The white adipose tissue used in lipotransfer procedures is a rich reservoir of CD34+ progenitors able to promote cancer progression

Running title: Adipose tissue progenitors promote cancer growth

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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 (EPCs). 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+ EPCs with endothelial differentiation potential, containing a mean of 263 times more CD45-CD34+ cells/mL than bone marrow. Compared to marrow-derived CD34+ cells mobilized in blood by G-CSF, purified WAT-CD34+ 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 anti-tumor immunity. In vitro, WAT-CD34+ cells generated mature endothelial cells and capillary tubes as efficiently as mature mesenchymal cells. The co-injection of human WAT-CD34+ 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+ cells lined the lumen of cancer vessels. These data indicate that CD34+ 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.

Precis

The white adipose tissue that is used in some cancer patients for autologous lipotransfer procedures is very rich in CD34+ progenitors that are able to promote tumor growth, angiogenesis and metastases in several orthotopic models of human breast cancer.
**Introduction**

The “catalytic” and quantitative roles of bone marrow (BM)-derived endothelial progenitor cells (EPCs) 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 BM transplants (2). Conflicting data have been obtained regarding the real relevance of BM-EPC-derived vessels in cancer growth in different preclinical models of neoplasia, with some models exhibiting a critical dependence on the presence of BM-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 1-12). One study has recently described that EPCs are present in tissues other than the BM, 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+ EPCs. Compared to BM-derived CD34+ cells mobilized in blood by G-CSF, purified human WAT-derived CD34+ 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 anti-tumor immunity (14). In vitro, WAT-CD34+ cells generated mature endothelial cells and endothelial tubes. In vivo, the co-injection of human WAT-CD34+ cells contributed to tumor vascularization and significantly increased tumor growth and metastases in orthotopic models of human breast cancer in NOD/SCID IL2R gamma null (NSG) mice.
Methods

Cell purification

Human WAT samples were obtained from lipotransfer procedures for breast reconstruction in breast cancer 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 1200g for 10 minutes to remove erythrocytes and leukocytes and subsequently digested in HBSS (Gibco, Paisley, UK) containing 2mg/ml collagenase type I (Sigma Aldrich, St. Louis, MO) and 3.5% bovine serum albumin (BSA, Sigma Aldrich) at 37°C in constant shaking for 60 min. The digestion was blocked with RPMI1640 supplemented by 20% FBS (Euroclone, Milan, Italy) and a cell pellet was obtained by centrifugation at 200g for 10 minutes at 4°C. The cell suspension was then filtered through a 100 µm mesh filter to remove non-digested tissue and washed twice with incubation buffer (PBS with 2mM EDTA and 0.5% BSA), working always on ice. An aliquot of these cells was labelled for flow cytometry analysis. Overall, 113 human WAT samples were studied in vitro and 38 of these were used to provide CD34+ cells that were used in 124 different individual mice studies.

After informed consent was obtained, CD34+ 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, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Final CD34+ cell purity was evaluated by flow cytometry and found always to be >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+ progenitor cells were evaluated by six-colour 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. 7AAD 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 -PeCy7 (clone 8G12), -CD31-PeCy7 (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 (Mountain View, CA); anti-VEGFR2-PE (clone 89106) and -VEGFR3-PE (clone 54733) were from R&D Systems (Minneapolis, MN); anti-CD44-APC (clone BJ18) was from Bio-Legend (San Diego, CA), anti-CD144-PE (TEA 1/31) was from Beckman-Coulter (Brea, CA). The nuclear staining 7-AAD and Syto16 were from Sigma (St. Louis, MO) and Invitrogen (Carlsbad, CA), 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 (San Diego, CA).

