured ASCs ($p = 0.094$). Osteonectin was detected in the cells after 7 days of osteogenic stimulation and intensity of staining increased in time (Color Plate 2B, 14 days). Matrix mineralization in the SVF cultures was demonstrated after 21 days (Color Plate 2C).

DISCUSSION

Regenerative cell-based therapies require an abundant source of adult stem cells that is readily available, preferably at the time of operation. The SVF fraction of adipose tissue is such an abundant source of adult stem cells. Although cultured ASCs have been characterized extensively, only little is known about the phenotypical and functional characteristics of the freshly isolated stem cells in the SVF. In this study, we demonstrated that freshly isolated ASCs in the SVF slightly differed in immunophenotype from cultured ASCs. In addition, we showed that, like cultured ASCs, freshly isolated ASCs were capable of differentiating into the osteogenic pathway.

In this study, we first investigated the expression of cell-surface markers of freshly isolated ASCs in more detail and compared the phenotypical marker profile with that of cultured ASCs. Contrary to cultured ASCs, freshly isolated ASCs were highly positive for CD34, positive for CD117 and HLA-DR, and expression of CD105 and especially CD166 was relatively low. Whereas the freshly isolated stem cells within the SVF express high levels of CD34, this expression of CD34 was lost upon culturing the cells, as was demonstrated in the FACS sort experiments and by confocal microscopy. Nevertheless, we show that this loss in CD34 expression did not imply a change in osteogenic differentiation capacity. Up to now, the phenotype of MSCs in fresh cell isolates was not completely clear, because of the low frequency of MSCs in the tissues from which they are retrieved. In the bone marrow, for example, MSCs represent only 0.001–0.01% of the total population of cells (10). In most studies, the capacity of the cells to adhere to plastic and expand in vitro was used to select for MSCs, and the phenotypical characterization was performed on these culture-expanded cells. Because the frequency of stem cells in adipose tissue is much higher, it is possible to isolate and phenotypically characterize these ASCs directly ex vivo. Only recently, ASCs from adipose tissue have been demonstrated to be $CD34^+$ (24,25,33). However, endothelial cells in the SVF are also $CD34^+$. These endothelial cells can be separated from the ASCs because they also express platelet endothelial cell adhesion molecule PECAM-1 or CD31. Hence, endothelial cells are $CD34^+CD31^+$, whereas ASCs are $CD34^+CD31^-$. Boquest et al. (24) and Miranville et al. (25) isolated these $CD34^+CD31^-$ cells from the SVF of adipose tissue and demonstrated that this cell population could differentiate into the chondrogenic, osteogenic, adipogenic, and neurogenic pathways, and into endothelial cells, respectively.

In this study, we first phenotypically characterized the $CD34^+CD31^-$ cell population in more detail in three-color stainings, using flow cytometry. The $CD34^{bright}$ $CD31^-$ cell fraction demonstrated a homogeneous expression of the different surface markers. In contrast to the study of Boquest et al., we did detect CD117 and HLA-DR on the freshly isolated ASCs, although the expression level of HLA-DR was low (MF = 32,9 ± 10; mean ± SD, $n = 4$) as compared to that on $CD34^+$ $CD31^+CD146^+$ endothelial cells (MF = 1,750 ± 957; $n = 4$; data not shown). Our findings, however, are in agreement with studies of Mangi et al. (43), who showed CD117 expression on $CD117^+CD90^+$ MSCs from the rat's bone marrow, of Prunet-Marcassus et al. (44), who identified $CD45^-CD117^+$ immature cells in the SVF of mice adipose tissue, and of Beltrami et al. (45), who reported the existence of $CD117^+$ multipotent stem cells in the heart, giving rise to myocytes, smooth muscle, and endothelial cells, thus demonstrating that CD117 is more widely distributed on progenitor/stem cells. In addition, HLA-DR expression on bone marrow-derived MSC lines was previously reported by Ishii et al. (46). The expression of HLA-DR might result in a rejection of the freshly isolated ASCs when used for cell-based therapies in an allogenic donor. However, in a pilot study, we did not detect proliferation of allogenic T cells induced by a FACS-sorted $CD34^{bright}CD31^-$ cell fraction (data not shown). In this study, freshly isolated and cultured ASCs showed expression of CD54 (ICAM-1), whereas no CD106 (VCAM-1) expression was detected. This expression pattern of CD54 and CD106 is reversed as compared to bone marrow-derived MSCs (9,26), indicating that there are differences between ASCs and bone marrow-derived MSCs.

Next we investigated whether the freshly isolated ASCs in the SVF had comparable functional properties

as cultured ASCs. However, the SVF of adipose tissue is a heterogeneous mixture of cells, and when SVF cells are seeded in plastic culture wells all these cell populations may potentially adhere. We were only interested in the ASC fraction of the SVF. In the study using confocal microscopy, we demonstrated that more than 85% of the SVF cells that initially adhered to the culture wells had an ASC-like phenotype, whereas only few endothelial cells, leukocytes, vascular smooth muscle cells, and pericytes adhered.

To answer the question of whether the freshly isolated ASCs were capable of differentiating into the osteogenic pathway, SVF cells were cultured in osteogenic medium containing BMP-2. After osteogenic stimulation, gene expression of *Runx-2*, osteopontin, and Collagen type I in the freshly isolated ASCs was increased, although the increase in osteopontin gene expression was not statistically significant. This was probably caused by the large differences in the amount of osteopontin mRNA produced between the SVF isolates tested. Freshly isolated ASCs showed increased ALP activity, stained positive for osteonectin, and showed matrix mineralization, demonstrating the potential to differentiate into an osteoblastic phenotype. The osteonectin response was detected throughout most of the cytoplasma, which is comparable to osteonectin staining in bone cells (47). Although we did find some differences in the kinetics of osteogenic gene expression between the different SVF isolates and between freshly isolated and cultured ASCs, we show that the SVF represents an abundant source of freshly isolated ASCs, capable of differentiating into the osteogenic pathway in vitro. However, to use the freshly isolated SVF cells for clinical application, differentiation of the SVF cells into the osteogenic pathway is required without in vitro expansion and selection steps. To achieve this, a possible scenario would be to stimulate the freshly isolated SVF cells with BMP-2 in suspension for a brief period of only 15 min, just prior to transplantation. Such a short stimulus is shown to be sufficient to induce the differentiation of freshly isolated ASCs into the osteogenic pathway (48). In addition, the biophysical and biochemical environment in vivo, which is provided by the scaffold and the surrounding tissue, may further induce or enhance the differentiation of the cells (49).

In conclusion, we phenotypically characterized freshly isolated ASCs in the SVF and demonstrated that freshly isolated ASCs slightly differed in immunophenotype from cultured ASCs. In addition, we demonstrated that the freshly isolated ASCs in the SVF were capable of differentiating into the osteogenic pathway. As such, the use of freshly isolated ASCs for tissue engineering purposes involving bone repair is potentially applicable, and may have the advantage over cultured ASCs in the way that the SVF cells are readily available, and thus no costly expansion or isolation steps of the stem cells is required.

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