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“ The ID4-dependent reprogramming of Tumor-Associated Macrophages in Triple Negative Breast Cancer ”

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SUMMARY

Breast cancer is one of the most common cancer affecting especially women worldwide and it shows a particularly aggressive behavior in the triple negative (TNBC) and basal-like breast cancer (BLBC) subtypes that are characterized by poor prognosis and by the lack of targeted therapies. Moreover, it is well established that the presence of a massive leukocyte infiltrate, is involved in the promotion of tumor progression, contributing in particular to the angiogenic switch that occurs in the early phases of tumor progression. Among the variety of cells infiltrating breast tumors, macrophages have been extensively shown to tightly control the angiogenic onset and progression to malignancy. Here, we investigated whether ID4 protein, previously reported to enhance the angiogenic potential of breast cancer cells, exerts its function also modulating the activity of tumor-associated macrophages. We first assessed the significant association between the expression of ID4 and the macrophages marker CD68 in series of triple negative breast tumors. Of note, high ID4 mRNA expression in presence of a high macrophage infiltrate (determined as the expression of 8

macrophage markers) in BLBC is a strong predictor of poor survival. *In vitro* and *in vivo* migration assays evidenced that expression of ID4 in breast cancer cells is able to influence macrophages motility. At gene expression level we observed induction of ID4 itself, in macrophages co-cultured with breast cancer cells, induction that was impaired when breast cancer cells were depleted of ID4 expression. The same ID4-dependent behavior was observed for HIF-1A and for an angiogenesis-related signature in macrophages. Expression of angiogenesis-related genes was further controlled by miR-107, down-regulated in macrophages in ID4-dependent manner. Altogether our results highlight a key role for ID4 in dictating the angiogenic behavior of tumor-associated macrophages in breast cancer.

1. INTRODUCTION

1.1 Breast cancer

Breast cancer is one of the most common cancers in women worldwide, and one of the principal cancer death cause. Over 1.5 million women (25% of all women with cancer) are diagnosed with breast cancer every year in the world [(1)]. Survival rates for breast cancer vary worldwide, but in general outcomes have improved first of all thanks to the widespread use of mammographic screening that has increased the rate of early detection, especially in most developed countries [(2)], and also thanks to the development of more effective adjuvant chemotherapeutic regimens and the extended use of endocrine therapies [(3)].

Age, reproductive factors [(4)], personal or family history of breast disease [(5)], genetic pre-disposition [(6)], environmental factors, diet, alcohol, obesity, lifestyle, physical inactivity, as well as endocrine factors (both endogenous and exogenous) have been associated with an increased risk for the development of female breast cancer [(7)] [(8)]. Despite significant advances in diagnosing and treating breast cancer, it remains a complicated disease to

treat due to its highly heterogeneous nature at both the molecular and clinical level [9] [10].

1.1.1 Breast cancer molecular classification

The traditional breast cancer classification system includes immunohistochemistry (IHC) markers such as estrogen receptor (ER), progesteron receptor (PR) and human epidermal growth factor receptor 2 (HER-2), together with traditional clinicopathological variables as tumor size, tumor grade and nodal involvement [11]. Although these parameters are used for patient management, however they are not sufficient to reflect the complexity of breast tumors, so many studies based on global gene expression analyses have provided additional insights, leading to the identification of four molecular subtypes of breast cancer that show different behavior for incidence, survival and response to treatment (Luminal A, Luminal B, HER2-enriched and Basal-like) [12] [13].

Indeed, the most robust distinction observed by microarray analysis is between the transcriptome of estrogen receptor-positive (ER+) and estrogen receptor negative (ER-) breast cancers [14]. Evaluation of ER status is indeed determinant to predict patients outcome and response to therapy, in particular it

is important to determine the candidacy for endocrine therapy (ET) that is suitable for ER+ breast cancer patients in association with Tamoxifen [15] [16](Table 1).

1.1.2 Estrogen receptor positive (ER+) tumors: Luminal A and Luminal B

Luminal A and Luminal B tumors are both positive for the expression of the estrogen receptor and show expression patterns similar to 'normal luminal epithelial cells' of the mammary gland, including low molecular weight cytokeratins 8/18, ER and genes associated with ER pathway . The principal characteristic of this group is the luminal expression signature, composed of *Estrogen receptor1* (ESR1), GATA3, *Forkhead Box A1* (FOXA1), *X-box binding protein 1* (XBP1), and cMYB [13] [17] [18].

Luminal A tumors have been demonstrated to have low levels of proliferation related genes, be usually of low histological grade and have an excellent prognosis; whereas luminal B cancers are more often of higher histological grade, have higher proliferation rates and a significantly worse prognosis than luminal A tumours [13] [17] [19]. Luminal A breast cancer subtypes

show frequent mutations for *phosphoinositide-3-kinase,catalytic,alpha polypeptide* (PIK3CA), *mitogen activated protein kinase kinase kinase- 1* (MAP3K1) and GATA-3, *Tumor protein 53* (TP53), and *cadherin 1* (CDH-1). The most frequent mutations in luminal B tumors are TP53, PIK3CA and GATA-3 [18].

1.1.3 Estrogen receptor negative tumors: HER2 and basal-like breast cancer

The ER-negative group is significantly more heterogeneous and comprises two different subtypes, HER-2 and Basal-like breast cancer:

1. HER2-positive cancers show amplification and high expression of the *Human Epidermal Growth Factor Receptor 2* (HER-2 or ERBB-2). The HER-2 gene encodes a 185 kDa transmembrane protein that can acquire an active conformation, dimerizing with other *epidermal growth factor receptors* (EGFR) in a ligand independent manner; its overexpression is associated with a more aggressive disease, higher recurrence rate, and shortened survival [20]. However, since its approval

in 1998, the HER-2 positive breast cancer can be treated with the monoclonal antibody Trastuzumab, that targets the HER-2 receptor and has become the standard of care for the treatment of HER-2 positive breast cancer improving outcomes for early stage as well as metastatic breast cancer [21] .

2. The Basal-like and Triple negative breast cancer subtypes (BLBC, TNBC), that comprise a heterogeneous group accounting for up to 15% of all breast cancers, is very clinically interesting for its high frequency, lack of effective targeted therapies, poor prognosis, and the tendency to affect younger patients [22]The BLBC and TNBC group is characterized by a high aggressiveness, with an high risk of recurrence in the first 3 years and an high percentage of deaths in the first 5 years following therapy [12].The Basal-like subtype is often referred to as Triple-Negative Breast Cancers (TNBC) because most Basal-like tumors are typically negative for ER, PR and HER-2 expression. However, ~75% of TNBC are basal-like with the other 25% comprised of all other mRNA subtypes including mostly HER2+ breast cancer including mostly HER2+ breast cancer [23]. 25% of all TNBCs lack ER, PR, and HER2 in IHC but do not

exhibit the features of the basal-like subtype [22]. Basal like breast cancer can be described by different combination of Immunohistochemical markers, in particular the lack of ER, PR, and HER-2 expression, associated with the expression of high-molecular weight basal cytokeratins (CK5/6, CK14, and CK17) or epidermal growth factor receptor (EGFR) [24]. The majority of basal-like breast cancers is of high histological grade, high mitotic indices, with the presence of central necrotic or fibrotic areas and massive leukocyte infiltration [25]. *TP53* gene mutation is observed in up to 85% of cases; alterations of the pRB and p16 G1/S cell-cycle checkpoints are remarkably prevalent in these cancers. There is also increasing evidence of the presence of BRCA1 dysfunctional pathway in BLBC; indeed TNBC are enriched for germ-line BRCA1 mutations [18] [26]. Because of the lack of approved targeted therapy, at present chemotherapy remains the mainstay of treatment for early and advanced disease [15].

Table 1.

Intrinsic subtype	cDNA microarrays	IHC	Treatment
Luminal A	High expression of Er α gene, GATA3, FOX1	ER+, PR+, HER-2 negative, Ki67<14%	Endocrine therapy
Luminal B	Low to moderate expression of luminal specific genes including ER cluster	ER+, PR+, HER-2 negative, Ki67> 14%	Endocrine therapy +/+ chemotherapy
		ER+, PR+, HER-2+ With any Ki67	Chemotherapy + anti- HER2 therapy + endocrine therapy
HER-2 enriched	High expression of genes in the ERBB2 amplicon	ER -, PR -, HER-2 +	Chemotherapy + anti-HER2 therapy
Basal-like	High expression of keratins 5 and 17, laminin and fatty acid binding protein 7	ER-, PR-, HER2-	Chemotherapy

Table 1 Breast cancer intrinsic subtypes with IHC profiles and treatment option. ER: estrogen receptor, PR: progesterone receptor, HER-2: human epidermal growth factor receptor2, IHC: immunohistochemistry.(Modified from Toss & Cristofanilli, 2015.)

1.2 Tumor Associated Macrophages

Solid tumors, such as breast cancer, are not considerable as single entities but are closely linked to their microenvironment that consists of a large variety of cellular and non-cellular components such as stromal cells, blood vessels and leukocyte infiltrate. Bidirectional interactions between tumor cells and the tumor microenvironment, that are necessary both for the normal tissue homeostasis and for tumor growth, affect cancer progression, response to treatment, patients prognosis [(27)].

One of the most prominent stromal component of tumor microenvironment are macrophages, commonly termed *tumor associated macrophages* (TAMs), that has been largely demonstrated to promote tumor progression supporting tumor-associated angiogenesis, tumor cell invasion, migration and metastatization.

Moreover, many different clinical studies, show that an increase in tumor-associated macrophages (TAMs) density is correlated with poor prognosis in breast cancer [(28)] [(29)], and subset of proliferating TAM populations are associated with high grade, hormone receptor negative breast cancers with poor

outcomes and can be considered predictors of recurrence and survival [30] [31].

During tumor progression, circulating monocytes, originating as well as other macrophages in bone marrow, are actively recruited into tumors by the release of chemokines, cytokines, growth factors and hypoxia; once there, they differentiate in macrophages that can be polarized in alternative phenotypes in response to stimuli received, contributing to alter the tumor microenvironment with the release of different stimuli in turn [32]. The most important and characterized chemokine for monocytes recruitment in breast cancer is monocyte chemotactic protein-1 (MCP-1 or CCL2); indeed inhibition of CCL2-CCR2 signaling impairs the recruitment of inflammatory monocytes as many studies demonstrate in breast cancer mouse model; also the depletion of tumor-derived CCL2 has been demonstrated to inhibit metastatization in murine models [33].

In addition to CCL2 other chemokines as CCL3, CCL4, CCL5, CCL22 and CXCL8 have also been shown to be important for TAMs recruitment [32]. Among the cytokines, macrophage colony stimulating factor 1 (M-CSF-1) plays an important role in regulate infiltration and polarization of TAMs. Indeed, an elevated CSF-1 level correlates with marked macrophage infiltration in human metastatic breast cancer; conversely, using

mice homozygous for CSF-1 null mutations, it has been demonstrated that CSF-1 depletion leads to a marked reduction of the macrophages infiltrate that in turn determine a slower progression of tumor growth and a better prognosis for mices both in lung and breast models [34] [35].

However, growth factors including vascular endothelial growth factor (VEGF), endothelin 2, and platelet-derived growth factor (PDGF) have also been reported to promote monocyte/macrophage recruitment [36].

Another important attractant for macrophages is the hypoxic environment in the tumor that, in internal areas, releases chemo-attractants such as VEGF-A, Endothelin and Endothelial-monocyte-activating Polypeptide II (EMAP-II) that stimulate macrophages to migrate in response to hypoxia. When macrophages reach hypoxic areas they are trapped in because of the hypoxic upregulation of Mitogen-activated Protein Kinase Phosphatase 1 (MKP-1) enzymes.

MKP-1 dephosphorylates p44/p42 mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK 1 and 2, respectively) and p38 MAPK that require phosphorylation and thus activation to mediate migration of monocytes and monocytic cell lines in response to chemokines [37].

1.2.1 Macrophages polarization

Depending on the stimuli received from microenvironment macrophages can adopt a large variety of phenotypes that allow us to classify them in:

1. Activated macrophages (AM), commonly referred as M1 type
2. Alternatively Activated Macrophages (AAM), commonly referred as M2 type

Each polarized macrophage displays a differential expression profile of cytokines, enzymes, and cell-surface markers.

Lipopolysaccharide (LPS) or Interferon gamma (IFN-γ) drive the macrophages differentiation in M1 phenotype that mainly participates in inflammatory response and antitumoral immunity. Indeed, M1 macrophages release pro-inflammatory cytokines as IL-6, IL-12, IL-23 and tumor necrosis factor α (TNF-α) and express high levels of major histocompatibility complex class I and II (MHC-I, MHC- II), so they can be considered potent effector cells of the immune system [(38)].

Conversely, the M2 macrophages exert anti-inflammatory and pro-tumorigenic activities; they also have poor ability to antigen

presenting and are involved in angiogenesis promotion, wound healing and tissue remodeling. In line with these functions, M2 cells display high levels of scavenger, mannose and galactose type receptors. They can be further divided into subsets named M2a, M2b, M2c on the basis of the stimuli received; IL-4 or IL-13 stimulate the polarization in M2a phenotype; the activation of Toll like receptors (TLRs) or IL-1 receptor (IL1-R) polarize macrophages in M2b phenotype; IL-10, glucorticoids and TGF-β induce the M2c phenotypes [32] [38] [39] (Fig.1).

Based on their functions within the tumor microenvironment, TAMs are generally characterized as M2-like macrophages, especially in the latter stage of cancer progression when they exert typical immunosuppressive, anti-inflammatory and pro-angiogenic functions of the alternative macrophages phenotype; however many subpopulation with different functional states can coexist within the tumors, and probably it depends on their localization in tumor microenvironment and on the kind of stimuli received [39] [40].

