Complementary Populations of Human Adipose CD34+ Progenitor Cells Promote Growth, Angiogenesis, and Metastasis of Breast Cancer

Stefania Orecchioni1, Giuliana Gregato1, Ines Martin-Padura1, Francesca Reggiani1, Paola Braidotti4, Patrizia Mancuso1, Angelica Calleni1, Jessica Quarna1, Paola Marighetti1, Chiara Aldeni1, Giancarlo Pruneri2, Stefano Martella3, Andrea Manconi3, Jean-Yves Petit3, Mario Rietjens3, and Francesco Bertolini1

Abstract

Obesity is associated with an increased frequency, morbidity, and mortality of several types of neoplastic diseases, including postmenopausal breast cancer. We found that human adipose tissue contains two populations of progenitors with cooperative roles in breast cancer. CD45−CD34+CD31−CD13−CCL2+ endothelial cells can generate mature endothelial cells and capillaries. Their cancer-promoting effect in the breast was limited in the absence of CD45−CD34+CD31−CD13−CD140b+ mesenchymal progenitors/adipose stromal cells (ASC), which generated pericytes and were more efficient than endothelial cells in promoting local tumor growth. Both endothelial cells and ASCs induced epithelial-to-mesenchymal transition (EMT) gene expression in luminal breast cancer cells. Endothelial cells (but not ASCs) migrated to lymph nodes and to contralateral nascent breast cancer lesions where they generated new vessels. In vitro and in vivo, endothelial cells were more efficient than ASCs in promoting tumor migration and in inducing metastases. Granulocyte colony-stimulating factor (G-CSF) effectively mobilized endothelial cells (but not ASCs), and the addition of chemotherapy and/or of CXCR4 inhibitors did not increase endothelial cell or ASC blood mobilization. Our findings suggest that adipose tissue progenitor cells cooperate in driving progression and metastatic spread of breast cancer. Cancer Res; 73(19): 1–12. ©2013 AACR.

Introduction

There is increasing evidence that obesity, an excess accumulation of adipose tissue occurring in mammalians when caloric intake exceeds energy expenditure, is associated with an increased frequency and morbidity of several types of neoplastic diseases, including postmenopausal breast cancer (reviewed in ref. 1). Disruption of the energy homeostasis results in obesity, inflammation, and alterations of adipokine signaling that may foster initiation and progression of cancer (2–4). Preclinical studies have suggested that differentiated cells of the white adipose tissue (WAT) and WAT-resident progenitors may also promote cancer growth and metastasis.

Materials and Methods

WAT collection and flow cytometry

As we previously described (5, 11), human WAT (>150 samples) was obtained after the signature of an informed consent from women undergoing breast reconstruction. In brief, samples were centrifuged at 1,200 g to remove erythrocytes and leukocytes and subsequently digested in Hank’s Balanced Salt Solution (Gibco) containing 2 mg/mL of collagenase type I (Sigma-Aldrich) and 3.5% bovine serum albumin (BSA; Sigma-Aldrich) at 37°C with constant shaking for 120 minutes. The digestion was blocked with RPMI-1640 supplemented by 20% FBS (Euroclone), and a cell pellet was obtained by centrifugation at 1,200 g for 10 minutes at 4°C.
The cell suspension was then processed through a 100-μm mesh filter to remove the 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-cytometric analysis. At least 500,000 total cells per sample were acquired on a flow cytometer equipped with 3 laser (Navios, Beckman Coulter), and analysis was conducted by a KALUZA software (Beckman Coulter), after selection of DNA− (Syto16+) and viable (7-aminoactinomycin D; 7-AAD+) cells. Analysis gates were set with the aid of "fluorescence minus outcomes" isotype controls. Monoclonal antibodies used in the study are described in Supplementary Table S1.

Hematopoietic CD45+CD34+ stem cells, endothelial cells, and ASCs were measured in the bone marrow and in the peripheral blood by flow cytometry as described above in more than 30 healthy volunteers and in patients with cancer (who signed an informed consent) before and after stem cell mobilization by granulocyte colony-stimulating factor (G-CSF), cyclophosphamide + G-CSF, or AMD3100 + G-CSF.