The absolute count of CD45-CD34+ 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, Lin. Again, the absolute cell count was obtained using reference beads in Trucount tubes.
RT-PCR and expression analysis

In magnetically labelled CD34+ cells, RNA isolation was performed using QI Amp RNA blood mini kit (Qiagen, Valencia CA) and cDNA was generated from 40 ng of RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA); quantitative PCR was performed 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 labelled targets, array hybridization (Affymetrix GeneChip Gene ST 1.0 Human array; Affymetrix, Santa Clara, CA), staining and scanning were performed according to Affymetrix standard protocols, starting from 500 ng of total RNA. Duplicate microarrays were hybridized with each cRNA sample. The MAS5 algorithm was used to determine the expression levels of mRNAs; the absolute analysis was performed using default parameters and scaling factor 500. Report files were extracted for each microarray chip, and performance of labelled target was evaluated on the basis of several values (scaling factor, background and noise values, percent present calls, average signal value, etc). The data were deposited at GEO (21). Results were confirmed by quantitative RT-PCR.

Cell lines and culture

MDA-MB-436 and HCC1937 triple negative breast cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as recommended by the manufacturer. Prior to injection in mice, cells (1 x 10^6 / 20 µL/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+ cells as previously described (9, 22). In brief, cells were plated in complete EGM-2 medium (Lonza, Visp, Switzerland) in 12- or 24-well plates pre-coated with rat tail collagen I. Plates were placed in a 37°C, 5% CO₂ humidified incubator. The seeding density ranged from 50x10³ to 500x10³ cells. The presence of EC and colonies was assessed using an inverted microscope. After 3-7 days of culture, endothelial cell colonies were identified morphologically, and subsequently picked out using cloning rings. Fibroblast contamination was avoided by depleting them from cell suspensions with the anti-fibroblast microbeads kit (Miltenyi). Endothelial cell surface antigen expression was assessed by flow cytometry and immunofluorescence staining of VE-Cadherin as previously described (20-22).

Capillary-like structures were obtained in culture using a commercial kit (Chemicon, Temecula, CA) as previously described (22). Briefly, matrix solution was thawed on ice, seeded on 24-well plates and incubated at 37°C to solidify. EC were harvested, resuspended in complete media, seeded at a final concentration of 5x 10⁴ cells per cell onto the polymerized EC Matrix and incubated at 37°C in a tissue incubator. After 17 hours tube formation was inspected under an inverted light microscope at 20x 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 cell/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 bFGF (BD Biosciences), and 4 mg/ml heparin (Sigma Aldrich) and maintained in a 5% CO₂-humidified incubator at 37°C. Six to eight days later, sphere formation was analyzed under an inverted light microscope.
Orthotopic xenograft in vivo studies

Female NOD/SCID IL2R gamma null (NSG) mice (24-25), 6- to 9-weeks old, were bred and housed under pathogen-free conditions in our animal facilities (IFOM-IEO campus, Milan, Italy). Mice were expanded from breeding pairs originally donated by Dr. Leonard Shultz. All animal experiments were carried 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 $10^6$ cells/13 μl. The cell suspension was then mixed with 5 μl growth factor-reduced Matrigel (BD Biocoat) and 2 μl trypan blue solution (Sigma Aldrich) and maintained on ice until injection. In cases where tumor cells were co-injected with $2 \times 10^5$ 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 μl cell suspension in Matrigel directly in the fourth mammary fat pad, through the nipple using a Hamilton syringe.

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

Bilateral studies

NSG mice were divided into two groups, a control group in which $1 \times 10^6$ MDA-MB-436 or HCC1937 cells were injected into the right fourth mammary fat pad (through the fourth nipple) and an experimental group in which $1 \times 10^6$ MDA-MB-436 or HCC1937 cells were co-injected with $2 \times 10^5$ human CD34+ WAT cells into the right fourth mammary fat pad.
Monolateral studies

1 x 10^6 MDA-MB-436 or HCC1937 cells were injected into the right fourth mammary fat pad and 1 x 10^6 MDA-MB-436 or HCC1937 cells were co-injected with 2 x 10^5 human CD34+ WAT cells into the left fourth mammary fat pad of the same NSG mouse. In both set of studies, tumors were measured at least once a week using digital calipers. Tumor and lung tissues were removed at experimental end on day 84. Tumors were measured and weighed. For histological evaluation of the tumors, one 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 & eosin (H&E) for the detection of metastases. The Scan Scope XT device and the Aperio Digital pathology system software (Aperio, Vista, CA) were used to detect metastases.

