Complementary populations of human adipose CD34+ progenitor cells promote growth, angiogenesis and metastasis of breast cancer

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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-CCRL2+ endothelial cells (ECs) 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 (ASCs) which generated pericytes and were more efficient than ECs in promoting local tumor growth. Both ECs and ASCs induced EMT gene expression in luminal breast cancer cells. ECs (but not ASCs) migrated to lymph nodes and to contralateral nascent breast cancer lesions where they generated new vessels. In vitro and in vivo, ECs were more efficient than ASCs in promoting tumor migration and in inducing metastases. G-CSF effectively mobilized ECs (but not ASCs), and the addition of chemotherapy and/or of CXCR4 inhibitors did not increase EC or ASC blood mobilization. Our findings suggest that adipose tissue progenitor cells cooperate in driving progression and metastatic spread of breast cancer.
Introduction

There is increasing evidence that obesity, an excess accumulation of adipose tissue occurring in mammals 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 1). Disruption of the energy homeostasis results in obesity, inflammation and alterations of adipokine signalling 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. We described that CD45-CD34+ progenitors from human white adipose tissue (WAT) may promote breast cancer growth and metastases in preclinical models (5). Other recent studies, some of which based on endogenous WAT expressing a transgenic reporter, demonstrated a significant level of adipose cell contribution to tumor composition (6-10). However, WAT contains several distinct populations of progenitors, and these data were obtained using crude or mixed cell populations. We therefore decided to purify by sorting the two quantitatively most relevant populations of WAT progenitors (endothelial cells, ECs, and adipose stromal cells, ASCs) and to investigate in vitro and in vivo their role in several orthotopic models of local and metastatic breast cancer.
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 1200g to remove erythrocytes and leukocytes and subsequently digested in HBSS (Gibco, UK) containing 2 mg/mL of collagenase type I (Sigma Aldrich, St. Louis, MO, USA) 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, Italy), and a cell pellet was obtained by centrifugation at 1200g for 10 minutes at 4°C.

The cell suspension was then processed through a 100 μm mesh filter to remove undigested tissue and washed twice with incubation buffer (PBS with 2 mM EDTA and 0.5% BSA), working always on ice. An aliquot of these cells was labeled for flow cytometry analysis. At least 500,000 total cells per sample were acquired on a flow cytometer equipped with 3 laser (Navios, Beckman Coulter, Pasadena, CA, USA) and analysis was performed by a KALUZA software (Beckman Coulter), after selection of DNA+ (Syto16+) and viable (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 1.

Hematopoietic CD45-CD34+ stem cells, ECs and ASCs were measured in the BM and in the PB by flow cytometry as described above in >30 healthy volunteers and in cancer patients (who signed an informed consent) before and after stem cell mobilization by G-CSF, cyclophosphamide+G-CSF or AMD3100+G-CSF.
**Cell sorting**

CD34+ microbeads-purified cells were labeled with sterile CD45FITC, CD13PE, CD31PeCy7 and CD34APC monoclonal antibodies, and resuspended in PBS1X/EDTA 2mM/FBS1% for cell sorting using a three laser Influx high speed cell sorter (BD, Mountain View, CA, USA) 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 performed to exclude cell clusters and doublets. A two way cell sorting procedure was performed with a 140μm nozzle with a 5.5 PSI pressure, and with an events rate of 1,000-1,500 events per second, using a sort pure mode. Samples were collected into sterile polypropylene tubes containing 20% bovine serum (FBS, Euroclone, Italy) and used for in-vitro and in vivo studies. Analysis was performed using a FACS-Software software (BD). Purity was always greater than 96% with a recovery of 70-80%.

**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 (Manassas, VA) and cultured as previously described (5). The cell lines were tested and authenticated by means of Stem Elite ID system (Promega, Fitchburg, WI) in January 2013. Prior to 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 (EC) cultures and capillary tubes were generated as previously described (5, 11).
Orthotopic xenograft transplantation

Female NOD SCID IL2RG null mice (NSG), 6 to 9 weeks old, were bred and housed under pathogen-free conditions in our animal facilities at the European Institute of Oncology–Italian Foundation for Cancer Research (FIRC) Institute of Molecular Oncology (IEO-IFOM, Milan, Italy). Mice were expanded from breeding pairs kindly donated by Dr. Leonard Shultz, The Jackson Laboratory, Bar Harbor, ME, USA. All animal experiments were carried out in accordance with the Italian Laws (D.L.vo 116/92 and following additions), and institutional guidelines.

