

## The White Adipose Tissue Used in Lipotransfer Procedures Is a Rich Reservoir of CD34<sup>+</sup> Progenitors Able to Promote Cancer Progression

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### Abstract

Previous studies have suggested a "catalytic role" in neoplastic angiogenesis and cancer progression for bone marrow-derived endothelial progenitor cells (EPC). However, preclinical and clinical studies have shown that the quantitative role of marrow-derived EPCs in cancer vascularization is extremely variable. We have found that human and murine white adipose tissue (WAT) is a very rich reservoir of CD45-CD34<sup>+</sup> EPCs with endothelial differentiation potential, containing a mean of 263 times more CD45-CD34<sup>+</sup> cells/mL than bone marrow. Compared with marrow-derived CD34<sup>+</sup> cells mobilized in blood by granulocyte colony-stimulating factor, purified WAT-CD34<sup>+</sup> cells expressed similar levels of stemness-related genes, significantly increased levels of angiogenesis-related genes, and increased levels of FAP- $\alpha$ , a crucial suppressor of antitumor immunity. *In vitro*, WAT-CD34<sup>+</sup> cells generated mature endothelial cells and capillary tubes as efficiently as mature mesenchymal cells. The coinjection of human WAT-CD34<sup>+</sup> cells from lipotransfer procedures contributed to tumor vascularization and significantly increased tumor growth and metastases in several orthotopic models of human breast cancer in immunodeficient mice. Endothelial cells derived from human WAT-CD34<sup>+</sup> cells lined the lumen of cancer vessels. These data indicate that CD34<sup>+</sup> WAT cells can promote cancer progression and metastases. Our results highlight the importance of gaining a better understanding of the role of different WAT-derived cells used in lipotransfer for breast reconstruction in patients with breast cancer. *Cancer Res*; 72(1); 325–34. ©2011 AACR.

### Introduction

The "catalytic" and quantitative roles of bone marrow-derived endothelial progenitor cells (EPC) in cancer growth have been intensively debated in the last decade (1–12). Donor-derived endothelial cells have been found, albeit in limited number, in patients who received allogeneic bone marrow transplants (2). Conflicting data have been obtained regarding the real relevance of bone marrow-EPC-derived vessels in cancer growth in different preclinical models of neoplasia, with

some models exhibiting a critical dependence on the presence of bone marrow-derived EPCs for cancer vessel growth and tumor development, and other models appearing, instead, to be insensitive to the presence of these cells (reviewed in refs. 1–12).

One study has recently described that EPCs are present in tissues other than the bone marrow, in particular in the adipose tissue of mice (13). Here, we report that human white adipose tissue (WAT) is a very rich reservoir of CD45-CD34<sup>+</sup> EPCs. Compared with bone marrow-derived CD34<sup>+</sup> cells mobilized in blood by granulocyte colony-stimulating factor (G-CSF), purified human WAT-derived CD34<sup>+</sup> cells were found to express similar levels of stemness-related genes and significantly increased levels of angiogenesis-related genes and of FAP- $\alpha$ , a crucial suppressor of antitumor immunity (14). *In vitro*, WAT-CD34<sup>+</sup> cells generated mature endothelial cells and endothelial tubes. *In vivo*, the coinjection of human WAT-CD34<sup>+</sup> cells contributed to tumor vascularization and significantly increased tumor growth and metastases in orthotopic models of human breast cancer in nonobese diabetic severe combined immunodeficient (NOD/SCID) interleukin-2 receptor  $\gamma$  (IL-2R $\gamma$ )-null (NSG) mice.

### Methods

#### Cell purification

Human WAT samples were obtained from lipotransfer procedures for breast reconstruction in breast cancer

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patients who signed an informed consent. Stromal-vascular cell fractions were obtained using a standard protocol (with few modifications), as previously described (15–17). In brief, samples were centrifuged at  $1,200 \times g$  for 10 minutes to remove erythrocytes and leukocytes and subsequently digested in HBSS (Gibco) containing 2 mg/mL of collagenase type I (Sigma Aldrich) and 3.5% bovine serum albumin (BSA; Sigma Aldrich) at  $37^\circ\text{C}$  with constant shaking for 60 minutes. The digestion was blocked with RPMI 1640 supplemented by 20% FBS (Euroclone), and a cell pellet was obtained by centrifugation at  $200 \times g$  for 10 minutes at  $4^\circ\text{C}$ . The cell suspension was then filtered through a 100- $\mu\text{m}$  mesh filter to remove undigested tissue and washed twice with incubation buffer (PBS with 2 mmol/L EDTA and 0.5% BSA), working always on ice. An aliquot of these cells was labeled for flow cytometry analysis.

Overall, 113 human WAT samples were studied *in vitro*, and 38 of these were used to provide CD34<sup>+</sup> cells that were used in 124 different individual mouse studies.

After informed consent was obtained, CD34<sup>+</sup> cells were purified from WAT samples and blood apheresis products of healthy donors undergoing stem cell collection after G-CSF administration by means of anti-CD34 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Final CD34<sup>+</sup> cell purity was evaluated by flow cytometry and found in each instance to be more than 95%.

Mouse WAT samples were obtained from the mammary and the ovary fat pads and processed as described above for human WAT.

### Flow cytometry

CD45-CD34<sup>+</sup> progenitor cells were evaluated by 6-color flow cytometry following an approach recently validated for the quantification of circulating EPCs and perivascular progenitors (18, 19). The nuclear staining Syto16 was used to discriminate between DNA containing cells, platelets, and cell debris. 7-Amino-actinomycin D (7-AAD) was used to determine the viability status of the cells.

We used the following anti-human antibodies: anti-CD45-APC-Cy7 (clone 2D1), -CD34-APC, and -PcCy7 (clone 8G12), -CD31-PcCy7 (custom product, clone L133.1), -CD13-APC (clone WM15), -CD10-APC (clone H10a), -CD140b-PE (clone 28D4), -CD29-PE (clone MAR4), and -CD90-PE (clone 5E10) from BD; anti-VEGF receptor (VEGFR)2-PE (clone 89106) and -VEGFR3-PE (clone 54733) were from R&D Systems; anti-CD44-APC (clone BJ18) was from Bio-Legend, anti-CD144-PE (TEA 1/31) was from Beckman-Coulter. The nuclear staining 7-AAD and Syto 16 were from Sigma and Invitrogen, respectively.

For murine studies, anti-CD45-APC-Cy7 (clone 30-F11) and -CD117-PE (clone ack45), were from BD. Anti-CD13-PE (clone WM15), -Sca-1 APC (clone D7), -CD34-PC-7 (clone RAM34), and -CD150-PE (clone BD1) were from Ebioscience.