Cell sorting

CD34+ microbead-purified cells were labeled with sterile CD45FITC, CD13PE, CD31 PeCy7, and CD34APC monoclonal antibodies, and resuspended in PBS1X/EDTA 2 mmol/L/FBS 1% for cell sorting using a three laser Infinium SORCE (BD) equipped with a class I biosafety cabinet. Samples were continuously cooled to 4°C and a forward scatter pulse height and side scatter analyses were conducted to exclude cell clusters and doublets. A two-way cell sorting procedure was conducted with a140-MicroSort nozzle with a pressure of 5.5 pounds per square inch and with an events rate of 1,000 to 1,500 events per second using a sort pure mode. Samples were collected into sterile polypropylene tubes containing 20% bovine serum (FBS, Euroclone) and used for logic evaluation of the tumors, one part of the tumor tissue was fixed in 4% phosphate-buffered formalin and embedded in paraffin. Tissue were removed. To conduct the assay, cells were labeled with sterile blue solution (Sigma-Aldrich, 25% and 10% in PBS, respectively) mixed with growth factor-reduced Matrigel (BD) and Trypan blue solution (Sigma-Aldrich, 25% and 10% in PBS, respectively). Samples were collected into sterile polypropylene tubes containing 20% bovine serum (FBS, Euroclone) and used for in vitro and in vivo studies. Analysis was conducted using a FACS Sortware software (BD). Purity was always more than 96% with a recovery of 70% to 80%.

Cell lines and cell cultures

MDA-MB-436 and HCC1937 (triple negative) and ZR75-1 (luminal) breast cancer cells were purchased from the American Type Culture Collection and cultured as previously described (5). The cell lines were tested and authenticated by means of Stem Elite ID system (Promega) in January 2013. Before injection in mice, cells (1 × 10⁶/20 μL/mouse) were mixed with growth factor-reduced Matrigel (BD) and Trypan blue solution (Sigma-Aldrich, 25% and 10% in PBS, respectively). Endothelial cell cultures and capillary tubes were generated by a KALUZA software (Beckman Coulter), after sorting the formula: W²L/2 = mm², where W represents the width and L the length of the tumor mass.

Breast cancer metastases model

Tumor resection (mammary) was done when the tumor size was 200 to 250 mm³ (about 45 days after tumor implant for MDA-MB-436 cell line and twice as long for HCC1937 cell line). After anesthetizing with Avertin, the tumor mass was gently removed and the incision closed with wound clips. For histologic evaluation of the tumors, one part of the tumor tissue was fixed in 4% phosphate-buffered formalin and embedded in paraffin. One month after cell injection, mice were sacrificed by carbon dioxide inhalation. Right axillary lymph node and lung tissue were removed. To confirm the presence of metastases, sections were cut and stained with hematoxylin and eosin (H&E) as previously described (5).

In brief, for detection of metastases, the axillary lymph node and lungs were fixed in 4% phosphate-buffered formalin and embedded in paraffin. Five-micron sections of the entire lungs and lymph node were made, and slides were counterstained with H&E for the detection of metastases. The Scan Scope XT device and the Aperio Digital pathology system software were used for the analysis.

RT-PCR and expression analysis

RNA isolations from ASCs and endothelial cells of 7 different patients were carried out using QIamp RNA Blood Mini Kit (Qiagen), and cDNAs were generated from 40 ng of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems); quantitative real-time (qRT)-PCR was carried out with an ABI Prism 7000 platform as previously described (Martin-padura and colleagues) using primers and probes from the TaqMan Gene Expression Assay (5).
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). Results were confirmed by qRT-PCR.