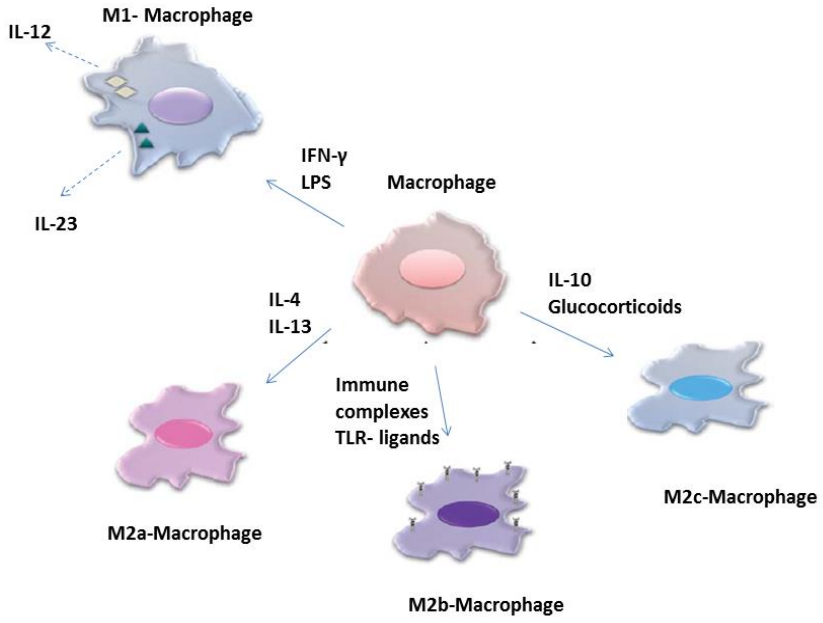

Figure 1 Macrophages polarization. Schematic representation of the polarization of macrophages: LPS or IFN-γ drive M1 macrophage polarization that in turn release IL-12 and IL-23. IL-4 and IL 13 polarize to M2a phenotype; activation of TLR by immune complexes or TLR-ligands polarize in M2b phenotype and IL-10, Glucocorticoids and TGFβ induce the M2c phenotype. (Modified from Obeid et al., 2013).

1.2.2 TAMs role in tumor progression

TAMs have been shown to be involved in tumor progression promoting angiogenesis, tumor growth, invasion and metastasis.

Angiogenesis:

It is now well established that TAMs play a pivotal role in promoting the angiogenic switch that consists on the development of a tumor vasculature necessary to sustain the increased metabolic demand of a growing tumor. A study by Lin et al., in a mouse model of breast cancer caused by the mammary epithelial cell restricted expression of the Polyoma middle T oncoprotein (PyMT mice), reported that the presence of macrophages in tumors, is positively associated with a high density of blood vessels; indeed depletion of macrophages, obtained using homozygous mice for a null mutation of CSF-1, determines a sensible reduction of vasculature in such tumor model [(41)].

Extensive studies have established the role of TAMs in promoting tumor angiogenesis or vascularization through the production and release of many pro-angiogenic growth factors and cytokines including VEGF, TNF-α, interleukin-8 (IL-8) and enzymes such as matrix metalloproteinase (MMP-2, MMP-7, MMP-9, MMP-12) that contribute to regulate the angiogenic process. In particular, macrophagic VEGF production is mainly driven by the hypoxic activation of Hypoxia inducible factor-1α (HIF-1A) that regulates the transcription of a large panel of angiogenesis related genes, including VEGF [(37) (41)].

VEGF expression itself is able to promote new vessels formation, even in CSF-1 null mice, but it also contributes to rescue macrophages recruitment that in turn stimulate the production of VEGF and other angiogenic factors underlining as macrophages play a pivotal role for angiogenesis. Macrophages are indeed responsible for VEGF supply both by its production and also by the production of MMP-9 that makes matrix-bound VEGF available, thus contributing to cell invasion [(42)].

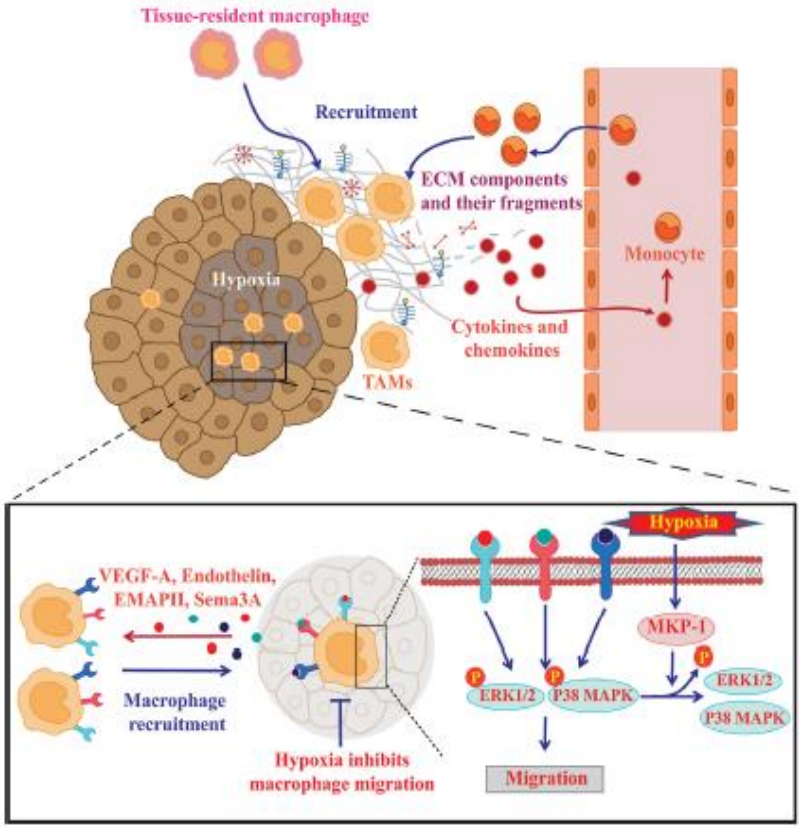

Figure2. Soluble Factors and Hypoxia Mediating Monocyte/Macrophage Mobilization into Tumors. Mechanisms of monocytes/macrophages recruitment into tumors mediated by environmental factors such cytokines, chemokines, ECM and hypoxia. Hypoxic areas enhance macrophage release in response to low oxygen concentration, releasing higher amount of chemoattractants such as EMAPII, endothelin, and VEGF-A; hypoxia also restrains macrophages by decreasing their mobility through the upregulation of MKP-1 enzymes and terminating the macrophage response to chemoattractants outside the hypoxic areas. **(**Modified from Chanmee et al.,2014)

Tumor growth, invasion and metastasis

Among TAMs contributions to tumor progression, there is also their ability to accelerate tumor growth by the expression and release of a large variety of factors that stimulate cell proliferation and survival, such as epithelial growth factor (EGF), PDGF, TGF-β and basic fibroblast growth factor (bFGF) [(43)].

TAMs are also involved in the metastatic process that represents a crucial phase of cancer progression, and it occurs when tumor cells acquire specific capabilities to leave the primary tumor, invade the surrounding matrix, and spread to other sites through blood vessels; TAMs are believed to directly and indirectly affect the metastatic process of tumor cells by modulating the tumor microenvironment.

The intense production of proteolytic enzymes like cathepsin B and S or MMPs, together with the evidence in PyMT induced mammary tumors of their tendency to localize along the basement membrane, represent important suggestions of their involvement in the degradation of the surrounding ECM allowing tumor cells to invade normal tissue crossing this barrier [44] [45].

Considering their important role in tumor progression TAMs are widely considered a potential biomarker for prognosis of cancer as well as potential therapeutic targets. The expression of macrophage markers in different tumor tissues is generally associated with worse clinical prognosis as well as a high density of infiltrated TAMs is associated with aggressive features [46] [47].

Therapeutic strategies against TAMs can be directed to restrict their recruitment into tumor tissue or their activation. Several ongoing clinical trials employ drugs against the CCL2/CCR2 axis that have proven to be effective in tumoral growth inhibition of several cancer models. CSF-1/CSF-1R signaling represent another target to limitate tumor recruitment and activation based on the evidence that Csf-1 null mice show slower progression of tumor growth and a better prognosis for mices in different kind of tumors [48] [49].

1.3 ID family of proteins (ID-1 to 4)

The ID family of proteins (Inhibitors of Differentiation, ID-1 to 4) is a group of dominant negative regulators of basic helix-loop-helix (bHLH) transcription factors. ID proteins lack the basic DNA-binding domain but maintain an intact HLH domain, so they can form heterodimers with bHLH transcription factors, inhibiting their ability to bind the DNA. Genes encoding ID proteins are paralogs well conserved amongst species, and they exert regulative role in different organisms maintaining high similarity in HLH domain [(50)].

While the HLH domain is highly conserved between the four proteins, the N- and C- terminal domains result to be different in ID4 respect the others ID proteins; this difference is due in particular to the presence of a poly-alanine rich tract, between residues 39 and 54 at the N-terminal, that in ID4 is independently evolved compared to its paralogs ID1, ID2, ID3 and that allow the ID4 N-terminal to adopt a helical conformation, differentiating ID4 from the others ID proteins.

Another structural peculiarity of ID4 protein is a proline-rich region present in the C-terminus that seems to be involved in

facilitating ID4 to form protein-protein interactions. It can be hypothesized that ID4 might exert unique functions through these structural features [51] [52]. ID proteins expression decreases during differentiation, becoming lower in mature tissues than in stem and progenitor cells; these proteins were described initially as inhibitors of differentiation and more recently as regulators of cell cycle progression [53], senescence, apoptosis [54] [55] and tumorigenesis [56]; moreover, depending on the cellular context in which they are expressed, ID proteins can exert opposite function, acting as oncoproteins or tumor suppressors [52].

1.3.1 Inhibitor of DNA binding protein 4 (ID-4)

Among ID proteins, ID4 in particular was shown to be involved in the differentiation of many cells including neurons [57] adipocytes [58] and osteoblasts, and in mammary gland development [59].

Its role as developmental regulator seems to contribute to confer stem-like properties such as strong renewal potential to transforming cells; such ability has been observed during the malignant transformation of primary murine astrocytes Ink4a/ARF−/−, where ID4 controls the expression of both Cyclin E and Jagged-1, driving the neural cells into a neural

stem-like state [60]. ID4 expression is found to be down-regulated, usually through its promoter hyper-methylation, in a large variety of tumours including leukaemia, prostate cancer, gastrointestinal carcinomas and breast cancer [51] [61]; while in such tumours it probably act as oncosuppressor, it has been reported to be up-regulated in many other tumours as glioblastoma multiforme (GBM), basal-like breast cancer and ovarian carcinoma [62] [63].

1.3.2 ID4 IN BREAST CANCER

Increasing evidences support a central role for ID4 in regulating mammary cell proliferation and lineage commitment. Transcriptome analyses by Lim et al. of mouse and human mammary cell subpopulations (e.g. luminal progenitors, committed/ mature luminal and basal cells) revealed that ID4 is one of the highest differentially expressed genes specific to basal cells [64].

ID4 controls luminal commitment and mammary stem/progenitor cell self-renewal, by suppressing the expression of several key pathways involved in appropriate luminal epithelial commitment. Breast Cancer Type 1 susceptibility

protein (BRCA1), Notch signaling, ERα and FOXA-1 are inhibited in their expression by direct interaction of ID4 with their promoter region, while ELF-5 is indirectly inhibited through the Notch signaling. Many of these factors, such as BRCA1 and ELF5 are well known to be involved in breast cancer, evidencing as ID4 plays an important role in mammary tumorigenesis [65] .

In particular, overexpression and amplification of ID4 has been reported in basal-like *BRCA-1*-mutated breast tumors and in ovarian cancer, and the complex regulatory network involving ID4, ERα and BRCA1 in these tumors still remains to be fully elucidated [66]. BRCA1 and ERα mRNA expression have been shown to correlate in sporadic breast cancers, while ID4 is negatively correlated to both BRCA1 and ERα in sporadic basal-like breast cancer [63] [67] .

However, in clinical breast cancer, ID4 expression is exclusive to ERα negative subtypes. Therefore, the role of ID4 in invasive breast cancer has been both tumor suppressive and oncogenic depending on the ER status [68].

Regulation of ID4 expression in breast cancer is a field of intensive study. The expression of ID4 can be induced by mutant p53 protein, that is recruited *in vivo* to ID4 gene promoter regulatory regions, in association with p65 (NF-kB)

and the transcription factor E2F-1, favouring the recruitment of the acetyltransferase p300 that indicate an active chromatin status. [(69)]. The activation of ID4 expression determines an enhancement of the angiogenic potential of mutant p53-carrying tumour cells. The mutant p53/ID4 axis promotes endothelial cells proliferation and migration *in vitro*. In addition, the analysis of human breast cancer cases revealed that a higher microvessels density is present in the ID4-positive population than in ID4-negative one. At the molecular level, ID4 protein is able to bind and stabilize the mRNAs of pro-angiogenic factors like CXCL8 (IL8) and CXCL1 (GRO-alpha), containing AU-rich (ARE) elements in their 3'UTR, resulting in a higher rate of translation of these transcripts [(69)] .

