Adipose progenitor cell secretion of GM-CSF and MMP9 promotes a stromal and immunological microenvironment that supports breast cancer progression

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Abstract

A cell population with progenitor-like phenotype (CD45-CD34+) resident in human white adipose tissue (WAT) is known to promote the progression of local and metastatic breast cancer and angiogenesis. However, the molecular mechanisms of the interaction have not been elucidated. In this study, we identified two proteins that were significantly up-regulated in WAT-derived progenitors after co-culture with breast cancer: granulocyte-macrophage colony-stimulating factor (GM-CSF) and matrix metalloproteinase 9 (MMP9). These proteins were released by WAT progenitors in xenograft and transgenic breast cancer models. GM-CSF was identified as an upstream modulator: breast cancer-derived GM-CSF induced GM-CSF and MMP9 release from WAT progenitors, and GM-CSF knockdown in breast cancer cells neutralized the pro-tumorigenic activity of WAT progenitors in preclinical models. GM-CSF neutralization in diet-induced obese mice significantly reduced immunosuppression, intratumor vascularization, and local and metastatic breast cancer progression. Similarly, MMP9 inhibition reduced neoplastic angiogenesis and significantly decreased local and metastatic tumor growth. Combined GM-CSF neutralization and MMP9 inhibition synergistically reduced angiogenesis and tumor progression. High-dose metformin inhibited GM-CSF and MMP9 release from WAT progenitors in in vitro and xenograft models. In obese syngeneic mice, metformin treatment mimicked the effects observed with GM-CSF neutralization and MMP9 inhibition, suggesting these proteins as new targets for metformin. These findings support the hypothesis that GM-CSF and MMP9 promote the pro-tumorigenic effect of WAT progenitors on local and metastatic breast cancer.
Introduction

Postmenopausal breast cancer is one of the leading causes of death in western countries (1). Epidemiological data indicate that in overweight/obese women breast cancer incidence is increased (2), prognosis is worsened (3), and the cancers themselves are more resistant to chemotherapy (4). White adipose tissue (WAT) promotes the growth of breast cancer in animal models, is abundant in breast tissue, and many of its cells secrete factors having paracrine and endocrine activity (5,6). In obese women adipocytes, inflammatory cells and the factors they secrete, are altered (7,8) and promote a breast microenvironment that favors cancer development, growth, migration and angiogenesis (9,10). Use of autologous adipose in breast reconstruction after mastectomy has been reported to increase the breast cancer relapse rate (11,12). It is therefore important to identify the molecules that mediate the tumor-promoting activity of WAT, not least because they may be targets for new breast cancer therapies.

WAT includes adult stem cells with progenitor-like phenotype (13,14). Progenitor cells have been found to support breast cancer growth and metastasis in preclinical models (15). Progenitors isolated from the WAT stromal vascular fraction consist of two sub-populations: mesenchymal progenitor cells or adipose stem cells (ASCs), and endothelial cells with a progenitor-like ultrastructure (ECs) (16). These have been found to have complementary effects in promoting breast cancer: ASCs support epithelial to mesenchymal transition (EMT), while ECs support local tumor growth and promote angiogenesis and metastasis (15-17).

However, notwithstanding the abundant evidence that adipocyte progenitors promote breast cancer, the molecular mechanisms mediating this effect have not been identified. In the present study we show that two proteins play key mediator roles: granulocyte-macrophage colony-stimulating factor (GM-CSF) and matrix metallopeptidase 9 (MMP9).

We found that both these proteins are up-regulated in WAT progenitors exposed to breast
cancer cells (in vitro and preclinical models). We also found that GM-CSF produced by breast cancer cells is an up-stream inducer of the aberrant up-regulation of GM-CSF and MMP9 in WAT progenitors.

We also investigated these proteins in diet-induced obese syngeneic mouse models of breast cancer, assessing their impact on the tumor microenvironment and tumor progression. Finally we investigated the effect of metformin on this newly-identified interaction. Metformin is a widely used anti-diabetic drug recently proposed as a treatment for breast cancer, since its administration is associated with reduced cancer incidence, particularly in obese patients or with metabolic syndrome (18-20). We have shown previously that metformin efficiently reduces tumor growth, metastasis, and angiogenesis in preclinical models of breast cancer and that it targets both breast cancer cells and WAT progenitors (21).
Materials and Methods

Collection and processing of WAT cells

Lipotransfer material was collected from 40-65 year old women undergoing breast reconstruction at the European Institute of Oncology, Milan, after giving informed consent. All patient studies were conducted in accordance with the Declaration of Helsinki, and performed after approval by the Institutional Review Board. WAT cells were obtained from lipotransfer material as described elsewhere (15,16). Cells were labeled magnetically with anti-human CD45 microbeads (Miltenyi Biotec, Germany) and separated on a magnetic column (LS columns, Miltenyi Biotec). The CD45- fraction was collected and its purity assessed by flow cytometry. This fraction was then labeled with anti-human CD31 microbeads (Miltenyi Biotec) to separate ASCs (CD34+CD31-) from ECs (CD34+CD31+) (16).

All mouse studies were approved by the Italian Ministry of Health and performed in accordance with the Institutional Animal Care and Use Committee. Subcutaneous and intra-peritoneal WAT was obtained from 8-12 week-old female mice, FVB/N-Tg(MMTV-PyVT)634Mul/J (MMTV-PyMT, Jackson Laboratories, Bar Harbor, ME, US), housed in our animal facilities at the IEO-FIRC Institute, Milan. WAT was purified with anti-mouse CD45 microbeads (Miltenyi Biotec).

Cell cultures

MDA-MB-436, MDA-MB-231, HCC1937, and 4T1 breast cancer cell lines were purchased in 2014-17 from ATCC, (Manassas, VA, US), tested every six months for Mycoplasma by means of the ATCC Universal Mycoplasma Detection Kit 30-1012, expanded and stored according to the producer’s instructions. Cells were tested and authenticated by StemElite ID (Promega, Fitchburg, WI) at the European Institute of Oncology, cultured for no more
than two weeks and used for no longer than 15 passages. MMTV-ErbB2+ breast cancer cells from FVB-NK1Mul/J mice were isolated and cultured as described elsewhere (22).

Breast cancer cells and WAT CD45-CD34+ cells were cultured together on collagen-coated 6-well plates (Corning, NY) (0.8x10^6 cells each) for 72 hours in EBM-2 medium (Lonza), supplemented with 0.5% gentamycin (Thermo Fisher Scientific). Transwell co-cultures were obtained using 0.4µm transwell permeable inserts (Corning). Alternatively, the different cell types were seeded together on collagen coating. After culturing, culture media were collected, briefly centrifuged to remove cell debris and stored at -20°C. The cells themselves were detached with Accutase (Sigma-Aldrich) and lysed in RLT Buffer (Qiagen).

The following drugs or monoclonal antibodies (MoAbs) were added directly to culture media: metformin hydrochloride (Sigma-Aldrich) 2mM-10mM; bortezomib (Sigma-Aldrich) 3nM-50nM; anti-human IL-1β MoAb (#MAB201, R&D systems, MN, US) 0.2µg/ml-0.5µg/ml; anti-human GM-CSF MoAb (#sc-377039, Santa Cruz Biotechnology, TX,US) 0.5µg/ml-1µg/ml; SB-3CT (#S1326, Sigma-Aldrich) 0.5µM-1µM. For each set of experiments, appropriate control solutions were added to the culture medium (IgG MoAb or dimethyl sulfoxide (DMSO) for bortezomib and SB-3CT).