In the second model of breast cancer metastases, 1 x 10^6 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 200-250 mm^3, i.e., about 45 days after tumor implant, tumor resection (mastectomy) was performed. 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 x 10^5 human CD34+ WAT cells were injected into the right third mammary fat pad (through the third nipple), a group in which 2 x 10^5 human CD34- 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 cells 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 63X 1.4NA oil immersion objective, zoom 3X, 0.3μm Z step. For the imaging of the red cells the 561 laser line was used and the auto-fluorescence of the cells was collected.

**Statistical analysis**

The Shapiro-Wilk test was used to test for normality. Considering that the very large majority of data were not normally distributed, statistical comparisons were performed using the non-parametric U test of Mann-Whitney. All reported p-values were two sided.
Results

*Human and murine WATs are very rich reservoirs of CD45-CD34+ EPCs.*

By means of flow cytometry, we counted the numbers of EPCs and other subsets of progenitors in the BM 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 BM was obtained from disease-free patients undergoing a follow-up involving BM investigation for haematological or solid neoplasia. The flow cytometry evaluation included a DNA staining procedure to exclude contamination with platelets and/or micro- and macro-particles, as previously described (18-19).

In humans, WAT was found to contain a large amount of CD45-CD34+ cells that fulfill the most recent criteria for EPC identification (9-12). These CD45-CD34+ cells included two subpopulations: CD34++ CD13+ CD140b+ CD44+ CD90++ cells and CD34+ CD31+CD105+ cells (Fig.1A-M). The immunomagnetic purification procedure used in the study led to a cell population which included 79-96% of CD45-CD34++ CD13+ CD140b+ CD44+ CD90++ cells and CD34+ CD31+CD105+ cells (Fig.1A-M). The immunomagnetic purification procedure used in the study led to a cell population which included 79-96% of CD45-CD34++ CD13+ CD140b+ CD44+ CD90++ cells, and 2-18% of CD45-CD34+ CD31+CD105+ cells. CD34- cells always made up less than 5% of the purified cell population (Fig.1N and supplementary Fig 1)

Quantitative studies indicated that human WAT contains about 263-fold more CD45-CD34+ EPCs/mL than BM (n=32, Fig. 1M). In particular, median human WAT CD45-CD34+CD31- cells were 181,046 /mL (range 35,970-465,357), and CD45-CD34+ CD31+ cells were 76,946/mL (range13,982-191,287). Correlations were found between the body
mass index and total CD34+ cells (r=0.608, p<0.001), and between WAT donor age and total CD34+ cells (r=0.387, p=0.035).

In mice, EPCs were defined as CD45-CD34+CD13+Sca-1+ cells, whereas hematopoietic progenitors were defined as Lin-Sca-1+CD150+ cells. Similarly to humans, mice WAT was also richer in EPC than BM, with WAT having 179-fold more EPCs/mL than BM. We also found CD45+CD34+ hematopoietic progenitor cells in human WAT (median 6,141/mL, range <1-161,338). On average, human WAT contained 87 times less CD45+CD34+ hematopoietic progenitor cells/mL than BM. The presence of human hematopoietic progenitors in WAT was confirmed by culture studies, where a mean of 1.4±0.2 CFU-GM and 3.4±1.8 BFU-E/10^5 seeded cells were obtained from WAT tissue.