The absolute count of CD45-CD34<sup>+</sup> cells was obtained using reference beads in Trucount tubes (BD). In murine studies, the panel of monoclonal antibodies used included Syto16, 7-AAD, anti-CD34, CD45, CD13, CD117, CD150, Sca-1, and Lin. Again, the absolute cell count was obtained using reference beads in Trucount tubes.

### RT-PCR and expression analysis

In immunomagnetically purified CD34<sup>+</sup> cells, RNA isolation was carried out using QIAmp RNA Blood Mini Kit (Qiagen), and cDNA was generated from 40 ng of RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems); quantitative real-time PCR (qRT-PCR) was carried out with an ABI Prism 700 platform as previously described (20) using primers and probes from the TaqMan Gene Expression Assay.

For microarray analysis, synthesis of labeled targets, array hybridization (Affymetrix GeneChip Gene ST 1.0 Human array; Affymetrix), staining, and scanning were carried out according to Affymetrix standard protocols, starting from 500 ng of total RNA. Duplicate microarrays were hybridized with each DNA sample. The MAS5 algorithm was used to determine the expression levels of mRNAs; the absolute analysis was carried out using default parameters and scaling factor 500. Report files were extracted for each microarray chip, and performance of labeled target was evaluated on the basis of several values (e.g., scaling factor, background and noise values, percentage of present calls, average signal value). The data were deposited at GEO (21; GSE31415). Results were confirmed by qRT-PCR.

### Cell lines and culture

MDA-MB-436 and HCC1937 triple-negative breast cancer cells were purchased from the American Type Culture Collection and cultured as recommended by the manufacturer. Prior to injection in mice, cells ( $1 \times 10^6/20 \mu\text{L}/\text{mouse}$ ) were mixed with Matrigel (BD) and trypan blue solution (Sigma Aldrich, 25% and 10% in PBS, respectively).

Endothelial cells (EC) and capillary tubes were obtained in Matrigel from cultures of purified human WAT-CD34<sup>+</sup> cells as previously described (9, 22). In brief, cells were plated in complete EBM-2 medium (Lonza) in 12- or 24-well plates precoated with rat tail collagen I. Plates were placed in a  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  humidified incubator. The seeding density ranged from  $50 \times 10^3$  to  $500 \times 10^3$  cells. The presence of ECs and colonies was assessed using an inverted microscope. After 3 to 7 days of culture, endothelial cell colonies were identified morphologically and were subsequently picked out using cloning rings. Fibroblast contamination was avoided by depleting them from cell suspensions with the Anti-Fibroblast Microbead kit (Miltenyi). Endothelial cell surface antigen expression was assessed by flow cytometry and immunofluorescence staining of VE-Cadherin was done as previously described (20–22).

Capillary-like structures were obtained in culture using a commercial kit (Chemicon) as previously described (22). Briefly, matrix solution was thawed on ice, seeded on 24-well plates, and incubated at  $37^\circ\text{C}$  to solidify. ECs were harvested, resuspended in complete media, seeded at a final concentration of  $5 \times 10^4$  cells per cell onto the polymerized Matrigel, and incubated at  $37^\circ\text{C}$  in a tissue incubator. After 17 hours, tube formation was inspected under an inverted light microscope at  $\times 20$  magnification.

Spheres were obtained in cultures as previously described (23). In brief, cells were plated onto ultraslow attachment plates (BD-Falcon) at a density of 40,000 viable cells/mL in a serum-free mammary epithelial basal medium (MEBM; Lonza), supplemented with 5 mg/mL insulin, 0.5 mg/mL hydrocortisone, B27 (Invitrogen), 20 ng/mL EGF and basic

fibroblast granulocyte factor (BD Biosciences), and 4 mg/mL heparin (Sigma Aldrich) and maintained in a 5% CO<sub>2</sub>-humidified incubator at 37°C. Six to 8 days later, sphere formation was analyzed under an inverted light microscope.

#### Orthotopic xenograft *in vivo* studies

Female NSG mice (24, 25), 6 to 9 weeks old, were bred and housed under pathogen-free conditions in our animal facilities (Institute of Molecular Oncology Foundation–European Institute of Oncology campus). Mice were expanded from breeding pairs originally donated by Dr. Leonard Shultz, The Jackson Laboratory, Bar Harbor, ME. All animal experiments were carried out in accordance with national and international laws and policies.

Prior to injection, tumor cells were trypsin detached, washed twice, and resuspended in PBS to a final concentration of 10<sup>6</sup> cells/13  $\mu$ L. The cell suspension was then mixed with 5- $\mu$ L growth factor–reduced Matrigel (BD Biocoat) and 2- $\mu$ L trypan blue solution (Sigma Aldrich) and maintained on ice until injection. In cases where tumor cells were coinjected with 2  $\times$  10<sup>5</sup> WAT-derived cells, cell suspensions were mixed before final suspension in Matrigel. Aseptic conditions under a laminar flow hood were used throughout the surgical procedure. Mice were anesthetized with 0.2% Avertin (Sigma Aldrich), laid on their backs, and injected with 20- $\mu$ L cell suspension in Matrigel directly in the fourth mammary fat pad through the nipple with a Hamilton syringe.

Tumor growth was monitored weekly using digital callipers, and tumor volume was calculated according to the formula:  $L \times W^2/2 = \text{mm}^3$ .

#### Bilateral studies

NSG mice were divided into 2 groups, a control group in which 1  $\times$  10<sup>6</sup> MDA-MB-436 or HCC1937 cells were injected into the right 4th mammary fat pad (through the 4th nipple) and an experimental group in which 1  $\times$  10<sup>6</sup> MDA-MB-436 or HCC1937 cells were coinjected with 2  $\times$  10<sup>5</sup> human CD34<sup>+</sup> WAT cells into the left fourth mammary fat pad.

#### Monolateral studies

A total of 1  $\times$  10<sup>6</sup> MDA-MB-436 or HCC1937 cells were injected into the right fourth mammary fat pad, and 1  $\times$  10<sup>6</sup> MDA-MB-436 or HCC1937 cells were coinjected with 2  $\times$  10<sup>5</sup> human CD34<sup>+</sup> WAT cells into the left fourth mammary fat pad of the same NSG mouse.

In both sets of studies, tumors were measured at least once a week using digital calipers.

Tumor and lung tissues were removed at the end of the experiment on day 70–100. Tumors were measured and weighed. For histologic evaluation of the tumors, 1 part of the tumor tissue was fixed in 4% phosphate-buffered formalin and embedded in paraffin. For detection of the pulmonary metastases, lungs were fixed in 4% phosphate-buffered formalin and embedded in paraffin. Five-micron sections of the entire lungs were made, and slides were counterstained with hematoxylin and eosin (H&E) for the detection of metastases. The Scan Scope XT device and the Aperio Digital pathology system software (Aperio) were used to detect metastases.

