

Large-Scale Production of Human Adipose Tissue from Stem Cells: A New Tool for Regenerative Medicine and Tissue Banking

Francesco D'andrea, M.D.,¹ Francesco De Francesco, M.D.,² Giuseppe A. Ferraro, M.D., Ph.D.,¹ Vincenzo Desiderio, B.Sc.,² Virginia Tirino, B.Sc.,^{2,3} Alfredo De Rosa, D.D.S., Ph.D.,² and Gianpaolo Papaccio, M.D., Ph.D.²

Adipose tissue is an easy, accessible, and abundant source of mesenchymal stem cells (MSCs) for the reconstruction and addition of soft tissue and for restoration of soft-tissue defects associated with trauma, tumor resections, and congenital deformities. A stable source of adipose cells or tissue is needed for autologous grafting. Therefore, the aim of this study was to obtain enough autologous adipose tissue for possible clinical applications. For this purpose, we isolated MSCs (CD34⁺/CD90⁺) from human lipoaspirated or resected fat, which differentiated into adipocytes when placed in culture. Human adipose tissue is a paramount source of autologous MSCs were capable of generating a complete adipose tissue *in vitro*. Differentiated adipocytes expressed a strong positivity for several specific antibodies, including adiponectin and peroxisome proliferator-activated receptor gamma. In addition, fibroblasts (~10% of the whole sorted-cell population) started to secrete an extracellular matrix after 60 days that was strongly positive for type I collagen and fibronectin. After long-term culture (4 months), an adipose tissue with collagenic fibers and vessels was obtained. This tissue was comparable with adult human adipose tissue and therefore may be a criterion standard for future tissue repair and regeneration and for therapeutic and transplantation purposes.

Introduction

MESENCHYMAL STEM CELLS (MSCs) are an excellent source for adipose tissue engineering.¹ Soft tissue is necessary for reconstructive and plastic surgery. The common clinical approach is to restore defects using free microvascular flaps and transferring adipose tissue grafts made of different alloplastic and allogenic products. In recent years, transplantation of autologous adipose tissue has been used for correction of soft-tissue defects, including trauma, tumor resection, and congenital and acquired anomalies, but autologous fat transplantation has unsatisfactory long-term results.^{2,3} Limitations of this method are due to mature adipocyte fragility, as well as to insufficient or unsuitable vascularization. Fat grafts are reabsorbed and replaced by fibrous tissue with formation of liponecrotic cysts.^{4,5} Moreover, cells located centrally within fat grafting undergo tissue necrosis, with volume loss and inflammation due to vascular failure.⁶

Stem and precursor adipose cells have been used for *in vitro* experiments, where they proliferate and differentiate;⁷

moreover, they are capable of differentiating *in vivo*.⁸ However, stem and precursor adipose cells have high long-term survival.^{9,10} For efficient adipose tissue regeneration, specific cell selection is necessary. Human adipose tissue-derived adult stem cells (hADASCs) have been previously identified;¹¹ these stem cells display multilineage developmental plasticity *in vitro* and *in vivo*. Furthermore, pools of hADASCs can be induced to express the biochemical profile of adipocytes, chondrocytes, and osteoblasts under appropriate *in vitro* culture conditions.^{12,13} In culture, these cells express cell-surface markers similar to those expressed by MSCs, including CD105, STRO-1, CD90, and CD44, and share many features with MSCs.¹⁴ Several researches have carried out adipose tissue engineering using the 3T3-L1 murine preadipose cell line,¹⁵ rat preadipocytes,¹⁶ or bone marrow-derived MSCs.¹⁷ The interest raised in adipose tissue as a source of adult multipotent stem cells has also generated results for their use in regenerative medicine;¹⁸ adipose tissue is an abundant and practical source of precursor cells for soft-tissue reconstruction. This

¹Department of Orthopedic, Traumatologic, Rehabilitative and Plastic-Reconstructive Science, Seconda Università di Napoli, Naples, Italy.

²Department of Experimental Medicine, Section of Histology and Embryology, Tissue Factory and Banking Laboratory, Seconda Università di Napoli, Naples, Italy.

³Department of Experimental Oncology, Istituto Nazionale Tumori, Naples, Italy.

stem cell population displays a high capability for self-expansion and differentiation into preadipocytes.

In this study, we isolated and selected hADASCs, which have several features that differ with respect to those previously discussed, mainly regarding their ability to produce a vascularized autologous adipose tissue. This tissue is suitable for clinical regeneration, transplantation, and implantation.