**GM-CSF knockdown in breast cancer cells**

Human GM-CSF (hGM-CSF; CSF-2 gene) shRNA lentiviral particles (sc-39391-V) and control particles (sc-108080) were purchased from Santa Cruz Biotechnology. The particles contain 3 target-specific shRNAs (19-25 nucleotides, plus hairpins) that knockdown GM-CSF gene expression. MDA-MB-436 cells were transduced according to the producer’s instructions using Polybrene (sc-134220, Santa Cruz Biotechnology). Quantitative reverse transcription PCR (qRT-PCR) and ELISA (#DGM00, R&D) were used to confirm knockdown in breast cancer cells, after puromycin selection.
**In vivo experiments**

In vivo experiments were carried out in accordance with Italian legislation and institutional guidelines. Mice were bred and housed in pathogen-free conditions. Xenografts were generated as described elsewhere (15). Six-to-eight week-old NODSCIDIL2Rγnull (NSG) female mice (12 per study arm) were injected (mammary fat pad) with $1 \times 10^6$ human breast cancer (hBC) cells (MDA-MB-436, MDA-MB-231 or HCC1937) alone or in combination with $0.2 \times 10^6$ human CD45-CD34+ WAT cells. Five mice were injected with human CD45-CD34+ cells alone as negative controls. In another set of experiments half the mice were given high-dose metformin (drinking water, 2mg/ml) three days after injection (12 per study arm) until sacrifice. Tumor growth was assessed weekly using digital calipers to determine width (W mm) and length (L mm): volume ($mm^3$) was calculated as $L \times W^2 / 2$.

Serum or plasma was prepared from blood collected every two weeks from the animals’ tail vein; EDTA was added to plasma. Plasma levels of hGM-CSF were determined with high sensitivity (HS) ELISA (#HSGM0, R&D). Serum levels of cholesterol, triglycerides and glucose were determined with Architect c8000 (Abbott, IL, US). When tumors reached 1.2 cm diameter, the animals were killed by CO$_2$ inhalation.

A syngeneic breast cancer model was generated in diet-induced obese mice. Six week-old FVB/Hsd or BALB/cOlaHsd females (Envigo, UK) had ad libitum access to a HFD (60% kcal fat, Brogaarden, Sweden). Weight was assessed weekly. After 30 days, the animals were injected orthotopically with breast cancer cells: $0.5 \times 10^6$ MMTV-ErbB2+ primary cells (FVB background) or $0.03 \times 10^6$ 4T1 cells (BALB/c background). As negative controls some mice were not injected with cancer cells (n=3). Three days after injection, inhibition of GM-CSF, MMP9, or both, was started by injecting the animals intraperitoneally (ip) (n=10 per study arm) with 50µg anti-mGM-CSF MoAb (#BE0259, BioXcells, West Lebanon, NH, US) dissolved in PBS, or 25mg/kg SB-3CT (#S1326, Sigma-Aldrich) after
dissolution in PBS containing 0.1%Tween20 and 10%DMSO. Injections were administered every other day until sacrifice. In controls, 50µg MoAb rat IgG2a (#BE0089, BioXcell) or PBS-0.1%Tween20-10%DMSO, or both, were administered (same schedule). Five mice were administered high-dose metformin (2mg/ml in drinking water) three days after tumor injection, until sacrifice.

Tumor growth was assessed weekly. Blood was collected monthly from the tail vein, and circulating immune cells were determined by multiparametric flow cytometry (Table S1). When tumors reached 1.2 cm the mice were either sacrificed or the tumor was removed (mastectomy). After mastectomy, the animals were sacrificed 30 days (MMTV-ErbB2+ breast cancer) or 15 days (4T1) later. Tumors, subcutaneous WAT, lungs and spleens were collected from sacrificed animals. Tissue samples were fixed in 4% phosphate-buffered formalin and embedded in paraffin. Other samples were stored for RNA (in RNAlater, Qiagen) or protein analysis (frozen in liquid nitrogen). Five-µm thick sections of lungs were stained with hematoxylin and eosin (H&E) to assess the presence of metastases. Images were acquired with a ScanScope XT scanner (Leica, Germany) and analyzed with Aperio Digital Pathology software

**Genotyping**

The genomic DNA of FVB/N-Tg(MMTV-PyVT)634Mul/J animals was analyzed by PCR of tail biopsies, using the Gentra Puregene Mouse Tail kit (#158267, Qiagen). Primers, internal positive controls, and cycling conditions were as recommended by Jackson Laboratory.
Protein analysis

Culture media were screened for 55 soluble factors involved in human angiogenesis using a Proteome Profiler Antibody Array (#ARY007, R&D). Most factors were further quantified by ELISA (#DGM00, #DMP900, #MGM00, #MMPT90, R&D).

For Western blot analyses, frozen tissue samples were first lysed in RIPA Buffer; culture media were used directly. Total protein concentrations in samples were measured by BCA assay (Thermo Fisher Scientific). Protein concentrations were equalized by appropriate dilution and the samples (10-15µg protein) run on 7.5% Mini Protean gels (BioRad), followed by transfer to nitrocellulose membranes (Amersham, GE Healthcare) using the Trans-Blot Turbo Transfer System (BioRad).

Membranes were blocked with 5% skimmed milk in PBS-0.1%Tween20 for 1 hour at room temperature. Immunostaining was performed overnight at 4°C with rabbit monoclonal anti-human MMP9 1:2000 (#ab76003, Abcam, UK) or rabbit polyclonal anti-human MMP9 1:500 (#10375-2-AP, Proteintech, Rosemond, US) and anti-human beta actin 1:5000 (#A5441, Sigma-Aldrich). Detection used horseradish peroxidase-conjugated secondary antibodies 1:5000 (Thermo Fisher Scientific), SuperSignal West Dura (Thermo Fisher Scientific) and the ChemiDOC (BioRad) imaging system. Bands were quantified with ImageJ software.

Zymography

Culture media or whole tumor lysates (15-20µg) were loaded 1:2 with Zymogram sample Buffer (BioRad) and run on 10% Zymogram gels (BioRad). Gels were then incubated 0.5 hour in Renaturation Buffer (BioRad), followed by overnight incubation in Developmental Buffer (BioRad) at 37°C, and staining with PageBlue (Thermo Fisher Scientific) for 5 hours at room temperature. Images were acquired with ChemiDOC.
qRT-PCR

RNA was extracted from samples with Qiamp Mini Blood kit (Qiagen). RNA quantity and quality were checked with NanoDrop 2000 (Thermo Fisher Scientific). 0.5-1µg of RNA was retro-transcribed with the Ipsogen RT kit (Qiagen). qRT-PCR was carried out on the ABI Prism 7000 platform (Thermo Fisher Scientific) using primers and probes from TaqMan Gene Expression Assays (Thermo Fisher Scientific). RT² Profiler PCR array for mouse inflammation and immunity cross-talk (#PAMM-181Z, Qiagen) was performed following the manufacturer’s instructions. The collected data were analyzed with web-based software (RT² Profiler PCR Array Data Analysis; SABiosciences, Qiagen).

