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Stromal cells in breast cancer microenvironment: molecular mechanisms involved in tumor progression and potential therapeutic targets

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ABSTRACT

Stromal cells in the tumor microenvironment (TME) play an important role in breast cancer progression, metastasis and therapeutic outcome. Among stromal cells, Tumor-Associated Macrophages (TAMs) and Mesenchymal Stem Cells (MSCs) have been shown to sustain breast tumor progression and worsen breast cancer prognosis. Elucidating the molecular mechanisms of epithelial/stromal cell interactions and discovering new therapeutic targets within the breast TME represent the main challenge of current research to increase the chances of successful treatment of breast cancer patients. Here, we firstly investigated the role of ligand-activated Peroxisome Proliferator Activator Receptor γ (PPARγ), a well-known tumor suppressor gene, to modulate breast TAM functional phenotype. We found that the treatment with natural and synthetic PPARγ ligands reduced the cytokine secretion by TAMs generated by exposure of conditioned media (CM) from breast cancer cells (BCCs). Interestingly, this effect was reversed by the PPARγ antagonist GW9662, suggesting the potential involvement of PPARγ in the attenuation of TAM polarization. Next, since it has been reported that soluble factors released in the TME mediate the tumor/stroma interactions, we mainly focused on the role of leptin which has been reported to sustain macrophage recruitment. Thus, we explored the impact of the leptin receptor knockdown (ObR sh) on BCCs in mediating the interaction between tumor cells and macrophages. In co-culture experiments between monocytes and BCCs, the absence of ObR reduced the recruitment of macrophages and affected their cytokine mRNA expression profile toward a less aggressive phenotype. We confirmed a decreased macrophage infiltration and reduced breast cancer growth in xenograft tumors of mice injected with ObR sh BCC.

Furthermore, we explored the interaction between BCCs and MSCs within the breast TME. To this aim, we generated BCCs engulfing MSCs which result in hybrid cancer cells characterized by a multinucleated phenotype with increased dormancy and chemoresistance. In mouse models of breast cancer metastasis, hybrid cells had a reduced ability to form metastasis, but upon doxorubicin treatment they acquired resistance, inducing the metastatic spread of breast cancer.

Collectively, our findings provide novel insights into the role of PPARγ and leptin signaling in modulating TAM polarization, opening new avenues for therapeutic intervention in breast cancer. Moreover, we identified and characterized a hybrid cell population, generated through

MSC engulfment by BBCs, with phenotypic features of malignancy, highlighting the potential of targeting stromal cells, to overcome drug resistance and metastasis in breast cancer.

INTRODUCTION

Breast cancer

Breast cancer is the most frequent malignancy diagnosed in women worldwide and the second most common cancer overall (Bray et al. 2018). Based on the expression of the three main molecular targets, the estrogen receptor alpha (ER-α), the progesterone receptor (PR) and the epidermal growth factor 2 (HER-2), breast cancer is classified into 4 different subtypes: luminal ER positive (luminal A and luminal B), HER2 enriched and basal-like (triple-negative) (Waks et al. 2019), each one clinically treated with a specific therapeutic approach. In particular, patients with hormone receptor positive tumors receive endocrine therapy consisting of antiestrogen medication, whereas patients with HER2+ tumors receive HER-2-target antibody or small-molecule inhibitor therapy combined with chemotherapy. In contrast, patients with triple negative tumors are treated with chemotherapy alone since they do not express the molecular therapeutic targets (Waks et al. 2019). Over the last years, numerous research reported that tumors consist not only of neoplastic cells, but also of significant alterations in the components of the surrounding tumor microenvironment (TME) (Artacho-Cordón et al. 2012). Moreover, it has been demonstrated that a dynamic interaction existing between breast cancer cells and the components of the TME impacts breast tumor progression influencing the effectiveness of the therapeutic treatment (Chen et al. 2015). Thus, understanding the interactions between tumor and stroma is needed for the development of more effective therapeutic strategies.

Tumor microenvironment

The TME is a high tissue complex, composed of cellular and non-cellular components, that sustains the development and progression of breast cancer. The cellular component consists of various stromal cells, including immune cells, adipocytes, endothelial cells, fibroblasts, pericytes, and mesenchymal cells, whereas the non-cellular component includes the extracellular matrix (ECM) and soluble substances, such as various cytokines and chemokines, growth factors, metabolites, exosomes and microvesicles (Zhong et al. 2020). During tumor initiation, breast cancer cells educate the surrounded non-malignant cells in the TME to acquire a new phenotype that promotes tumorigenesis. In turn, the transformed cells in TME, release cytokines, chemokines, growth factors, inflammatory mediators and matrix remodeling enzymes, creating a

favorable milieu for tumor progression (Place et al. 2011; Roma-Rodrigues et al. 2019; Baghban et al. 2020; Hill et al. 2020) (Figure 1).

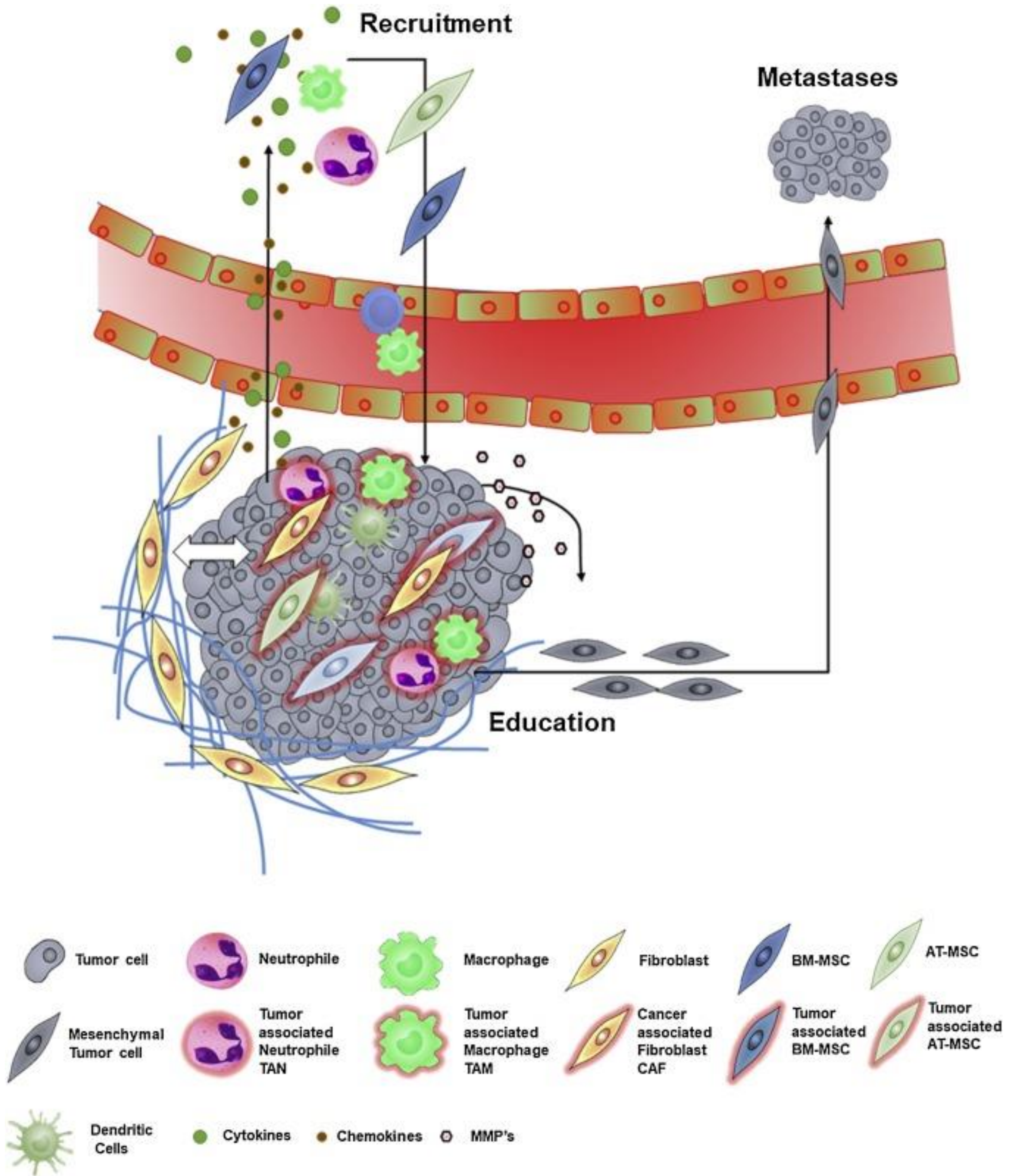


Figure 1. Schematic illustration of cancer cells and tumor microenvironment interplay. Stromal cells are recruited into the tumor site and educated into a pro-tumoral phenotype though a bidirectional communication with breast cancer cells. BM-MSCs: bone marrow mesenchymal stem cells, AT-MSCs: adipose tissue mesenchymal stem cells

Tumor Associated Macrophages

Macrophages are one of the most abundant immune cells in the TME and may constitute over 50% of the solid tumor mass (Solinas et al. 2009). In the inactive state (M0), macrophages are highly plastic and can change their phenotypes under influence of environmental signals to generate a population with different properties and functions (Mantovani et al. 2002). Mirroring T helper cell classification, activated macrophages are often classified in two opposite classes: pro-inflammatory M1 macrophages and pro-tumoral M2 macrophages. The M1 macrophages are induced by Th1 cytokines, such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFNγ) producing cytokines, such as interleukin (IL)-6, IL-12, IL-23, matrix metallopeptidase 9 (MMP-9) and TNF-α, associated with microbicidal and pro-inflammatory activities. The M2-like macrophages, on the other hand, are polarized by Th2-derived cytokines, such as IL-4, IL-10, IL-13, and secrete factors, including vascular epithelial growth factors (VEGFs), transforming growth factor β (TGFβ), IL-1 Receptor antagonist (IL-1RA) and IL-10 that support tumor growth and metastasis (Qiu et al. 2018; Jayasingam et al. 2020). M1 and M2 macrophages can also be distinct based on the regulation of the L-arginine metabolism. In particular, M1 macrophages are characterized by a high expression of the nitric oxide synthase 2 (NOS2 or iNOS), which converts the L-arginine into L-citrulline and nitric oxide (NO), important for the microbicidal activity. In contrast, M2 macrophages express high amount of arginase-1, which metabolized L-arginine into urea and ornithine, essential for collagen synthesis, cell proliferation and tissue remodeling (Biswas and Mantovani 2012). During breast cancer initiation, macrophages are recruited into the tumor site through the release of chemokines and growth factors by breast cancer cells, such as C–C motif chemokine ligand 2 (CCL2) /monocyte chemoattractant protein 1 (MCP-1) and colony-stimulating factor 1 (CSF-1) (Figure 2) (Genard et al. 2017). In breast TME, M0 macrophages acquire the phenotype of Tumor-Associated Macrophages (TAMs) (Qiu et al. 2018). In particular, in the early stage of tumorigenesis, tumors are characterized by a predominance of proinflammatory M1 macrophages that should destroy the tumor cells. Unfortunately, the inflammation-driven cancer response is usually weak because the tumor-associated antigens are considered "self". As result, the pro-inflammatory milieu is not enough to eradicate tumor cells and, in contrast, support tumor growth and progression (Whiteside 2008). Once malignancy has been established, most TAMs belong to the M2 phenotype and contribute to worsening breast cancer prognosis, enhancing cancer cell resistance

to chemotherapy, tumor migration, invasion and metastasis (Whiteside 2008). However, signals generated from tumor cells or other components of the TME can skew macrophages to different phenotypes that do not respect the classical M1/M2 polarized cell features. Indeed, the TAM phenotype is characterized by the expression of both M1 and M2 markers, suggesting that in the context of the TME fully polarized macrophages have to be considered as extremes in a spectrum of activation states (Mantovani et al. 2017).

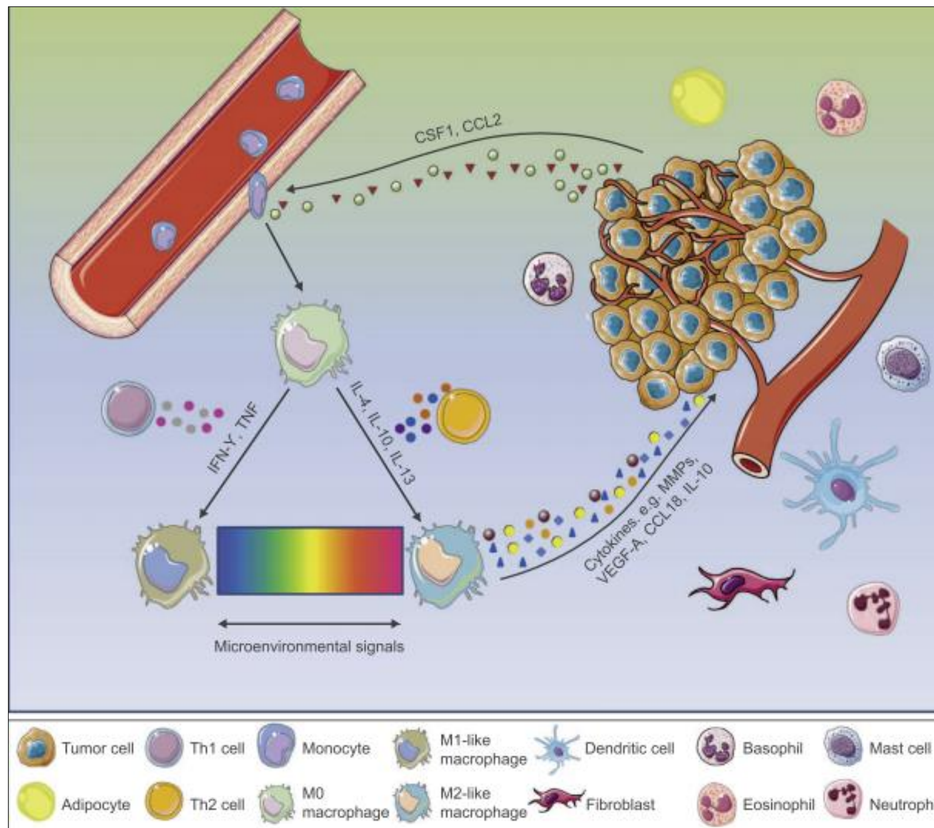


Figure 2. Macrophage recruitment and polarization in the breast tumor microenvironment. Breast cancer cells secrete colony stimulating factor 1 (CSF1) and chemokine (C-C motif) ligand 2 (CCL2) to recruit monocytes into the tumor microenvironment. Under the influence of microenvironmental signals, monocytes differentiate into M0 macrophages and polarized into M1-like and M2-like tumor associated macrophages (TAMs), which represent two extremes polarization states of the TAM population.
Interleukin (IL)-4, -10, -13; matrix metalloproteases (MMPs), vascular endothelial growth factor A (VEGF-A), interferon-γ (IFN-γ), tumor necrosis factor (TNF), Chemokine (C-C motif) ligand 18 (CCL18)

Regardless TAM phenotype, TAM infiltration has a negative prognostic relevance in breast cancer since it is correlated with high grade, lack of hormone receptors and worst outcome (Campbell et al. 2011). Thus, reprogramming macrophage phenotype or removal of all

macrophage populations regardless of polarization state has emerged as a potential therapeutic option to reduce primary and metastatic breast carcinogenesis (Ostuni et al. 2015).