Materials and Methods

Adipose tissue extraction and digestion

Subcutaneous adipose tissue of abdominal and mammary origin was obtained with informed consent from female patients aged 18 to 43 who had undergone elective procedures for plastic surgery. Adipose tissue was obtained by lipectomy or liposuction in the Plastic and Reconstructive Surgery Clinic of the Second University of Naples. The adipose tissue was placed in a physiological solution (sodium chloride (NaCl) 0.9%), washed twice in phosphate buffered saline (PBS) 1× (137 mM NaCl, 2.7 mM potassium chloride, 10 mM disodium phosphate, 1.8 mM monopotassium phosphate), scraped, and placed in a digestion solution (collagenase type I (3 mg/mL) and dispase (4 mg/mL), supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL) and clarythromycin (500 µg/mL)) in PBS 1× at 37°C in agitation for 60 min. The digest was filtered through 70-µm filters (Becton & Dickinson, Sunnyvale, CA).

Cell culture

After filtration and washing, the pellet was resuspended in erythrocyte lysis buffer (155 mM ammonium chloride; 10 mM potassium bicarbonate, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.3) for 10 min at room temperature. The cell suspension was centrifuged at 1300 rpm for 7 min and the pellet resuspended in 5 mL of Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin and seeded in 25-cm² flasks. Flasks were incubated at 37°C with 5% carbon dioxide and the medium changed twice a week. Cells reached confluence in 5 to 7 days. Experiments were performed in quadruplicate.

In vitro differentiation

We studied three methods of stimulation of adipose differentiation: basal culture medium supplemented with 20% FBS for the whole experiment;^{19,20} culture medium supplemented with 10% FBS plus dexamethasone (1 µM, Sigma, Milan, Italy), human recombinant insulin (10 µM, Sigma), indomethacin (200 µM, Fluka, Milan, Italy), and 3-isobutyl-1-methyl-xanthine (IBMX) (0.5 mM, Sigma); and hADASCs cocultured with sorted fibroblasts (10%) and grown in basal culture medium supplemented with 20% FBS for 4 months. The media were changed two times per week. Cells cultured in a basal medium without additions were used as controls.

Limited dilution assay and proliferative potential of hADASC

To assess clonogenic and proliferative ability of cells, a suspension of hADASCs (CD34⁺/CD90⁺/CD45⁻) was diluted and single cells seeded in multiwells. Two weeks later, cells were stained with Toluidine blue to assess their proliferation rate.

Flow cytometry

Cells were detached using a solution of trypsin-EDTA (200 mg/L EDTA, 500 mg/L trypsin, Cambrex, Verviers, Belgium). At least 200,000 cells were incubated with primary antibody for 30 min at 4°C, washed twice in PBS 1×, and incubated with a secondary antibody. Alternatively, cells were incubated directly with fluorescent-conjugated antibodies for 30 min at 4°C, washed, and resuspended in 0.6 mL PBS 1×. Samples were analyzed at day 0 (day of withdrawal), day 15, and day 30. Antibodies used in this study were anti-CD117 phycoerythrin (PE) (c-kit) (Miltenyi-Biotech, Calderara di Reno, Italy), anti-CD34 fluorescein isothiocyanate (FITC) and PE (Miltenyi-Biotech), anti-CD90 FITC (Chemicon, Prodotti Gianni, Milan, Italy), anti-CD105 FITC (Santa Cruz, CA), anti-CD29 Cy (Miltenyi-Biotech), anti-CD31 FITC (Miltenyi-Biotech), anti-CD133 PE (Miltenyi-Biotech), anti-human vascular endothelial growth factor (hVEGF); Santa Cruz Biotechnology, Santa Cruz, CA), anti-VEGF receptor 2 (VEGFR-2; Santa Cruz Biotechnology), anti-CD54 PE (Miltenyi-Biotech), anti-CD44 FITC (Miltenyi-Biotech), anti-CD45 Cy and PE (Becton & Dickinson, Franklin Lakes, NJ), and anti-CD14 PE (Miltenyi-Biotech).

A FACS Vantage (Becton Dickinson) was used, and data were analyzed using CellQuest Software (Becton Dickinson).

Immunofluorescence and Immunohistochemical staining

Cells in multiwells were washed in PBS 1× and fixed with 4% paraformaldehyde for 30 min at 4°C, washed three times in PBS 1× for 10 min, and incubated in PBS\5% FBS for 60 min at 4°C. After another double washing in PBS 1× for 10 min at room temperature, cells were incubated overnight at 4°C with monoclonal antihuman antibodies (diluted 1:100 in PBS 1×). Wells were washed in PBS 1× three times for 10 min at room temperature and incubated for 90 min at 4°C with the secondary FITC- or PE-conjugated antibody (diluted 1:200 in PBS 1×) (Santa Cruz Biotechnology). These cells were also treated with 4', 6-diamidino-2-phenylindole (Invitrogen, San Giuliano Milanese, Italy) diluted 1:10000 (5 µg/mL) in PBS 1× for 7 min at room temperature. Cells incubated for 90 min at 4°C with conjugated secondary antibodies were used as negative control. Cells were then observed under a fluorescence microscope (Nikon Instruments Italia, Calenzano, Italy).