Flow cytometry

At least 500,000 cells per sample were acquired using a 3-laser flow cytometer (Navios, Beckman Coulter). Viable cells (negative for 7-aminoactinomycin, 7AAD) were labeled with a panel of antibodies (Beckman Coulter or BD Biosciences, San Diego, CA) to analyze immune cell populations and WAT-derived progenitors (Table S1). Lymphocytes, macrophages, granulocytes and dendritic cells were characterized using standard markers (23, 24). Myeloid derived suppressor cells (MDSCs) and inflammatory monocytes were identified as a recent classification (25). Tumor-associated macrophages (TAMs) were identified according to Su et al. (26). We have previously described the complete characterization of WAT-derived progenitors, including ASCs and ECs (15,16).

Immunofluorescence and confocal microscopy

Immunofluorescence studies were performed as described previously (21). Samples were incubated with rabbit polyclonal anti-mouse CD31 (1:20, #ab28364, Abcam) and mouse monoclonal anti-alpha smooth muscle actin (αSMA, 1:3000, #A2547, Sigma-Aldrich). Secondary antibodies were Alexa Fluor 555 goat anti-mouse IgG2a, and Alexa Fluor 488
donkey anti-rabbit IgG2a (1:200, Thermo Fisher Scientific). Nuclei were revealed with 4′,6-diamino-2-phenylindole (DAPI, Sigma-Aldrich). As negative controls primary antibodies were omitted. Images were captured with a SP5 II confocal microscope (Leica), x20 objective, and optical configuration as described previously (21). Blood vessel density was quantified with ImageJ software.

**Statistical analysis**

Results are summarized as means and SEMs. The Shapiro-Wilk test was used to assess normality. Most data were not normally distributed so all statistical comparisons used the nonparametric Mann-Whitney U test of. All p values are two sided. Differences were considered significant for p<0.05 after Bonferroni correction. The statistical analyses were performed with GraphPad Prism software.
Results

GM-CSF and MMP9 are up-regulated in WAT progenitors co-cultured with breast cancer cells

Co-cultures of purified WAT progenitors (CD45-CD34+) with breast cancer cell lines were analyzed for soluble factors. Two secreted molecules were found up-regulated compared to single cultures: GM-CSF and MMP9 (Fig. 1A). Up-regulation was confirmed by ELISA (GM-CSF) and Western blot (MMP9) in WAT progenitors from 15 women cultured with various human triple-negative breast cancer (TNBC) cell lines (Fig. 1B-D). TNBC cells expressed considerably higher baseline levels of GM-CSF than WAT cells cultured alone (Fig. 1B). Zymography revealed increased MMP9 activity in direct co-cultures, consistent with the greater expression found on Western blot (Fig. 1C). Transcriptional analysis showed that WAT progenitors were the primary source of GM-CSF and MMP9 in co-cultures (Fig. 1E,F). Co-culture of murine WAT (mWAT) CD45-CD34+ progenitors with human breast cancer (hBC) further supported these findings since only murine transcripts were up-regulated (Fig. S1A,B).

To assess GM-CSF and MMP9 specific induction in WAT progenitors, in additional experiments we co-cultured human WAT-derived hematopoietic cells (CD45+CD34-) and WAT progenitors (CD45-CD34+) with hBC cells. The CD45+CD34- population, which constitutes the main part of the stromal vascular fraction, is increased in WAT from obese persons (9). Up-regulation of GM-CSF and MMP9 mRNA did not occur in CD45+CD34- cells on exposure to breast cancer cells, but did occur in CD45-CD34+ cells (Fig. S1C). As we reported previously (16), two progenitor sub-populations are present in the CD45-CD34+ fraction of WAT: ASCs (CD45-CD34+CD31-) and ECs (CD45-CD34+CD31+). When cultured alone ASCs and ECs did not express detectable levels of GM-CSF, but on
co-culturing with breast cancer cells, GM-CSF was significantly up-regulated in both ASCs and ECs (Fig. S1D).

Although ECs expressed higher baseline levels of MMP9 than ASCs, MMP9 release was significantly increased when both progenitors were co-cultured with breast cancer cells (Fig. S1E). We also found that transcripts of GM-CSF and MMP9 were up-regulated in ECs and ASCs when co-cultured with breast cancer cells (Fig. S1F). Thus both ASCs and ECs contribute to greater production of GM-CSF and MMP9 in WAT progenitors when exposed to breast cancer cells.

Inhibition of GM-CSF and MMP9 release from co-cultured WAT progenitors

Monoclonal antibodies or inhibitors affecting putative upstream regulatory pathways, as identified in previous studies (27-29), were investigated. These were added to co-cultures to determine whether they inhibited GM-CSF or MMP9 release (Fig. 2). The NF-κB regulatory pathway was inhibited by bortezomib using concentrations that did not affect cell viability (data not shown). IL-1β signaling was blocked by anti-human IL-1β MoAb. SB-3CT (irreversible inhibitor MMP9) and anti-hGM-CSF MoAb were used to exclude reciprocal regulation. Bortezomib was found not to impair GM-CSF or MMP9 expression in co-cultures of breast cancer cells and WAT progenitors (Fig. 2A-E). However, IL-1β neutralization impaired MMP9 release in direct co-cultures (Fig. 2B) and MMP9 transcript expression in WAT progenitors (Fig. 2E). Neither IL-1β neutralization (Fig. 2A,D) nor SB-3CT (Fig. 2A) had any effect on GM-CSF release. However GM-CSF release was impaired by GM-CSF neutralization in co-cultures (Fig. 2A). GM-CSF neutralization also resulted in dose-dependent reduction in MMP9 release (Fig. 2C). qRT-PCR provided additional support for this finding since GM-CSF and MMP9 transcripts were reduced in WAT progenitors after GM-CSF neutralization (Fig. 2D,E). These results suggest that GM-
CSF, produced by breast cancer cells, may be an up-stream regulator of GM-CSF/MMP9 release by WAT progenitors.

**GM-CSF and MMP9 are up-regulated in xenograft and spontaneous breast cancer models**

The expression of human GM-CSF (hGM-CSF) and human MMP9 (hMMP9) was assessed in xenograft models of TNBC. NSG mice were injected with TNBC cells alone or with hWAT CD45-CD34+ progenitors (Fig. S2A-C). Circulating hGM-CSF was quantified by ELISA and found to be significantly higher in co-injected (hWAT+hBC) than single-injected (hBC) mice (Fig. 3A; Fig. S2D). Mice injected with hWAT progenitors were used as negative controls to support the specificity of the assay.

hMMP9 expression was also investigated in tumors and was found at higher levels in co-injected (hBC+hWAT) than single-injected tumors (Fig. 3B; Fig. S2E). The full-length precursor (pro-MMP9, 92kDa) and cleaved (active) MMP9 forms (82kDa or 67kDa) were up-regulated in whole tumor lysates from co-injected mice, with the biologically-active 67kDa form expressed most prominently. Zymography showed that MMP9 had greater enzymatic activity in co-injected tumors (Fig. 3B).

qRT-PCR of breast cancer cells isolated from mouse tumors did not indicate significant induction of GM-CSF or MMP9 transcripts, suggesting that WAT progenitors were secreting these proteins (Fig. 3C). To provide support for this supposition, mWAT CD45-CD34+ progenitors were isolated from transgenic tumor-bearing mice (MMTV-PyMT). These cells were compared to mWAT progenitors collected from wild type (WT) mice of the same age and sex. It was found the GM-CSF and MMP9 transcripts were significantly up-regulated in WAT from transgenic mice compared to WAT from WT mice (Fig. S3A). Protein release was also significantly greater in WAT from transgenic mice (Fig. S3B),
indicating that in vivo, up-regulation of these proteins in adipose progenitors depends on the presence of breast cancer.