To produce orthotopic primary tumors, 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 as previously described (5).

Briefly, prior to injection tumor cells were trypsin detached, washed twice, and resuspended in PBS to a final concentration of 10^6 cells/13 µL. The cell suspension was then mixed with 5 µL of Matrigel and 2 µL of trypan blue solution and maintained on ice until injection.

When tumor cells were coinjected with 2 × 10^5 purified CD34+ WAT cells (or total WAT, or CD34- WAT cells, or purified ECs or ASCs), cell suspensions were mixed together before adding Matrigel. Surgical procedure was performed in aseptic conditions under a laminar flow hood.

Mice were anesthetized with 2.5% 2-2-2-tribromoethanol (Avertin; Sigma Aldrich), laid on their backs, and injected with 20 µL of cell suspension in Matrigel directly in the fourth mammary fat pad through the nipple with a Hamilton syringe.

Tumor growth was monitored at least once a week using digital callipers, and tumor volume was calculated according to the formula: \( V = \frac{L \times W^2}{2} \) mm\(^3\), where \( W \) represents the width and \( L \) the length of the tumor mass.
Breast cancer metastases model

Tumor resection (mastectomy) 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 anesthesing 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 ECs of 7 different patients were carried out using QIAamp RNABlood Mini Kit (Qiagen, the Netherlands), and cDNAs were generated from 40 ng of RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA); quantitative real-time PCR (qRT-PCR) was carried out with an ABI Prism 700 platform as previously described (Martin-padura et al., 2012) 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, Santa Clara, CA, USA), 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 ECs on tumor angiogenesis, immunofluorescence analysis of CD31, α-SMA (1A4, Sigma Aldrich) and CENP-A (ab13939, AbCam, Boston, MA, USA) was performed on 5 μm-thick FFPE sections obtained after sacrifice of mice. Sections were collected on superfrost plus slides (Thermo Scientific, Waltham, MA, USA). After deparaffinization and hydration, antigen retrieval was accomplished by heating (at 97°C) in 10 mM sodium citrate buffer/0.05% Tween 20 (Sigma-Aldrich) (pH 6.0) for 30 min. 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, Cambridge, UK; 1:200 dilution), anti-α-SMA (1A4, Sigma Aldrich; 1:1000 dilution) and anti-CENPA (ab13939, Abcam) were applied for 16 h 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, Carlsbad, CA, USA; and Alexa Fluor® 555 Goat Anti-Mouse IgG2A) for 1 h at RT in PBS/10% BSA. Slides were stained with 49,6-
diamino-2-phenylindole (DAPI) (Sigma-Aldrich) and mounted with Fluorashield™ (Sigma-Aldrich). Negative controls without primary antibodies were performed for all reactions. Images were acquired using a Leica TCS SP5 confocal microscope, and sequential Z-stacks were performed using 40X (1.4NA) oil immersion objective.

For confocal Imaging, all the z-stacks were collected on a Lieca SP5 II confocal microscope (Leica Microsystems, Exton, PA) using a 40X 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 in order to minimize the cross talk between channels and to maximize the signal arising from the sample: PMT1, 407-485 nm (Dapi); PMT2, 492-556 nm (Alexa 488); PMT3, 579-684 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 1024x1024 pixel² (about 228x228 um²) while the z-step between two images within a stack is 0.8 um resulting in a voxel size of 0.223x0.223x0.8 um³.

The acquisition parameters, the microscope and all the detectors are controlled by means of the software LAS-AF (Leica Microsystems, Exton, PA).