Mesenchymal Stem Cells

Over the last years, Mesenchymal Stem Cells (MSCs) have attracted much interest because of their important role in many pathological processes, including cancer (Galland and Stamenkovic 2020). MSCs are self-renewing and multipotent progenitor cells, mainly found in the bone marrow and in adipose tissue, characterized by the expression of stromal cell markers such as CD73, CD90, and CD105, but not hematopoietic and endothelial cell markers, such as CD45, CD34, CD14 or CD11b, CD79α, CD19 (Whiteside 2008). It has been reported that MSCs are recruited in the tumor site by the cytokines and chemokines released by cancer cells to support each step of tumor progression (Nwabo et al. 2017) (Figure 3). Indeed, MSCs can release cytokines and chemokines, such as C-X-C motif chemokine 12 (CXCL12) and C-X-C motif chemokine 1 (CXCL1), that interact in a paracrine manner on breast cancer cells enhancing cancer cell proliferation, migration and invasion. Moreover, MSCs secrete a wild spectrum of molecules involved in tissue repair, such as the vascular endothelial growth factor (VEGF), which contributes to protect cancer cells from the effects of chemotherapy drugs. In the TME, MSCs also exert immunomodulatory actions. Indeed, they can establish contact to contact interactions with the immune cells or release mediators that inhibit the inflammatory responses, protecting the tumor cells from detection and destruction by the adaptive immune system (Cuiffo and Karnoub 2012). Interestingly, it has been reported that MSCs support breast tumorigenesis differentiating in other cell types (Dominici et al. 2006; Nwabo et al. 2017;). In particular, MSCs have been recognized to be an important source of cancer-associated fibroblasts (CAFs), that are well known for their role in enhancing cancer cell survival, stemness and chemoresistance (Nwabo et al. 2017). Moreover, MSCs can differentiate into pericytes or endothelial-like cells promoting breast cancer angiogenesis (Lin et al. 2019). In the TME, MSCs can also fuse with other cells in a process known as trans-differentiation (Nwabo et al. 2017) or be cannibalized by breast cancer cells supporting tumor dormancy (Bartosh et al. 2016). Recently, it has been reported that breast cancer cells can engulf MSCs generating a more aggressive breast cancer phenotype, with an upregulated gene signature consisting of Macrophage scavenger receptor 1 (MSR1), WNT5A, (Engulfment And Cell Motility 1) ELMO1, Interleukin 1 Receptor Like 2

(IL1RL2), Zona Pellucida Like Domain Containing 1 (ZPLD1) and SIRB1 (Chen et al. 2019). In particular, it has been reported that MSC engulfment increases the epithelial-mesenchymal transition (EMT), migration, invasion and self-renewal traits of breast cancer cells to potentiate breast cancer metastasis (Chen et al. 2019). However, the phenotypic features of MSC engulfed by breast cancer cells are still unclear.

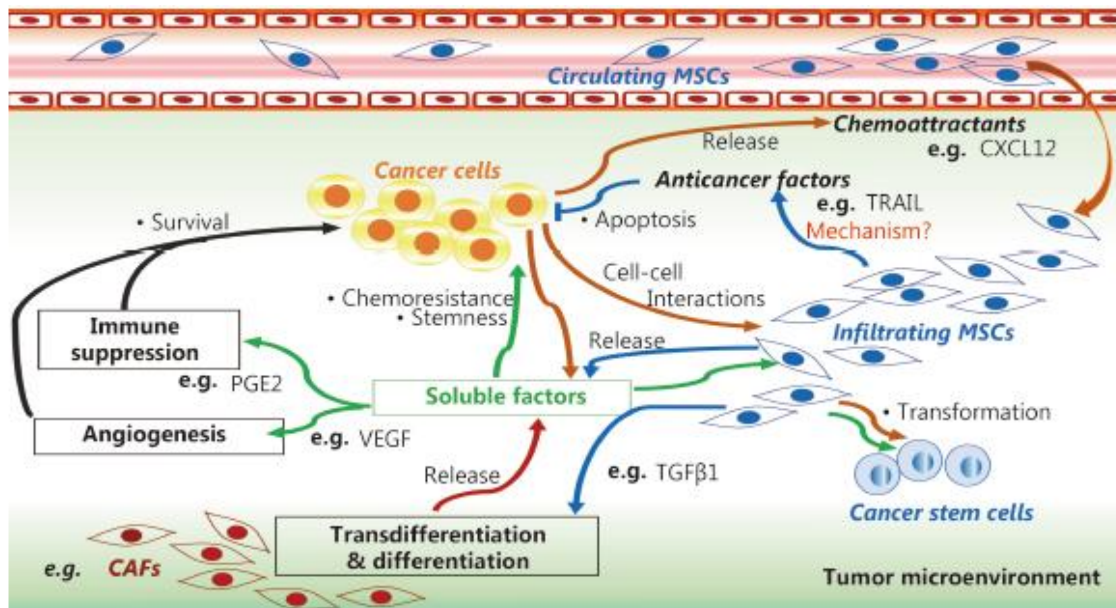


Figure 3. Mesenchymal stem cells role in breast tumor microenvironment. Circulating Mesenchymal Stem Cells (MSCs) are recruited into the tumor microenvironment through chemoattractant molecules released by breast cancer cells and support tumor survival, stemness and chemoresistance. CAFs: cancer associated fibroblasts, PGE2: prostaglandin E_2; VEGF: vascular endothelial growth factor; TGFβ1: Transforming growth factor beta 1; CXCL12: C-X-C motif chemokine 12.

Therapeutic targets

PPARγ

Peroxisome Proliferator Activator Receptor (PPAR) γ is a nuclear receptor belonging to the PPARs subfamily, which includes PPARα and PPARβ/δ (Desvergne and Wahli 1999; Guan and Breyer 2001). As the other members of the nuclear receptor family, the structural organization of PPARγ consists of five different domains: the N-terminus domain (A/B domain) comprises the ligand-independent activation factor 1 (AF-1) region, that once phosphorylated regulates PPARγ activity (Quintão et al. 2019); the central DNA-binding domain (C domain) is involved in the binding of PPARγ to the PPAR response elements (PPRE) in the promoter region of PPAR-response genes, regulating their transcription (Guan and Breyer 2001; Quintão et al. 2019;); the D domain is a flexible hinge region connecting the C domain to the ligand-binding domain (E

domain), which, in turn, regulates the ligand recognition and binding (Guan and Breyer 2001); the F domain, localized in the C-terminus, contains the ligand-dependent activation domain (AF-2), involved in the docking of coactivator proteins in response to ligand stimulation (Tontonoz and Spiegelman 2008; Quintão et al. 2019). In the inactive state, PPARγ is localized into the cytoplasm, bound to a complex of co-repressor proteins, such as the nuclear receptor corepressor complexes (N-CoR) or silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), which prevent PPARγ activation (Powell et al. 2007; Kroker and Bruning 2015). After the binding to its agonists, PPARγ forms a heterodimer with the Retinoid X Receptor (RXR) and translocates into the nucleus to bind the PPRE in the PPARγ target genes. Moreover, the transcriptional activity of ligand-activated PPARγ is enhanced by coactivator proteins, such as PPARγ coactivator 1-α (PGC-1α), binding protein p300 (EP300), vitamin D receptor-interacting protein (DRIP) or thyroid hormone receptor-associated protein (TRAP), which allow the initiation of the genetic transcription remodeling the chromatin structure and facilitating the binding of the RNA polymerase to the promoter region of PPARγ target genes (Zieleniak et al. 2008). More than 100 genes mainly involved in the lipid and glucose metabolism are PPARγ-regulated factors. In addition, ligand-activated PPARγ modulates the expression of different genes involved in tumorigenesis, counteracting the progression of several types of cancer, including breast carcinoma (Fujimura et al. 1998; Tsubouchi et al. 2000; Kotta-Loizou et al. 2010). In breast cancer, activation of PPARγ by its natural or synthetic ligands, which include omega-3 polyunsaturated fatty acids and their derivatives and thiazolidinediones, respectively, induces breast cancer cell death and reduces cell growth, motility and invasion (Grommes et al. 2004; Bonofiglio et al. 2009; Catalano et al. 2011; Augimeri et al. 2020). Interestingly, PPARγ is not only expressed in epithelial breast cancer cells, but also in other components of the breast TME, including CAFs and TAMs (Herwig et al. 2013; Rovito et al. 2016). It has been reported that the activation of PPARγ in different cells of the breast TME results in a reduction of the breast tumor progression (Cheng et al. 2016; Rovito et al. 2016). Although the tumor suppressor role of PPARγ in the breast cancer microenvironment has been suggested (Figure 4, manuscript under review *International Journal of Molecular Sciences* Augimeri et al. 2020), further investigations are needed to fully understand the potential of its activation in different cells of the TME, including macrophages.

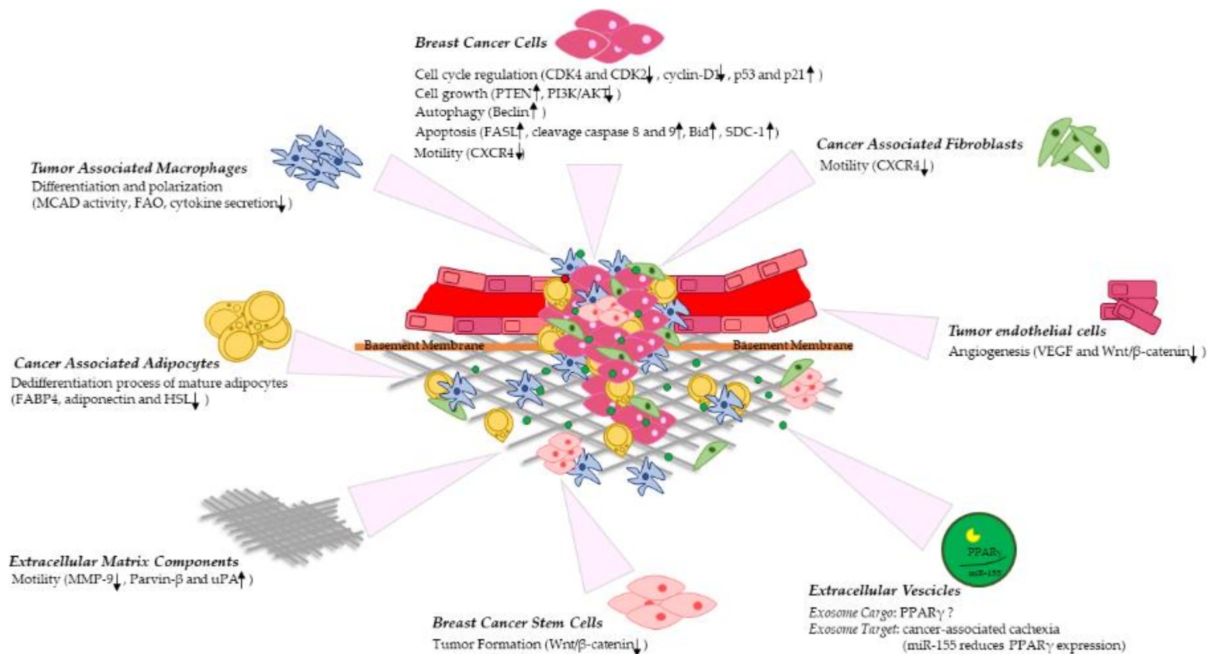


Figure 4. The potential tumor suppressor role of PPARγ in breast cancer microenvironment. PPARγ activation in several cells of the tumor microenvironment, including cancer associated-fibroblasts, -adipocytes, -macrophages, breast cancer stem cells, exosomes and extracellular matrix components induces anti-tumoral effects through the modulation of pro-tumorigenic pathways and genes involved in tumor death, invasion and migration. CDK: cyclin-dependent kinase; PTEN: phosphatase and tensin homolog on chromosome ten; PI3K/AKT: phosphatidylinositol 3-kinase/protein kinase B; FASL: FAS Ligand; SDC-1: syndecan-1; CXCR4: C-X-C chemokine receptor type 4; MCAD: medium-chain acyl-CoA dehydrogenase; FAO: fatty acid oxidation; FABP4: fatty acid binding protein 4; HSL: hormone sensitive lipase; VEGF: vascular endothelial growth factor; MMP9: Matrix metallopeptidase 9; uPA: urokinase plasminogen activator.

Leptin

Leptin is a 16 kDa polypeptide molecule mainly produced by the adipocytes of the white adipose tissue and involved in the control of feeding, metabolism and body weight (Margetic et al. 2002; 2002; Pan and Myers 2018). The molecular action of leptin depends on its binding to the leptin receptor (ObR), which exists in two different isoforms, namely long and short isoforms (Pan and Myers 2018). The long isoform is mainly expressed in the brain, but it is also present in all types of immune cells, whereas the short isoform is highly expressed in various cells and tissue, including kidney, liver and macrophages (Pan and Myers 2018). Alterations of the leptin signal in the central nervous system result in hyperleptinemia which is often correlated with obesity and the development of breast cancer in postmenopausal women (Gonzalez et al. 2006; Yang and Barouch 2007). It has been reported that leptin and its receptor are overexpressed in breast carcinoma and their expression is associated with a worst prognosis (Wu et al. 2009; Guo et al.