Immunohistochemical analyses were performed using a DAKO CYTOMATION kit (En Vision + System-HRP-AEC, Dako Italia, Milan, Italy) according to the manufacturer's protocol.

RNA isolation and polymerase chain reaction

RNA was extracted using TRI Reagent (Sigma, Milan, Italy). cDNA synthesis was carried on total RNA using SuperScript II reverse transcriptase (Invitrogen, San Giuliano Milanese, Italy). Primer sequences used were glyceraldehyde-3-phosphate dehydrogenase: fw AGCCGCATCTTCTTTTGC GTC; rw TCATATTTGGCAGGTTTTTCT; CD34: fw AAA GACCCTGATTGCACTGG rw GCCCTGAGTCAATTTCA CTT; CD90: fw CCCAGTGAAGATGCAGGTTT; rw GAC AGCCTGAGAGGGTCTTG; CD44: fw TCCAAAGGTTTT CCATCCTG; rw AGGGCCAGCCTCTATGAAAT; CD54: fw

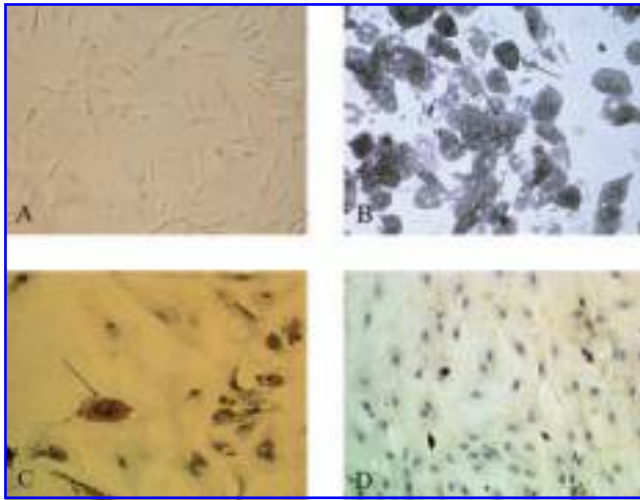


FIG. 1. (A) Human adipose tissue-derived adult stem cells (hADASCs) in 20% fetal bovine serum-supplemented medium at day 1 exhibit an elongated fibroblast-like morphology (original magnification, $\times 200$). (B) hADASCs in medium containing adipogenic inducing factors differentiate into adipocytes with lipid droplets (*arrow*) (original magnification, $\times 400$). (C) Adipocytes stained with adiponectin (*arrow*) (original magnification, $\times 400$). (D) Undifferentiated cells, showing negativity for adiponectin (original magnification, $\times 200$).

CAGTTGTAACACTGCAGGAGAG; rw ATTGTGAACAC
TGGCAGAAATG; VEGF: fw TGACAGGGAAGAGGAGGA
GA; rw CGTCTGACCTGGGGTAGAGA.

Results

Stem cell isolation was performed using enzymatic digestion as described in the Materials and Methods section, and three different methods of differentiation were used and compared. By day 1, cells cultured in medium supplemented with 20% FBS were adherent and quickly expanding; they exhibited an elongated fibroblast-like morphology (Fig. 1A). Confluence was obtained after 5 to 7 days. When hADASCs were cultured in the medium containing adipogenic-inducing factors (insulin, dexamethasone, and IBMX), approximately 80% of the cells differentiated into adipocytes containing multilocular lipid droplets within the cytoplasm (Fig. 1B). These adipocytes were strongly positive for adiponectin (Fig. 1C). Undifferentiated cells were negative (Fig. 1D). To assess the proliferation rate and the clonogenic potential of cells, we performed a limiting dilution assay. After 2 week of culture, most wells (16/24), which had been initially plated with one cell each, contained colonies with a doubling time of approximately 5 days (showing high clonogenic capability). After 7 days of culture, the proliferation rate of $CD34^+$ cells was higher than that of $CD34^-$ cells (82%), as shown by Ki-67 expression (Fig. 2A). The proliferation rate of $CD34^-$ cells was low (18%) or negligible (Fig. 2B). Cell cycle events can be distinguished based on the expression of Ki-67. Cells in S phase or G_2/M phase are Ki-67⁺ (Fig. 2C). Using the FACS sorter, we analyzed $1000,000 \pm 100,000$ cell samples using specific stem-cell antigens (see below). At day 0, we detected three stem or progenitor cell populations: stromal stem cells that coexpressed CD117 (5%) and CD34 (7%); mesenchymal stem or progenitor cells, whose markers were CD29 (β -integrin) (72%), CD34 (25%), CD90 (Thy-1)