**WAT-CD34+ cells express stemness-related genes and very high levels of angiogenesis-related genes**

CD34+ cells from human WAT were purified and their gene expression profile compared (by Affymetrix human gene 1.0 ST) with that of purified BM CD34+ cells mobilized in the blood of healthy donors by G-CSF administration (n=5 per study arm). Data were confirmed by quantitative real-time PCR (Suppl. Table1). When compared to BM-derived CD34+ cells (Fig.2A-C), human WAT CD34+ cells expressed significantly higher levels of genes associated with angiogenesis (eg VEGFR-1 and -2, NRP1, TEK, VE-Cadherin, VCAM-1, ALK1, etc), adipogenesis (eg LPL, FABP4, PPARG, etc) endothelial and mesenchymal (eg RGS5, IGF1, PDGFRbeta, etc) differentiation (1-13). A large majority of the panel of genes associated with stemness (e.g. SOX2, LIF, WNT3A, Nanog, etc) and hematopoiesis (eg RUNX, IL-6, CSF-1, etc) was expressed in WAT and BM-derived CD34+ cells at similar levels (Fig.2B).
WAT-derived CD34+ cells expressed higher levels of FAP-alpha, a crucial suppressor of anti-tumor immunity (14), and of genes of the brain/adipocyte-BDNF/leptin axis that has recently been suggested to play a relevant role in cancer progression (27).

**WAT-CD34+ cells generate mature endothelial cells and capillary tubes**

When cultured in vitro in appropriate endothelial-differentiation media, human WAT-derived CD34+ 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+ cells, but not WAT CD34- 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+ cells. By flow cytometry, these spheres were found to be made of CD45-CD13+CD34+ (16% of cells in spheres) CD44+CD90+ cells (Supplementary Fig. 2).

**The co-injection of human WAT-CD34+ cells from lipotransfer procedures significantly increases tumor growth and metastases in breast cancer models**

The role of purified CD34+ cells from human WAT was investigated in different models of human breast cancer (Fig. 4-6 and Supplementary Fig. 3-5). Triple negative human breast cancer MDA-MB-436 and HCC1937 cells were injected in the mammary fat pad alone or co-injected with WAT-derived human cells (N=124, Fig. 4A). Breast cancer cells generated tumors in the mammary fat pad. Purified human CD34+ WAT cells, when injected in the mammary fat pad in the absence of tumor cells, did not generate tumors. The co-injection of breast cancer cells and unprocessed nucleated cells from human WAT significantly
increased tumor growth. The co-injection of breast cancer cells and purified CD34+ 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+ WAT cell fraction (Fig. 4B). The co-injection of CD34- WAT cells was less effective than the co-injection of CD34+ WAT cells in promoting tumor growth (Fig. 4B). Similar results were obtained in NSG mice injected with NCC1937 breast cancer cells alone, or in combination with human CD34+ WAT cells (Fig. 4C). Histology studies ruled out the possibility that the larger size of tumors in animals co-injected with CD34+ WAT cells was due to the generation of adipocytes (Supplementary Fig. 5).

We performed two separate studies to examine the in vivo involvement of CD34+ WAT cells in promoting tumor growth. In bi-lateral studies, human CD34+ WAT cells were co-injected with breast cancer cells in one of the two lateral mammary fat pads (with the contralateral mammary fat pad injected with breast cancer cells alone, as control). In mono-lateral studies, human CD34+ WAT cells were co-injected 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 bi-lateral studies compared to mono-lateral studies (Fig. 4D). These data suggest that human WAT CD34+ cells exert most (if not 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 co-injected with human WAT CD34+ 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-F, and supplementary Fig. 3-4). The number of lung metastases was significantly increased in mice co-injected with
breast cancer and CD34+ WAT cells compared to mice injected with breast cancer cells alone or mice injected with breast cancer cells and CD34- 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 200-250 mm$^3$, i.e., about 45 days after tumor implant, the tumor was surgically removed. Mice were then divided into an experimental group in which CD34+ WAT cells were injected, an experimental group in which CD34- WAT cells were injected, and a control group without WAT cell injection. Two months after cell injection, mice injected with CD34+WAT cells had significantly more axillary and lung metastases, compared to mice injected with CD34- cells and to controls (Fig. 4H).

Immunohistochemistry and confocal/z-stack microscopy studies demonstrated the presence of human CD31+, CD34+, CD105 endothelial vessels and perivascular cells in the mammary fat pad of mice co-injected with breast cancer cells and CD34+ human WAT cells (Fig.5). Panels 5c to 5h show representative images illustrating the incorporation of human cells generated from WAT-derived CD34+ cells lining cancer blood vessels, some of which contain red blood cells (panels G and H). Confocal microscopy (Fig. 6 and supplementary movie 1 made from 42 consecutive slices) confirmed the presence of a lumen and of circulating red blood cells in human CD34+ and CD31+ vessels in mice injected with human CD34+ WAT cells. We were never able to observe this effect in our previous studies using BM-derived cells, thus these results demonstrate an important bona fide functional difference between WAT- and BM-derived progenitors.

