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IFATS Collection: Identification of Hemangioblasts in the Adult Human Adipose Tissue

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Key Words. Hematopoietic stem cells • Hemangioblast • Adipose tissue

ABSTRACT

The stromal-vascular fraction (SVF) of human adipose tissue contains, among other cell types, mesenchymal stem cells and precursors of adipocyte and endothelial cells. Here we show that, in addition, the nonhematopoietic fraction of the SVF has hematopoietic activity, since all types of hematopoietic colony-forming units (CFUs) developed when cultured in methylcellulose-based medium. This hematopoietic activity was restricted to the $CD45^-CD105^+$ cell subset, well correlated with KDR^+ cell content, and increased after culture with a combination of early-acting hematopoietic cytokines. Most of the $CD45^-KDR^+CD105^+$ cells were nonadherent and did not express CD31, and this subset included both $CD34^-$ and $CD34^+$ cells. Moreover, these nonadherent cells migrated in response to KDR gradient, and when they were cultured in the presence of both hematopoietic and endothelial growth factors, a wave of CFUs was followed by a wave of mixed colonies comprising adherent elongated and nonadherent round hematopoietic cells. These mixed hematopoietic-endothelial (Hem-End) colonies were able to generate secondary Hem-End colonies and exhibited both hematopoietic and endothelial activity, as demonstrated by in vitro functional assays. These findings demonstrate for the first time the existence of primitive mesodermal progenitors within the SVF of human adipose tissue that exhibit in vitro hematopoietic and hemangioblastic activities, susceptible to being used in cell therapy and basic cell research. STEM CELLS *2008;26:2696–2704*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

White human adipose tissue is composed of adipocytes and a heterogeneous cell population surrounding them that is termed the stromal-vascular fraction (SVF). This fraction has emerged as a source of stromal cells for regenerative medicine, as they exhibit immunosuppressive properties [1] and a capacity for multilineage differentiation comparable to that of mesenchymal stem cells from bone marrow [2, 3]. Adipose tissue is an attractive tissue because of its enormous capacity for expansion and remodeling during the adult life that results from adipocyte hypertrophy, the recruitment of cell precursors from the SVF, and the development of a capillary network [4, 5]. Whereas adipocyte differentiation has been extensively studied, adipose tissue angiogenesis is poorly understood, in spite of the facts that fat cell development in the embryo is spatially and temporally related to capillary development and that vascularization is critical to the physiological function of adipose tissue [4]. SVF is highly enriched in $CD34^+$ cells, from which the $CD34^+CD31^+$ cell subset corresponds to endothelial cells from the microvasculature, and the $CD34^+CD31^-$ cell fraction contains adipocyte and endothelial progenitor cells [6]. More recently, adipogenesis has been reported to be restricted to $CD34^+CD31^-$ cells negative for CD105 [7]. In human, the presence of CD34 and CD105 is considered to be restricted to hematopoietic and endothelial lineages, although CD105 is also expressed in mesenchymal stem cells. In addition, during embryonic stem cell differentiation, CD105 is expressed on KDR^+ precursors with hematopoietic potential [8]. Considering that both endothelial and hematopoietic lineages arise in mesoderm from the bipotent progenitor, the hemangioblast, identified only in hematopoietic tissues and cord blood [9, 10], it is tempting to speculate that this progenitor could be preserved in adipose tissue from adult human, thus contributing to the maintenance of such a pool of endothelial progenitor cells. If this assumption is true, then hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) might also be present in adipose tissue. Supporting this hypothesis, the SVF from mouse adipose tissue has been shown to reconstitute hematopoiesis in lethally irradiated animals [11] and to support the complete differentiation of HPCs but not their self-renewal [12]. However, it remains to be determined whether the in vivo hematopoietic activity was hematopoietic in origin or not, as no depletion of $CD45^+$ cells was performed. In this study, we analyzed the $CD45^-$ cell fraction contained in the SVF from human adipose

Author contributions: M.-D.M.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; F.C.-U. and A.E.: collection of data, data analysis and interpretation, final approval of manuscript; V.M.: collection of data; S.M.: provision of study patients, collection of data.

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tissue and identified a KDR^+ cell subset that gives rise to hematopoietic colony-forming units (CFUs) in clonogenic assays and also to mixed colonies of both hematopoietic and endothelial cells. These findings demonstrate for the first time the existence of cells with hemangioblastic activity in the nonhematopoietic compartment of a mesodermal tissue.

MATERIALS AND METHODS

Cell Purification

Human adipose tissue samples were obtained from abdominal lipectomies or lipoaspiration procedures from consenting healthy donors, ages 30–40 years. Stromal cells were obtained as described [7]. Briefly, samples were digested in phosphate-buffered saline (PBS) containing 2 mg/ml collagenase type I (Gibco, Paisley, U.K., http://www.invitrogen.com) and 2% bovine serum albumin (BSA) at 37°C under constant shaking for 60–95 minutes, filtered through 100-μm filters to remove nondigested tissue, and centrifuged to discard adipocytes. Cells remaining after lysing of contaminating erythrocytes with ammonium chloride solution constituted the SVF.

Cells from the SVF were magnetically labeled with CD45 microbeads (magnetic cell sorting [MACS]; Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com), and CD45-negative cells were isolated. When indicated, $CD45^-$ cells were then incubated with CD105 microbeads (Miltenyi Biotec) and collected both populations $CD105^+$ and $CD105^-$ cells. Immunomagnetic cell separation was carried out using the MACS system (Miltenyi Biotec). The purity of selected cell subsets was always >95%.

Cell Culture

Hematopoietic Serum-Free Liquid Culture. Immunomagnetically isolated cells were cultured in 24-well plates (4×10^4 to 10×10^4 cells per milliliter) in serum-free medium [13] containing the following human recombinant cytokines: 50 ng/ml stem cell factor (SCF), 50 ng/ml flt3-ligand, and 10 ng/ml thrombopoietin, plus interleukin-6 (IL-6), IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF) at 20 ng/ml each (R&D Systems Inc., Minneapolis, http://www.rndsystems.com). The cultures were incubated at 37°C in a humidified atmosphere for 7 days, and cytokines were replaced every 3 days. After 7 days of culture, cells were counted, characterized by flow cytometry, and seeded for CFU generation.