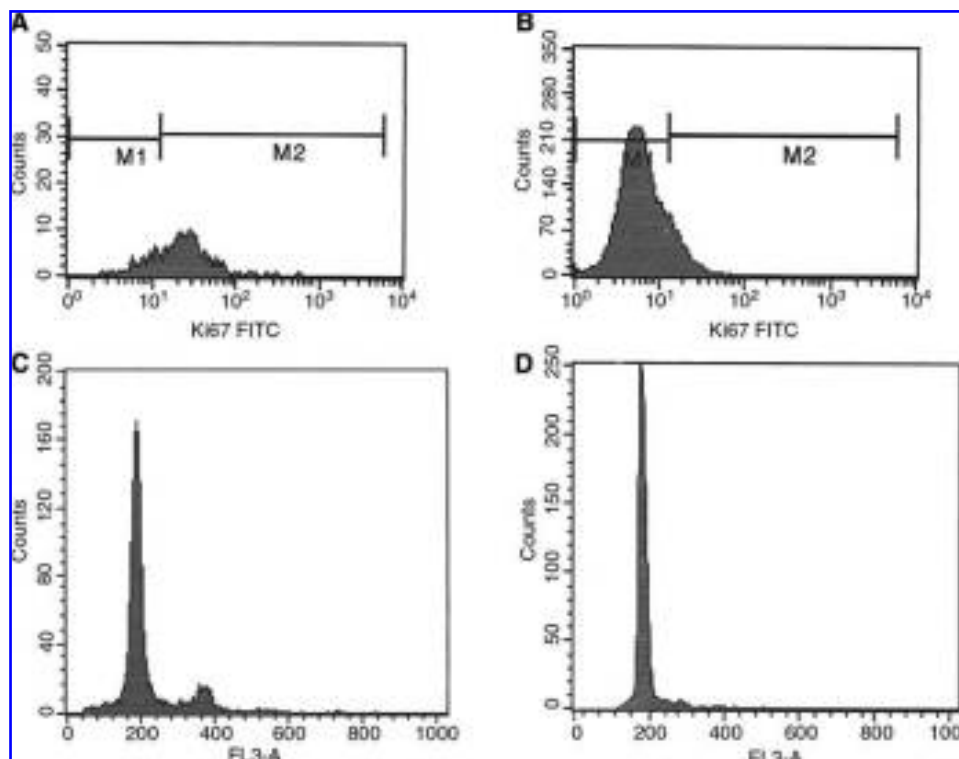


FIG. 2. (A) Proliferation rate of $CD34^+$ at day 7. (B) Proliferation rate of $CD34^-$ at days 7. (C) Cell cycle of $CD34^+$ cells. (D) Cell cycle of $CD34^-$ cells.