In the second model of breast cancer metastases, 1  $\times$  10<sup>6</sup> MDA-MB-436 cells were injected into the right fourth mammary fat pad (through the fourth nipple) of NSG mice to produce orthotopic primary tumors. When the tumor size was 200 to 250 mm<sup>3</sup>, that is, about 45 days after tumor implant, tumor resection (mastectomy) was done. The tumor mass was gently removed and the incision closed with wound clips. Three days after mastectomy, mice were divided into an experimental group in which 2  $\times$  10<sup>5</sup> human CD34<sup>+</sup> WAT cells were injected into the right third mammary fat pad (through the third nipple), a group in which 2  $\times$  10<sup>5</sup> human CD34<sup>-</sup> WAT cells were injected into the right third mammary fat pad, and a control group without WAT cell injection ( $n = 6$  per study group). Two months after cell injection, mice were sacrificed and right axillary lymph node and lung tissue were removed. To confirm the presence of metastases, sections were cut and stained with H&E.

For high-fat diet (HFD) studies, mice were bred and fed as previously described (26).

#### Confocal microscopy

Images were acquired using a Leica TCS SP5 confocal microscope, and sequential Z-stacks were performed using a  $\times 63$  1.4NA oil immersion objective, zoom  $\times 3$ , 0.3- $\mu$ m Z step. For imaging of the red cells, the 561 laser line was used and the autofluorescence of the cells was collected.

#### Statistical analysis

The Shapiro–Wilk test was used to assess for normality. Considering that the very large majority of data were not normally distributed, statistical comparisons were carried out using the nonparametric *U* test of Mann–Whitney. All reported *P* values were 2 sided.

## Results

#### Human and murine WATs are very rich reservoirs of CD45-CD34<sup>+</sup> EPCs

By means of flow cytometry, we counted the numbers of EPCs and other subsets of progenitors in the bone marrow and in the WAT of humans and mice. Human WAT tissue was obtained from lipotransfer/lipofilling procedures for breast reconstruction in breast cancer patients at the European Institute of Oncology in Milan, Italy. Most of these procedures involved WAT collection from the abdomen. Human bone marrow was obtained from disease-free patients undergoing a follow-up involving bone marrow investigation for hematologic or solid neoplasia. The flow cytometry evaluation included a DNA staining procedure to exclude contamination with platelets and/or micro- and macroparticles, as previously described (18, 19).

In humans, WAT was found to contain a large amount of CD45-CD34<sup>+</sup> cells that fulfil the most recent criteria for EPC identification (9–12). These CD45-CD34<sup>+</sup> cells included 2 subpopulations: CD34<sup>++</sup> CD13<sup>+</sup> CD140b<sup>+</sup> CD44<sup>+</sup> CD90<sup>++</sup> cells and CD34<sup>+</sup> CD31<sup>+</sup>CD105<sup>+</sup> cells (Fig. 1A–M). The immunomagnetic purification procedure used in the study led to a



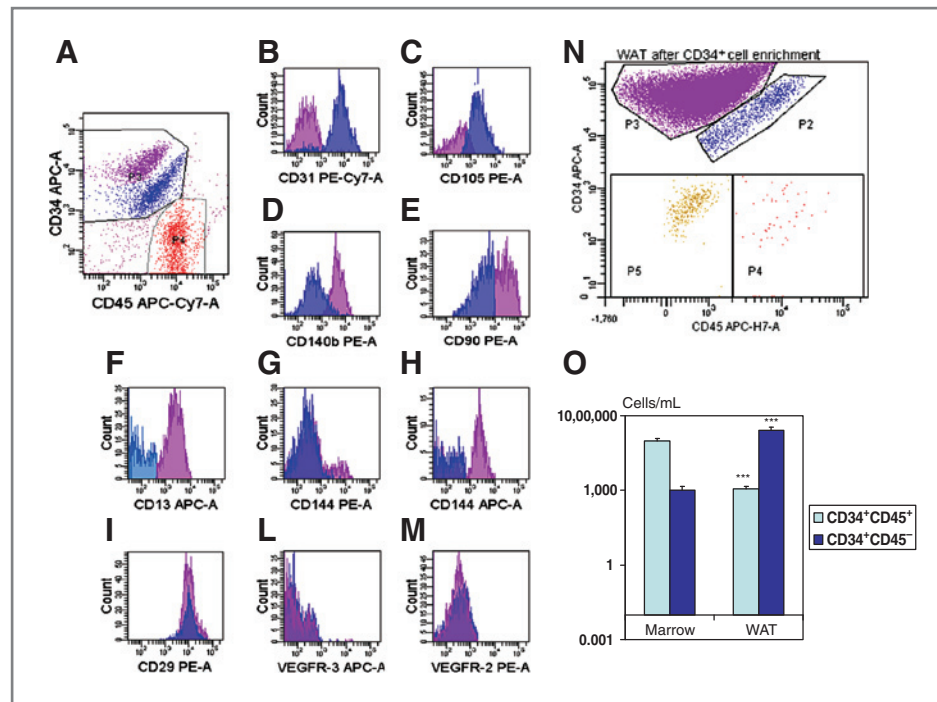


Figure 1. Flow cytometry evaluation and count of CD45-CD34<sup>+</sup> cells in human WAT. Representative evaluation of CD45-CD34<sup>+</sup> cells in human WAT tissue from lipotransfer procedures. A, gate used to investigate CD34<sup>+</sup>CD45<sup>-</sup> cells. B–M, expression of CD31, CD105, CD140b (PDGFR), CD90, CD13, CD144 (VE-cadherin), CD44, CD29, VEGFR3, and VEGFR2 in the 2 populations of CD34<sup>+</sup>CD45<sup>-</sup> and CD34<sup>+</sup>CD45<sup>+</sup> cells. N, a representative evaluation of the CD34<sup>+</sup> cell population obtained by the immunomagnetic purification procedure (79%–96% of CD45-CD34<sup>+</sup> CD13<sup>+</sup> CD140b<sup>+</sup> CD44<sup>+</sup> CD90<sup>+</sup> cells, 2%–18% of CD45-CD34<sup>+</sup> CD31<sup>+</sup>CD105<sup>+</sup> cells and always <5% of CD34<sup>+</sup> cells). O, the quantitation of CD34<sup>+</sup>CD45<sup>+</sup> (hematopoietic) and CD34<sup>+</sup>CD45<sup>-</sup> (endothelial) cells in human bone marrow and WAT ( $n = 32$ ). \*\*\*,  $P < 0.0005$  versus marrow by Mann-Whitney  $U$  test.