**Immunofluorescence and confocal microscopy analysis**

To assess the effects of ASCs and endothelial cells on tumor angiogenesis, immunofluorescence analysis of CD31, α-smooth muscle actin (SMA; 1A4, Sigma-Aldrich), and CENPA-A (ab13939, Abcam) was conducted on 5 μm thick formalinfixed paraffin-embedded sections obtained after sacrifice of mice. Sections were collected on superfrost plus slides (Thermo Scientific). After deparaffinization and hydration, antigen retrieval was accomplished by heating (at 97°C) in 10 mmol/L sodium citrate buffer/0.05% Tween 20 (Sigma-Aldrich; pH 6.0) for 30 minutes. The sections were permeabilized with 1% Triton X-100 in PBS (Sigma-Aldrich) and blocked with PBS/10% BSA (Sigma-Aldrich). Mouse monoclonal primary antibodies, anti-CD31 (2F7B2, Novus Biologicals; 1:200 dilution), anti-α-SMA (1A4, Sigma Aldrich; 1:1000 dilution), and anti-CENPA (ab13939, Abcam), were applied for 16 hours at 4°C in PBS/10% BSA. Sections were then incubated with the secondary antibody (Alexa Fluor 488 Goat Anti-Mouse IgG1, 1:200, Invitrogen; and Alexa Fluor 555 Goat Anti-Mouse IgG2A) for 1 hour at room temperature in PBS/10% BSA. Slides were stained with 4′, 6-diamino-2phenylindole (DAPI; Sigma-Aldrich) and mounted with Fluorashield (Sigma-Aldrich). Negative controls without primary antibodies were conducted for all reactions.

Images were acquired using a Leica TCS SP5 confocal microscope, and sequential Z-stacks were conducted using 40× (1.4NA) oil immersion objective. For confocal imaging, all the z-stacks were collected on a Leica SP5 II confocal microscope (Leica Microsystems) using a 40× oil immersion objective with a numerical aperture of 1.3 at zoom 1.7. Fluorochromes were excited using a 405-nm diode laser for DAPI, an argon 488 laser for Alexa 488 and a 561 diode laser for Alexa 555. Detector slits were configured to minimize the cross-talk between channels and to maximize the signal arising from the sample: PMT1, 407 to 485 nm (DAPI); PMT2, 492 to 556 nm (Alexa 488); PMT3, 579 to 684 nm (Alexa 555); the cross-talk is also limited by the fact that the stacks were collected in “between frame mode,” i.e., the blue and red fluorescence (spectrally well separated) are collected simultaneously while the green one by itself.

Every single image of the collected z-stacks is 1,024 × 1,024 pixel² (about 228 × 228 μm²), whereas the z-step between 2 images within a stack is 0.8 μm resulting in a voxel size of 0.223 × 0.223 × 0.8 μm³. The acquisition parameters, the microscope, and all the detectors are controlled by means of the software LAS-AF (Leica Microsystems).

**EMT assay**

Epithelial-to-mesenchymal transition (EMT) expression pathway of cells was studied using 12 Transwell chambers with 0.4-μm pores (Corning). The Transwell membranes were precoated with collagen. A total of 8 × 10⁴ cells from WAT (total, CD34⁺, CD34⁻, ASCs, endothelial cells) in 500 μL of EBM2 were added to the upper chamber of each Transwell unit. The lower chamber was seeded with 4 × 10⁵ ZR75-1 (luminal) or MDA-MB-436 (triple negative) breast cancer cells in 1.5 mL of EBM2. After one week of coculture, without any changing of medium, the cells in both chambers were collected in RTL buffer (Qiagen) for RNA extraction. RNA isolation was carried out using QIAamp RNA Blood Mini Kit (Qiagen), and cDNA was generated from 1 μg of RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems); qRT-PCR was carried out with an ABI Prism 700 platform using the EMT PCR Array following the manufacturer instruction. The analysis was conducted with RT² Profiler PCR Array Data Analysis Web base.

**Time-lapse assays**

WAT-derived human cells were plated in EGM-2 medium (Lonza) 5 days before the experiment, in a 6-well plates precoated with rat tail collagen. Plates were placed in a 37°C, 5% CO₂ humidified incubator. Adherent cells were trypsin detached, washed once, and resuspended in EBM-2 medium (Lonza) without growth factors to a final volume of 80 μL of 50,000 WAT-cells and 50,000 MDA-MB-436 cells. Selected growth factors (100 ng/mL hepatocyte growth factor, 10 μg/mL insulin, 10 ng/mL PDGF, 100 ng/mL EGF) were polymerized in highly concentrated 3D type I collagen gels (10.21 mg/mL); on top of these gels, the suspension of MDA-MB-436 cells alone or in the presence of WAT-derived human cells was seeded and allowed to invade 3D collagen matrices as previously described (12). The invasive migration of tumor cells was then monitored by taking pictures every 5 minutes for 48 hours.