2012). Indeed, immunohistochemical staining has revealed that leptin expression is positively correlated with grade, stage, lymph node involvement and recurrence in breast carcinoma (Khabaz et al. 2017). Leptin produced by breast cancer cells binds to the long form of ObR and induces the activation of the janus kinase (JAK)/signal transducer and activator of transcription (STAT), the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3 kinase (PI3K) signaling pathways, supporting breast cancer cell proliferation, differentiation, survival, migration, and invasion (Margetic et al. 2002). Interestingly, it has been demonstrated that ligand activated PPARγ antagonizes the leptin signaling through the inhibition of the leptin-induced MAPK/STAT3/ Protein kinase B (Akt) pathway, thus reducing tumor proliferation in *vitro* and in *vivo* models (Catalano et al. 2011). Leptin is not only produced by the epithelial breast cancer cells, but also by other stromal cells and it affects breast cancer progression through endocrine, paracrine and autocrine mechanisms (Figure 5, modified from Park and Scherer 2011) (Andò and Catalano 2011; Andò et al. 2014; Barone et al. 2016; Giordano et al. 2016; Barone et al. 2012). In particular, it has been reported that the activation of MAPK/STAT3/Akt pathways induced by leptin in endothelial cells supports their migration, enhancing angiogenesis and neovascularization (Bouloumié et al. 1998; Sierra-Honigmann et al. 1998). Moreover, it has been demonstrated that leptin promotes breast cancer stem cell (CSC) formation and chemoresistance through the activation of the STAT3 signaling. Interestingly, treatment with a leptin receptor antagonist showed to reverse the breast CSC phenotype (Giordano et al. 2016). Recently, we have reported that leptin induces the generation and release of exosomes, extracellular vesicles involved in cell-to-cell communication, from breast cancer cells (Giordano et al. 2019). More importantly, leptin has been found to be involved in macrophage recruitment (Andò and Catalano 2011). In fact, in *vivo* studies have demonstrated that adipose tissue within the mammary TME of obese mice exhibited higher numbers of macrophages and crown-like structures than that of lean tumor-bearers (Santander et al. 2015; Ip et al. 2017). Furthermore, it was shown that leptin stimulates the secretion of interleukins (IL)-8 and -18 by TAMs, thus promoting the malignant phenotype of breast cancer cells (Ruffell et al. 2014; Cao et al. 2016). More recently, it has been also reported that enhanced production of leptin from anastrozole-resistant MCF-7 breast cancer cells impacts macrophage behavior within the TME (Gelsomino et al. 2020). However, definitive conclusions on the biological significance of ObR in mediating tumor/stroma crosstalk deserve further investigation.

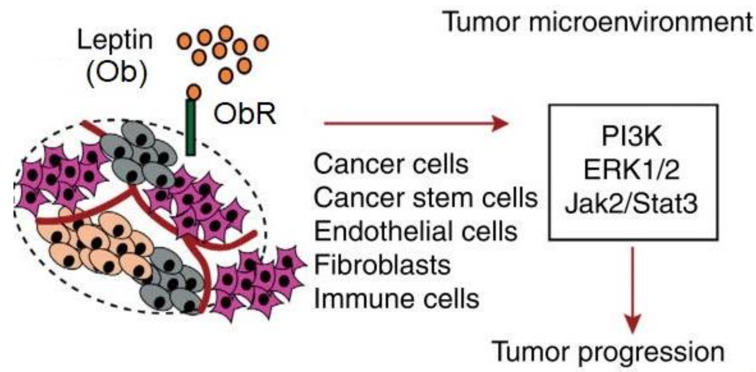


Figure 5. Role of leptin in breast tumor microenvironment. Leptin (Ob) binds to leptin receptor (ObR) expressed in different cells within the tumor microenvironment, including epithelial cancer cells, cancer stem cells, immune cells, endothelial cells and fibroblasts. Ob/ObR-mediated pathways include activation of downstream kinases, such as Phosphoinositide 3-kinases (PI3K), extracellular signal-regulated kinases 1/2 (ERK1/2), Janus kinase 2 (Jak2)/ Signal transducer and activator of transcription 3 (Stat3). These pathways contribute to various steps of tumor progression, from cancer stem cell survival and proliferation to metastatic tumor growth.

AIMS OF THE THESIS

The overall aim of this thesis is to elucidate the mechanisms underlying the interplay between breast cancer cells (BCCs) and its surrounding stromal cells and to identify new potential therapeutic targets for breast cancer patients. Firstly, we evaluated the role of the PPARγ and leptin signaling in affecting functional TAM phenotype, thus impacting breast cancer progression. Then, we characterized a cell population generated by the interaction between BCCs and MSCs which may contribute to chemoresistance and metastasis in breast cancer.

MATERIALS AND METHODS

Reagents and Antibodies

Rosiglitazone (BRL49653, BRL) was obtained from Alexis. GW9662 (GW), Phorbol 12-myristate 13-acetate (PMA), Lipopolysaccharide (LPS), Doxorubicin (DOXO) were obtained from Sigma Aldrich. IL-4 was obtained from R&D system. Docosahexaenoyl serotonin (DHA-5-HT) and docosahexaenoyl ethanolamide (DHEA) were purchased from Cayman Chemical. Puromycin (#A1113803) was acquired from Thermo Fisher Scientific. Santa Cruz antibodies: anti-PPARγ (sc7196), anti-GAPDH (sc25778), anti-β-Actin-HRP (#47778). Cell signaling Technology antibodies: anti-p21(2947,), anti-p27 (3686), anti-cleaved caspase-3 (9661S). anti-PE-CD80 (# 557227) was obtained from Becton Dickinson Italia. Anti-FITCH-CD206 (# 321103) was obtained from BioLegend. Anti-F4/80 (#ab16911) was obtained from Abcam.

Cell cultures

Human THP-1 monocytic cell line, human ERα-positive MCF7 and the triple-negative (ER-, PR and HER2-negative) MDA-MB-231 breast cancer epithelial cells were acquired from American Type Culture Collection (Manassas, VA, USA). All cell lines were authenticated, stored according to supplier's instructions, and used within 4 months after recovery of the frozen aliquots. Mesenchymal stem/stromal cells (MSCs) were isolated from human breast cancer metastasis to a supraclavicular lymph node and characterized as described (M. E. Gonzalez et al. 2017).

Lentiviral Transfection

We established stable MCF-7 and MDA-MB-231 cell lines using Control shRNA lentiviral particles-A (#sc-108080, Santa Cruz Biotechnology) and ObR shRNA lentiviral particles (#sc-36115-V, Santa Cruz Biotechnology) following manufacturer's instructions. Cells were selected with 1.5 µg/mL (MCF-7) and 3 µg/mL (MDA-MB-231) puromycin overtime to eliminate uninfected cells. *LepR* mRNA expression in stable clones was evaluated by real-time RT-PCR.

Macrophage Differentiation and Polarization

One million THP-1 cells were seeded in 6-well plates in RPMI media plus 100 nM PMA for 24 h followed by 1 day of rest in medium without PMA to obtain THP-1 macrophage-like cells (M0).

M0 Macrophages were stimulated for 6 h with 10 ng/mL LPS or with 20 ng/mL IL4 for 72 h to generate M1 or M2 macrophages, respectively.

Co-culture Systems

To evaluate the effects of PPARγ activation in modulating TAM polarization, MCF7 and MDA-MB-231 breast cancer cells (BCCs) were cultured until 80–90% confluence and then incubated with serum-free media for 48 h in order to obtain the BCC-conditioned media (CM), which were collected and used in co-culture experiments with M0 macrophages upon treatment with BRL, DHEA, DHA-5HT and GW as described.

To evaluate the impact of the integrity of the leptin signaling in modulating TAM polarization, MCF-7 and MDA-MB-231 clones were plated in full media (4 x 10^6) in 10 cm dish for 24 h, washed twice and cultured with 5% charcoal-stripped serum medium for 24 h in order to obtain the conditioned media, which were collected and used in co-culture experiments with M0 macrophages.

To generate BCCs engulfing MSCs, 2x10^5 MSCs and 1x10^5 MDA-MB-231 BCCs were plated in 6-multiwell plate and cultured for 72 h with mixed 1:1 MDA-MB-231 BCCs and MSC full media.

Transmigration/Chemiotaxis Assays

MCF-7 and MDA-MB-231 cells were placed in the upper compartments of Boyden Chamber (8 µm membranes, Corning Costar, Corning, NY, USA) and transmigration assay was performed as described (Catalano et al. 2015). THP1 cells (10^5 cells) in 200 µL of phenol-red-free RPMI 1640 were added to the top chamber of a 24-transwell apparatus (5 µm membranes, Corning Costar), while 500 µL of 5% charcoal-stripped serum or of conditioned media derived from breast cells were added to the lower compartment. Cells were incubated for 5 h at 37 ̊C in an atmosphere containing 5% CO_2. Migrated cells were fixed and stained with 40,6-diamidino-2-phenylindole dihydrochloride (DAPI) and quantified by viewing five separate fields per membrane at 20x magnification, using ImageJ.

Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of Interleukin-6 (IL6), Interleukin-1 receptor antagonist (IL1Ra), were measured in supernatants from macrophages. Specifically, in mixed-medium culture systems cells were maintained in serum-free medium for another 24 h, and supernatants were collected and used for analyses. Levels of IL6, C-C Motif Chemokine Ligand 2 (CCL-2), Osteopontin (OPN), Thrombospondin-1(THBS-1), Urokinase-type plasminogen activator receptor (uPAR) were measured in supernatants derived from single culture of BCCs and MSCs diluted 1:1 or in co-culture. Levels of C-C Motif Chemokine Ligand 2 (CCL-2) were measured in in supernatants from Obr sh and Control sh MCF-7 and MDA-MB-231 after 24 h. All ELISAs were performed using human ELISA kits according to manufacturer’s instructions (R&D Systems).

Real Time RT-PCR Assays

Gene expression was evaluated by real-time reverse transcription (RT)-PCR assessed using SYBR Green Universal PCR Master Mix (Bio-Rad, Hercules, CA, USA). Each sample was normalized on 18S mRNA content. Relative gene expression levels were calculated as previously described (Panza et al. 2016). Primers used are:

5’-GATAGAGGCCCAGGCATTTTTTA-3’ (*LepR long-forward*)
5’- CACCACTCTCTCTCTTTTTGATTGA-3’ (*LepR long-reverse*)
5’-ATTGTGCCAGTAATTATTTCCTCTTCC-3’ (*LepR short-forward*)
5’-CCACCATATGTTAACTCTCAGAAGTTCAA-3’ (*LepR short-reverse*)
5’-CCCACTCCTCCACCTTTGAC-3’ (*18s-forward*)
5’-TGTTGCTGTAGCCAAATTCGTT-3’ (*18s-reverse*)
5’-CAGCCAGATGCAATCAATGCC-3’ (*MCP-1 /CCL-2-forward*)
5’-TGGAATCCTGAACCCACTTCT-3’ (*MCP-1 /CCL-2-reverse*)
5’-AGTTCCCGGAGTGAGTTGAA-3’ (*MMP-9-forward*)
5’-CTCCACCCTCCCTTTCCTC-3’ (*MMP-9-reverse*)
5'-GAGATGAGCT TCCTACAGCAC-3’ (*VEGF-forward*)
5'-TCACCGCCTCGGCTTGTCACAT-3’ (*VEGF-reverse*)
5’-GCCCAGGCAGTCAGATCATC-3’ (*TNF-α-forward*)
5’-GGTTTGCTACAACATGGGCTA-3’ (*TNF-α-reverse*)
5’-CCAGGAGCCCAGCTATGAAC-3’ (*IL-6-forward*)

5’-CCCAGGGAGAAGGCAACTG-3’ (*IL-6-reverse*)
5’-TCTCCGAGATGCCTTCAGCAGA-3’ (*IL-10-forward*)
5’-TCAGACAAGGCTTGGCAACCCA-3 (*IL-10-reverse*)

Immunofluorescence

Cells were fixed with 4% paraformaldehyde, permeabilized with PBS + 0.2% Triton X-100 followed by blocking with 5% bovine serum albumin for 30 min and incubated overnight with anti-MCP-1 antibody (dilution 1:100) in Phosphate-buffered saline (PBS) at 4 ̊C. The day after the cells were washed three times with PBS and incubated with the secondary antibody anti mouse IgG-fluorescein isothio-cyanate (dilution 1:200) for 1 h at room temperature. To check the specificity of immunolabeling the primary antibody was replaced by normal mouse serum (negative control). Fluorescence was photographed with an Olympus BX51 microscope (Tokyo, Japan), 100x objective.

Immunoblot analysis

Equal amount of protein extracts was subjected to SDS-PAGE as described (Bonofiglio, Cione, et al. 2009). Images were acquired using Odissey FC (Licor, Lincoln, NB, USA) or ChemiDoc MP Imaging System (Bio-rad Laboratories, California, USA).

Flow Cytometry

THP-1 cells were seeded in 60 mm dishes, differentiated and treated as indicated. Cells were washed with cold PBS; detached with versine, pelleted, resuspended in a total of 100 µL of cold PBS containing 5 µL of PE anti-CD80 antibody or FITC anti-CD206 antibody and incubated 15 min at room temperature in the dark. After incubation, cells were washed with 1 x PBS and centrifuged at 500x g for 5 min and then re-suspended in 500 µL of 1 x PBS. Cells were analyzed by FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and the data acquired using CellQuest software (version 3.3). Unstained cells were used to determine the background autofluorescence to set the negative population allowing cells stained with anti-CD80 (or anti-CD206) antibody to be visualized.

MSCs labeled with DsRed (DsRed-MSC) and MDA-MB-231 labeled with GFP (GFP-231) were cultured and treated as indicated. Single cell pictures were taken from the DsRed+/GFP+

population using Life Imaging Stream Flow Cytometry. Flow cytometry analyses were completed using the UM Flow Cytometry Core.

Cell cycle analysis using flow cytometer

MSCs and BCCs were cultured and treated as indicated. Cells were collected by trypsinization, re-suspended in ice cold PBS and fixed by adding ice cold ethanol. After 20 min of incubation, cells were centrifuged, re-suspended in 0.5 ml PBS/RNAse solution containing 50 µg/ml DAPI for 20 min in the dark and FACs analyzed. To determine the % of $Ki67^{low}$ cells in G_0-G_1 phase of the cell cycle, cells were fixed in ethanol as described and stained in 100 µl of BD Horizon Brilliant Stain Buffer with anti-KI67 for 30 min in the dark. After two washes in the Brilliant Stain Buffer, cells were resuspended in regular medium, stained with Vybrant DyeCycle Ruby and FACs analyzed by Bio-Rad ZE5 #2 Cell Analyzer (Bio-rad, Laboratories, California, USA) at the UM Flow Cytometry Core.

Motility assay

Random motion cell motility assays were completed as previously described (Rosenthal et al. 2011). Briefly, cells were plated on collagen-coated chambered coverslips at low density attaching overnight. Next day, cells were imaged every 10 minutes at 37 °C for 24 h using the DeltaVision RT Live Cell Imaging System (Applied Precision, GE Healthcare) equipped with a UPlanAo 20X/0.7 NA lens at the University of Michigan Microscopy and Image analysis Laboratory. DIC images were acquired using SoftWoRx 3.5.1 software and cell movements were quantified using MTrackJ /ImageJ software.

Cytokine Array

Human XL Cytokine Array Kits, obtained from R&D Systems, were used to analyze the secreted proteins in the conditioned medium derived from single culture of MSCs and BCCs diluted 1:1 or co-culture. The intensity of selected spots was quantified using ImageJ software.