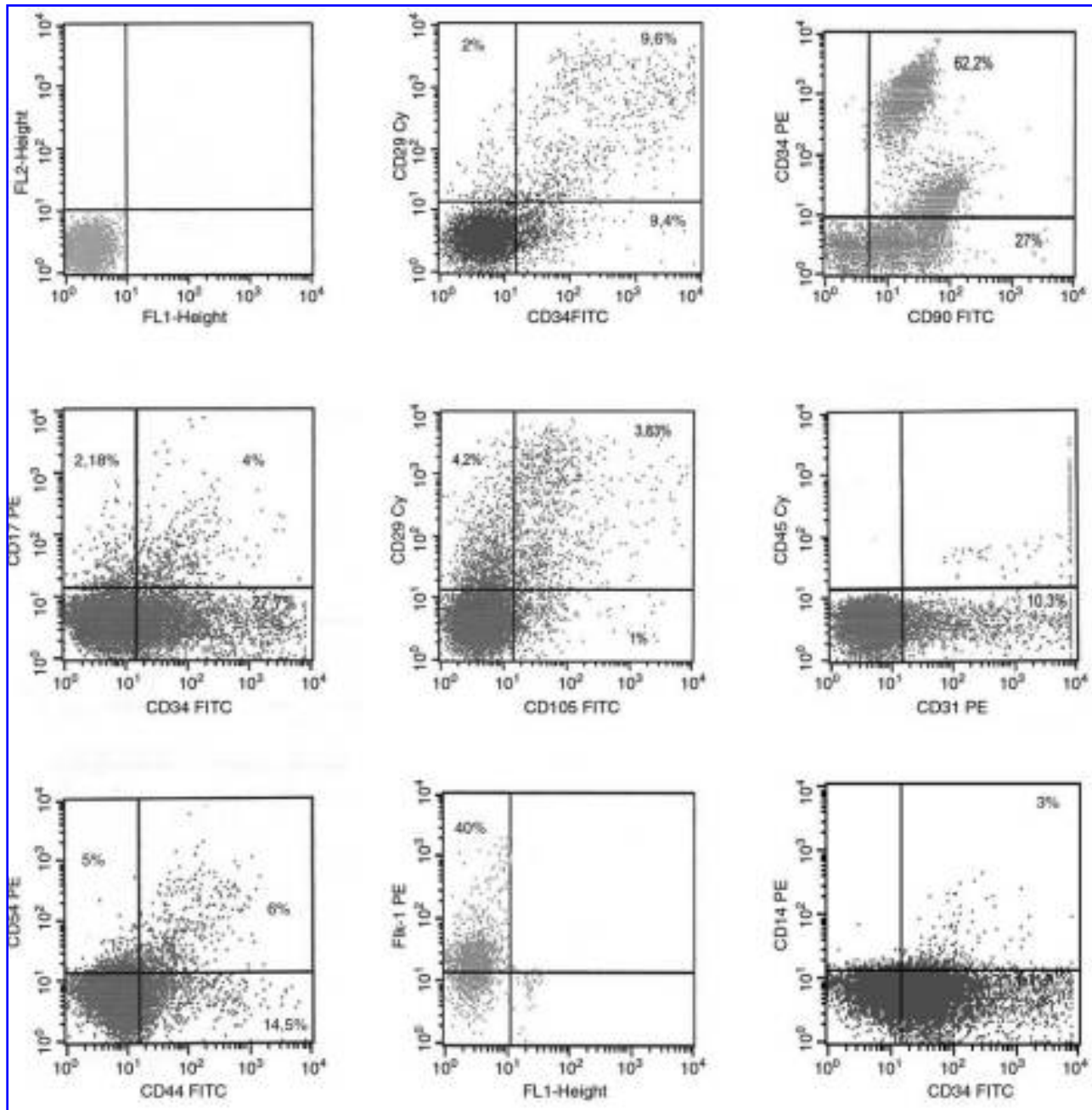


FIG. 3. Representative fluorescence-activated cell sorting performed at day 0 of culture. A significant number of hADASCs are clearly positive for mesenchymal markers, including CD29 (72%), CD34 (25%), CD90 (30%), CD117 (7%), and CD105 (5%), and for endothelial markers, including CD31 (6%), CD133 (8%), CD44/CD54 (5%), and Flk-1 (45%). Control phycoerythrin-conjugated and fluorescein isothiocyanate-conjugated isotypes were negative.

(30%), and CD105 (endoglin) (5%); and endothelial stem or progenitor cells, whose markers were CD31 (6%), CD133 (8%), CD44/CD54 (5%), and Flk-1 (VEGFR-2) (45%) (Fig. 3). Reverse transcriptase polymerase chain reaction (RT-PCR) analysis confirmed previous data (Fig. 4). Only CD34⁺/CD90⁺ cells were sorted and cultured for the experiments.

Cell differentiation was followed by observing the kinetics of marker expression after sorting. Flow cytometry revealed that the CD34⁺/CD90⁺ population gradually lost positivity for CD34 but not for CD90. Analyses performed at day 15 of culture showed that the initial CD34⁺/CD90⁺ cell population

comprised only approximately 2% CD34 positive elements and 35% CD90 positive elements at this time (Fig. 5A); after 30 days of culture, the percentage of CD90 increased to 40% (Fig. 5B). At this later time point, these cells acquired an endothelial morphology and expressed high levels of endothelial markers, including CD44⁺/CD54⁺ (80%) and VEGF⁺ (20%) (Fig. 5C, D). Isotypes and nonprobed cells, used as controls, were negative.

We induced adipocyte differentiation also by adding dexamethasone, insulin, and IBMX to cultured cells, as specified above. Cells cultured in this adipogenic medium differenti-

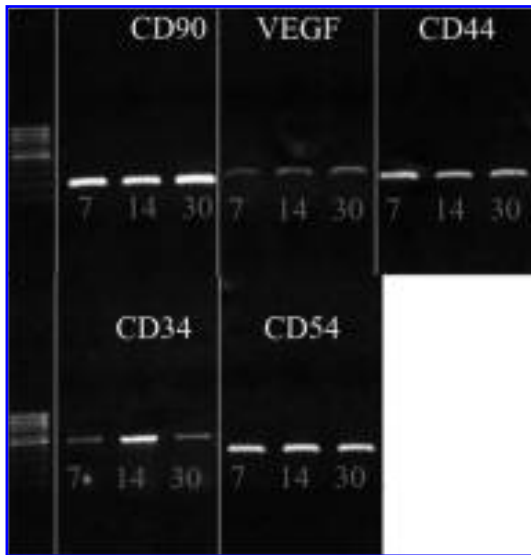


FIG. 4. Representative figure of reverse transcriptase polymerase chain reaction showing mRNA transcript expression of CD90, CD34, CD44, CD54, and vascular endothelial growth factor (VEGF) at 7, 14 and 30 days of culture.

ated into adipocytes, as assessed using specific staining for lipids. Diffuse adipocyte differentiation was obtained with this method. After 72 to 96 h of digestion, approximately 80% of cells differentiated into adipocytes containing a lipid content positive for immunohistochemistry staining. The adipogenic medium resulted in multivacuolar adipose cell formation in a shorter time and with higher quantity than with the medium of 20% FBS only.