Time-lapse imaging of cell migration was conducted on an Eclipse TE2000-E inverted full-motorized microscope (Nikon) equipped with an incubation chamber (OKOLab) for temperature and CO₂ control. Movies were acquired by a Cascade II 512 CCD camera (Photometrics) controlled by Metamorph Software (Molecular Devices) using a 10×/0.30 NA magnification objective.

Tracking of cells was conducted using the “Manual Tracking” plugin distributed with ImageJ software. Single cells were followed and the rate of invasion, the speed of motion, the directionality, and the distance covered by tumoral cells were measured under the different experimental conditions.

**Renal capsule invasion assay**

As previously described by Walter and colleagues (13), either MDA-MB-436- cells (2 × 10⁶ cells) alone or mixed with an equal
number of WAT cells (CD34+, CD34−, ASCs, or endothelial cells) were injected under the renal capsule. Two weeks after cell transplantation, xenografts were fixed in 4% paraformaldehyde and processed by conventional methods. Penetration of the invading cell front into the host kidney was measured under ×100 magnification.

Results
Ultrastructural and molecular characterization of CD45−CD34+CD31+CD13+CCRL2+ (WAT endothelial cells) and CD45−CD34+CD31+CD140b+ (WAT-ASC) progenitors

As shown in Fig. 1, the multiparametric flow-cytometric investigation of nonhematopoietic (i.e., CD45−) cells present in human WAT showed the presence of 3 major cell populations: (i) CD34+/CD31−CD13−CCRL2− endothelial cells, (ii) CD34+/CD31−CD13−CD140b− ASCs, and (iii) mature CD34−CD13−CD140b− pericyte/fibroblasts. In more than 150 human WAT samples collected and processed as we previously described (5, 11), the percentage of nonhematopoietic (CD45−) cells was 55 ± 14%, and among those endothelial cells, ASCs and mature pericyte/fibroblasts were 50 ± 14, 42 ± 16, and 8 ± 7%, respectively.

As shown in Fig. 2, the very large majority of sorted human WAT CD34− endothelial cells were small, undifferentiated progenitors (Fig. 2A). These findings were consistent with previous data from Zimmerlin and colleagues (14), indicating that in human WAT mature endothelial cells are not CD34−. In fact, we found by flow cytometry, a small population (<5% of all WAT cells) of CD45−DNA(Syto)−CD31−CD34− cells that were likely mature endothelial cells. Among sorted endothelial cells, a few cells showed initial hallmarks of endothelial differentiation such as Weibel-Palade microtubulated bodies (Fig. 2B and C) and a few formed microcapillaries (Fig. 2D). At the present time, we believe that WAT CD45−CD34−CD31+ endothelial cells are a mixed population of endothelial cells enriched for endothelial progenitors. When investigated at the molecular level (data...
on file at www.ncbi.nlm.nih.gov/geo, NCBI tracking system #16724781), sorted endothelial cells (>96% purity) overexpressed endothelial-specific genes such as VE-cadherin, Claudin 5, Tie-2, VEGFR-1, 2, 3, ICAM-2, Dll4, and 16 out of the 18 genes were recently discovered in developing endothelial cells (15). Sorted endothelial cells, but not ASC, generated VE-cadherin-expressing endothelial cells in vivo and in vitro (Fig. 1D–F).

Array and confirmatory qPCR data indicated that purified endothelial cells expressed 3 to 16 times more CCRL2 mRNA compared than purified ASCs.

As shown in Fig. 3A, the large majority of human WAT ASCs were small, undifferentiated mesenchymal progenitors. A few of these cells showed hallmarks of initial adipose cell differentiation such as lipid droplets (Fig. 3B–E). Gene expression studies indicated that purified ASCs overexpressed perivascular cell genes such as Endosialin, Adam12, PDGFR, TGFβ, CD44, and RUNX1. Decreasing levels of CD34 expression among some ASCs (Supplementary Fig. S1) suggested that WAT CD45−CD34+CD31+CCRL2+ cells might derive from CD45+CD34+CD140b+ ASC progenitors. Further studies are ongoing to validate the hypothesis that CD34+ pericytes are generated by CD34+ ASCs.