**WAT-CD34+ 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 a high fat diet
to that in control mice fed on a control diet. Both EPCs and hematopoietic progenitor cells were significantly increased in the WAT of mice receiving a 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 BM-derived EPCs in cancer growth has been intensively debated in the last decade (1-12). Our present work offers new insight into the controversy over 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+ (or otherwise genetically labelled) BM. This approach excluded the quantification of the role of WAT-derived EPCs, that in our work were found to be in numbers significantly higher than in the BM.

Considering that our in vitro and in vivo studies were performed using a cell population containing 79-96% of CD45-CD34++ CD13+ CD140b+ CD44+ CD90++ 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 pro-tumorigenic and pro-metastatic potential might reside in this cell population. The lack of bright CD31 expression of this cells population (see Fig. 1) suggests that these cells are not mature endothelial cells. Moreover, as reported by Zimmerlin et al. (28), mature endothelial cells in WAT do not seem to express CD34. Along a similar line, the work of Yoder, Ingram and Case has repeatedly indicated that the putative EPC phenotype is CD45-CD34+, 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+ cells within the population of CD45-CD34+ cells, more work is currently needed to understand which population (the CD144+ or CD144-) has endothelial differentiation, pro-tumorigenic and pro-metastatic potential. It also remains to be discovered whether the functional vessels expressing human antigens in mice injected with CD34+ WAT cells are entirely derived from WAT-derived CD34+ cells or whether a fraction of WAT-derived CD34+ cells differentiated and incorporated into the murine tumor vessels.

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

Controversies have been also reported regarding the role of WAT-derived mesenchymal progenitors (MPs) in cancer growth, with some authors reporting that MPs promote tumor growth and some other reporting that MPs suppress tumor growth (reviewed in 33). Most of these studies, however, were performed in mice injected with crude suspensions of MPs obtained from cell culture. To our knowledge, our study is one of the first reporting the tumor promoting activity of fresh human WAT-derived purified CD34+ cells. As shown by our data in mice receiving breast cancer and WAT cells in one of the two mammary fat pads and breast cancer cells alone in the other mammary fat pad, the cancer promoting activity of WAT CD34+ cells is likely to be exerted through a local, rather than systemic, activity. These data complement the recent observation from the Kolonin laboratory (34) that, in mouse models, WAT cells are mobilized and recruited by experimental tumors to promote cancer progression.

In the present study, HFD was associated in mice with a significant increase in WAT-CD34+ cell numbers. HFD might also interfere with the characteristics of WAT CD34+ 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 increased also WAT hematopoietic progenitors. This, in turn, might increase the mobilization of hematopoietic pro-angiogenic cells already described by other studies (37).

A novel brain/adipocyte-BDNF/leptin axis has been recently proposed to play a potentially relevant role in cancer progression (27). Although more studies are clearly needed to reach robust conclusions, WAT-derived CD34+ progenitors seem to express high levels of the receptors involved in this axis. Along a similar line, WAT-CD34+ cells express very high levels of FAP-alpha, a crucial suppressor of anti-tumor 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 operated for primary breast cancer between 1997 and 2008, who subsequently underwent a lipotransfer procedure for esthetic purposes, compared with two matched patients with 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 to 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 be of 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 pharmaceutical
strategy, which can be assessed in preclinical models and clinical studies, to reduce any “cancer-promoting” risk of WAT EPCs.

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Figure legends

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

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

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

Fig. 4: Orthotopic models of breast cancer.

Panel A shows representative pictures of the growth of human MDA-MB-436 breast cancer cells in the mammary fat pad of NSG mice injected with breast cancer cells alone (red arrow) or breast cancer cells plus CD34+ WAT cells (blue arrow).