In addition, we performed long-term cultures for 4 months in which cells cocultured with 10% fibroblasts formed aggregated centers at confluence and produced an extracellular matrix made of collagenic fibers (Fig. 6A). The tissue continued to grow, forming a layer of human univacuolar adipocytes. FACS sorting identified a mixed population of differentiated cells, including fibroblasts (10%), as assessed according to specific antibodies (anti-fibroblast growth factor), and univacuolar adipocytes. Analyses of the newly formed tissue using histological staining revealed that adipose cells surrounded a central area of fibrous connective fibers (collagenic fibers) (Fig. 6B). This extracellular matrix was strongly positive to markers usually present in adipose tissue, including type I collagen and fibronectin (Fig. 6C, D), and endothelial markers, including VEGF, CD31 (platelet-endothelial cell adhesion molecule-1) (Fig. 6E, F), and slightly positive for VEGFR-2. In addition, adiponectin and PPAR γ were expressed more than in human adult adipose tissue (Fig. 6G, H).

Discussion

In this study, we isolated and characterized stem and progenitor cells from adult adipose tissue taken from mammary (lipectomy) and abdominal (lipectomy and liposuction) regions. We did not observe significant differences between the two sites and demonstrated the ability of *ex vivo* expanded human adipose tissue-derived stem cells to form adipose tissue. The literature on stem cells has expanded tremendously during the past 5 years, but studies have increased

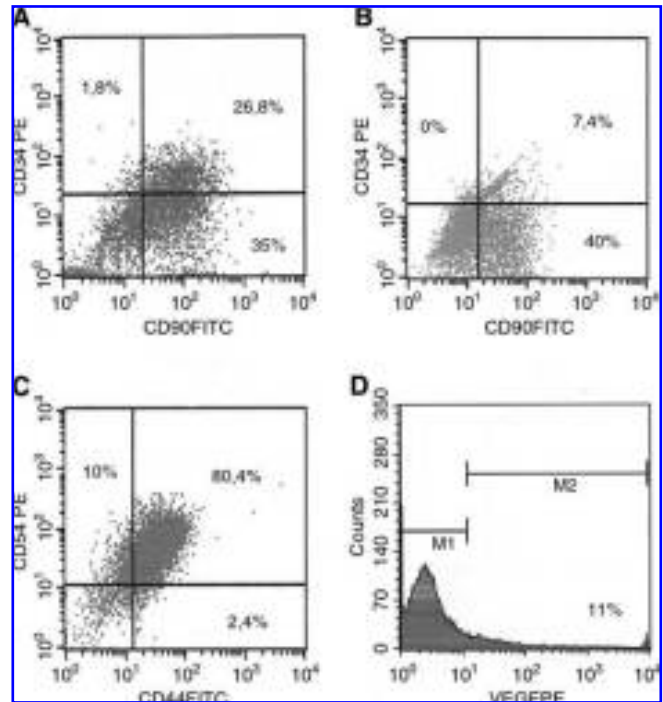


FIG. 5. (A) Representative fluorescence-activated cell sorting (FACS) of cells performed at day 15 of culture showing that CD34⁺/CD90⁺ cells are CD34⁺ only in 2% and CD90⁺ in 35% of cells. (B) Representative FACS of cells performed at day 30 of culture showing that the CD34⁺/CD90⁺ cell population has lost its positivity for CD34 but not for CD90. (C) Representative FACS of cells performed at day 30 of culture showing CD34⁺/CD90⁺ cell differentiation. These cells acquire an endothelial morphology expressing high levels of endothelial markers including CD44/CD54 (80%). (D) Representative FACS of cells performed at day 30 of culture showing CD34⁺/CD90⁺ cell differentiation. These cells acquire an endothelial morphology expressing high levels of endothelial markers including VEGF (20%).

more the understanding and theoretical capabilities of stem cells rather than their effective tissue production and regeneration capacities.

One of the main challenges in medicine is regeneration and repair of damaged tissues. The field of regenerative medicine is growing, thanks to the implementation of stem cell discoveries. Of great importance in this field is the use of human stem cells rather than rat, mice, or other animal cells, because they often differ, starting with their biology. Furthermore, it is necessary to obtain a completely formed tissue *in vitro* that is ready for use in human transplantation. In this way, a sort of tissue factory for banking and quick use in relationship to the patient's demand could be obtained. The aim of this study was to obtain an adipose tissue *in vitro* displaying the same characteristics of human adipose tissue, through the use of stem cells harvested from human fat, and suitable for transplantation and use or banking. To obtain this result, we designed three culture methods in an attempt to mimic the embryology and physiology of human adipose tissue.