Moreover, we recently identified ID4 to be part of a ribonucleoprotein complex containing mutant p53, the splicing factor SRSF1 and the long non-coding RNA Malat-1. Malat-1 usually localizes to nuclear speckles where it modulates the activity of many splicing factors, including SRSF1 that has also been previously reported to control the expression of specific isoforms of VEGFA, a major player in tumour angiogenesis. SRSF1 bridges MALAT1 to mutant p53 and ID4 proteins in breast cancer cells, and mutant p53 and ID4 in turn delocalize MALAT1 from nuclear speckles and favor its association with

chromatin. This enables aberrant recruitment of MALAT1 on VEGFA pre-mRNA and modulation of pro-angiogenic 121 e 165 VEGFA isoforms expression [70]

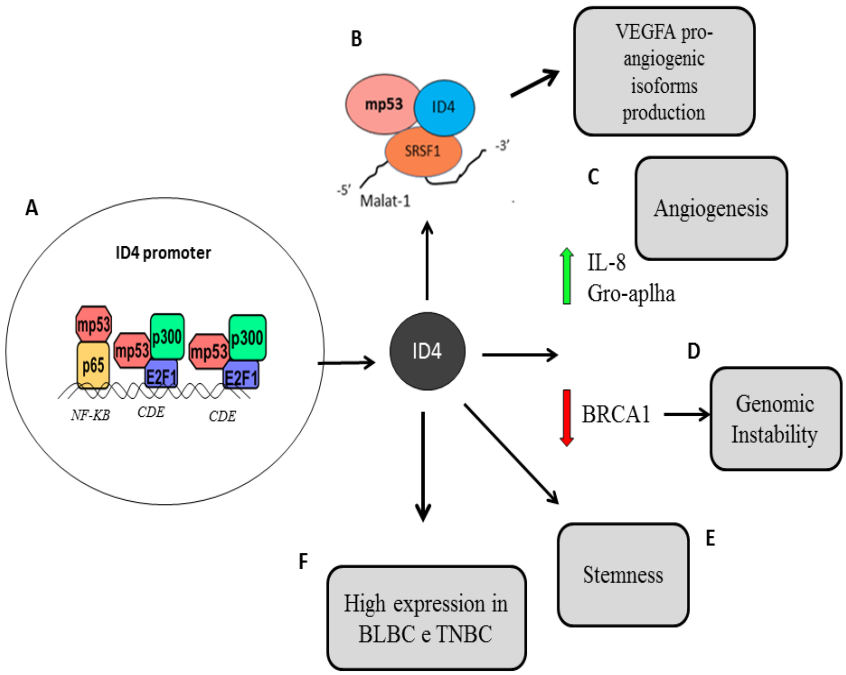

Figure 3. **Recapitulation of ID4 functions in Breast cancer. A** Inhibitor of differentiation 4 (ID4) promoter can be transcriptionally activated *in vivo* by mutant-p53 in association with p65, E2F-1 and p300 [69] **B** ID4 exert angiogenic functions as part of a molecular complex, comprising mut-p53, lnc-Malat-1 and SRSF1, that drives the alternative splicing of VEGFA isoforms favouring the production of the pro-angiogenic 165 e 121 [70] **C** Once synthesized ID4 protein is able to bind and stabilize the mRNAs of IL8 and CXCL1 (GRO-alpha), resulting in a higher rate of translation of these pro-angiogenic cytokines transcripts [69]. **D** ID4 expression is inversely correlated to the expression of BRCA1 leading to the enhancement of genomic instability [63]. **E** As reported in Junankar et.al, 2015 ID4 is a key controller of mammary stem/progenitor cell self-renewal, acting upstream of Notch signalling to repress luminal fate commitment [65]. **F** TNBC and BLB C are the breast cancer subtypes presenting the highest ID4 expression [71]

2. AIM OF THE PROJECT

Breast cancer is a heterogeneous disease, comprising a large variety of immune-histological, clinicopathological and molecular subtypes that show different behavior for incidence, survival and response to treatment. Among these subtypes, BLBC and TNBC are the most aggressive, due to the absence of targeted therapies and for the high probability of metastatization and recurrence in 3 years after therapy.

It has been well established that the presence of a massive leukocytes infiltration, especially of TAMs, which characterizes BLBC and TNBC, is associated with a poor prognosis. Among the most characterized abilities of TAMs there is the acceleration of tumor progression through the activation of angiogenic pathways that contribute to the development of a tumor vasculature necessary to sustain the growing tumor.

ID4 is a protein associated to stemness, proliferation and neo-angiogenesis in human cancers and it is highly expressed and prognostic in TNBC and BLBC.

The purpose of this study is to clarify the mechanisms through which ID4 controls angiogenesis in breast cancer. Starting from the observation that high ID4 expression in breast cancer cells is able to influence macrophage behavior in paracrine manner, we here investigated the molecular networks, comprising both mRNAs and microRNAs, controlling the ID4-dependent angiogenic program in tumor-associated macrophages.

3. RESULTS

3.1 ID4 expression in BC cells influences macrophages recruitment

We have previously shown that ID4 is able to drive the enhancement of angiogenic potential in breast cancer cells, as its expression is associated with an increase in the production of pro-angiogenic cytokines, as CXCL-1 (also known as GRO-alpha) and IL-8, involved in the proliferation and migration of endothelial cell, and it is also associated with high microvessels density in BC [(69) (70)].

As previously reported, macrophages are considered determinants of the onset of the angiogenic switch that is necessary for tumor progression [(41) (43)], therefore based on this and on the evident role of ID4 in angiogenesis, we wondered whether ID4 promotes angiogenesis also by influencing macrophages behavior and recruitment.

We first evaluated if the expression of ID4 in breast cancer cells is able to influence the migratory capacity of murine bone marrow-derived macrophages; to this end we isolated CD34+

progenitors from mouse bone marrow, differentiated *in vitro* to macrophages (Fig 4A) and allowed them to migrate in response to breast cancer cells depleted or not of ID4 expression (si- ID4 or si- SCR) (Fig 4B). We observed that a lower number of macrophages migrated in response to si-ID4 breast cancer cells compared to control si-SCR cells (Fig 4C). We then evaluated whether ID4 expression in breast cancer cells is able to induce the recruitment of macrophages also *in vivo*. To this end we performed matrigel assays, by the use of matrigel plugs containing conditioned medium (CM) from MDA-MB-468 breast cancer cells, transfected with an expression vector for HA-tagged ID4 or an empty vector as control, and inoculating plugs subcutaneously in mice flanks (Fig 4D-E). Matrigel plugs have been recovered after 7 days and stained by immunohistochemistry with mouse monocyte/macrophage marker F4/80, according to what previously reported [(72)]. We observed a higher number of F4/80+ cells in plugs containing CM from ID4-overexpressing cells compared to control plugs (Fig 4F-G).

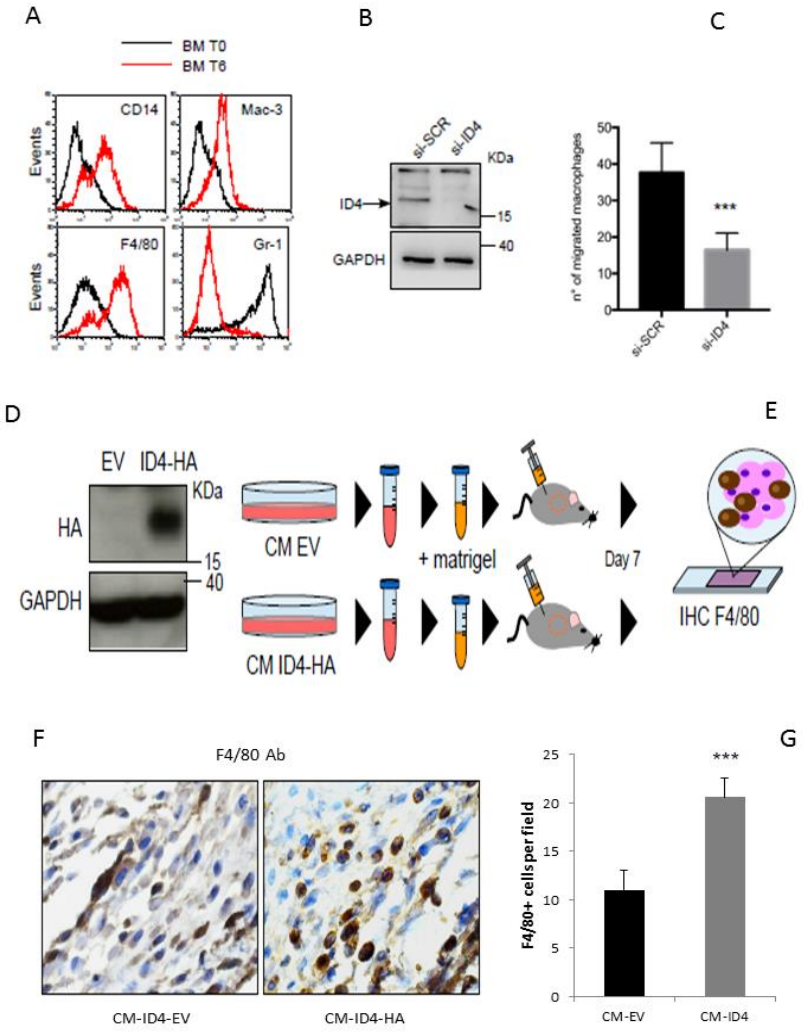

Figure 4 ID4 expression in breast cancer cells controls macrophages recruitment. **A** FACS analysis of differentiation markers in mouse bone marrow-derived before (T0) and after (T6) culturing in CSF-1 rich medium for 6 days.

B Western blot analysis of ID4 si-RNA interference in SKBR3 cells used for migration assay. **C** Trans-well migration assay of mouse bone marrow-derived macrophages in response to SKBR3 cells depleted (si-ID4) or not (si-SCR) for ID4 expression. **D** Western blot analysis showing efficacy of transfection of ID4 vector HA-tagged compared with an empty vector transfection in MDA-MB468 cells used to prepare conditioned media (CM). **E** Schematic representation of Matrigel assay **F** Immunohistochemical analysis of mouse macrophage marker F4/80 on matrigel plugs containing MDA-MB468 CM, overexpressing or not ID4-HA. **G** Counts of F4/80+ cells from at least 3 biological replicates as mean +/- SEM ***p<0.0005 calculated by two-tailed t test.

3.2 ID4 expression correlates with macrophage recruitment in triple-negative breast cancer

On the basis of the observed correlation between ID4 expression in breast cancer and tumor associated macrophages recruitment, we decided to evaluate whether any association existed between ID4 protein expression and infiltrating tumor-associated macrophages in human breast cancer. To this end we stained a series of 62 triple-negative breast cancers (TNBC), the breast cancer subtype showing the higher ID4 expression level among all breast cancer subtypes [(71)] (Fig 5 A-B) and in which high ID4 expression is associated with low probability of survival [(68)] for ID4 protein and for the widely used macrophage marker CD68 [(73)]. Based on the expression of ID4 protein, detectable as expected in 75% of the specimens, we divided the analyzed population in ID4-low (comprising negative and scored as 1+ tumors) and ID4-high (comprising scored 2+ and 3+ tumors); CD68 staining was scored as the infiltration density and was evaluated as follows: 0 absent, 1+ mild, 2+ moderate, 3+ dense. We interestingly observed that high CD68 protein expression was significantly associated with the ID4-high group (P= 0.028) (Figure 5C). Moreover, we didn't find any association between

ID4 and CD68 and other clinical or pathological variables in this group of patients. Representative images of TNBC showing high or low protein levels of ID4 and CD68 are shown in Figure 5D.

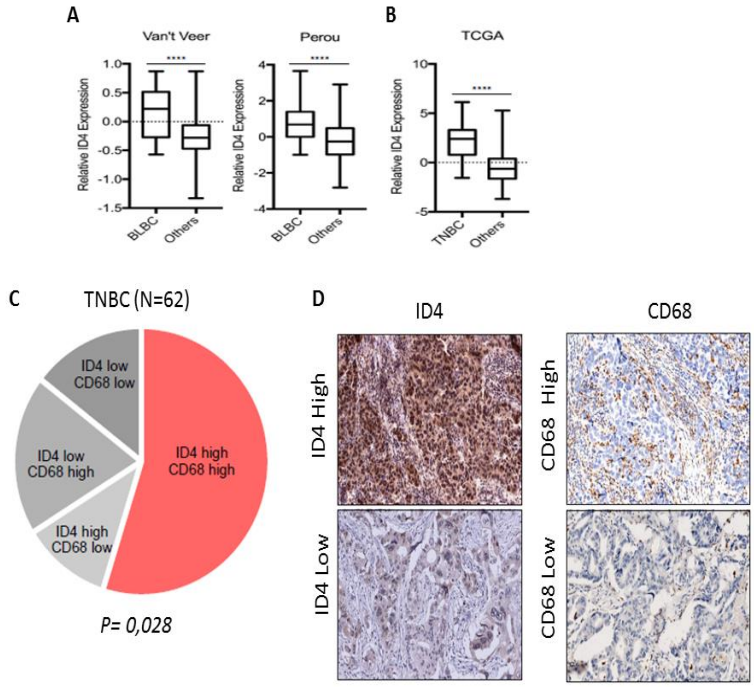

Figure 5 Inhibitor of differentiation 4 (ID4) protein and macrophage marker CD68 are significantly associated in triple-negative breast cancer (TNBC). A-B Comparison of ID4 mRNA expression in BLBC and TNBC versus all other breast cancer subtypes(others) **C-D** Staining of 62 TNBC for ID4 protein and for the macrophage marker CD68. Fisher's exact test evidenced that ID4 and CD68 expressions are significantly associated (P=0.028). **D** Representative images of TNBC showing high or low protein levels of ID4 or CD68.

3.3 ID4 expression predicts survival in tumors highly infiltrated by macrophages

Both ID4 high expression and macrophages infiltrate have been correlated with angiogenesis and have been shown to have a prognostic value in breast cancer; but if ID4 expression in TNBC and BLBC has been associated with decreased survival, the prognostic value of macrophages is contradictory, probably for the existence of different populations infiltrated in the tumor with different properties [33] [65] [68].

On this basis, we decided to investigate whether the expression of ID4 and the presence of macrophage infiltration in the tumor, are correlated with survival in BLBC cohorts of patients. To this end we interrogated the Kaplan- Meier Plotter database (www.kmplot.com), which contains a compendium of studies with gene expression and relative survival data for BLBCs.

Interestingly, we observed that high ID4 expression was strongly associated with low probability of DMFS (n = 232) specifically in the group of tumors characterized by high expression of CD68 (and therefore highly infiltrated by macrophages) (Fig 6 A), whereas no association of ID4 with survival was present in the low-CD68 group (Fig. 6 B).

A similar result was obtained when a macrophage signature comprising a subset of eight widely used markers (CD14, CD105, CD11b, CD68, CD93, CD33, IL-4R and CD163) for the mononuclear phagocyte system was used to identify tumors highly infiltrated by macrophages (Fig 6 C-D).

