UNIVERSITÀ DELLA CALABRIA

Dipartimento di Biologia, Ecologia e Scienze Della Terra

Dottorato di Ricerca in Scienze della Vita

(XXXIV CICLO)

Novel mechanisms involved by G-protein estrogen receptor (GPER) in the progression of estrogen receptor-negative breast cancer

Settore scientifico Disciplinare: MED/04

Supervisore

Prof. Marcello Maggiolini

[redacted] Firma oscurata in base alle linee guida del Garante della privacy [redacted]

Coordinatrice del Dottorato

Prof.ssa Maria Carmela Cerra

Firma oscurata in base alle linee guida del Garante della privacy

Dottoranda

Dott.ssa Marianna Talia

Firma oscurata in base alle linee guida del Garante della privacy [redacted]

ANNO ACCADEMICO 2020/2021

Index

Abstract

The G protein-coupled estrogen receptor (GPER) is a seven transmembrane receptor that mediates estrogen signals in both normal and malignant cells, including breast cancer. In particular, GPER activation triggers diverse transduction pathways prompting transcriptional and biological pro-tumorigenic responses. In this context, we aimed to perform in-silico analysis that show a correlation of GPER expression levels with worse clinical-pathological features of breast cancer. By gene expression correlation, gene set enrichment analysis (GSEA) and KEGG pathway enrichment analysis on the transcriptomics data of ER-negative breast tumors provided by TCGA and METABRIC datasets, we also ascertained that the levels of GPER are associated with pro-migratory and metastatic genes. In particular, a strong association was found between the expression of GPER and that of genes belonging to cell adhesion molecules (CAMs), extracellular matrix (ECM)-receptor interaction and focal adhesion (FA) signaling pathways. Accordingly, we found that high GPER levels are predictive of a shorter disease-free interval (DFI) in ER-negative and HER2-negative breast cancer patients. Overall, our results may pave the way to further dissect the network triggered by GPER in breast malignancies lacking ER. Next, starting from a further bioinformatics analysis on TCGA and METABRIC triple negative breast cancer (TNBC) cohorts of patients, we determined that the expression of the pro-inflammatory cytokine interleukin-1β (IL-β) correlates with the levels of the hypoxia inducible factor-1α (HIF-1α) as well as with a hypoxia related gene signature. Then, through gene and protein expression studies, immunofluorescence analysis, co-immunoprecipitation, ChiP and ELISA assays we demonstrated that hypoxia triggers a functional liaison among HIF-1α, GPER and the IL-1β/IL1R1 signaling toward a metastatic gene signature and a feed-forward loop of IL-1β. Cell spreading, invasion and spheroid formation assays showed that IL-1β/IL1R1 axis leads to proliferative and invasive responses in TNBC cells. Furthermore, we found that the IL-1β released in the conditioned medium of TNBC cells exposed to hypoxic conditions promotes an invasive phenotype of breast cancer-associated fibroblasts (CAFs). Together, these findings may contribute to unveil the mechanisms involved in the hypoxic activation of the IL-1β/IL1R1 signaling toward aggressive features of both TNBC cells and CAFs, suggesting novel targets needed for innovative and more comprehensive therapeutic strategies in TNBC patients.

1 Introduction

1.1 Breast tumor

Breast cancer is the most frequent malignancy among the female population worldwide: one in eight to ten women will get breast cancer during their lifetime (Harbeck et al., 2017). As a result of the early detection and improved treatments, breast cancer mortality is decreasing in developed countries (Britt et al., 2020). Nevertheless, the incidence of this malignancy is gradually increasing likely due to an implementation of mammographic screening (Britt et al., 2020). Breast cancer is a complex heterogeneous disease comprising biologically different entities with distinct pathological features and clinical implications (Yeo et al., 2017). Accumulating evidence has suggested that breast cancer displays a large degree of inter- and intra- tumoral heterogeneity, thus the stratification of tumors is essential to achieve tailored approaches toward better clinical outcomes (Yeo et al., 2017). Two major categories of breast cancer are:

- Non-invasive breast cancer: carcinoma (in situ) characterized by the proliferation of malignant epithelial cells that cannot pass the basal membrane, thus not reaching lymph nodes. This event can occur at lobular level (*lobular carcinoma in situ*: it is a sign of increased risk of forming tumors in both breasts) or can involve the duct of the gland (*ductal carcinoma in situ*: it is an initial form of breast cancer limited to cells that form the wall of the ducts. If not cured, it can become invasive). This type of breast cancer can be additionally subdivided into: intraepithelial ductal neoplasia (DIN) (carcinoma in situ) and lobular intraepithelial neoplasia (LIN).

- Invasive breast cancer: infiltrating carcinoma characterized by the crossing of the basal membrane and the presence of stromal invasion and diffusion at the lymphatic level. It is possible to distinguish *infiltrating ductal carcinoma* (it passes the duct wall and represents the largest percentage of all forms of breast cancer) and *infiltrating lobular carcinoma* (the tumor surpasses the lobule wall; it can simultaneously attack both breasts or appear in multiple spots in the same breast).

Several risk factors drive breast cancer development, including:

- Gender/sex: epidemiological data showed that women present a risk of developing breast cancer 100 times higher than men (Leon-Ferre et al., 2018).

- Age: in industrialized countries the risk become higher as the age increases, ranging from 1:5900 to 1:290 between the third and the eighth decade of life. The frequency of breast carcinoma seems to be progressively reaching a peak at the age of 50, which corresponds to the beginning of menopause. From 60 to 65 years there is a stasis and from 65 on the incidence rises again. (De Carli et al., 1993).

- Age of the first pregnancy: the risk is double for women who deal with their first pregnancy in advanced age and for women who have not completed any pregnancy. A pregnancy carried out before the age of 30 is a protective factor for breast carcinoma. (Hulka and Moorman, 2001).

- Ovulation span: a weak increase of risk has been recorded in case of early menarche (before the age of 12) and late menopause (age of onset after 55) (Bernstein, 2002).

- Hormone replacement therapy: several studies reported an increase of the risk of breast cancer in women subjected to hormone replacement therapy based on estrogen or estrogen-progesterone combination in post-menopausal age. In the process of neoplastic transformation of the breast gland, an important role is played by hormones as estrogens as well as further mitogenic growth factors (e.g., insulin-like growth factor, epidermal growth factor) that activating the cognate receptors stimulate tumorigenic cellular signals (Dickson and Lippman, 1996).

- Ionizing radiation: their carcinogenic effect is directly linked not only to the cumulative dose but also to the age of exposure (Helm and Rudel, 2020). In addition, women who have been subjected to radiotherapy during young age have a greater risk of developing breast tumor (Biglia et al., 2004).

- Familiarity: first-degree family members of women who have been affected by breast cancer have nearly twice the risk of developing breast cancer over those who have no affected family members (Thompson, 1994).

- Personal history: the risk to develop a breast carcinoma is particularly high for a woman who previously suffered from it. Moreover, women who have been subjected to breast biopsy, even with a positive outcome, present a higher risk of developing breast tumor (Armstrong et al., 2000).

- Genetic factors: about 5-10% of breast cancer cases is attributable to hereditary factors such as pathogenic mutations in cancer-related genes (i.e. BRCA1, BRCA2, checkpoint kinase 2 (CHEK2)) and breast cancer-associated common single-nucleotide polymorphisms (SNPs) (Britt et al., 2020). The risk of developing breast cancer for people who carry a mutation on BRCA1 is between 36% and 87%, whereas mutations of BRCA2, which are present in most dominant transmissible breast carcinomas, are associated with a risk ranging between 45 and 84% (Fodor et al., 1998; Antoniou et al., 2003; Zografos et al., 2004).

- Dietary and metabolic factors: it has been demonstrated that a high-lipid diet can promote the development of different spontaneous, transplanted or chemically induced neoplasms in laboratory animals (Kushi et al., 1992; Lubin et al., 1996; van der Brandt et al., 1993; Byrne et al., 2002).

- Smoking: it represents a risk factor for the majority of malignancies, including breast cancer (Baron et al., 1996; Marcus et al., 2000; Egan et al., 2002).

Breast cancer can be diagnosed with mammography and breast ultrasound, while in specific circumstances magnetic resonance imaging may be a further option. The potential identification of nodules and suspicious formations may suggest a biopsy that allows establishing the nature of the disease and its biological features. Almost every woman with breast cancer, regardless of the stage, undergoes surgery in order to remove the neoplastic tissues. Surgery has to be followed by radiotherapy, necessary to protect the remaining mammary gland both from the risk of local recurrence and from the appearance of a new breast cancer. Several advanced breast tumors are treated removing the whole breast: the technique used (radical mastectomy) is based on the removal of the gland, the sentinel lymph node and/or all the axillary lymph nodes, rarely part or all of the pectoral muscle and often also the overlying skin. After the surgery, an accurate histological and biological evaluation is necessary to define precautionary therapies in order to minimize the risk to affect other organs (remote metastases). Chemotherapy is helpful, but not

always necessary and it has to be prescribed after a personalized evaluation. It is also prescribed for the initial forms for precautionary purposes and the benefit, in terms of years of survival, is greater than the most advanced tumor forms. In the last few years it became diffused the use of neoadjuvant chemotherapy, administered before the surgery to reduce the dimension and the aggressiveness of the tumor.

Breast tumors are classified in five stages (Figure 1.1.1).

- Stage 0: also called carcinoma in situ comprising lobular carcinoma in situ and ductal carcinoma in situ.
- Stage I: it is a tumor at the initial phase; it has a diameter less than 2 cm and it do not involve lymph nodes.
- Stage II: it is a tumor at the initial phase with less than 2 cm of diameter but it already reached axillary lymph nodes; it could also be a tumor bigger than 2 cm without the implication of lymph nodes.
- Stage III: it is a locally advanced tumor of variable dimensions that already got to the axillary lymph nodes or that involves the tissues near the breast (for example the skin).
- Stage IV: Is a metastatic cancer that has involved other organs outside the breast.

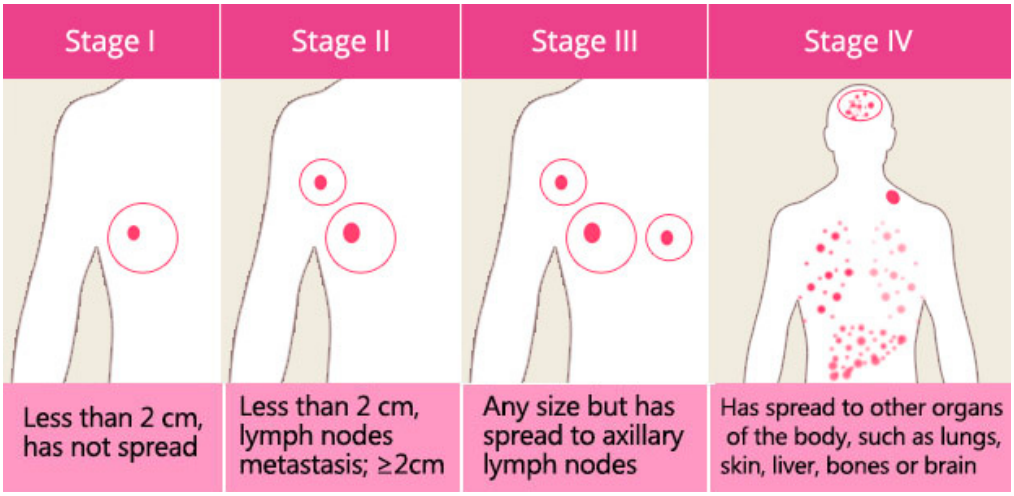

Figure 1.1.1 Breast tumor staging.

Whether the tumor is identified at stage 0, the survival percentage in five years of treated women is approximately 98%, even if the relapses may vary between 9 and 30% of the cases depending on the therapy chosen. If lymph nodes include neoplastic cells, the survival in five years is 75%. For metastatic diseases (cancers that have already affected organs outside the breast), the average survival of patients treated with chemotherapy is generally around two years. However,

this time can be longer as in some cases the survival time is higher, even up to ten years. The recent advancement and extensive application of high-throughput and cost-effective 'omics' technologies (for instance genomics, transcriptomics or proteomics) has provided extraordinary insights and novel understanding of breast cancer molecular portraits (Bianchini et al., 2016). The pioneer studies conducted by Sørlie et al. classified breast tumors into five intrinsic subtypes, based on variations in gene expression patterns derived from cDNA microarrays: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) over-expression, basal and normal-like, (Sørlie et al., 2001). In spite of this complexity, patient prognosis and management mostly rely on the immunoistochemical evaluation of the endocrine receptors for estrogen and progesterone (ER and PR, respectively), and HER2 (Bianchini et al., 2016). Tumors that lack the expression of all the aforementioned receptors are defined triple negative breast cancers (TNBC). Due to a more aggressive clinical behavior and the lack of recognized therapeutic molecular targets, TNBC patients are characterized by high rates of tumor recurrence and poor overall compared to those with other breast cancer subtypes (Bianchini et al., 2016; Garrido-Castro et al., 2019).

1.2 The tumor microenvironment

The tumor microenvironment (TME), also known as tumor stroma, represents an intricate network of non-cancer components that surround cancer cells, including cancer associated fibroblasts (CAFs), both innate immune (macrophages, mast cells, neutrophils, dendritic cells, myeloid derived suppressor cells, and natural killer cells) and adaptive immune cells (T and B lymphocytes), adipocytes, ECM, endothelial cells and pericytes (Hinshaw and Shevde, 2019; Chen and Song, 2018) (Fig. 1.2.1). Despite extensive efforts have been dedicated on targeting tumor cells, recent advances in immunotherapy revealed that targeting the TME is a potent tool to hamper cancer development and progression (Houthuijzen and Jonkers, 2018). The strong interaction existing between cancer cells and the TME makes the latter the supportive essential 'soil' for the development of metastasis by the 'seeds' (malignant cells) (Chen and Song, 2018). Notably, upon stimulation the stromal cells can be recruited to a secondary site, distant from the primary tumor, in order to foster malignancy through metastasis development (Chen and Song, 2018). In fact, stromal cells influence the behavior of cancer cells by producing and secreting ECM proteins, chemokines, cytokines and growth factors, thus aberrantly activating autocrine and paracrine loops (Mittal et al., 2018).

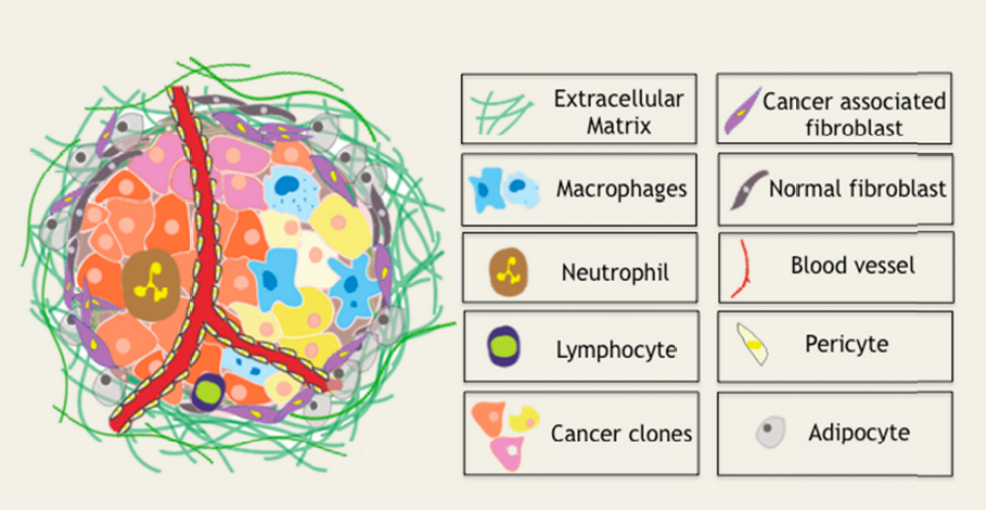

Figure 1.2.1 The major components of the tumor microenvironment.
(Mittal et al., 2018)

Immune cells may influence the tumor behavior and its response to treatments by interacting with malignant cells through direct contact or through the release of chemokines and cytokines (Mittal et al., 2018). In this vein, immune cells can either support or hamper therapeutic effectiveness. In this regard, tumor-associated macrophages (TAMs) are key regulators of therapeutic response in the TME and orchestrate tumor-associated inflammation (Chen and Song, 2018; Chang et al., 2005; Farmer et al., 2009). TAMs originate from monocytes that spread to the tumor site and differentiate into M1 or M2 macrophages subtypes based on their polarization status. M1 macrophages seem to have tumoricidal properties, while M2 macrophages stimulate the growth and survival of tumor cells (Chang et al., 2005; Farmer et al., 2009). In vitro and in vivo evidence demonstrated the ability of TAMs to mediate resistance to diverse chemotherapeutic agents (5-fluorouracil, doxorubicin, gemcitabine, paclitaxel, platinum compounds, etc.) as well as anti-angiogenic therapies (Tice et al., 2008; Farmer et al., 2009).

The ECM comprises an intricate network of diverse components, such as collagen, fibronectin, laminin, glycoproteins and polysaccharides, among others (Mittal et al., 2018). In solid tumors, collagens and fibronectin provide the mechanical strength that regulates the different stages of tumor development and differentiation, whereas proteoglycans exhibit growth factor- and cytokine-binding properties (Ozbek et al., 2010; Kim et al., 2011). A well-organized interconnected ECM operates as a physical barrier for drug delivery (Grantab et al., 2006; Netti et al., 2000), the binding components of the ECM are able to sequestrate drugs, inhibiting the diffusion to hidden tumor regions (Berk et al., 1997).

Endothelial cells, which form the lining of tumor blood vessels, have a pivotal role in the process of tumor angiogenesis (Carmeliet and Jain, 2000). In response to the vascular endothelial growth factor A (VEGFA), endothelial cells proliferate to form new blood vessels which are essential for the tumor constant supply of oxygen and nutrients and for the successful migration and invasion of neoplastic cells (De Palma et al., 2017). Nevertheless, tumor vessels are abnormal, disorganized, leaky and immature, deficient of pericytes required for vascular maturation. This may lead to radiation and chemotherapy-enhanced sensitivity, suggesting that pericytes may endorse therapeutic-resistance (Mittal et al., 2018). In this vein, endothelial cells release the platelet-derived growth factor-β (PDGFβ) necessary to recruit pericytes that ensure microvessel stabilization (Armulik et al., 2011).

Cancer-associated fibroblasts (CAFs) constitute the largest proportion of stromal cells within the TME and provide critical signals toward tumor progression, therefore representing a conspicuous target in many solid tumors (Kalluri and Zeisberg, 2006; Kalluri, 2016). Extensive evidence indicates that a high amount of CAFs within the TME correlates with poor clinical prognosis in diverse tumors, including breast cancer (Lappano et al., 2020). Accordingly, CAFs play a main role in tumor progression by enhancing cell proliferation and survival, angiogenesis, immune suppression, maintenance of stemness, ECM production and remodeling, and therapy resistance (Fig.1.2.2) (Chen and Song, 2018; Houthuijzen and Jonkers, 2018; Lappano et al., 2020).

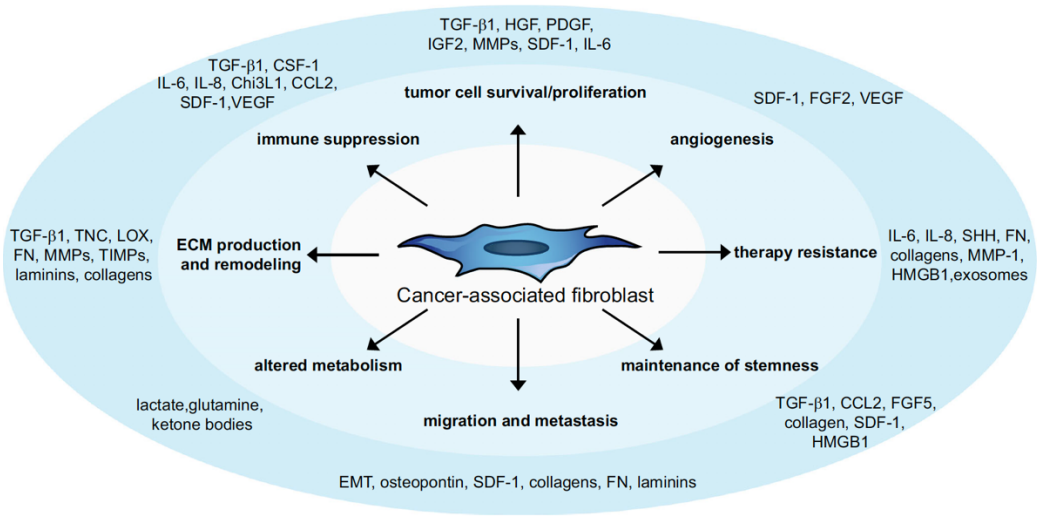

Figure 1.2.2 Pro-tumorigenic effects elicited by CAFs.
(Houthuijzen and Jonkers, 2018)

Due to the high heterogeneity of biological, molecular and functional properties of CAFs, their origin is still under debate (Ishii et al., 2016). CAFs are considered as mesoderm-derived cells

exhibiting mesenchymal-like features (Kalluri, 2016). Moreover, CAFs are thought to derive from normal fibroblasts which, evolving with cancer cells, acquire a pro-tumorigenic phenotype contributing to the growth and evolution of the tumor mass (Öhlund et al., 2014). CAFs are spindle-shaped blast-like cells similar to their normal counterparts, but are larger, harbor multiple branches of cytoplasm and have indented nuclei (De Wever et al., 2008). In particular, it has been shown that breast CAFs stem from diverse cell types comprising normal fibroblasts, vascular smooth cells and pericytes as well as cancer stem cells (Ishii et al., 2016; Kalluri, 2016; LeBleu and Kalluri, 2018). The identification of unique fibroblast markers has enabled live-cell sorting for CAFs subpopulations as well as in vivo mechanistic studies (Su et al., 2018). For instance, markers commonly used for the identification of CAFs are the Fibroblast Activating Protein (FAP) and the combination of Platelet-Derived Growth Factor Receptor alpha (PDGFR-α) and alpha-Smooth Muscle Actin (α-SMA) (LeBleu and Kalluri, 2018). Furthermore, single cell high throughput analysis of CAFs, isolated from breast cancer patients, revealed a deeper layer of complexity of these cells, showing peculiar features such as loss of Caveolin-1 (Cav-1), increased α-SMA, Snail1, Tenascin-C and ALDH1A3 (Aldehyde Dehydrogenase 1 A3) (Busch et al., 2017). In this scenario, further investigations aimed to define each subpopulation at the molecular and functional levels are warranted. CAFs produce and secrete various ECM proteins (collagens I, III, IV), proteoglycans (fibronectin, laminin, TN), chemokines (CXCL and CCL), cytokines (interleukin (IL)-6 and IL-8) and other tumor-promoting factors which affect vascularization (PDGF, vascular endothelial growth factor (VEGF), stromal-derived factor-1 (SDF-1), matrix metalloproteinase (MMPs)), proliferation, invasiveness and survival (TGF-β, EGF, hepatocyte growth factor (HGF) or FGF) (Shimoda et al., 2010; Paraiso and Smalley, 2013; Bremnes et al., 2011; Gonda et al., 2010). Recent advances in immunotherapy demonstrate that targeting CAFs represents a powerful tool in controlling tumor progression and predicting therapeutic responses (Torre et al., 2015). For instance, tumor infiltration by CAFs expressing the zinc-dependent metalloproteinase CD10 and the G protein-coupled receptor 77 is predictive of chemotherapy response and patient survival of ER-negative and HER2-negative breast tumors (Torre et al., 2015; Su et al., 2018). The expression of Snail1 together with myofibroblast markers was shown to be a requisite for CAFs activation and paracrine effects toward breast tumor invasiveness (Stanisavljevic et al., 2015). In this scenario, various preclinical studies tempting to hamper the pro-tumorigenic function exerted by CAFs have been reported, some of which have moved into the clinic (Chen and Song, 2018).

1.3 Hypoxia in the tumor microenvironment

Hypoxia is a low non-physiological level of oxygen (O_2) tension within a tissue. This condition has been recognized as one of the hallmarks of cancer, including breast tumor, altering cell metabolism, leading to the acquisition of epithelial-to-mesenchymal transition phenotype and contributing to therapy resistance by inducing cell quiescence (Schito and Semenza, 2016; Gilkes et al., 2014 A). O_2 availability decreases as the distance from the nearest blood vessel increases. Rapidly dividing cancer cells, which outgrow the vascular network, require a constant O_2 uptake that cause an advanced but dysfunctional vascularization and an imbalance of O_2 supply and demand (Gilkes et al., 2014 A). Malignant cells are able to adapt to the hypoxic environment through the transcriptional action of the hypoxia-inducible factors (HIFs) that regulate the expression of several genes involved in angiogenesis, metabolism, cancer cell invasion and metastasis (Hubbi and Semenza, 2015). HIFs function as heterodimers and consist of an O_2 regulated HIF-α subunit and a stable HIF-1β subunit. Mammals possess three diverse HIF-α isoforms: HIF-1α, HIF-2α and HIF-3α. The most well characterized are HIF-1α and HIF-2α which are structurally similar (Hubbi and Semenza, 2015; Semenza, 2019). HIF-1α is expressed in all cells, while HIF-2α is expressed only in certain cell types, including vascular endothelial cells, renal interstitial cells and cells of the myeloid lineage (Hubbi and Semenza, 2015). HIF-2α shares 48% amino acid sequence identity with HIF-1α and binds to HIF-1β to form HIF-2, which activates the transcription of some, but not all, HIF-1 target genes (Tian et al., 1997; Wiesener et al., 1998), furthermore, in breast cancer, HIF-1α is the predominantly (over)expressed isoform (Schödel et al., 2013; Smythies et al., 2019). HIF-3α exists as multiple splice variants, some of which heterodimerize with HIF-1β and others that bind to and inhibit the activity of HIF-1α and HIF-2α, though HIF-3α role in cancer progression remains to be elucidated (Yang et al., 2015). HIF transcription factors exert their transcriptional activity by binding to hypoxia responsive elements ("HRE") consensus sequence, 5′-RCGTG-3′, located within or near their target genes (Fig. 1.3.1) (Semenza et al., 1996).