Adipose tissue is a greatly diffused tissue that can be harvested with minimal morbidity from humans. During life, this tissue undergoes considerable changes in the number

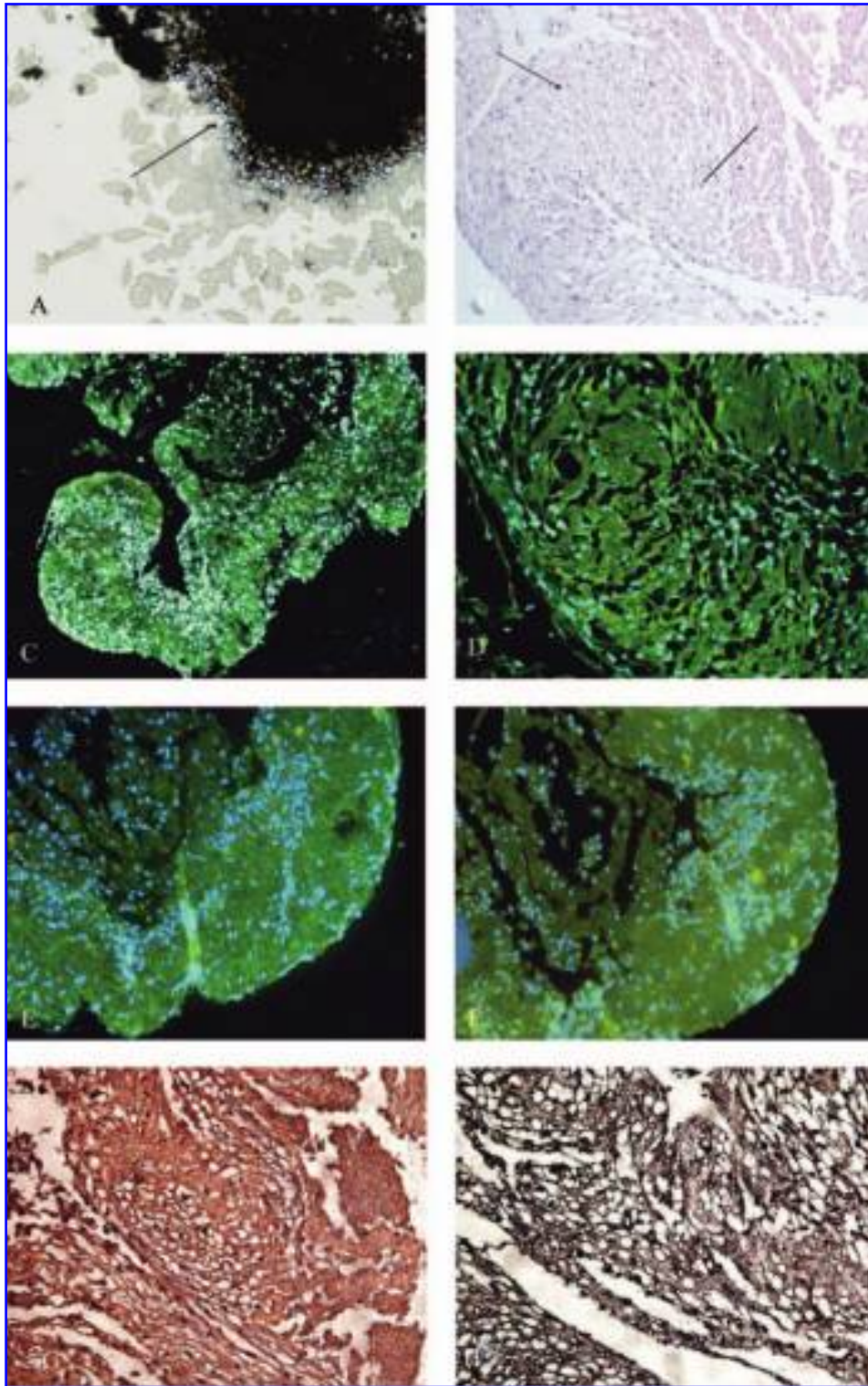


FIG. 6. (A) Differentiated cells that, after 4 months of culture, form aggregated centers (*arrow*) and produce an extracellular matrix (original magnification, $\times 200$). (B) Newly formed adipose tissue, stained with hematoxylin and eosin; adipose univacuolar cells (*arrow*) surround a central area made of fibrous connective (*thick arrow*) (original magnification, $\times 100$). Immunofluorescence images of the adipose tissue showing positivity for several specific antibodies for adipose matrix protein including: (C) type I collagen (original magnification, $\times 100$); (D) fibronectin (*arrow*) (original magnification, $\times 400$). Immunofluorescence images of the adipose tissue showing positivity for specific endothelial markers including: (E) vascular endothelial growth factor (*arrow*) (original magnification, $\times 200$); (E) CD31 (platelet-endothelial cell adhesion molecule-1) (*arrow*) (original magnification, $\times 200$). Immunofluorescence images of the adipose tissue showing positivity for adipose cells markers, including: (F) adiponectin (original magnification, $\times 200$) and (G) peroxisome proliferator-activated receptor gamma (original magnification, $\times 400$).