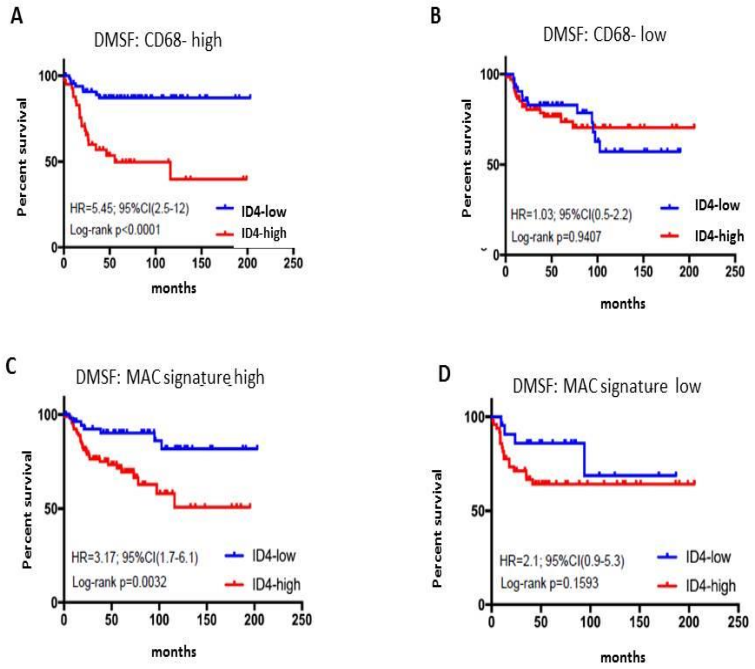

Figure 6. ID4 expression predicts survival in tumors highly infiltrated by macrophages. Kaplan-Meier analysis on kmplot database (**A-D**) of the predictive power of ID4 mRNA expression for distant metastasis-free survival (DMFS) (N = 232) evaluated in basal-like breast cancer (BLBC) showing high or low CD68 (A-B)or macrophage signature (MacSig) levels (C-D**)** composed of eight widely used markers for the mononuclear phagocyte system (CD14, CD105, CD11b, CD68, CD93, CD33, IL4R, and CD163

Analysis of gene expression data from The Cancer Genome Atlas (TCGA) cohort of BLBCs confirmed that high ID4 expression is associated to low probability of overall survival specifically in the CD68-high and macrophage signature (MacSig)-high groups (Fig 7 A-D).

The TCGA cohort allowed us also to assess that ID4 and CD68 do not associate with the clinical variables T, N and G, whereas ID4 significantly associates with mutated TP53 status. Moreover, because none of the considered patients from the TCGA cohort received neoadjuvant treatment, we can assert that the observed associations are independent of particular treatment regimens. These results indicated that the combination of ID4 and macrophage markers represents a powerful predictive indicator in BLBC.

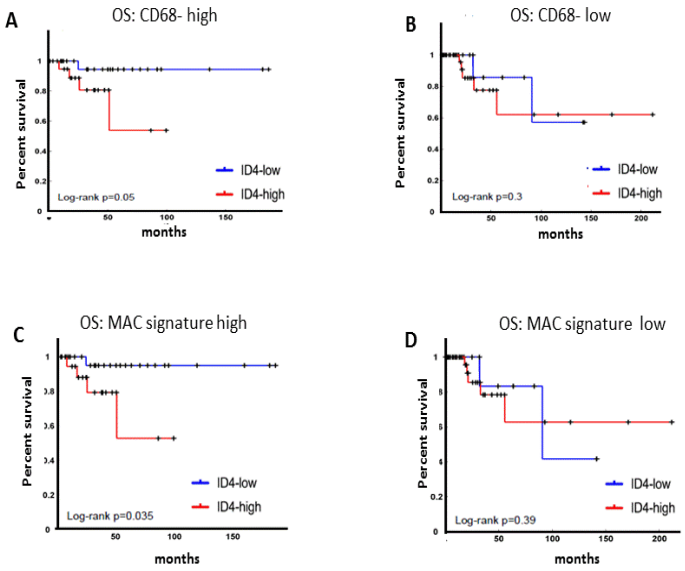

Figure 7 ID4 expression predicts survival in tumors highly infiltrated by macrophages Predictive power of *ID4* mRNA expression for overall survival (OS) was evaluated by Kaplan–Meier analysis on the TCGA cohort in BLBCs showing high or low CD68 (A-B) or macrophage signature (MacSig) (C-D) levels. Macrophage signature is composed of eight widely used markers for the mononuclear phagocyte system (CD14, CD105, CD11b, CD68, CD93, CD33, IL4R, and CD163)

3.4 ID4 expression in breast cancer cells determines ID4 induction in macrophages

We next decided to evaluate whether the expression of ID4 in BC was able to reprogram the gene expression of macrophages co-cultured with BC cells.

We first evaluated whether ID4 expression levels changed in monocytes/macrophages co-cultured with breast cancer cells compared to their monoculture.

As shown in Figure 8A, ID4 mRNA was induced in a time-dependent manner in human peripheral blood-derived monocytes (PBDM) grown in co-culture with SKBR3 breast cancer cells. Similar results have been obtained culturing U937 monocytic cells, with conditioned medium (CM) from two BC cell lines (SKBR3 and MDA-MB-468) (Fig 8 B-C). Moreover the induction of ID4 expression was assessed in HL60 cell line, after 72 hours of differentiation in VitD3 [(74) (75)], cultured with SKBR3 and MDA-MB-468 CM, both by mRNA quantification and by immunocytochemistry (Fig 8 D-E). Interestingly using other macrophage stimuli (LPS, TNF-alpha, IL-4/IL-13) to culture the HL60 cell line, we didn't observe any induction of the expression of ID4, indicating that the effect is specifically dependent on the CM from breast cancer cells (Fig 8F). Moreover, the modulation of ID4 expression is specific of macrophages, as no modulation of ID4 was observed in human fibroblasts cultured in CM from BC cells. (Fig 8G-H).

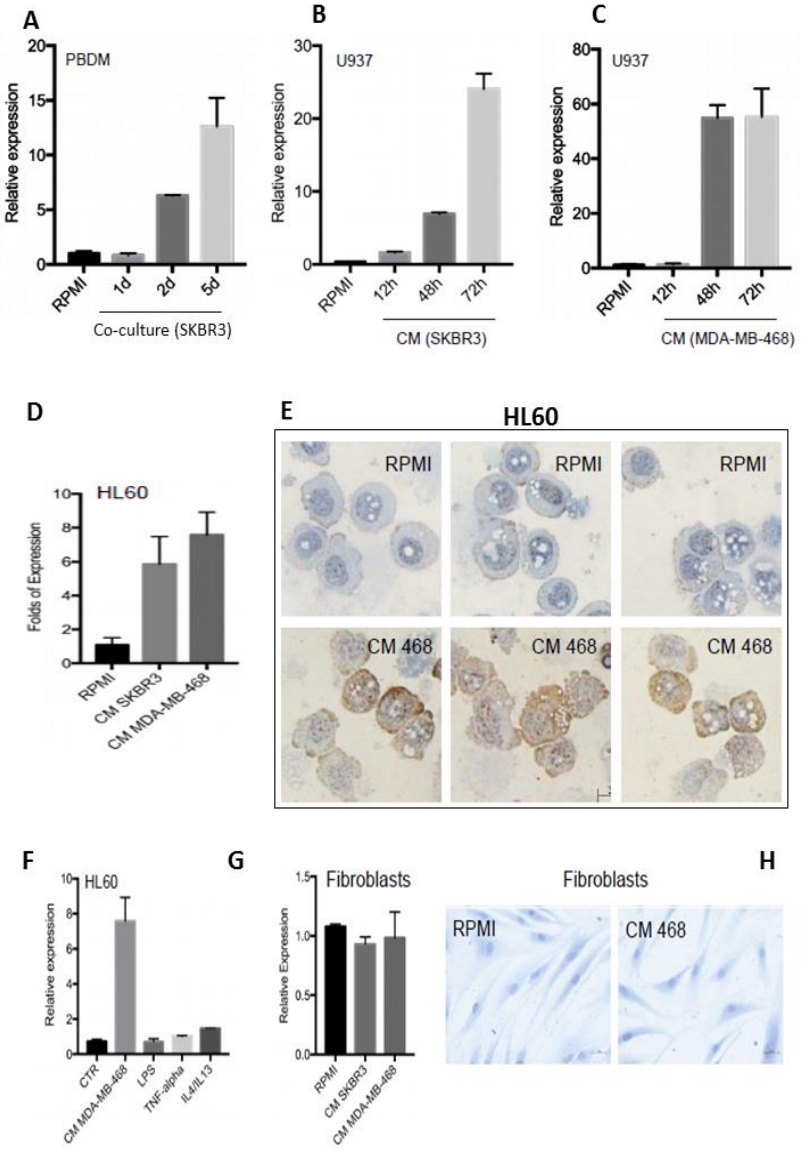

Figure 8 ID4 expression in macrophages is induced by CM from breast cancer cells. A. Time course of ID4 mRNA expression evaluated by RT-qPCR in PBMD co-cultured with BC cells (SKBR3). **B-C.** Expression of ID4 mRNA in U937 cells after culturing in RPMI or BC cell lines CM. **D**. ID4 mRNA expression in HL60 cells cultured in RPMI medium or in CM from SKBR3 or MDA-MB468 cell lines for 24h. **E.** ID4 protein was detected by immunocytochemistry (ICC) in HL60 cells cultured in RPMI medium or in CM from MDA-MB-468 (CM 468) cells for 24h. **F.** ID4 mRNA expression in HL60 cells cultured in CM from MDA-MB-468 cells or in RPMI medium containing the indicated macrophage-activating compounds [LPS 1µg/mL; TNF-alpha 50ng/mL; IL4/IL13 20ng/mL each]**G-H.** Human fibroblasts were grown

in presence of CM from MDA-MB-468 and SKBR3 cells and analyzed for ID4 mRNA (**G**) and protein (**H**) expression.

Moreover, a reduced ID4 induction in macrophages has been observed by culturing various macrophage experimental systems in conditioned medium from BC cells ID4-depleted by si-RNA transfection (si-ID4- 1 and si-ID4-2) compared to control CM (si-SCR) (Fig 9A-E). On the contrary, using CM from ID4-overexpressing breast cancer cells we observed stronger induction of ID4 mRNA in PBDM than in the control CM (empty vector, EV) in a time dependent manner (Fig 9F); in the same experimental conditions also HL60 cell line showed similar induction both at mRNA and protein level. (Figure 9G-H). Altogether these results demonstrated that breast cancer cells determined an induction of ID4 expression in monocytes/macrophages that is strictly dependent on the ID4 expression levels in breast cancer cells.

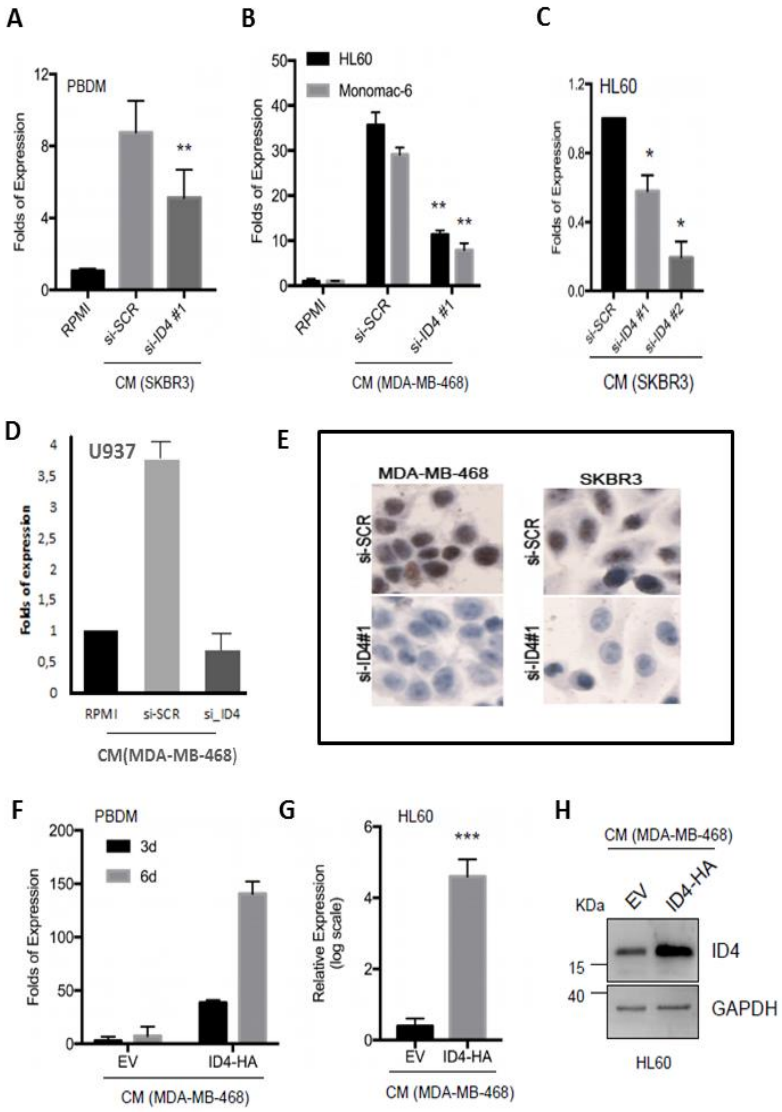

Figure 9 ID4 expression in breast cancer cells determines ID4 expression induction in macrophages. **A** ID4 mRNA expression on PBDM co-cultured with control (si-SCR) or ID4-depleted (si-ID4) SKBR3 cells for 48h. **B-C** ID4 mRNA expression in HL60 and Monomac cells cultured in RPMI medium or in CM from si-SCR or si-ID4 MDA-MB-468 and SKBR3 cells. **D** ID4 mRNA expression in U937 cultured in CM from si-ID4 MDA-MB-468 cells, versus si-SCR. **E** Immunocytochemistry analysis of ID4 protein levels in si- SCR vs. si-ID4 MDA-MB-468 and SKBR3 cells used for CM preparation **F** ID4 mRNA expression on PBDM cultured in CM from control (EV) and ID4-HA-overexpressing MDA-MB-468 cells evaluated after 3 days or 6 days of culture. **G-H** ID4 mRNA (G) and protein (H) expression evaluated on macrophages obtained from HL60 differentiation and

cultured in CM from control (EV) or ID4-HA-overexpressing (ID4-HA) MDA-MB-468 cells. **(P<0.005) P-values were calculated by two-tailed t-test.