Clonogenic Culture. HPCs were determined as CFUs in semisolid medium. Cells were cultured in the methylcellulose-based medium Methocult GF H4435 (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) containing human recombinant cytokines SCF, GM-CSF, IL-3, IL-6, granulocyte colony-stimulating factor, and EPO. Cultures were made in duplicate in 35-mm Petri dishes or in 24-well plates and incubated at 37°C in a humidified atmosphere. Hematopoietic CFUs were identified by morphologic criteria, using an inverted microscope (CK40; Olympus, Tokyo, http://www.olympus-global.com), as colony-forming unit-granulocyte, macrophage (CFU-GM); colony-forming unit-erythroid (CFU-E); and colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM).

For the generation of mixed hematopoietic-endothelial (Hem-End) colonies, hematopoietic-endothelial growth medium (HEGM) consisting of hematopoietic clonogenic medium supplemented with 2 ng/ml insulin-like growth factor, 5 ng/ml epidermal growth factor, 50 ng/ml basic fibroblast growth factor (bFGF), 50 ng/ml vascular endothelial growth factor (VEGF), and 10 U/ml heparin (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) was used. Moreover, wells were coated with 30 μg/ml collagen and 10 μg/ml fibronectin (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com).

Endothelial Cell Culture. Adherent cells from mixed Hem-End colonies were dissociated with trypsin-EDTA (Gibco) and seeded onto fibronectin-coated well plates in EGM-2MV medium (Cambrex Bio Science, Verviers, Belgium, http://www.cambrex.com). Subconfluent cells were passaged following trypsinization.

CFUs and adherent-derived endothelial cells were visualized and recorded using a Leica DM IRB or a Leica DM 6000 B microscope (Leica Microsystems, Wetzlar, Germany) for May-Grünwald-Giemsa cytospins. Images were taken with a digital camera (Leica DFC480) using either a ×10 (numeric aperture, 0.25) or a ×20 (numeric aperture, 0.40) objective lens or with a Leica DFC350 FX digital camera using a ×63 (numeric aperture, 1.60). Images were analyzed using the Leica IM 500 4.0 image processing program (Leica Microsystems).

Cell Analysis

Flow Cytometry. For fluorescence-activated cell sorting (FACS) analysis, six-color cytometric analysis was performed using standard procedures on a FACSCanto (BD Biosciences) using the FACSDiva software (BD Biosciences). Cells were stained in PBS containing 1 mM EDTA and 1% BSA with either isotype controls or antigen-specific antibodies for 30 minutes at 4°C. The antibodies used were CD45, CD34, CD31, CD90, CXCR4 (CD184), CD14, GFA (CD235a), CD43, CD13, CD117 (BD Biosciences), CD133/2 (Miltenyi Biotec), KDR, Tie2 (R&D Systems), and CD105 (eBioscience, San Diego, CA) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), PE-cyanin7 (PE-Cy7), or APC-cyanin7 (APC-Cy7). 7-Aminoactinomycin D (Sigma-Aldrich) was used for discarding nonviable cells in the analysis. As controls, the matched labeled isotypes were used.

Immunocytochemistry. Cells cultured on fibronectin- or fibronectin/collagen-coated wells containing coverslips were washed in 1× PBS, fixed with cold methanol for 10 minutes at −20°C, and incubated for 45 minutes at room temperature in 1× PBS containing 0.5% Tween, 0.5% BSA (Sigma-Aldrich), and 10% fetal bovine serum (FBS) (Gibco). After washing, samples were incubated overnight at 4°C with primary antibody in 1× PBS containing 2% BSA and then washed five times with in 1× PBS supplemented with 2% BSA and 0.5% Tween and incubated with corresponding secondary antibodies for 1 hour in the dark at 37°C. The coverslips were washed five times, and then cell nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich). Primary antibodies used were anti-human von Willebrand factor (VWF), anti-human CD45 (Sigma-Aldrich), and anti-human CD31 (DakoCytomation, Glostrup, Denmark, http://www.dakocytomation.com). Secondary antibodies used were anti-mouse IgG labeled with Cy3 (Jackson Immunoresearch Laboratories, Newmarket, U.K., http://www.jacksonimmuno.com) or with FITC (Sigma-Aldrich), and anti-rabbit IgG labeled with tetramethyl rhodamine isothiocyanate (Sigma-Aldrich) or with Cy2 (Jackson Immunoresearch Laboratories). The antibodies were used at concentrations recommended by the manufacturers.

Fluorescence was visualized using a Leica DM IRB microscope, and images were recorded using a digital camera (Leica 480) and analyzed by using the Leica IM 500 4.0 image processing program. Images were taken using a ×40 (numeric aperture, 0.60) objective lens. In some cases, images were acquired with a Leica TCS SP2 AOBS (Leica Microsystems) inverted laser scanning confocal microscope using a ×63 Plan-Apochromat-Lambda Blue (numeric aperture, 1.4) oil objective.

Endothelial Cell Assay. For in vitro tube formation, Matrigel (BD Biosciences) was added to fibronectin-coated wells of a 96-well plate in a volume of 50 μl. After the Matrigel solidified, 5×10^3 adherent-derived endothelial cells were added in 100 μl of endothelial medium. The cells were incubated at 37°C with humidified atmosphere. Capillary-like structures were visualized and recorded after 16 hours of incubation using a Leica DM IRB microscope, and images were taken with a ×20 (numeric aperture, 0.40) objective lens. For acetylated low-density lipoprotein (Ac-LDL) uptake, adherent-derived endothelial cells seeded onto fibronectin-coated well-plate containing coverslips or adherent cells from Hem-End colonies were cultured in endothelial medium for 2 days and then incubated for 4 hours or overnight, respectively, with 5 μg Dil Ac-LDL (Molecular Probes, Paisley, U.K., http://probes.invitrogen.com). Noninternalized Dil Ac-LDL was washed with 1× PBS. Cells were fixed with 4% paraformaldehyde, 0.4 M sucrose in 1× PBS for 30 minutes at room temperature and then counterstained to detect VWF expression. Cells were visualized and