Cell Proliferation

20.000 GFP-231 were cultured alone or with 40.000 DsRed-MSCs in 24-multiwell plate for 48 h and then exposed to treatments as indicated. GFP pixels were quantified by viewing three separate fields at 10x magnification, using ImageJ software.

Animal studies

Female 45-d-old athymic nude mice (Envigo, Milan, Italy) were maintained in a sterile environment and were injected orthotopically with Control sh and shObR MCF-7 and MDA-MB-231 clones (5×10^6). At day 0, estradiol pellets (0.72 mg/pellet, 90-d release; Innovative Research of America, Sarasota, FL, USA) were subcutaneously implanted into the intrascapular region of the mice receiving an inoculation of ERα-positive MCF-7 cells. The next day, cells in 0.1 mL of Matrigel (BD Biosciences, Bedford, MA, USA) were injected orthotopically into the mammary fat pad. Xenograft tumor growth was monitored twice a week by caliper measurements, and tumor volumes (mm3) were estimated using the following formula: TV = a x (b2)/2, where a and b are tumor length and width, respectively, in millimeters. At day 20, the animals were euthanized following standard protocols. The tumors were dissected from the neighboring connective tissue, frozen in nitrogen, and stored at -80 °C for further analyses (Mauro et al. 2018). All animals were maintained and handled in accordance with the recommendation of the Guidelines for the Care and Use of Laboratory Animals and experiments were approved by the Animal Care Committee of University of Calabria (OPBA), Italy (ethic code: 533/2019-PR, approved on 19 July 2019).

In another set of experiments, eight-week old severe combined immunodeficiency mice (The Jackson Laboratories) were used for examining breast cancer metastasis. To this aim, GFP-231 labeled with Firefly-luciferase, were cultured alone or with DsRed-MSCs for 72 h and injected intracardially in anesthetized mice at a concentration of 1×10^5cells (GFP-231 alone) or 1.5×10^5 (GFP-213 in co-culture) resuspended in 50 µl of PBS (n = 10 mice per group). At day 20, mice were divided into 2 groups (n=5 mice per group) and treated every three days i.p with doxorubicin dissolved in saline at doses of 4 mg/kg or vehicle. Metastases were monitored using bioluminescence imaging as previously described (Chen et al. 2019). Bioluminescence images were acquired using the IVIS imaging system (Xenogen) within approximately 2–5 minutes after injection. Analysis was performed using the Living Image software platform (Xenogen) by measuring photon flux, measured in photons/s/cm2/sr, by using a region of interest (ROI) drawn

around the bioluminescence signal to be measured and subtracting background measurements. Mice were sacrificed and necropsied at day 27. Metastases were identified by GFP fluorescence microscopy right after collecting the tissues at necropsy. The number of metastasis per mice per group was quantified using ImageJ (M. E. Gonzalez et al. 2017).

Immunohistochemistry

For immunohistochemistry, antigen retrieval was performed on 5 μm paraffin sections in 0.01 mol/L citrate buffer (pH 6) in a microwave at low setting. Incubations with primary antibodies were performed at room temperature overnight in a humidified chamber. Primary antibodies used were anti-F4/80 and anti-MCP-1. Normal horse or goat serum was used as blocking agent. Biotinylated horse anti-mouse/rabbit (1:100) or biotinylated goat anti-rat (1:100) was used as the secondary antibody and revealed with a Vectastain ABC Kit Elite (Vector Laboratories, Burlingame, CA, USA, PK-6200) and a Peroxidase Substrate Kit DAB (Vector Laboratories, Burlingame, CA, USA, SK-4100). All stained slides were visualized using an Olympus BX41 microscope and the images were taken with CSV1.14 software, using a CAM XC-30 for image acquisition. Immunoreactivity was evaluated by a pathologist in a blinded fashion and scored as: 0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive; and 4, very strongly positive (Panza et al. 2019).

Statistical analysis

Each datum point represents the mean ± SD of three different experiments. Experimental data were analyzed for statistical significance by one-way, 2-way mixed ANOVA test or Student's t test using the GraphPad Prism5 software program as described.

In the set of experiments in which we investigated MSC engulfment, data represent the mean ± SEM of three different experiments. Experimental data were analyzed by Student's t test. $^*P < 0.05$ was considered as statistically significant.

RESULTS

Results 1: Tumor Associated Macrophage phenotype is affected by PPARγ and leptin signaling

TAM polarization is modulated by PPARγ activation

TAM generation

Macrophages are an intrinsically heterogeneous population classically designated as pro-inflammatory M1 and protumoral M2 macrophages. Here, we wanted to investigate the ability of molecular regulators to modulate the polarization state of macrophages. To this aim, in our previous work, we examined the effects of the conjugate of eicosapentaenoic acid (EPA) with dopamine (EPDA) in M1 macrophages, which represent the main macrophage phenotype in the initial phase of tumorigenesis (Whiteside 2008). We demonstrated that EPDA reduces gene expression and protein secretion of several M1 markers in LPS-derived human and murine macrophages, suggesting that this compound exerts anti-inflammatory effects and may counteract inflammation-driven cancer (Augimeri et al. 2019). Based on these findings, we investigated whether omega-3 conjugates as PPARγ ligands can also influence the TAM polarization induced by the breast cancer cell secreted factors. In order to generate breast TAMs, human THP-1 monocytes were stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA), to obtain M0 macrophages, which were, then, incubated with the breast cancer cell (BCC) conditioned media (CM) collected from two different types of BCCs, MCF7 (CM MCF-7) and MDA-MB-231 (CM MDA) cells for 72 h (Figure 1).

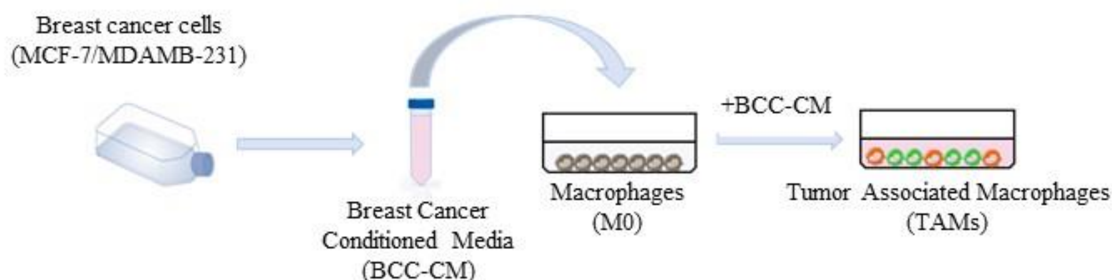


Figure 1. Schematic representation of Tumor Associated Macrophage generation. Conditioned media from breast cancer cells (BCC-CM) were added to M0 macrophages for 72 h to obtain Tumor-Associated Macrophages (TAMs).

In line with previous studies (Stewart et al. 2012), we found that macrophages treated with BCC-CM display features of both M1 and M2 phenotypes. Indeed, we revealed that CM MCF-7 was able to induce a significant upregulation of both CD80 and CD206, which are M1 and M2 typically surface markers, respectively, whereas CM MDA enhanced only the expression for CD80 marker (Figure 2 A). Moreover, we observed a significant BCC-induced secretion of both IL6 (M1 marker) and IL1Ra (M2 marker) (Figure 2 B).

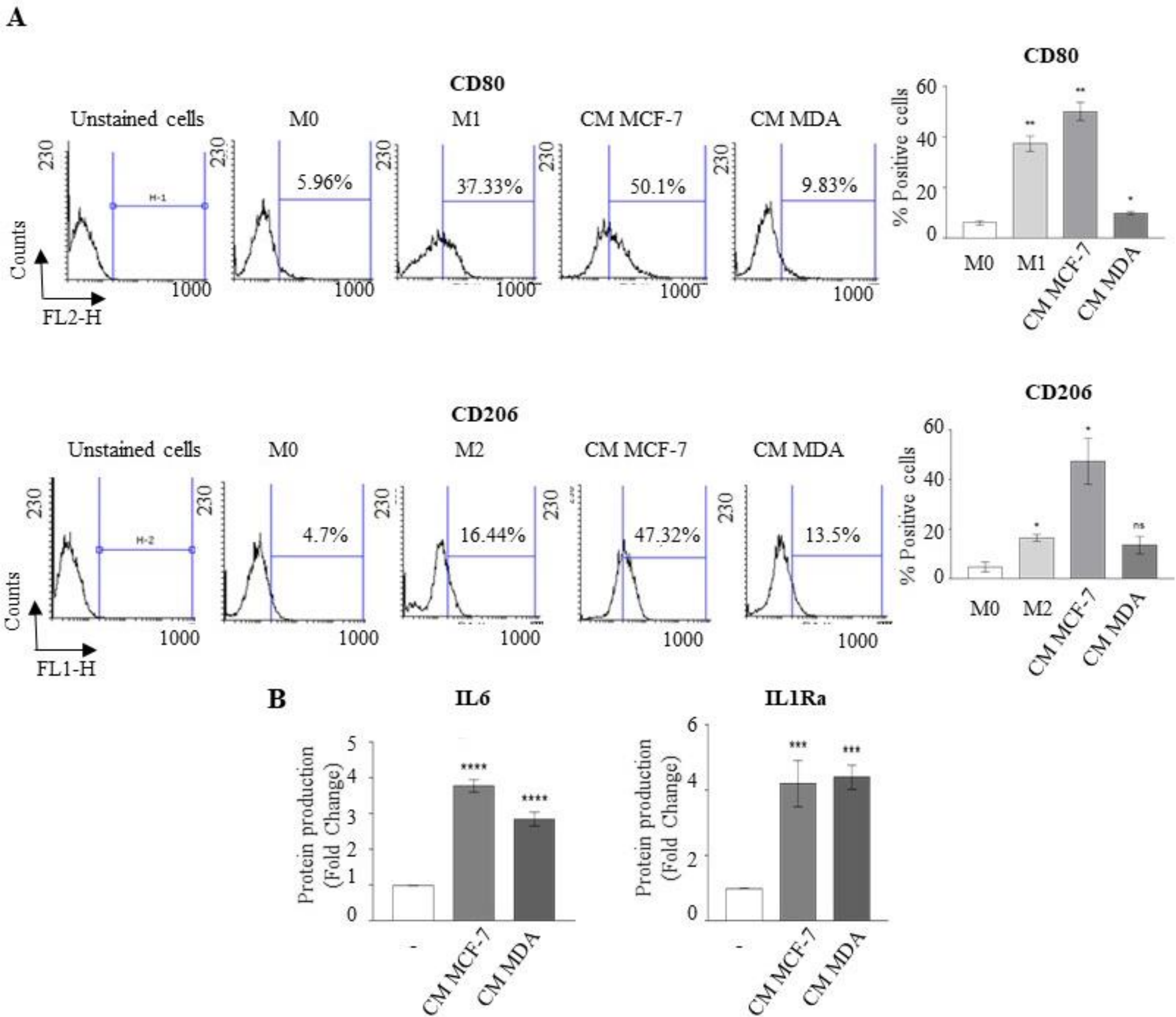


Figure 2. Macrophage polarization induced by breast cancer cells. **A.** Flow cytometry analyses of M1 marker CD80 and M2 marker CD206 in M0, M1, M2 macrophages and in M0 cells incubated with CM MCF-7 or CM MDA for 72 h. The histograms represent the percentage of positive cells **B.** ELISA analyses of IL6 and IL1Ra in M0 macrophages (-) incubated with CM MCF-7 or CM MDA for 72 h. Values represent the mean ± SD of three different experiments, each performed with duplicate samples. The results are expressed as fold change with respect to differentiated cells. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$, ns: not significant.

PPARγ activated by ligands counteracts TAM phenotype

To explore the ability of PPARγ activation to affect macrophage polarization induced by BCCs, we tested the effects of the docosahexaenoic acid (DHA) conjugates that we have previously reported to inhibit breast cancer progression and development (Rovito et al. 2013; Rovito et al. 2015). To this aim, we firstly evaluated the expression of PPARγ in TAMs generated by exposure to CM MCF-7 or CM MDA (Figure 3).

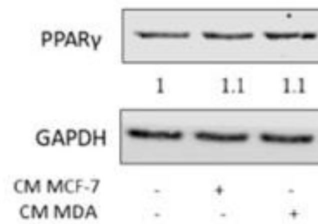


Figure 3. Immunoblotting of PPARγ in M0 macrophages (-) incubated with CM MCF-7 or CM MDA for 72 h. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. The blot is representative of three independent experiments, while the numbers below the blots represent the average fold change between PPARγ and GAPDH protein expression with respect to M0 macrophages.

Next, we cultured TAMs in presence of DHA conjugates with ethanolamine or serotonin, DHEA and DHA-5HT, respectively for 72 h and protein secretion was analyzed by ELISAs. As shown in Figure 4A, DHA-5-HT significantly reduced IL6 production by both TAMs which was completely upregulated by the PPARγ antagonist GW9662 (GW). A similar but not significant decrease in the IL1Ra levels was observed after DHA-5-HT treatment, whereas GW treatment resulted in a marked and significant upregulation (Figure 4 A). DHEA stimulation strongly downregulated IL1Ra secretion only in macrophages cultured with CM MCF7, once again GW was able to reverse this effect and also caused a higher production of both cytokines, IL6 and IL1Ra in macrophages treated with CM-MDA (Figure 4 B). As expected, treatment with rosiglitazone (BRL), the synthetic PPARγ ligand, was able to decrease IL6 and IL1Ra production by macrophages exposed to BCC-CM of both tumor cell lines in a PPARγ dependent manner (Figure 4 C).