**GM-CSF knockdown in breast cancer cells prevents the pro-tumorigenic effects of WAT progenitors**

GM-CSF was identified *in vitro* as a potential up-stream regulator of both GM-CSF and MMP9 production in WAT progenitors. To provide further evidence on this, lentiviral vectors were used to knockdown GM-CSF in the MDA-MB-436 TNBC cell line. shGM-CSF hBC cells were injected into NSG mice alone or together with hWAT progenitors. GM-CSF knockdown in tumors was demonstrated by qRT-PCR (*Fig. 3D*). Tumor growth in co-injected (hBC+hWAT) mice was significantly impaired when GM-CSF knockdown cells were used, compared to scramble, and did not differ from that in single-injected (hBC) mice (*Fig. 3E*). There were also fewer metastatic foci in lungs in GM-CSF knockdown hBC cells (*Fig. 3F*). These data indicate that breast cancer-derived GM-CSF supports local tumor growth and metastatic progression in preclinical models. Plasma hGM-CSF was significantly reduced after GM-CSF knockdown in both single- and co-injected mice (*Fig. 3G*). Thus, GM-CSF released from breast cancer cells is required to support GM-CSF production in WAT progenitors. Our data also indicate that GM-CSF is an up-stream regulator of MMP9 since we found that hMMP9 protein levels in tumor lysate were significantly lower in GM-CSF knockdown tumors compared to scramble in co-injected mice (*Fig. 3H*).
Combined GM-CSF neutralization and MMP9 inhibition reduce tumor growth and metastatic spread in an obese syngeneic breast cancer model

The preclinical effects of inhibiting GM-CSF and MMP9 were investigated in immunocompetent obese mice injected with TNBC (4T1 cells, in BALB/c background) or MMTV-ErbB2+ (in FVB/Hsd background). Since WAT progenitors are significantly increased (per unit weight of WAT) in obese mice (15,17), before injection of breast cancer cells, the mice were fed a HFD and rapidly gained body weight (Fig. S4A). BALB/c mice have been reported as more resistant to obesity than FVB mice, although they have similar adiposity (30). After tumor injection, the animals were administered anti-mGM-CSF MoAb, SB-3CT (MMP9 inhibitor), or both. Mouse weight was unaffected by these treatments (data not shown). Preliminary experiments (using IgG2a MoAb or vehicle as controls) showed that both anti-mGM-CSF MoAb and SB-3CT reduced tumor volume and metastatic spread in obese mice (Fig. S4B,C). Administration of GM-CSF and MMP9 inhibitors together revealed they acted synergistically to reduce local breast tumor growth in MMTV-ErbB2+ breast cancer (Fig. 4A). Metastatic spread to lungs was investigated 30 days after mastectomy. Administration of both inhibitors together had the best efficacy in reducing metastatic spread (p<0.001, Fig. 4B).

In the other obese model of breast cancer (4T1-injected Balb/c) tumor growth was significantly reduced by GM-CSF neutralization and MMP9 inhibition (p<0.001, Fig. 4C). SB-3CT was the most effective in reducing lung metastases in this model (p<0.01, Fig. 4D). Spleens from these mice were less enlarged (lower weight) than those from IgG2a+vehicle controls (p<0.01, Fig. 4E). These findings indicate that GM-CSF and MMP9 are involved in the local and metastatic growth of triple negative and ErbB2 overexpressing breast cancers in diet-induced obese mice.
GM-CSF neutralization and MMP9 inhibition impair neoplastic angiogenesis in vivo

GM-CSF and MMP9 are known modulators of angiogenesis. GM-CSF influences angiogenesis by regulating the coordinated expression of VEGF (31); MMP9 triggers the angiogenic switch during carcinogenesis (32). To investigate whether angiogenesis is affected by GM-CSF neutralization or MMP9 inhibition, we performed double immune-fluorescence analysis, staining endothelial cells (CD31+ cells) and pericytes (αSMA+ cells) in tumors from obese mice. In both models, GM-CSF neutralization and MMP9 inhibition resulted in strong inhibition of intratumoral angiogenesis, with significantly (p<0.01) lower microvessel density (MVD) (Fig. 5A-C). Use of both inhibitors together further impaired tumor angiogenesis, suggesting that both factors are crucially involved in breast cancer neovascularization. Use of both inhibitors did not affect the ratio of αSMA+ to CD31+ blood vessels, but did preferentially target αSMA+ cells (Fig. 5D).

GM-CSF promotes an immunosuppressive microenvironment, possibly leading to tumor immune escape

Multiparametric flow cytometry was used to investigate immune cells (for gating strategy see Fig. S4D). Peripheral blood, tumors, and peritumoral WAT were collected from obese tumor-bearing mice. A month after starting GM-CSF neutralization, the number of circulating monocytes was found to be significantly lowered in GM-CSF-neutralized mice, compared to controls (CTR) administered IgG2a MoAb (Fig. 5E). Immunosuppressive cells were also significantly reduced by GM-CSF neutralization in tumors and peritumoral WAT: T-regulatory (T-reg) cells and granulocytic (G)-MDSCs were reduced in tumors and WAT (Fig. 5F-G), whereas monocytic (M)-MDSCs were reduced in tumors only (Fig. 5F). Macrophage and TAM infiltration was also significantly reduced by GM-CSF neutralization.
Taken together, these findings suggest that GM-CSF is able to promote the recruitment of immunosuppressive cells to the tumor microenvironment, to thereby promote immune escape by tumor cells. By contrast, MMP9 inhibition (SB-3CT) had no effect on immune cells in peripheral blood, WAT or tumors (data not shown).

To further investigate immunosuppression, WAT and tumor cells from GM-CSF-neutralized mice were analyzed by qRT-PCR. In comparison to IgG2a controls, transcripts of several immunosuppressive factors were downregulated, including IL-10, IL-5, CXCL5, CCL22, CCL4, CXCR5 and CD274 (PD-L1) (Fig. S4E). GM-CSF neutralization more profoundly reduced the expression of these genes in WAT than tumor cells. It is noteworthy that GM-CSF and IL-1β gene expression was also downregulated in WAT and tumors, providing in vivo support to the GM-CSF regulation observed in vitro.