**EMT assay**

EMT expression pathway of cells was studied using 12 Transwell chambers with 0.4-μm pores (Corning, Corning, NY, USA). The Transwell membranes were precoated with collagen. 8x10⁴ cells from WAT (total, CD34+, CD34-, ASCs, ECs) in 500 μl of EBM2 were added to the upper chamber of each Transwell unit. The lower chamber was seeded with 4x10⁵ ZR75-1 (luminal) or MDA-MB-436 (triple negative) breast cancer cells in 1.5ml of...
EBM2. After one week of coculture, without any changing of medium, the cells in both chambers were collected in RTL buffer (Qiagen, the Netherlands) for RNA extraction. RNA isolation was carried out using QIAmp RNA Blood Mini Kit (Qiagen), and cDNA was generated from 1µg 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 using the EMT PCR Array following the manufacture instruction. The analysis were performed 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 (HGF 100ng/ml, insulin 10µg/ml, PDGF 10ng/ml, EGF 100ng/ml) 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 performed on an Eclipse TE2000-E inverted full motorized microscope (Nikon, Japan) equipped with an incubation chamber (OKOlab) for temperature and CO₂ control. Movies were acquired by a Cascade II 512 CCD camera (Photometrics, Japan) controlled by Metamorph Software (Molecular Devices, Sunnyvale, CA, USA) using a 10×/0.30 NA magnification objective.
Tracking of cells was performed 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 et al. (13), either MDA-MB-436 cells (2 × 10^6 cells) alone or mixed with an equal number of WAT cells (CD34+, CD34-, ASCs or ECs) 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 100x magnification.
Results

Ultrastructural and molecular characterization of CD45-CD34+CD31+CD13-CCRL2+ (WAT-ECs) and CD45-CD34+CD31-CD13+CD140b+ (WAT-ASC) progenitors

As shown in Fig. 1, the multiparametric flow cytometry investigation of non-hematopoietic (ie CD45-) cells present in human WAT showed the presence of three major cell populations: a) CD34+CD31+CD13-CCRL2+ ECs, b) CD34+CD31-CD13+CD140b+ ASCs, and c) mature CD34-CD13+CD140b+ pericyte/fibroblasts. In >150 human WAT samples collected and processed as we previously described (5, 11), the percentage of non hematopoietic (CD45- cells) was 55±14%, and among those ECs, 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+ ECs were small, undifferentiated progenitors (Fig. 2A). These findings were consistent with previous data from Zimmerlin et al (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 ECs, a few cells showed initial hallmarks of endothelial differentiation such as Weibel Palade microtubulated bodies (Fig 2 B-C) and a few formed microcapillaries (Fig. 2D). At the present time, we believe WAT CD45-CD34+CD31+ ECs 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 ECs (>96% purity) overexpressed endothelial-specific genes such as VE-Cadherin, Claudin 5, Tie-2, VEGFR1,2,3, ICAM-2, Dll4, and 16 out of the 18 genes recently discovered in developing endothelial cells (15). Sorted ECs, but not ASC, generated VE-Caderin-expressing endothelial cells in vivo and in vitro (Fig. 1D-F).
Array and confirmatory quantitative PCR data indicated that purified ECs expressed 3-16 times more CCRL2 mRNA compared to 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, PDGF receptors, TGFbeta, CD44 and RUNX1. Decreasing levels of CD34 expression among some ASCs (Supplementary Fig. 1) suggested that WAT CD45-CD34-CD140b+ pericyte/fibroblasts 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 from human WAT, more than 98% of ECs and ASCs were Ki-67 negative and in 2N (data not shown). Levels of CD34 gene expression were similar in WAT ECs and ASCs.

**WAT-ECs and ASC have different trafficking potential**

We enumerated ECs and ASCs in human lymph nodes (LNs), peripheral blood (PB) and bone marrow (BM) by 10-color multiparametric flow cytometry following a validated approach with a sensitivity of $10^{-4}$ (5, 11, 16). Since LNs are rich in high endothelial venule (HEV) cells (17), we also investigated the HEV-specific MECA 79 antigen to discriminate between HEV cells and ECs (Supplementary Fig. 2). As shown in Fig. 4A-B, human LNs were rich in ECs, but ASCs were <1%.

In human BM and PB, ECs with a phenotype similar to WAT ECs were always <0.1/µL and ASCs were always <0.1/µL. These data suggest that WAT ECs and ASCs are extremely rare in human BM and are not likely to traffic to PB in healthy subjects.
We measured the number of cells with a phenotype similar to WAT ECs (CD45-CD34+CD31+CCRL2+ cells) and ASCs (CD45-CD34+CD31-CD13+CD140b+ cells) in the PB of >30 healthy subjects and in the PB of 15 cancer patients before and after several different procedures for the mobilization of hematopoietic stem cells for autologous transplant (Fig 4C). CD45+CD34+ hematopoietic progenitors, ECs and ASCs/µL blood were similar in healthy subjects and in cancer patients before mobilization. EC count (always <0.1/µL PB before mobilization), was increased to 2-800/mL 5 days after G-CSF administration. The addition of chemotherapy and/or the CXCR4 inhibitor AMD3100 did not increase EC mobilization in the PB.