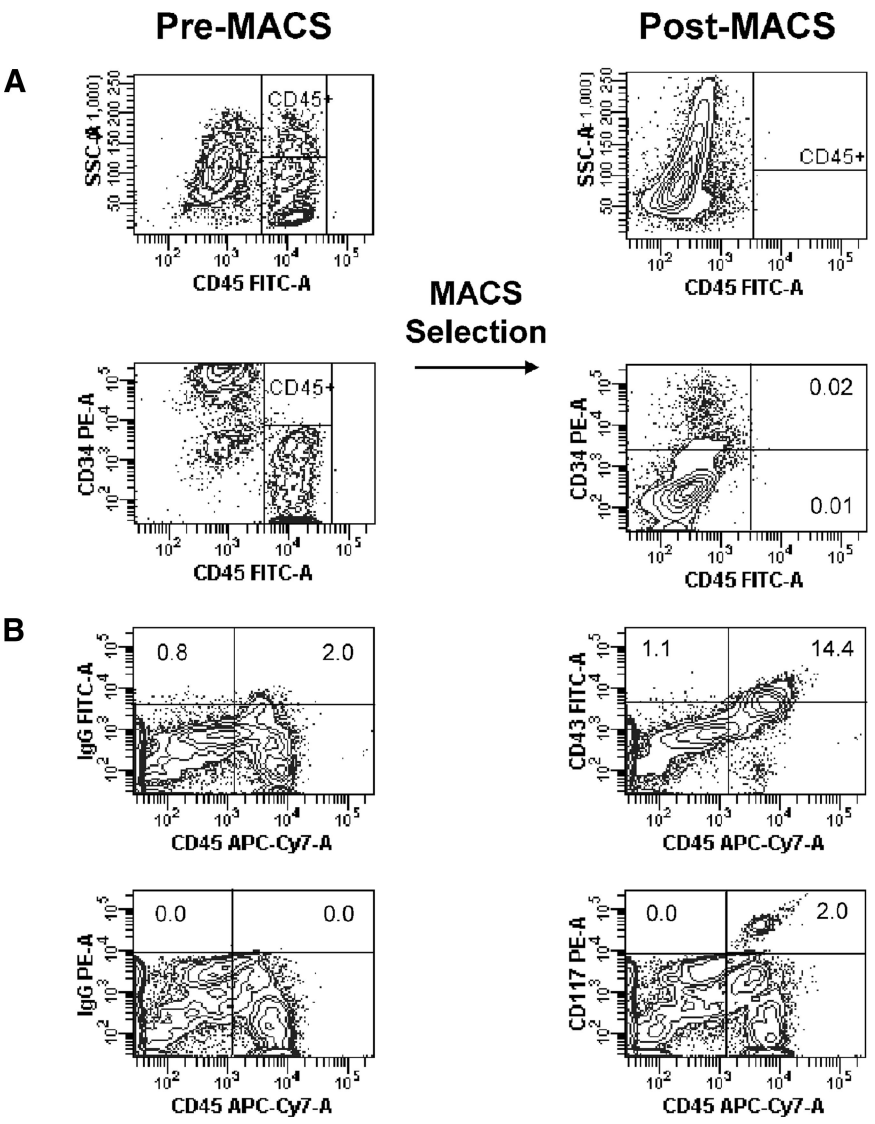

Figure 1. Immunomagnetically isolated CD45$^-$ cells from the stromal-vascular fraction (SVF) do not express markers of hematopoietic progenitor cells. **(A):** Fluorescence-activated cell sorting analysis of cells from the SVF before and after isolation of CD45-negative cells by immunomagnetic methods. **(B):** The expression of CD43 and CD117 antigens were restricted to the hematopoietic CD45$^+$ cell subset. A representative cytometric analysis is shown. Abbreviations: A, area scaling detector; FITC, fluorescein isothiocyanate; MACS, magnetic cell sorting; PE, phycoerythrin.

recorded with a Leica DM IRB microscope, and images were taken with a ×40 (numeric aperture, 0.60) objective lens.

Migration Assay. Freshly isolated CD45$^-$ cells were seeded on Petri dishes in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 5% FBS, 0.5% BSA, 1 ng/ml transforming growth factor-β, 25 ng/ml VEGF, 25 ng/ml bFGF, and 5 U/ml heparin (Sigma-Aldrich). After overnight incubation, nonadherent and adherent cells were collected and analyzed by flow cytometry. To study migration of CD45$^-$KDR$^+$ cells, nonadherent CD45$^-$ cells at 5×10^5 cells per well in 1.5 ml of migration medium (DMEM containing 0.5% BSA) were placed on the insert of an 8-μm pore size Costar transwell six-well plate (Corning Enterprises, Corning, NY, http://www.corning.com). Immediately, 100 ng/ml $VEGF_{165}$ (Sigma-Aldrich) was added to the lower chamber, and after 3 hours of incubation, the number of CD45$^-$KDR$^+$ cells migrated was quantified by flow cytometry.

RESULTS

CD45$^-$ Cells from SVF Generate Hematopoietic CFUs

To determine whether nonhematopoietic compartment of SVF could display hematopoietic activity, CD45$^-$ cells comprising approximately 75% of total cells were selected by immunomagnetic methods (Fig. 1A). In the first series of experiments, freshly isolated CD45$^-$ cells were seeded in Petri dishes at a density of 80×10^3 to 500×10^3 cells per 3 ml in clonogenic medium to evaluate their ability of generating hematopoietic CFUs. As expected, a stromal cell monolayer developed rapidly, but after 10–12 days of culture, CFUs emerged, and a frequency of one CFU per 140,000 CD45$^-$ cells was estimated by Poisson statistics, thus demonstrating the existence of hemogenic precursors in the SVF.

Hematopoietic Activity Is Restricted to the CD45$^-$KDR$^+$CD105$^+$ Cell Subset

To identify the cell subset responsible for hematopoietic potential, we evaluated expression of some primitive hematoendothelial cell markers, some of them also expressed in mesenchymal stem cells. As shown in Figure 1B, the CD45-negative cell fraction did not contain hematopoietic progenitor cells, as no expression of CD43 or CD117 was observed. Within the CD45$^-$ subset, 90% ± 7%, 27% ± 8%, and 3.7% ± 1.2% of cells were CD34$^+$, CD31$^+$, and CD133$^+$, respectively. In addition, CD90 was uniformly expressed by 84% ± 8% of cells, indicating their mesodermal origin, whereas KDR, Tie2, and CD105 were ex-

pressed by only 4.6% ± 3.3%, 9.4% ± 4.9%, and 13.3% ± 6.5% of cells, respectively.