When collected form human WAT, more than 98% of endothelial cells and ASCs were Ki-67 negative and in 2N (data not shown). Levels of CD34 gene expression were similar in WAT endothelial cells and ASCs.

**WAT endothelial cells and ASC have different trafficking potential**

We enumerated endothelial cells and ASCs in human lymph nodes, peripheral blood, and bone marrow by 10-color multiparametric flow cytometry following a validated approach with a sensitivity of 10⁻⁴ (5, 11, 16). Because lymph nodes are rich in high endothelial venule (HEV) cells (17), we also investigated the HEV-specific MECA 79 antigen to discriminate between HEV cells and endothelial cells (Supplementary Fig. S2). As shown in Fig. 4A and B, human lymph nodes were rich in endothelial cells, but ASCs were less than 1%.

In human bone marrow and peripheral blood, endothelial cells with a phenotype similar to WAT endothelial cells were always less than 0.1/mL and ASCs were always less than 0.1/μL. These data suggest that WAT endothelial cells and ASCs are extremely rare in human bone marrow and are not likely to traffic to peripheral blood in healthy subjects.

We measured the number of cells with a phenotype similar to WAT endothelial cells (CD45−CD34+CD31+CCRL2+ cells) and ASCs (CD45−CD34+CD31−CD140b− cells) in the peripheral blood of more than 30 healthy subjects and in the
peripheral blood of 15 patients with cancer before and after several different procedures for the mobilization of hematopoietic stem cells for autologous transplant (Fig. 4C). CD45\(^+\)CD34\(^+\) hematopoietic progenitors, endothelial cells, and ASCs/\(mL\) blood were similar in healthy subjects and in patients with cancer before mobilization. Endothelial cell count (always <0.1/\(\mu L\) peripheral blood before mobilization) was increased from 2 to 800/mL 5 days after G-CSF administration. The addition of chemotherapy and/or the CXCR4 inhibitor AMD3100 did not increase endothelial cell mobilization in the peripheral blood.

At variance with endothelial cells, G-CSF administration was associated only to a minimal mobilization of ASCs (from <0.1/\(\mu L\) peripheral blood before mobilization to a peak of 10–70/mL peripheral blood), and this mobilization was observed only in a minority (3 out of 15) of the patients investigated. Also in the case of ASCs, the addition of chemotherapy and/or AMD3100 to G-CSF did not increase endothelial cell mobilization in the peripheral blood.

Human WAT endothelial cells, when injected in murine mammary fat pads, generated vascular structures (Fig. 5A and B), migrated toward lymph nodes (Fig. 5C) and to contralateral nascent breast cancer lesions where they generated new vessels (Fig. 5D). Conversely, human ASCs did not generate vascular structures and did not migrate (data not shown).

**WAT endothelial cells and ASC have different roles in promoting breast cancer angiogenesis, growth, and metastases**

As shown in Fig. 6, WAT endothelial cells and ASCs increased local breast cancer growth in 2 different models of orthotopic breast cancer (5). In both the models, tumor growth was significantly increased by the coadministration of crude WAT cells. The coadministration of purified CD34\(^+\) WAT cells increased tumor volume as crude WAT cells, whereas CD34\(^-\) WAT cells were significantly less effective in increasing local tumor growth. These data suggest that in these models, the large majority (if not all) of the activity of WAT was due to cells included in the CD34\(^+\) fraction. The coadministration of purified endothelial cells or ASCs did not increase ASC mobilization in the peripheral blood.

Human WAT endothelial cells, when injected in murine mammary fat pads, generated vascular structures (Fig. 5A and B), migrated toward lymph nodes (Fig. 5C) and to contralateral nascent breast cancer lesions where they generated new vessels (Fig. 5D). Conversely, human ASCs did not generate vascular structures and did not migrate (data not shown).
whereas the number of lung metastases enumerated after the cocoadministration of human ASCs was significantly inferior.

Along a similar line, coculture with WAT endothelial cells significantly increased breast cancer cell invasion in the renal capsule assay (Supplementary Fig. S3).