At variance with ECs, G-CSF administration was associated only to a minimal mobilization of ASCs (from <0.1/µL PB before mobilization to a peak of 10-70/mL PB), 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 ASC mobilization in the PB.

Human WAT ECs, when injected in murine mammary fat pads, generated vascular structures (Fig. 5A-B), migrated toward LNs (Fig. 5C) and to contra-lateral 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-ECs and ASC have different roles in promoting breast cancer angiogenesis, growth and metastases

As shown in Fig. 6, WAT ECs and ASCs increased local breast cancer growth in two different models of orthotopic breast cancer (5). In both models, tumor growth was significantly increased by the co-administration of crude WAT cells. The co-administration 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 co-administration of purified ECs or ASCs increased tumor volumes, but in both models purified ASCs were slightly more efficient than purified ECs in promoting local tumor growth (Fig. 6A-B).

The role of human WAT CD34+ cells, ECs and ASCs was then studied in a preclinical orthotopic model of breast cancer metastatic spreading in the lung after mastectomy (5). As shown in Fig. 6C, the co-injection of CD34+ cells significantly increased the number of lung metastases in mice over controls and mice injected with human CD34- WAT cells. The co-injection of human ECs increased the number of lung metastases as efficiently as CD34+ cells, whereas the number of lung metastases enumerated after the co-administration of human ASCs was significantly inferior. Along a similar line, co-culture with WAT ECs significantly increased breast cancer cell invasion in the renal capsule assay (Supplementary Fig. 3).

When human breast cancer cells were co-injected 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 supplemental Fig. 4-5). When human ECs (and not ASCs) were co-injected with tumors, we observed human CD31+ endothelial cells (but not human CD140b+ pericytes) participating into nascent functional tumor vasculature in the first 2-3 weeks after injection (Fig. 6D). Conversely, when human ASCs (and not ECs) were co-injected 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-ECs and ASC induce epithelial to mesenchymal transition (EMT) 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 Tab. 2). Interestingly, EMT gene overexpression 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 to triple negative breast cancer cells. When co-cultured alone with breast cancer cell lines, both ECs and ASCs increased the expression of crucial EMT genes, albeit with different efficiency. For instance, ECs induced a more relevant overexpression of FOXC2 and VIM, while ASCs induced a more relevant overexpression of FGFBP1 and WNT11. In culture studies, both ASCs and ECs induced a significant switch towards a mesenchymal morphology in luminal breast cancer cells (Supplementary Fig. 6).

**WAT-ECs 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 ECs and ASCs had significantly different activity in this assay. Purified ECs 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. 7, breast cancer cells (and not ECs) 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 signalling may foster neoplasia (reviewed in 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 et al. (7) reported that cancer-associated WAT cells exhibit an activated phenotype and contribute to breast cancer invasion. Kidd et al. (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 BM-derived cells. Chandler et al. (9) demonstrated 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 et al (19) reported that breast WAT cells stimulate progression of basal-type breast cancer. These studies, though, were carried out using poorly defined WAT cell populations. Our current data indicate that two WAT progenitor cell populations, namely ASCs and ECs, 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 ECs can migrate to LNs and PB, 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 to more aggressive triple negative cell lines. Our present preclinical studies shed light on clinical data from our Institution indicating that breast cancer patients 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 to controls (11, 20-21).

In a previous study (5), we demonstrated 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 ECs and ASCs cooperate in this mechanism, acting as a source for pericytes (ASCs) and endothelial cells (ECs). It would be relevant to investigate in the future whether anti-VEGF drugs would reduce the level of WAT-derived ECs in preclinical models of breast cancer.

Communication between ASCs and ECs 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 angiopoietin-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 angiopoietin-1 and several related molecules such as ANGPLT 1, 2 and 4, and ECs 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 ECs to breast cancer progression.

We observed in WAT ECs an overexpression of CCRL2 (Fig. 1 and Supplementary Fig. 8). 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 to ECs, and we are currently studying whether targeting CCRL2 and/or chemerin might impair EC trafficking towards WAT and/or the tumor and metastases promoting role of ECs and
ASCs. Interestingly, another known chemerin receptor, CMKLR1, was overexpressed in WAT ASCs.