cell population which included 79% to 96% of CD45-CD34<sup>+</sup> CD13<sup>+</sup> CD140b<sup>+</sup> CD44<sup>+</sup> CD90<sup>+</sup> cells, and 2% to 18% of CD45-CD34<sup>+</sup> CD31<sup>+</sup>CD105<sup>+</sup> cells. CD34<sup>-</sup> cells always made up less than 5% of the purified cell population (Fig. 1N and Supplementary Fig. S1)

Quantitative studies showed that human WAT contains about 263-fold more CD45-CD34<sup>+</sup> EPCs/mL than bone marrow ( $n = 32$ , Fig. 1M). In particular, median human WAT CD45-CD34<sup>+</sup>CD31<sup>-</sup> cells were 181,046/mL (range, 35,970–465,357), and CD45-CD34<sup>+</sup> CD31<sup>+</sup> cells were 76,946/mL (range, 13,982–191,287). Correlations were found between the body mass index and total CD34<sup>+</sup> cells ( $r = 0.608$ ,  $P < 0.001$ ), and between WAT donor age and total CD34<sup>+</sup> cells ( $r = 0.387$ ,  $P = 0.035$ ).

In mice, EPCs were defined as CD45-CD34<sup>+</sup>CD13<sup>+</sup>Sca-1<sup>+</sup> cells, whereas hematopoietic progenitors were defined as Lin-Sca-1<sup>+</sup>CD150<sup>+</sup> cells. Similarly to humans, mouse WAT was also richer in EPC than was bone marrow, with WAT having 179-fold more EPCs/mL than bone marrow.

We also found CD45<sup>+</sup>CD34<sup>+</sup> hematopoietic progenitor cells in human WAT (median 6,141/mL, range <1–161,338). On average, human WAT contained 87 times less CD45<sup>+</sup>CD34<sup>+</sup> hematopoietic progenitor cells/mL than did bone marrow. The presence of human hematopoietic progenitors in WAT was confirmed by culture studies, where a mean of  $1.4 \pm 0.2$  granulocyte macrophage colony-forming

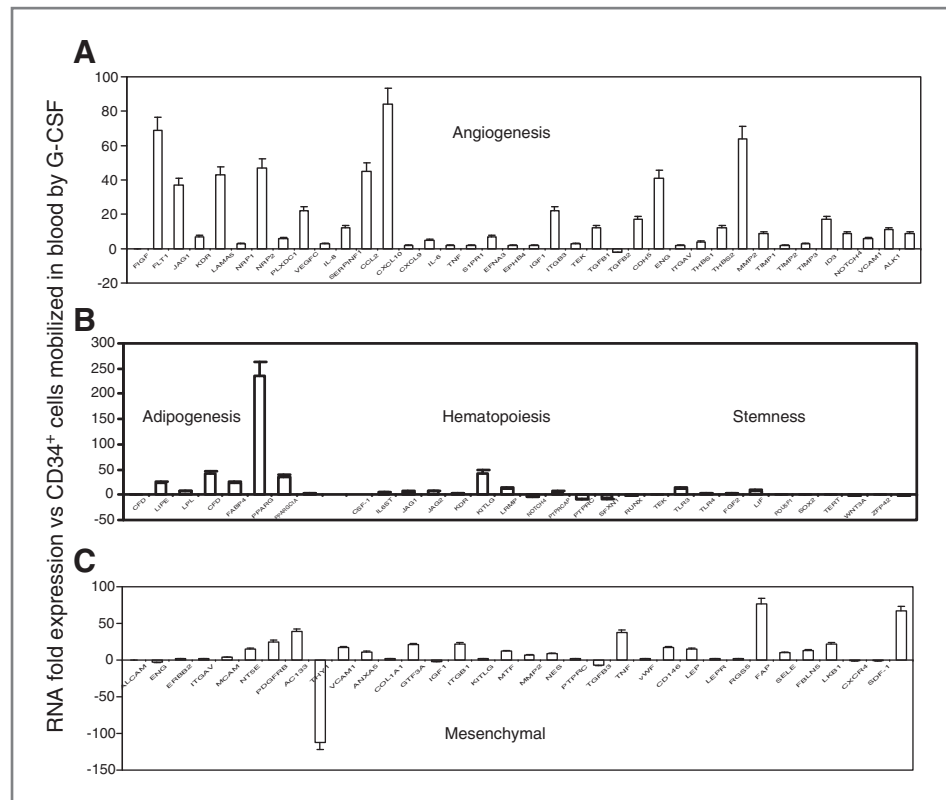
units and  $3.4 \pm 1.8$  BFU-E/ $10^5$  seeded cells were obtained from WAT tissue.

#### WAT-CD34<sup>+</sup> cells express stemness-related genes and very high levels of angiogenesis-related genes

CD34<sup>+</sup> cells from human WAT were purified and their gene expression profile compared (by Affymetrix human gene 1.0 ST) with that of purified bone marrow CD34<sup>+</sup> cells mobilized in the blood of healthy donors by G-CSF administration ( $n = 5$  per study arm). Data were confirmed by qRT-PCR (Supplementary Table S1). When compared with bone marrow-derived CD34<sup>+</sup> cells (Fig. 2A–C), human WAT CD34<sup>+</sup> cells expressed significantly higher levels of genes associated with angiogenesis (e.g., VEGFR-1 and -2, NR1, TEK, VE-Cadherin, VCAM-1, ALK1), adipogenesis (e.g., LPL, FABP4, PPARG) endothelial and mesenchymal (e.g., RGS5, insulin-like growth factor I, platelet-derived growth factor receptor  $\beta$ ) differentiation (1–13). A large majority of the panel of genes associated with stemness (e.g., SOX2, LIF, WNT3A, Nanog) and hematopoiesis (e.g., RUNX, IL-6, CSF-1) was expressed in WAT and bone marrow-derived CD34<sup>+</sup> cells at similar levels (Fig. 2B).

WAT-derived CD34<sup>+</sup> cells expressed higher levels of FAP- $\alpha$ , a crucial suppressor of antitumor immunity (14), and of genes of the brain/adipocyte-BDNF/leptin axis, which

**Figure 2.** Gene expression profile in purified CD34<sup>+</sup> WAT cells versus CD34<sup>+</sup> cells mobilized in the blood from G-CSF. Y axes show RNA fold expansion versus CD34<sup>+</sup> cells mobilized by G-CSF. WAT CD34<sup>+</sup> cells expressed significantly higher levels of (A) angiogenesis- and adipogenesis-related genes (B), similar levels of hematopoietic- and stemness-related genes (B), and higher levels of mesenchymal-related genes (C).



has recently been suggested to play a relevant role in cancer progression (27).