Vascular endothelial growth factor receptor-2 (KDR) is expressed by early hematopoietic precursors [14]. Consistent with this notion, a direct relationship between the number of CFUs generated and the number of KDR^+ plated cells, with a slope approaching 1, was obtained (Fig. 2A). Moreover, a frequency of 2.4 CFUs per 1×10^3 KDR^+ cells could be established. Among CFUs generated, 42.5% corresponded to CFU-E, 47.5% to CFU-GM, and 10.0% to CFU-GEMM (Fig. 2B). No correlation between CFU number and the other analyzed antigens was observed. To further assess whether the clonogenic progenitor capacity of $CD45^-$ cells could be induced, $CD45^-$ cells were cultured in hematopoietic serum-free liquid medium previously shown to support $CD34^+$ HPC. After 7 days of culture, no cell expansion was observed, and in fact only 81% ± 26% of cells were recovered, but total KDR^+ cells were 4 ± 3-fold increased ($n = 11$). These culture conditions allowed the cells to generate vigorous CFUs starting at 6–7 days of hematopoietic clonogenic culture, beginning with the detection of small clusters and acquiring, in most cases, the morphology of high proliferative potential colony-forming cells (Fig. 2C) that, when replated, gave rise to secondary CFUs, indicating the presence of multipotent progenitor cells. Again, the numbers of both CFUs and KDR^+ plated cells were well correlated (Fig. 2A). Importantly, ex vivo cultured cells exhibited a higher clonogenic capacity, reaching a value of four CFUs per 1×10^3 KDR^+ cells, which indicates that early-acting cytokines are required for $CD45^-KDR^+$ cell proliferation and stimulation. To define KDR^+ precursors with hematopoietic potential, we characterized the KDR^+ subset on the basis of the surface expression of antigens associated with early progenitors. Cytometric analysis revealed that $CD45^-KDR^+$ cells were small and agranular cells, and they were $CD105^+$, with low or negative expression of CD34 and CD31 (Fig. 2D). In addition, they were $Tie2^+$ and had a variable proportion of cells positive for CXCR4 (data not shown). Therefore, we took advantage of their positivity for CD105, and $CD45^-$ isolated cells were fractioned into $CD105^+$ and $CD105^-$ populations and assayed for CFUs, directly or after 7 days of culture in serum-free medium as described above. Only the $CD105^+$ cell subset accounted for the hematopoietic potential. Moreover, frequencies of CFUs per KDR^+ cells were similar to those previously obtained using isolated $CD45^-$ cells.

$CD45^-KDR^+$ Cells Exhibit Hemangioblastic Properties

KDR is a molecular determinant of the hemangioblast in embryonic stem cell differentiation [15, 16]. Therefore, the presence of hemogenic precursors in a nonhematopoietic tissue joined to the intimate association between fat cell and capillary development in embryo led us to investigate the hemangioblastic potential of $CD45^-KDR^+$ cells. To address this issue, freshly isolated $CD45^-$ cells were incubated overnight in the presence of growth factors critical for the maintenance of a stem cell reserve [14, 17, 18]. This incubation resulted in approximately 85% recovery of KDR^+ cells. This lack of adherence allowed us to test their capacity to respond to chemotactic stimulus by a simple transmigration assay. In response to VEGF, 65% ± 7% of KDR^+ cells migrated to the lower chamber. These migrated cells, with a high nucleus-to-cytoplasm ratio (Fig. 3A), were assayed for the ability to form mixed Hem-End colonies and also CFUs.

In HEGM, a wave of hematopoiesis emerged first, and all types of CFUs, appearing at 9–12 days of culture, were developed with a frequency of 10.3 ± 1.6 CFUs per 10×10^3 KDR^+ plated cells ($n = 4$). Approximately 1 week after the CFU