MFG8, a gene recently involved in follicular dendritic cell (FDC) maturation from mural cell (26), was overexpressed in human WAT ASCs (data not shown). This indicate that WAT ASC might be studied in the future as a source of FDC trafficking (possibly when differentiated) towards neoplasia and attracting cancer-specific B cells.

The close anatomical association between LNs and WAT deposits (17) and the possible metastatic spreading of breast cancer cells through LNs prompted us to investigate the trafficking of WAT ECs and ASCs towards LNs. Benezech et al. (27) showed that embryonic mesenchymal cells with characteristics of adipocyte precursors present in the LN microenvironment can give rise to LN 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 Pparγ and Cebpα. Our current data indicate that ECs have a robust LN and PB migration potential, whereas the LN and PB 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 ECs is in clear contrast to the potential of both these agents to mobilize BM-derived ECs, and deserves further mechanistic investigation. We have preliminary evidence (Orecchioni et al, manuscript in preparation) that purified human WAT ECs, when injected in murine mammary fat pads, can migrate to LNs, and controlateral breast cancer. Cells with this phenotype were found in LNs and WAT, but not in other organs including the hematopoietic system. We can not rule out at the present time that CD45-CD34+CD31+CCRL2 cells can be mobilized by G-CSF from organs other than WAT and...
LNs.

During development and in a variety of morphogenetic events, epithelial cells can undergo EMT (reviewed in 28). In this process, the cells lose their epithelial characteristics, including their polarity and specialized cell-cell contacts, and acquire a migratory behaviour, allowing them to traffic and to integrate into surrounding tissue, even at remote locations. EMT can be recapitulated under pathological conditions such as fibrosis, invasion and metastasis of carcinomas. Both ECs 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 TGF-beta-related bone morphogenetic proteins (BMPs). We found that ASCs (and, to a slightly lesser extent, ECs) overexpressed several BMPs and related receptors such as BMP1, 2, and 4 and BMPR1A. 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 ECs to tumor vasculature previously observed by some groups (reviewed in 29-31) may have been due in large part to the exclusive use of BM-derived cell populations. Based on the results summarized in this paper, one would assume that ECs may indeed make a meaningful contribution to new blood vessel formation but this would be unappreciated when doing only BM transfer experiments using fluorescent labeled or genetically tagged donor BM cells in irradiated mice, and then looking for incorporation of such cells into new blood vessels."

Future studies will investigate a) the role of WAT progenitors in neoplasia originating in other WAT-rich (eg colon) and WAT-poor (eg lung) organ and tissues, and b) validate at the preclinical and clinical level the therapeutic potential of targeting WAT progenitors.
References


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Figure Legends

Fig. 1: Multiparamentric flow cytometry investigation of non-hematopoietic (ie CD45-) cells present in human WAT indicating the presence of three major cell populations.

Representative 10-color flow cytometry evaluation of CD45-CD34+ cells in human WAT tissue from lipotransfer procedures. Panel A shows the gate used to investigate viable (7AAD-) DNA+ (Syto16+) cells. Panel B shows the gate on viable DNA+CD45-CD34+ cells. Panels C-F show the expression of CD13, CD31, CD140b (PDGFR), and CCRL2 in the three populations of CD34+CD45-CD31+CCRL2+CD140b- (ECs), CD34+CD45-CD31-CD140b+ (ASCs) and CD34-CD45-CD140b+ cells (mature pericyte/fibroblasts). As depicted by immunofluorescence (Blue=DNA; Green=VE-cadherin), only ECs (D), but not ASCs (E) or mature pericyte/fibroblasts (F) generated in vitro VE-Cadherin+ endothelial cells.

Fig. 2: Electron microscopy investigation of purified CD45-CD34+CD31+ WAT ECs.