#### WAT-CD34<sup>+</sup> cells generate mature endothelial cells and capillary tubes

When cultured *in vitro* in appropriate endothelial-differentiation media, human WAT-derived CD34<sup>+</sup> cells generated mature endothelial cells (depicted by the expression of the endothelial-restricted VE-cadherin antigen, Fig. 3A–C). Endothelial capillary tubes were also generated using the appropriate culture procedure in Matrigel (Fig. 3D–E). Purified WAT CD34<sup>+</sup> cells, but not WAT CD34<sup>−</sup> cells, generated spheres (13, 23) in appropriate culture conditions (Fig. 3F–G). We obtained a mean of 1,200 cells/sphere in cultures generated from 40,000 purified WAT CD34<sup>+</sup> cells. By flow cytometry, these spheres were found to be made of CD45-CD13<sup>+</sup>CD34<sup>+</sup> (16% of cells in spheres) CD44<sup>+</sup>CD90<sup>+</sup> cells (Supplementary Fig. S2).

#### The coinjection of human WAT-CD34<sup>+</sup> cells from lipotransfer procedures significantly increases tumor growth and metastases in breast cancer models

The role of purified CD34<sup>+</sup> cells from human WAT was investigated in different models of human breast cancer (Figs. 4–6 and Supplementary Fig. S3–5). Triple-negative human breast cancer MDA-MB-436 and HCC1937 cells were injected in the mammary fat pad alone or coinjected with WAT-derived human cells ( $N = 124$ , Fig. 4A). Breast cancer cells generated tumors in the mammary fat pad. Purified

human CD34<sup>+</sup> WAT cells, when injected in the mammary fat pad in the absence of tumor cells, did not generate tumors. The coinjection of breast cancer cells and unprocessed nucleated cells from human WAT significantly increased tumor growth. The coinjection of breast cancer cells and purified CD34<sup>+</sup> WAT cells increased tumor growth to a similar extent, suggesting that the large majority of the tumor-promoting activity in human WAT cells resides in the CD34<sup>+</sup> WAT cell fraction (Fig. 4B). The coinjection of CD34<sup>−</sup> WAT cells was less effective than the coinjection of CD34<sup>+</sup> WAT cells in promoting tumor growth (Fig. 4B). Similar results were obtained in NSG mice injected with HCC1937 breast cancer cells alone, or in combination with human CD34<sup>+</sup> WAT cells (Fig. 4C). Histology studies ruled out the possibility that the larger size of tumors in animals coinjected with CD34<sup>+</sup> WAT cells was due to the generation of adipocytes (Supplementary Fig. S5).

We conducted 2 separate studies to examine the *in vivo* involvement of CD34<sup>+</sup> WAT cells in promoting tumor growth. In bilateral studies, human CD34<sup>+</sup> WAT cells were coinjected with breast cancer cells in one of the lateral mammary fat pads (with the contralateral mammary fat pad injected with breast cancer cells alone, as control). In monolateral studies, human CD34<sup>+</sup> WAT cells were coinjected with breast cancer cells in a single mammary fat pad of a series of animals (with another series of mice being injected with breast cancer cells alone in the corresponding mammary fat pads, as control). Tumor growth was slightly (albeit not significantly) higher in bilateral studies compared with monolateral studies (Fig. 4D). These data suggest that human WAT CD34<sup>+</sup> cells exert most (if not

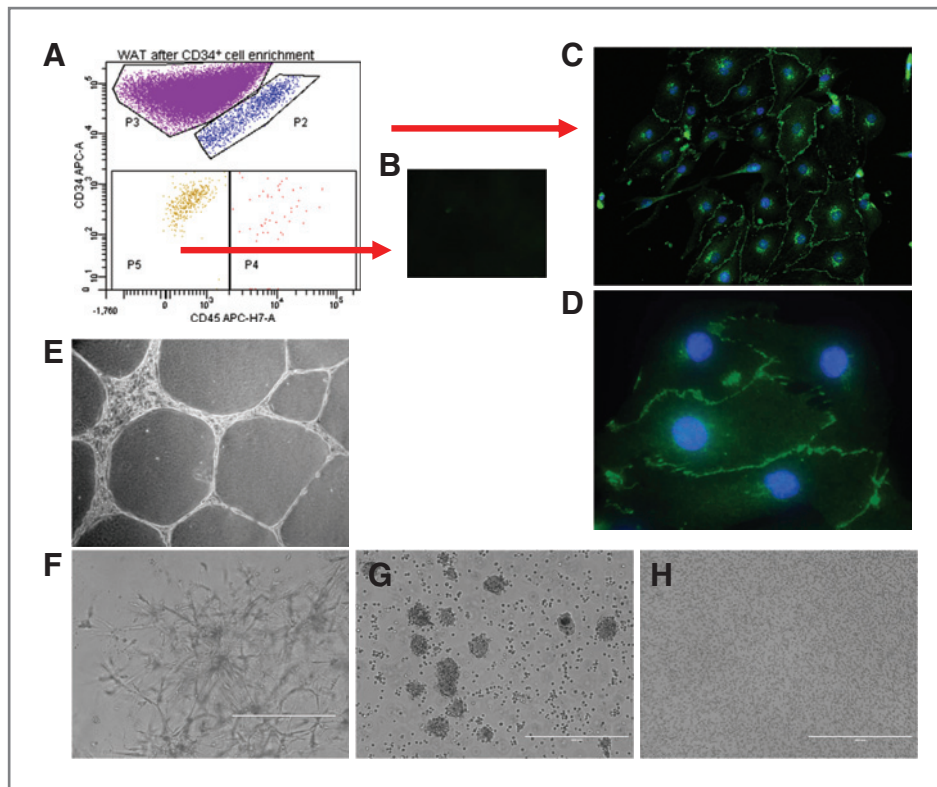


Figure 3. *In vitro* endothelial differentiation of CD34<sup>+</sup>CD45<sup>-</sup> cells from human WAT. A, CD45<sup>-</sup>CD34<sup>+</sup> cells obtained by the immunomagnetic purification procedure. B, lack of endothelial cell generation *in vitro* when CD34<sup>-</sup> cells were cultured. C and D, representative *in vitro* generation of endothelial cells (depicted by immunofluorescence expression of the endothelial-restricted VE-cadherin antigen) when CD34<sup>+</sup> cells were cultured. E and F, *in vitro* generation in Matrigel of endothelial capillary tubes from CD34<sup>+</sup> cells. G, presence of spheres in the culture of CD34<sup>+</sup> WAT cells. H, lack of spheres in the cultures of CD34<sup>-</sup> WAT cells.

all) of their tumor-promoting activity locally and not via soluble factors that are released in circulation, which would also have promoted the growth of tumors in the opposite mammary fat pad that was not coinjected with human WAT CD34<sup>+</sup> cells.