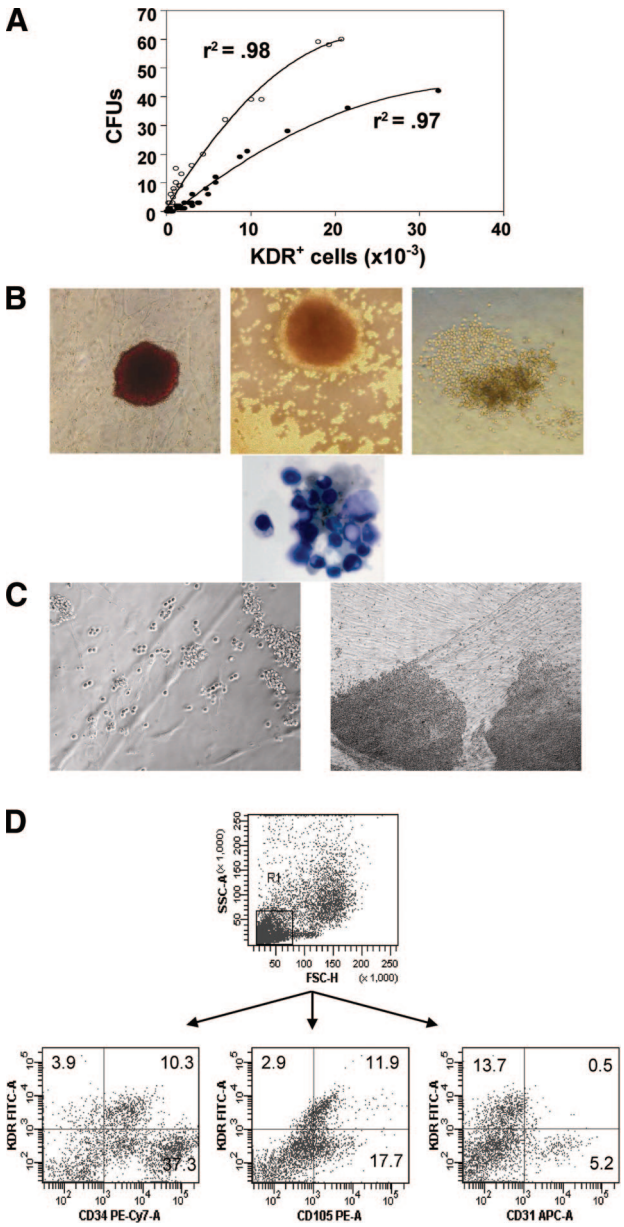

Figure 2. Hematopoietic activity of $CD45^-$ cells from the stromal-vascular fraction is dependent on KDR^+ cell content. **(A):** Freshly isolated $CD45^-$ cells (●) or $CD45^-$ cells previously cultured in serum-free hematopoietic medium (○), containing 500–35,000 KDR^+ cells or 200–20,000 KDR^+ cells, respectively, were cultured in methylcellulose-based medium for hematopoietic progenitor cell determination, and CFUs were scored by morphological criteria. Values of total CFUs obtained per number of KDR^+ plated cells are shown. $n = 12$ samples, in triplicate. **(B):** Morphological aspect of CFUs derived from $CD45^-$ isolated cells, at 16 days of culture: CFU-E, original magnification, ×10 (top left); CFU-GEMM, original magnification, ×10 (top middle); CFU-GM, original magnification, ×20 (top right); Giemsa staining of hematopoietic CFU-GEMM, original magnification, ×63 (bottom). **(C):** Morphological aspect of hematopoietic cells derived from $CD45^-$ cells, previously cultured in hematopoietic serum-free medium, at 8 days (left) or 20 days (right) of clonogenic culture. Original magnification, ×20 (left) and ×10 (right). **(D):** Immunophenotypic characterization of $CD45^-KDR^+$ cells. Freshly isolated $CD45^-$ cells were stained with the following monoclonal antibodies: KDR-FITC, CD105-PE, CD31-APC, and CD34-PE-Cy7; they were then analyzed by flow cytometry. R1 (55% of total cells) corresponds to cells with low forward and side scatter. Percentages of KDR^+ cells gated on R1 are given. Abbreviations: A, area scaling detector; APC, allophycocyanin; CFU, colony-forming unit; FITC, fluorescein isothiocyanate; FSC, forward scatter; H, height; PE, phycoerythrin; SSC, side scatter.

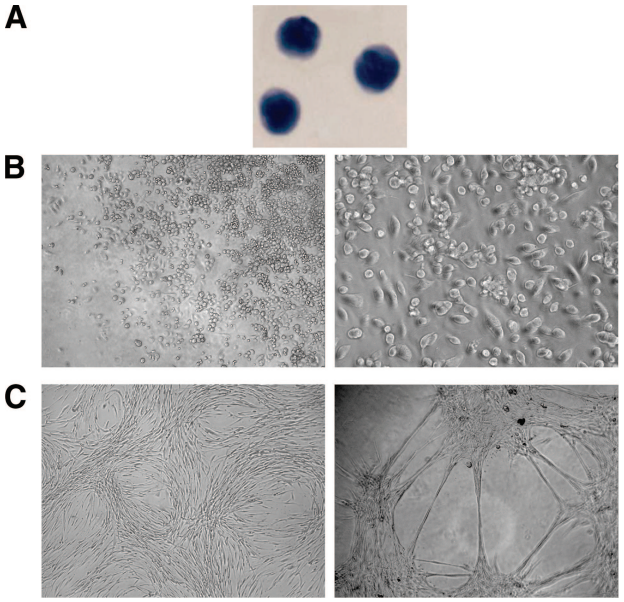

Figure 3. $CD45^-KDR^+$ cells in culture generate mixed hematopoietic-endothelial colonies that give rise to endothelial cells. **(A):** May-Grünwald-Giemsa staining of $CD45^-KDR^+$ migrated cells; original magnification, ×63. **(B):** Phase-contrast images of mixed hematopoietic-endothelial (Hem-End) colonies generated by $CD45^-KDR^+$ migrated cells in semisolid medium containing hematopoietic and endothelial growth factors. Images of a mixed colony at 16 days of culture (original magnification, ×10 [left]) and at 22 days of culture (original magnification, ×20 [right]) are shown. **(C):** Adherent cells from Hem-End colonies when grown in endothelial medium gave rise to endothelial cells (left) that were able to form capillary-like structures when seeded in Matrigel (right); original magnification, ×10.

appearance, mixed colonies comprising adherent elongated cells and nonadherent round hematopoietic cells emerged (Fig. 3B), rapidly expanding for 10–15 days and containing 3×10^5 to 9×10^5 cells ($n = 6$). A frequency of 3.1 ± 0.7 Hem-End colonies per 10×10^3 KDR^+ cells was estimated ($n = 4$). Surprisingly, hematopoietic clonogenic medium did not support CFU generation, which indicates that growth factors present in HEGM are necessary to support hematopoiesis. Moreover, as no stromal cells were grown in both culture conditions, the lower clonogenic efficiency obtained could be due to the need for other factors released by these cooperating cells.

We used two strategies to determine the bipotential capacity of Hem-End colonies. In the first strategy, we handpicked nonadherent cells and analyzed them by flow cytometry. Hematopoietic colonies were multilineage, as $CD45^+$ and $CD45^-$ were generated. Moreover, these colonies were essentially composed of lineage-restricted hematopoietic precursors. Therefore, primitive erythrocytes, as demonstrated by the presence of nucleated cells positive for GlyA, were observed. In addition, among $CD14^+$ generated cells, a similar proportion of $CD14^+CD34^-$ and $CD14^+CD34^+$ cells, previously identified as dendritic cell precursors [19], was observed (Fig. 4A). Moreover, when these colonies were replated in hematopoietic clonogenic medium, they formed secondary CFUs, probably because of the high percentage of $CD45^-CD34^+$ hematopoietic precursor cells within the cell colony.

The remaining adherent cells were trypsinized and then analyzed by flow cytometry, tested for endothelial cell generation, or directly immunostained. Interestingly, FACS analysis of single-cell suspensions revealed the presence of $KDR^+CD105^+CD34^-$ primitive precursors and $CD34^-CD133^+$ cells whose phenotype corresponds to that of HSCs [20] (Fig. 4B). These adherent cells comprised approximately 10% of $CD45^+$ cells, probably corresponding to contaminating monocytes/macrophages. When adherent cells were processed for the generation of endothelial cells or for immunocytochemistry, they were intensively washed to avoid hematopoietic contamination. As can be seen in Figure 3C, adherent cells growing in endothelial medium gave rise to endothelial cells that formed capillary-like structures when seeded in Matrigel. Furthermore, immunostaining analysis of adherent cells from Hem-End colonies demonstrated the presence of cells coexpressing CD45, CD31, and VWF, with a low capacity to uptake Ac-LDL (Fig. 5A), that became in $CD31^+VWF^+$ endothelial cells with a high ability to uptake Ac-LDL (Fig. 5B). Immunocytochemical and functional properties of endothelial cells generated in culture were preserved for at least five serial passages.

In the second approach, individual Hem-End colonies were tested for secondary Hem-End colony formation. As soon as 6–7 days after replating in HEGM, both secondary Hem-End colonies and CFUs appeared. When nonadherent cells from Hem-End colonies were seeded for CFU generation, multilineage hematopoietic colonies appeared, containing immature and mature myelomonocytic cells. In fact, unlike cells from primary Hem-End colonies, approximately 70% of $CD45^+$ cells corresponded to $CD14^+$ monocytes positive for CD13, CD90, and CD105 and also for CD31 and Tie2 (Fig. 6). However, among $CD45^-$ cells, approximately 40% of cells still had an immature phenotype, as they were $CD13^+CD105^+CD34^-$. Moreover, when adherent cells were incubated in endothelial medium, endothelial cells were generated that exhibited the same immunocytochemical and functional properties as those exhibited by cells derived from a primary Hem-End colony.