Metformin inhibits GM-CSF and MMP9 release in vitro

The antidiabetic drug metformin has been reported to inhibit the onset of several breast cancer subtypes in diabetic patients, especially those who are obese (18,19). The drug targets neoplastic and microenvironment cells, including endothelial cells and WAT-derived progenitors (17,21). Metformin was added directly to in vitro co-cultures to determine whether it affects GM-CSF and MMP9 release. Metformin was added at the concentration 5mM – a level at which cells remained viable at 72 hours (Fig. S5A,B). GM-CSF and MMP9 release was reduced in co-culture media to which metformin had been added, but not in single cell cultures (Fig. 6A,B). As expected, zymography showed reduced MMP9 enzymatic activity in the presence of metformin (Fig. 6C). GM-CSF and MMP9 transcript levels were unaffected by metformin (data not shown), suggesting that metformin does not affect the expression of these proteins at the transcriptional level.
**Metformin reduces hGM-CSF and hMMP9 expression in xenograft models**

Xenograft NSG mice, injected with hBC cells alone or with CD45-CD34+ hWAT progenitors, were given metformin in drinking water (2mg/ml) at a higher dosage than that used in diabetic patients, but found to be effective and non-toxic in preclinical models of breast cancer (21). Neither mouse weight, nor serum levels of glucose, cholesterol or triglycerides were significantly affected by the drug (data not shown). As expected in this model (21), tumor growth was significantly reduced (Fig. S5C). Plasma levels of hGM-CSF were reduced in metformin-administered mice, compared to controls (Fig. 6D). hMMP9 expression in whole tumor lysates, previously shown to be up-regulated in hBC-hWAT co-injected mice, was reduced in metformin-treated mice (Fig. 6E). By contrast, metformin did not reduce hMMP9 expression in tumor lysates from single-injected (hBC) mice, in fact pro-MMP9 levels were higher in metformin-administered mice (Fig. 6E).

**Metformin reduces angiogenesis and breast cancer progression in obese syngeneic models**

Since metformin reduces GM-CSF and MMP9 release in vitro and in vivo, we investigated the effect of the drug in obese syngeneic models of breast cancer. Mice were administered either metformin, anti-mGM-CSF MoAb or SB-3CT (Fig. 7). These treatments significantly reduced local breast cancer growth (tumor volume) compared to control, with GM-CSF-neutralization achieving a greater (not significant) reduction (Fig. 7A). Metastases in lungs were significantly reduced in treated mice (p<0.01, Fig. 7B). Quantification of intratumoral CD31+αSMA+ blood vessels indicated that metformin was more effective (p<0.001) in reducing angiogenesis than GM-CSF neutralization or MMP9 inhibition (p<0.01) (Fig. 7C). Flow cytometry of tumors and peritumoral WAT indicated that
metformin had similar effects to GM-CSF neutralization (Fig. 7D): several immunosuppressive populations, including T-regs and TAMs, were downregulated in peritumoral WAT, whereas G-MDSCs were strongly downregulated in tumors. Metformin significantly reduced the presence of inflammatory monocytes in both tumors and peritumoral WAT, providing further evidence of its anti-inflammatory activity. These findings suggest that GM-CSF and MMP9 might be previously unrecognized targets of metformin, which would explain some of its anti-tumor effects in breast cancer preclinical models.
Discussion

We investigated interactions between breast cancer cells and CD45-CD34+ WAT progenitors because the latter promote tumor growth, metastasis and angiogenesis in preclinical breast cancer models (15,16). We found that the proteins GM-CSF and MMP9 were significantly upregulated in murine and human WAT progenitors on exposure to a variety of breast cancer cell types in vitro and in vivo. These proteins were not upregulated in other WAT cells (e.g. hematopoietic cells). Both ASCs and ECs – constituents of the CD45-CD34+ WAT fraction – produced GM-CSF and MMP9 when co-cultured with breast cancer cells, consistent with previous findings that ASCs and ECs cooperate to support breast cancer growth, angiogenesis and metastatic spread (16).

GM-CSF is a growth factor for hematopoietic and immune cells, that mobilizes stem cells and induces macrophage/granulocyte differentiation (33). Other roles include the regulation of inflammation and autoimmunity (34). MMP9 is a type IV collagenase whose increased expression has been reported associated with higher grade, metastasis, and angiogenesis in several cancers (29,35).

We investigated various pathways to clarify breast cancer-dependent GM-CSF/MMP9 upregulation. Our findings indicate that GM-CSF produced by breast cancer cells induces GM-CSF and MMP9 release from WAT progenitors; IL-1β might be also induced by GM-CSF, as our and previous data suggest (28), possibly implicating it in MMP9 release by WAT progenitors.

In mice co-injected with breast cancer cells and WAT progenitors we found greater tumor volume and more metastases than in mice injected with breast cancer cells alone. However, when GM-CSF was knocked down in breast cancer cells, there was no increase in tumor growth. Thus tumor-derived GM-CSF seems to be essential for triggering the pro-tumorigenic actions of WAT-progenitors. Other studies support the existence of such
regulation. Thus, GM-CSF has been reported to up-regulate MMP9 in squamous cell carcinoma (29), and GM-CSF feedback regulation has been reported in myeloid cells (36). Considering that CD116, the GM-CSF receptor, is expressed on myeloid cells (36), we are now investigating why WAT progenitors do not produce GM-CSF and MMP9 in isolation but only when co-cultured, and the role of CD116 expression in obesity and WAT-embedded tumors.

To further explore the roles of GM-CSF and MMP9 in promoting breast cancer we examined the effects of inhibiting these proteins in a syngeneic breast cancer model generated in immune-competent obese mice. Obese mice were used because WAT progenitors are markedly increased in adipose tissue in obesity (17). Use of immune-competent animals allowed us to investigate the effect of GM-CSF on immune cells. We found that both GM-CSF neutralization and MMP9 inhibition significantly reduced tumor volume and number of metastases. However these anti-tumor effects may not be due (entirely) to direct interaction with tumor cells, but to microenvironment modulation, since GM-CSF neutralization reduced angiogenesis and immunosuppressive cells within the tumor, and MMP9 inhibition reduced angiogenesis and metastasis. MMP9 may exert its cancer-promoting effects by multiple mechanisms including degrading basement membranes (35), thus MMP9 inhibition could reduce tumor invasion into surrounding tissues.

Some recent data suggest that while angiogenesis inhibitors may suppress growth of primary tumors, they might also push the tumor into a more aggressive metastatic state (37). Accordingly, more studies in different models would be useful to better investigate the role of GM-CSF and MMP9 blockade in cancer local and metastatic progression.

Myeloid cells are known to be targeted by GM-CSF, which has been identified as a macrophage chemoattractant in the presence of WAT inflammation (28). In our models, GM-CSF neutralization was associated with reduced numbers of monocytes in PB, and
reduced macrophages and MDSCs in tumors and peritumoral WAT, while TAMs were reduced in tumors (TNBC model) and also peritumoral WAT – supporting the finding of Su et al. (26) that GM-CSF as a TAM inducer in breast cancer. Macrophage accumulation has been found to promote tumor growth in obesity by increasing chronic inflammation, immune escape, and angiogenesis (38). Thus it is possible that some of the anti-tumor effects of GM-CSF neutralization found in our study might be due to reduced macrophage activation. We note also that GM-CSF can expand MDSCs in breast cancer (39).