In our model of breast cancer, where MDA-MB-436 cells were injected in the mammary fat pad, lung metastases were observed around day 70 (Fig. 4E and F, and Supplementary Fig. S3 and S4). The number of lung metastases was significantly increased in mice coinjected with breast cancer and CD34<sup>+</sup> WAT cells compared with mice injected with breast cancer cells alone or mice injected with breast cancer cells and CD34<sup>-</sup> WAT cells (Fig. 4G).

In another model of breast cancer metastasis, MDA-MB-436 breast cancer cells were injected into the mammary fat pad of NSG mice to produce orthotopic primary tumors. When the tumor size was 200 to 250 mm<sup>3</sup>, that is, about 45 days after tumor implant, the tumor was surgically removed. Mice were then divided into an experimental group in which CD34<sup>+</sup> WAT cells were injected, an experimental group in which CD34<sup>-</sup> WAT cells were injected, and a control group without WAT cell injection. Two months after cell injection, mice injected with CD34<sup>+</sup> WAT cells had significantly more axillary and lung metastases, compared with mice injected with CD34<sup>-</sup> cells and controls (Fig. 4H).

Immunohistochemistry and confocal/Z-stack microscopy studies showed the presence of human CD31<sup>+</sup>, CD34<sup>+</sup>, CD105<sup>+</sup> endothelial vessels, and perivascular cells in the mammary fat pad and in tumors of mice coinjected with breast cancer cells

and CD34<sup>+</sup> human WAT cells (Fig. 5). Fig. 5C–H shows representative images illustrating the incorporation of human cells generated from WAT-derived CD34<sup>+</sup> cells lining cancer blood vessels, some of which contain red blood cells (Fig. 5G and 5H). Confocal microscopy (Fig. 6 and Supplementary Movie S1 made from 42 consecutive slices) confirmed the presence of a lumen and of circulating red blood cells in human CD34<sup>+</sup> and CD31<sup>+</sup> vessels in mice injected with human CD34<sup>+</sup> WAT cells. We were never able to observe this effect in our previous studies using bone marrow-derived cells; consequently, these results show an important bona fide functional difference between WAT- and bone marrow-derived progenitors.

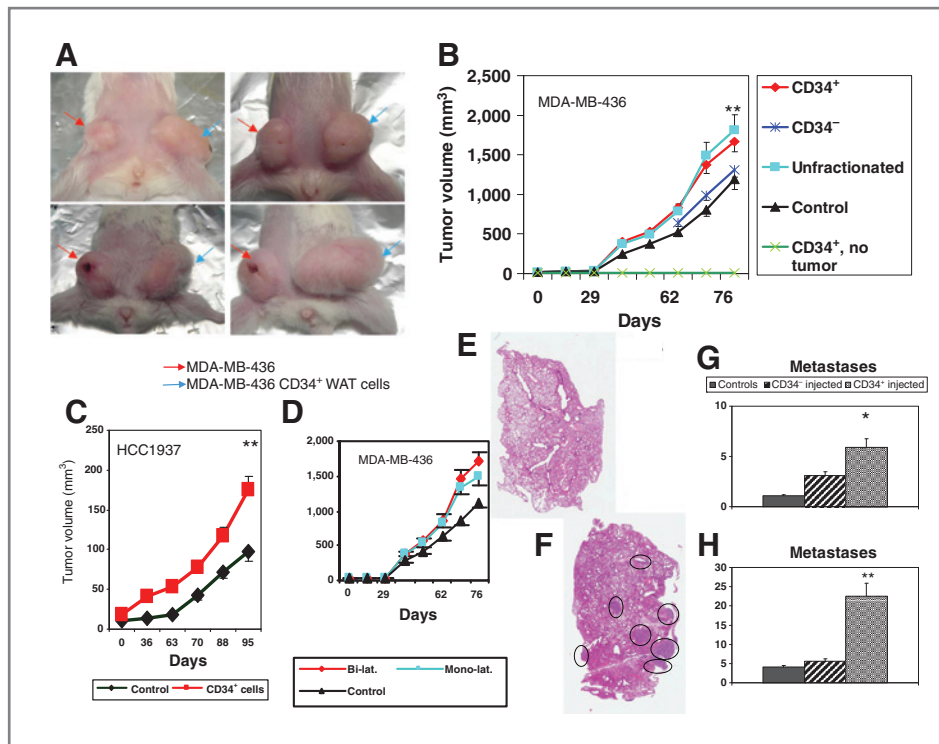
#### WAT-CD34<sup>+</sup> and hematopoietic progenitor cell kinetics in mice receiving high-fat diet

Considering the known correlation between obesity and breast cancer, we compared the number of EPCs in the mammary and ovarian WAT tissue of mice receiving an HFD ( $n = 12$ ) with that in mice fed a control diet. Both EPCs and hematopoietic progenitor cells were significantly increased in the WAT of mice receiving an HFD (Fig. 7A–C). Hematopoietic progenitors, but not EPCs, were significantly decreased in the femurs of mice receiving HFD (Fig. 7D).

#### Discussion

The role of bone marrow-derived EPCs in cancer growth has been intensively debated in the past decade (1–12). Our present work offers new insight into the controversy over





**Figure 4.** Orthotopic models of breast cancer. A, representative pictures of the growth of human MDA-MB-436 breast cancer cells in the mammary fat pads of NSG mice injected with breast cancer cells alone (red arrow) or breast cancer cells plus CD34<sup>+</sup> WAT cells (blue arrow). B, tumor growth in NSG mice injected with WAT CD34<sup>+</sup> cells alone, MDA-MB-436 cells alone, MDA-MB-436 cells plus unfractionated WAT cells, MDA-MB-436 cells plus CD34<sup>+</sup> WAT cells, and MDA-MB-436 cells plus CD34<sup>-</sup> WAT cells. C, tumor growth in NSG mice injected with HCC1937 breast cancer cells alone and in NSG mice injected with the same number of breast cancer cells plus human CD34<sup>+</sup> WAT cells. D, tumor growth in NSG mice injected with MDA-MB-436 breast cancer cells in mono- and bilateral studies. E and F, representative pictures of breast cancer metastatic spots (depicted by the black rings) in the lungs of NSG mice that were not coinjected with human WAT CD34<sup>+</sup> cells (E) or coinjected with human WAT CD34<sup>+</sup> cells (F). E and F are reproduced in larger size in Supplementary Fig. S3. G and H, the number of metastases in NSG mice injected with MDA-MB-436 breast cancer cells in models evaluated in the absence (G) of tumor surgical removal or after tumor surgical removal (H). \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  versus control by Mann-Whitney  $U$  test.