Finally, to determine whether Hem-End colonies originated from a common bipotent progenitor cell, we performed experiments at limiting dilution containing 2,000, 1,000, 500, 250, 125, 62.5, and 32 KDR^+ migrated cells per well in HEGM and plated them in 24-well plates. In addition, 1 week after plating, cells corresponding to two wells containing 2,000 KDR^+ cells and to one well with 1,000 KDR^+ cells were replated in three 48-well plates (each well in one plate). As expected, colony development was cell density-dependent, with poor development at low cell numbers. Only four mixed Hem-End colonies developed from a total of 26,700 plated KDR^+ cells, three colonies emerging from initially plated wells containing 2,000 and 1,000 KDR^+ cells and one from the 2,000 KDR^+ serially replated cells. Altogether, the results obtained indicate that the in vitro Hem-End colonies generated from $CD45^-KDR^+$ cells exhibit functional properties of the bipotent precursor, the hemangioblast. However, one must be cautious given the low frequency of mixed colony development and the difficulty of obtaining them from single KDR^+ cells.

DISCUSSION

HSCs have been studied extensively both in vitro and in vivo, but the development of early stages of hematopoiesis has recently been possible due to the advances in embryonic stem cell differentiation model, allowing the generation and expansion of the hemangioblast, the common ancestor of hematopoietic and endothelial lineages. Although the identification of the hemangioblast is very elusive in the human adult, several groups have reported the existence of $CD34^+$ cells [9, 21] in cord blood and $CD133^+$ cells from mobilized peripheral blood, hematopoietic or endothelial in origin [22], that exhibit hemangioblastic properties.

In the present study, we report that the nonhematopoietic fraction from the SVF contains a rare KDR^+ cell subset en-

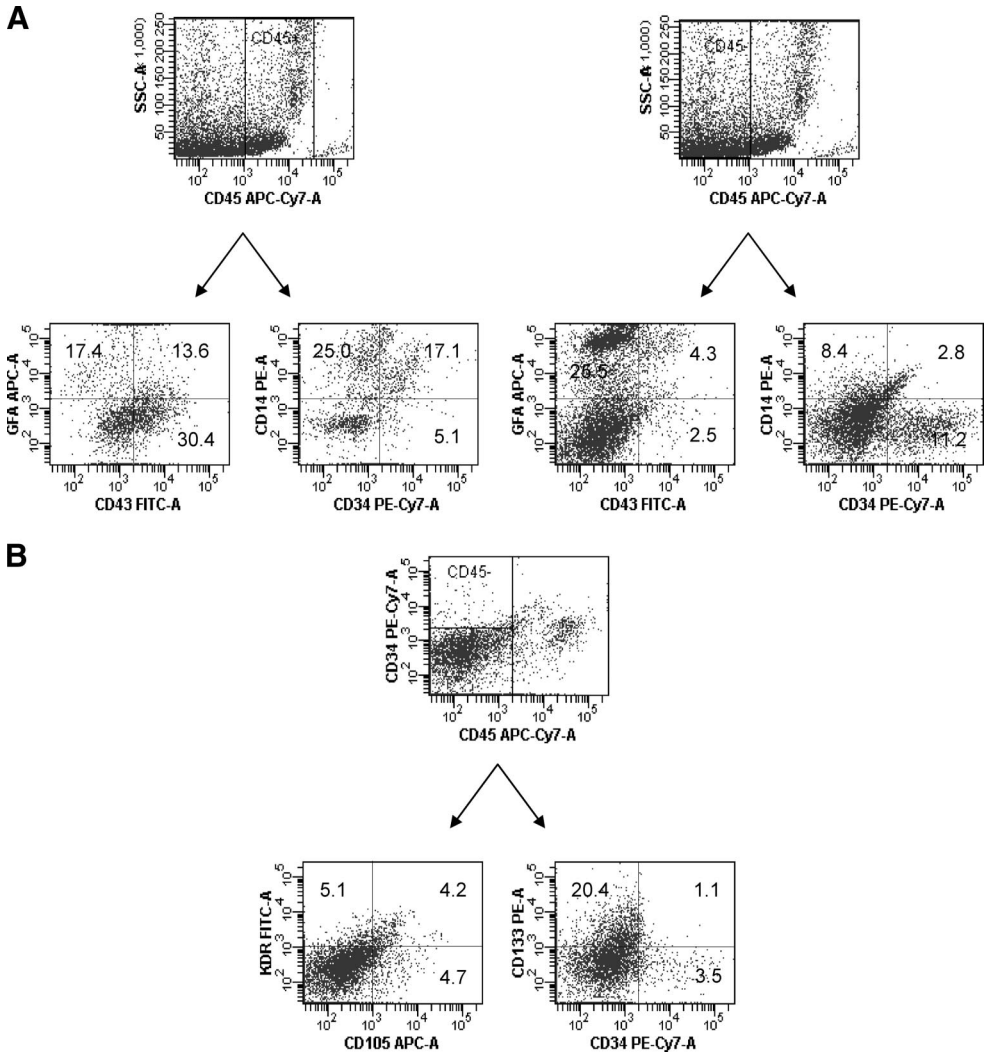

Figure 4. Fluorescence-activated cell sorting (FACS) analysis of a primary mixed hematopoietic-endothelial colony. **(A):** Nonadherent cells were hand-picked and stained with the selected conjugated monoclonal antibodies. Six-color cytometric analysis was performed using standard procedures. Cells gated on the basis of their positivity for CD45 were analyzed separately. $CD45^+$ and $CD45^-$ regions represent 20% and 80% of total cells, respectively. **(B):** The remaining adherent cells were trypsinized and stained for FACS analysis. Cells gated on $CD45^-$ region (90% of total cells) were analyzed. Numbers in quadrants represent the percentage of cells within the indicated gates. Abbreviations: APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

riched in hemogenic precursors and hemangioblasts. Considering that hematopoietic activity was restricted to $CD105^+$ cells and that KDR^+ cells did not express CD31, it can be assumed that hematopoietic CFUs developed from KDR^+CD31^- $CD105^+$ cell fraction. This result is apparently in contrast to the lack of hematopoietic activity of the selected $CD34^+CD31^-$ $CD105^+$ cell fraction from human SVF that was previously reported [7]. There are at least two possible explanations for this discrepancy: first, very few $CD34^+CD31^-$ cells were seeded in clonogenic assays, given the frequency of CFUs in KDR^+ cells; and second, CFU generation was dependent on the presence of endothelial growth factors, among other things, as $CD45^-$ KDR^+ migrated cells, unlike unfractionated $CD45^-$ cells or $CD45^-CD105^+$ cells, were not able to induce CFUs development. In addition, it is important to note that $CD31^-CD105^+$ cells were selected from $CD34^+$ isolated cells [7], and as demonstrated by FACS analysis, the $CD45^-KDR^+$ cell subset includes both $CD34^+$ and $CD34^-$ cells.