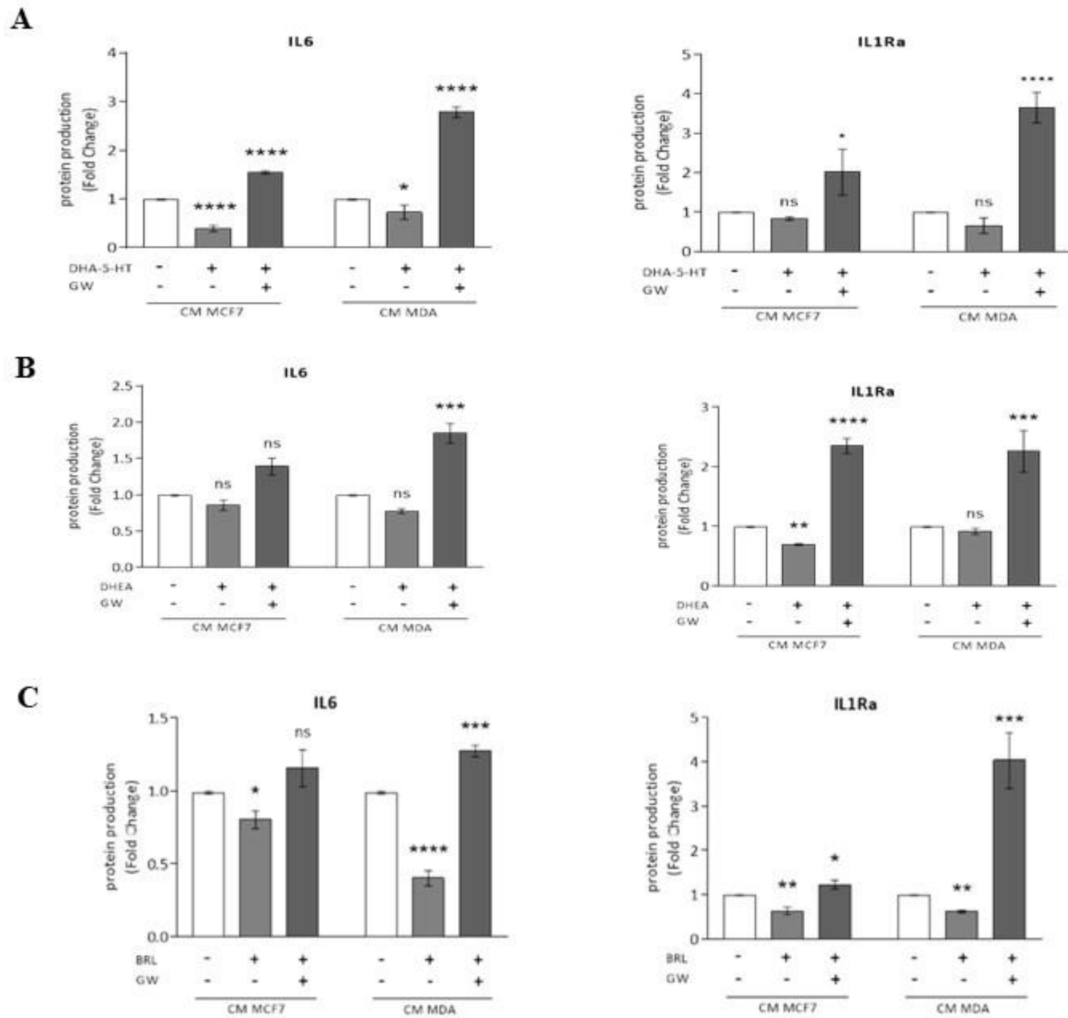


Figure 4. PPARγ activation counteracts TAM cytokine secretion induced by MCF-7 and MDA-MB-231 breast cancer cell conditioned media. ELISA analyses of IL6 and IL1Ra in M0 macrophages incubated with CM MCF7 or CM MDA and treated with DHA-5-HT 1 µM (**A**), DHEA 5 µM (**B**) or BRL 10 µM (**C**) along or in combination with GW9662 (GW) for 72 h. Data are expressed as means ± SD. Each experiment was performed three times with duplicate samples. The results are expressed as fold change respect to vehicle-treated cells (-). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.0001$, ns= nonsignificant.

Taken together, these data show that PPARγ activation by its natural and synthetic ligands simultaneously attenuate M1 and M2 macrophage phenotypes, which are known to promote a pro-tumorigenic milieu in breast TME. These results were recently pubblished in *Cells* (Gionfriddo et al. 2020).

Knockdown of leptin receptor affects functional phenotype in breast tumor microenvironment

Generation of leptin receptor knockdown

The biological significance of the integrity of leptin/leptin receptor in breast cancer cells as well as in breast tumor immune microenvironment is still not completely elucidated. To dissect the functional role of the leptin receptor (ObR), we stably knocked-down the endogenous expression of ObR (ObR sh) in MCF-7 and MDA-MB-231 BCCs using lentiviral delivered short hairpin RNA (shRNA). *Ob* mRNA levels, as measured by quantitative RT-PCR, were significantly down-regulated in ObR sh clones as compared to cells stably transfected with a vector shRNA (Control sh) in both cell lines (Figure 5 A and B).

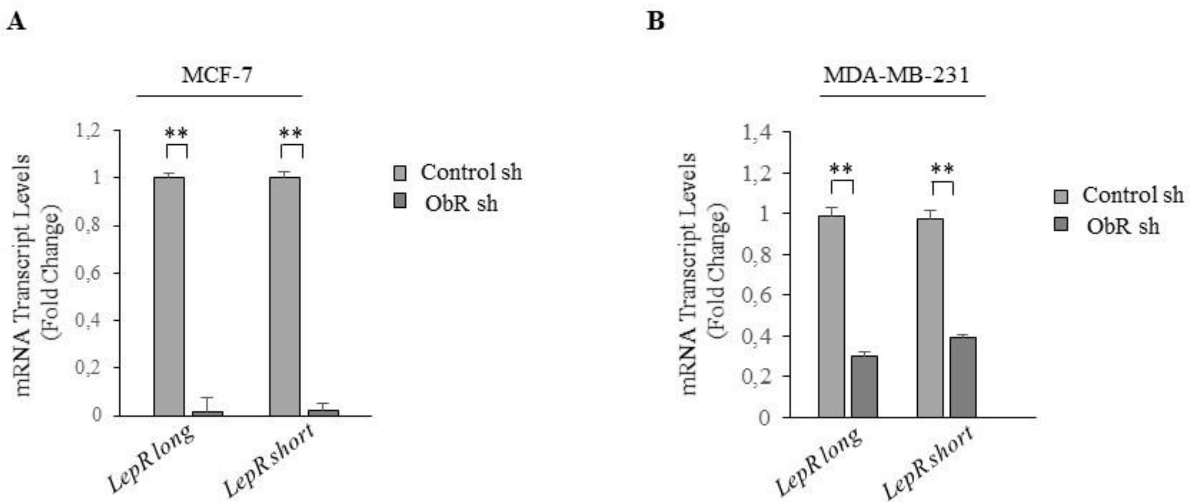
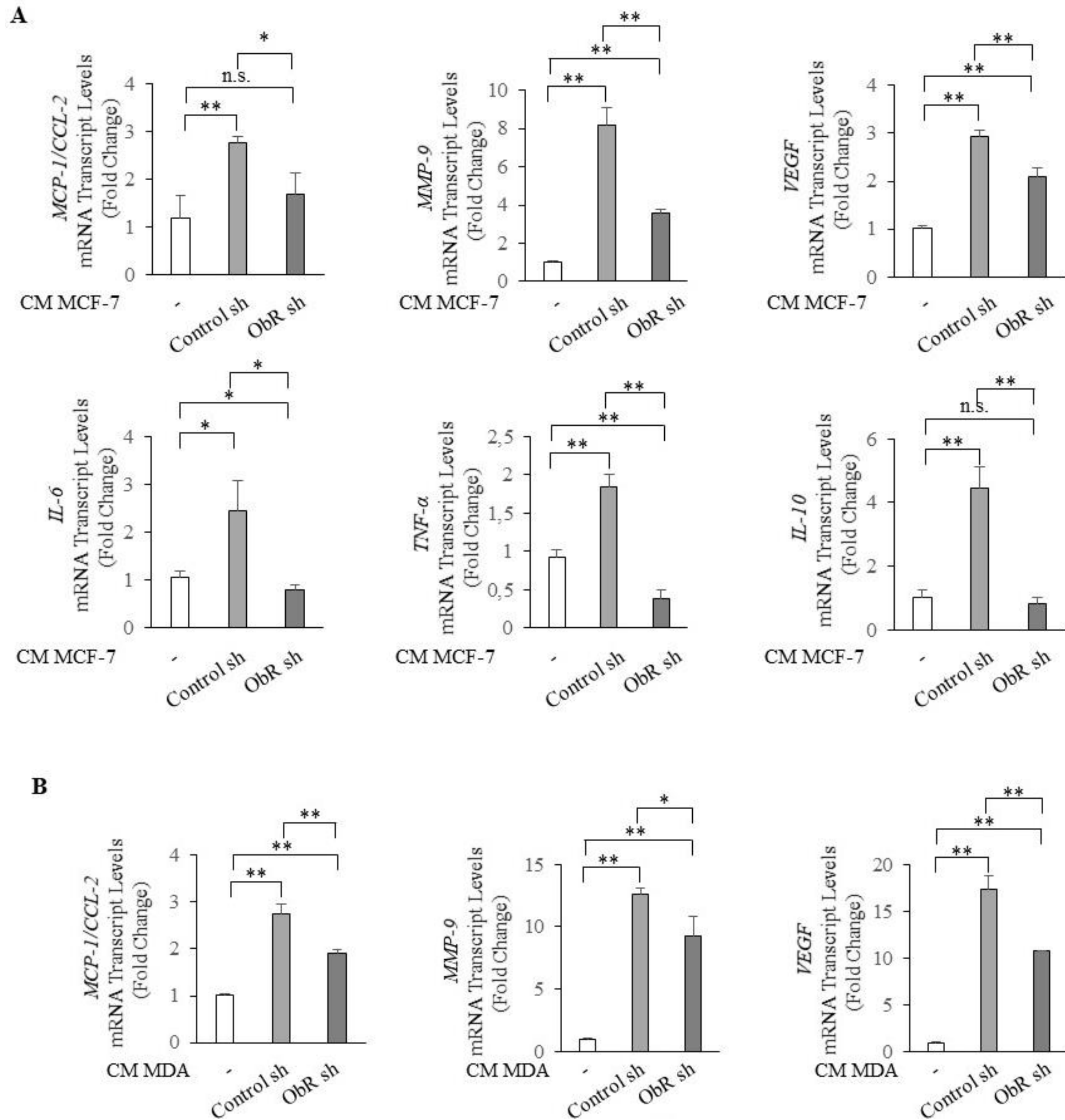


Figure 5. Expression of leptin receptor in ObR sh MCF-7 and MDA-MB-231 breast cancer cells. Real time RT-PCR assay for leptin receptor (long and short isoforms) mRNA expression in control sh and ObR sh MCF-7 (**A**) and MDA-MB-231 (**B**) breast cancer cells. The values represent the mean ± SD of three different experiments, each performed in triplicate. ** $p < 0.005$.

Leptin/leptin receptor signaling knockdown influences the functional macrophage phenotype in breast cancer

Next, we used this experimental model to investigate whether the integrity of the ObR signaling may influence TAM behavior. To this aim, THP-1-derived M0 macrophages were incubated with control media, CM from ObR sh and Control sh clones. As revealed by RT-PCR, we showed increased mRNA levels of several genes usually associated with the TAM phenotype,

such as CCL-2/MCP1, MMP-9, VEGF, IL-6, TNF-α and IL-10 in M0 incubated with both CM from Control sh clones with respect to control media (Figure 6 A and B). Interestingly a clearly lower induction of these genes was observed in M0 incubated with CM from ObR sh clones compared to Control sh-derived CM (Figure 6 A and B), suggesting that the knockdown of Ob/ObR signaling impacts TAM functional phenotype.



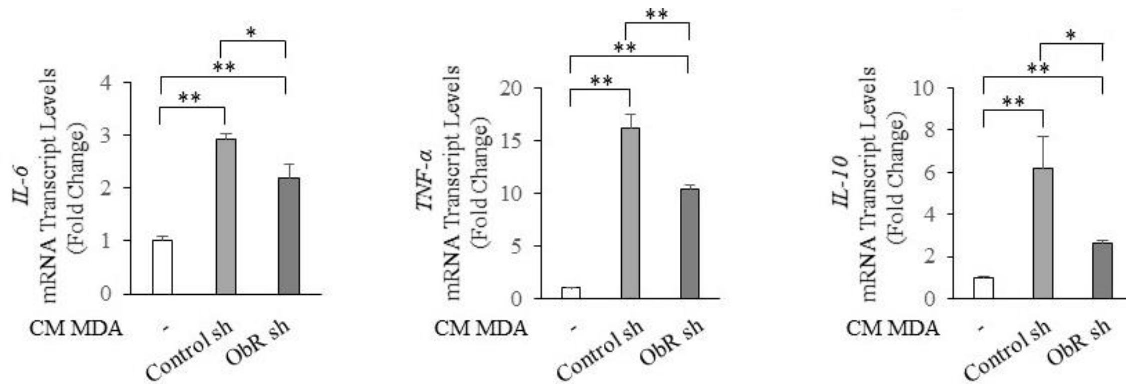


Figure 6. ObR knockdown in breast cancer cells affects the mRNA expression profile of tumor-associated macrophages. Real-time RT-PCR assay for indicated proteins in M0 treated with 5% charcoal stripped media (-) or incubated with conditioned media (CM) derived from Control sh and ObR sh MCF-7 breast cancer cells for 5 days (**A**) and from MDA-MB-231 breast cancer cells for 3 days (**B**). The values represent the mean ±SD of three different experiments, each performed in triplicate. n.s., nonsignificant; * $p < 0.05$; ** $p < 0.005$.

Leptin/leptin receptor signaling knockdown hampers macrophage recruitment in breast cancer

TAMs derive from circulating bone marrow monocytes which were recruited into the tumor site through the release of chemoattractant molecules by breast cancer cells. Among them, the monocyte chemoattractant protein-1 (MCP-1) is one of the key chemokines that facilitates the recruitment and accumulation of TAMs in tumors (Williams et al. 2016). Thus, we further investigated whether the ablation of ObR may also modulate TAM recruitment in breast TME. Firstly, we evaluated MCP-1 expression in Control sh and ObR sh clones. We found that MCP-1 was significantly reduced in terms of mRNA expression, protein content and secretion in ObR sh clones compared to control, addressing that an impairment of Ob/ObR signaling negatively interferes with MCP-1 expression (Figure 7 A,F).