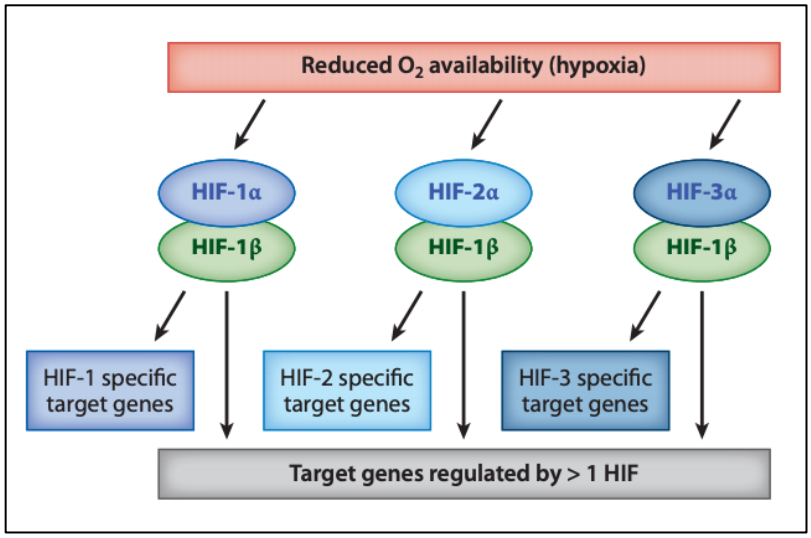

Figure 1.3.1 Transcriptional responses to reduced O_2 availability are mediated by hypoxia-inducible factors (HIFs).
(Semenza, 2019)

HIF-1 consists of an oxygen-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit, which are basic-helix-loop-helix-PER-ARNT-SIM homology domain (PAS) domain proteins (Wang et al., 1995; Wang and Semenza, 1995; Prabhakar and Semenza, 2015). Under normoxic condition, HIF-1α is synthetized at basal rate and undergoes rapid proteasomal degradation. In particular, HIF-1α subunits are modified by the prolyl hydroxylase domain-containing protein (PHD) 1, PHD2 and PHD3 enzymes (also termed EGLN2, EGLN1 and EGLN3, respectively), and the asparaginyl hydroxylases (FIH1) that are dependent on O_2 and α-ketoglutarate as substrates to hydroxylate proline and asparagine residues (P402, P564, and N804 in human HIF-1α) (Samanta and Semenza, 2018). The asparagine hydroxylation blocks the recruitment of the co-activator protein P300 whereas the proline hydroxylation prompts the interaction with the von Hippel-Lindau (VHL) tumor suppressor protein. Subsequently, VHL recruits an E3 ubiquitin-ligase (BCCRE2) consisting of Elongin B, Elongin C, Cullin 2, RBX1, and an E2 ubiquitin ligase that targets HIF-1α subunits for proteasomal degradation Samanta and Semenza, 2018). Under hypoxic conditions, hydroxylation is inhibited, PHD and FIH-1 activities are diminished, thus leading to HIF-1α accumulation, translocation into the nucleus and heterodimerization with HIF-1β (Semenza, 2003). The HIF-1 complex is able to bind to the HREs of target genes, thus prompting their transcriptional upregulation (Fig.1.3.2) (Vito et al., 2020).

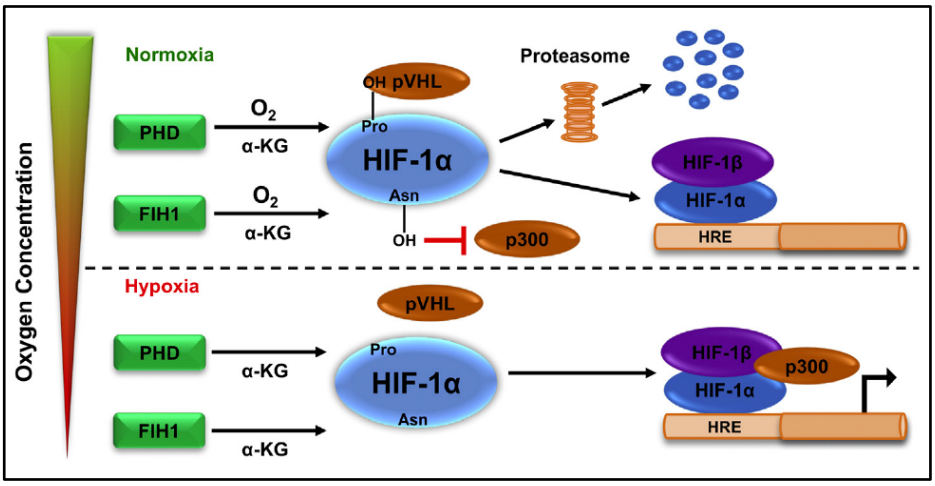

Figure 1.3.2 Hypoxia-inducible factor (HIF) signaling pathway.
(Samanta and Semenza, 2018)

It has been widely reported that high HIF-1α protein levels correlate with decreased survival rates in diverse malignancies including bladder, brain, breast, cervix, colon, endometrium, esophagus, head and neck, larynx, oropharynx, ovary, liver, lung, pancreas, prostate, and stomach cancers, as well as melanoma and acute lymphoid and myeloid leukemias (Semenza, 2010; Deeb et al., 2011; Frolova et al., 2012; Morine, 2011; Zheng et al., 2013). Moreover, in breast tumors, HIF-1α levels strongly correlate with tumor grade and invasion (Bos, 2001) and its accumulation is prevalent around necrotic areas such as the hypoxic tumor core. In breast cancers cells an increase of HIFα activity may also be caused by common genetic mutations and oncogenic alterations, including the loss of the tumor suppressors PTEN, p53, BRCA1 or VHL, as well as hyper-activation of the PI3K- pathway (Maxwell et al., 1999; Zundel et al., 2000; Akakura et al., 2001; Zhong et al., 2000; de Heer et al., 2020). Likewise, the over-expression of HIF-1α in breast malignancies and the consequent hyperactivation of its target genes have been indicated as key drivers especially in the TNBC aggressive subtype (Samanta et al., 2014). In this regard, it is well acknowledged the transcriptional activity of HIFα toward the activation of a large battery of genes involved in tumor vascularization and growth, stromal cell recruitment, extracellular matrix remodeling, cell motility, local tissue invasion and metastasis in breast and other tumors (Schito and Semenza, 2016). For instance, vascular pro-angiogenic factors such as VEGF-A, the chemokine CXCL-12, angiopoietin (ANGPT)2, placental growth factor (PGF), PDGFβ and stem cell factor (SCF) are up-regulated by HIFα in response to hypoxia thus altering extra- and intra-cellular responses by binding to their cognate receptors (Schito and Semenza, 2016; Zhang et al., 2021). In addition, HIFα coordinates several other signaling pathways involved in the transition from mesenchymal to epithelial phenotype (EMT) of cancer cells which drives clonal expansion and metastatic colonization (Schito and

Semenza, 2016). HIFα down-regulates E-cadherin, the major component of the adherents junctions (Schito and Semenza, 2016), up-regulates EMT genes (SNAIL1, SNAIL2, ZEB1, ZEB2, TWIST, and TCF3), MMP (MMP2, MMP9, and MMP14), procollagen prolyl-4-hydroxylases (P4HA1 and P4HA2), lysyl hydroxylases (PLOD1 and PLOD2), and lysyl oxidases (LOX, LOXL2, and LOXL4), which are required for cancer invasion and metastasis (Inoue et al., 1989; Gilkes et al., 2013 A; Gilkes et al., 2013 B; Wong et al., 2011). Additionally, it has been widely reported that in a persistent hypoxic condition, healthy cells undergo programmed cell death, whereas tumor cells succeed in surviving (Zhang et al., 2021; Schito and Semenza, 2016). Some of the mechanisms through which cancer cells adapt to low O_2 tension are mediated through VEGF and include the decrease of BAX/BCL-2 factors ratio, the reduction of cytochrome C release and the inhibition of caspase 3 activity (Baek et al., 2000). The ability to escape apoptosis leads to a dramatic loss of response to radiotherapy and chemotherapy in cancer patients (Zhang et al., 2021). Of note, advances in cancer metabolomic studies revealed that HIF-1-mediated transcriptional activity is essential for the shift of cell metabolism from oxidative phosphorylation to glycolysis in order to increase the ATP production under low O_2 tension. Both the maximal glucose uptake and its utilization lay the basis for glycolytic respiration, which enables tumor cells to adapt and proliferate under such conditions (Schito and Semenza, 2016). HIF-1 boosts the efficiency of glycolysis, up-regulating the expression of glycolytic enzymes as SLC2A1 and SLC2A3 that encode for the glucose transporters GLUT1 and GLUT3, required for increased uptake of glucose (Schito and Semenza, 2016), hexokinases (HK1 and HK2), phosphofructokinases (PFKL and PFKP), aldolases (ALDOA and ALDOC), phosphoglycerate kinase 1 (PGK1), enolases (ENO1 and ENO2), pyruvate kinase M (PKM), and lactate dehydrogenase A (LDHA) that catalyzes the conversion of pyruvate to lactate, the terminal product of glycolysis (Duan, 2016; Lee et al., 2009; Carmeliet and Jain, 2011; Rapisarda and Melillo, 2012; Luo et al., 2011). Furthermore, HIF-1 down-regulates the oxidative phosphorylation enhancing the expression of genes encoding pyruvate dehydrogenase kinase 1 (PDK1) (Pescador et al., 2010; Favaro et al., 2012) and the BCL2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) in order to reduce the O_2 request by tumor cells within hypoxic tissues (Chiche et al., 2010;; Favaro et al., 2012).

1.4 G protein-coupled estrogen receptor (GPER)

Estrogens regulate diverse physiological processes in several tissues throughout the body, such as development, reproduction and homeostasis. On these bases, they have a pivotal role in the development of hormone-sensitive tumors, including breast cancer (MacGregor and Jordan,

1998; Ruggiero and Likis, 2002; Liang and Shang, 2013). The multiple biological effects exerted by estrogens are mainly mediated by the activation of the classical estrogen receptor (ER)α, identified in the 1960s (Jensen and Jacobson, 1962; Jensen and DeSombre, 1973), and ERβ, a second highly homologous estrogen receptor discovered in 1996 (Böttner et al., 2014). Upon ligand activation, ERα and ERβ translocate to the nucleus where they can act as transcriptional factors (Marino et al., 2006). In particular, by binding to estrogen responsive elements (EREs), ERs regulate the expression of target genes involved in cell growth, invasion and survival (Björnström and Sjöberg, 2005). Beyond the aforementioned "genomic" signaling, that is characterized by changes in gene transcription occurring in the frame of hours, it has been reported the capability of 17β-Estradiol (E_2) to trigger rapid "non-genomic" effects (Prossnitz and Maggiolini, 2009). In this scenario, it has been recognized the involvement of the G-protein-coupled estrogen receptor (GPER, formerly known as GPR30) in the rapid estrogen effects (Maggiolini and Picard, 2010). GPER is a seven transmembrane receptor firstly identified in the 1990s (Carmeci et al., 1997; Takada et al., 1997) that belongs to the rhodopsin-like receptor superfamily (Carmeci et al., 1997), its gene is mapped to chromosome 7p22.3 (Albanito et al., 2007). GPER has a peculiar cellular distribution pattern as it localizes on the plasma membrane, but also in the endoplasmic reticulum, Golgi apparatus and in specific cellular contexts also in the nucleus (Filardo et al., 2007; Madeo and Maggiolini, 2010; Sandén et al., 2011; Revankar et al., 2019). GPER has been shown to mediate estrogenic signals in diverse normal and malignant contexts, including breast cancer, by activating a network of transduction pathways and transcriptional changes involved in tumor progression (Rouhimoghadam et al., 2020). These rapid cellular effects include the activation of the epidermal growth factor receptor (EGFR) and multiple kinases, such as extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K), the production of cyclic adenosine monophosphate (cAMP), the mobilization of intracellular calcium, as well as the activation of ion channels and endothelial nitric oxide synthase (eNOS) (Maggiolini and Picard, 2010). In particular, previous studies on breast cancer cells showed that GPER-mediated ERK1/2 activation results from the Gβγ subunit-dependent transactivation of EGF receptor (EGFR), which occurs through the cleavage and the release of heparan-bound EGF (HB-EGF) by metalloproteinases (MMPs) (Prossnitz and Maggiolini, 2009). The stimulation of this pathway leads to diverse cellular responses such as the activation of the phospholipase C (PLC), PI3K and AKT, as well as MAPK (Maggiolini and Picard, 2010). As a result of GPER activation, a specific downstream gene signature has been characterized and has been shown to contribute to the stimulation of aggressive features in breast cancer cells. Of note, transcription

factors including SRF, CREB, JUN, FOS, CTGF, EGR1, C/EBPd and NR4A2 are prompted by estrogenic GPER signaling in breast cancer cells (Pandey et al., 2009) toward the upregulation of several target genes such as cyclin A, D1 and E, EGR-1, HIF-1, VEGF (Fig.1.4.1) (De Francesco et al., 2013; Lappano et al., 2014).

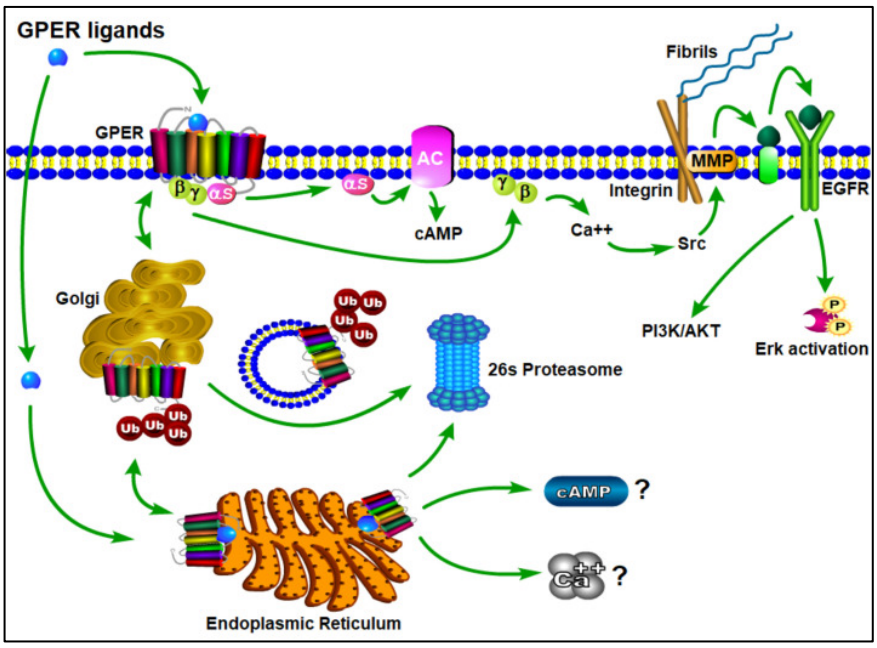

Figure 1.4.1 GPER trafficking and signaling.
(Rouhimoghadam et al., 2020)

GPER exhibits many of the expected characteristics of an estrogen receptor, including the capability to bind to a variety of estrogens, phyto- and xeno-estrogens as well as to the ERα antagonists 4-hydroxytamoxifen (OHT) and fulvestrant (ICI 182 780) (Thomas et al., 2005; Revankar et al., 2005; Thomas et al., 2006; Lappano et al., 2010; Rouhimoghadam et al., 2020; Maggiolini et al., 2004; Dong et al., 2011; Pupo et al., 2012). Nevertheless, it should be mentioned that these compounds may exert equal or opposite functions through the action of these receptors. For instance, in ER-negative breast cancer cells, E2 and two major phytoestrogens, genistein and quercetin, induced in a GPER-dependent manner the rapid up-regulation of *c-fos* (Maggiolini et al. 2001; Maggiolini et al. 2004), whereas the well-known ERα agonist estriol elicits inhibitory effects on GPER-mediated signaling (Lappano et al., 2010). Furthermore, the ERα antagonist OHT and ICI 182 780 have a strong binding affinity for GPER and act as GPER agonists (Filardo et al., 2000; Revankar et al., 2005; Vivacqua et al., 2006; Pandey et al., 2009). In particular, the most strongly GPER target gene, namely connective tissue growth factor (CTGF), has been implicated in pro-tumorigenic features of ER-negative breast cancer cells upon both E2 or OHT stimulation through GPER transduction

pathway (Pandey et al., 2009). In order to better characterize the role of GPER in both normal and breast cancer cells, selective ligands that specifically bind to this receptor, such as the agonists G-1, GPER-L1 and GPER-L2 and the antagonists G15 and G36 have been discovered (Bologa et al., 2006; Ariazi et al., 2010; Lappano et al., 2010; Lappano et al., 2012 A). Of note, a further GPER antagonist, the indole derivative compound named MIBE, has shown the peculiar property to inhibit both GPER and ERα-mediated signaling (Lappano et al., 2012 B). Recently, diverse indole-thiazole derivates have been identified to elicit selective GPER agonism (O'Dea et al., 2018). Importantly, the activities exerted by GPER ligands provided the opportunity to deeply characterize the role of GPER in biological responses driving breast cancer progression including proliferation, migration and invasion (Pandey et al., 2009; De Francesco et al., 2013; Lappano et al., 2014; Marjon et al., 2014; Barton et al., 2018). For instance, it has been reported that GPER activation stimulates breast cancer cell migration through the up-regulation of CTGF (Pandey et al., 2009), cyclin E (Li et al., 2013), the notch pathway (Pupo et al., 2014) and the CXC receptor-1 (CXCR1) (Jiang et al., 2013). In this regard, we recently found that the GPER-mediated activation of the FA kinase (FAK) stimulates the formation of FA in TNBC cells (Rigiracciolo et al., 2019). Furthermore, *in vivo* data derived from breast cancer mouse models demonstrated a positive correlation between GPER expression and the metastatic process (Filardo et al., 2006), whereas GPER-deficiency has been shown to revert this effect (Marjon et al., 2014). The role of GPER has also been assessed in CAFs derived from breast tumor samples, suggesting that the pro-tumorigenic actions of GPER may also be elicited through these key players of the TME (Prossnitz and Maggiolini, 2009; Barton et al., 2018). In particular, GPER mediates the up-regulation of VEGF expression in breast CAFs through a HIF-1α-dependent mechanism (De Francesco et al., 2014; De Francesco et al., 2017). Moreover, the role of GPER in mediating a feedforward FGF2/FGFR1 paracrine loop involving CAFs and breast cancer cells has been recently reported (Santolla et al., 2019).

1.5 Omics approaches in breast cancer research

Cancer is considered a highly heterogenous disease, so far more than 200 forms of cancer, characterized by different molecular features, have been described (Tomczak et al., 2015). This heterogeneity inevitably leads to the requirement of tailored strategies aimed to face the tumors dynamic changes (Tomczak et al., 2015). Indeed, each type of tumor present unique genetic aberrations such as somatic mutations, copy number variations (CNVs), changed gene and protein expression profiles, and diverse epigenetic alterations. Recently, ‘omics approaches and computational science became powerful tools in handling and extracting meaningful

information from large multiomic datasets (Gao et al., 2017; Parsons and Francavilla, 2020). In particular, multi-dimensional data are increasingly being generated in routine care, high throughput techniques and big data analysis methods have allowed deep investigations of the whole genome, epigenome, transcriptome, proteome and metabolome of different types of samples, shedding new light on cancer molecular insights (Vamathevan et al., 2019; Parsons and Francavilla, 2020). In this framework, genomic approaches based on next generation sequencing (NGS) technologies played a pivotal role in redefining breast cancer subtypes (Cancer Genome Atlas Network, 2012), helped to identify genetic alterations as single nucleotide polymorphisms (SNPs) or driver mutations (Nik-Zainal et al., 2016), served as screening for clinical trials patients (Curtis et al., 2012), provided novel markers for breast cancer prognosis (Davalos et al., 2017) and for metastasis detection (Lim et al., 2020). Transcriptome analyses were first enabled by microarray technologies, nevertheless with some limitations only to known genes and cross-hybridization problems (Soon et al., 2013). NGS has thereafter facilitated the global mapping of the transcriptome through RNA-sequencing (RNA-seq), providing a comprehensive profile of cellular phenotypes through the examination of the genes expressed in specific physiological and pathological conditions with very high precision (Chambers et al., 2019). Transcriptomics approaches allowed the classification of breast cancer molecular subtypes in cell lines (Neve et al., 2006) as well as the comparison of normal breast and tumor or primary breast cancers and their metastases expression landscapes (Varešlija et al., 2018), potentially paving roads to tailored individualized therapies. Data generated from patients and sophisticated machine learning are revolutionizing breast health care allowing the extraction of patterns and correlations that leads to ultimately produce valuable insights (Ibnouhsein et al., 2018). For instance, genomic driver alterations, that can be targeted with matched drugs, arise from the sequencing of patient's tumors, in this vein, targeted therapies directed against identified driver events are often successful in inducing tumor regression (Birkbak and McGranahan, 2020). Data are produced in any number of settings: by individual investigators or labs, by charities or philanthropies that host and may curate data, by national projects and by international consortia (Clare and Shaw, 2016).

The Cancer Genome Atlas (TCGA) is a public funded project that aims to collect and uncover the major cancer-causing genomic alterations in order to create a comprehensive “atlas” of the diverse cancer genomic profiles. The Cancer Genome Atlas (TCGA) is a project, initiated in 2005 by the National Cancer Institute that includes over 2.5 petabytes of genomic, epigenomic, transcriptomic, and proteomic data from matched tumors and normal tissues of about 11.000 patients representing 33 diverse human cancer types. TCGA data extended current knowledge

of tumorigenesis, helped improving diagnostic methods, treatment standards, and cancer prevention. Diverse centers, responsible for the advanced bioinformatics data analyses, cooperate in diverse steps, as the collection and the processing of samples as well as high-throughput sequencing, to obtain TCGA well-structured data. Required biospecimens (blood, tissue) from eligible cancer patients are collected by the Diverse Tissue Source Sites (TSSs) and thereafter catalogued, processed, and verified by the Biospecimen Core Resource (BCR), which guarantee the quality and quantity of samples. The clinical data and metadata generated is then submitted to the Data Coordinating Center (DCC) that provide molecular analytes for the Genome Characterization Centers (GCCs) and Genome Sequencing Centers (GSCs) for further genomic characterization and high-throughput sequencing and next it is deposited in the DCC. The Genome Characterization Centers also submit trace files, sequences, and alignment mappings to NCI's Cancer Genomics Hub (CGHub) secure repository. The generated data is made public and easy to manage by the Genome Data Analysis Centers (GDACs) that generate new processing, analysis, and visualization methods. The information provided are entered into public free-access datasets (TCGA Portal, NCBI's Trace Archive, CGHub, UCSC Xena, cBioportal). The breast cancer cohort of TCGA provides integrated information of array-based messenger RNA (mRNA) expression, sequencing-based mRNA expression, DNA methylation, single-nucleotide polymorphisms, array-based microRNA expression, and protein/phosphoprotein expression along with the clinical data of the patients (Cancer Genome Atlas Network, 2012).

The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) is a Canada-United Kingdom project integrating genomic, copy number variations and transcriptomic profiles as well as SNP genotypes of over two thousand primary breast tumors along with long-term clinical follow-up (Curtis et al., 2012). A large body of evidence denotes the fundamental contribution of TCGA and METABRIC datasets in defining new insights into the molecular portraits of breast cancer patients (Shimizu et al., 2019; Craven et al., 2021). Notably, a comprehensive analysis of the TCGA data, carried out by Ciriello and colleagues, profiled 817 breast tumors with the aim to discover peculiar features of invasive lobular carcinomas (ILC) (Ciriello et al., 2015). METABRIC dataset analysis contributed to a definitive framework for understanding how gene copy number aberrations affect gene expression in breast cancer and reveals novel subgroups that should be the target of future investigations (Curtis et al., 2012). Furthermore, a recent functional analysis of dysregulated genes (DEGs) of TCGA breast cancer patients led to the development of a stromal and immune signatures,

toward a better understanding of the potential genes behind the regulation of tumor microenvironment and cells infiltration (Xu et al., 2020).

1.6 Aim of the study

We aimed to analyze the GPER-associated gene expression network as well as its prognostic value in large cohorts of ERα-negative breast cancer patients. In particular, performing in silico analysis we evaluated the association of GPER levels with pro-tumorigenic genes and clinical outcomes in the aforementioned subset of patients. Subsequently, further bioinformatics analyses and *in vitro* studies were carried out in order to establish the involvement of GPER, along with HIF-1α, in mediating the IL-1β/IL1R1-dependent pro-metastatic phenotype in hypoxic TNBC cells and CAFs.

2 Materials and Methods

2.1 Publicly available molecular datasets

Gene expression analyses were performed using the publicly available The Cancer Genome Atlas (TCGA) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) datasets (Curtis et al., 2012; Ciriello et al., 2015). The clinical information of the patients along with the mRNA expression data (RNA Seq V2 RSEM) reported in the Invasive Breast Cancer Cohort of the TCGA project (n. 1247) were downloaded from UCSC Xena (https://xenabrowser.net/). The clinical information and the microarray gene expression data (Log2 transformed intensity values) of the METABRIC cohort (n. 2509) were retrieved from cBioPortal for Cancer Genomics (http://www.cbioportal.org/). Samples of the TCGA cohort were filtered by the "sample type" in order to exclusively obtain the tumor tissues (n. 1101). Thereafter, patients of both TCGA and METABRIC were classified on the basis of the presence or absence, detected by immunohistochemistry, of the estrogen receptor (ER), the progesterone receptor (PR) and HER2. Gene expression and clinical information were also filtered for missing values. The final filtering resulted in 771 patients of TCGA and 1904 patients of METABRIC.

2.2 Correlation analysis

The Pearson correlation coefficients (r-values) between the expression levels of GPER and the other genes of the TCGA (n. 20,530) and METABRIC (n. 24,367) datasets were assessed in ER-negative BC patients using the *cor.test()* function and setting the method as "Pearson" in R Studio (version 3.6.1). The first 1000 most correlated genes of each dataset were intersected with the *intersect()* function in order to obtain the most correlated genes shared by the two datasets. Thereafter, the r-values between the mRNA levels of HIF-1α and the other genes of the TCGA dataset in the TNBC cohort of patients were calculated. The first 250 HIF-1α most correlated genes were selected for the next evaluations. The statistical analyses were performed by using the t-tests, considering significant the coefficients obtained with $p < 0.001$.

2.3 Pathway enrichment analysis

Aiming to cluster the selected genes in pathways, we uploaded the lists obtained on the Database for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation analysis website (https://david.ncifcrf.gov/). We analyzed the genes selecting the functional annotation tool and the option Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, choosing the official gene symbol as "select identifier" and gene list as "list type" in the upload options and selecting a limit species of "Homo sapiens" in the background.

2.4 Gene Set Enrichment Analysis (GSEA)

GSEA was performed using the *gsea()* function of the phenoTest package (https://bioconductor.org/packages/release/bioc/html/phenoTest.html) in R Studio in order to test the association between the predefined groups of genes and a specific phenotype. The gene lists used for the analysis concerning GPER expression, derived from DAVID functional annotation tool, are the CAM pathway (KEGG entry = hsa04514), the ECM-receptor interaction pathway (KEGG entry = hsa04512), and the FA signaling pathway (KEGG entry = hsa04510). The genes belonging to the aforementioned pathways were ranked in accordance with the differential expression within GPER high and low (median expression value as threshold assessment) samples in the ER-negative subgroup of BC patients, verifying if the selected set of genes were enriched at the bottom or the top of the ranked list. Considering the subsequent analyses on HIF-1α and IL-1β, the KEGG "Cytokine-cytokine receptor interaction" pathway (KEGG entry = hsa04060) and "HIF-1 signaling" pathway (KEGG entry = hsa04066) were selected as the reference genes sets. We ranked the genes of the "Cytokine-cytokine receptor interaction" pathway in accordance with the differential expression within HIF-1α high and low (median expression value as threshold assessment) samples, and the "HIF-1 signaling" pathway genes in accordance with the differential expression within IL-1β high and low (median expression value as threshold assessment) samples. The HIF-1α/ IL-1β analyses were performed in the TNBC subgroup of patients, verifying if the selected genes sets were enriched at the bottom or the top of the ranked lists. For all the aforementioned analyses we calculated the enrichment score (ES) that reflects the degree to which a set of genes is overrepresented at the extremes of the entire ranked list. The score was calculated by walking down a list of genes ranked by their correlation with the selected phenotype (high or low GPER/HIF-1α and IL-1β levels), increasing a running-sum statistic when a gene of the gene set is encountered (each vertical line underneath the enrichment plot) and decreasing it when a gene that isn't in the gene set is encountered. The magnitude of the increment depends on the correlation of one gene with

the phenotype. In these analyses, 20,000 simulations were used (B = 20,000). $p < 0.05$ was considered significant.