Given that a wave of hematopoietic clonogenic progenitors preceded that of the Hem-End colony formation, it can be assumed that $CD45^-KDR^+CD105^+CD31^-$ cells include two different cell precursors. Embryonic stem cell differentiation models have provided information to elucidate the origins of hematopoiesis, and so it has been demonstrated that both murine and human blast colonies with hematopoietic and vascular potential developed from KDR^+ cells [14–16, 23] prior to the expression of CD31 and CD34 and the onset of primitive erythropoiesis [16, 23], although peripheral blood $CD34^+CD133^+$ cells with hemangioblastic activity did not express KDR [22], and in a more recent study, Lu et al. [24] described that blast cells derived from human embryonic stem cells do not yet express KDR. On the other hand, cytometric analysis demonstrated that CD34 emergence was strictly correlated with the appearance of the first CFU-erythroid and that multilineage CFUs were generated later as $CD34^+CD45^-$ KDR^+CD105^+ [25]. Our results demonstrated that both $CD34^-$ cells and $CD34^+$ cells with a different intensity in CD34 expression were present within $CD45^-KDR^+$ cell population.

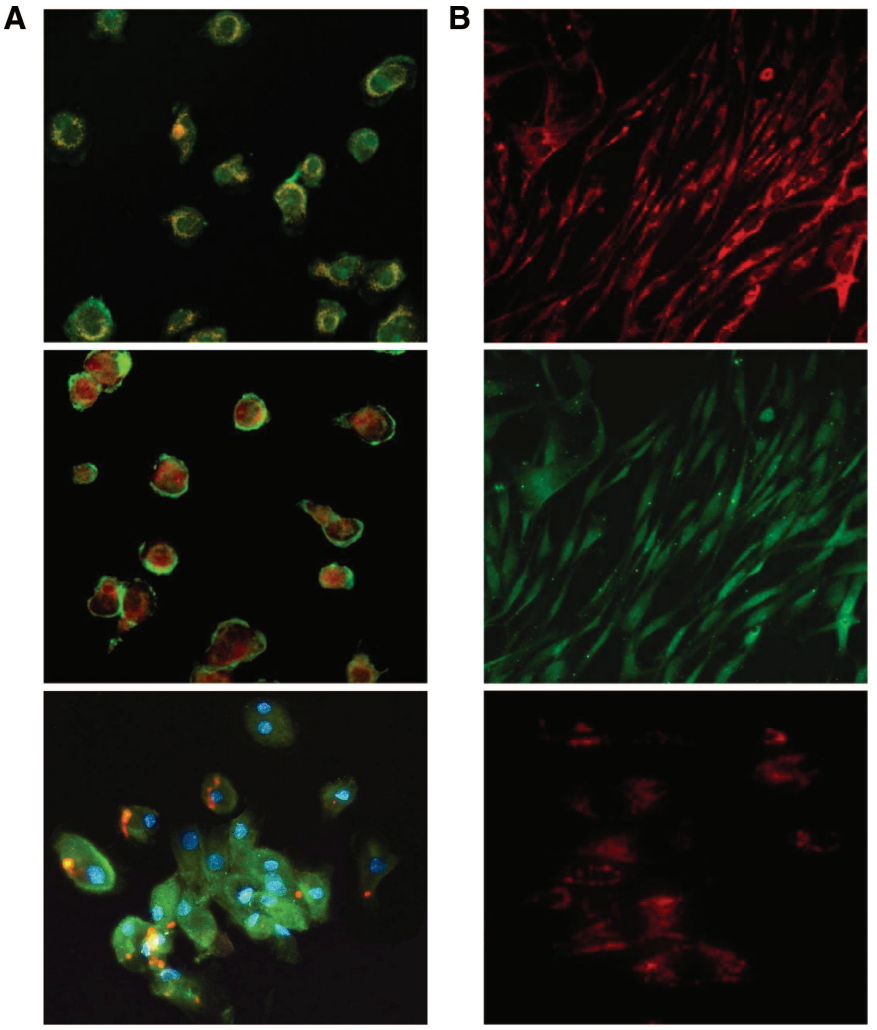

Figure 5. Immunocytochemistry characterization of adherent cells from hematopoietic-endothelial (Hem-End) colonies and adherent-derived endothelial cells. **(A):** Immunostaining and acetylated low-density lipoprotein (Ac-LDL) uptake of adherent cells generated from a single primary mixed Hem-End colony. Top panel, expression of CD31 (red) and CD45 (green). Middle panel, expression of von Willebrand factor (VWF) (red) and CD45 (green). Bottom panel, cells showing their ability to uptake Ac-LDL (red) and then immunostained against CD45 (green), and finally nuclei visualized with 4′,6-diamidino-2-phenylindole (blue). Top and middle fluorescence photographs were taken with an inverted laser scanning confocal microscope. Original magnifications, ×63 (top and middle panels) and ×40 (bottom panel). **(B):** Immunostaining and Ac-LDL uptake of endothelial cells generated from adherent cells from mixed Hem-End colonies. Expression of CD31 is shown in red (top), VWF in green (middle), and Ac-LDL uptake in red (bottom). Original magnification, ×40.

Therefore, it is possible that $CD45^-KDR^+$ cells include cell precursors in different stages of maturation, and thus hematopoietic clonogenic progenitors developed from $CD45^-KDR^+CD34^+$ cells, whereas $CD45^-KDR^+CD34^-$ cells would be the responsible for the mixed Hem-End colony generation. Nevertheless, we cannot rule out the possibility that Hem-End colonies can be developed from more mature KDR^+CD34^+ cells. In this respect, in human embryonic stem cells in a more advanced stage of differentiation when CD34 is already expressed, $CD34^+CD43^-KDR^+$ cells give rise a monolayer of endothelial cells, reflecting their endothelial potential [25]. Moreover $CD34^+KDR^+$ cells from cord blood have been reported to yield Hem-End colonies in culture [9].