of adipocytes it contains; the generation of new adipocytes brings about this hyperplasia, which remodeling and expansion of the vasculature coordinates.²¹ This process is mediated through differentiation of stem or progenitor cells contained within the adipose tissue itself. Many researchers have described the presence of a large population of stem or progenitor cells within this tissue²² and have found that it contains a multipotent cell population with properties similar to MSCs.^{23,24}

In this study, we identified the presence of three cell populations: stromal stem cells (c-Kit⁺, CD34⁺, CD45⁻), mesenchymal stem or progenitor cells (CD90⁺, CD34⁺, CD29⁺, CD105⁺, CD44⁺, CD14⁻, CD45⁻), and endothelial progenitor cells (CD44⁺, CD54⁺, VEGF⁺ and Flk-1⁺). The CD34 marker is an antigen on the surface of hematopoietic progenitors,¹³ but it is well expressed on stromal stem cells, fibroblasts, and endothelial progenitors.⁷ The combination of CD34 with markers such as CD14 and CD45 (hematopoietic progenitors), CD117, CD90 and CD105 (MSCs), and CD31 or CD133 (endothelial progenitors) is a useful tool for the characterization of stem progenitor cells. In addition, the finding that these stem cells become CD34⁻ shows that they are different from circulating bone marrow-derived fibroblasts, which remain positive for this surface marker antigen.²⁵ Up to now, CD90 (Thy1) has never been used for hADASC detection, although it is a useful stem or progenitor cell marker. The exact property and activity of CD90 has not been fully elucidated. It has been speculated that it may exert a role in cell-to-cell and cell-to-matrix interactions.

The relative expendability of adipose tissue, the ease with which it can be obtained in large amounts with minimal risk, and the possibility of easily removing contaminating mature adipocytes lead to large yields. In addition, confluence is achieved quickly for hADASCs, similar to MSCs.²⁶ Studies on stem cells from adipose tissue have focused on two main directions: adipocyte differentiation²⁷ and the osteogenic potential of these cells.²⁸

Adipose stem cells are often difficult to distinguished and select from preadipocytes and the osteogenic lineage; although it is possible, other stem cell sources are much more affordable. In this regard, our research group has achieved interesting results in bone regeneration^{19,20} and bone engineering using dental pulp stem cells.^{29,30}

Therefore, we think that the use of adipose stem cells is mainly, although not only, devoted to the building of new adipose tissue.

Another interesting point is that ADASCs, like MSCs, are able to suppress a mixed lymphocyte reaction in a dose-dependent and time-dependent manner.³¹ In addition, it has been demonstrated that clonally derived, multipotent cells from adipose tissue are immunoprivileged *in vitro* or *in vivo*,³² suggesting that they, like MSCs, can be universal donor cells with the ability to be used in allogenic transplantation, reducing rejection risks.³³ This is of paramount importance because of its potentially widespread use; ADASCs contain the capability to functionally act as a vascular building block of adipose and other tissues. We have focused our attention in building a whole adipose tissue for quick use in transplantation. Although underappreciated, this is a valuable resource for biotechnologies.²³

Selected cells, under specific inductive stimuli, differentiate into multivacuolar adipocytes, as confirmed using immu-

nohistochemical staining and RT-PCR for adiponectin and PPAR- γ . The strong PPAR γ expression suggests that this gene has a predominant role during cell differentiation.^{34,35}

Adipose tissue formation was achieved by adding 20% FBS to cultures.^{19,20} FBS plays an essential role in differentiation and proliferation.³⁶

Our results are effectively in favor of its use. Although many studies have been carried out on adipose stem cells, no one has gained the result of obtaining an adult adipose tissue for future clinical applications. The majority of studies have been devoted to understanding the capability of these cells to differentiate into various cell types or to hypothesize their use in biotechnological or tissue engineering, although results were more hypothetical than realistic. The need for adipose tissue in reconstructive medicine, plastic lipofilling, and regenerative medicine increases daily.

In conclusion, our study has provided evidence that adipose tissue represents an abundant source of stem cells capable of forming, *in vitro*, an adipose tissue that could be the criterion standard for other studies, leading to its use in reconstructive surgery applications and tissue-based clinical therapies.

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Address reprint requests to:
 Prof. Gianpaolo Papaccio M.D., Ph.D.
 Department of Experimental Medicine
 Section of Histology and Embryology
 TESLab
 2nd University of Naples
 via L. Armanni 5
 80138 Naples, Italy

E-mail: gianpaolo.papaccio@unina2.it

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