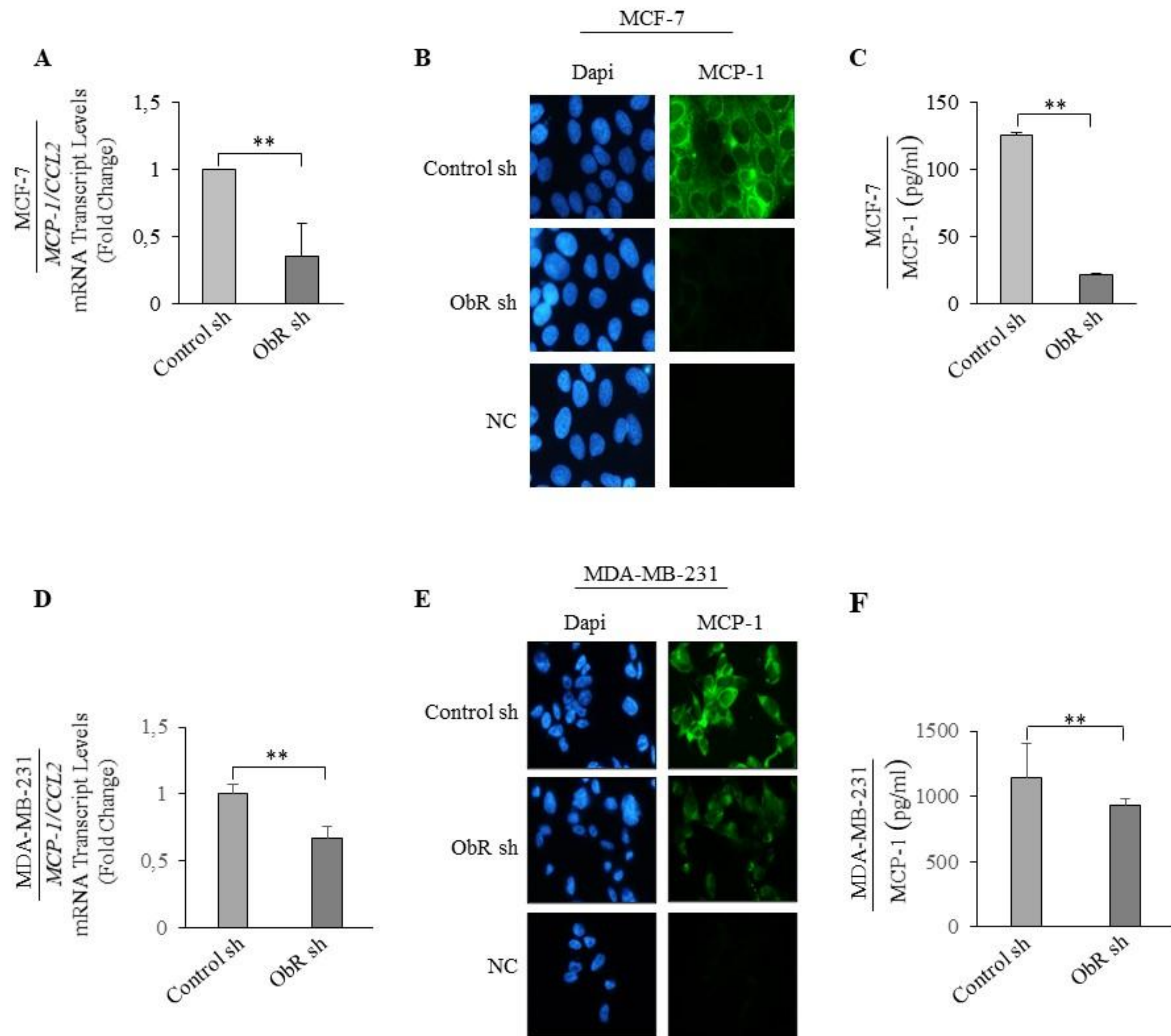


Figure 7. ObR knockdown effects on MCP-1 expression. **A**,**D** Real-time RT-PCR assay for MCP-1/C-C Motif Chemokine Ligand 2 (MCP-1/CCL2) mRNA expression in Control sh and ObR sh MCF-7 and MDA-MB-231 breast cancer cells. **B**,**E** Immunofluorescent staining of MCP-1 protein expression in Control sh and ObR sh MCF-7 and MDA-MB-231 breast cancer cells. DAPI staining was used for nuclei detection (100 magnification). **C**,**F** Enzyme linked immunosorbent assay (ELISA) for MCP-1 protein secretion in Control sh and ObR sh MCF-7 and MDA-MB-231 breast cancer cells. The values represent the mean ± SD of three different experiments, each performed in triplicate. * $p < 0.05$; ** $p < 0.005$.

Then, we incubated THP-1 monocytic cells with 5% charcoal stripped media, or CM collected from ObR sh and Control sh MCF-7 and MDA-MB-231 clones, used as chemo-attractants. We found that CM from Control sh clones increased the recruitment of monocytes over basal medium controls; while the migration of monocytes was completely inhibited in the presence of

CM derived from ObR sh clones, confirming that knockdown of Ob/ObR signaling impacts the recruitment of TAMs (Figure 8 A and B).

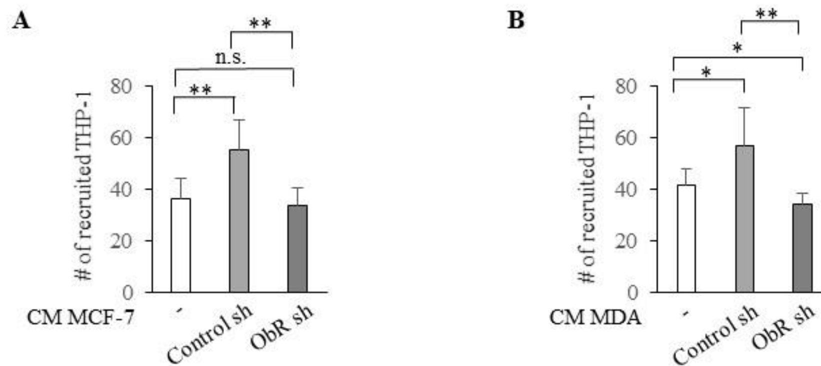


Figure 8. ObR knockdown effects on monocyte recruitment. Trans-well migration of THP-1 in response to 5% charcoal stripped media (-) and the conditioned medium (CM) derived from Control sh and ObR sh MCF-7 (**A**) and MDA-MB-231 (**B**) breast cancer cells was assessed after 5 h incubation. The migrated monocytes were stained with DAPI and six random fields were captured per well with Olympus microscope at 10 magnification. The values represent the mean ± SD of three different experiments, each performed in triplicate. n.s., nonsignificant; * $p < 0.05$; **, $p < 0.005$.

Based on these "in vitro" results, mouse xenograft models were used to investigate whether ObR knockdown modulates macrophage recruitment "in vivo". Either ObR sh or Control sh MCF-7 and MDA-MB-231 cells were injected into the mammary fat pad of female nude athymic mice and tumor growth was monitored. We observed a significant reduction in tumor growth of both ObR sh clones compared to the control one (data not shown). Interestingly, we found a reduced macrophage infiltration within xenograft tumors from mice injected with either ObR sh MCF-7 or MDA-MB-231 cells with respect to the control group as revealed by immunohistochemical staining of F4/80 expression, a unique murine monocyte-macrophage marker (Figure 9 A and B). Moreover, decreased expression of MCP-1 was detected in ObR sh MCF-7 and MDA-MB-231 xenograft tumors compared to the Control sh ones (Figure 9 C and D), confirming that the ablation of ObR reduces TAM recruitment also in *vivo* models.

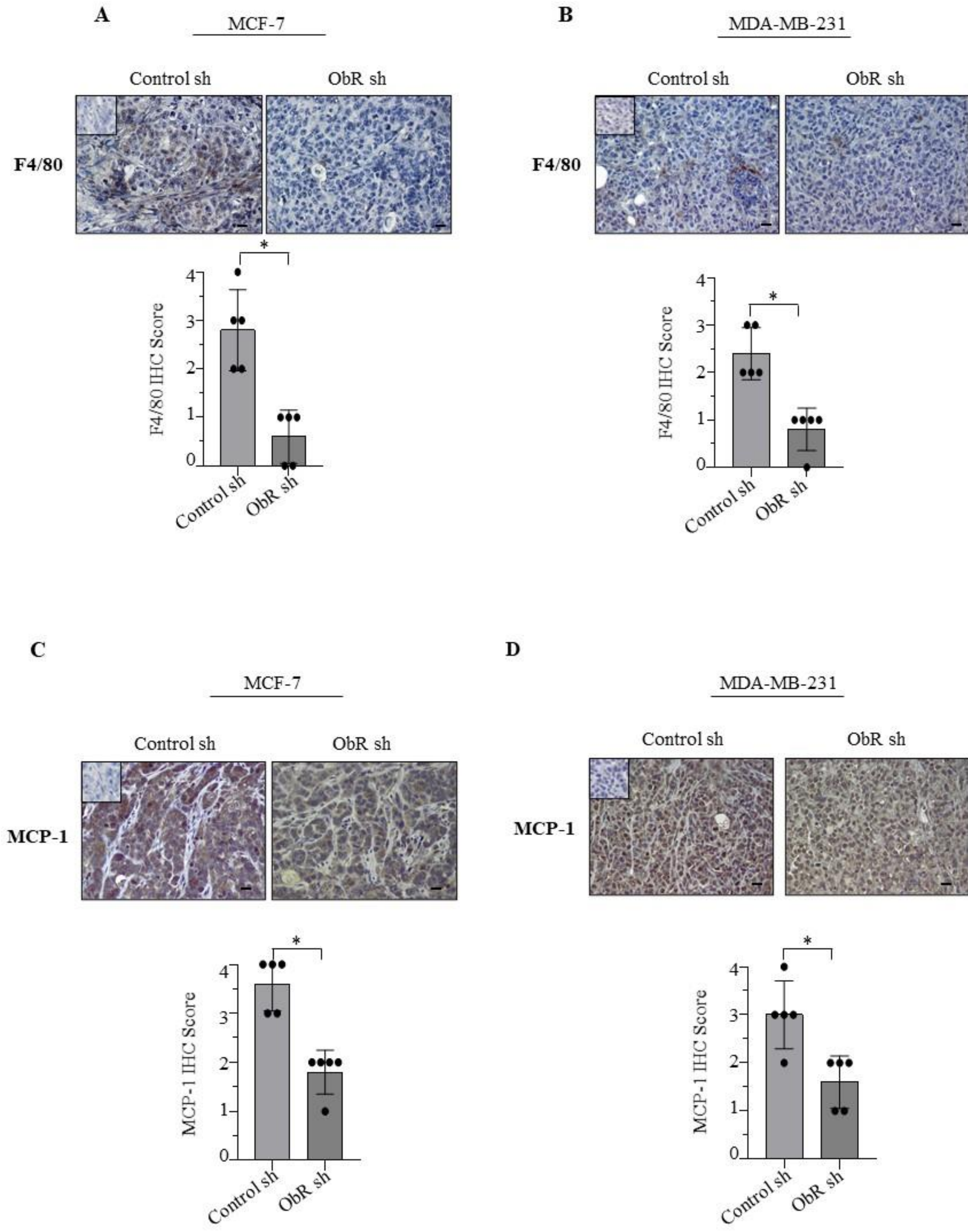


Figure 9. Influence of the lack of ObR on macrophage infiltration and monocyte chemoattractant protein 1 (MCP-1) expression into MCF-7 and MDA-MB-231 xenograft tumors. **A, B** Immunohistochemical staining and relative score of F4/80 and **C, D** MCP-1 in Control sh and ObR sh MCF-7 and MDA-MB-231 xenograft tumor sections. Inset, negative control. Scale bar = 25 µm. * $p < 0.05$.

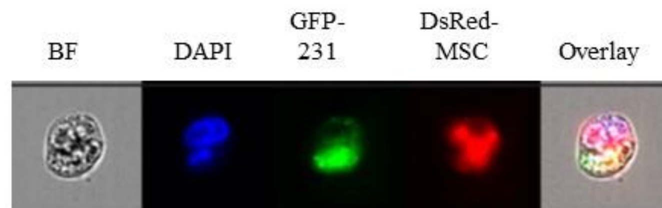
In conclusion, these data demonstrated that Ob/ObR signaling knockdown contributes to modulate TAM behavior in a less aggressive macrophage phenotype and to reduce macrophage recruitment in the breast tumor site. These findings were recently published in *Cancer* (Gelsomino et al. 2020).

Results 2: Multinucleated polyploid hybrid cell population generated by Mesenchymal Stem/Stromal Cells engulfment drives dormancy and resistance to chemotherapy in breast cancer

Generation and characterization of hybrid cells

Mesenchymal Stromal/Stem Cells (MSCs) are considered an important stromal cell source within the TME because of their ability to communicate with other cells in the TME through chemical signals modulating tumor progression. In the last years, it has been reported that MSCs can be engulfed by breast cancer cells (Chen et al. 2019) in a process known as MSC engulfment which leads to a more aggressive breast cancer phenotype. However, the phenotypic features of BCCs engulfing MSCs are still unclear. Thus, we wondered to characterize MSCs engulfed by BCCs using as model system co-cultures of GFP-labeled MDA-MB-231 BCCs (GFP-231) with DsRed-labeled MSCs (DsRed-MSCs) isolated from fresh human breast cancer metastasis to a supraclavicular lymph node (Gonzalez et al. 2017). After 72 h, flow cytometry Live Imaging Stream single-cell analyses revealed that MSC engulfment by BCCs generates a hybrid multinucleated population identified by the co-expression of GFP+ and DsRed+ markers (Figure 10 A). Emerging evidence has highlighted that multinucleated cells are also characterized by aneuploidy (Lu and Kang 2009). Thus, we investigated whether multinucleated hybrid cells generated by MSC engulfment display DNA copy-number changes. We found an increased content in DNA in GFP+/DS-Red+ hybrid cells (> 4N+) compared to GFP+ cells (Figure 10 B). Moreover, we revealed an upregulated expression of p21 and a reduced expression of p27 in hybrid cells compared to GFP-231 (Figure 10 C), indicating that MSC engulfment generates a population of polyploid hybrid cells.