2.5 Survival Analysis

Comprehensive survival analysis was conducted using TCGA gene expression data of GPER along with the disease-free interval (DFI) information. Patients were filtered for missing values, and the ER and the HER2 statuses were used to divide the population. The survivALL package was employed to examine Cox proportional hazards for all possible points-of-separation (low-high cut-points), selecting the cut-point with the lowest p-value (Pearce et al., 2018) and separating the patients into high (n. 27) and low (n. 93) GPER expression levels. The Kaplan–Meier survival curves were generated using the survival and the survminer R packages.

2.6 Reagents

The ROS scavenger N-acetyl-L-cysteine (NAC) (used at a 300 μM concentration) and the proteasome inhibitor MG132 (used at a 10 μM concentration) were purchased from Merck Life Science (Milan, Italy). PD98059 (PD) and LY294,002 (LY) (both used at a 1 μM concentration) were obtained from Calbiochem (Milan, Italy). All compounds were dissolved in DMSO, except NAC that was solubilized in water. Recombinant human IL-1β (used at a 10 ng/mL concentration) was purchased from Thermo Fisher Scientific (Life Technologies Italia, Monza, Italy) and solubilized in PBS with 1% BSA. The IL1R1 antagonist (IL1R1a) human recombinant protein (used at a 50 ng/mL concentration) was purchased from Thermo Fisher Scientific and solubilized in 20mM TBS, pH 8, with 50% glycerol. Anti-IL-1β neutralizing antibody (MAB601) was purchased from R&D Systems (Bio- Techne, Milano, Italy).

2.7 Cell cultures

The TNBC MDA-MB 231 breast cancer cells were provided by ATCC (Manassas, VA, USA), used less than 6 months after resuscitation, routinely tested and authenticated according to the ATCC suggestions. MDA-MB 231 cells were maintained in DMEM/F12 (Dulbecco's modified Eagle's medium) with phenol red, supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/ streptomycin (Thermo Fisher Scientific). CAFs were isolated, cultured and characterized as previously described (Cirillo et al., 2019) from 10 invasive mammary ductal carcinomas and pooled for the subsequent studies. Briefly, specimens were cut into 1–2mm diameter pieces, placed in a digestion solution (400 IU collagenase, 100 IU hyaluronidase, 10% FBS, antibiotics and antimycotics) (Thermo Fisher Scientific) and incubated overnight at 37

°C. Cells were then separated by differential centrifugation at 90×g for 2 min. The supernatant containing fibroblasts were centrifuged at 485×g for 8 min, the pellet obtained was suspended in fibroblasts growth medium (Medium 199 and Ham's F12 mixed 1:1 and supplemented with 10% FBS and 1% penicillin) (Thermo Fisher Scientific) and cultured at 37 °C, 5% CO_2. CAFs were then expanded into 10-cm Petri dishes and stored as cells passaged for three population doublings within total 7 to 10 days after tissue dissociation. Primary cell cultures of fibroblasts were characterized by immunofluorescence with human anti-vimentin (V9; 1:500) and human anti-cytokeratin 14 (LL001) (Santa Cruz Biotechnology, DBA, Milan, Italy; 1:250). FAPα antibody (H-56; Santa Cruz Biotechnology, DBA, Milan, Italy; 1:500) was used to assess fibroblast activation (data not shown). We used CAFs passaged for up to 10 population doublings for the experiments, to minimize clonal selection and culture stress, which could occur during extended tissue culture. All cell lines were grown in a 37 °C incubator with 5% CO_2 and switched to medium without serum and phenol red the day before treatments to be processed for immunoblot and RT-PCR assays.

2.8 Gene expression studies and PCR arrays

Total RNA was extracted, and cDNA was synthesized by reverse transcription as previously described (Lappano et al., 2012 B). The expression of selected genes was quantified by real-time PCR using platform Quant Studio7 Flex Real-Time PCR System (Thermo Fisher Scientific). Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems) and are as follows: 5′-ACCTATGACCTGCTTGGTGC-3′ (HIF-1α forward) and 5′-GGCTGTGTCGACTGAGGAAA-3′ (HIF-1α reverse); 5′-TTAAAGCCCGCCTGACAGA-3′ (IL-1β forward) and 5′-GCGAATGACAGAGGGTTTCTTAG-3′ (IL-1β reverse); 5′-TTACAGAGGGAAAACGACACCT-3′ (GPER forward) and 5′-GTGGGTCTTCCTCAGAAGGG-3′ (GPER reverse); 5′-AGTCCCTGAGCATCTACGGT-3′ (COX2 forward) and 5′-CATCATCAGACCAGGCACCA-3′ (COX2 reverse); 5′-AAGCCACCCCACTTCTCTCTAA-3′ (ACTB forward) and 5′-CACCTCCCCTGTGTGGACTT-3′ (ACTB reverse). Assays were performed in triplicate and the results were normalized for actin beta (ACTB) expression and then calculated as fold induction of RNA expression. PCR arrays were performed using a TaqMan™ Human Tumor Metastasis Array (Thermo Fisher Scientific) according to the manufacturer's instructions. The amplification reaction and the results analysis were carried out using platform Quant Studio7 Flex Real-Time PCR System (Thermo Fisher Scientific).

2.9 Gene silencing experiments and plasmids

Cells were transfected using X-treme GENE 9 DNA Transfection Reagent (Roche Diagnostics, Merck Life Science) for 24 h before treatments with a control vector and a specific shRNA sequence or antisense vector for each target gene. The short hairpin (sh) RNA constructs to knock down the expression of HIF-1α and the control shRNA construct were purchased form SABioscience Corporation. Antisense vector for GPER (AsGPER), which was generated by cloning of the entire open reading frame of the receptor cDNA in the reverse orientation in pcDNA3.1 Hygro (−), was a kind gift from Eric R Prossnitz (University of New Mexico Health Science Center, Albuquerque, USA). The plasmid DN/c-fos, which encodes for c-fos mutant that heterodimerizes with c-fos dimerization partners but does not allow DNA binding, was a kind gift from Dr. C. Vinson (NIH, Bethesda, MD, USA).

2.10 Chromatin Immunoprecipitation (ChIP) assay

Cells were grown in 10-cm dishes, exposed to treatments for 16 h, and then cross-linked with 1% formaldehyde and sonicated. Supernatants were immuno-cleared with salmon DNA/protein A-agarose (Merck Life Science) and immunoprecipitated with anti-HIF-1α or anti-GPER antibody or nonspecific IgG. Pellets were washed, eluted with a buffer consisting of 1%SDS and 0.1 mol/L NaHCO3, and digested with proteinase K. DNA was obtained by phenol/chloroform extractions and precipitated with ethanol. The yield of target region DNA in each sample after ChIP was analyzed by real-time PCR. The primers used to amplify a region containing a HRE site located into the GPER promoter sequence were: 5′-TGCAGCACTTCAAAACAATAACC − 3′ (Fw) and 5′-GGGTTTGAGTTGTTTTTCCTTTGG-3′ (Rv); the primers used to amplify a region containing a HRE site located into the IL-1β promoter sequence were: 5′-ACAGACAGGGAGGGCTATTG-3′ (Fw) and 5′-GGGCAAGGAGTAGCAAACTA-3′ (Rv). Data were normalized to the input for the immunoprecipitation and the results were reported as fold changes respect to nonspecific IgG.

2.11 Western blot analysis

Cells were grown in 10-cm dishes, exposed to treatments, and then lysed as previously described (Santolla et al., 2018). Equal amounts of whole-protein extract were resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences, Sigma-Adrich, Milan, Italy), which were probed with primary antibodies (1:1000) against HIF-1α and IL-1β (R&D Systems, Bio-Techne, Milano, Italy), GPER (AB137479)

(Abcam, DBA, Milan, Italy), phosphorylated ERK1/2 (E-4), ERK2 (C-14), p-1/2/3 (Ser 473)-R, /1/2/3 (H-136), c-fos (E-8) and β-actin (AC-15; 1:4000) (Santa Cruz Biotechnology, DBA, Milan, Italy) and then revealed using the chemiluminescent substrate for western blotting Westar Nova 2.0 (Cyanagen, Biogenerica, Catania, Italy). For nuclear extracts, cells were lysed using 300 µl of cytosolic buffer (50mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 1.5mM $MgCl_2$, 1mM EGTA, pH 7.5, 10% glycerol) with protease inhibitors (1.7 mg/ml aprotinin, 1 mg/ml leupeptin, 200 mmol/liter phenylmethylsulfonyl fluoride, 200 mmol/liter sodium orthovanadate and 100 mmol/liter sodium fluoride). Following centrifugation (14,000 g, 4 °C, 10 min), the supernatant was referred to as cytoplasmic fraction and the pellet containing nuclei was resuspended in high salt buffer (20mM HEPES pH 7.9, 25% [v:v] glycerol, 420 mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA and protease inhibitors). For the extraction of nuclear proteins, the obtained solution was vortexed thoroughly, incubated overnight with agitation and centrifugated at 14000 g, 4 °C for 10 min. Equal amounts of the collected supernatant, which represent the nuclear fraction, were then run on 10% SDS-PAGE and western blot analysis was performed as described above. The purity of the nuclear fraction was confirmed by immunoblotting with primary antibodies against β-actin (AC-15; 1:4000) and anti- LMNB/Lamin (M-20; 1:2000) (Santa Cruz Biotechnology, DBA, Milan, Italy).

2.12 Co-immunoprecipitation assay

After exposure to treatments, cells were washed and lysed using 500 µl RIPA buffer with protease inhibitors (1.7 mg/ml aprotinin, 1 mg/ml leupeptin, 200 mmol/liter phenylmethylsulfonyl fluoride, 200 mmol/liter sodium orthovanadate and 100 mmol/liter sodium fluoride). Samples were then centrifuged at 13,000 rpm for 10 min and protein concentrations were determined using Coomassie (Bradford) protein assay. Proteins (200 µg) were then incubated for 2 h with 900 µl of immunoprecipitation buffer with inhibitors, 2 µg of anti-c-fos or anti- GPER antibodies and 20 µl of Protein A/G agarose immunoprecipitation reagent (Santa Cruz Biotechnology, DBA, Milan, Italy). Samples were then centrifuged at 13,000 rpm for 5 min at 4 °C to pellet beads. Pellets were washed four times with 500 µl of PBS and centrifuged at 13,000 rpm for 5 min at 4 °C. Supernatants were collected, resuspended in 20 µl RIPA buffer with protease inhibitors, 2X SDS sample buffer and heated to 95 °C for 5 min. Samples were then run on 10% SDS-PAGE, transferred to nitrocellulose and probed with primary antibodies. Western blot analysis and ECL detection were performed as described above.

2.13 Immunofluorescence microscopy

Cells were grown on a cover slip, serum deprived for 18 h and then exposed to treatments for 16 h. Next, cells were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100, washed 3 times with PBS and incubated at 4 °C overnight with a primary antibody (1:250) against GPER (AB137479) (Abcam, DBA, Milan, Italy) or Phospho-Myosin Light Chain 2 (Ser19) (p-MLC) (Cell Signaling, Euroclone, Milan, Italy). After incubation, the slides were extensively washed with PBS, probed with alexa Fluor 594 goat anti-rabbit IgG (1:300, Thermo Fisher Scientific) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (1:1000; Sigma-Aldrich). Then, the images were obtained using the Cytation 3 Cell Imaging Multimode reader (BioTek, AHSI, Milan Italy) and analyzed by the Gen5 software (BioTek, AHSI, Milan Italy).

2.14 Phalloidin staining

Cells were exposed to treatments for 16 h, washed twice with PBS, fixed in 4% paraformaldehyde in PBS for 10 min, washed briefly with PBS, then incubated with Phalloidin-Fluorescent Conjugate (Santa Cruz Biotechnology). The images were obtained using the Cytation 3 Cell Imaging Multimode reader (BioTek, AHSI, Milan Italy) and analyzed by the Gen5 software (BioTek, AHSI, Milan Italy).

2.15 Enzyme-linked Immunosorbent assay

The concentrations of IL-1β in supernatants from hypoxia-treated MDA-MB-231 cells were measured using human IL-1β ELISA Kit (Thermo Scientific, Monza Italy), according to the manufacturer's instructions. The plates were read at 450 nm on a Microplate Spectrophotometer Epoch™ (BioTek, AHSI, Milan Italy).

2.16 DCFDA fluorescence measurement of ROS

The non-fluorescent 2′,7′-dichlorofluorescin diacetate (DCFDA) probe was used to evaluate intracellular ROS production. Briefly, cells were incubated with 10 μM DCFDA (Sigma-Aldrich) at 37 °C for 30 min, washed with PBS, and then exposed to treatments for 15 min, as indicated. Cells were then washed with PBS, and the images were obtained using the Cytation 3 Cell Imaging Multimode reader (BioTek, AHSI, Milan Italy) and analyzed by the Gen5 software (BioTek, AHSI, Milan Italy).

2.17 Conditioned medium

MDA-MB-231 cells were cultured under normal or low oxygen tension (2%) for 16 h. Thereafter, the supernatants were collected, centrifuged at 3500 rpm for 5 min to remove cell debris and used as conditioned medium (CM) in the appropriate experiments.

2.18 Cell spreading assay

Cells were treated for 16 h, trypsinized and seeded onto fibronectin (5 μg/ml) coated 96-well plate at a density of 2×10^5 cells/ml and incubated at 37 °C in a humidified incubator containing 5% CO_2. Phase-contrast images were captured after 15 min and 60 min. As previously reported (Pijuan et al., 2019), round bright cells were considered unspread, whereas spread cells were defined as those cells that had lost their phase-bright appearance and had readily distinguishable nucleus and cytoplasm and quantified by counting six randomly selected fields in each well under a phase-contrast microscope.

2.19 Invasion assay

Transwell 8 μm polycarbonate membrane (Costar, Sigma-Aldrich, Milan, Italy) was used to evaluate in vitro cell invasion. 5×10^4 cells in 300 μL serum-free medium were seeded in the upper chamber, coated with Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Biogenerica, Catania, Italy) (diluted with serum-free medium at a ratio of 1:3). For invasion assays in MDA-MB-231 cells, complete medium was added to the bottom chambers in the presence of treatments where required, then cells were cultured under normoxia or hypoxia for 16 h. For invasion assays in CAFs, CM collected from MDA-MB-231 cells previously exposed to hypoxia for 16 h was added to the bottom chambers in the presence of treatments, where required. Cells on the upper surface of the membrane were then removed by wiping with Q-tip, and invaded cells were fixed with 100% methanol, stained with Giemsa (Sigma-Aldrich, Milan, Italy), photographed using Cytation 3 Cell Imaging Multimode Reader (BioTek, AHSI, Milan Italy) and counted using the WCIF ImageJ software.

2.20 Spheroid formation assay

For spheroid generation, 100 μL/well of MDA-MB 231 cell suspensions (1×10^4) were dispensed into 2% agar-coated 24-well plates. Three days after seeding, tumor spheroids (a single spheroid per well) were exposed to treatments and a 50% medium and treatment replenishment was performed every 2 days. Images were obtained on day 20 using a conventional inverted microscope, thereafter cell number per spheroid was determined by

trypsinizing three different spheroids, mixing the cell suspension with trypan blue and counting the number of viable cells. The total number of cells obtained was divided by the number of trypsinized spheroids.

2.21 Statistical analysis

The statistical analysis was performed using ANOVA followed by Newman-Keuls' test to determine differences in means. The bioinformatics analyses, including t-tests, box plots scatter plots, were performed using the R tidyverse package (https://www.tidyverse.org/packages/). Heatmaps were performed with the R pheatmap package. Gene expression values of both TCGA and METABRIC datasets were normalized by calculating their respective normalized z-scores. $p < 0.05$ and $p < 0.01$ were considered statistically significant.

3 Results

3.1 GPER expression correlates with pro-metastatic pathways in ER-negative breast cancer patients

GPER and ER are considered to act as estrogen receptors on the basis of their genetic, biochemical, biological and pharmacological properties. Indeed, these receptors possess estrogen binding characteristics and employ different intracellular signaling mechanisms (Filardo, 2018). Considering that GPER-mediated signaling has been involved in breast cancer development and aggressiveness (Lappano et al., 2014; Marjon et al., 2014; Filardo, 2018), we aimed to deepen the role of this receptor in breast malignancies lacking ER, which are characterized by a worse prognosis (Eroles et al., 2012; Bray et al., 2018). We began our investigations by correlating the expression levels of GPER and the genes of TCGA and METABRIC datasets in ER-negative breast tumor patients. Next, we ranked the genes by their Pearson correlation coefficient, listing the first 1000 genes positively correlated with GPER either in TCGA or METABRIC cohorts. Hence, we obtained 277 shared genes between the two datasets, as shown in figure 1a. In order to investigate the biological significance of the aforementioned 277 genes, we then performed KEGG (The Kyoto Encyclopedia of Genes and Genomes) pathway analysis using the online Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov), founding that the 277 genes were enriched in a number of pathways schematically reported in figure 1b.

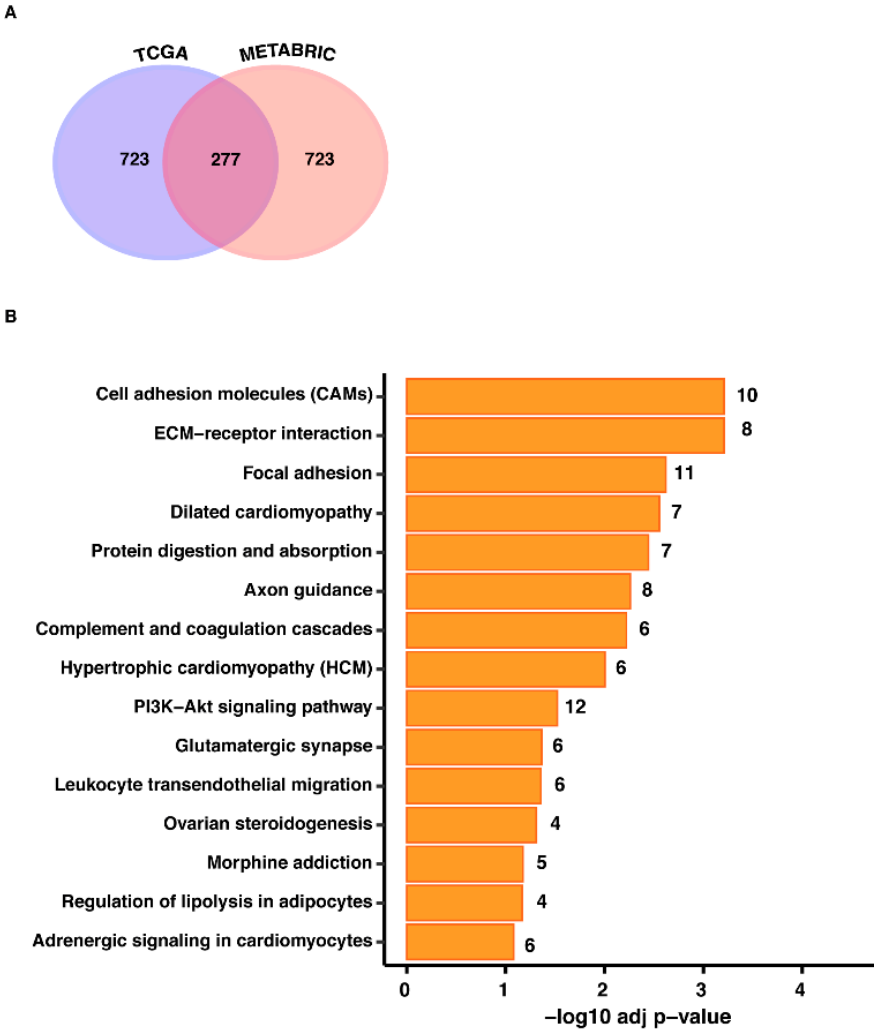

Figure 1. (a) Intersection of the top 1000 G protein-coupled estrogen receptor (GPER) correlated genes in estrogen receptor (ER)-negative breast cancer patients querying The Cancer Genome Atlas (TCGA) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) datasets. (b) GPER expression is correlated with pro-metastatic pathways in ER-negative breast cancer samples, as evaluated by The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the 277 genes shared by the TCGA and METABRIC datasets and their positive correlation with GPER in ER-negative breast cancer patients. The −log10 adjusted values are displayed along the x-axis, while the different KEGG pathways are shown along the y-axis. The number of the genes included in the identified pathways is plotted on the right of each bar.

Notably, transduction pathways that characterize aggressive features of cancer cells, such as CAM, ECM-receptor interaction and FA appeared to be the most significantly correlated with GPER expression levels, as indicated by their respective -log10 adj p-value. Thereafter, we performed GSEA in order to explore the expression profile of the genes belonging to the CAMs, ECM-receptor interaction, and the FA pathways in high and low GPER phenotypes of the TCGA and METABRIC cohorts of ER-negative breast cancer patients. It is worth noting that

the genes included in these signaling pathways were found enriched in the subgroup of patients displaying high GPER expression levels (Fig. 2 a-f).

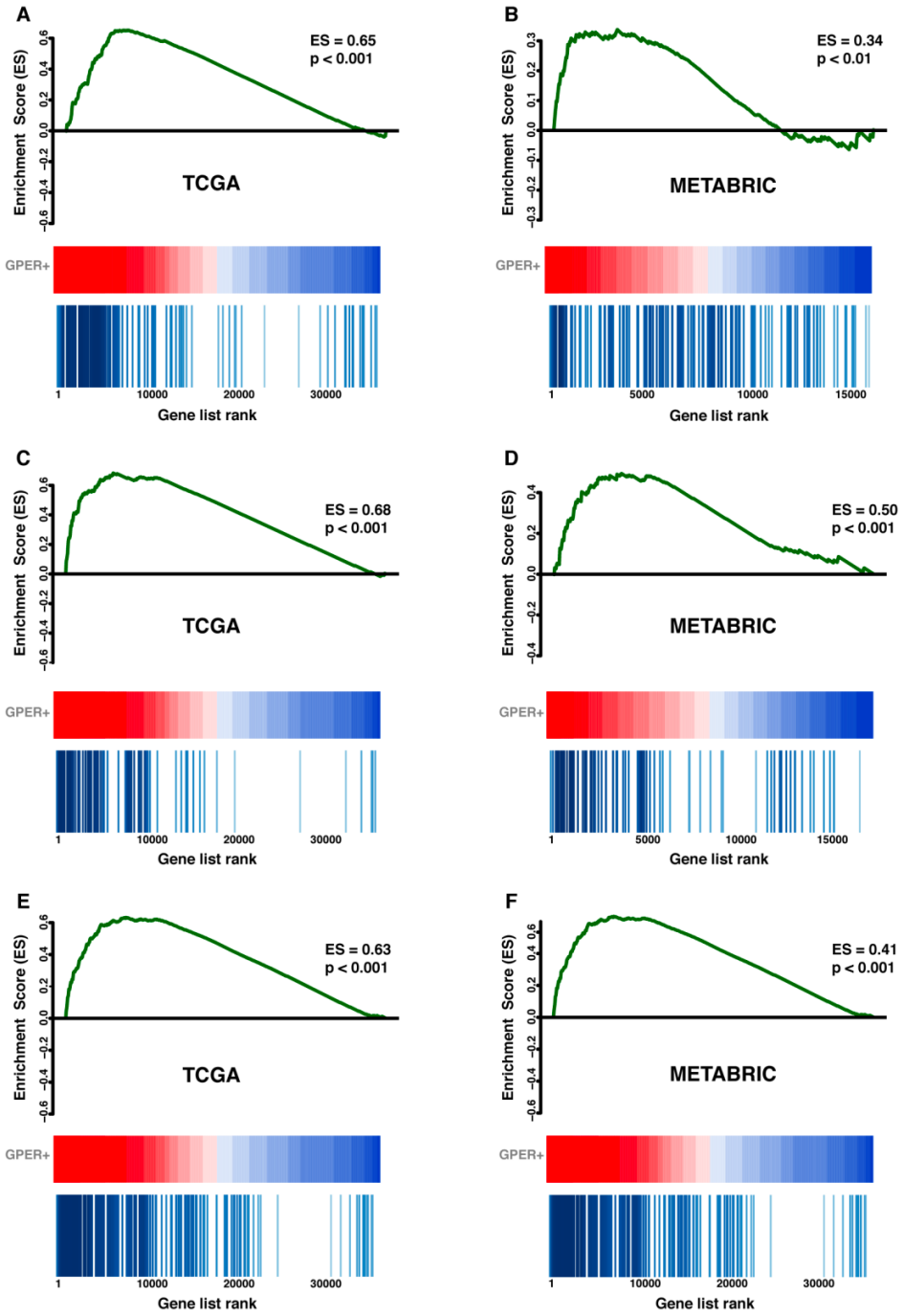

Figure 2. Enrichment plots of KEGG CAMs (A, B) ECM-receptor interaction (C, D) and FA pathway genes (E, F) by GSEA in ER-negative BC patients of TCGA and METABRIC datasets. Enrichment scores (ES) and relative p-values are plotted. A positive enrichment score (ES) indicates an enrichment of the selected gene set at the top of the ranked list. The score is calculated by walking down a list of genes ranked by their correlation with the selected phenotype (high or low GPER levels), increasing a running-sum statistic when a gene in that gene set is encountered (each black vertical line underneath the enrichment plot) and decreasing it when a gene that isn't in the gene set is encountered.

These results are in accordance with previous findings showing the engagement of FAK by GPER signaling toward the migration and invasion of TNBC cells (Rigiracciolo et al., 2019). Moreover, we assessed the profile of the most GPER-correlated genes shared by the TCGA and METABRIC datasets. In this regard, we identified pro-tumorigenic genes belonging to the CAMs pathway like cell adhesion molecule 3 (CADM3), CD34, cadherin 5 (CDH5), claudin 5 (CLDN5), endothelial cell-selective adhesion molecule (ESAM) and the junctional adhesion molecules JAM2 and JAM3 (Fig. 3a). Additionally, we evidenced further pro-tumorigenic genes belonging to the ECM-receptor interaction and FA pathways including for instance caveolin 1 (CAV1), alpha(α)1(VI) and the alpha(α)2(VI) chain of type VI collagen (COL6A1 and COL6A2, respectively), insulin like growth factor 1 (IGF1), the integrin subunits alpha 5 and alpha 7 (ITGA5 and ITGA7, respectively), the laminin subunit beta 2 (LAMB2), PDGFRβ, PGF and von Willebrand factor (VWF) (Fig. 3b).