Three different differentiation’s stage of CD 34+CD31+CD13- cells from a precursor (A) to capillary forming cell (D). A Weibel Palade microtubulated body is evident in B and in C where the enlargement of the boxed area is shown. A,B,D scale bar = 1μm, D scale bar = 200 nm

Fig. 3: Electron microscopy investigation of purified CD45-CD34+CD31-CD13+ WAT ASCs

A, B, C: Three different differentiation’s stage of CD 34+ CD13+ cells from mesenchymal precursor (A) toward adipose cell (B, C) bearing lipid droplets (L) D,E: Enlargements of the boxed areas in B and C respectively. Scale bars = 1μm.
Fig. 4: Traffic and mobilization of cells with CD45-CD34+CD31+CCRL2+ (EC) and CD45-CD34+CD13+CD140b+ (ASC) phenotype.

Panels A and B show the percentages of cells with ECs, ASCs, HEVs, and pericyte/fibroblasts phenotype among non-hematopoietic (CD45-) human LNs (A) and WAT (B). Human LNs were rich in ECs (about 35% of all CD45- non-hematopoietic LN cells), ASCs were <1%.

Panel C shows PB cells with EC (CD45-CD34+CD31+CCRL2+) and ASCs (CD45-CD34+CD31-CD13+CD140b+) phenotype before and after several different procedures for the mobilization of hematopoietic stem cells in cancer patients.

*=p<0.001 vs control by Mann-Whitney U test

Fig. 5: Migration of WAT ECs.

When injected in murine mammary fat pads, purified human WAT ECs generated vascular structures and were able to migrate to contra-lateral neoplasia. Panel A shows a murine mammary fat pad before the injection of human ECs. Panel B shows the mammary fat pad 7 days after the injection of human WAT ECs, which were depicted by human specific CD34 antibodies. Panel C shows the migration of human ECs 10 days after injection (arrow), and panel D shows human ECs incorporating the vasculature of contra-lateral nascent breast cancer lesions 21 days after injection. Panel E shows a control from a tumor-bearing mice not injected with ECs.

Fig. 6: WAT ECs and ASCs in models of orthotopic breast cancer and metastatic spreading.

Panel A shows 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
unfractionated WAT cells, MDA-MB-436 cells plus CD34+ (or CD34-) WAT cells and MDA-MB-436 cells plus purified ECs or ASCs WAT cells.

Panel B shows 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, ECs or ASCs.

Panel C shows show the number of lung metastases in NSG mice injected with MDA-MB-436 breast cancer cells in models evaluated after tumor surgical removal.

Panel D shows the frequency of human vs murine endothelial cells (as depicted by human-specific anti-CD31 staining in green) in the tumor of NSG mice injected with human purified ECs.

Panel E shows the frequency of human vs murine pericytes 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 vs control by Mann-Whitney U test

Fig. 7: *Time lapse culture studies showing breast cancer migration toward chemoattractants.*

As shown in representative top panels, human WAT cells increase breast cancer MDA-MB-436 cell migration and velocity towards chemoattractants. When on their own (lower panels), purified ECs and ASCs had significantly different activity in this assay. Purified ECs were significantly more efficient than total CD34- cells and purified ASCs in increasing the mean distance run by breast cancer cells in the assay and in increasing breast cancer cell velocity in the assay.

*=p<0.01 vs control by Mann-Whitney U test
**Fig. 1**

A: Total Cells

B: Viable and Nucleated Cells

C: CD45+.cells

D: EPCs

E: ASCs

F: CD34-CD31- cells

pericytes/fibroblasts
Fig. 4

Lymph node

<table>
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<tr>
<th>EPCs</th>
<th>ASCs</th>
<th>Pericytes</th>
<th>High endothelial venules</th>
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Adipose tissue

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<tr>
<th>EPCs</th>
<th>ASCs</th>
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<th>High endothelial venules</th>
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Peripheral blood

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* * *
Fig. 6

(A) Tumor volume (mm³) vs. Days
(B) Tumor volume (mm³) vs. Days

Lung Metastases

C

Controls
CD34- injected
CD34+ injected
ASCs injected
EPCs injected

D

Human vessels
Murine vessels

E

Human pericyte
Murine pericyte

Days
0 29 62 76

0 36 63 70 88 95

0 10 20 30 40

0 5 10 15 20

0 1 2 3 4 5

0 10 20 30 40

0 2 4
Breast cancer cells

WAT

Breast cancer cells + WAT

Y forward migration index

Mean accumulated distance (µm)

Mean velocity (µm/min)

Fig. 7
Complementary populations of human adipose CD34+ progenitor cells promote growth, angiogenesis and metastasis of breast cancer

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