3.5 ID4 induction in macrophages depends on ID4 promoter activation

To assess if the modulation of ID4 expression in macrophages, co-cultured with, or growth in the presence of CM from breast cancer cells, relies on the activation of endogenous ID4 promoter, we evaluated the status of chromatin on the promoter of ID4 in macrophages by Chromatin Immunoprecipitation (ChIP) experiments.

We cultured RAW 264.7 cells for 16 hours in CM from ID4 over-expressing BC cells (CM ID4-HA) and in CM from BC cells transfected with an empty vector (CM EV) as control, and we then analyzed the ID4 promoter by ChIP analysis using antibodies recognizing acetylation of histone H4 (H4Ac), correlated with an open chromatin status, and methylation of histone H3 lysine 9 (H3K9me3), highly correlated with constitutive heterochromatin.

As well as the induction of ID4 mRNA in RAW 264.7 cultured in presence of CM from ID4-HA breast cancer cells (Fig 10 A),

we also observed a reduction in the methylation status of the lysine 9 of the histone H3 and an induction of the acetylation of the histone H4; altogether these results are indicative of an activation of the ID4 promoter in macrophages in dependence of the expression of ID4 in BC cells (Fig. 10B).

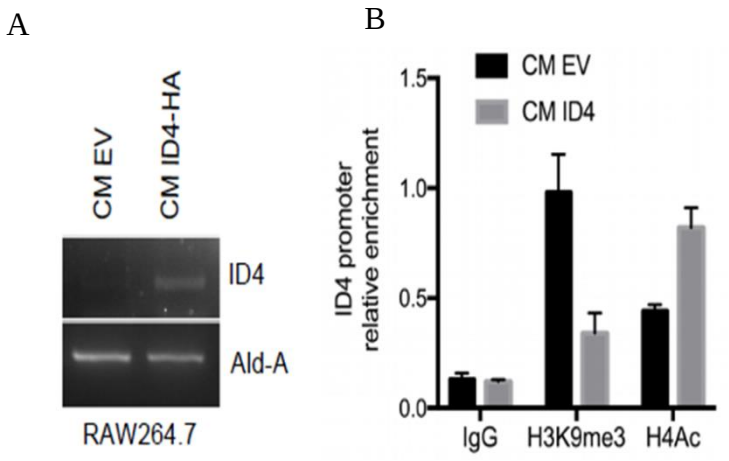

Figure 10 The induction of ID4 expression in macrophages is an endogenous event occurring on the promoter of ID4. A ID4 mRNA induction in RAW264.7 cells in presence of the conditioned medium from BC cells ID4-HA over-expressing compared with the expression of ID4 in presence of CM from EV transfected BC cells. **B** ChIP analysis of the ID4 promoter in RAW 264.7 cultured as in (D) using antibodies against the H3K9me3 and the H4Ac and normalizing the enrichment on a negative control genomic region.

3.6 ID4 expression in breast cancer cells determines the activation of a pro-angiogenic program in macrophages

As one of the main activities of TAMs is to promote angiogenesis, we next explored whether ID4 expression in BC cells affects the expression of angiogenic genes in macrophages, using a TaqMan Low Density Array (TLDA) containing probes for a panel of 94 angiogenesis-related genes. Macrophages, obtained from HL60 differentiation, cultured with CM from MDA-MB-468 cells transfected with an ID4 expression vector (ID4) or an empty vector (EV), as well as control macrophages cultured in RPMI medium, were evaluated. In this experimental setting we detected 36 expressed genes amongst which 11 genes modulated in ID4-dependent manner and we validated in the same experimental conditions some of them (Ephrin B2, Midkine, Granulin, Edil3) by RT-qPCR (Fig. 11 A) and Western Blot (Fig. 11 C). Interestingly, the same subset of genes didn't result modulated in MDA-MB-468 cells overexpressing ID4 (Fig 11 B).

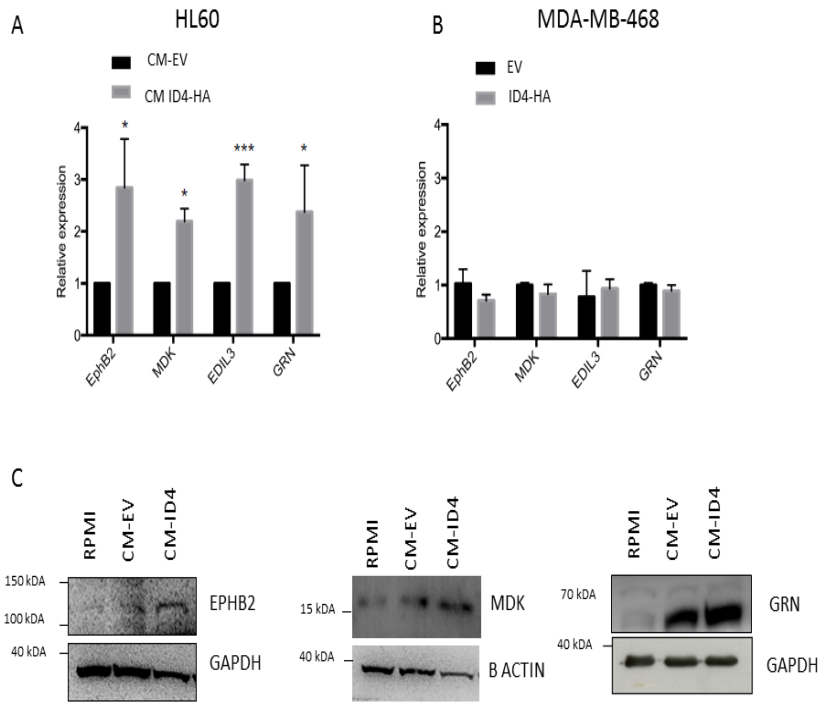

Figure 11. A. Validation of subset of angiogenic genes modulated in macrophages in ID4-dependent manner (A-C) A Validation by RT-qPCR of subset of angiogenic genes modulated in TLDA in differentiated HL60 cells cultured in CM from ID4 overexpressing MDA-MB-468 cells or control (CM EV). **B** Subset of genes as in (**A**) analysed in MDA-MB-468 cells transfected with ID4 vector or empty vector (EV). **C** Western blot analysis for EphB2, MDK and GRN in HL60 cells cultured as in (A).

Coherently, we observed an opposite effect in ID4-interference condition analyzing the expression a subset of the genes modulated in TLDA, comprising GRN, EphB2, NRP2; in addition to these we evaluated the expression of HIF1-A, a master regulator of angiogenesis [(76) (77)], and the expression of CXCL1 that we previously reported to be modulated by ID4

expression in breast cancer [69]. Specifically, in U937 cells cultured with CM from MDA-MB-468 cells depleted of ID4 expression (si-ID4) we observed a reduction of the mRNA induction of the selected genes (Fig. 12A) and a lower induction of the protein of GRN, HIF1A and EphB2, compared to control CM (si-SCR) (Fig. 12B-C). Interestingly, these results showed that ID4 expression in breast cancer cells is determinant to the activation of an angiogenic program in macrophages.

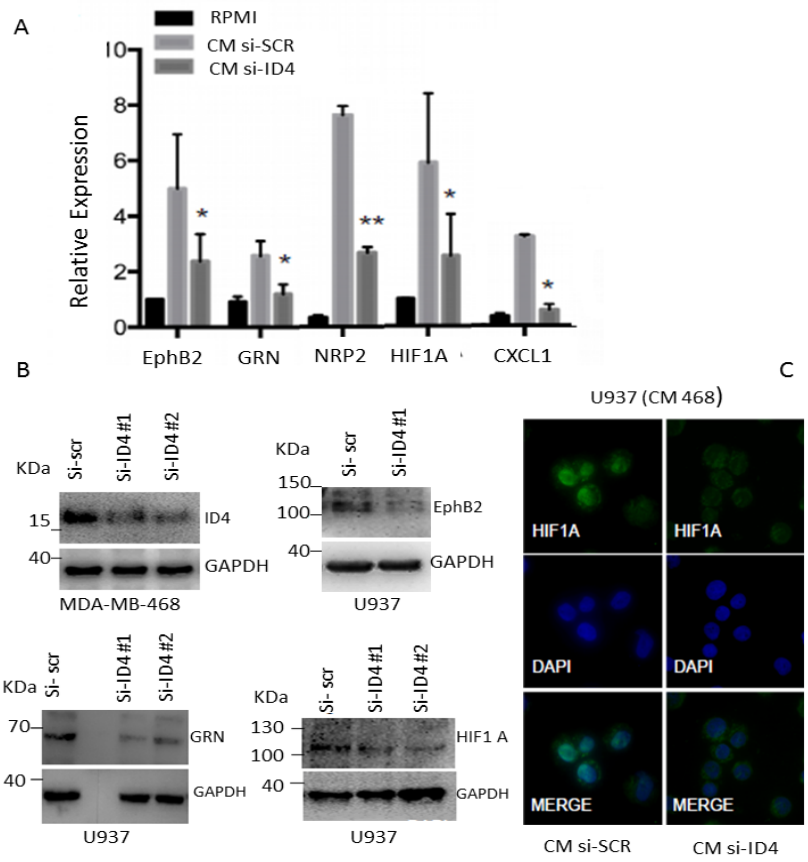

Figure 12 ID4 depletion in breast cancer cells determines down-regulation of angiogenic genes. **A** Relative mRNA expression evaluated by RT-qPCR of a subset of genes modulated in the arrays comprising EphB2, GRN, NRP2, HIF- 1A, CXCL1, in U937 cells cultivated in control medium (RPMI) or in conditioned medium from breast cancer cells ID4-depleted (si-ID4) or not (si-SCR). **B** Western blot analysis showing ID4 protein level after its depletion in MDA-MB-468 cells used to prepare the conditioned medium and the protein levels of EphB2, GRN and HIF- 1A in u937 cells cultured in breast cancer cells si-SCR or si-ID4 conditioned medium. **C** Immunofluorescence analysis of HIF-1A protein performed in differentiated U937 cells cultured in the presence of CM si-SCR or CM si-ID4 from MDA-MB-468 cells.

Based on the evidence that the expression of ID4 in breast cancer cells is able to drive the activation of the expression of angiogenesis related genes in macrophages, we hypothesized

that the crosstalk between BC cells and macrophages could be exerted by a soluble factor released from breast cancer cells in ID4-dependent manner.

In this regard, we recently reported that ID4 protein is involved in the synthesis of pro-angiogenic VEGFA isoforms in BC cells [70], so we postulated that the VEGFA could be responsible for the observed effects. To explore this hypothesis we first cultured differentiated U937 cells in CM from VEGFA-depleted (si-VEGFA) or control (si-SCR) BC cells and analyzed a panel of angiogenesis-related factors evidencing a partial decrease of their expression after VEGFA depletion (Fig.13A - B). Next, we observed that the addition of VEGFA blocking antibody to the CM from BC cells subsequently used to culture U937 cells, partially impaired the induction of this panel of angiogenesis-related factors (Fig.13C). These results indicated that ID4-dependent gene expression modulation in macrophages is at least in part under the control of VEGFA signaling.

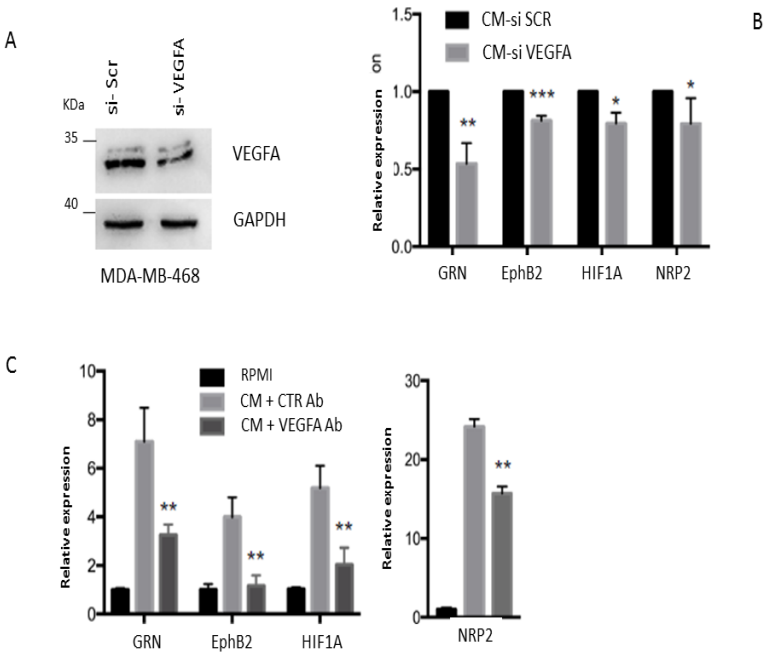

Figure 13 VEGFA partially mediates the crosstalk ID4 dependent between BC cells and macrophages. A Western blotting showing the efficiency of vascular endothelial growth factor A (VEGFA) depletion by si-RNA transfection in MDA-MB-468 cells used to prepare CM used in experiments shown in **B** RT-qPCR analysis of the indicated messenger RNAs in U937 macrophages cultivated in the presence of CM from control (si-SCR) or VEGFA-depleted (si-VEGFA) MDA-MB-468 cells. **C** RT-qPCR analysis of the indicated genes in differentiated U937 cells cultivated in RPMI medium or in CM from MDA-MB-468 cells in the presence of VEGFA blocking antibody (Ab) or a control Ab. Specifically, VEGFA blocking Ab or control Ab were incubated with CM for 30 minutes at room temperature and CM plus Ab was subsequently used to culture U937 cells for 48 hours. Results from at least three biological replicates are shown. Data are presented as mean ± SEM. *P < 0.05, **P < 0.005, ***P < 0.0005 calculated by two-tailed t test.