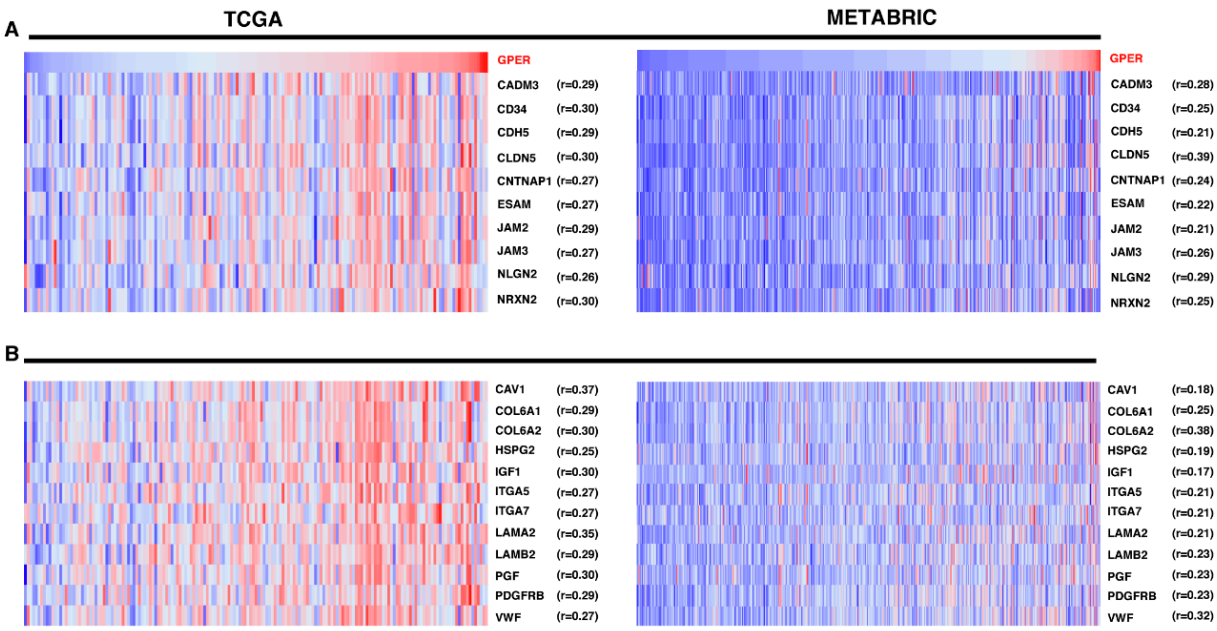

Figure 3. GPER correlates with the expression of cell adhesion molecules (CAMs), extracellular matrix (ECM)-receptor interaction, and focal adhesion (FA) pathway genes as determined querying the TCGA and the METABRIC datasets**.** The heatmaps, ranked from left to right, show the most GPER correlated genes belonging to the CAMs pathway (a) and to the ECM-receptor interaction and FA molecular pathways (b) in ER-negative breast tumor samples. Colors are z-score normalized values, red indicates high and blue indicates low.

3.2 High GPER expression levels are associated with a worse outcome in ER-negative breast cancer patients

Previous immunohistochemical studies on breast tumors have shown that the expression levels of GPER are associated with increased tumor size, distant metastasis, recurrence and poor prognosis of patients (Filardo et al., 2006; Liu et al., 2009; Ye et al., 2020). Conversely, Martin and co-workers showed that low expression levels of GPER are associated with aggressive features in a large cohort of primary invasive breast cancer patients (Martin et al., 2018).

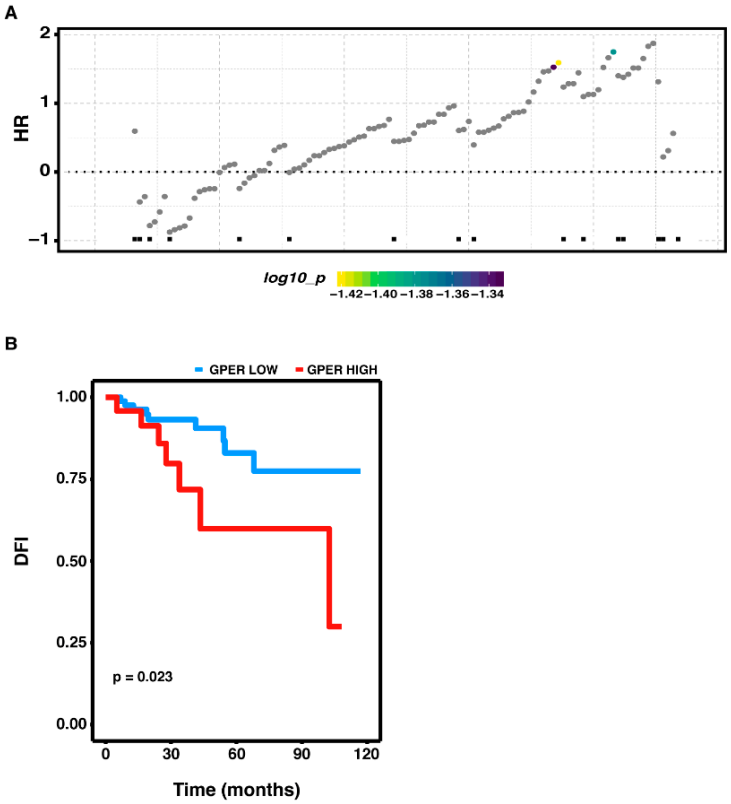

Figure 4. Clinical outcome on the basis of GPER expression in ER-negative and HER2-negative breast cancer patients. (a) ER-negative and HER2-negative breast cancer patients of the TCGA cohort divided into high and low expression levels of GPER on the basis of the established cut-point. The color bar gradient stands for the range of the most significant points-of-separation of the population (low-high significance = blue-yellow gradient) based on GPER expression and survival of each patient. The x-axis represents the patients ordered by the increasing expression of GPER. (b) Correlation between GPER expression and disease-free interval (DFI) of ER-negative breast cancer patients in the TCGA cohort.

On these bases, we aimed to evaluate whether GPER expression would be a prognostic indicator for the outcome of the aggressive breast cancer subtype lacking the expression of both ER and HER2. Taking advantage of the disease-free interval (DFI) data, a significant cut-point was predictable only from the TCGA cohort. Ranking the gene expression data according to the low

and high GPER levels, all possible points-of-separation and their significance were reported in the survivALL plot by which the most significant cut-point was calculated (Fig. 4a). Afterward, the Kaplan-Meier survival curve revealed that a worse DFI characterizes breast cancer patients exhibiting high GPER levels (Fig. 4b).

3.3 The expression levels of HIF-1α and IL-1β are correlated in TNBC

A large body of evidence indicate that malignant cells may adapt to hypoxia mainly through the action of the HIF-1α (Semenza, 2012 A; Semenza, 2012 B), which has been largely involved in tumor growth and vascularization, stromal cell recruitment, remodeling, premetastatic niche formation, invasion and metastasis (Semenza, 2016). In this regard, high expression levels of HIF-1α have been associated with the aggressiveness and poor clinical outcomes of breast cancer (Semenza, 2012 A; Semenza, 2012 B). Likewise, the over-expression of HIF-1α and the consequent hyperactivation of its target genes have been indicated as key drivers in TNBC (Cancer Genome Atlas Network, 2012; Samanta et al., 2014). Hence, we evaluated the gene signature associated with HIF-1α in TNBC patients characterized by a poor prognosis (Waks and Winer, 2019). Exploring the TCGA cohort of TNBC patients, the 250 genes mostly correlated with HIF-1α were first ranked by their Pearson correlation coefficient. Aiming to explore the biological implication of these genes, KEGG pathway analysis was then performed using DAVID. The top 250 HIF-1α correlated genes were enriched in a set of pathways, as schematically reported in figure 5a. The "Cytokine-cytokine receptor interaction" transduction pathway, which includes the IL1B, IL1A, IL1R, IL6, IL6ST, IL7R, IL1RAP, INHBA, OSMR and TGFBR2 genes that are strongly associated with breast cancer progression (Jin et al., 2018; Weng et al., 2019; Tulotta and Ottewell, 2018; Qin et al., 2018; Lei et al., 2020; Nagaraja et al., 2017; Salamanna et al., 2019), was found significantly correlated with HIF-1α. Next, we performed GSEA in order to investigate the "Cytokine-cytokine receptor interaction" expression profile in accordance with the high and low HIF-1α phenotypes of both TCGA and METABRIC cohorts of TNBC patients. Of note, the genes included in the "Cytokine-cytokine receptor interaction" pathway were found enriched in the subgroup of patients showing high HIF-1α levels (Fig. 5b). As IL-1β may have a role in breast cancer development and metastasis (Tulotta and Ottewell, 2018) and may contribute to the migratory phenotype of TNBC cells through the action of HIF-1α (Naldini et al., 2010; Filippi et al., 2015) we sought to assess whether IL-1β could be involved in the activation of a hypoxia-related gene signature in TNBC patients. Hence, we ascertained that the genes included in the "HIF-1 signaling pathway" of the

KEGG database are significantly enriched in the TNBC cohorts displaying high IL-1β expression of both TCGA and METABRIC datasets (Fig. 5c).

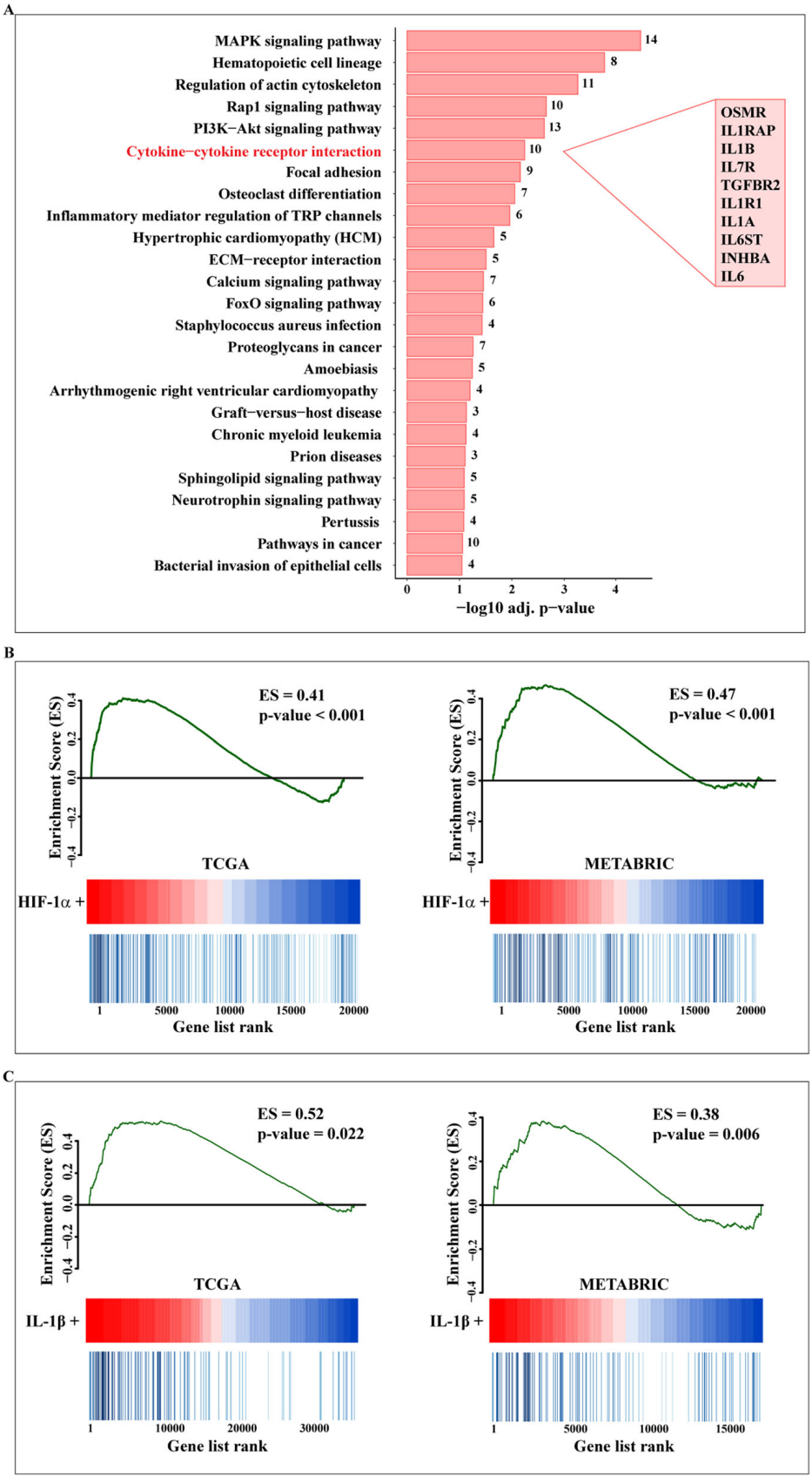

Figure 5. (a) The "Cytokine-cytokine receptor interaction" signaling is a prominent transduction pathway associated with the expression of HIF-1α in TNBC patients. The top 250 genes correlated with HIF-1α in the TCGA dataset were clustered using the KEGG pathway enrichment analysis. The x-axis and the y-axis indicate respectively the −log10-adjusted p-value and the different KEGG pathways. The number of the genes represented in the identified pathways is plotted on the right of each bar. The genes belonging to the "Cytokine-cytokine receptor interaction" pathway (listed in the pink box) were ordered by their HIF-1α correlation coefficients (from high to low). (b) GSEA confirms an enrichment of the genes belonging to the "Cytokine-cytokine receptor interaction" pathway in the TNBC patients with high HIF-1α levels, as indicated. The patients of the TCGA and METABRIC datasets were ranked in accordance with the differential HIF-1α expression levels. (c) GSEA reveals an enrichment of the "HIF-1 signaling" genes in the TNBC patients with high IL-1β expression. Patients of the TCGA and METABRIC datasets were ranked in accordance with the differential IL-1β expression levels. Enrichment scores (ES) and relative p-values are plotted.

In line with these findings, we found in TNBC patients of both datasets a positive correlation between IL-1β and HIF-1α expression (Fig. 6a). Furthermore, we assessed that the levels of IL-1β are significantly higher in TNBC compared to non-TNBC (Fig. 6b). Overall, the aforementioned data suggest that IL-1β may be engaged by HIF-1α toward the development of the aggressive TNBC.

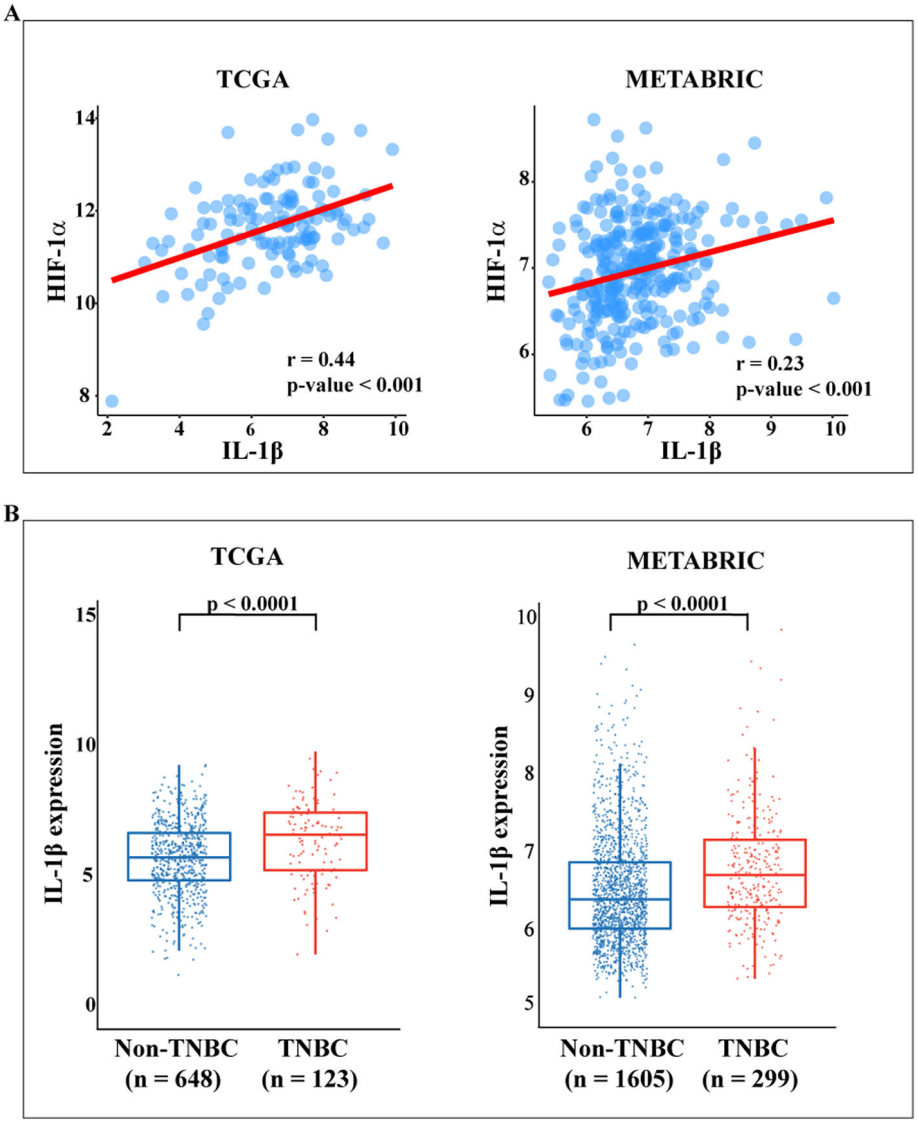

Figure 6. (a) Scatter plots depicting the correlation between HIF-1α expression and IL-1β levels in TNBC patients of the TCGA and METABRIC cohorts. The Pearson correlation coefficients (r) and the relative p-values are shown in each panel. (b) Box plots showing the differential IL-1β expression levels in non-TNBC and TNBC patients, as found in the TCGA and METABRIC datasets. The number of patients and p-values are reported in each panel.

3.4 HIF-1α and GPER cooperate toward the regulation of IL-1β upon hypoxic conditions

On the basis of the aforementioned findings and in order to provide novel insights on the regulation of IL-1β by hypoxia in TNBC, we ascertained that a low oxygen tension (2% O_2, thereafter mentioned as hypoxia) up-regulates the mRNA (Fig. 7a) and protein (Fig. 7b) levels of IL-1β in MDA-MB-231 cells, which are a well-recognized model system of TNBC. Upon hypoxic conditions, the mRNA levels of HIF-1α did not show any modification (Fig. 7a) whereas an accumulation of the protein was observed (Fig. 7b), in accordance to the known ability of hypoxia to decrease the HIF-1α ubiquitination and degradation (Semenza, 2012 A). Then, ELISA confirmed an increased IL-1β secretion in the medium of MDA-MB-231 cells

exposed to hypoxia (Fig. 7c). Remarkably, the silencing of HIF-1α abrogated the expression of IL-1β driven by hypoxia, suggesting the involvement of HIF-1α in the up-regulation of IL-1β in MDA-MB-231 cells under hypoxia (Fig. 7d).

Previous studies have demonstrated that GPER may contribute to HIF-1α action in breast tumor growth and angiogenesis prompted by hypoxia (Recchia et al., 2011; De Francesco et al., 2013; De Francesco et al., 2014; Rigiracciolo et al., 2015; De Francesco et al., 2017). In this framework, we assessed in MDA-MB-231 cells that hypoxia up-regulates the mRNA (Fig. 7e) and protein (Fig. 7f) levels of GPER in a time-dependent manner. By ChIP assay, we next ascertained the recruitment of HIF-1α to the GPER promoter region in MDA-MB-231 cells exposed to hypoxia (Fig. 7g). Accordingly, the silencing of HIF-1α prevented the protein induction of GPER upon a 16 h exposure to hypoxic conditions (Fig. 7h), indicating that HIF-1α is required for the transcription of GPER in MDA-MB-231 cells exposed to hypoxia. Next, the silencing of GPER did not alter the hypoxia-induced HIF-1α levels, while it abolished IL-1β induction (Fig. 7i). Overall, these findings suggest the involvement of HIF-1α in the hypoxic up-regulation of GPER, which in turn cooperates with HIF-1α toward the regulation of IL-1β.

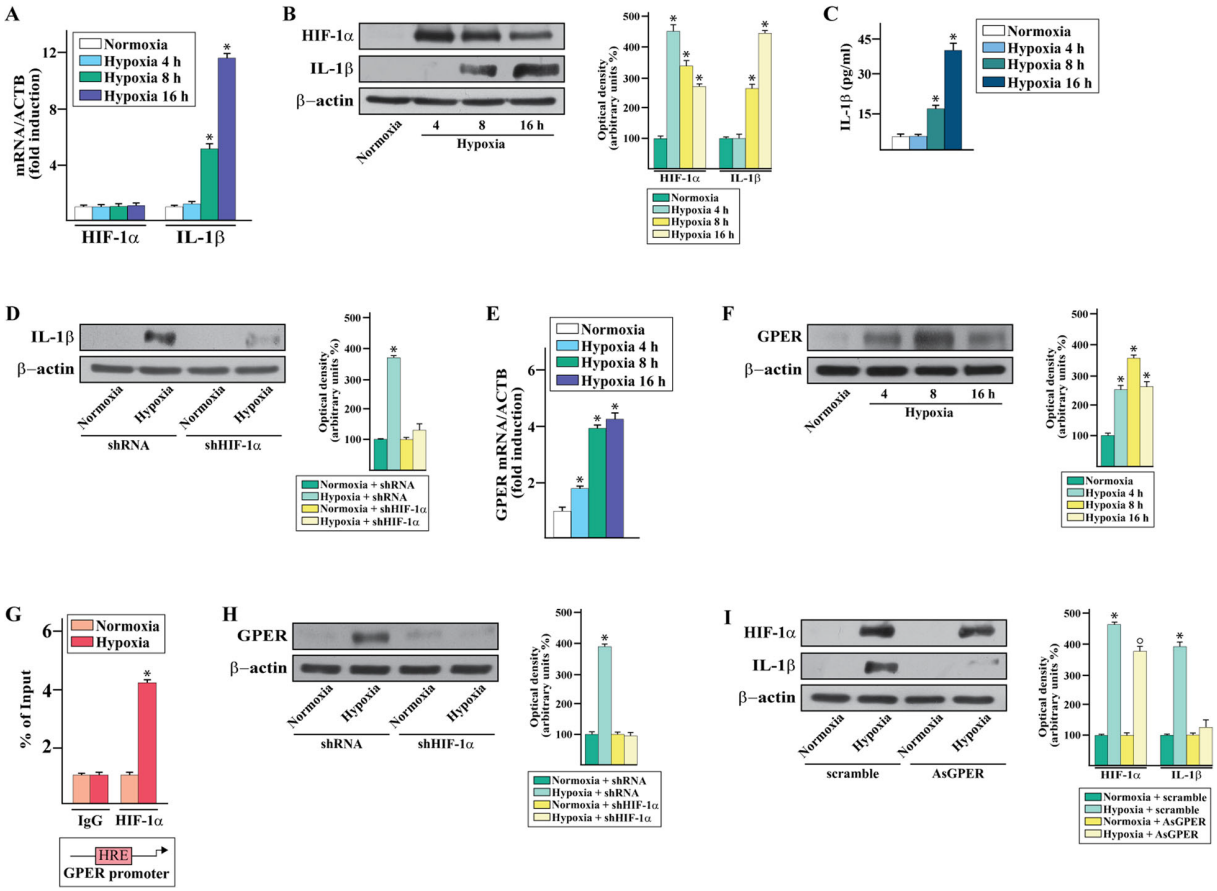

Figure 7. The HIF-1α/GPER signaling is involved in the expression of IL-1β induced by hypoxia. mRNA (a) and protein (b) expression of HIF-1α and IL-1β evaluated respectively by real-time PCR and immunoblotting in MDA-MB-231 breast cancer cells cultured upon normoxia or hypoxia (2% O_2). In RNA experiments, values are normalized to the actin beta (ACTB) expression and shown as fold changes of mRNA expression induced by hypoxia compared to normoxic cells. (c) IL-1β levels evaluated by ELISA in the supernatants collected from MDA-MB-231 cells cultured upon normoxia or hypoxia. (d) The up-regulation of IL-1β observed in MDA-MB-231 cells cultured upon hypoxia is no longer evident silencing HIF-1α. mRNA (e) and protein (f) expression of GPER evaluated in MDA-MB-231 cells cultured upon normoxia or hypoxia, as evaluated by real-time PCR and immunoblotting, respectively. (g) Recruitment of HIF-1α to the HRE site located within the GPER promoter sequence in MDA-MB-231 cells cultured upon hypoxia. In control samples nonspecific IgGs were used instead of the primary antibody. The amplified sequences were evaluated by real-time PCR. (h) The up-regulation of GPER observed in MDA-MB-231 cells exposed to hypoxia was abrogated silencing HIF-1α. (i) Immunoblots of HIF-1α and IL-1β from GPER-silenced MDA-MB-231 cells exposed to hypoxia. Side panels show densitometric analysis of the blots normalized to β-actin. Values represent the mean ± SD of three independent experiments performed in triplicate. (*) and (○) indicate $p < 0.05$ for cells cultured under normoxia versus cells cultured under hypoxia.

Then, we sought to evaluate the transduction mechanism mediating the aforementioned responses. We assessed that a short exposure of MDA-MB-231 cells to hypoxia does increase the levels of reactive oxygen species (ROS) (Fig. 8a), which are required for the stabilization of HIF-1α and the subsequent transduction of the hypoxia-mediated signaling (Bell et al., 2007). In the aforementioned experimental conditions, we next ascertained that the activation of two main transduction regulators of HIF-1α, namely ERK1/2 and AKT (Sutton et al., 2007; Yang et al., 2009), is prevented in the presence of the ROS scavenger NAC (Fig. 8b). As c-fos expression is a suitable molecular sensor of both GPER and HIF-1α transduction signaling (Maggiolini et al., 2004; De Francesco et al., 2014; Rigiracciolo et al., 2019), we also determined that the induction of c-fos protein levels observed upon a 4 h exposure to hypoxia is no longer evident in the presence of the MEK inhibitor PD, the PI3K inhibitor LY (Fig. 8c) and NAC (Fig. 8d). Likewise, the increase of HIF-1α, GPER and IL-1β protein levels upon a 16 h exposure to hypoxia using either NAC (Fig. 8e) or PD and LY (Fig. 8f), suggested that the ROS activity triggers the ERK1/2//c-fos transduction signaling toward the hypoxic regulation of HIF-1α, GPER and IL-1β expression. In line with these results, the transfection of MDA-MB-231 cells with a plasmid encoding a for a mutant dominant/negative form of c-fos (DN/c-fos), abolished the up-regulation of HIF-1α, GPER and IL-1β triggered by a 16 h exposure to hypoxia (Fig. 8g). Aiming to evaluate whether the reduction of HIF-1α protein levels prompted in the aforementioned experimental condition by the DN/c-fos construct is associated with the HIF-1α proteasome-dependent degradation, MDA-MB-231 cells were treated for 16 h with the

proteasome inhibitor MG132. Notably, MG132 rescued the effects of the DN/c-fos construct on HIF-1α protein levels (Fig. 8h), suggesting the involvement of c-fos in the complex HIF-1α biological regulation at the proteasomal level. By co-immunoprecipitation studies, we also ascertained that low O_2 tension stimulates a direct interaction between c-fos and HIF-1α in MDA-MB-231 cells (Fig. 8i), suggesting that the action of c-fos on HIF-1α stabilization may occur, at least in part, through a physical interaction between these two proteins. Next, taking into account previous findings showing that the functional cooperation between HIF-1α and GPER triggers the expression of certain genes in hypoxic conditions (De Francesco et al., 2013), we first determined that HIF-1α co-immunoprecipitates with GPER in MDA-MB-231 cells exposed to hypoxia (Fig. 8j). Thereafter, we evidenced the accumulation of GPER in the nuclear compartment of MDA-MB-231 cells exposed to hypoxia, as evidenced by both immunofluorescence (Fig. 8k) and subcellular fractionation studies (Fig. 8l). Intriguingly, by ChIP assays we then demonstrated that the exposure of MDA-MB-231 cells to hypoxia induces the recruitment of both HIF-1α and GPER to an HRE site located within the IL-1β promoter sequence (Fig. 8m). Overall, these data suggest that a functional interplay between HIF-1α and GPER may be stimulated by hypoxia toward the transcription of IL-1β in MDA-MB-231 cells.