Lymphoid cells were not affected by GM-CSF neutralization in our experiments, consistent with reported lack of expression of GM-CSF receptors on T-cells, B-cells and natural killer cells (40). Nevertheless, T-regs were downregulated in GM-CSF-neutralized mice, so GM-CSF may modulate these cells, as suggested elsewhere (41). Several immunosuppressive interleukins and cytokines have also been reported downregulated in WAT and tumors from GM-CSF-neutralized mice, including IL-10, CCL22 and PD-L1 (42-43).

The prominent pro-tumorigenic effect of GM-CSF found in our obese syngeneic models is consistent with recent findings that GM-CSF ablation in in vitro primary pancreatic cancer cells resulted in defeated immune escape (44). Nevertheless the tumor-promoting effects of GM-CSF are surprising when considered alongside current therapeutic applications of GM-CSF such as hematopoietic stem cell mobilization (45) and immunotherapy (46). However many GM-CSF effects are dose- and context-dependent (47), Eubank et al (48) have reported opposite effects of GM-CSF in a different breast cancer model, thus the quantity of GM-CSF present in the tumor microenvironment may determine its biological effects on WAT progenitors.

As regards MMP9 inhibition, achieved by administration of SB-3CT, we found that this was associated with significant reduction in tumor progression in obese preclinical models. However, in general MMP inhibitors have failed to produce beneficial effects in cancer patients (49). This may be due to enrollment of patients with late-stage disease, whereas
MMP9 inhibition might be more efficient in early stage disease or in the presence of concomitant obesity.

We used metformin to disrupt the interaction between WAT progenitors and breast cancer cells. We and others have shown that metformin delays tumor progression by effects on both breast cancer cells and WAT progenitors (17,21). Metformin also reduces hyperglycemia and hyperinsulinemia, which are frequently associated with inflammation of breast WAT and breast cancer in humans (50). We found that metformin significantly reduced GM-CSF and MMP9 release in co-cultures, but not in single cell cultures, without affecting transcription. In vivo, in xenograft mice, high-dose metformin reduced levels of hGM-CSF and hMMP9 proteins. Metformin probably reduced the translation of these proteins in association with mTOR inhibition and decreased phosphorylation of S6 kinase (51). When we administered high-dose metformin to obese breast cancer models, it significantly reduced local tumor growth and metastasis, in a similar way to GM-CSF/MMP9 inhibition. The drug was also effective in reducing intratumoral angiogenesis and intratumoral immunosuppressive cells (TAMs, G-MDSCs, T-regs). Metformin blocks GM-CSF and MMP9 production only in co-cultured cells. As there is an evidence that – depending upon models and drug dosage – metformin can target a variety of pathways other than mTOR (17, 21, 50-51) , we are currently investigating multiple potential effects of metformin in the presence or in the absence of concomitant GM-CSF or MMP9 blockade.

Overall, these findings suggest that GM-CSF and MMP9 may be new targets of metformin in breast cancer, and add support to use of the drug in clinical studies on breast cancer, particularly in women who are obese or have insulin resistance. Nevertheless, further studies are required to clarify the mechanisms by which metformin prevents GM-CSF/MMP9 up-regulation in WAT progenitors.
Although in our model GM-CSF/MMP9 blockade had an effect also in WAT-poor organs such as the lungs, it would be crucial to further investigate how metformin and GM-CSF/MMP9 blockade can be effective in metastases arising in organs where there is little or no WAT, like – for instance - the brain or the bone, and how metformin and GM-CSF/MMP9 blockade can be effective in inhibiting primary BC cells collected from these metastatic sites. Another relevant field for investigation will involve the study of the pro-tumorigenic role of WAT cells from mice of different ages and different strains.
Acknowledgements

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References


Figure legends

Fig. 1

GM-CSF and MMP9 are up-regulated in WAT progenitors co-cultured with triple-receptor-negative breast cancer cell lines

A number of TNBC cell lines were co-cultured with primary human WAT (hWAT) progenitors (CD45-CD34+) isolated from 15 women. Cells (0.8x10^6) were seeded alone, in transwell (0.4µm), or in direct contact on collagen-coated 6-well plates, and cultured in EBM-medium for 72 hours.

A. Screening of angiogenesis-related factors by proteomic antibody array in media collected from single and co-cultures of MDA-MB-436 (breast cancer) with hWAT (CD45-CD34+) cells.

B. ELISA quantitation shows that GM-CSF release is higher in co-cultures (BC+WAT) than in breast cancer cells cultured alone (BC control, CTR) (n=15; * p<0.05, ** p<0.01, *** p<0.001).

C. Top: representative Western blots showing MMP9 in culture media. Band at 92kDa corresponds to pro-MMP9 (1:2000, anti-hMMP9). Bottom: representative MMP9 activity in culture media as assessed by zymography. WAT CD45-CD34+ cells were obtained from 2 patients.

D. Quantitation of MMP9 on Western blot using ImageJ software to determine average band density (n=15). MMP9 levels are significantly higher (*** p<0.001) in direct co-cultures compared to single MDA-MB-436 breast cancer and WAT single cultures. MMP9 release also increased in transwell co-cultures (*p<0.05).

E-F. qRT-PCR of GM-CSF (E) and MMP9 (F) transcripts from WAT progenitors and different TNBC cells co-cultured in transwell. Cells co-cultured in transwell were analyzed separately and compared to cells from respective single control cultures. Values are
normalized to ABL expression (housekeeping gene) and expressed as fold changes compared to WAT cells seeded alone (n=15; *** p<0.001 transwell vs. single culture).

**Fig. 2**

Up-stream regulation of GM-CSF/MMP9 release in co-cultures

Bortezomib (3nM), anti-human IL-1β MoAb (0.4µg/ml), anti-human GM-CSF MoAb (0.5-1µg/ml) or SB-3CT (1µM) were added to co-cultures of MDA-MB-436 (breast cancer) with WAT CD45-CD34+ progenitors, to assess their ability inhibit the production of GM-CSF and MMP9. No differences in GM-CSF or MMP9 expression were observed in the various controls, which are thus shown as single control in the figure.

**A.** ELISA quantitation of human GM-CSF in transwell and direct co-cultures of MDA-MB-436 (hBC) cells with WAT progenitors after addition of inhibitor, compared to MDA-MB-436 control (n=3; ** p<0.01, *** p<0.001).

**B.** Representative Western blots of pro-MMP9 (92kDa, 1:2000 anti-hMMP9) in culture media to which bortezomib or anti-human IL-1β MoAb was added.

**C.** Representative Western blots of pro-MMP9 (92kDa, 1:2000 anti-hMMP9) in culture media to which various quantities of anti-hGM-CSF MoAb were added.

**D-E.** qRT-PCR of GM-CSF (D) and MMP9 (E) transcripts from cells co-cultured in transwell system compared to single cell cultures (BC cultured in transwell with WAT vs. BC alone; WAT cultured in transwell with BC vs. WAT alone). Values are normalized to ABL expression (housekeeping gene) and expressed as fold changes compared to breast cancer cells cultured alone. GM-CSF transcription was significantly inhibited by GM-CSF neutralization (n=3; *** p<0.001). Anti-human IL-1β and anti-GM-CSF MoAbs prevented MMP9 up-regulation in WAT progenitors (n=3; ** p<0.01, *** p<0.001).
Fig. 3

GM-CSF and MMP9 in xenograft models co-injected with breast cancer and WAT-derived progenitors.