3.7 ID4 expression in breast cancer cells down-regulates anti-angiogenic microRNAs in macrophages

To fully explore the molecular mechanisms involved in the activation of the angiogenic program in macrophages, we hypothesized the presence of a post-transcriptional level of control by micro-RNAs; so we focused our attention on the members of the miR-15/107 group that has been previously correlated to angiogenesis and reported to target GRN and HIF-1B [78] [79] [80]; so we evaluated their expression in macrophages cultured with BC cells conditioned medium.

We observed that miR-107, miR-15b and miR-195 are down-regulated in macrophages cultured with CM from ID4-overexpressing BC cells (CM ID4) compared with macrophages cultured with CM from BC cells with control empty vector (CM EV) (Fig 14A). On the contrary, expression of these miRNAs was recovered in the presence of CM from si-ID4 BC cells in two macrophage cell lines U937 e HL60 (Fig 14B-G). We also evaluated the expression of miR-96, which exhibits oncogenic activity in BC [81], as a control of the specificity of the effect for our selected miRNAs, and we observed that it behaves in opposite manner, compared to miR-107 family members (Fig 14E) indicating that the effect observed was specific for our selected miRNAs.

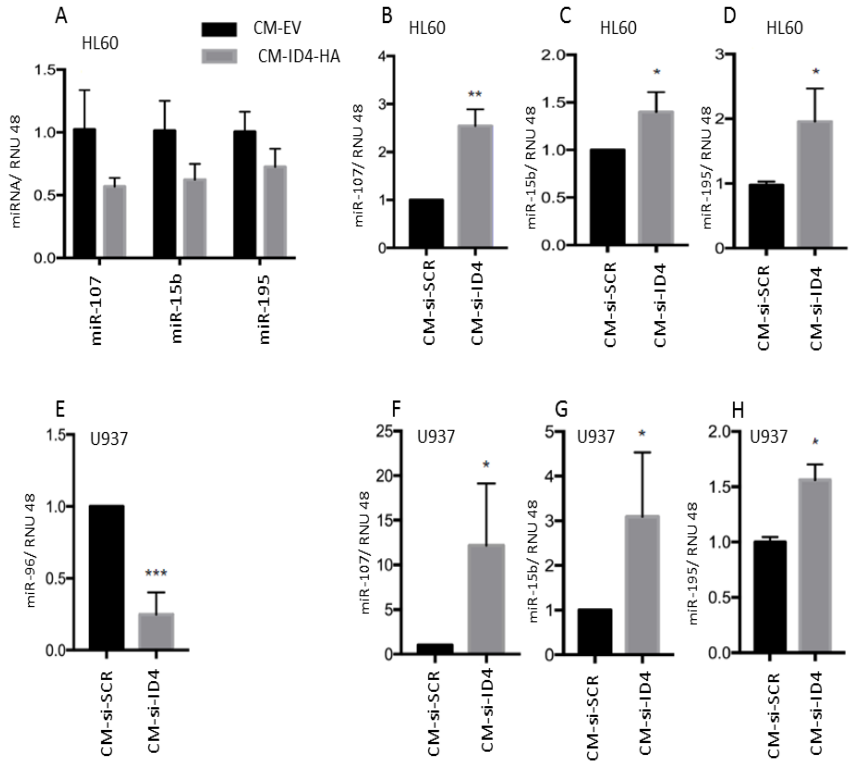

Figure 14 A Expression of miR-107, miR-15b and miR-195 in differentiated HL60 cells cultured with CM from control (CM EV) or ID4-overexpressing (CM ID4) MDA-MB-468 cells. **B, C, D, F, G, H** Expression of miR-15b and miR-195 in HL60 and U937 cells cultured with CM from control (si-SCR) or ID4-depleted (si-ID4) BC cells. **E** RT-qPCR for miR-96 in U937-derived macrophages as in B, C,D,F,G,H

Recovery of miR-107, miR-15b and miR-195 expression was also observed in U937 cells cultured in the presence of CM from VEGFA-depleted BC cells indicating that VEGFA signaling also controls, at least in part, miRNAs expression in TAMs (Fig. 15A). Next, we focused on miR-107, which shows the strongest ID4-dependent paracrine down-regulation in macrophages, and evaluated whether it affects the expression of GRN and HIF-1B,

two well-established targets [79] [80] [82]. To this end, we inhibited miR-107 in U937 cells by transfecting an LNA oligonucleotide (Fig.15B). As shown in Fig.14 C miR-107 inhibition recovered GRN and HIF-1B protein expression, mimicking the effect of the CM derivated from breast cancer cells.

We also observed induced protein expression of EphB2 and HIF-1A (Fig.15C), which, as the majority of the angiogenesis-related factors that are activated in an ID4-dependent paracrine manner in macrophages, are predicted to be targeted by the miR-15/107 group members. To further investigate the relevance of miR-107 down-regulation associated with CM, we overexpressed miR-107 using mimic oligonucleotides in macrophages cultured with CM from MDA-MB-468 BC cells (Fig.15D). The forced expression of miR-107 led to decreased GRN protein levels (Fig.15E) Similar results were observed for HIF-1A as showed by immunofluorescence analysis in Fig 15F. Our results indicated that the expression of angiogenesis-related genes is strictly controlled by the activity of the ID4-dependent miR-107 in macrophages.

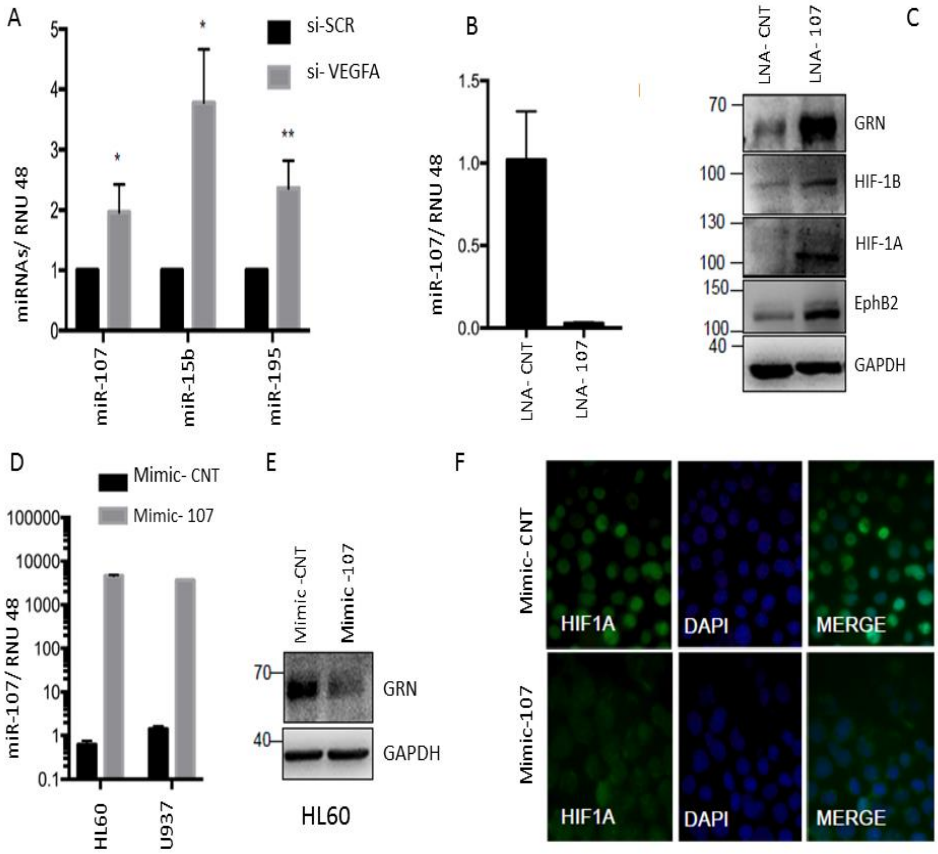

Figure 15 A miR-107, miR-15b and miR-195 expression, evaluated by RT-qPCR, in differentiated U937 cells cultured with CM from MDA-MB-468 cells depleted or not of VEGFA expression. VEGFA interference efficiency is shown in Figure 12A. **B** RT-qPCR analysis of miR-107 levels in differentiated U937 cells transfected with locked nucleic acid (LNA) antisense oligonucleotide directed to miR-107. **C** Western blot analysis of the indicated proteins in differentiated U937 cells transfected with LNA antisense oligonucleotide directed to miR-107. **D** miR-107 expression levels evaluated by RT-qPCR in HL60 and U937 cells transfected with control mimic or miR-107 mimic oligonucleotides. **E** Western blot analysis of GRN in HL60 transfected with control mimic or miR-107 mimic oligonucleotides. Results from at least three biological replicates are shown. Data are presented as mean ± SEM. *P < 0.05,**P < 0.005, ***P < 0.0005 calculated by two-tailed t test. **F** HIF1A expression evaluated by immunofluorescence in differentiated U937 cells transfected with control mimic or miR-107 mimic and cultured in CM from MDA-MB-468 cells for 48h

4. Materials and methods

4.1 Cell culture and reagents

The SKBR3, MDA-MB-468, HL60 and U937 cell lines were obtained from the ATCC (Manassas, US) and cultured in RPMI; RAW 264.7 in DMEM (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat inactivated FBS (Life Technologies), 100 Units/mL Penicillin and 100 µg/mL Streptomycin (Life Technologies). Cell lines were grown at 37°C in 5% CO2. DPBS (EuroClone, Milano, Italy) and Trypsin 0.05% (GE Healthcare Hyclone, Little Chalfont, UK) were used to wash and detach cells. HL60 and U937 cells were differentiated by treatment with 1,25-dihydroxyvitamin D3 (VitD3) (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 250 ng/ml.

Mouse bone marrow–derived macrophage precursors were obtained from rodents by flushing the femurs and tibias with 2% FBS in PBS. Differentiation was obtained by culturing precursors in CSF1-rich conditioned medium derived from L929 fibroblasts cell culture. Differentiation was evaluated by FACS

analysis using the following antibodies: anti-mouse F4/80 antigen APC (17-4801; eBioscience), Ly-6G (Gr-1) APC (17-5931; eBioscience, San Diego, CA, USA), CD14 PE (12-0141; eBioscience, San Diego, CA, USA) and CD107b (Mac-3) PE (12-5989; eBioscience, San Diego, CA, USA).

Human peripheral blood derived monocytes (PBDM) were isolated from blood donors using Lymphoprep solution (AXIS-SHIELD) followed by isolation of CD14+ cells with the Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Differentiation was obtained through a 1-week culturing in RPMI medium containing recombinant human CSF1.

Breast cancer cells and macrophages were co-cultured using 0.4 μm-pore 6-well Boyden chambers (Corning). Conditioned media (CM) from breast cancer cells were prepared by culturing cells for 24h in serum-free RPMI medium. CM were centrifuged to eliminate cell residues, before preparation of aliquots and storage at -80°C.

Monocytic differentiation was assessed by fluorescence-activated cell sorting (FACS) as previously reported [83] using allophycocyanin (APC) anti-human CD11b (BD Biosciences, San Jose, CA, USA), PerCP-Cy5.5 (peridinin chlorophyll protein complex-cyanine 5.5) anti-human CD14 (BD

Biosciences) and phycoerythrin-immunoglobulin G1 (PE-IgG1) isotype control (eBioscience Inc., San Diego, CA, USA) antibodies for the evaluation of CD11b-CD14 co-expression as a marker of monocytic differentiation. A minimum of 10,000 events were collected for each sample with a flow cytometer (CyAN ADP; Beckman Coulter Life Sciences, Brea, CA, USA) using Summit 4.3 software (Beckman Coulter Life Sciences) for data acquisition and analysis.

4.2 Cell transfection

An expression vector containing a hemagglutinin (HA)-tagged ID4 coding sequence or control empty vector was transfected in cancer cells using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) in ID4 overexpression experiments. RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to transfect small interfering RNAs (siRNAs) in BC cells. siRNAs were purchased from Eurofins MWG (Ebersberg, Germany). Monocytic cell lines were transfected with mimic oligonucleotides and locked nucleic acid (LNA) oligonucleotides (Dharmacon, Lafayette,

CO,USA) using TransIT- X2 Dynamic Delivery System (Mirus Bio LLC, Madison, WI, USA) following manufacturer's instructions. Si-RNA sequences used for ID4 silencing as follow:

Scr- CTATAACGGCGCTCGATAT,

ID4_1-GATCCTGCAGCACGTTATC,

ID4_2 TTACAGAGCTCTTGATATC

VEGFA si-RNA sequences are TriFecta, synthesized from Integrated DNA technologies (IdT).

4.3 In vitro and in vivo macrophage migration assays

Migration of mouse bone marrow–derived macrophages in response to SKBR3 cells was evaluated using 3 μm-pore Boyden chambers (Corning Inc., Corning, NY, USA). Infiltration of F4/80+ macrophages in Matrigel plugs containing CM from breast cancer MDA-MB-468 cells was evaluated by subcutaneous inoculation of a solution composed by 500 μl of matrigel (BD Bioscience) and 50 μl of 10X-concentrated CM. In the negative control, the CM was replaced with SFM. Plugs

were recovered at day 7, fixed for 18–24 hours in 4% (v/v) buffered formaldehyde and then processed through to paraffin wax. Immunohistochemistry was performed using F4/80 antibody (MA5-16363; Pierce Biotechnology, Rockford, IL, USA). All procedures involving animals and their care were conducted in conformity with the institutional guidelines, which are in compliance with national and international laws.