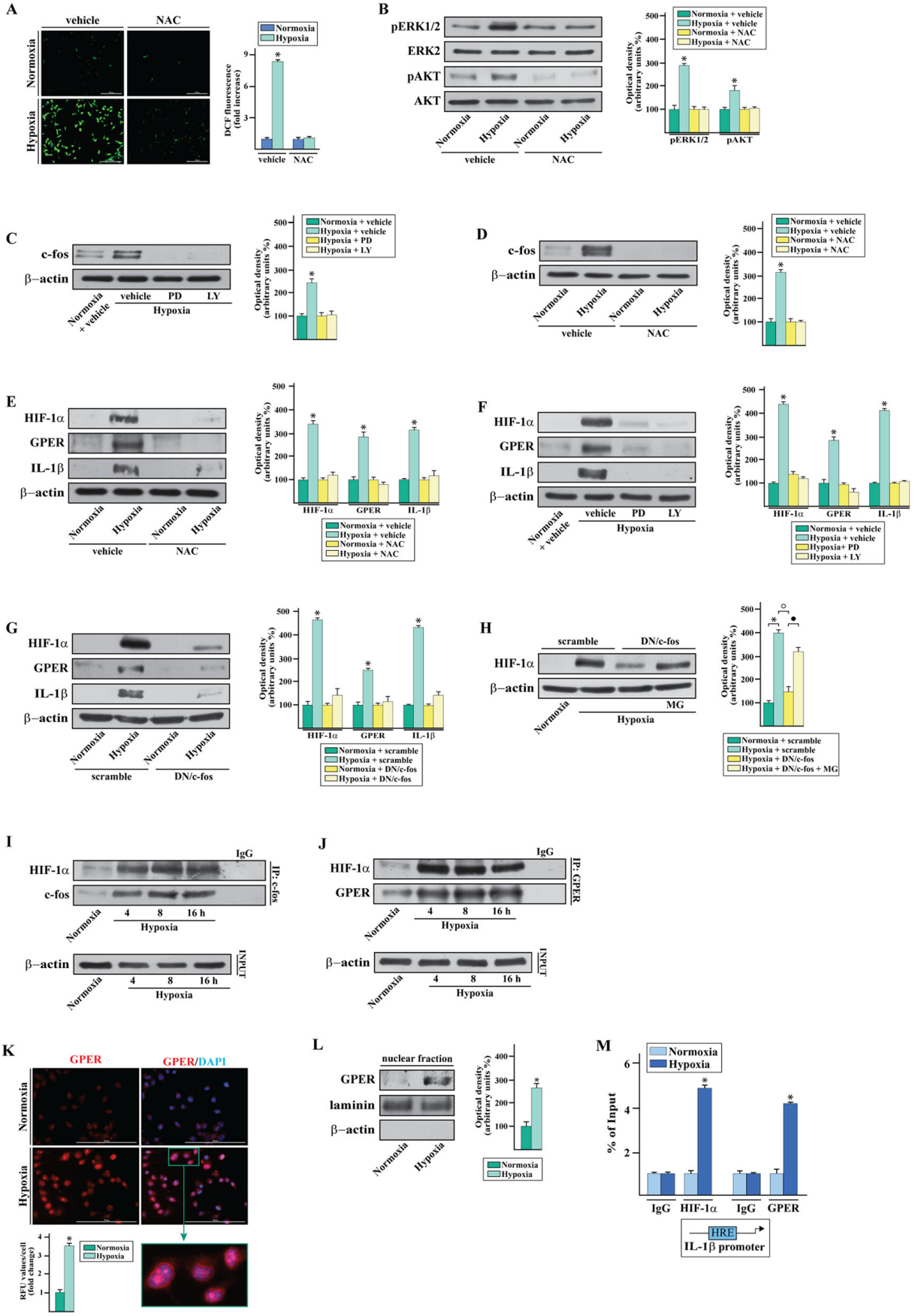

Figure 8. The ERK1/2 and transduction pathways along with c-fos, HIF-1α and GPER are involved in the up-regulation of IL-1β induced by hypoxia. (a) ROS generation in MDA-MB-231 cells cultured upon normoxia or 15

min hypoxia (2% O_2) in the presence or absence of the free radical scavenger NAC, as evaluated using the fluorescent probe DCF-DA. Scale bar 200 μM. Side panel shows the quantitative measurement of intracellular ROS (DCF fluorescence obtained in normoxic cells was set as one-fold induction upon which ROS levels induced by hypoxia was calculated). Values represent the mean ± SD of three independent experiments performed in triplicate. (b) The ERK1/2 and activation induced by 15 min hypoxia in MDA-MB-231 cells is no longer evident in the presence of NAC. Side panels show densitometric analysis of the blots normalized to ERK2 and that served as loading control, as indicated. (c) Immunoblot of c-fos from MDA-MB-231 cells cultured upon normoxia or hypoxia and in the presence of MEK inhibitor PD98059 (PD) or PI3K inhibitor LY294,002 (LY). Immunoblots of c-fos (d), HIF-1α, GPER and IL-1β (e) in MDA-MB-231 cells upon normoxia or hypoxia in the presence or absence of NAC. (f) Immunoblots of HIF-1α, GPER and IL-1β in MDA-MB-231 cells upon normoxia or hypoxia and in the presence of PD or LY. (g) Immunoblots of HIF-1α, GPER and IL-1β in MDA-MB-231 cells transfected with a scramble or a dominant-negative c-fos construct (DN/c-fos) and thereafter exposed to normoxia or hypoxia. (h) The 26S proteasome inhibitor MG132 rescued the HIF-1α repression observed in MDA-MB-231 cells transfected for 24 h with the DN/c-fos construct and exposed to low oxygen tension (2%). Side panels show densitometric analysis of the blots normalized to β-actin. Values represent the mean ± SD of three independent experiments performed in triplicate. (i-j) Co-immunoprecipitation assays performed in MDA-MB-231 cells cultured in normoxia or hypoxia, as indicated. Cell lysates were immunoprecipitated with anti-c-fos (i) or anti-GPER (j) antibodies. Immunocomplexes were analyzed by immunoblot with antibodies against the indicated proteins. In control samples, nonspecific IgG was used instead of the primary antibody. An equal amount of the total lysates (input) was blotted for β-actin as loading control. k GPER expression evaluated by immunofluorescence assays in MDA-MB-231 cells cultured upon normoxia or hypoxia. Nuclei were stained by DAPI (blue signal). Fluorescence intensities were quantified in 20 random fields for each condition and results are expressed as fold change of relative fluorescence units (RFU) over cells cultured upon normoxia (set as one-fold induction). Enlarged details are shown in the separate box. (l) Immunoblots of nuclear fraction lysates derived from MDA-MB-231 cells cultured upon normoxia or hypoxia. Side panel shows densitometric analysis of the blots normalized to laminin, which served as a nuclear marker. β-actin served as a cytoplasmic marker. (m) Recruitment of HIF-1α and GPER to the HRE site located within the IL-1β promoter sequence in MDA-MB-231 cells exposed to hypoxia. In control samples nonspecific IgG was used instead of the primary antibody. The amplified sequences were evaluated by real-time PCR. Values represent the mean ± SD of three independent experiments performed in triplicate. (*), (○) $p < 0.05$ for cells cultured upon normoxia versus cells cultured upon hypoxia.

3.5 Hypoxia triggers a functional cooperation among HIF-1α, GPER and IL-1β/IL1R1 axes toward a metastatic gene expression profile

Considering accumulating evidence that recognize IL-1β as a cytokine with a key role in angiogenesis, tumor growth and metastasis (Elaraj et al., 2006; Wu et al., 2018) and in order to provide further insights into the metastatic gene expression profile triggered by hypoxia through the IL-1β/IL1R1 axis, we performed a TaqMan Gene Expression Assay consisting of a Human Tumor Metastasis Array. In particular, MDA-MB-231 cells were exposed for 16 h either to

hypoxic conditions (Fig. 9a) or the recombinant human IL-1β protein (Fig. 9b), in the presence or absence of IL1R1a.

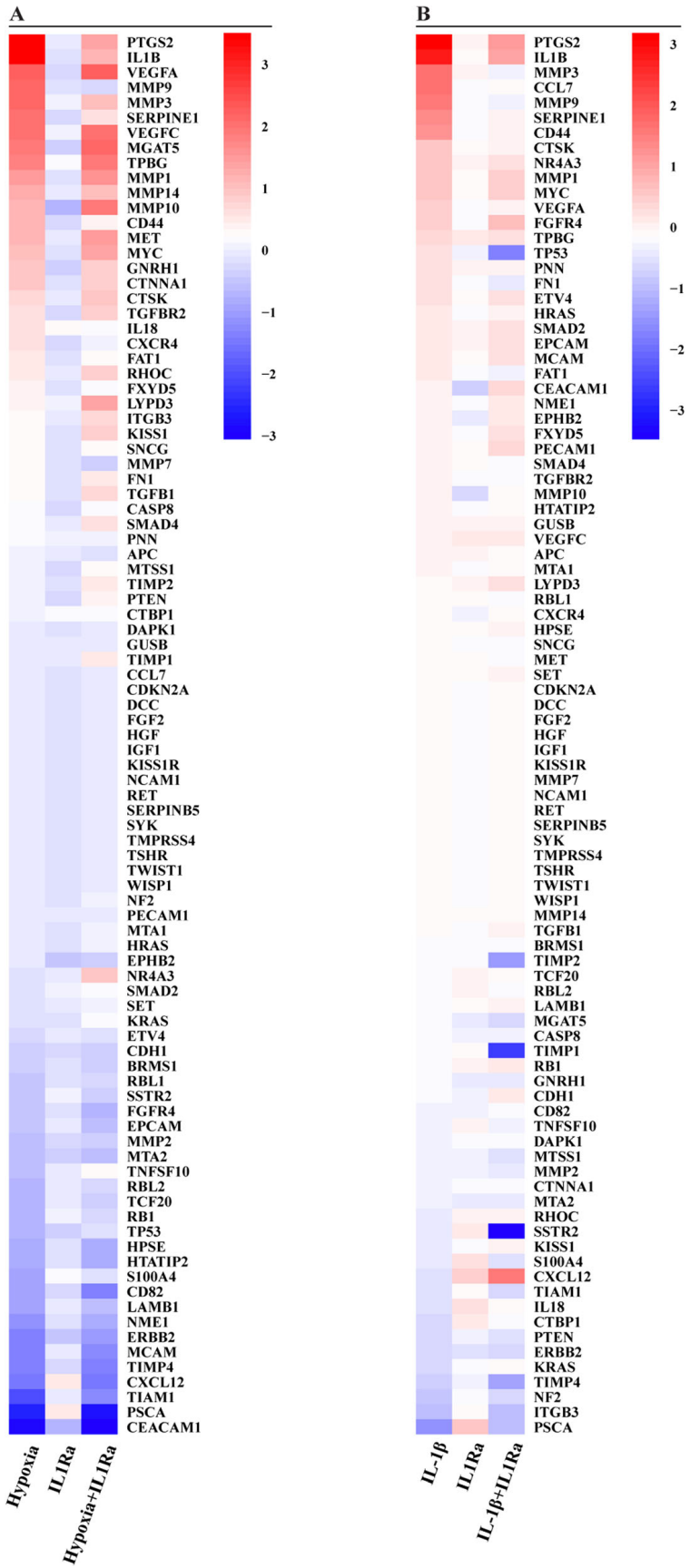

Figure 9. Consistent expression changes of metastasis-related genes are induced by hypoxia and IL-1β in MDA-MB-231 cells, as evaluated by TaqMan™ Human Tumor Metastasis Array. (a) MDA-MB-231 cells were cultured upon normoxia or hypoxia (2% O_2) in the presence or absence of the IL1R1 antagonist IL1R1a. (b) MDA-MB-231 cells were treated with vehicle or IL-1β alone or in combination with IL1R1a. Values were normalized to the Glucuronidase Beta (GUSB) expression, the colors indicate the log2 fold changes of gene expression upon the indicated conditions.

Considering the genes resulting with at least a 0.25 log2 fold change upon either hypoxia with respect to normoxia-exposed cells (Fig. 9a) or IL-1β with respect to vehicle-treated cells (Fig. 9b), we identified 31 and 15 metastatic genes induced respectively by hypoxia and IL-1β (Fig. 10a). Among the 11 genes up-regulated by either hypoxia or IL-1β as portrayed in the Venn diagram (Fig. 10a), we then focused on the regulation of IL-1β and its target gene cyclooxygenase 2 (COX2) considering that their increase was abrogated using IL1R1a (Fig.9a-b). First, we found that a positive correlation does exist between IL-1β and COX2 expression levels in TNBC patients of the METABRIC dataset (Fig. 10b). Subsequently, we confirmed by real-time and western blotting assays that IL1R1a prevents the mRNA (Fig. 10c) and protein (Fig. 10d-e) inductions of both IL-1β and COX2 upon a 16 h exposure to either hypoxia or IL-1β treatment, in accordance with previous evidence revealing that IL-1β may auto-regulate its own production (Manson et al., 1989). Furthermore, we demonstrated that the COX2 protein induction by a 16 h hypoxic condition is abrogated by silencing GPER (Fig. 610f), suggesting the involvement of GPER not only in the above described IL-1β expression but also in the increase of COX2 upon hypoxia.

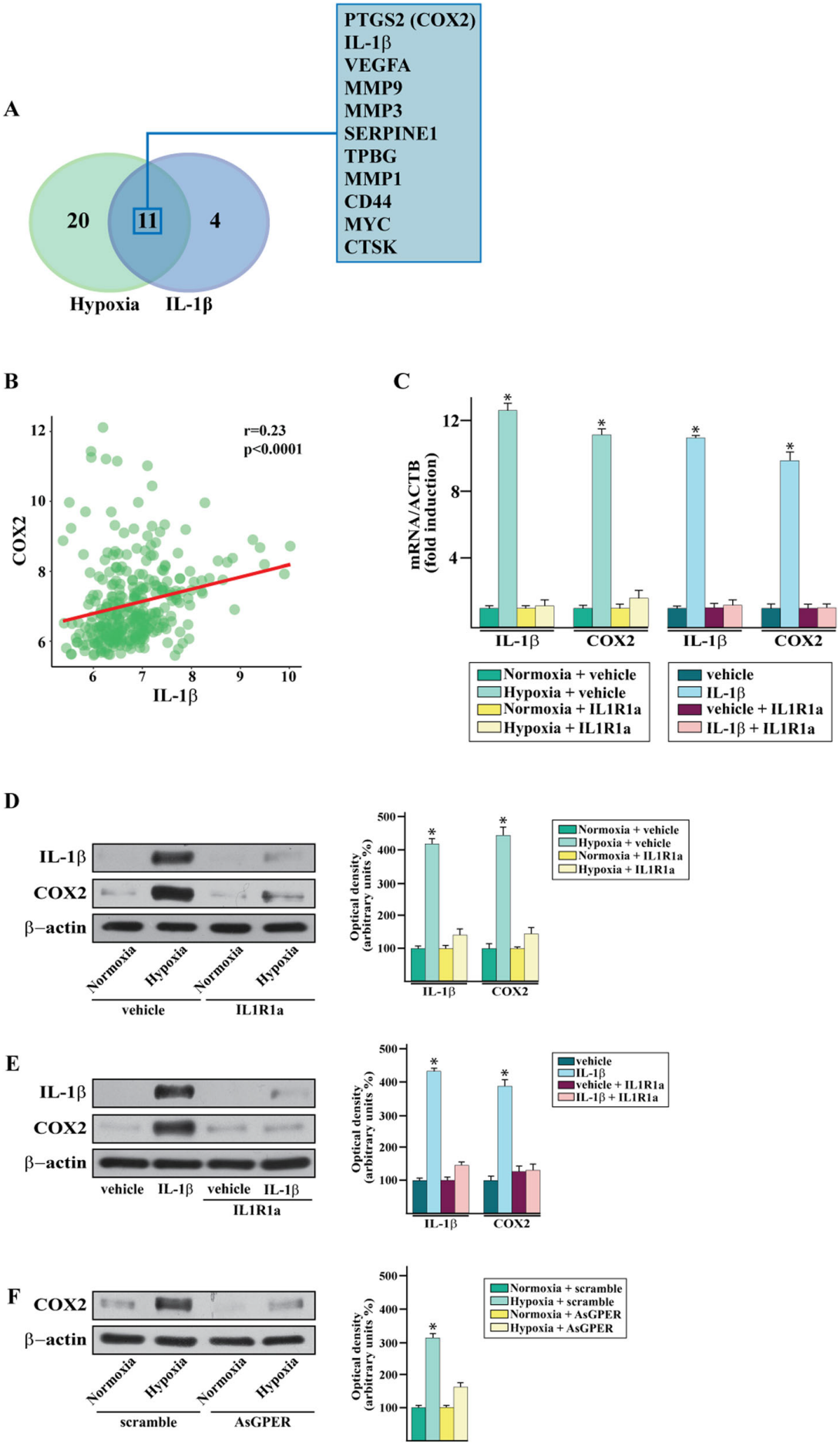
A
PTGS2 (COX2)
IL-1β
VEGFA
MMP9
MMP3
SERPINE1
TPBG
MMP1
CD44
MYC
CTSK
20
11
4
Hypoxia
IL-1β
B
r=0.23
p<0.0001
COX2
IL-1β
C
mRNA/ACTB (fold induction)
IL-1β
COX2
IL-1β
COX2
Normoxia + vehicle
Hypoxia + vehicle
Normoxia + IL1R1a
Hypoxia + IL1R1a
vehicle
IL-1β
vehicle + IL1R1a
IL-1β + IL1R1a
D
IL-1β
COX2
β–actin
Normoxia
Hypoxia
Normoxia
Hypoxia
vehicle
IL1R1a
Optical density (arbitrary units %)
IL-1β
COX2
Normoxia + vehicle
Hypoxia + vehicle
Normoxia + IL1R1a
Hypoxia + IL1R1a
E
IL-1β
COX2
β–actin
vehicle
IL-1β
vehicle
IL-1β
IL1R1a
Optical density (arbitrary units %)
IL-1β
COX2
vehicle
IL-1β
vehicle + IL1R1a
IL-1β + IL1R1a
F
COX2
β–actin
Normoxia
Hypoxia
Normoxia
Hypoxia
scramble
AsGPER
Optical density (arbitrary units %)
Normoxia + scramble
Hypoxia + scramble
Normoxia + AsGPER
Hypoxia + AsGPER

Figure 10. (a) Venn diagram of metastatic genes up-regulated in MDA-MB-231 cells in an exclusive and shared manner upon hypoxia (2% O_2) and IL-1β. The genes up-regulated by both hypoxia and IL-1β are listed in the box according to the gene expression changes induced by hypoxia (from high to low). (b) mRNA expression of IL-1β and COX2 in MDA-MB-231 cells cultured upon normoxia and hypoxia or vehicle and IL-1β in the presence or absence of the IL1R1 antagonist IL1R1a, as evaluated by real-time PCR. Values are normalized to the actin beta (ACTB) expression and shown as fold changes of the mRNA expression induced by hypoxia respect to normoxic cells. (c) Scatter plot depicting the correlation between the expression of COX2 and IL-1β in TNBC patients of the METABRIC cohort of patients. The Pearson correlation coefficient (r) and the relative p-value are indicated. (d) Immunoblots of IL-1β and COX2 in MDA-MB-231 cells cultured upon normoxia or hypoxia in combination with IL1R1a, as indicated. (e) Immunoblots of IL-1β and COX2 in MDA-MB-231 cells treated with vehicle or IL-1β and in combination with IL1R1a. (f) Immunoblots of COX2 in MDA-MB-231 cells upon normoxia and hypoxia silencing GPER. Side panels show densitometric analysis of the blots normalized to β-actin. Values represent the mean ± SD of three independent experiments performed in triplicate. (*) $p < 0.05$ for cells cultured under normoxia versus cells cultured under hypoxia or cells treated with IL-1β versus vehicle-treated cells

3.6 HIF-1α/GPER and IL-1β/IL1R axes are involved in invasive and metastatic properties of hypoxic TNBC cells through IL-1β auto-regulatory loop

A hypoxic milieu may lower cell-ECM adhesion encouraging cell motility and EMT toward aggressive breast cancer phenotypes (Petrova et al., 2018; Liu et al., 2015). Therefore, we evaluated the involvement of GPER and IL-1β/IL1R1 mediated signaling in the metastasis-related responses induced by IL-1β under hypoxia. In this regard, we demonstrated that MDA-MB-231 cells cultured under hypoxic conditions present a reduced spreading ability on fibronectin 60 min after seeding respect to cells grown under normoxic conditions (Fig. 11a-b). Of note, this effect was no abrogated silencing GPER as well as using IL1R1a (Fig. 11a-b). Similar results were observed by treating cells with IL-1β (Fig. 11c-d). Together, these findings indicate that both GPER and IL-1β/IL1R axis contribute to hypoxia decreased cancer cell spreading on the ECM protein fibronectin, according to previous data correlating the hypoxia-lowered cell-ECM adhesion to an increased invasion, intravasation and metastasis (Misra et al., 2007; Gilkes et al., 2014 A). Of note, the invasive effects prompted by both hypoxia and IL-1β treatment were no longer evident by either silencing GPER or using IL1R1a, as evaluated by Transwell Matrigel invasion assays (Fig. 11e-f). Moreover, IL1R1a reduced the spheroid expansion observed upon hypoxia and IL-1β treatment (Fig. 11g). Overall, these findings may suggest that the signaling network of both HIF-1α/GPER and IL-1β/ IL1R may lead to an auto-regulatory loop of IL-1β, toward invasive and metastatic properties of hypoxic TNBC cells.

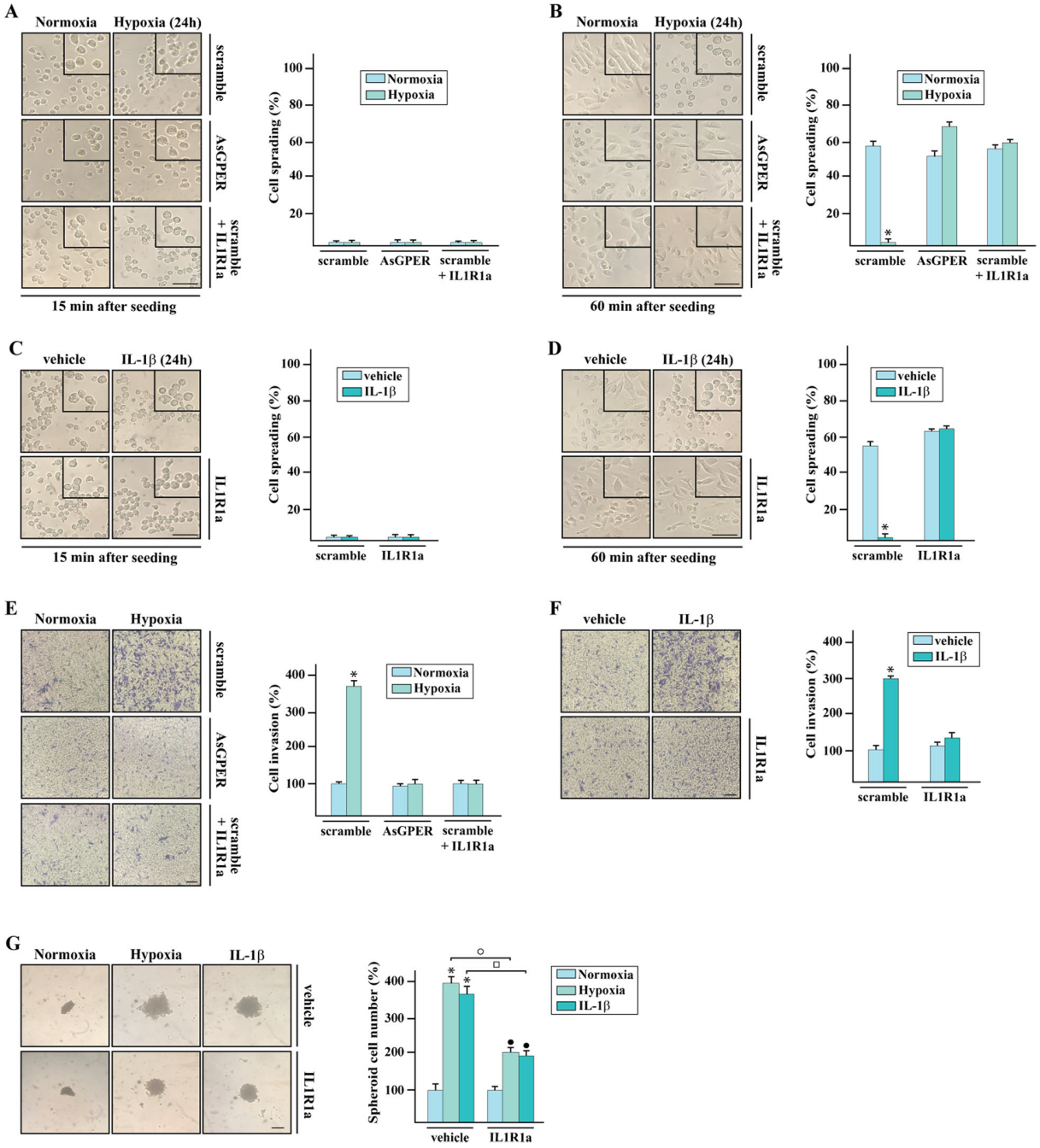

Figure 11. The IL-1β/IL1R1 system is involved in the invasive and proliferative features induced by hypoxia in MDA-MB-231 cells. (a-b) Cell spreading was evaluated in MDA-MB-231 cells transfected with scramble or AsGPER and exposed to normoxia or hypoxia (2% O_2) in the presence or absence of IL1R1a. Cells were then trypsinized, plated onto coverslips coated with fibronectin and the morphology was recorded after 15 min (a) or 60 min (b). (c-d) Cell spreading was evaluated in MDA-MB-231 cells treated with vehicle and IL-1β alone or in combination with IL1R1a, as indicated. Cells were then trypsinized, plated onto coverslips coated with fibronectin and the morphology was recorded after 15 min (c) or 60 min (d). Scale bar 50 μM. Side panels show quantification of cell spreading, as indicated. Data are representative of three independent experiments performed in triplicate. (e-f) Transwell Matrigel invasion assay in MDA-MB-231 cells transfected with scramble or AsGPER and then cultured in normoxia or hypoxia (e) or treated with vehicle or IL-1β (f) alone or in combination with IL1R1a, as indicated. Cells were counted in at least 10 random fields in three independent experiments performed in triplicate,

as quantified in the side panels. Scale bar 200 μM. (g) Spheroid formation assay in MDA-MB-231 cells exposed to hypoxia and IL-1β alone or in combination with IL1R1a. Scale bar 100 μm. Side panel shows the quantification of cell growth. Values represent the mean ± SD of three independent experiments performed in triplicate. (*), (○), (□) $p < 0.05$ for cells cultured under normoxia versus cells cultured under hypoxia or treated with IL-1β versus vehicle-treated cells.