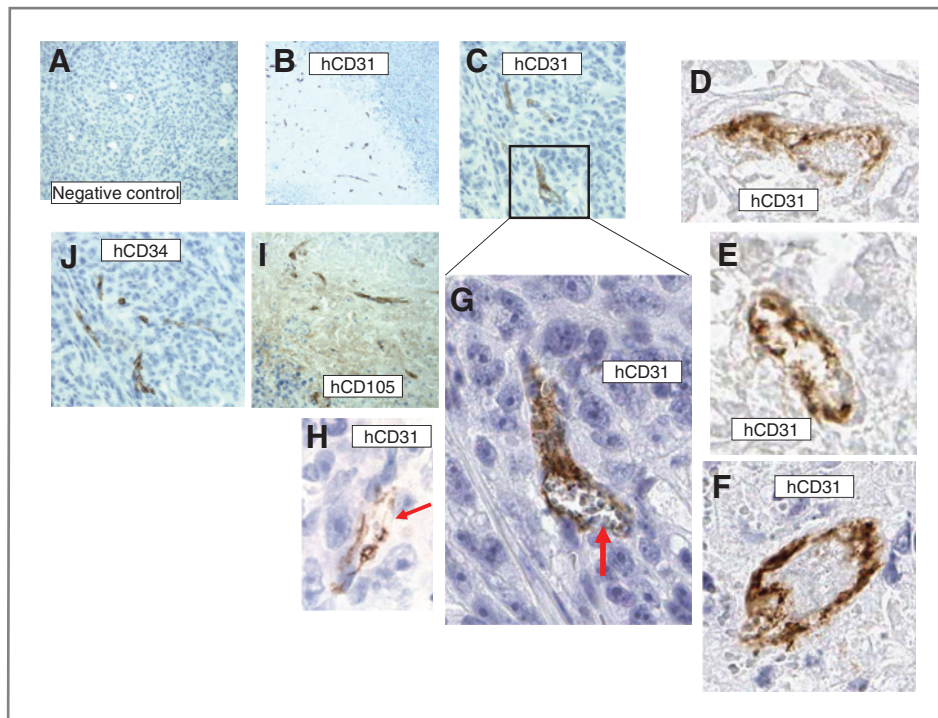
the quantitative and the "catalytic" role of EPCs in cancer growth. All previous studies investigating this topic enumerated the role of EPCs in mice carrying GFP<sup>+</sup> (or otherwise genetically labeled) bone marrow. This approach excluded quantification of the role of WAT-derived EPCs, which in our work were found to be in numbers significantly higher than in the bone marrow.

Considering that our *in vitro* and *in vivo* studies were carried out using a cell population containing 79% to 96% of CD45-CD34<sup>++</sup> CD13<sup>+</sup> CD140b<sup>+</sup> CD44<sup>+</sup> CD90<sup>++</sup> cells, and that CD144 was expressed by a subpopulation of these cells (Fig. 1G), it is likely that most of the endothelial differentiation potential and of the protumorigenic and prometastatic potential might reside in this cell population. The lack of bright CD31 expression of this cell population (Fig. 1) suggests that these cells are not mature endothelial cells. Moreover, as reported by Zimmerlin and colleagues (28), mature endothelial cells in WAT do not seem to express CD34. Along a similar line, other researchers have repeatedly indicated that the putative EPC phenotype is CD45-CD34<sup>+</sup>, and that VEGFR2 expression in immature EPCs is highly controversial, given the present lack of validated reagents (29–32).

Considering the presence of a minute subpopulation of CD144<sup>+</sup> cells within the population of CD45-CD34<sup>+</sup> cells, more work is needed to understand which population (the CD144<sup>+</sup> or CD144<sup>-</sup>) has endothelial differentiation, protumorigenic, and prometastatic potential. It also remains to be discovered whether the functional vessels expressing human antigens in mice injected with CD34<sup>+</sup> WAT cells are entirely derived from WAT-derived CD34<sup>+</sup> cells or if a fraction of WAT-derived CD34<sup>+</sup> cells differentiated and incorporated into the murine tumor vessels.

The present array studies show that WAT-derived CD34<sup>+</sup> cells express significantly higher levels of angiogenesis-related genes compared with bone marrow-derived, mobilized CD34<sup>+</sup> cells. More work with mice with GFP<sup>+</sup> WAT is currently ongoing to elucidate the precise role of WAT-EPCs in the tumor promotion and metastatic process.

Controversies have also been reported regarding the role of WAT-derived mesenchymal progenitors (MP) in cancer growth, with some authors reporting that MPs promote tumor growth and others reporting that MPs suppress tumor growth (reviewed in ref. 33). Most of these studies, however, were conducted in mice injected with crude suspensions of MPs obtained from cell culture. To our knowledge, our study is one



**Figure 5.** Immunohistochemistry evaluation of human CD34<sup>+</sup> WAT cell engraftment in breast cancer-bearing NSG mice. Clockwise, from top left: A, negative control (human tumor in NSG mouse not injected with human WAT cells, stained with anti-human CD31). B, low-resolution presence of human CD31<sup>+</sup> vessels. C, high-resolution presence of perivascular human CD31<sup>+</sup> cells and CD31<sup>+</sup> vessels. D and H, representative images illustrating the incorporation of human cells (depicted by the expression of human CD31) generated from WAT-derived CD34<sup>+</sup> cells lining cancer blood vessels, some of which contain red blood cells (G and H, red blood cells indicated by the red arrows). I–J, expression of human CD105 (I) and human CD34 (J) in perivascular cells and vessels.

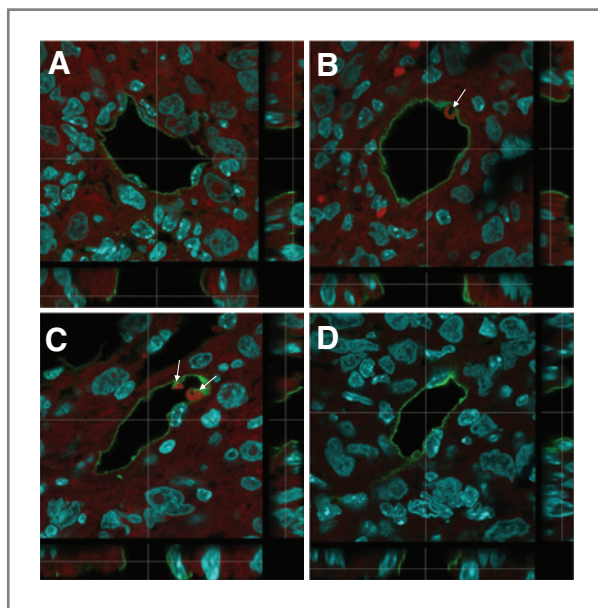
of the first reporting the tumor-promoting activity of fresh human WAT-derived purified CD34<sup>+</sup> cells. As shown by our data in mice receiving breast cancer and WAT cells in the mammary fat pad and breast cancer cells alone in the other

mammary fat pad, the cancer-promoting activity of WAT CD34<sup>+</sup> cells is likely to be exerted through a local, rather than systemic, activity. These data complement the recent observations from Zhang and colleagues (34) that, in mouse models, WAT cells are mobilized and recruited by experimental tumors to promote cancer progression.