NSG mice were injected with MDA-MB-436 (hBC) cells, primary hWAT CD45-CD34+ cells, or both (n=12 per study arm, with hWAT from 3 different women). GM-CSF knockdown (shGM-CSF) in MDA-MB-436 cells was also evaluated in this xenograft model, in single- and co-injected mice (n=5 per study arm).

A. HS ELISA of plasma collected at various times after tumor injection. Concentrations normalized to tumor volume. Circulating hGM-CSF was significantly higher (n=12; ** p<0.01; *** p<0.001) in co-injected (hBC+hWAT) mice than mice injected only with hBC. Mice injected with hWAT only were negative controls (n=5).

B. Top: representative Western blots showing increased hMMP9 from whole tumor lysates of co-injected mice (1:500 anti-hMMP9). The bands at 92kDa is pro-MMP9. Bands at 67 and 82kDa, correspond to alternative cleaved active forms of hMMP9. Beta actin was loading control (42kDa; 1:5000). Bottom: representative zymography of whole tumor lysates. Increased hMMP9 proteolytic activity is evident in co-injected (hBC+hWAT) compared to single-injected (hBC) mice.

C. qRT-PCR of hGM-CSF and hMMP9 transcripts in breast cancer cells from xenograft mice. No up-regulation was found in co-injected mice compared to those injected hBC alone. Values are normalized to ABL expression and expressed as relative fold changes compared to single-injected tumors (n=9; ns: not significant).

D. GM-CSF knockdown (sh-GMCSF) in hBC cells grown in NSG mice but previously transduced with lentivirus carrying shRNAs. The hGM-CSF transcript was assessed by qRT-PCR on hBC cells isolated from tumors. Values are normalized to ABL expression and expressed as relative fold changes compared to mice injected with hBC transduced with scrambled shRNA (n=5; ** p<0.01 shGM-CSF vs. scramble; 82% knockdown).
E. Tumor size as measured by digital calipers. GM-CSF knockdown in single-injected mice (shGM-CSF hBC) did not alter tumor growth, compared to scramble-hBC (single) injected mice. In co-injected mice (hBC + hWAT), GM-CSF knockdown is associated with a significantly lower tumor volume than in scramble co-injected mice, but no difference in volume compared to single-injected mice (n=5; ** p<0.01 vs. scramble co-injected mice).

F. Lung metastases in H&E-stained sections from single- or co-injected mice using scrambled- or shGM-CSF-transduced hBC cells. The Aperio digital system was used to quantify the number of metastatic foci (n=5; * p<0.05, ** p<0.01 sh-GMCSF vs. scramble).

G. Effect of GM-CSF knockdown on plasma hGM-CSF (HS ELISA) from single- (hBC) and co-injected (hBC + hWAT) mice. Values normalized to tumor volume. Mice injected with sh-GM-CSF hBC have significantly lower plasma hGM-CSF than mice injected with scrambled shRNA hBC (n=5; * p<0.05, ** p<0.01, *** p<0.001).

H. Representative Western blots showing pro-MMP9 (92kDa) and active MMP9 (67kDa) in whole tumor lysates (1:500 anti-hMMP9, Proteintech). Beta actin (42kDa; 1:5000, Sigma-Aldrich) was loading control. Active MMP9 levels are lower in shGM-CSF hBC-injected mice than in scramble-hBC-injected controls.

Fig. 4

Neutralization of GM-CSF and inhibition of MMP9 in obese syngeneic models of breast cancer

A. Combined administration of anti-mGM-CSF MoAb (#BE0259, 50µg/mouse ip every other day) and the MMP9 inhibitor SB-3CT (25mg/kg/mouse ip every other day) had a synergic effect on local breast cancer growth in obese MMTV-ErbB2+ FVB/Hsd mice. Tumor growth was measured weekly with digital calipers (n=5 per study arm; * p<0.05, ** p<0.01, *** p<0.001). Control mice were administered both IgG2a MoAb (#BE0089) and SB-3CT vehicle.
B. Left: lung metastases assessed 30 days after mastectomy (n=5 per study arm; **
p<0.01, *** p<0.001). Right: representative images of H&E-stained lung from obese mice infected with MMTV-ErbB2+ breast cancer cells (Aperio digital system).

C. Tumor growth is significantly reduced in obese 4T1-injected BALB/c and administered anti-mGM-CSF MoAb, SB-3CT or both (n=10, *** p<0.001).

D. Evaluation of lung metastases 15 days after mastectomy in obese BALB/c mice: representative of H&E-stained images of lungs, acquired with Aperio digital system (n=10; ** p<0.01).

E. Spleen from obese BALB/c mice, 15 days after mastectomy: weight measurement and representative images (n=10; * p<0.05, ** p<0.01, *** p<0.001).

Fig. 5

Inhibition of GM-CSF and MMP9 in obese model of breast cancer reduces neoplastic angiogenesis and tumor-induced immunosuppression

A. Representative confocal microscope images of tumors (20x magnification, scale bars 100µm). Staining is anti-mCD31 (green, 1:20, Abcam) for endothelial cells, anti-αSMA (red, 1:3000) for pericytes, and DAPI for nuclei (blue).

B-C. Analysis of intratumoral microvessel density (MVD) in (B) obese 4T1-injected BALB/c and (C) obese FVB/Hsd mice injected with primary MMTV-ErbB2+ breast cancer cells. Blood vessels were quantified with ImageJ software. MVD is significantly reduced in all groups that received GM-CSF/MMP9 inhibitors (n=5, ** p<0.01, *** p<0.001).

D. Quantitation of relative amounts of CD31+ and αSMA+ blood vessels, normalized to tumor area in MMTV-ErbB2+ injected mice (n=5; ns: not significant).

E. Flow cytometry to quantitate (cells/µl) immune cells in peripheral blood (PB), collected 30 days after breast cancer injection. Circulating cells were analyzed in obese 4T1-
injected Balb/c and MMTV-ErbB2+-injected mice, treated with anti-GM-CSF MoAb (#BE0259) or IgG2a control (#BE0089) (n=5; * p<0.05, ** p<0.01, *** p<0.001).

**Flow cytometry to quantitate immune cells (F) in tumors and (G) in peritumoral WAT (cells/g).** In both tumors and WAT, macrophages (F4/80+CD11b+Gr1-), TAMs (CD206+ MHCII<sub>low</sub> macrophages) and G-MDSCs (CD11b+ F4/80+/ Ly6C<sub>low</sub> Ly6G+) infiltration was reduced. Peritumoral WAT had reduced levels of T-regs (CD4+ CD25<sup>bright</sup> CD127<sup>low</sup>) in GM-CSF-neutralized mice compared to IgG2a controls (n=5; * p<0.05, *** p<0.001, ** p<0.01).

**Fig. 6**

**Metformin reduces GM-CSF and MMP9 release in vitro and in xenograft mice**

**A.** ELISA quantitation of hGM-CSF in culture media with added metformin (5mM).

Metformin inhibited GM-CSF release in co-cultures of breast cancer (MDA-MB-436) and WAT-progenitors, compared to no metformin controls (n=8; * p<0.05,** p<0.01).