Panel B shows tumor growth in NSG mice injected with WAT CD34+ cells alone, MDA-MB-436 cells alone, MDA-MB-436 cells plus unfractionated WAT cells, MDA-MB-436 cells plus CD34+ WAT cells, and MDA-MB-436 cells plus CD34- WAT cells.

Panel C shows 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+ WAT cells.

Panel D shows tumor growth in NSG mice injected with MDA-MB-436 breast cancer cells in mono- and bi-lateral studies.

Panels E and F shows representative pictures of breast cancer metastatic spots (depicted by the black rings) in the lungs of NSG mice which were not co-injected with human WAT CD34+ cells (panel E) or co-injected with human WAT CD34+ cells (panel F). Panels E and F are reproduced in larger size in Supplementary Fig. 3.

Panels G and H show the number of metastases in NSG mice injected with MDA-MD-436 breast cancer cells in models evaluated in the absence (panel G) of tumor surgical removal or after tumor surgical removal (panel H).
*=p<0.05, **=p<0.005 vs control by Mann-Whitney U test.

Fig. 5: *Immunohistochemistry evaluation of human CD34+ WAT cell engraftment in breast cancer-bearing NSG mice.*

Clockwise, from top left:


Panels D to H show representative images illustrating the incorporation of human cells (depicted by the expression of human CD31) generated from WAT-derived CD34+ cells lining cancer blood vessels, some of which contain red blood cells (panels G and H, red blood cells indicated by the red arrows).

Panels I-J: Expression of human CD105 (I) and human CD34 (J) in perivascular cells and vessels.

Fig. 6: *Confocal-Z-Stack evaluation of cancer vessels.*

Confocal laser-scanning of human CD34 (A, B, C) and human CD31 (D) antigen distribution in a tumor section in mice injected with MB-MDA436 tumor cells plus WAT-derived CD34+ human cells. For the imaging of the red cells the 561 laser line was used and the auto-fluorescence of the cells was collected (arrows). Snapshot images are orthogonal sections of the z-stacks taken at points along the vessel cavity.
Fig. 7  *Endothelial progenitor cells (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 vs. controls of CD45-CD34+CD13+Sca1+ EPCs (A), Lin-Sca1+CD150+ HSCs (B) and CD45-CD31+Sca1- endothelial mature cells (C) in the mammary and ovarian WAT tissue of mice receiving HFD (white bars) vs. controls (black bars). Total numbers of EPCs and HSCs in the femurs of mice receiving normal vs. HFD (D).

*=p<0.05, **=p<0.005 by Mann-Whitney U test.
Fig. 1

Marrow WAT

CD34+CD45+
CD34+CD45-

CD13 APC-A
CD144 PE-A
CD44 APC-A

CD29 PE-A
VEGFR-3 APC-A
VEGFR-2 PE-A

1000000 Cells/mL

1000

0.001

Marrow WAT

CD34+CD45+ CD34+CD45-

***

0.001

1000

1000000

Fig. 1
Fig. 2

RNA fold expression vs CD34+ cells mobilized in blood by G-CSF

A. Angiogenesis

B. Adipogenesis, Hematopoiesis, Stemness

C. Mesenchymal
Fig. 3
**Fig. 4**

A. Tumor growth curves for MDA-MB-436 and MDA-MB-436 CD34+ WAT cells.

B. Tumor volume (mm³) over time for MDA-MB-436 cells. ** indicates significant difference.

C. Tumor growth curves for HCC1937 cells.

D. Metastases in lungs for MDA-MB-436 cells.

E. Histological sections of metastases in lungs.

F. Metastases in lungs for HCC1937 cells.

G. Metastases in lungs for MDA-MB-436 cells.

H. Metastases in lungs for HCC1937 cells.

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Fig. 5
Fig. 7

A. Fold expansion vs control

B. Fold expansion vs control

C. Fold expansion vs control

D. Fold expansion vs control

Fold expansion vs control

Cells/mL

Control  High Fat Diet
The white adipose tissue used in lipotransfer procedures is a rich reservoir of CD34+ progenitors able to promote cancer progression

Ines Martin-Padura, Giuliana Gregato, Paola Marighetti, et al.

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