In this study, HFD was associated in mice with a significant increase in WAT-CD34<sup>+</sup> cell numbers. HFD might also interfere with the characteristics of WAT CD34<sup>+</sup> cells. This, in turn, may be one of the explanations for the higher incidence of breast cancer in postmenopausal obese individuals (35, 36). So far, most studies on the role of obesity in cancer growth have focused on soluble factors, whereas our data underline the role of cellular players. In addition to EPCs, HFD also increased WAT hematopoietic progenitors. This, in turn, might increase the mobilization of hematopoietic proangiogenic cells already described by other studies (37).

A novel brain/adipocyte-BDNF/leptin axis has recently been proposed to play a potentially relevant role in cancer progression (27). Although more studies are clearly needed to reach robust conclusions, WAT-derived CD34<sup>+</sup> progenitors seem to express high levels of the receptors involved in this axis. Along a similar line, WAT-CD34<sup>+</sup> cells express very high levels of FAP- $\alpha$ , a crucial suppressor of antitumor immunity (14).

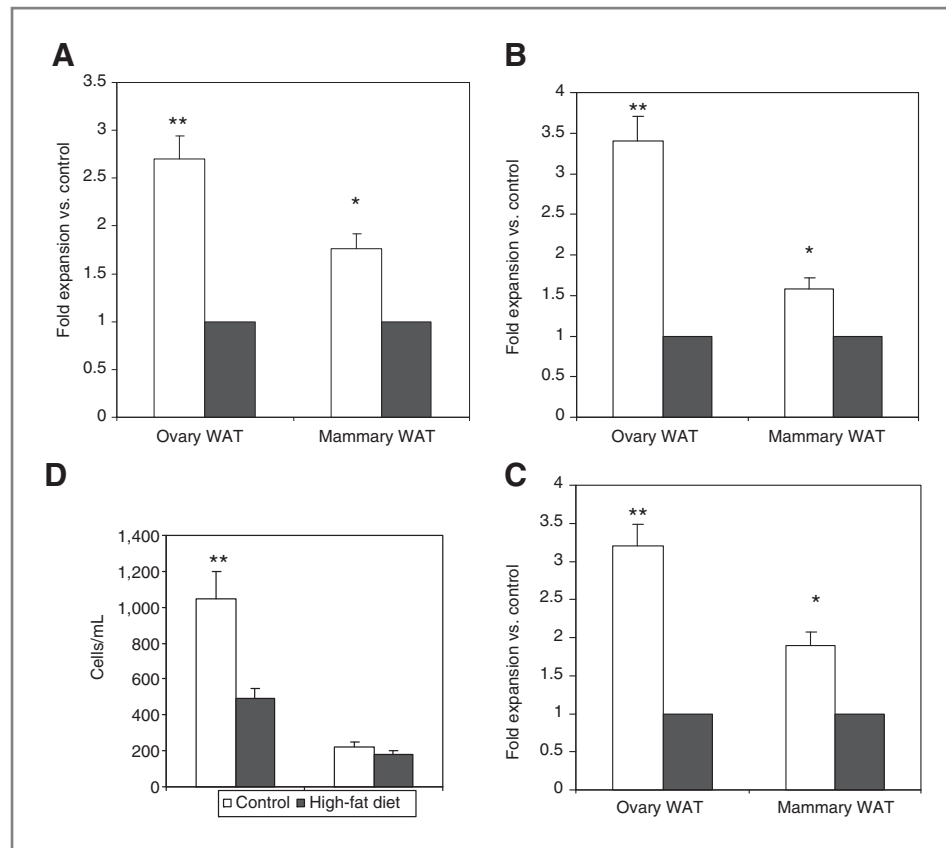
Our data suggest that caution is warranted in the clinical use of lipotransfer-derived WAT cells for breast reconstruction in patients with breast cancer (15, 16). We have recently reported a study of 321 consecutive patients who underwent surgery for primary breast cancer between 1997 and 2008, who subsequently underwent a lipotransfer procedure for aesthetic purposes, compared with 2 matched patients with



**Figure 6.** Confocal-Z-stack evaluation of cancer vessels. Confocal laser scanning of human CD34 (A, B, and C) and human CD31 (D) antigen distribution in a tumor section in mice injected with MB-MDA436 tumor cells plus WAT-derived CD34<sup>+</sup> human cells. For the imaging of the red cells the 561 laser line was used and the autofluorescence of the cells was collected (arrows). Snapshot images are orthogonal sections of the Z-stacks taken at points along the vessel cavity.



**Figure 7.** EPCs and hematopoietic stem cells (HSC) in the mammary and ovarian WAT tissue and in the femurs of mice receiving high-fat diet. Clockwise, from top left, fold expansion versus controls of CD45-CD34<sup>+</sup>CD13<sup>+</sup>Sca1<sup>+</sup> EPCs (A), Lin-Sca1<sup>+</sup>CD150<sup>+</sup> HSCs (B), and CD45-CD31<sup>+</sup>Sca1<sup>-</sup> endothelial mature cells (C) in the mammary and ovarian WAT tissue of mice receiving HFD (white bars) versus controls (black bars). Total numbers of EPCs and HSCs in the femurs of mice receiving normal versus HFD (D). \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  by Mann-Whitney  $U$  test.



similar characteristics who did not undergo lipotransfer (38). In this study, to be confirmed by prospective trials enrolling a larger series of patients, the lipotransfer group exhibited a higher risk of local events (4 events) compared with the control group (no event) when the analysis was limited to intraepithelial neoplasia.

The dissection of the different roles of purified populations of WAT-derived progenitors and mature cells will help to clarify which WAT cell populations can be used safely for breast reconstruction and which are associated with a risk linked to their capacity to support the growth of otherwise quiescent cancer cells still resident after surgery. In this context, the recent observation that zoledronic acid inhibits the interaction between mesenchymal stem cells and breast cancer cells (39) indicates a possible pharmacologic strategy, which can be assessed in preclinical models and clinical studies, to reduce any "cancer-promoting" risk of WAT EPCs.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## The White Adipose Tissue Used in Lipotransfer Procedures Is a Rich Reservoir of CD34<sup>+</sup> Progenitors Able to Promote Cancer Progression

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