When human breast cancer cells were coinjected with human WAT CD34\(^+\) cells, z-stack 3D studies identified functional vessels (containing red blood cells) with human endothelial cells and pericytes (Fig. 5 and Supplementary Figs. S4 and S5). When human endothelial cells (and not ASCs) were coinjected with tumors, we observed human CD31\(^+\) endothelial cells (but not human CD140b\(^+\) pericytes) participating into nascent functional tumor vasculature in the first 2 to 3 weeks after injection (Fig. 6D). Conversely, when human ASCs (and not endothelial cells) were coinjected with tumors, we observed human CD140b\(^+\) pericytes (but not human CD31\(^+\) endothelial cells) participating into tumor vasculature for more than one month after injection (Fig. 6E).

**WAT endothelial cells and ASC induce epithelial-to-mesenchymal transition gene expression in breast cancer luminal cells**

WAT CD34\(^+\) cells (but not CD34\(^-\) cells) induced the overexpression of several genes crucial for EMT in breast cancer cells (Supplementary Table S2). Interestingly, EMT gene expression was significantly enhanced in breast cancer cell lines of the luminal subtype such as ZR75-1, and less significant in breast cancer cell lines of more undifferentiated and aggressive triple-negative subtype such as MDA-MB-436 (data not shown). This suggests that WAT CD34\(^+\) cells might enhance the metastatic potential of luminal breast cancer cells, which, per se, have a poorer metastatic potential when compared with triple-negative breast cancer cells. When cocultured alone with breast cancer cell lines, both endothelial cells and ASCs increased the expression of crucial EMT genes, albeit with different efficiency. For instance, endothelial cells induced a more relevant overexpression of FOXC2 and VIM, whereas ASCs induced a more relevant overexpression of FGFBP1 and WNT11. In culture studies, both ASCs and endothelial cells induced a significant switch toward a mesenchymal morphology in luminal breast cancer cells (Supplementary Fig. S6).

**WAT endothelial cells and ASC cooperate in inducing breast cancer cell migration**

In time-lapse culture studies (Fig. 7), WAT CD34\(^+\) cells (but not CD34\(^-\) cells) induced a significant increase in breast cancer cell migration toward chemoattractants such as HGF, insulin, PDGF, and EGF. When on their own, purified endothelial cells and ASCs had significantly different activity in this assay. Purified endothelial cells were as efficient as total CD34\(^+\) WAT
cells and significantly more efficient than purified ASCs in increasing all the migration parameters (Y forward migration index, mean accumulated distance, mean Euclidean distance, mean velocity) measured in the assay. As shown in Supplementary Fig. S7, breast cancer cells (and not endothelial cells) were found to migrate toward chemoattractants.

**Discussion**

According to Varmus and Harlow (18), deciphering how WAT and obesity promote cancer progression is among the most urgent and provocative questions in cancer research. It is known that adipokine signaling may foster neoplasia (reviewed in ref. 1). Recent studies have also suggested that WAT and obesity can support cancer onset and progression by means of WAT-residing cells. For instance, Dirat and colleagues (7) reported that cancer-associated WAT cells exhibit an activated phenotype and contribute to breast cancer invasion. Kidd and colleagues (8) have shown in syngenic models of ovarian and breast tumors that most vascular and fibrovascular stroma (including pericytes and endothelial cells) originates from neighboring WAT and not from bone marrow-derived cells. Chandler and colleagues (9) showed that WAT cells can act as physicochemical regulators of breast cancer cells. The Kolonin laboratory (6, 10) indicated that endogenous WAT progenitors contribute to tumor pericytes and adipocytes, regardless of the type of diet administered to the mice. Zhao and colleagues (19) reported that breast WAT cells stimulate progression of basal-type breast cancer. These studies were carried out using poorly defined WAT cell populations. Our current data indicate that two WAT progenitor cell populations, namely ASCs and endothelial cells, have separate and complementary roles in breast cancer angiogenesis, local progression, and metastatic spreading. ASCs can incorporate into tumor vasculature as pericytes and promote local tumor growth and EMT. WAT endothelial cells can migrate to lymph nodes and peripheral blood, generate endothelial cells in tumor vessels, induce EMT, and increase breast cancer spreading and metastases. Notably, the induction of EMT gene overexpression was more relevant in breast cancer cell lines representative of the luminal subtype when compared with more aggressive triple-negative cell lines. Our present preclinical studies shed light on clinical data from our institution, indicating that patients with breast cancer with intraepithelial neoplasia who received autologous WAT cells for breast reconstruction after surgical removal of breast cancer show an increased risk of recurrence of local events when compared with controls (11, 20, 21).