A



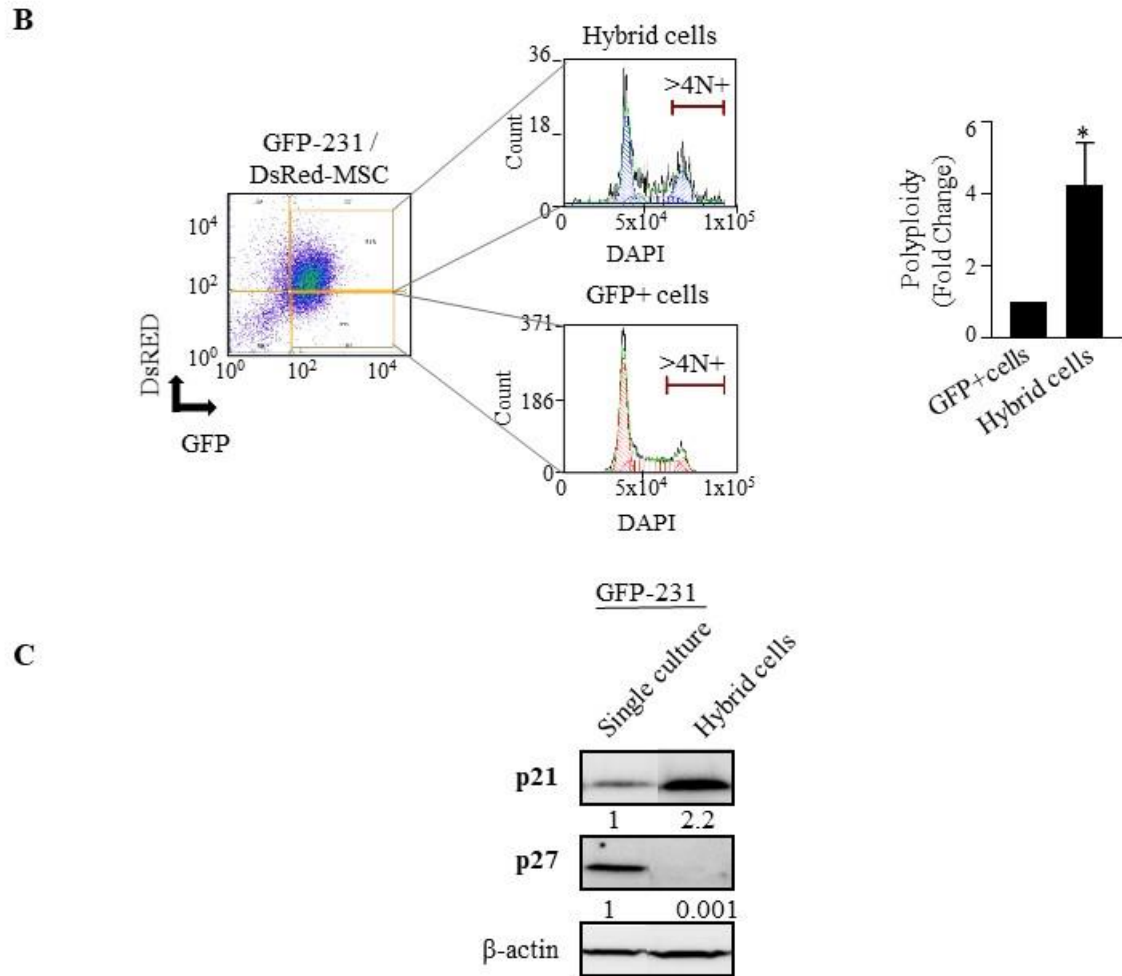
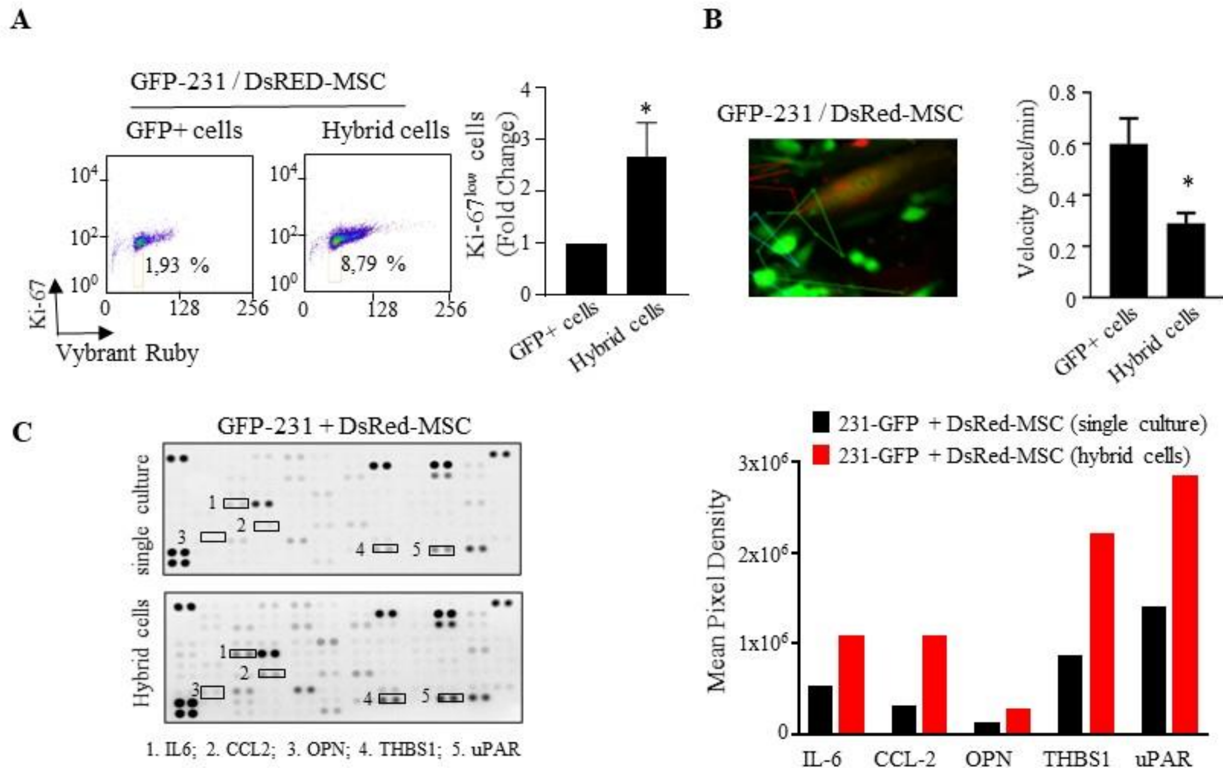


Figure 10. MSC engulfment generates a hybrid multinucleate polyploid population. A. Representative flow cytometry Live Imaging Stream pictures showing hybrid cells. GFP-labeled MDA-MB-231 BCCs (GFP-231) were cultured with Ds-Red-labeled MSCs (DsRed-MSCs) for 72 h. Cells were fixed and nuclei were stained with DAPI. An overlay of all fluorescence channels and bright field (BF) shows a multinucleated double positive cell. Cell phase image is included to display cell morphology. **B.** Flow cytometry analysis of cell-cycle in the co-culture of GFP-231 with DsRed-MSCs showing the emergency of polyploidy population in GFP+/DsRed+ cells (hybrid cells) compared to GFP+ cells. Bar-graph shows the fold change of polyploidy in GFP+/Ds-Red+ population (hybrid cells) versus GFP+ population. The values represent the average fold change ± SEM of three different experiments. **C**. Immunoblotting of indicated proteins in GFP-231 cells in single cult. or cultured with MSCs (hybrid cells) after 72 h. β-Actin was used as a control for equal loading and transfer. Numbers below blots represent the average fold change between p21 or p27 and β-actin with respect to GFP-231 single culture. *$p < 0.05$.

Polyploidy is closely related to dormancy (Storchova and Pellman 2004; Recasens and Munoz 2019), defined as a property of cells entering in a low proliferative G_o state (Cheung and Rando 2013; Coward and Harding 2014). Thus, we queried if polyploid hybrid cells may exhibit dormant features. Firstly, we investigated the presence of low-proliferative cells in G_0-G_1 phase of the cell cycle in the hybrid population evaluating the Ki67 expression, which is a well-known

proliferation marker. We observed a higher percentage of Ki-67^{low} cells in G_0-G_1 in GFP+/DsRed+ hybrid cells (> 4N+) compared to GFP+ cells (Figure 11 A). Moreover, studies of random cell motion using live cell imaging with time lapse microscopy showed that the hybrid cells significantly decreased the average cell velocity compared to GFP+ cells, supporting that these cells are in an inactive state (Figure 11 B). Furthermore, by human cytokine array, we analyzed the secretome profile of co-cultured cells respect to that of BCCs and MSCs collected as single culture. We observed that hybrid cells secrete an increased amount of several proteins, such as IL-6, CCL2, OPN, THBS1 and uPAR (Figure 11 C), belonging to the senescence-associated secretory phenotype (SASP) factors, which are known to support dormancy (Pazolli et al. 2012, Bartosh et al. 2016, Coppè et al. 2016). The upregulated secretion of the SAPS factors by hybrid cells was further validated by ELISA assays (Figure 11 D). Overall, these data demonstrate that MSC engulfment generates a hybrid population of polyploid cells that display a dormant phenotype.



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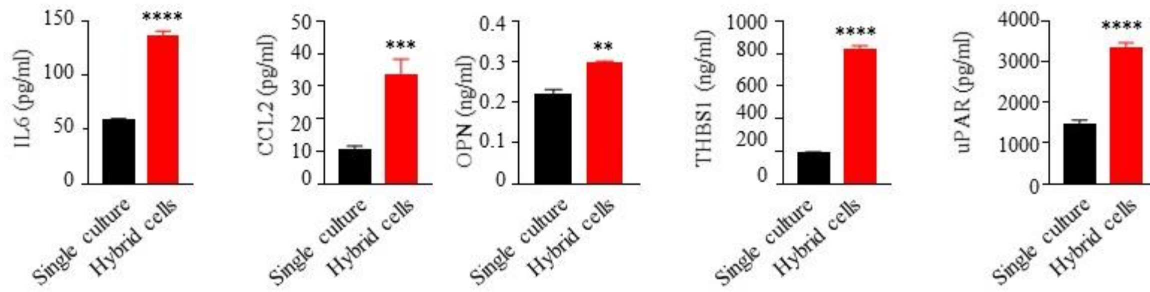


Figure 11. Hybrid cells generated by MSC engulfment display a dormant phenotype. A. Cell cycle analysis of Ki-67 marker in the GFP+ cells and hybrid cells obtained from co-cultured GFP-231 with DsRed-MSCs. Bar-graph shows the fold change of Ki-67^{low} cells in G1. The values represent the mean ± SEM of three different experiments. **B**. Representative images displaying MTrackJ individual cell tracks, colored dots and connecting lines, from 24 h time-lapse videos of GFP-231 cells and GFP+/ DsRed+ hybrid cells [200X magnification]. Each dot represents a 10-minute time span. Bar graph shows the average cell velocity ± SEM. **C**. *Left* Human cytokine arrays for the detection of secreted proteins in the conditioned media (CM) derived from single culture of BCCs and MSCs diluted 1:1 (single culture) or in co-culture (hybrid cells) collected after 72 h. *Right,* Raw numerical densitometry data were extracted, and the background subtracted. Results were shown as mean pixel density**. D.** Enzyme linked immunosorbent assay (ELISA) analyses for indicated proteins in CM collected as in C. Data are expressed as mean ± SEM of three independent experiments. * $p < 0.05$; ** $p < 0.005$, *** $p < 0.0005$, **** $p< 0.00001$

Chemoresistant properties of hybrid cells in vitro

Since dormant cells are known for their resistance to drug treatment (Rossari et al., 2020), we further investigated the sensitivity of hybrid cells to doxorubicin (DOXO), which represents one of the most widely used chemotherapeutic agents in breast cancer therapy (Hernandez-Aya and Gonzalez-Angulo 2013). To this end, GFP-231 BCCs cultured with DsRed-MSCs were treated with DOXO for 24 h and then the percentage of GFP+ cells was analyzed by flow cytometry analysis. We revealed that DOXO decreases the percentage of GFP+ cells in the co-culture of BCCs with MSCs (Figure 12 A), while enhances the percentage of hybrid cells, which are characterized by the expression of both GFP and DsRed markers. To further confirm this result, we measured the GFP signal in GFP-231 BCCs and in hybrid cells after DOXO treatment for 24 h. As expected, we revealed a higher GFP signal in hybrid cells compared to GFP-231 in single culture upon DOXO treatment (Figure 12 B). Moreover, we measured the protein levels of cleaved caspase-3 to estimate cell death in GFP-231 and hybrid cells after DOXO exposure. We observed that hybrid cells express lower levels of cleaved caspase-3 compared to GFP-231, confirming that they acquired resistance to drug treatment (Figure 12 C). In conclusion, these data provide evidence that the MSC engulfment confers resistant to doxorubicin treatment supporting dormancy features in the hybrid cell population.

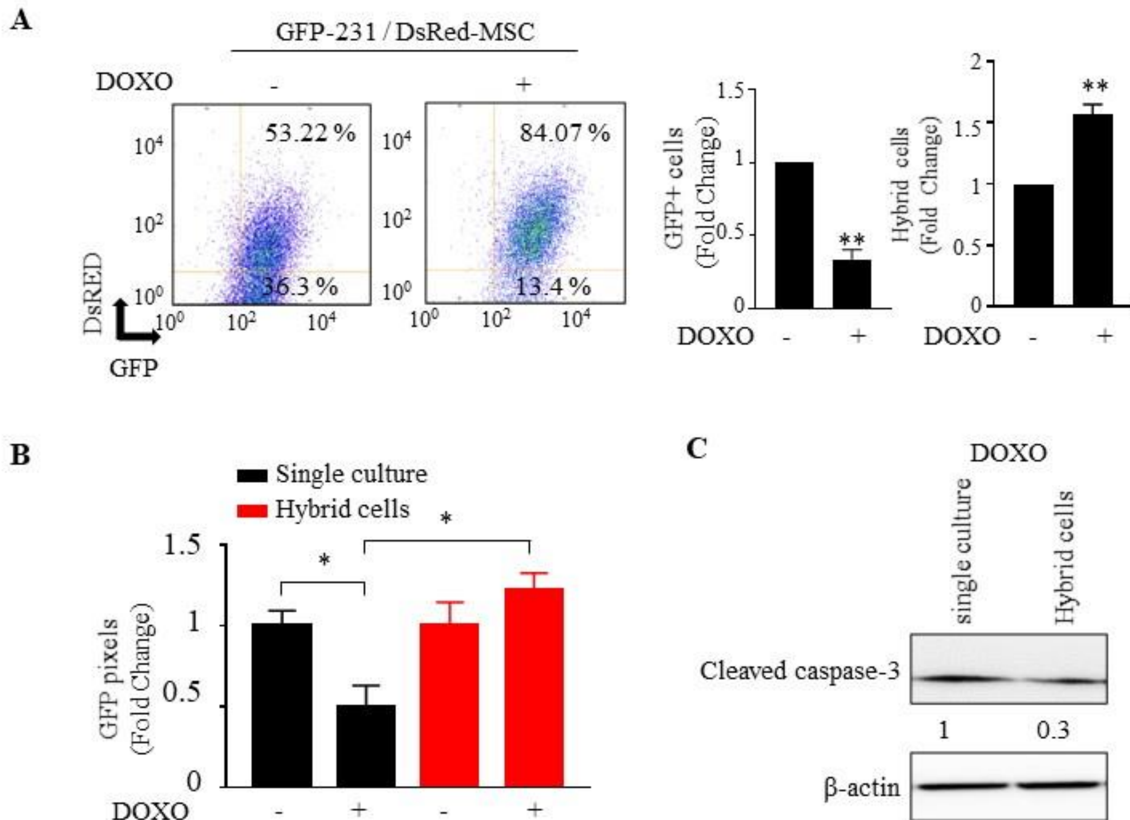


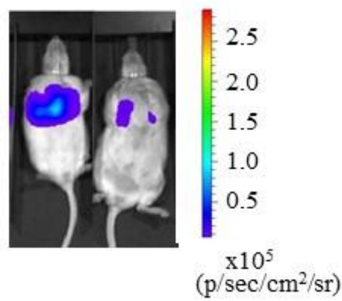
Figure 12. Hybrid cells exhibit resistance to doxorubicin. **A.** Flow cytometry analysis in GFP-231 cultured with DsRed-MSCs for 48 h and treated with vehicle (-) or doxorubicin (DOXO) 1 μM for 24 h. Percentage of GFP+ or GFP+/DsRed+ (hybrid) cells are shown in the lower and higher right quadrants of each dot plot, respectively. Bar-graph shows the fold change of GFP+ or hybrid cells treated with DOXO respect to vehicle treated (-). The values represent mean ± SEM of three different experiments. **B.** GFP signal in GFP-231 cells cultured alone or with DsRed-MSC (hybrid cells) for 48 h and treated with DOXO 1 μM for 24 h. GFP was quantified in three different fields per condition using Image J in triplicate. **C**. Immunoblotting of cleaved caspase-3 in GFP-231 cells in single culture or hybrid cells after treatment with DOXO 1 μM for 24 h. β-actin was used as a control for equal loading and transfer. Numbers below represent the average fold change between cleaved caspase-3 and β-Actin with respect to GFP-231 single culture. *$p < 0.05$; ** $p < 0.005$.