There are striking differences between $CD45^-KDR^+$ cells from human adipose tissue and other adult cells with hemangioblastic properties. Thus, $CD34^+KDR^+$ cells from cord blood only were able to give CFU-GM [9], and $CD34^+CD133^+$ cells from peripheral blood uniformly expressed CD31 [22]. In contrast, $CD45^-KDR^+$ cells from adipose tissue exhibited an immunophenotype more similar to that of KDR^+ cells derived from embryonic stem cells or KDR^+ cells from fetal bone marrow, with hemangioblastic properties, which also were negative for CD31 and CD34 [26].

Finally, when mixed Hem-End colonies were analyzed, we observed cells positive for CD31 and VWF with the ability to take up Ac-LDL but also cells coexpressing CD45, indicating their endothelial or bilineage potential, respectively. Interestingly, our studies revealed that a significant proportion of adherent cells did not stain for any endothelial- or hematopoietic-specific markers, thus suggesting their mesodermal origin. This result suggests that mixed Hem-End colonies arise from primitive mesodermal progenitors capable of generating endothelial cells, erythromyeloid cells, and mesenchymal stem cells. Although the plasticity of mesenchymal stem cells is well reported, the contribution of HSCs seems to be restricted to hematopoietic cells present in bone marrow, and although some cells from nonhematopoietic tissues have been found to contribute to hematopoietic reconstitution, they were hematopoietic in origin [27, 28]. Nonetheless, it has recently been reported that Oct4-expressing multipotent adult progenitor cells from murine bone marrow, negative for CD45, CD105, and CD31 expression, were able to reconstitute hematopoiesis in irradiated NOD-SCID mice [29], leading to the authors to hypothesize that these cells must precede hematopoietic stem cells during ontogeny and that commitment to an HSC fate might be required before establishing hematopoiesis.

In this respect, it is important to mention that the SVF must be a good niche for HSCs, as SVF-derived cells support long-term hematopoiesis in vitro when cocultured with $CD34^+$ cells from cord blood [12], probably by their ability to secrete a broad repertoire of cytokines, including those implicated in hematopoiesis [30]. Our findings extend these

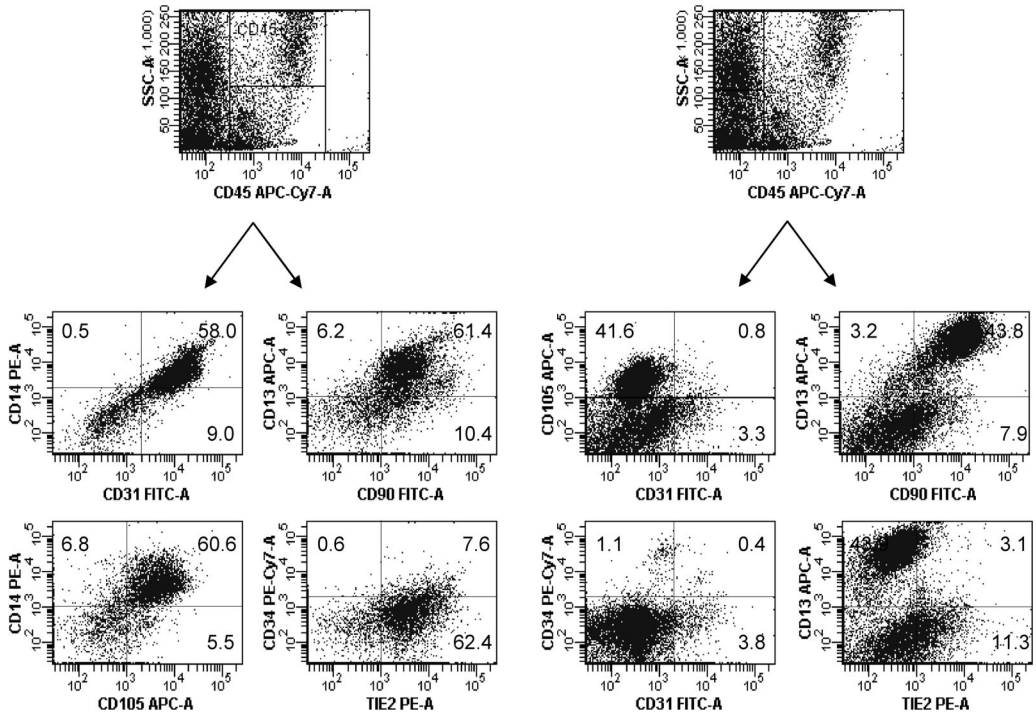

Figure 6. Fluorescence-activated cell sorting analysis of nonadherent cells derived from a secondary mixed hematopoietic-endothelial colony. Nonadherent cells were hand-picked and stained with the selected conjugated monoclonal antibodies. Subpopulations of $CD45^+$ and $CD45^-$ cells, representing 25% and 75% of total cells, respectively, were analyzed separately. Numbers in quadrants represent the percentages of cells within the indicated gates. Abbreviations: APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

previous reports and support the existence in human adipose tissue SVF of a rare population of $CD45^-KDR^+$ cells enriched in hemogenic precursors and hemangioblasts that could represent a reservoir for endothelial and hematopoietic progenitors in adult life. Whether these precursors contribute to hematopoiesis in vivo remains to be elucidated. However, importantly, they constitute a nonembryonic hemogenic cell source that is useful for studying the earlier stages of hematopoiesis, and they also provide a new cell source for vascular repair.

Conclusion

The SVF from adipose tissue contains a rare population of $CD45^-KDR^+$ mesodermal precursors that exhibit in vitro both hematopoietic and hemangioblastic activities. These findings support the idea that a pool of bipotent stem cells is maintained during adult life that is susceptible of being used in regenerative medicine.

Acknowledgments

This work was supported by Grants A.P. 049/05 and A.P. 075/06 from the Consellería de Sanidad, Generalitat Valenciana. We thank C. Riol at Centro de Transfusión de la Comunidad Valenciana for technical assistance in flow cytometry assays and Dr. J.M. Soria and A. Navarro at Fundación Hospital General Valencia for excellent help with immunofluorescence images and Giemsa stainings.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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