In a previous study (5), we showed that human WAT CD34⁺ cells can generate functional vessels in orthotopic models of breast cancer. For a proper maturation and function of the vascular wall, interdependency between endothelial cells and mural cells (including pericytes and vascular smooth muscle cells) is required. Current data indicate that in our breast cancer models, both endothelial cells and ASCs cooperate in this mechanism, acting as a source for pericytes (ASCs) and mature endothelial cells/capillaries (WAT endothelial cells). It would be relevant to investigate in the future whether anti-VEGF drugs would reduce the level of WAT-derived endothelial cells in preclinical models of breast cancer.

Communication between ASCs and endothelial cells can take place via direct cellular or via paracrine interactions (22). Possible candidates in the paracrine interplay are angiopoietins and their Tie receptors. The receptor tyrosine kinase, Tie2, is expressed in endothelial cells and is stimulated by

**Figure 5.** Migration of WAT endothelial cells. When injected in murine mammary fat pads, purified human WAT endothelial cells generated vascular structures and were able to migrate to contralateral neoplasia. A, a murine mammary fat pad before the injection of human endothelial cells. B, the mammary fat pad 7 days after the injection of human WAT endothelial cells, which were depicted by human-specific CD34 antibodies. C, the migration of human endothelial cells 10 days after injection (arrow). D, human endothelial cells incorporating the vasculature of contralateral nascent breast cancer lesions 21 days after injection. E, a control from a tumor-bearing mouse not injected with endothelial cells.
angiopoietin-1 (Ang-1) secreted by surrounding mesenchyme and perivascular cells (23). Ang-1 is believed to be involved in vessel maturation (24). In our current studies, WAT ASCs overexpressed Ang-1 and several related molecules such as ANGPT1, 2, and 4, and endothelial cells overexpressed related receptors such as Tie 1 and 2. We are currently investigating whether targeting these axes can significantly impair the contribution of ASCs and/or endothelial cells to breast cancer progression.

We observed in WAT endothelial cells, an overexpression of CCRL2 (Fig. 1 and Supplementary Fig. S8). This molecule has been described as a receptor for the chemokine/adipokine chemerin, involved in autocrine and paracrine signaling for adipocyte differentiation, and found to stimulate chemotaxis of dendritic cells and macrophages to the site of inflammation (25). Chemerin was overexpressed in WAT ASCs when compared with endothelial cells, and we are currently studying whether targeting CCRL2 and/or chemerin might impair endothelial cell trafficking toward WAT and/or the tumor and metastases promoting the role of endothelial cells and ASCs. Interestingly, another known chemerin receptor, CMKLR1, was overexpressed in WAT ASCs.

MFGE8, a gene recently involved in follicular dendritic cell (FDC) maturation from mural cell (26), was overexpressed in human WAT ASCs (data not shown). This indicates that WAT ASCs might be studied in the future as a source of FDC trafficking (possibly when differentiated) toward neoplasia and attracting cancer-specific B cells.

The close anatomic association between lymph nodes and WAT deposits (17) and the possible metastatic spreading of breast cancer cells through lymph nodes prompted us to investigate the trafficking of WAT endothelial cells and ASCs toward lymph nodes. Bénézech and colleagues (27) showed that embryonic mesenchymal cells with characteristics of

Figure 6. WAT endothelial cells and ASCs in models of orthotopic breast cancer and metastatic spreading. A, orthotopic tumor growth in the mammary fat pad of NSG mice injected with WAT CD34+ cells alone, triple-negative MDA-MB-436 cells alone, MDA-MB-436 cells plus CD34+ (or CD34−) WAT cells, and MDA-MB-436 cells plus purified endothelial cells or ASC WAT cells. B, orthotopic 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 total CD34+ WAT cells, endothelial cells, or ASCs. C, the number of lung metastases in NSG mice injected with MDA-MD-436 breast cancer cells in models evaluated after tumor surgical removal. D, the frequency of human versus murine endothelial cells (as depicted by human-specific anti-CD31 staining in green) in the tumor of NSG mice injected with human purified endothelial cells. E, the frequency of human versus murine pericyte cells (as depicted in red by human-specific anti-CD140b staining) in the tumor of NSG mice injected with human purified ASCs.