4.4 Immunohistochemistry, immunocytochemistry and immunofluorescence

BC specimens for Immunohistochemistry analysis were fixed for 18–24 hours in 4% (v/v) buffered formaldehyde and then processed through to paraffin wax. Anti-ID4 (MAB4393, EMD Millipore, Billerica, MA, USA), anti-ER (clone 6F11, Novocastra, Menarini, Florence, Italy) anti-PgR (clone 1A6, Novocastra), anti-HER-2 (A0485; Dako, Milan, Italy), were evaluated by immunohistochemistry on 5-µm-thick paraffin-embedded tissues. Monoclonal antibody directed against ID4 was incubated at a dilution of 1:200 overnight at 4°C, and monoclonal antibodies (MoAb) anti-ER, anti-PgR and the

polyclonal antibody anti-HER-2 were incubated for 60 minutes at room temperature. Immunoreactions were revealed by a streptavidin-biotin enhanced immunoperoxidase technique (Super Sensitive MultiLink; BioGenex, Fremont, CA, USA) in an autostainer (Bond III; Leica Biosystems, Wetzlar, Germany). Diaminobenzidine (DAB) was used as a chromogenic substrate. Evaluation of the IHC data was performed independently and in blinded manner by 2 investigators (E.G. and M.E.).

For immunocytochemistry assay, cells were seeded onto glass coverslips (Paul Marienfeld, Lauda-Königshofen, Germany) in 6-well dishes (Corning Inc.) at 4×10^4 cells/ well, cultured with RPMI or CM, and fixed with 4% formaldehyde in PBS for 15 minutes at room temperature. Cells were permeabilized with 0.25% Triton X-100 in PBS for 10 minutes. After washing with PBS, the coverslips were incubated with anti-ID4 antibody diluted in 5% bovine serum albumin (BSA)/PBS for 2 hours at room temperature. Cells were incubated with peroxidase inhibitor before primary antibody incubation. Protein staining was revealed through DAB enzymatic reaction, and nuclei were counterstained with haematoxylin.

For immunofluorescence, cells grown in the presence of RPMI or CM (48 hours), as well as cells transfected with mimic oligonucleotides (48 hours), were concentrated onto microscope

slides using cytospin and fixed and permeabilized as already described. Slides were blocked for 30 minutes in 5% BSA/PBS at room temperature and then incubated with an anti-HIF-1Aantibody (A300-286A; Bethyl Laboratories, Montgomery, TX, USA) diluted in 5% BSA/PBS for 2 hours at room temperature. Cells were incubated with secondary antibody Alexa Fluor 594 (1:500; Thermo Fisher Scientific) for 45 minutes. Nuclei were stained with DAPI (Thermo Fisher Scientific).

4.4 Western blotting and antibodies

For the Western blot analysis, cells were lysed on ice for 30 minutes in radioimmunoprecipitation assay buffer (RIPA), supplemented with protease and phosphatase inhibitors (1 mM CI, 5 mM PMSF, 1 mM DTT, 1 mM Na_2VO_4), or in urea 8M at room temperature. Cell lysates were sonicated and the protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The lysate was mixed with 4 × Laemmli buffer. Equal amounts of total protein extracts were resolved on 10% or 15% denaturing SDS polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membrane. Membranes were blocked with 5%-

milk-TBS 0.05% Tween-20 for 2 h and incubated overnight with specific primary antibodies. The following primary antibodies were used: Gapdh (sc-32,233), ID4 (H70) sc-13047, ID4 (B5) sc-365656, HA (12CA5) sc-57592 (Santa Cruz Biotechnology, Dallas, TX, USA); HIF-1A (A300-286A; Bethyl Laboratories); GRN (PA5-29909), EphB2 (PA5-14607), Mdk (PA5-30601; Thermo Fisher Scientific), HIF-1B (clone 1A1, Origene). Secondary antibody fused with horseradish peroxidase was used for chemiluminescence detection on a UVITEC instrument (Uvitec, Cambridge, UK). VEGFA blocking antibody (AF-293-NA; R&D Systems, Minneapolis, MN, USA) was added to CM and incubated for 30 minutes at room temperature before being used to culture macrophages, following the manufacturer's instructions.

4.5 RNA isolation, RT-qPCR and TaqMan Low Density Arrays (TLDA)

RNA was isolated with TRIzol reagent (Sigma-Aldrich), following the manufacturer's instructions and its concentration was measured using a NanoDrop 2000 instrument (NanoDrop Technologies, Wilmington,DE, USA). Reverse transcription was

performed with Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific). Rt- qPCR was carried out on an ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The expression values of mRNAs were calculated by the standard curve method and normalized with housekeeping control genes (GAPDH, β-actin, H3). RT- qPCR using TaqMan Low Density Arrays (TLDA) Human Angiogenesis (4378725; Thermo Fisher Scientific) was carried out following the manufacturer's instructions on an ABI PRISM 7900HT Sequence Detection System. The following primers were used:

Table 2

ID4	F	5' GTGCGATATGAACGACTGCT 3'
	R	5' CAGGATCTCCACTTTGCTGA 3'
EphB2	F	5': CGTGGAAGAAACGCTAATGG 3'
	R	5'- : TGACTCAAACACGTTGCACA -3'
NRP2	F	5' GTCTCCTACAGCCTAAACGGCA-3'
	R	5' GGGTCAAACCTTCGGATGTCAG 3'
HIF 1A	F	5' ATCTGCAGGTCCCCATTCAA 3'
	R	5' ATGTGTGTGTGTCGTGTGTG 3'

VEGFA (all isoforms)	F	5’ CTTCCTACAGCACAACAAATGTG 3’
	R	5’ GTCTTGCTCTATCTTTCTTTGG 3’
RPL19	F	5’- CGGAAGGGCAGGCACAT -3’
	R	5’- GGCGCAAAATCCTCATTCTC- 3’
GAPDH	F	5’ -GAGTCAACGGATTTGGTCGT -3’
	R	5’- GACAAGCTTCCCGTTCTCAG-3’
Beta-ACTIN	F	5’- GGCATGGGTCAGAAGGATT- 3’
	R	5’- CACACGCAGCTCATTGTAGAAG-3’
CXCL1	F	5’-GCGCCCAAACCGAAGTC-3’
	R	5’- TGCAGGATTGAGGCAAGCTT- 3’
GRN	F	5’- CAGTGGGAAGTATGGCTGCT- 3’
	R	5’- TTAGTGAGGAGGTCCGTGGT- 3’
MDK	F	5’- CCTGCAACTGGAAGAAGGAG- 3’
	R	5’- CTGGCACTGAGCATTGTAGC- 3’
H1H2 BA	F	5’-ACTCTCCTTACGGGTCCTCTTG- 3’
	R	5’-AGTGCTGTGTAACCCTGGAAAA- 3
ID4 murine promoter	F	5’-AAGCAAATTGCGGGCGGGGA- 3’
	R	5’- CGGGCTCACCGCCTTCATCG- 3’

Table 2 RT-PCR primers

4.6 Chromatin immunoprecipitation assay

Cells were cross-linked using 1% formaldehyde for 10 min at room temperature before the reaction was stopped by the addition of 0.125M Glycine, and washed three times with ice-cold PBS. Cells were lysed in Buffer A (5 mM Pipes pH 8, 85 mM KCl, 0.5% NP-40, 1 mM CI), centrifuged at 400 RCF for 10 min at 4°C, resuspended in Buffer B (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8) and sonicated to shear DNA to lengths of approximately 200 bp. The chromatin solution was immunoprecipitated with rabbit anti-H3K9Me3 (Abcam), H4Ac (Abcam) or IgG (Santa Cruz) as negative control. IPs were performed using Protein A/G magnetic beads (Thermo Fisher Scientific, Rockford,IL, USA). The immunoprecipitated and purified chromatin was subjected to quantitative PCR analysis (qPCR). Data were normalized to the amount of Input chromatin.

5. DISCUSSION

TAMs are considered important players in solid tumor progression and, in particular in breast cancer, their presence as part of the leukocyte infiltrate can be considered predictor of poor outcome, recurrence, survival and it has been associated with high grade, hormone receptor negative breast cancers [31] [73]. ID4 high levels have been previously correlated with decreased survival in BLBC and TNBC [23] [65]. Here we demonstrated that the presence of high ID4 expression is predictor of poor outcome for overall and distant metastasis free survival specifically in BLBC cohorts showing high macrophage infiltration (Fig. 6e 7).

In this study we also demonstrated that ID4 in is able to induce macrophage migration and recruitment in BC and that its expression in breast cancer cells determined a reprogramming of macrophages gene expression, leading to induction of angiogenesis related genes.

Interestingly, we observed that ID4 was able to modulate its own expression in macrophages co-cultured with breast cancer cells, and we determined that it was a macrophage-specific

event that did not involve other stromal cells such as fibroblasts, and that it occurred through an activation of ID4 promoter. This enhancement in ID4 expression in macrophages could lead to the recapitulation of one of the main ID4 functions in breast cancer cells, the pro-angiogenic one, that has been previously reported to be exerted by ID4 through both favoring the production of CXCL-1 and IL-8 cytokines and the production of proangiogenic VEGFA isoforms, finally enhancing micro-vessels density [(69)]; so it will be interesting to further investigate whether also ID4-expressing macrophages recapitulate ID4-dependent functions.

In parallel to ID4 expression induction in macrophages, we observed a strong modulation of a panel of angiogenesis-related genes, among which there are some important regulators of inflammation and angiogenesis as Granulin, CXCL-1 and HIF-1A.

In particular Granulin captured our interest because since its initial discovery it has been revealed to be an important molecule in a wide variety of disease processes. GRN level is considered a prognostic biomarker for many forms of cancer as PGRN overexpression is associated with cancer cell proliferation and migration [(84)].

Moreover, it is a growth factor that is specifically expressed in TNBC and BLBC where it has been positively correlated to aggressiveness and chemoresistance [85].

In macrophages, GRN has been reported to control cytokines production, but its effect on the angiogenic potential of these cells has been only characterize to its function in wounds repairs in mice, where it has been reported to increase fibroblasts, macrophages and capillaries that enter the wounds [86], and only recently it has been reported to contribute to angiogenesis in mesothelioma [87].

It has been extensively explored GRN's role in inflammation since it is a potent inhibitor of the inflammatory cytokine tumor necrosis factor-a (TNF-alpha); it has been indeed reported that GRN directly binds to tumor-necrosis-factor receptors (TNFR-1 and TNFR-2) and counteracts the TNF-mediated inflammatory signaling pathway. GRN also induces T-cell populations [88] and IL-10 production and inhibits CXCL-9 and CXCL-10 chemokines release [89] [90]

Of note we determined that also HIF-1A shows an ID4-dependent behavior in macrophages. HIF-1 A is the most important transcription factor involved in the cellular response to low oxygen concentration, and it has been previously reported to be up-regulated by hypoxic macrophages to in turn up-

regulate a broad array of genes, including VEGF, whose production promotes angiogenesis [91] [92]. HIF-1A has been also demonstrated to be induced by other stimuli than hypoxia, such as growth factor, hormones, and viral proteins [93] and in macrophages by LPS stimulation [94]. Here, we depicted an ID4 dependent modulation of HIF-1A in macrophages in an hypoxia- independent manner underlining as ID4 could promote neo-angiogenesis even in non-hypoxic regions of the tumor. Interestingly, the majority of the angiogenesis-related factors presents HIF-1A consensus sequences in their promoter regions as we assessed by the study of the promoter sequences using the algorithm to predict transcription factors binding sites, TFBs LASAGNA 2.0 (Table 3).

Of note we identified as soluble mediator of breast cancer cells and tumor-associated macrophages cross-talk, the VEGFA, that we demonstrated to be almost in part responsible to the activation of the angiogenic program in macrophages co-cultured with breast cancer cells. We have previously reported that ID4 participate to the control of VEGF isoforms production, in association with a molecular complex comprising mut-p53, SRSF1 and the lncMALAT-1 in breast cancer cells; this ribonucleoprotein complex drives the alternative splicing of VEGFA to the enhancement of pro-angiogenic VEGFA

isoforms, VEGF121 and VEGF165 [70]. We depleted VEGFA both by si-RNA silencing in breast cancer cells used to prepare the conditioned medium, and by the use of blocking antibodies in the CM from BC cells. Using both this conditioned medium to culture macrophages, we observed a significant reduction in the expression of a panel of angiogenesis related genes, in particular of GRN, EphB2, NRP2 and HIF-1A. As that the depletion of both VEGFA and ID4, reduced the CM dependent activation of these genes in macrophages, it probably demonstrate that in macrophages the angiogenic program could depend on the ribonucleoprotein complex that mediate the VEGF isoforms production in BC cells.

Interestingly, we showed that blocking of VEGFA prevents CM-dependent activation of EphB2 and NRP2, among others.

Of note, EphB2 has been shown to control VEGFR-2 internalization that is necessary for activation and downstream signaling of the receptor [95]; NRP2 has been also demonstrated to be necessary to potentiate the activity of pro-angiogenic cytokines including VEGF-A_{165} acting as co-receptor for VEGFR [96]. Activation of EphB2 and NRP2 then could represent a mechanism for VEGFA signaling amplification in macrophages, because an increase of these molecules will probably lead to a more efficient response to the VEGFA

present in the CM (in our experimental system) and in the in vivo tumor microenvironment.

Moreover, we identified a post-transcriptional level of control of the angiogenesis related genes involving the miR-15/107 group of miRNA, that has been previously reported to regulate angiogenesis in particular through miR-107 [97].

MiR-107 has been previously reported, in colon cancer, to inhibit HIF-1beta (also known as aryl hydrocarbon receptor nuclear translocator, ARNT) that act as dimerization partner of HIF-1A in controlling cellular response to hypoxia [79]; it has also been reported to target GRN [78] [82] in neuroglioma cells with implications to neurodegenerative disease such as Alzheimer disease, and in human cancer.