Metastatic properties of chemoresistant hybrid cells in vivo

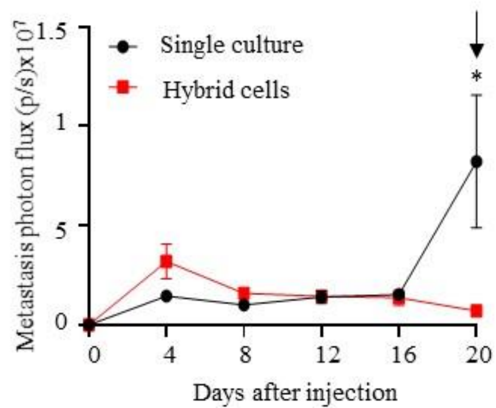
Based on our in *vitro* studies showing that hybrid cells have dormant and chemo-resistant properties, we tested their ability to form metastasis and acquire resistance to drug treatment in *vivo*. Thus, GFP-231 cells in single culture or hybrid cells labeled with firefly luciferase were intracardially injected in NOD/SCID mice and monitored for metastases by bioluminescence imaging (BLI). After 20 days, we found that hybrid cells established less metastasis compared to control (Figure 13 A and B). Thus, mice were divided into two groups and were treated with

DOXO 4 mg/kg every three days. After a week of DOXO treatment, we observed a higher qualitative BLI intensity in mice injected with hybrid cells compared to GFP-231 cells, suggesting that hybrid cells were resistant to DOXO treatment, as evidenced by a significant ability to form metastasis (Figure 13 C and D). Moreover, the number of lung metastasis in the DOXO-treated mice injected with hybrid cells was significantly higher than the DOXO-treated mice inoculated with single culture (Figure 13 E). Taken together, these data, suggesting that hybrid cells acquire resistance to DOXO treatment and enhance the metastatic spread of breast cancer cells, are included in a manuscript in preparation.

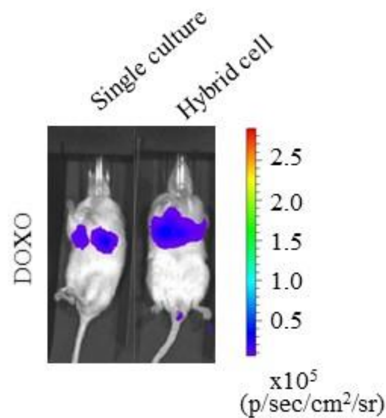
A



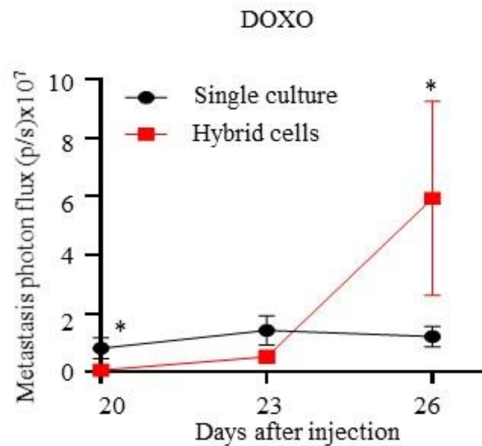
B



C



D



E

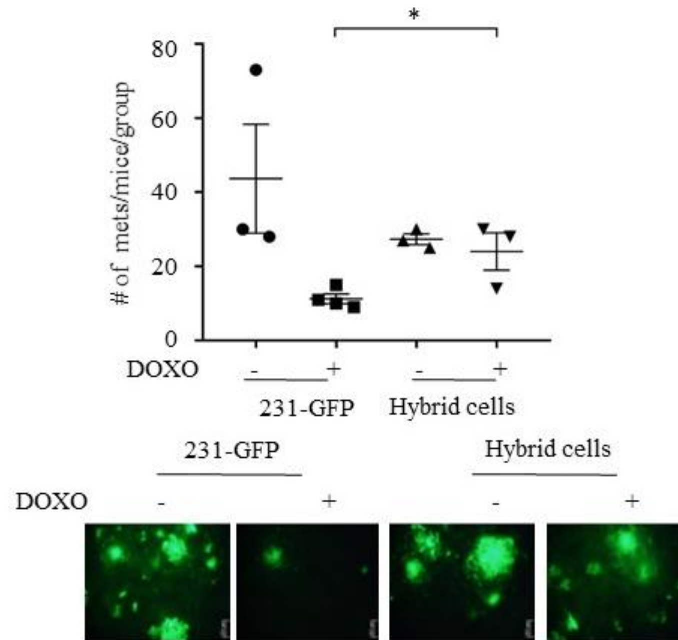


Figure 13. Hybrid cells exhibit increased ability to form metastasis upon doxorubicin treatment. A. Representative bioluminescence image of distant metastases at day 20 after intracardiac injection of hybrid cells (1.5 $\times$ 10^5 cells) and controls (1.0×10^5) in NOD/SCID mice (n = 10 mice per group). **B.** Metastatic burden assessed by measuring photon flux at the indicated times post-intracardiac injections. The arrow indicates the first day of DOXO treatment. **C**. Representative bioluminescence image of distant metastasis at day 26 after intracardiac injection of single culture of hybrid cells in mice treated with DOXO 4 mg/kg (n = 5 mice per group) every three days. **D.** Metastatic burden assessed by measuring photon flux at the indicated times post-intracardiac injections after DOXO treatment in mice treated as in C. **E.** Number of lung metastasis in mice injected with single culture or hybrid cells treated with vehicle (-) or DOXO at day 27. Representative images of the lungs of mice showing GFP-metastases are shown (scale bar: 200 µm). Data are presented as mean ± SEM. $p^* < 0.05$.

DISCUSSION

In the TME, BCCs communicate with the stromal cells in a dynamic interaction that supports breast cancer progression. Among stromal cells, TAMs representing over 50% of the tumor mass has been shown to correlate with tumor aggressiveness and to predict poorer prognosis in almost all tumors, including breast carcinoma. Studies on macrophage–cancer cell interactions have emphasized that TAMs, classically divided into M1 and M2 polarized macrophages, are plastic cells with different functions and cytokine production in response to various micro-environmental switching signals (Qian and Pollard 2010; Sica and Mantovani 2012). Thus, strategies aiming to affect macrophage recruitment and/or to educate macrophages in a less aggressive phenotype promise therapeutic benefits.

In the first part of this thesis, we explored the phenotypic features of breast TAMs generated by exposure to the conditioned media of MCF7 or MDA-MB-231 BCCs, obtaining a population of TAMs with features of both M1 and M2 polarized cells. Next, in order to search new TAM-modulating agents, we tested the effects of natural and synthetic PPARγ agonists to modulate macrophage polarization induced by breast cancer cell secretome. We observed that the synthetic and specific PPARγ agonist rosiglitazone reduced the secretion of M1 pro-inflammatory cytokines. Moreover, the two conjugates of omega-3 polyunsaturated fatty acid docosahexaenoic acid (DHA) with ethanolamine and serotonin, DHEA and DHA-5-HT, respectively, exerted similar inhibitory effects on macrophage polarization. It has been reported that DHEA possesses anti-inflammatory- and (or) general immune-modulating properties (Berger et al. 2001; Artmann et al. 2008; Balvers et al. 2010; Brown et al. 2010). Also, DHA-5-HT has been shown to modulate inflammation in macrophages by reducing levels of key mediators involved in cytokine signaling pathways (Poland et al. 2016). Our data revealed that the decreased secretion of M1 IL-6 cytokine exerted by DHEA and DHA-5-HT in TAMs was prevented by the PPARγ antagonist GW9662, suggesting the potential involvement of PPARγ. More interestingly, synthetic and natural PPARγ agonists significantly reduced also the secretion of the M2-cytokine IL1Ra, simultaneously attenuating both M1 and M2 macrophage phenotypes. We have previously demonstrated that ligand-activated PPARγ induces cell growth inhibition, triggering autophagy and apoptosis in breast cancer cells (Bonofiglio et al. 2009; Bonofiglio et al. 2009, Rovito et al. 2013; Rovito et al. 2015). Taken together, our previous findings and these data highlight that natural or synthetic PPARγ agonists may offer leads to novel strategies that target both epithelial neoplastic cells and the breast TME.

In order to identify other targets in the immune breast cancer microenvironment, we next investigated the potential role of the signal of leptin, which has been well recognized as a molecular player involved in breast cancer development and prognosis. Indeed, hyperactive leptin signaling affects different aspects of breast cancer biology by both modulating the phenotype of neoplastic epithelial cells as well as the behavior of the different components within the TME, including macrophages (Guo and Gonzalez-Perez 2011; Barone et al. 2012; Napoleone et al. 2012; Giordano et al. 2016). Nevertheless, how Ob/ObR signaling in breast epithelial cancer cells may influence the behavior of TAMs remains to be properly focused. In our search for evaluating the impact of the integrity of leptin signaling on the interaction between

tumor cells and TAMs, we incubated TAMs in the presence of CM obtained from ObR sh MCF-7 and MDA-MB-231 BCCs. The loss of the ObR signaling decreased the expression of cytokines sustaining the multistep development of breast malignancy involved in tumor angiogenesis, invasiveness and metastasis (e.g., MCP-1, VEGF, MMP-9)(Leek et al. 1996; Leek et al.2000; Saji et al. 2001; Nagakawa et al. 2002), in EMT and stemness (TNF-α, IL-6) (Neumark et al. 1999; Asiedu et al. 2011) and in immunosuppressive effects (IL-10) (Vinogradov et al. 2014). Unlike tissue-resident macrophages, which are derived largely from the yolk sac in embryogenesis (Lahmar et al. 2016), TAMs derive from circulating monocytes that are recruited in the tumor site through the secretion of chemo-attractant molecules such as MCP-1. Interestingly, we found that MCP-1 expression and secretion were significantly reduced in both ObR sh breast cancer cell lines. Moreover, the in vitro chemotaxis assay that mimics the microenvironment milieu, showed a reduced capability to recruit human monocytes in the presence of the CM-derived from ObR sh clones. The translation of our *in vitro* data into animal studies demonstrated a lower content of infiltrating macrophages concomitantly with a lesser expression of MCP-1 in ObR sh xenografts. Moreover, both ObR sh MCF-7 and MDA-MB-231 xenografts displayed a markedly reduced growth rate with respect to the control Sh tumors, suggesting that the reduced macrophage infiltration may impact breast cancer growth.

Collectively, our findings demonstrate that Ob/ObR signaling knockdown reduces macrophage infiltration and contributes reprogramming the recruited macrophages by BCCs in a less aggressive phenotype, thus reducing tumor growth.

Another crucial component of the TME is represented by MSCs, which are an important stromal cell source characterized by self-renewal and pluripotency properties (Whiteside 2008). In the TME, MSCs can directly interact with other cells in the TME, such as BCCs and immune cells, supporting tumor progression (Jiang and Xu 2020). Recently, it has been reported that BCCs can engulf MSCs acquiring a more aggressive breast cancer phenotype that supports breast cancer metastasis (Chen et al. 2019). Here, we generated and characterized the phenotypic features of BCCs engulfing MSCs, which result in a hybrid cell population expressing both MSC and BCC markers. Hybrid cells showed multiple nuclei and increased DNA content, that are features of polyploid cells, usually formed by defective cell cycle or cell fusion processes (Spies and van Wyk 1995; Fox and Duronio 2013). In addition, polyploid cells may have reduced ability to proliferate and are defined as dormant cells (Banys-Paluchowski et al. 2020), characterized by

G0-G1 cell cycle arrest, lack of proliferative (such as Ki67 and PCNA) and pro-apoptotic markers (Gao et al. 2017; Phan and Croucher 2020). Moreover, dormant cells are also reprogramming to survive and escape to immunosurveillance. In this context, it has been reported that dormant cells can secrete senescence-associated secretory phenotype (SASP) factors, creating a permissive pro-tumorigenic microenvironment (Bartosh 2016; Bartosh et al. 2016; Triana-Martínez et al. 2020). According to these findings, we observed an increased percentage of $Ki67^{low}$ cells in the G0-G1 phase of the cell-cycle. We found that hybrid cells have a SASP secretome, characterized by the production of several factors including uPAR, CCL2, OPN, THBS-1 and IL6. These factors may also regulate the interaction between BCCs and MSCs. Indeed, THBS-1 has been described as a bridge molecule (Krispin et al. 2006) that may allow the physical interaction between MSCs and BCCs in the initial phases of MSC engulfment. Moreover, BCC-derived OPN, uPAR, IL-6 and CCL2 may stimulate the migration of MSC toward BCCs (Gutova et al. 2008; Mi et al. 2011). The mechanism by which disseminated cancer cells enter in a dormant state is not fully understood. However, physical factors, such as limited availability of oxygen (angiogenic dormancy) or secretion of specific soluble molecules by the stromal cells, such as THBS-1, are responsible for the entering of cancer cells in a slowly or not proliferative state within the cell niche (Ghajar et al. 2013; Phan and Croucher 2020). As a consequence, dormant cells become not responsive to the classical chemotherapeutic drugs that target proliferating cells (Phan and Croucher 2020). In line with these observations, we found that hybrid cells acquire resistance to doxorubicin and more interestingly also animal studies revealed that hybrid cells acquire the ability to form metastasis upon doxorubicin treatment. Taken together, we showed for the first time the existence of a hybrid cell population derived from MSC engulfment by BCCs that drives dormancy and drug resistance in breast cancer. However, further investigations are needed to understand the molecular mechanisms underlying the dormant hybrid cell formation. To date, although different dormancy-target strategies have been proposed, including the maintenance of cancer cells in a dormant state, the reactivation of dormant cells to improve their sensibility to chemotherapeutic drugs and the eradication of dormant cells, the research is still ongoing and further clinical studies are required to test the efficacy of these approaches.

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