3.7 The hypoxic regulation of the IL-1β/IL1R1 signaling in TNBC cells promotes an invasive phenotype of CAFs

It is well acknowledged that the strong interaction existing between cancer cells and the component of the TME contributes to worse patient outcomes (Chen and Song, 2019). Indeed, cancer cells through the release of various factors educate fibroblasts toward their activation and acquisition of a pro-tumorigenic phenotype (Hanahan and Coussens, 2012; Balkwill and Mantovani, 2012; Yoshida, 2020). In particular, CAFs sustain the malignant properties of cancer cells by synthesizing ECM components, cytokines and growth factors, which in turn contribute to the metastatic cascade (Zhang et al., 2013). Moreover, the dynamic network occurring between breast cancer cells and CAFs relies on the production of many effectors, such as diverse chemokines exerting tumor-promoting effects (Mishra et al., 2011). In this scenario, we aimed to provide insights into the paracrine IL-1β feedback on CAFs as key components of the TME. Thus, CAFs were exposed to CM collected from MDA-MB-231 cells, which were previously cultured under hypoxia. Interestingly, the up-regulation of both IL-1β and COX2 occurring in CAFs at the protein level upon these experimental conditions (Fig. 12a) was abrogated using IL1R1a (Fig. 12a). Similar findings were observed treating CAFs with IL1β (Fig. 12b). Since hypoxia may stimulate breast cancer cells toward invasive features as cell motility, formation of stress fibers and matrix contraction (Gilkes et al., 2014 B), the CM from MDA-MB-231 cells exposed to hypoxia promoted in CAFs the phosphorylation of the myosin light chain (MLC) on serine-19 (pMLCS19) (Fig. 12c), which is known to be required for the coordination of the actin-myosin contractility (Kimura et al., 1996). In accordance with these results, an increased MLC phosphorylation was observed in CAFs treated with IL1β (Fig. 12d). Moreover, immunofluorescent staining of polymerized actin (F-actin) using FITC-conjugated phalloidin revealed an enhanced formation of stress fibers upon exposure of CAFs to the CM from hypoxia-stimulated MDA-MB- 231 cells (Fig. 12e) as well as upon treatment with IL-1β (Fig. 12f). Notably, both pMLCS19 phosphorylation and the increased formation of actin stress fibers triggered in CAFs by the CM from hypoxic MDA-MB-231 cells (Fig. 12c, e) and IL-1β treatment (Fig. 12d, f) were abolished in the presence of IL1R1a. Then, we observed

that the invasive capacity of CAFs promoted by the CM from hypoxia-exposed MDA-MB-231 cells (Fig. 12g) or by IL-1β treatment (Fig. 12h) was impaired in the presence of IL1R1a. Altogether, these results show that the production of IL-1β by hypoxic TNBC cells may trigger a paracrine feed-forward signaling that stimulates malignant features in main components of the breast TME as CAFs.

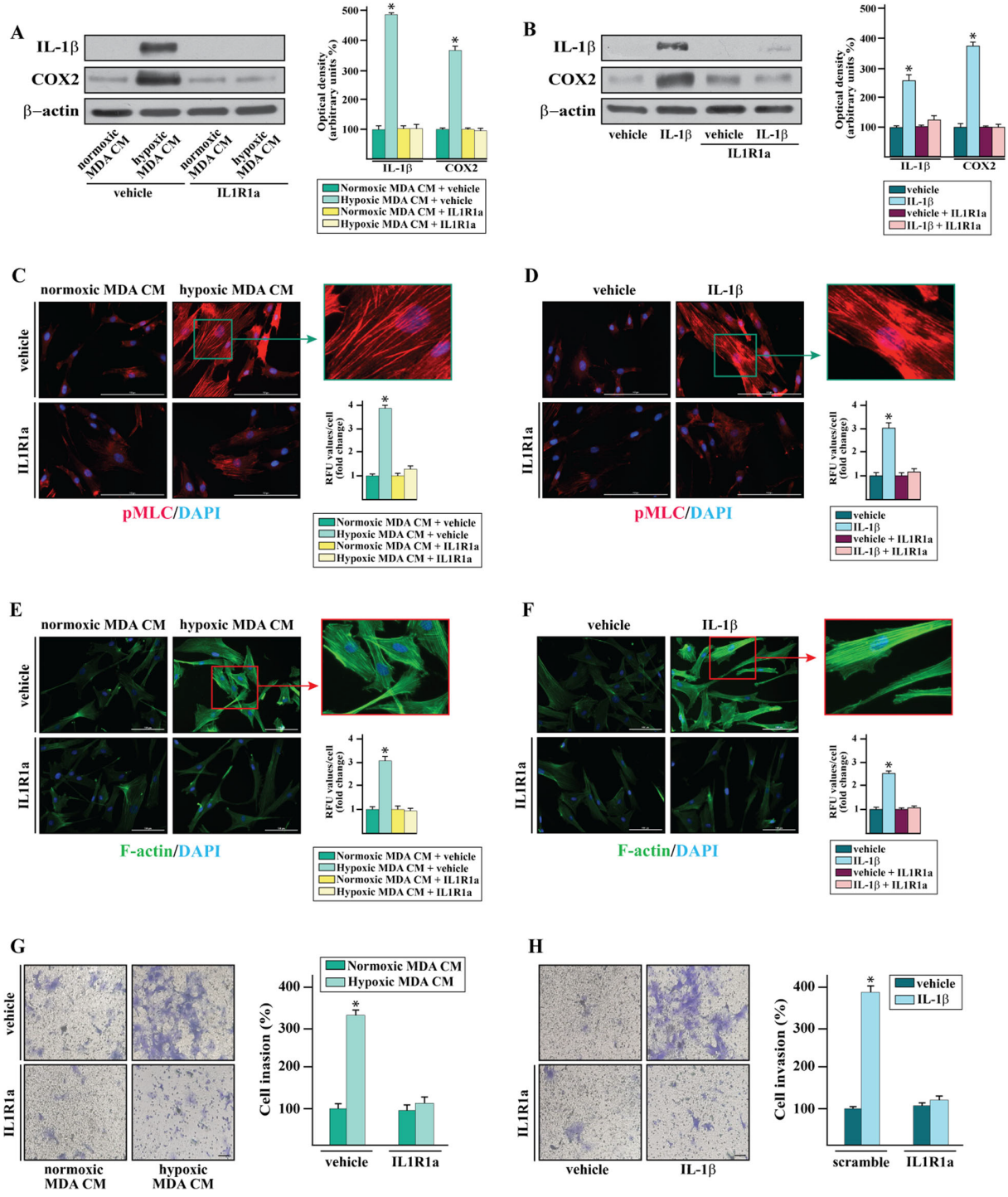

Figure 12. The paracrine action of the IL-1β/IL1R1 axis promotes the actin-myosin contractility and the invasion of CAFs. (a-b) Immunoblots of IL-1β and COX2 in CAFs exposed to conditioned medium (CM) collected from

MDA-MB-231 cells cultured upon normoxia or hypoxia (2% O_2) (a) and in CAFs treated with vehicle or IL-1β (b), in the presence or absence of IL1R1a. (c-d) Phosphorylation of MLC in CAFs cultured with CM collected from MDA-MB-231 cells exposed to normoxia and hypoxia (c) or IL-1β (d), in the presence or absence of the IL1R1 antagonist IL1R1a. Nuclei were stained by DAPI (blue signal). (e-f) CAFs exposed to CM from MDA-MB-231 cells grown upon normoxia and hypoxia (e) or treated with IL-1β (f), in the presence or absence of IL1R1a were stained with FITC-phalloidin to detect F-actin stress fibers (green) and with DAPI to detect nuclei (blue). Fluorescence intensities of pMLC and the number of stress fibers/cell was quantified based on F-actin staining in 20 random fields for each condition; results are expressed as fold change of relative fluorescence units (RFU). Enlarged details are shown in the separate boxes. Scale bar 100 μM. (g-h) Transwell Matrigel invasion assay in CAFs exposed to CM collected from MDA-MB-231 cells grown upon normoxia or hypoxia (g) or treated with IL-1β (h), in the presence or absence of IL1R1a. Cells were counted in at least 10 random fields in three independent experiments performed in triplicate, as quantified in side panels. Scale bar 200 μM. Values represent the mean ± SD of three independent experiments performed in triplicate. (*) $p < 0.05$.

4 Discussion

The great amount of data on cancer-related molecular networks and gene expression patterns have challenged the use of comprehensive information highlighting the multifaceted functions driving tumor progression. In this vein, big data analysis methods and large-scale computational studies have allowed to handle and analyze open-source multi-omics biological datasets. As a better understanding of key regulatory networks involved in cancer biology may strongly boost the identification of new targets and innovative therapeutic approaches, the use of multi-dimensional data regarding the gene expression landscape in large cohorts of cancer patients could represent a promising perspective. By querying the TCGA and METABRIC cohorts of patients, the first aim of our study was to analyze the GPER-associated gene expression network as well as its prognostic significance in breast cancer. In particular, we focused on the gene expression profile and signaling pathways associated with GPER in ER-negative breast cancer patients. Of note, the correlation and pathway analyses revealed an association of GPER with the expression of pro-metastatic CAMs, ECM-receptor interaction and FA genes in breast cancer patients lacking ER, suggesting the potential contribution of GPER to the metastatic outgrowth of breast cancer cells, as previously reported (Marjon et al., 2014; Filardo et al., 2018; Deng et al., 2018; Castillo Sanchez et al., 2016; Zhou et al., 2015; Filardo et al., 2008). CAMs are cell surface glycoproteins implicated in the establishment of normal tissue structure and function, hence contributing to several physiological processes as morphogenesis, embryogenesis, organogenesis, immunological function, wound healing, and inflammation (Makrilia et al., 2009). CAMs are classified in four major groups, cadherins, integrins, selectins, and members of the immunoglobulin superfamily, mainly involved in signal transduction, cytoskeletal organization, and gene regulation upon cell-to-cell and cell-to-ECM interactions (Theocharis et al., 2016; Humphrey et al., 2014). Hence, alterations in CAMs expression may lead to processes related to neoplastic transformation, as the loss of cellular morphology and tissue architecture (He et al., 2016; Bonnans et al., 2014) as well as cell invasion, migration, EMT, trans-endothelial migration, intra- and extra-vasation, tumor angiogenesis and organ-specific metastasis (Li and Feng, 2011). FAs are protein complexes that connect the cytoskeleton to the ECM, thus acting as scaffolds in outside-in transduction signaling (Shen et al., 2018; Nardone et al., 2017; Katoh, 2020). In particular, the FAs-mediated intracellular

pathways cooperate with receptor tyrosine kinases toward the regulation of cell shape, polarity, adhesion, migration, differentiation, survival and proliferation (Stutchbury et al., 2017). As far as tumorigenesis is concerned, an altered expression and function of both ECM and FAs is critical for the motility of breast cancer cells and the acquisition of further malignant features (Rigiracciolo et al., 2019; Nardone et al., 2017; Montagner and Dupont, 2020; Lin et al., 2020; Raab-Westphal et al., 2017). The association of GPER expression with clinicopathological determinants of breast cancer progression, including survival, tumor size, number of positive lymph nodes and vascular invasion, still remains to be fully clarified (Liu et al., 2009; Martin et al., 2018; Aiad et al., 2014; Broselid et al., 2013; Ye et al., 2019; Ignatov et al., 2013; Ignatov et al., 2014). Here, we determined that the subgroup of ER and HER2-negative breast cancer patients displaying high expression levels of GPER are characterized by a worse DFI, in line with our previous findings showing the association of GPER with a pro-invasive gene expression landscape. To date, controversial findings on the prognostic role of GPER in breast cancer have been reported. For instance, a recent survival analysis demonstrated an association of high expression of GPER with low overall survival of breast cancer patients (Ye et al., 2019). Moreover, the expression of GPER was indicated as an independent unfavorable factor for relapse-free survival in breast cancer patients treated with tamoxifen (Ignatov et al., 2014). Accordingly, the involvement of GPER in the resistance to tamoxifen was suggested in previous studies (Catalano et al., 2014; Ignatov et al., 2010). Moreover, immunohistochemical investigations related the lack of GPER in the plasma membrane to an improved long-term prognosis of tamoxifen-treated patients (Sjöström et al., 2014). Overall, these findings indicate the requirement for a better understanding of the role exerted by GPER in breast cancer.

Previous findings suggested that hypoxia may regulate the expression levels of GPER in breast cancer as well as in CAFs obtained from breast cancer biopsies (De Francesco et al., 2013). Accordingly, it has been demonstrated that GPER activation may induce the up-regulation and secretion of VEGF toward neo-angiogenesis and tumor progression (De Francesco et al., 2013). In the framework of these findings, we provided novel evidence regarding the involvement of GPER into the hypoxia-mediated regulation of the IL-1β/IL1R1 signal transduction in TNBC cells and CAFs derived from breast cancer patients. First, we found an association of the genes belonging to the “Cytokine-cytokine receptor interaction” pathway with HIF-1α expression in human TNBC samples. Then we ascertained that the genes belonging to the aforementioned pathway were enriched in TNBC patients showing high HIF-1α levels. Next, we assessed that the genes included in the “HIF-1 signaling pathway” are enriched in the TNBC cohort displaying high levels of IL-1β, which is a main component of the “Cytokine-cytokine receptor

interaction" pathway. Thereafter, we found that IL-1β expression levels are significantly higher in TNBC respect to non-TNBC samples and are positively correlated with HIF-1α in TNBC patients. Mechanistically, we then assessed the involvement of the HIF-1α/GPER signaling in the hypoxic regulation of IL-1β, which triggers a metastatic gene signature as well as invasive features in TNBC cells. Aiming to characterize the functional interaction occurring between tumor cells and the surrounding stroma, we found that hypoxic TNBC secrete IL-1β, which in turn may promote a feed-forward loop by binding to the cognate receptor IL1R1 in CAFs. Accordingly, we then determined that the CM collected from TNBC cells, previously exposed to hypoxia, stimulates in CAFs the actin polymerization and the MLC phosphorylation, which are both implicated in the attainment of invasive phenotypes. Intra-tumoral hypoxia is a common feature in solid cancers including breast tumor and is associated with an increased risk of distant metastasis and worse clinical outcomes (Semenza, 2016). Notably, malignant cells may adapt to hypoxia through the activation of hypoxia-inducible factors that mainly prompt a peculiar gene expression signature implicated in critical aspects of cancer biology as angiogenesis, stemness, metabolic reprogramming, EMT, invasion, metastasis and quiescence, leading to therapy resistance (Semenza, 2010; Semenza, 2012 A; Semenza 2012 B Farina et al., 2020). Hypoxia-activated HIF-1α plays a role in the tumor-related inflammation leading to worse clinical outcomes in diverse cancers including breast tumors (DiGiacomo and Gilkes, 2018). These events may occur through the HIF-1α dependent regulation of signaling mediators and inflammatory genes in both cancer and neighboring cells within the tumor microenvironment (DiGiacomo and Gilkes, 2018). Moreover, the pro-tumorigenic action of HIF-1α may occur through its interaction with various signaling pathways like transforming growth factor-β, Wnt/β-catenin, Notch and GPCRs (Lappano et al., 2016; Mallikarjuna et al., 2019; Kaufman, 2010; De Francesco et al., 2018). In this regard, it has been previously reported that both GPER and HIF-1α are involved in the cell adaptation to low oxygen tension in diverse tumors including breast cancer (De Francesco et al., 2013; De Francesco et al., 2014). In particular, the hypoxia-induced stabilization of HIF-1α was shown to cooperate with GPER in breast cancer cells and CAFs toward gene expression changes and relevant biological responses (De Francesco et al., 2013). Interestingly, GPER activation was also shown to prompt the IL-1β/IL1R1 transduction signaling in CAFs, which in turn promoted inflammatory responses and aggressive features of breast cancer cells (De Marco et al., 2016). In line with these findings, we demonstrated that a functional interplay between HIF-α and GPER regulates the expression levels of IL-1β in TNBC cells exposed to hypoxia. In this framework, we first ascertained that the recruitment of HIF-1α to the HRE site, located within the GPER promoter sequence, is

involved in the up-regulation of GPER levels in hypoxic TNBC cells. Then, we found that HIF-1α and GPER are both recruited to the HRE site located within the IL-1β promoter sequence. Aiming to identify the transduction mechanisms involved in the aforementioned findings, we demonstrated that the ROS-dependent ERK1/2 and AKT activation, together with the subsequent c-fos induction, contribute to the stabilization of HIF-1α as well as the up-regulation of GPER and IL-1β in hypoxic TNBC cells. Co-immunoprecipitation assays revealed that c-fos interacts as well as protects HIF-1α from the proteasomal degradation.

Corroborating previous data showing the role of IL-1β as an important effector of the transduction pathways linking inflammation to cancer (Wu et al., 2018; Tulotta and Ottewell, 2018; Naldini et al., 2010; Mantovani et al., 2008; Mantovani et al., 2019; Holen et al., 2016; Kaplanov et al., 2019), we provided novel evidence on the hypoxia-induced regulation of IL-1β expression toward growth and invasive properties of TNBC cells. In particular, by gene expression assays we demonstrated that either hypoxia or IL-1β are able to induce a strong increase of COX2, which was abrogated in the presence of a specific inhibitor of IL1R1. COX2 is the key enzyme involved in the biosynthesis of pro-inflammatory eicosanoids such as the prostaglandin E2 (PGE-2) (Funk, 2001). Worthy, the constitutive expression of COX2 and the sustained biosynthesis of PGE-2 are both linked to breast cancer development due to their stimulatory actions on mitogenesis, invasion, metastasis, angiogenesis and immunosuppression (Subbaramaiah and Dannenberg, 2003; Harris et al., 2014; Gupta et al., 2007). Additionally, previous evidences pointed out that high expression levels of COX2 characterize approximately 50% of breast cancers and are associated with reduced disease-free and overall survival as well as the primary tumor size (Howe, 2007; Denkert et al., 2003). In line with previous studies showing that MDA-MB-231 cells display a lipidomic profile characterized by high levels of polyunsaturated fatty acids that are known to contribute to breast cancer progression, we demonstrated that hypoxia-dependent activation of the IL-1β/COX2 signaling facilitates pro-tumorigenic features of TNBC cells (Giudetti et al., 2019; Vona-Davis and Rose, 2013;Rose, 1997).

In the breast TME, the bidirectional communication of neoplastic cells with CAFs, tumor-associated macrophages, endothelial and immune infiltrating cells, may support tumor progression by stimulating tumor cell proliferation, invasion, angiogenesis and metastasis (Hanahan and Coussens, 2012; Balkwill and Mantovani, 2012). In particular, CAFs may facilitate the establishment of a conducive microenvironmental niche for the recruitment and maintenance of disseminated tumor-initiating cells (De Francesco et al., 2018; Wels et al., 2008; Joyce and Pollard, 2009). Likewise, malignant features as proliferative, migratory and invasive

potential of tumor cells may be endorsed by CAFs (Lappano et al., 2020). In this respect, it has been shown that IL-1β may be involved in the intricate network connecting breast cancer cells and mesenchymal stem cells (MSCs), which are considered an additional source of CAFs beyond fibroblasts (Mishra et al., 2008; Escobar et al., 2015). Worthy, the IL-1β-dictated cellular interaction may stimulate the production of diverse chemokines by MSCs as well as an increased motility of TNBC cells (Escobar et al., 2015). Nicely reminiscing these observations, we have demonstrated that TNBC cells exposed to low oxygen tension activate IL-1β/IL1R1-mediated paracrine signals that prompt cytoskeletal changes like the MLC-driven formation of actin stress fibers toward pro-invasive properties of breast CAFs. Overall, these results provide novel evidence on the mechanisms through which hypoxia may trigger the IL-1β/IL1R1 signaling, which in turn generates a feed-forward stimulatory loop in both TNBC cells and CAFs. Therefore, our findings may be considered in more comprehensive therapeutic strategies targeting TNBC progression.

References

Aiad HA, Wahed MM, Asaad NY, El-Tahmody M, Elhosary E. Immunohistochemical expression of GPR30 in breast carcinoma of Egyptian patients: An association with immunohistochemical subtypes. APMIS. 2014;122, 976–984.

Akakura N, Kobayashi M, Horiuchi I, Suzuki A, Wang J, Chen J, Niizeki H, Kawamura Ki, Hosokawa M, Asaka M. Constitutive expression of hypoxia-inducible factor-1alpha renders pancreatic cancer cells resistant to apoptosis induced by hypoxia and nutrient deprivation. Cancer Res. 2001;61(17):6548-54.

Albanito L, Madeo A, Lappano R, Vivacqua A, Rago V, Carpino A, Oprea TI, Prossnitz ER, Musti AM, Andò S, Maggiolini M. G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17beta-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. Cancer Res. 2007;67(4):1859-66.

Antoniou A, Pharoah PD, Narod S, Risch HA, Eyfjord JE, Hopper JL, Loman N, Olsson H, Johannsson O, Borg A, Pasini B, Radice P, Manoukian S, Eccles DM, Tang N, Olah E, Anton Culver H, Warner E, Lubinski J, Gronwald J, Gorski B, Tulinius H, Thorlacius S, Eerola H, Nevanlinna H, Syrjäkoski K, Kallioniemi OP, Thompson D, Evans C, Peto J, Lalloo F, Evans DG, Easton DF. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. Am J Hum Genet. 2003;72(5):1117-30.

Ariazi EA, Brailoiu E, Yerrum S, Shupp HA, Slifker MJ, Cunliffe HE, Black MA, Donato AL, Arterburn JB, Oprea TI, Prossnitz ER, Dun NJ, Jordan VC. The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. Cancer Res. 2010;70(3):1184-94.

Armstrong K, Eisen A, Weber B. Assessing the risk of breast cancer. N Engl J Med. 2000;342(8):564-71.

Armulik A, Genové G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell. 2011;21(2):193-215.

Baek JH, Jang JE, Kang CM, Chung HY, Kim ND, Kim KW. Hypoxia-induced VEGF enhances tumor survivability via suppression of serum deprivation-induced apoptosis. Oncogene. 2000;19(40):4621-31.

Balkwill FR, Mantovani A. Cancer-related inflammation: common themes and therapeutic opportunities. Semin Cancer Biol. 2012;22:33–40.

Baron JA, Newcomb T, Longnecker G, Mittendorf B, Storer KM, Clapp PN, Bogdan T, Yuen F. Cigarette Smoking and Breast Cancer. Cancer Epidemiology Biomarkers and Prevention. 1996;5:399-403.

Barton M, Filardo EJ, Lolait SJ, Thomas P, Maggiolini M, Prossnitz ER. Twenty years of the G protein-coupled estrogen receptor GPER: Historical and personal perspectives. J Steroid Biochem Mol Biol. 2018;176:4-15.

Bell EL, Klimova TA, Eisenbart J, Moraes CT, Murphy MP, Budinger GR, Chandel NS. The Qo site of the mitochondrial complex III is required for the transduction of hypoxic signaling via reactive oxygen species production. J Cell Biol. 2007;177(6):1029-36.

Berk DA, Yuan F, Leunig M, Jain RK. Direct in vivo measurement of targeted binding in a human tumor xenograft. Proc Natl Acad Sci U S A. 1997;94(5):1785-90.

Bernstein L. Epidemiology of endocrine-related risk factor for breast cancer. J. Mammary Gland Biol Neoplasia 2002;7:3-15.

Bianchini G, Balko JM, Mayer IA, Sanders ME, Gianni L. Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. Nat Rev Clin Oncol. 2016;13(11):674-690.

Biglia N, Defabiani E, Ponzone R, Mariani L, Marenco D, Sismondi P. Management of risk of breast carcinoma in postmenopausal women. Endocrine-Related Cancer. 2004;11:69-83.

Birkbak NJ, McGranahan N. Cancer Genome Evolutionary Trajectories in Metastasis. Cancer Cell. 2020;37(1):8-19.

Björnström L, Sjöberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol. 2005;19(4):833-42.

Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, Parker MA, Tkachenko SE, Savchuck NP, Sklar LA, Oprea TI, Prossnitz ER. Virtual and biomolecular screening converge on a selective agonist for GPR30. Nat Chem Biol. 2006;2(4):207-12.

Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. Nat. Rev. Mol. Cell Biol. 2014, 15, 786–801.

Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, Pinedo HM, Abeloff MD, Simons JW, van Diest PJ, van der Wall E. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst. 2001;93(4):309-14.

Böttner M, Thelen P, Jarry H. Estrogen receptor beta: tissue distribution and the still largely enigmatic physiological function. J Steroid Biochem Mol Biol. 2014;139:245-51.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.

Bremnes RM, Dønnem T, Al-Saad S, Al-Shibli K, Andersen S, Sirera R, Camps C, Marinez I, Busund LT. The role of tumor stroma in cancer progression and prognosis: emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer. J Thorac Oncol. 2011;6(1):209-17.

Britt KL, Cuzick J, Phillips KA. Key steps for effective breast cancer prevention. Nat Rev Cancer. 2020;20(8):417-436.

Broselid S, Cheng B, Sjöström M, Lövgren K, Klug-De Santiago HL, Belting M, Jirström K, Malmström P, Olde B, Bendahl PO, Hartman L, Fernö M, Leeb-Lundberg LM. G protein-coupled estrogen receptor is apoptotic and correlates with increased distant disease-free survival of estrogen receptor-positive breast cancer patients. Clin Cancer Res. 2013;19(7):1681-92.

Busch S, Andersson D, Bom E, Walsh C, Ståhlberg A, Landberg G. Cellular organization and molecular differentiation model of breast cancer-associated fibroblasts. Mol Cancer. 2017;16(1):73. doi: 10.1186/s12943-017-0642-7.

Byrne C, Rockett H, Holmes MD. Dietary fat, fat subtypes, and breast cancer risk: lack of an association among postmenopausal women with no history of benign breast disease. Cancer Epidemiol Biomarkers Prev. 2002;11(3):261-5.

Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490(7418):61-70. doi: 10.1038/nature11412.

Carmeci C, Thompson DA, Ring HZ, Francke U, Weigel RJ. Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. Genomics. 1997;45(3):607-17.

Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000;407(6801):249-57.

Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nat Rev Drug Discov. 2011;10(6):417-27.

Castillo Sanchez R, Gomez R, Perez Salazar E. Bisphenol A Induces Migration through a GPER-, FAK-, Src-, and ERK2-Dependent Pathway in MDA-MB-231 Breast Cancer Cells. Chem. Res. Toxicol. 2016, 29, 285–295.

Catalano S, Giordano C, Panza S, Chemi F, Bonofiglio D, Lanzino M, Rizza P, Romeo F, Fuqua SA, Maggiolini M, Andò S, Barone I. Tamoxifen through GPER upregulates

aromatase expression: a novel mechanism sustaining tamoxifen-resistant breast cancer cell growth. Breast Cancer Res Treat. 2014;146(2):273-85.