**B.** Representative Western blot of pro-MMP9 (92kDa; 1:2000 anti-hMMP9) in co-culture media with added metformin. MMP9 release was lowered in both direct and transwell co-cultures (MDA-MB-436 breast cancer).

**C.** Zymography shows reduced MMP9 activity in media of direct co-cultures with added metformin (MDA-MB-436 breast cancer).

**D.** Influence of metformin administration on plasma levels of hGM-CSF in NSG mice injected with MDA-MB-436 breast cancer (hBC) and WAT progenitors. hGM-CSF was quantified by HS ELISA (#HSGM0) 42 and 56 days after injection of hBC. Concentrations normalized to tumor volume. Values are fold changes compared to hBC mice not given metformin. All mice given metformin had lower plasma hGM-CSF than untreated mice (n=12 per study arm; * p<0.05, ** p<0.01, *** p<0.001).
E. Representative Western blots of whole tumor lysates from single- (hBC) or co-injected (hBC+hWAT) mice (1:500, anti-hMMP9) treated or not treated with metformin. Precursor (92kDa) and active (67kDa) hMMP9 levels are reduced in lysates from metformin-treated mice. Beta actin was loading control (42kDa; 1:5000).

**Fig. 7**

**Metformin reduces breast cancer growth and metastases in obese syngeneic mice**

FVB/Hsd mice on HFD, were injected orthotopically with $0.5 \times 10^6$ MMTV-ErbB2+ cells and given high-dose (2mg/ml) metformin in drinking water, or anti-mGM-CSF MoAb (#BE0259, 50µg ip every other day) or SB-3CT (25mg/kg ip every other day). Control mice were administered IgG2a MoAb (#BE0089) and SB3CT vehicle.

A. Tumor volume, measured weekly with digital calipers, was significantly reduced (n=5 per study arm; * p<0.05, ** p<0.01) in metformin-treated animals compared to controls.

B. Metastases were investigated 30 days after mastectomy in lungs. H&E sections were acquired with the Aperio digital system. Metastases were significantly reduced in metformin-treated mice (n=5; ** p<0.01).

C. Intratumoral staining with anti-mCD31 (green, 1:20) for endothelial cells, anti-αSMA (red, 1:3000, Sigma-Aldrich) for pericytes, and DAPI for nuclei (blue). Images acquired with Leica confocal microscope (20x magnification, scale bars represent 100µm). Metformin had greater anti-angiogenic activity than inhibition of MMP9 or neutralization of GM-CSF (n=3; ** p<0.01, *** p<0.001).

D. Multiparametric flow cytometry of immune cell infiltration of tumors (top) and peritumoral WAT (bottom). Only cell populations altered by the treatments (metformin, GM-CSF neutralization) are shown; units are cells/g tissue. Metformin had similar effects to GM-CSF neutralization, downregulating immunosuppressive cells (TAMs, G-MDSCs, T-regs) (n=5 per study arm; * p<0.05, ** p<0.01, *** p<0.001).
Figure 1

A

BC (MDA-MB-436) WAT CD45/CD34

BC + WAT direct BC + WAT transwell

GM-CSF MMP9 IL-8 VEGF SerpinE1

TIMP-1 uPA

B

GM-CSF protein

HCC1937 MDA-MB-231 MDA-MB-436 WAT

BC CTR (direct) BC + WAT direct BC CTR (transwell) BC + WAT transwell

C

MMP9 protein

Patient 1 Patient 2

MMP9 (92kDa)

D

MMP9 protein

MDA-MB-231 HCC1937 MDA-MB-436 WAT

BC CTR (direct) BC + WAT direct BC CTR (transwell) BC + WAT transwell

WAT

E

GM-CSF mRNA

HCC1937 MDA-MB-231 MDA-MB-436

F

MMP9 mRNA

WAT CTR WAT transwell BC CTR BC transwell
Figure 2

A. GM-CSF protein

B. MMP9 protein

C. MMP9 protein

D. GM-CSF mRNA

E. MMP9 mRNA

Note: The figures illustrate the expression levels of GM-CSF and MMP9 proteins and mRNAs under different conditions, including the use of bortezomib, anti-IL-1β MoAb, and anti-GM-CSF MoAb. The graphs show the effects on cellular levels, with bars indicating the control (CTR), bortezomib treatment, anti-IL-1β MoAb, and anti-GM-CSF MoAb at different concentrations.
Figure 3

A. Plasma hGM-CSF in NSG mice (MDA-MB-436)

B. hMMP9 in tumor microenvironment (NSG mice)

C. mRNA of hBC from NSG mice

D. GM-CSF knockdown in NSG mice

E. Tumor growth (GM-CSF knockdown mice)

F. Lung metastases (GM-CSF knockdown mice)

G. Plasma hGM-CSF (GM-CSF knockdown)

H. hMMP9 in tumor microenvironment (GM-CSF knockdown)
**Figure 4**

A. MMTV-ErbB2

- FVB/Hsd 6-week old females
- HFD, MMTV-ErbB2+ injection, Start of treatment, Mastectomy, Sacrifice
- Tumor volume measurement

B. MMTV-ErbB2+ Lung metastases

- Mean number of metastatic foci
- CTR, Anti-MMP9, Anti-GM-CSF, Anti-GM-CSF + Anti-MMP9

C. 4T1

- BALB/cOlaHsd 6-week old females
- HFD, 4T1 injection, Start of treatment, Mastectomy, Sacrifice
- Tumor volume measurement

D. 4T1 Lung metastases

- Metastases mean area (mm²)
- CTR, Anti-GM-CSF, Anti-MMP9, Anti-GM-CSF + Anti-MMP9

E. 4T1 Spleen

- Splenic weight (g)
- CTR, Anti-GM-CSF, Anti-MMP9, Anti-GM-CSF + Anti-MMP9
- Not injected, CTR, Anti-MMP9
Figure 5

A

CTR

Anti-MMP9

Anti-GM-CSF

Anti-GM-CSF + Anti-MMP9

B

4T1 tumor angiogenesis

MMTV-ErbB2+ tumor angiogenesis

C

4T1 tumor angiogenesis

MMTV-ErbB2+ tumor angiogenesis

Ratio of αSMA to CD31 cells

Number of vessels

Mean ratio αSMA+/CD31+

D

E

Circulating immune cells

F

Intratumoral immune cells

G

Peritumoral WAT immune cells

Cells/µl of PB

Monocytes

Granulocytes

Inflammatory monocytes

Macrophages

TAMs

Inflammatory M-MDSCs G-MDSCs

T-regs

Cells/g of tumor

Cells/g of WAT

Mean ratio αSMA+/CD31+

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**Figure 6**

**A** In vitro MDA-MB-436 (BC)

- CTR
- METFORMIN 5mM

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**B** In vitro MDA-MB-436 (BC)

- Metformin 5mM

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**C**

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**D** In vivo xenograft

- MDA-MB-436 (hBC)
- hBC + hWAT

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**E** In vivo xenograft

- Metformin
- Pro-MMP9 (92kDa)
- Cleaved MMP9 (67kDa)
- Beta actin

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Adipose progenitor cell secretion of GM-CSF and MMP9 promotes a stromal and immunological microenvironment that supports breast cancer progression

Francesca Reggiani, Valentina Labanca, Patrizia Mancuso, et al.

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