\[P < 0.01\] versus control by Mann-Whitney U test.
adipocyte precursors present in the lymph node microenvironment can give rise to lymph node organizer cells. Signaling through the lymphotoxin-β receptor controlled the fate of WAT progenitors by blocking adipogenesis and instead promoting lymphoid tissue stromal cell differentiation. This effect involved activation of the NF-κB2-RelB signaling pathway and inhibition of the expression of the key adipogenic factors Ppar and Cebp. Our current data indicate that endothelial cells have a robust lymph node and peripheral blood migration potential, whereas the lymph node and peripheral blood migration potential of ASCs is limited, at least in our murine preclinical models of breast cancer and in humans receiving stem cell mobilization stimuli such as G-CSF, chemotherapY, and/or CXCR4 inhibitors. The failure of some chemotherapy drugs and of CXCR4 inhibitors to mobilize WAT endothelial cells is in clear contrast with the potential of both these agents to mobilize bone marrow-derived endothelial progenitor cells, and deserves further mechanistic investigation. We have preliminary evidence (Orecchioni and colleagues, manuscript in preparation) that purified human WAT endothelial cells, when injected in murine mammary fat pads, can migrate to lymph nodes and controlateral breast cancer. Cells with this phenotype were found in lymph nodes and WAT, but not in other organs including the hematopoietic system. We cannot rule out at the present time that CD45+CD34+CD31+CCL2 cells can be mobilized by G-CSF from organs other than WAT and lymph nodes.

During development and in a variety of morphogenetic events, epithelial cells can undergo EMT (reviewed in ref. 28). In this process, the cells lose their epithelial characteristics, including their polarity and specialized cell–cell contacts, and acquire a migratory behavior, allowing them to traffic and to integrate into surrounding tissue, even at remote locations. EMT can be recapitulated under pathologic conditions such as fibrosis, invasion, and metastasis of carcinomas. Both endothelial cells and ASCs were found to induce an overexpression of several EMT genes in breast cancer cells, and particularly in luminal breast cancer cells. EMT and epithelial plasticity can be regulated and induced by TGFβ-related bone morphogenetic proteins (BMP). We found that ASCs (and, to a slightly lesser extent, endothelial cells) overexpressed several BMPs and related receptors such as BMP1, 2, and 4, and BMPRIA. Studies are ongoing to investigate whether the inhibition of these BMPs and related receptors might impair the EMT-promoting activity of WAT progenitors in vitro and in vivo.

Taken together, our data suggest that WAT progenitors can cooperate in the local progression and metastatic spreading of cancer in the breast, which is usually rich in WAT.
The low contribution of endothelial progenitor cells to tumor vasculature previously observed by some groups (reviewed in refs. 29–31) may have been due, in large part, to the exclusive use of bone marrow-derived cell populations. On the basis of the results summarized in this paper, one would assume that endothelial progenitor cells may indeed make a meaningful contribution to new blood vessel formation but this would be unappreciated when doing only bone marrow transfer experiments using fluorescent-labeled or genetically tagged donor bone marrow cells in irradiated mice, and then looking for incorporation of such cells into new blood vessels. Future studies will investigate (i) the role of WAT progenitors in neoplasia originating in other WAT-rich (e.g., colon) and WAT-poor (e.g., lung) organ and tissues, and (ii) validate at the preclinical and clinical level the therapeutic potential of targeting WAT progenitors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


29. Yoder MC, Ingram DA. The definition of EPCs and other bone marrow cells contributing to neoangiogenesis and tumor growth: is there common ground for understanding the roles of numerous marrow-derived cells in the neoangiogenic process? Biochim Biophys Acta 2009;1796:50–4.

30. Shaked Y, Voest EE. Bone marrow derived cells in tumor angiogenesis and growth: are they the good, the bad, or the evil? Biochim Biophys Acta 2009;1796:1–4.

Complementary Populations of Human Adipose CD34+ Progenitor Cells Promote Growth, Angiogenesis, and Metastasis of Breast Cancer

Stefania Orecchioni, Giuliana Gregato, Ines Martin-Padura, et al.

Cancer Res Published OnlineFirst August 5, 2013.