Chambers DC, Carew AM, Lukowski SW, Powell JE. Transcriptomics and single-cell RNA-sequencing. Respirology. 2019;24(1):29-36.

Chang HY, Nuyten DS, Sneddon JB, Hastie T, Tibshirani R, Sørlie T, Dai H, He YD, van't Veer LJ, Bartelink H, van de Rijn M, Brown PO, van de Vijver MJ. Robustness, scalability, and integration of a wound-response gene expression signature in predicting breast cancer survival. Proc Natl Acad Sci U S A. 2005;102(10):3738-43.

Chen X, Song E. Turning foes to friends: targeting cancer-associated fibroblasts. Nat Rev Drug Discov. 2019;18(2):99-115.

Chiche J, Brahimi-Horn MC, Pouysségur J. Tumour hypoxia induces a metabolic shift causing acidosis: a common feature in cancer. J Cell Mol Med. 2010;14(4):771-94.

Ciriello G, Gatza ML, Beck AH, Wilkerson MD, Rhie SK, Pastore A, Zhang H, McLellan M, Yau C, Kandoth C, Bowlby R, Shen H, Hayat S, Fieldhouse R, Lester SC, Tse GM, Factor RE, Collins LC, Allison KH, Chen YY, Jensen K, Johnson NB, Oesterreich S, Mills GB, Cherniack AD, Robertson G, Benz C, Sander C, Laird PW, Hoadley KA, King TA; TCGA Research Network, Perou CM. Comprehensive Molecular Portraits of Invasive Lobular Breast Cancer. Cell. 2015;163(2):506-19.

Cirillo F, Lappano R, Bruno L, Rizzuti B, Grande F, Guzzi R, Briguori S, Miglietta AM, Nakajima M, Di Martino MT, Maggiolini M. AHR and GPER mediate the stimulatory effects induced by 3-methylcholanthrene in breast cancer cells and cancer-associated fibroblasts (CAFs). J Exp Clin Cancer Res. 2019;38(1):335.

Clare SE, Shaw PL. "Big Data" for Breast Cancer: Where to look and what you will find. NPJ Breast Cancer. 2016;2:16031–.

Craven KE, Gökmen-Polar Y, Badve SS. CIBERSORT analysis of TCGA and METABRIC identifies subgroups with better outcomes in triple negative breast cancer. Sci Rep. 2021;11(1):4691.

Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, Gräf S, Ha G, Haffari G, Bashashati A, Russell R, McKinney S; METABRIC Group, Langerød A, Green A, Provenzano E, Wishart G, Pinder S, Watson P, Markowetz F, Murphy L, Ellis I, Purushotham A, Børresen-Dale AL, Brenton JD, Tavaré S, Caldas C, Aparicio S. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature. 2012;486(7403):346-52.

Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, Gräf S, Ha G, Haffari G, Bashashati A, Russell R, McKinney S; METABRIC Group, Langerød A, Green A, Provenzano E, Wishart G, Pinder S, Watson P,

Markowetz F, Murphy L, Ellis I, Purushotham A, Børresen-Dale AL, Brenton JD, Tavaré S, Caldas C, Aparicio S. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature. 2012;486(7403):346-52.

Davalos V, Martinez-Cardus A, Esteller M. The Epigenomic Revolution in Breast Cancer: From Single-Gene to Genome-Wide Next-Generation Approaches. Am J Pathol. 2017;187(10):2163-2174.

De Carli A, La Vecchia C, Negri E, Cislaghi C. Cancer mortality in Italy and overview of trend from 1955 to 1998. Tumori. 1993;79:151-165.

De Francesco EM, Lappano R, Santolla MF, Marsico S, Caruso A, Maggiolini M. HIF-1α/GPER signaling mediates the expression of VEGF induced by hypoxia in breast cancer associated fibroblasts (CAFs). Breast Cancer Res. 2013;15(4):R64.

De Francesco EM, Maggiolini M, Anna Maria Musti AM. Crosstalk between notch, HIF-1α and GPER in breast Cancer EMT. Int J Mol Sci. 2018;19:2011.

De Francesco EM, Pellegrino M, Santolla MF, Lappano R, Ricchio E, Abonante S, Maggiolini M. GPER mediates activation of HIF1α/VEGF signaling by estrogens. Cancer Res. 2014;74(15):4053-64.

De Francesco EM, Sims AH, Maggiolini M, Sotgia F, Lisanti MP, Clarke RB. GPER mediates the angiocrine actions induced by IGF1 through the HIF-1α/VEGF pathway in the breast tumor microenvironment. Breast Cancer Res. 2017;19(1):129.

De Francesco EM, Sotgia F, Lisanti MP. Cancer stem cells (CSCs): metabolic strategies for their identification and eradication. Biochem J. 2018;475:1611–34.

de Heer EC, Jalving M, Harris AL. HIFs, angiogenesis, and metabolism: elusive enemies in breast cancer. J Clin Invest. 2020;130(10):5074-5087.

De Marco P, Lappano R, De Francesco EM, Cirillo F, Pupo M, Avino S, Vivacqua A, Abonante S, Picard D, Maggiolini M. GPER signalling in both cancer-associated fibroblasts and breast cancer cells mediates a feedforward IL1β/IL1R1 response. Sci Rep. 2016;6:24354.

De Palma M, Biziato D, Petrova TV. Microenvironmental regulation of tumour angiogenesis. Nat Rev Cancer. 2017;17(8):457-474.

De Wever O, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. Int J Cancer. 2008;123(10):2229-38.

Deeb G, Vaughan MM, McInnis I, Ford LA, Sait SN, Starostik P, Wetzler M, Mashtare T, Wang ES. Hypoxia-inducible factor-1α protein expression is associated with poor survival in normal karyotype adult acute myeloid leukemia. Leuk Res. 2011;35(5):579-84.

Deng Q, Jiang G, Wu Y, Li J, Liang W, Chen L, Su Q, Li W, Du J, Wong CKC, Chen Z, Wang H. GPER/Hippo-YAP signal is involved in Bisphenol S induced migration of triple negative breast cancer (TNBC) cells. J Hazard Mater. 2018;355:1-9.

Denkert C, Winzer KJ, Müller BM, Weichert W, Pest S, Köbel M, Kristiansen G, Reles A, Siegert A, Guski H, Hauptmann S. Elevated expression of cyclooxygenase-2 is a negative prognostic factor for disease free survival and overall survival in patients with breast carcinoma. Cancer. 2003;97(12):2978-87.

Dickson R and Lippman ME. Disease of breast cancer. Cell 1996;13:272-273.

DiGiacomo JW, Gilkes DM. Tumor Hypoxia As an Enhancer of Inflammation-Mediated Metastasis: Emerging Therapeutic Strategies. Target Oncol. 2018 Apr;13(2):157-173.

Dong S, Terasaka S, Kiyama R. Bisphenol A induces a rapid activation of Erk1/2 through GPR30 in human breast cancer cells. Environ Pollut. 2011;159(1):212-218.

Duan C. Hypoxia-inducible factor 3 biology: complexities and emerging themes. Am J Physiol Cell Physiol. 2016;310(4):C260-9.

Egan KM, Stampfer MJ, Hunter D, Hankinson S, Rosner BA, Holmes S, Willet J, Colditz A. Active and passive smoking in breast cancer: prospective results from the Nurses' Health Study. Epidemiology. 2002;13:138-145.

Elaraj DM, Weinreich DM, Varghese S, Puhlmann M, Hewitt SM, Carroll NM, Feldman ED, Turner EM, Alexander HR. The role of interleukin 1 in growth and metastasis of human cancer xenografts. Clin Cancer Res. 2006;12(4):1088-96.

Eroles P, Bosch A, Pérez-Fidalgo JA, Lluch A. Molecular biology in breast cancer: intrinsic subtypes and signaling pathways. Cancer Treat Rev. 2012;38(6):698-707.

Escobar P, Bouclier C, Serret J, Bièche I, Brigitte M, Caicedo A, Sanchez E, Vacher S, Vignais ML, Bourin P, Geneviève D, Molina F, Jorgensen C, Lazennec G. IL-1β produced by aggressive breast cancer cells is one of the factors that dictate their interactions with mesenchymal stem cells through chemokine production. Oncotarget. 2015;6(30):29034-47.

Farina AR, Cappabianca L, Sebastiano M, Zelli V, Guadagni S, Mackay AR. Hypoxia-induced alternative splicing: the 11th Hallmark of Cancer. J Exp Clin Cancer Res. 2020;39:110.

Farmer P, Bonnefoi H, Anderle P, Cameron D, Wirapati P, Becette V, André S, Piccart M, Campone M, Brain E, Macgrogan G, Petit T, Jassem J, Bibeau F, Blot E, Bogaerts J, Aguet M, Bergh J, Iggo R, Delorenzi M. A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. Nat Med. 2009;15(1):68-74.

Favaro E, Bensaad K, Chong MG, Tennant DA, Ferguson DJ, Snell C, Steers G, Turley H, Li JL, Günther UL, Buffa FM, McIntyre A, Harris AL. Glucose utilization via glycogen

phosphorylase sustains proliferation and prevents premature senescence in cancer cells. Cell Metab. 2012;16(6):751-64.

Filardo E, Quinn J, Pang Y, Graeber C, Shaw S, Dong J, Thomas P. Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. Endocrinology. 2007;148(7):3236-45.

Filardo EJ, Graeber CT, Quinn JA, Resnick MB, Giri D, DeLellis RA, Steinhoff MM, Sabo E. Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. Clin Cancer Res. 2006;12(21):6359-66.

Filardo EJ, Quinn JA, Bland KI, Frackelton AR Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol. 2000;14(10):1649-60.

Filardo EJ, Quinn JA, Sabo, E. Association of the membrane estrogen receptor, GPR30, with breast tumor metastasis and transactivation of the epidermal growth factor receptor. Steroids 2008, 73, 870–873.

Filardo EJ. A role for G-protein coupled estrogen receptor (GPER) in estrogen-induced carcinogenesis: Dysregulated glandular homeostasis, survival and metastasis. J Steroid Biochem Mol Biol. 2018;176:38-48.

Filippi I, Carraro F, Naldini A. Interleukin-1β affects MDA-MB-231 breast cancer cell migration under hypoxia: role of HIF-1α and NFκB transcription factors. Mediat Inflamm. 2015;2015:789414.

Fodor FH, Weston A, Bleiweiss IJ, Mc Curdy LD, Walsh MM, Tarter PI, Brower ST, Eng CM. Frequency and carrier risk associated with common BRCA1 and BRCA2 mutations in Ashkenazi Jewish breast cancer patients. American Journal of Human Genetics 1998;63:45-51.

Frolova O, Samudio I, Benito JM, Jacamo R, Kornblau SM, Markovic A, Schober W, Lu H, Qiu YH, Buglio D, McQueen T, Pierce S, Shpall E, Konoplev S, Thomas D, Kantarjian H, Lock R, Andreeff M, Konopleva M. Regulation of HIF-1α signaling and chemoresistance in acute lymphocytic leukemia under hypoxic conditions of the bone marrow microenvironment. Cancer Biol Ther. 2012;13(10):858-70.

Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science. 2001;294:1871–5.

Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal. 2013;6(269):pl1.

Garrido-Castro AC, Lin NU, Polyak K. Insights into Molecular Classifications of Triple-Negative Breast Cancer: Improving Patient Selection for Treatment. Cancer Discov. 2019;9(2):176-198.

Gilkes DM, Bajpai S, Chaturvedi P, Wirtz D, Semenza GL. Hypoxia-inducible factor 1 (HIF-1) promotes extracellular matrix remodeling under hypoxic conditions by inducing P4HA1, P4HA2, and PLOD2 expression in fibroblasts. J Biol Chem. 2013 A;288(15):10819-29.

Gilkes DM, Bajpai S, Wong CC, Chaturvedi P, Hubbi ME, Wirtz D, Semenza GL. Procollagen lysyl hydroxylase 2 is essential for hypoxia-induced breast cancer metastasis. Mol Cancer Res. 2013 B;11(5):456-66.

Gilkes DM, Semenza GL, Wirtz D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. Nat Rev Cancer. 2014 A;14(6):430-9.

Gilkes DM, Xiang L, Lee SJ, Chaturvedi P, Hubbi ME, Wirtz D, Semenza GL. Hypoxia-inducible factors mediate coordinated RhoA-ROCK1 expression and signaling in breast cancer cells. Proc Natl Acad Sci U S A. 2014; B111(3):E384-93.

Giudetti AM, De Domenico S, Ragusa A, Lunetti P, Gaballo A, Franck J, Simeone P, Nicolardi G, De Nuccio F, Santino A, Capobianco L, Lanuti P, Fournier I, Salzet M, Maffia M, Vergara D. A specific lipid metabolic profile is associated with the epithelial mesenchymal transition program. Biochim Biophys Acta Mol Cell Biol Lipids. 2019;1864(3):344-357.

Gonda TA, Varro A, Wang TC, Tycko B. Molecular biology of cancer-associated fibroblasts: can these cells be targeted in anti-cancer therapy? Semin Cell Dev Biol. 2010;21(1):2-10.

Grantab R, Sivananthan S, Tannock IF. The penetration of anticancer drugs through tumor tissue as a function of cellular adhesion and packing density of tumor cells. Cancer Res. 2006;66(2):1033-9.

Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C, Gomis RR, Manova-Todorova K, Massagué J. Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. Nature. 2007;446(7137):765-70.

Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell. 2012;21:309–22.

Harbeck N, Gnant M. Breast cancer. Lancet. 2017;389(10074):1134-1150.

Harris RE, Casto BC, Harris ZM. Cyclooxygenase-2 and the inflammogenesis of breast cancer. World J Clin Oncol. 2014;5:677–92.

He X, Lee B, Jiang Y. Cell-ECM Interactions in Tumor Invasion. Adv. Exp. Med. Biol. 2016, 936, 73–91.

Helm JS, Rudel RA. Adverse outcome pathways for ionizing radiation and breast cancer involve direct and indirect DNA damage, oxidative stress, inflammation, genomic instability, and interaction with hormonal regulation of the breast. Arch Toxicol. 2020;94(5):1511-1549.

Hinshaw DC, Shevde LA. The Tumor Microenvironment Innately Modulates Cancer Progression. Cancer Res. 2019;79(18):4557-4566.

Holen I, Lefley DV, Francis SE, Rennicks S, Bradbury S, Coleman RE, Ottewell P. IL-1 drives breast cancer growth and bone metastasis in vivo. Oncotarget. 2016;7(46):75571-75584.

Houthuijzen JM, Jonkers J. Cancer-associated fibroblasts as key regulators of the breast cancer tumor microenvironment. Cancer Metastasis Rev. 2018;37(4):577-597.

Howe LR. Inflammation and breast cancer. Cyclooxygenase/prostaglandin signaling and breast cancer. Breast Cancer Res. 2007;9:210.

Hubbi ME, Semenza GL. Regulation of cell proliferation by hypoxia-inducible factors. Am J Physiol Cell Physiol. 2015;309(12):C775-82.

Hulka BS and Moorman PG. Breast cancer: hormones and other risk factors. Maturitas. 2001;38:103-116.

Humphrey JD, Dufresne ER, Schwartz MA. Mechanotransduction and extracellular matrix homeostasis. Nat. Rev. Mol. Cell Biol. 2014, 15, 802–812.

Ibnouhsein I, Jankowski S, Neuberger K, Mathelin C. The Big Data Revolution for Breast Cancer Patients. Eur J Breast Health. 2018;14(2):61-62.

Ignatov A, Ignatov T, Roessner A, Costa SD, Kalinski T. Role of GPR30 in the mechanisms of tamoxifen resistance in breast cancer MCF-7 cells. Breast Cancer Res Treat. 2010;123(1):87-96.

Ignatov A, Ignatov T, Weissenborn C, Eggemann H, Bischoff J, Semczuk A, Roessner A, Costa SD, Kalinski T. G-protein-coupled estrogen receptor GPR30 and tamoxifen resistance in breast cancer. Breast Cancer Res Treat. 2011;128(2):457-66.

Ignatov T, Weißenborn C, Poehlmann A, Lemke A, Semczuk A, Roessner A, Costa SD, Kalinski T, Ignatov A. GPER-1 expression decreases during breast cancer tumorigenesis. Cancer Invest. 2013;31(5):309-15.

Inoue H, Ichinose M, Miura M, Katsumata U, Takishima T. Sensory receptors and reflex pathways of nonadrenergic inhibitory nervous system in feline airways. Am Rev Respir Dis. 1989;139(5):1175-8.

Ishii G, Ochiai A, Neri S. Phenotypic and functional heterogeneity of cancer-associated fibroblast within the tumor microenvironment. Adv Drug Deliv Rev. 2016;99(Pt B):186-196.

Jensen EV, DeSombre ER. Estrogen-receptor interaction. Science. 1973;182(4108):126-34.

Jensen EV, Jacobsen HI: Basic guides to the mechanism of estrogen action. Recent Prog Horm Res. 1962;18: 387-414.

Jiang QF, Wu TT, Yang JY, Dong CR, Wang N, Liu XH, Liu ZM. 17β-estradiol promotes the invasion and migration of nuclear estrogen receptor-negative breast cancer cells through cross-talk between GPER1 and CXCR1. J Steroid Biochem Mol Biol. 2013;138:314–24.

Jin K, Pandey NB, Popel AS. Simultaneous blockade of IL-6 and CCL5 signaling for synergistic inhibition of triple-negative breast Cancer growth and metastasis. Breast Cancer Res. 2018;20:54.

Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer. 2009;9:239–52.

Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer. 2006;6(5):392-401.

Kalluri R. The biology and function of fibroblasts in cancer. Nat Rev Cancer. 2016;16(9):582-98.

Kaplanov I, Carmi Y, Kornetsky R, Shemesh A, Shurin GV, Shurin MR, Dinarello CA, Voronov E, Apte RN. Blocking IL-1β reverses the immunosuppression in mouse breast cancer and synergizes with anti-PD-1 for tumor abrogation. Proc Natl Acad Sci U S A. 2019;116(4):1361-1369.

Katoh K. FAK-Dependent Cell Motility and Cell Elongation. Cells. 2020;9, E192.

Kaufman DS. HIF hits Wnt in the stem cell niche. Nat Cell Biol. 2010;12:926–7.

Kim SH, Turnbull J, Guimond S. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. J Endocrinol. 2011;209(2):139-51.

Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science. 1996;273(5272):245-8.

Kushi LH, Sellers TA, Potter JD, Nelson CL, Munger RG, Kaye SA, Folsom AR. Dietary fat and postmenopausal breast cancer. J Natl Cancer Inst. 1992;84(14):1092-9.

Lappano R, Pisano A, Maggiolini M. GPER Function in Breast Cancer: An Overview. Front Endocrinol (Lausanne). 2014;5:66.

Lappano R, Rigiracciolo DC, Belfiore A, Maggiolini M, De Francesco EM. Cancer associated fibroblasts: role in breast cancer and potential as therapeutic targets. Expert Opin Ther Targets. 2020;24(6):559-572.

Lappano R, Rigiracciolo DC, De Marco P, Avino S, Cappello AR, Rosano C, Maggiolini M, De Francesco EM. Recent advances on the role of G protein-coupled receptors in hypoxia-mediated signaling. AAPS J. 2016;18:305–10.

Lappano R, Rosano C, De Marco P, De Francesco EM, Pezzi V, Maggiolini M. Estriol acts as a GPR30 antagonist in estrogen receptor-negative breast cancer cells. Mol Cell Endocrinol. 2010;320(1-2):162-70.

Lappano R, Rosano C, Santolla MF, Pupo M, De Francesco EM, De Marco P, Ponassi M, Spallarossa A, Ranise A, Maggiolini M. Two novel GPER agonists induce gene expression changes and growth effects in cancer cells. Curr Cancer Drug Targets. 2012 A;12(5):531-42.

Lappano R, Santolla MF, Pupo M, Sinicropi MS, Caruso A, Rosano C, Maggiolini M. MIBE acts as antagonist ligand of both estrogen receptor α and GPER in breast cancer cells. Breast Cancer Res. 2012 B;14(1):R12.

LeBleu VS, Kalluri R. A peek into cancer-associated fibroblasts: origins, functions and translational impact. Dis Model Mech. 2018;11(4):dmm029447.

Lee K, Zhang H, Qian DZ, Rey S, Liu JO, Semenza GL. Acriflavine inhibits HIF-1 dimerization, tumor growth, and vascularization. Proc Natl Acad Sci U S A. 2009;106(42):17910-5.

Lei B, Wang D, Zhang M, Deng Y, Jiang H, Li Y. miR-615-3p Promotes the Epithelial-Mesenchymal Transition and Metastasis of Breast Cancer by Targeting PICK1/TGFBRI Axis. J Exp Clin Cancer Res. 2020;39:71.

Leon-Ferre RA, Giridhar KV, Hieken TJ, Mutter RW, Couch FJ, Jimenez RE, Hawse JR, Boughey JC, Ruddy KJ. A contemporary review of male breast cancer: current evidence and unanswered questions. Cancer Metastasis Rev. 2018; 37(4):599-614.

Li DM, Feng YM. Signaling mechanism of cell adhesion molecules in breast cancer metastasis: Potential therapeutic targets. Breast Cancer Res. Treat. 2011, 128, 7–21.

Li Y, Chen Y, Zhu ZX, Liu XH, Yang L, Wan L, Lei TW, Wang XD. 4-Hydroxytamoxifen-stimulated processing of cyclin E is mediated via G protein-coupled receptor 30 (GPR30) and accompanied by enhanced migration in MCF-7 breast cancer cells. Toxicology. 2013;309:61–5.

Liang J, Shang Y. Estrogen and cancer. Annu Rev Physiol. 2013;75:225-40.

Lim B, Lin Y, Navin N. Advancing Cancer Research and Medicine with Single-Cell Genomics. Cancer Cell. 2020;37(4):456-470.

Lin T, Yang C, Cheng L, Chang W, Lin Y, Cheng H. Fibronectin in Cancer: Friend or Foe. Cells. 2020;9, 27.

Liu Q, Li JG, Zheng XY, Jin F, Dong HT. Expression of CD133, PAX2, ESA, and GPR30 in invasive ductal breast carcinomas. Chin Med J (Engl). 2009;122(22):2763-9.

Liu ZJ, Semenza GL, Zhang HF. Hypoxia-inducible factor 1 and breast cancer metastasis. J Zhejiang Univ Sci B. 2015;16(1):32-43.

Lubin F, Wax Y, Modan B. Role of fat, animal protein, and dietary fiber in breast cancer etiology: a case-control study. J Natl Cancer Inst. 1986;77(3):605-12.

Luo W, Hu H, Chang R, Zhong J, Knabel M, O'Meally R, Cole RN, Pandey A, Semenza GL. Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. Cell. 2011;145(5):732-44.

MacGregor JI, Jordan VC. Basic guide to the mechanisms of antiestrogen action. Pharmacol Rev. 1998;50(2):151-96. PMID: 9647865.

Madeo A, Maggiolini M. Nuclear alternate estrogen receptor GPR30 mediates 17beta-estradiol-induced gene expression and migration in breast cancer-associated fibroblasts. Cancer Res. 2010;70(14):6036-46.

Maggiolini M, Bonofiglio D, Marsico S, Panno ML, Cenni B, Picard D, Andò S. Estrogen receptor alpha mediates the proliferative but not the cytotoxic dose-dependent effects of two major phytoestrogens on human breast cancer cells. Mol Pharmacol. 2001;60(3):595-602.

Maggiolini M, Picard D. The unfolding stories of GPR30, a new membrane-bound estrogen receptor. J Endocrinol. 2010;204(2):105-14.

Maggiolini M, Vivacqua A, Fasanella G, Recchia AG, Sisci D, Pezzi V, Montanaro D, Musti AM, Picard D, Andò S. The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-estradiol and phytoestrogens in breast cancer cells. J Biol Chem. 2004;279(26):27008-16.

Makrilia N, Kollias A, Manolopoulos L, Syrigos, K. Cell adhesion molecules: Role and clinical significance in cancer. Cancer Invest. 2009, 27, 1023–1037.

Mallikarjuna P, Raviprakash TS, Aripaka K, Ljungberg B, Landström M. Interactions between TGF-β type I receptor and hypoxia-inducible factor-α mediates a synergistic crosstalk leading to poor prognosis for patients with clear cell renal cell carcinoma. Cell Cycle. 2019;18:2141–56.

Manson JC, Symons JA, Di Giovine FS, Poole S, Duff GW. Autoregulation of interleukin 1 production. Eur J Immunol. 1989;19:261–5.

Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454:436–44.

Mantovani A, Dinarello CA, Molgora M, Garlanda C. Interleukin-1 and related cytokines in the regulation of inflammation and immunity. Immunity. 2019;50:778–95.

Marcus PM, Newman B, Millikan RG, Moorman PG, Baird DD, Qaqish B. The association of adolescent cigarette smoking, alcoholic beverage consumption, environmental tobacco smoke, and ionizing radiation with subsequent breast cancer risk (United States). Cancer Causes and Control. 2000;11:271-278.

Marino M, Galluzzo P, Ascenzi P. Estrogen signaling multiple pathways to impact gene transcription. Curr Genomics. 2006;7(8):497-508.

Marjon NA, Hu C, Hathaway HJ, Prossnitz ER. G protein-coupled estrogen receptor regulates mammary tumorigenesis and metastasis. Mol Cancer Res. 2014;12(11):1644-1654.

Martin SG, Lebot MN, Sukkarn B, Ball G, Green AR, Rakha EA, Ellis IO, Storr SJ. Low expression of G protein-coupled oestrogen receptor 1 (GPER) is associated with adverse survival of breast cancer patients. Oncotarget. 2018;9(40):25946-25956.

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature. 1999;399(6733):271-5.

Mishra P, Banerjee D, Ben-Baruch A. Chemokines at the crossroads of tumor-fibroblast interactions that promote malignancy. J Leukoc Biol. 2011; 89:31–9.

Mishra PJ, Mishra PJ, Humeniuk R, Medina DJ, Alexe G, Mesirov JP, Ganesan S, Glod JW, Banerjee D. Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. Cancer Res. 2008;68(11):4331-9.

Misra A, Lim RP, Wu Z, Thanabalu T. N-WASP plays a critical role in fibroblast adhesion and spreading. Biochem Biophys Res Commun. 2007;364:908–12.

Mittal S, Brown NJ, Holen I. The breast tumor microenvironment: role in cancer development, progression and response to therapy. Expert Rev Mol Diagn. 2018;18(3):227-243.

Montagner M, Dupont S. Mechanical Forces as Determinants of Disseminated Metastatic Cell Fate. Cells. 2020;9, 250.

Morine Y, Shimada M, Utsunomiya T, Imura S, Ikemoto T, Mori H, Hanaoka J, Kanamoto M, Iwahashi S, Miyake H. Hypoxia inducible factor expression in intrahepatic cholangiocarcinoma. Hepatogastroenterology. 2011;58(110-111):1439-44.

Nagaraja AS, Dood RL, Armaiz-Pena G, Kang Y, Wu SY, Allen JK, Jennings NB, Mangala LS, Pradeep S, Lyons Y, Haemmerle M, Gharpure KM, Sadaoui NC, Rodriguez-Aguayo C, Ivan C, Wang Y, Baggerly K, Ram P, Lopez-Berestein G, Liu J, Mok SC, Cohen L,

Lutgendorf SK, Cole SW, Sood AK. Adrenergic-mediated increases in INHBA drive CAF phenotype and collagens. JCI Insight. 2017;2(16):e93076.

Naldini A, Filippi I, Miglietta D, Moschetta M, Giavazzi R, Carraro F. Interleukin-1β regulates the migratory potential of MDA-MB-231 breast cancer cells through the hypoxia-inducible factor-1α. Eur J Cancer. 2010; 46(18):3400–8.

Nardone G, Oliver-De La Cruz J, Vrbsky J, Martini C, Pribyl J, Skládal P, Pešl M, Caluori G, Pagliari S, Martino F, Maceckova Z, Hajduch M, Sanz-Garcia A, Pugno NM, Stokin GB, Forte G. YAP regulates cell mechanics by controlling focal adhesion assembly. Nat Commun. 2017;8:15321.

Netti PA, Berk DA, Swartz MA, Grodzinsky AJ, Jain RK. Role of extracellular matrix assembly in interstitial transport in solid tumors. Cancer Res. 2000;60(9):2497-503.

Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, Gray JW. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell. 2006;10(6):515-27.

Nik-Zainal S, Davies H, Staaf J, Ramakrishna M, Glodzik D, Zou X, Martincorena I, Alexandrov LB, Martin S, Wedge DC, Van Loo P, Ju YS, Smid M, Brinkman AB, Morganella S, Aure MR, Lingjærde OC, Langerød A, Ringnér M, Ahn SM, Boyault S, Brock JE, Broeks A, Butler A, Desmedt C, Dirix L, Dronov S, Fatima A, Foekens JA, Gerstung M, Hooijer GK, Jang SJ, Jones DR, Kim HY, King TA, Krishnamurthy S, Lee HJ, Lee JY, Li Y, McLaren S, Menzies A, Mustonen V, O'Meara S, Pauporté I, Pivot X, Purdie CA, Raine K, Ramakrishnan K, Rodríguez-González FG, Romieu G, Sieuwerts AM, Simpson PT, Shepherd R, Stebbings L, Stefansson OA, Teague J, Tommasi S, Treilleux I, Van den Eynden GG, Vermeulen P, Vincent-Salomon A, Yates L, Caldas C, van't Veer L, Tutt A, Knappskog S, Tan BK, Jonkers J, Borg Å, Ueno NT, Sotiriou C, Viari A, Futreal PA, Campbell PJ, Span PN, Van Laere S, Lakhani SR, Eyfjord JE, Thompson AM, Birney E, Stunnenberg HG, van de Vijver MJ, Martens JW, Børresen-Dale AL, Richardson AL, Kong G, Thomas G, Stratton MR. Landscape of somatic mutations in 560 breast cancer whole-genome sequences. Nature. 2016;534(7605):47-54.

O'Dea A, Sondergard C, Sweeney P, Arnatt CK. A Series of Indole-Thiazole Derivatives Act as GPER Agonists and Inhibit Breast Cancer Cell Growth. ACS Med Chem Lett. 2018;9(9):901-906.

Öhlund D, Elyada E, Tuveson D. Fibroblast heterogeneity in the cancer wound. J Exp Med. 2014;211(8):1503-23.

Ozbek S, Balasubramanian PG, Chiquet-Ehrismann R, Tucker RP, Adams JC. The evolution of extracellular matrix. Mol Biol Cell. 2010;21(24):4300-5.

Pandey DP, Lappano R, Albanito L, Madeo A, Maggiolini M, Picard D. Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF. EMBO J. 2009;28(5):523-32.

Paraiso KH, Smalley KS. Fibroblast-mediated drug resistance in cancer. Biochem Pharmacol. 2013;85(8):1033-41.

Parsons J, Francavilla C. 'Omics Approaches to Explore the Breast Cancer Landscape. Front Cell Dev Biol. 2020;7:395.

Pearce DA, Nirmal AJ, Freeman TC, Sims AH. Continuous Biomarker Assessment by Exhaustive Survival Analysis. 2018, bioRxiv 208660.

Pescador N, Villar D, Cifuentes D, Garcia-Rocha M, Ortiz-Barahona A, Vazquez S, Ordoñez A, Cuevas Y, Saez-Morales D, Garcia-Bermejo ML, Landazuri MO, Guinovart J, del Peso L. Hypoxia promotes glycogen accumulation through hypoxia inducible factor (HIF)-mediated induction of glycogen synthase 1. PLoS One. 2010;5(3):e9644.

Petrova V, Annicchiarico-Petruzzelli M, Melino G, Amelio I. The hypoxic tumour microenvironment. Oncogenesis. 2018;7:10.

Pijuan J, Barceló C, Moreno DF, Maiques O, Sisó P, Marti RM, Macià A, Panosa A. *In vitro* Cell Migration, Invasion, and Adhesion Assays: From Cell Imaging to Data Analysis. Front Cell Dev Biol. 2019;7:107.

Prabhakar NR, Semenza GL. Oxygen Sensing and Homeostasis. Physiology (Bethesda). 2015;30(5):340-8.

Prossnitz ER, Maggiolini M. Mechanisms of estrogen signaling and gene expression via GPR30. Mol Cell Endocrinol. 2009;308(1-2):32-8.

Pupo M, Pisano A, Abonante S, Maggiolini M, Musti AM. GPER activates notch signaling in breast cancer cells and cancer-associated fibroblasts (CAFs). Int J Biochem Cell Biol. 2014; 46:56–67.

Pupo M, Pisano A, Lappano R, Santolla MF, De Francesco EM, Abonante S, Rosano C, Maggiolini M. Bisphenol A induces gene expression changes and proliferative effects through GPER in breast cancer cells and cancer-associated fibroblasts. Environ Health Perspect. 2012;120(8):1177-82.

Qin J-J, Yan L, Zhang J, Zhang W-D. STAT3 as a potential therapeutic target in triple negative breast Cancer: a systematic review. J Exp Clin Cancer Res. 2019;38(1):195.

Raab-Westphal S, Marshall JF, Goodman SL. Integrins as Therapeutic Targets: Successes and Cancers. Cancers. 2017;9, 110.

Rapisarda A, Melillo G. Overcoming disappointing results with antiangiogenic therapy by targeting hypoxia. Nat Rev Clin Oncol. 2012;9(7):378-90.

Recchia AG, De Francesco EM, Vivacqua A, Sisci D, Panno ML, Andò S, Maggiolini M. The G protein-coupled receptor 30 is up-regulated by hypoxia-inducible factor-1alpha (HIF-1alpha) in breast cancer cells and cardiomyocytes. J Biol Chem. 2011;286(12):10773-82.

Revankar CM, Bologa CG, Pepermans RA, Sharma G, Petrie WK, Alcon SN, Field AS, Ramesh C, Parker MA, Savchuk NP, Sklar LA, Hathaway HJ, Arterburn JB, Oprea TI, Prossnitz ER. A Selective Ligand for Estrogen Receptor Proteins Discriminates Rapid and Genomic Signaling. Cell Chem Biol. 2019;26(12):1692-1702.e5.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science. 2005;307(5715):1625-30.

Rigiracciolo DC, Santolla MF, Lappano R, Vivacqua A, Cirillo F, Galli GR, Talia M, Muglia L, Pellegrino M, Nohata N, Di Martino MT, Maggiolini M. Focal adhesion kinase (FAK) activation by estrogens involves GPER in triple-negative breast cancer cells. J Exp Clin Cancer Res. 2019;38(1):58.

Rigiracciolo DC, Scarpelli A, Lappano R, Pisano A, Santolla MF, De Marco P, Cirillo F, Cappello AR, Dolce V, Belfiore A, Maggiolini M, De Francesco EM. Copper activates HIF-1α/GPER/VEGF signalling in cancer cells. Oncotarget. 2015;6(33):34158-77.

Rose DP. Dietary fatty acids and cancer. Am J Clin Nutr. 1997;66:998S–1003S.

Rouhimoghadam M, Lu AS, Salem AK, Filardo EJ. Therapeutic Perspectives on the Modulation of G-Protein Coupled Estrogen Receptor, GPER, Function. Front Endocrinol (Lausanne). 2020;11:591217.

Ruggiero RJ, Likis FE. Estrogen: physiology, pharmacology, and formulations for replacement therapy. J Midwifery Womens Health. 2002;47(3):130-138.

Salamanna F, Borsari V, Contartese D, Costa V, Giavaresi G, Fini M. What is the role of interleukins in breast cancer bone metastases? a systematic review of preclinical and clinical evidence. Cancers (Basel). 2019;11:2018.

Samanta D, Gilkes DM, Chaturvedi P, Xiang L, Semenza GL. Hypoxia-inducible factors are required for chemotherapy resistance of breast cancer stem cells. Proc Natl Acad Sci U S A. 2014;111(50):E5429-38.

Samanta D, Semenza GL. Metabolic adaptation of cancer and immune cells mediated by hypoxia-inducible factors. Biochim Biophys Acta Rev Cancer. 2018;1870(1):15-22.

Sandén C, Broselid S, Cornmark L, Andersson K, Daszkiewicz-Nilsson J, Mårtensson UE, Olde B, Leeb-Lundberg LM. G protein-coupled estrogen receptor 1/G protein-coupled

receptor 30 localizes in the plasma membrane and traffics intracellularly on cytokeratin intermediate filaments. Mol Pharmacol. 2011;79(3):400-10.

Santolla MF, Lappano R, Cirillo F, Rigiracciolo DC, Sebastiani A, Abonante S, Tassone P, Tagliaferri P, Di Martino MT, Maggiolini M, Vivacqua A. miR-221 stimulates breast cancer cells and cancer-associated fibroblasts (CAFs) through selective interference with the A20/c-Rel/CTGF signaling. J Exp Clin Cancer Res. 2018;37(1):94.

Santolla MF, Lappano R, De Marco P, Pupo M, Vivacqua A, Sisci D, Abonante S, Iacopetta D, Cappello AR, Dolce V, Maggiolini M. G protein-coupled estrogen receptor mediates the up-regulation of fatty acid synthase induced by 17β-estradiol in cancer cells and cancer-associated fibroblasts. J Biol Chem. 2012;287(52):43234-45.

Santolla MF, Vivacqua A, Lappano R, Rigiracciolo DC, Cirillo F, Galli GR, Talia M, Brunetti G, Miglietta AM, Belfiore A, Maggiolini M. GPER Mediates a Feedforward FGF2/FGFR1 Paracrine Activation Coupling CAFs to Cancer Cells toward Breast Tumor Progression. Cells. 2019;8(3):223.

Schito L, Semenza GL. Hypoxia-Inducible Factors: Master Regulators of Cancer Progression. Trends Cancer. 2016;2(12):758-770.

Schödel J, Mole DR, Ratcliffe PJ. Pan-genomic binding of hypoxia-inducible transcription factors. Biol Chem. 2013;394(4):507-17.

Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, Giallongo A. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem. 1996;271(51):32529-37.

Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene. 2010;29(5):625-34.

Semenza GL. Hypoxia-inducible factors in physiology and medicine. Cell. 2012 A;148(3):399-408.

Semenza GL. Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. Trends Pharmacol Sci. 2012 B;33(4):207-14.

Semenza GL. Oxygen Homeostasis. Wiley Interdiscip Rev Syst Biol Med. 2010;2:336–61.

Semenza GL. Pharmacologic Targeting of Hypoxia-Inducible Factors. Annu Rev Pharmacol Toxicol. 2019;59:379-403.

Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer. 2003;3(10):721-32.

Semenza GL. The hypoxic tumor microenvironment: A driving force for breast cancer progression. Biochim Biophys Acta. 2016;1863(3):382-391.

Shen J, Cao B, Wang Y, Ma C, Zeng Z, Liu L, Li X, Tao D, Gong J, Xie D. Hippo component YAP promotes focal adhesion and tumour aggressiveness via transcriptionally activating THBS1/FAK signalling in breast cancer. J. Exp. Clin. Cancer Res. 2018, 37, 175.

Shimizu H, Nakayama KI. A 23 gene-based molecular prognostic score precisely predicts overall survival of breast cancer patients. EBioMedicine. 2019;4:150-159.

Shimoda M, Mellody KT, Orimo A. Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression. Semin Cell Dev Biol. 2010;21(1):19-25.

Sjöström M, Hartman L, Grabau D, Fornander T, Malmström P, Nordenskjöld B, Sgroi DC, Skoog L, Stål O, Leeb-Lundberg LM, Fernö M. Lack of G protein-coupled estrogen receptor (GPER) in the plasma membrane is associated with excellent long-term prognosis in breast cancer. Breast Cancer Res Treat. 2014;145(1):61-71.

Smythies JA, Sun M, Masson N, Salama R, Simpson PD, Murray E, Neumann V, Cockman ME, Choudhry H, Ratcliffe PJ, Mole DR. Inherent DNA-binding specificities of the HIF-1α and HIF-2α transcription factors in chromatin. EMBO Rep. 2019;20(1):e46401.

Soon WW, Hariharan M, Snyder MP. High-throughput sequencing for biology and medicine. Mol Syst Biol. 2013;9:640.

Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lønning PE, Børresen-Dale AL. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;98(19):10869-74.

Stanisavljevic J, Loubat-Casanovas J, Herrera M, Luque T, Peña R, Lluch A, Albanell J, Bonilla F, Rovira A, Peña C, Navajas D, Rojo F, García de Herreros A, Baulida J. Snail1-expressing fibroblasts in the tumor microenvironment display mechanical properties that support metastasis. Cancer Res. 2015;75(2):284-95.

Stutchbury B, Atherton P, Tsang R, Wang D, Ballestrem C. Distinct focal adhesion protein modules control different aspects of mechanotransduction. J. Cell Sci. 2017;130, 1612–1624.

Su S, Chen J, Yao H, Liu J, Yu S, Lao L, Wang M, Luo M, Xing Y, Chen F, Huang D, Zhao J, Yang L, Liao D, Su F, Li M, Liu Q, Song E. CD10+GPR77+ Cancer-Associated Fibroblasts Promote Cancer Formation and Chemoresistance by Sustaining Cancer Stemness. Cell. 2018;172(4):841-856.e16.

Subbaramaiah K, Dannenberg AJ. Cyclooxygenase 2: a molecular target for cancer prevention and treatment. Trends Pharmacol Sci. 2003;24:96–102.

Sutton KM, Hayat S, Chau NM, Cook S, Pouyssegur J, Ahmed A, Perusinghe N, Le Floch R, Yang J, Ashcroft M. Selective inhibition of MEK1/2 reveals a differential requirement for

ERK1/2 signalling in the regulation of HIF-1 in response to hypoxia and IGF-1. Oncogene. 2007;26(27):3920-9.

Takada Y, Kato C, Kondo S, Korenaga R, Ando J. Cloning of cDNAs encoding G protein-coupled receptor expressed in human endothelial cells exposed to fluid shear stress. Biochem Biophys Res Commun. 1997;240(3):737-41.

Theocharis AD, Skandalis SS, Gialeli C, Karamanos NK. Extracellular matrix structure. Adv. Drug Deliv. Rev. 2016, 97, 4–27.

Thomas P, Dong J. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. J Steroid Biochem Mol Biol. 2006;102(1-5):175-9.

Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology. 2005;146(2):624-32.

Thompson WD. Genetic epidemiology of breast cancer. Cancer. 1994;74(1 Suppl):279-87.

Tian H, McKnight SL, Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev. 1997;11(1):72-82.

Tice JA, Cummings SR, Smith-Bindman R, Ichikawa L, Barlow WE, Kerlikowske K. Using clinical factors and mammographic breast density to estimate breast cancer risk: development and validation of a new predictive model. Ann Intern Med. 2008;148(5):337-47.

Tomczak K, Czerwińska P, Wiznerowicz M. The Cancer Genome Atlas (TCGA): an immeasurable source of knowledge. Contemp Oncol (Pozn). 2015;19(1A):A68-77.

Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65(2):87-108.

Tulotta C, Ottewell P. The role of IL-1B in breast Cancer bone metastasis. Endocr Relat Cancer. 2018;25:R421–34.

Vamathevan J, Clark D, Czodrowski P, Dunham I, Ferran E, Lee G, Li B, Madabhushi A, Shah P, Spitzer M, Zhao S. Applications of machine learning in drug discovery and development. Nat Rev Drug Discov. 2019;18(6):463-477.

van den Brandt PA, van't Veer P, Goldbohm RA, Dorant E, Volovics A, Hermus RJ, Sturmans F. A prospective cohort study on dietary fat and the risk of postmenopausal breast cancer. Cancer Res. 1993;53(1):75-82.

Varešlija D, Priedigkeit N, Fagan A, Purcell S, Cosgrove N, O'Halloran PJ, Ward E, Cocchiglia S, Hartmaier R, Castro CA, Zhu L, Tseng GC, Lucas PC, Puhalla SL, Brufsky AM, Hamilton RL, Mathew A, Leone JP, Basudan A, Hudson L, Dwyer R, Das S, O'Connor DP, Buckley PG, Farrell M, Hill ADK, Oesterreich S, Lee AV, Young LS. Transcriptome

Characterization of Matched Primary Breast and Brain Metastatic Tumors to Detect Novel Actionable Targets. J Natl Cancer Inst. 2019;111(4):388-398.

Vito A, El-Sayes N, Mossman K. Hypoxia-Driven Immune Escape in the Tumor Microenvironment. Cells. 2020;9(4):992.

Vivacqua A, Bonofiglio D, Recchia AG, Musti AM, Picard D, Andò S, Maggiolini M. The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17beta-estradiol and hydroxytamoxifen in endometrial cancer cells. Mol Endocrinol. 2006;20(3):631-46.

Vona-Davis L, Rose DP. The obesity-inflammation-eicosanoid axis in breast cancer. J Mammary Gland Biol Neoplasia. 2013;18:291–307.

Waks AG, Winer EP. Breast Cancer Treatment: A Review. JAMA. 2019;321(3):288-300.

Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A. 1995;92(12):5510-4.

Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem. 1995;270(3):1230-7.

Wels J, Kaplan RN, Rafii S, Lyden D. Migratory neighbors and distant invaders: tumor-associated niche cells. Genes Dev. 2008;22:559–74.

Weng YS, Tseng HY, Chen YA, Shen PC, Al Haq AT, Chen LM, Tung YC, Hsu HL. MCT-1/miR-34a/IL-6/IL-6R signaling Axis promotes EMT progression, Cancer Stemness and M2 macrophage polarization in triple-negative breast Cancer.Mol Cancer. 2019;18:42.

Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, Ratcliffe PJ, Maxwell PH. Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. Blood. 1998;92(7):2260-8.

Wong CC, Gilkes DM, Zhang H, Chen J, Wei H, Chaturvedi P, Fraley SI, Wong CM, Khoo US, Ng IO, Wirtz D, Semenza GL. Hypoxia-inducible factor 1 is a master regulator of breast cancer metastatic niche formation. Proc Natl Acad Sci U S A. 2011;108(39):16369-74.

Wu TC, Xu K, Martinek J, Young RR, Banchereau R, George J, Turner J, Kim KI, Zurawski S, Wang X, Blankenship D, Brookes HM, Marches F, Obermoser G, Lavecchio E, Levin MK, Bae S, Chung CH, Smith JL, Cepika AM, Oxley KL, Snipes GJ, Banchereau J, Pascual V, O'Shaughnessy J, Palucka AK. IL1 Receptor Antagonist Controls Transcriptional Signature of Inflammation in Patients with Metastatic Breast Cancer. Cancer Res. 2018;78(18):5243-5258.

Xu M, Li Y, Li W, Zhao Q, Zhang Q, Le K, Huang Z, Yi P. Immune and Stroma Related Genes in Breast Cancer: A Comprehensive Analysis of Tumor Microenvironment Based on the Cancer Genome Atlas (TCGA) Database. Front Med (Lausanne). 2020;7:64.

Yang SL, Wu C, Xiong ZF, Fang X. Progress on hypoxia-inducible factor-3: Its structure, gene regulation and biological function (Review). Mol Med Rep. 2015;12(2):2411-6.

Yang XM, Wang YS, Zhang J, Li Y, Xu JF, Zhu J, Zhao W, Chu DK, Wiedemann P. Role of PI3K/Akt and MEK/ERK in mediating hypoxia-induced expression of HIF-1alpha and VEGF in laser-induced rat choroidal neovascularization. Invest Ophthalmol Vis Sci. 2009;50(4):1873-9.

Ye S, Xu Y, Li J, Zheng S, Sun P, Wang T. Prognostic role of GPER/Ezrin in triple-negative breast cancer is associated with menopausal status. Endocr Connect. 2019;8(6):661-671.

Ye S, Xu Y, Wang L, Zhou K, He J, Lu J, Huang Q, Sun P, Wang T. Estrogen-Related Receptor α (ERRα) and G Protein-Coupled Estrogen Receptor (GPER) Synergistically Indicate Poor Prognosis in Patients with Triple-Negative Breast Cancer. Onco Targets Ther. 2020;13:8887-8899.

Yeo SK, Guan JL. Breast Cancer: Multiple Subtypes within a Tumor? Trends Cancer. 2017;3(11):753-760.

Yoshida GJ. Regulation of heterogeneous Cancer-associated fibroblasts: the molecular pathology of activated signaling pathways. J Exp Clin Cancer Res. 2020;39(1):112.

Zhang XH, Jin X, Malladi S, Zou Y, Wen YH, Brogi E, Smid M, Foekens JA, Massagué J. Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma. Cell. 2013;154(5):1060-1073.

Zhang Y, Zhang H, Wang M, Schmid T, Xin Z, Kozhuharova L, Yu WK, Huang Y, Cai F, Biskup E. Hypoxia in Breast Cancer-Scientific Translation to Therapeutic and Diagnostic Clinical Applications. Front Oncol. 2021;11:652266.

Zheng SS, Chen XH, Yin X, Zhang BH. Prognostic significance of HIF-1α expression in hepatocellular carcinoma: a meta-analysis. PLoS One. 2013;8(6):e65753.

Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, Semenza GL. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res. 2000;60(6):1541-5.

Zhou X, Wang S, Wang Z, Feng X, Liu P, Lv XB, Li F, Yu FX, Sun Y, Yuan H, Zhu H, Xiong Y, Lei QY, Guan KL. Estrogen regulates Hippo signaling via GPER in breast cancer. J Clin Invest. 2015;125(5):2123-35.

Zografos GC, Panou M, Panou N. Common risk factors of breast and ovarian cancer: recent view. Int J Gynecol Cancer. 2004;14(5):721-40.

Zundel W, Schindler C, Haas-Kogan D, Koong A, Kaper F, Chen E, Gottschalk AR, Ryan HE, Johnson RS, Jefferson AB, Stokoe D, Giaccia AJ. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev. 2000;14(4):391-6.

Publications

1. Cirillo F., Pellegrino M., **Talia M.**, Perrotta I.D., Rigiracciolo D.C., Spinelli A., Scordamaglia D., Muglia L., Guzzi R., Miglietta A.M., De Francesco E.M., Belfiore A., Maggiolini M., Lappano R. Estrogen receptor variant Erα46 and insulin receptor drive in primary breast cancer cells growth effects and interleukin 11 induction prompting the motility of cancer-associated fibroblasts. Clinical and Translational Medicine. 2021.

2. Santolla M.F., **Talia M.**, Maggiolini M. S100A4 Is Involved in Stimulatory Effects Elicited by the FGF2/FGFR1 Signaling Pathway in Triple-Negative Breast Cancer (TNBC) Cells. Int J Mol Sci. 2021.

3. Rigiracciolo D.C., Cirillo F., **Talia M.**, Muglia L., Gutkind J.S., Maggiolini M., Lappano R. Focal Adhesion Kinase Fine Tunes Multifaced Signals toward Breast Cancer Progression. Cancers (Basel). 2021.

4. Muoio M.G., **Talia M.**, Lappano R., Sims A.H., Vella V., Cirillo F., Manzella L., Giuliano M., Maggiolini M., Belfiore A., De Francesco E.M. Activation of the S100A7/RAGE Pathway by IGF-1 Contributes to Angiogenesis in Breast Cancer. Cancers (Basel). 2021.

5. Iacopetta D., Lappano R., Mariconda A., Ceramella J., Sinicropi M.S., Saturnino C., **Talia M.**, Cirillo F., Martinelli F., Francesco Puoci F., Rosano C., Longo P., Maggiolini M. Newly Synthesized Imino-Derivatives Analogues of Resveratrol Exert Inhibitory Effects in Breast Tumor Cells. Int. J. Mol. Sci. 2020.

6. Lappano R., **Talia M.**, Cirillo F., Rigiracciolo D.C., Scordamaglia D., Guzzi R., Miglietta A.M., De Francesco E.M., Belfiore A., Sims A.H., Maggiolini M. The IL1β-IL1R signaling is involved in the stimulatory effects triggered by hypoxia in breast cancer cells and cancer-associated fibroblasts (CAFs). J Exp Clin Cancer Res. 2020.

7. Clericuzio M., Hussain F.H.S., Amin H.I.M., Bona E., Gamalero E., Novello G., Lappano R., **Talia M.**, Maggiolini M., Bazzicalupo M., Cornara L. Cytotoxic, Anti-bacterial, and Wound-healing Activity of Prenylated Phenols from the Kurdish Traditional Medicinal Plant Onobrychis Carduchorum (Fabaceae). Planta Medica International Open. 2020.

8. Rigiracciolo D.C., Nohata N., Lappano R., Cirillo F., **Talia M.**, Scordamaglia D., Gutkind J.S., Maggiolini M. IGF-1/IGF-1R/FAK/YAP Transduction Signaling Prompts Growth Effects in Triple-Negative Breast Cancer (TNBC) Cells. Cells. 2020.

9. **Talia M.**, De Francesco E.M., Rigiracciolo D.C., Muoio M.G., Muglia L., Belfiore A., Maggiolini M., Sims A.H., Lappano R. The G Protein-Coupled Estrogen Receptor (GPER) Expression Correlates with Pro-Metastatic Pathways in ER-Negative Breast Cancer: A Bioinformatics Analysis. Cells. 2020.

10. Santolla M.F., Vivacqua A., Lappano R., Rigiracciolo D.C., Cirillo F., Galli G.R., **Talia M.**, Brunetti G., Miglietta A.M., Belfiore A., Maggiolini M. GPER mediates a feedforward FGF2/FGFR1 paracrine activation coupling CAFs to breast cancer cells. Cells, 2019.

11. Rigiracciolo D.C., Santolla M.F., Lappano R., Vivacqua A., Cirillo F., Galli G.R., **Talia M.**, Muglia L., Pellegrino M., Nohata N., Di Martino M.T., Maggiolini M. Focal adhesion kinase (FAK) activation by estrogens involves GPER in triple-negative breast cancer cells. J Exp Clin Cancer Res, 2019.

12. Vivacqua A., Sebastiani A., Miglietta A.M., Rigiracciolo D.C., Cirillo F., Galli G.R., **Talia M.**, Santolla M.F., Lappano R., Giordano F., Panno M.L., Maggiolini M. miR-338-3p Is Regulated by Estrogens through GPER in Breast Cancer Cells and Cancer-Associated Fibroblasts (CAFs). Cells, 2018.

13. Mon M., Bruno R., Ferrando-Soria J., Bartella L., Di Donna L., **Talia M.**, Lappano R., Maggiolini M., Armentano D., Pardo E. Crystallographic snapshots of host–guest interactions in drugs@metal–organic frameworks: towards mimicking molecular recognition processes. Materials Horizons, 2018.