Here we observed that miR-107, miR-15b and miR-195 are downregulated in macrophages co-cultured with BC cells. This downregulation is stronger in macrophages co-cultured with breast cancer cells expressing high levels of ID4 and is indeed impaired with the CM from ID4 depleted BC cells. We also demonstrated that VEGFA signaling in part controls miRNAs expression in macrophages as observed by the recovery of miR-107, miR-195, miR-15b in U937 cells cultured with CM from breast cancer cells VEGFA depleted.

We also confirmed in macrophages that GRN, HIF-1A and HIF-1beta are post-transcriptionally controlled by miR-107 and we also demonstrated EphB2 to be subjected to the same miR-107 control. Our study elucidates a novel role for these miRNAs in the control of the angiogenic program in TAMs.

Table 3

Gene Symbol	HIF-1 consensus on promoter	n° of databases predicting miR-107 dependence	n° of databases predicting miR-15b dependence	n° of databases predicting miR-195 dependence
ANGPTL4	Yes	3(CDS)	-	-
ECGF1	Yes	-	-	-
EDIL3	Yes	6(3'UTR), 2(CDS)	2(CDS)	-
EPHB2	Yes	4(3'UTR), 2(CDS)	4(3'UTR), 4(CDS)	5(3'UTR), 4(CDS)
FN1	No	5(CDS)	4(CDS)	5(CDS)
GRN	Yes	3(CDS)	6(CDS)	6(CDS)
MDK	Yes	-	-	
NRP2	Yes	8(3'UTR), 2(CDS)	9(3'UTR), 6(CDS)	9(3'UTR), 5(CDS)
PRL	Yes	3(CDS)	5(CDS)	4(CDS)
VEGFB	Yes	2(CDS)	3(3'UTR), 5(CDS)	4(3'UTR), 5(CDS)
VASH1	Yes	6(3'UTR)	-	3(3'UTR)

Table 3 . Presence of HIF-1 consensus sequences on promoters of angiogenic-genes modulated in HL60 in ID4 dependent manner as assessed by TLDA, was evaluated using the web tool http://biogrid-lasagna.engr.uconn.edu. Presence of putative binding sites for miR-107. miR-15b and miR-195 on 3'-UTR or coding (CDS) sequences of mRNAs was evaluated using the miRWalk analysis tool (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) by selecting the following databases: 1) 3'-UTR analysis= miRWalk, miRanda, miRDB, miRNAMap, Pictar2, RNA22, RNAhybrid, Targetscan; 2) CDS analysis= miRWalk, miRanda, RNA22, RNAhybrid, Targetscan.

6. CONCLUSION

Altogether our results give additional insights to ID4 ability to control angiogenesis in breast cancer not only by the enhancement of the production of pro-angiogenic cytokines as we previously reported [69] but also by the reprogramming of tumor associated macrophages. We identified a paracrine signaling between breast cancer cells and tumor associated macrophages that could represent a promising basis for the development of targeted therapies aimed at blocking the cross talk between BC cells and the tumor microenvironment.

7. ABBREVIATIONS

APC: Allophycocyanin

BC: Breast cancer

BLBC: Basal-like breast cancer;

BRCA1: Breast cancer susceptibility genes 1;

ChIP: Chromatin Immunoprecipitation analysis;

CM: Conditioned media;

CSF-1: Colony stimulating factor-1

DAB: Diaminobenzidine;

DMFS: Distant metastasis free survival;

ECM: Extracellular matrix;

ER: Estrogen receptor;

EV: Empty vector;

FACS: Fluorescence-activated cell sorting;

GRN: Granulin;

HA: Hemagglutinin;

HER-2: human epidermal receptor growth factor- 2

HIF: Hypoxia-inducible factor;

ID: Inhibitors of differentiation;

IFN: Intefereron;

IHC: Immunohistochemistry

IL-n : Interleuchin

LNA: Locked nucleic acid;

LncRNA: long non coding RNA

LPS: Lipopolysaccharide

MacSig: Macrophage signature;

miRNA, miR: MicroRNA;

MMP: Matrix metalloproteinase;

mut-p53: Mutant p53;

mRNA: Messenger RNA;

PBDM: ePripheral blood-derived monocytes;

PDGF: Platelet-derived growth factor;

PR: Progesteron receptor;

OS: Overall survival;

RT: Room temperature;

RT-qPCR: Realtime quantitative polymerase chain reaction

si-ID4: ID4-depleted breast cancer cells;

si-RNA: Small interfering RNA;

si-SCR: Control breast cancer cells;

SRSF1: Serine/arginine-rich splicing factor 1;

TAM: Tumor-associated macrophage;

TGF-β: Transforming growth factor;

TLDA: TaqMan Low Density Array;

TLR: Toll-like Receptor;

TNBC: Triple-negative breast cancer;

TNF: Tumor necrosis factor;

TP53: tumor protein 53;

UTR: Untranslated region;

VEGFA: Vascular endothelial growth factor A;

VitD3: 1,25-dihydroxyvitamin D3

8. Appendix (list of publications):

1. Tempestilli M, **Milano E,** D'Offizi G, Montalbano M, D'Avolio A, Gasperi T, Narciso P, Ascenzi P, Pucillo LP **(**2013) Determination of telaprevir in plasma of HCV-infected patients by HPLC-UV. *IUBMB life*,**65**(9),800-5.

2. Pruszko M, **Milano E**, Forcato M, Donzelli S, Ganci F, Di Agostino S, Bates DO, Bicciato S, Zylicz M, Żylicz A, Blandino G and Fontemaggi G. (2017). The mutant p53-ID4 complex controls VEGFA isoforms production by recruiting lncRNA MALAT1. *EMBO Rep*. 1**8**(8):1331-1351

3. Pruszko M, **Milano E**, Żylicz A, Zylicz M, Blandino G and Fontemaggi G. (2018). Zebrafish as experimental model to estabilish the contribution of mutant p53 and ID4 to breast cancer angiogenesis. *J Thorac Dis*, **10**(3),231-233.

4. Donzelli S*, **Milano E***, Pruszko M, Sacconi A, Masciarelli S, Iosue I, Melucci E, Gallo E, Terrenato I, Mottolese M, Zylicz M, Zylicz A, Fazi F, Blandino G, and Fontemaggi G. Expression of ID4 protein in breast cancer cells induces reprogramming of tumour-

associated macrophages (2018). *Breast Cancer Research,* **20**(59).

Posters:

1. SPECIAL CONFERENCE EACR AACR SIC 2015.
   "ID4- driven cross-talk between breast cancer cells and tumor associated macrophages". Firenze , 2015.

2. XIV FISV CONGRESS, 2016.
   "ID4-driven cross-talk between breast cancer cells and tumor associated macrophages". **Elisa Milano,** Sara Donzelli, Ilaria Iosue, Elisa Melucci, Enzo Gallo, Irene Terrenato, Marcella Mottolese, Giovanni Blandino, Francesco Fazi and Giulia Fontemaggi. Roma, 20- 23 settembre 2016, Università La Sapienza. (FISV Programme and Abstracts book pag 66 P5.13 in the Oncogenes and Tumor suppressors section)

3. 17TH INTERNATIONAL P53 WORKSHOP, 2017.
"The mutant p53-ID4 complex controls VEGFA isoforms production by recruiting lncRNA MALAT1" Pruszko M., **Milano E**., Forcato M, Donzelli S, Ganci F, Di Agostino S., Bates D.O., Bicciato S., Zylicz M, Żylicz A., Blandino G and Fontemaggi G. 8-12 luglio 2017, Biopolis, Singapore. P034 pag 99 Programme and abstract book

Bibliography

1. **WHO.**(2016) WHO:Geneva, Switzerland. Breast Cancer. *http://www.who.int/cancer/prevention/diagnosis-screening/breast-cancer/en/.*

2. Kalager M, Haldorse T, Bretthauer M, Hoff G, Thoresen S O, Adami H-O (2009). Improved breast cancer survival following introduction of an organized mammography screening program among both screened and unscreened women: a population-based cohort study. *Breast Cancer Res* **11**.

3. Siegel RL, Miller KD, Jemal A (2017) Cancer Statistics, 2017. *CA: A cancer journal for clinicians* **67**.

4. Rosner B, Colditz GA, Willet WC. (1994) Reproductive risk factors in a prospective study of breast cancer: the Nurses' Health Study. *Ann J Epidemiol* **139**, 819-835.

5. Colditz GA, Kaphingst KA, Hankinson SE, Rosner B. (2012) Family history and risk of breast cancer: nurses' health studyBreast *Cancer Res Treat* **133**, 1097-1104.

6. Lalloo F, Evans DG. (2012) Familial breast cancer. *Clin Genet* **82**, 105-114.

7. Shah R, Rosso K, and Nathanson SD. (2014) Pathogenesis, prevention, diagnosis and treatment of breast cancer. *World Journal of Clinical Oncology* **5**, 283-298.

8. Danaei G, Vander Hoorn S, Lopez AD, Murray CJ, Ezzati M. (2005).Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. *Lancet* **366**, 1784-1793.

9. Prat A., Perou C.M. (2010) Deconstructing the molecular portraits of breast cancer. *Molecular Oncology* **5**, 5-23.

10. Sørlie T, Wang Y, Xiao C, Johnsen H, Naume B, Samaha RR and Børresen-Dale AL. (2006) Distinct molecular mechanisms underlying clinically relevant subtypes of breast cancer: gene expression analyses across three relevant platforms. *BMC Genomics*, **7**.

11. Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, Wesseling J. (2010) Subtyping of Breast Cancer by Immunohistochemistry to Investigate a Relationship between Subtype and Short and Long Term Survival: A Collaborative Analysis of Datafor 10.159 Cases from 12 Studies. *Plos Medicine*, **7**.

12. Millikan RC, Newman B, Tse C-K, Moorman PG, Conway K, Smith LV, Labbok MH, Geradts J, Bensen JT, Jackson S, Nyante S, Livasy C, Carey L, Earp HS, and Perou CM. (2008) Epidemiology of basal-like breast cancer, *Breast cancer research and treatment,* **109**, 123-139.

13. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA. (2000) Molecular portraits of human breast tumours. *Nature*, **406**, 747-752.

14. Gruvberger S, Ringnér M, Chen Y, Panavally S, Saal LH, Borg A, Fernö M, Peterson C, Meltzer PS **(**2001) Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. *Cancer research* **61**, 5979-84.

15. Pusztai L**.** (2008) Current Status of Prognostic Profiling in Breast Cancer. *The Oncologist*, **13**.

16. Toss A, Cristofanilli M. (2015) Molecular characterization and targeted therapeutic approaches in breast cancer. *Breast cancer research*, **17**(60).

17. Weigelt B, Baehner FL and Reis-Filho JS. (2010) The contribution of gene expression profiling to breast cancer

classification, prognostication and prediction: a retrospective of the last decade. *Journal of Pathology* **220**, 263-280.

18. TCGA. (2012) Comprehensive molecular portraits of human breast tumors. *Nature* **490** (7418),61-70.

19 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, Borresen-Dale. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *PNAS* **98** (19), 10869–10874.

20. Mitri Z, Constantine T, and O'Regan R. (2012) The HER2 Receptor in Breast Cancer: Pathophysiology, Clinical Use, and New Advances in Therapy. *Chemoterapy research and practice*, **Vol. 2012.**

21. Spector NL, Blackwell KL.(2009) Understanding the mechanisms behind trastuzumab therapy for human epidermal growth factor receptor 2-positive breast cancer. *Journal of Clinical Onocology* **27**(34)

22. Hubalek M, Czech T, Müllerb H. (2017). Biological Subtypes of Triple-Negative Breast Cancer. *Breast Care* **12**(1), 8-14.

23 Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, Fox SB, Ichihara S, Jacquemier J, Lakhani SR, Palacios J, Rakha EA, Richardson AL, Schmitt FC, Tan P, Tse GM, Weigelt B, Ellis IO and Reis-Filho JS. (2011) Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists.**.** *Modern Pathology* **24**, 157-167.

24. Perou CM (2011) Molecular stratification of triple-negative breast cancers. *Oncologist* **16**, 61-70.

25 Cheang MC1, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, Perou CM, Nielsen TO. (2008) Basal-Like Breast Cancer Defined by Five Biomarkers Has Superior Prognostic Value than Triple-Negative Phenotype. *Clinical cancer research*, **14**(5).

26. Turner NC and Reis-Filho JS (2006). Basal-like breast cancer and the BRCA1 phenotype. *Oncogene* **25**, 5846–5853.

27. Quail DF and Joyce JA (2013). Microenvironmental regulation of tumor progression and metastasis. *Nature Medicine*, **19**(11), 1423–1437.

28. Bingle L, Brown N, Lewis CE (2002). The role of tumour-associated macrophages in tumourprogression: implications for new anticancer therapies. *Journal of Pathology*. **196**, 254-265.

29. Pollard, J Condeelis and J W. (2006.) Macrophages: Obligate Partners for Tumor Cell Migration, Invasion, and Metastasis *ElsevierCell*. **124**(1).

30. Campbell MJ, Wolf D, Mukhtar RA, Tandon V, Yau C, Au A, Baehner F, van't Veer L, D Berry, Esserman LJ. (2013). The Prognostic Implications of Macrophages Expressing Proliferating Cell Nuclear Antigen in Breast Cancer Depend on Immune Context. *PLOS ONE*. **8**(10).

31 Mukhtar RA, Moore AP, Tandon VJ, Nseyo O, Twomey P, Adisa CA, Eleweke N, Au A, Baehner FL, Moore DH, McGrath MS, Olopade OI, Gray JW, Campbell MJ, Esserman LJ.(2012) Elevated levels of proliferating and recently migrated tumor-associated macrophages confer increased aggressiveness and worse outcomes in breast cancer. *Annals of surgery oncology*. **19**(12).

32. Chanmee T, Ontong P, Konno K and Itano N. (2014) Tumor-Associated Macrophages as Major Players in the tumor microenviroment. *Cancers*. **6**, 1670-1690.

33. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, Kaiser EA, Snyder LA, and Pollard JW (2012) CCL2 recruits inflammatory monocytes to facilitate breast tumor metastasis. *Nature*, **475,** 222-225.

34. Lin EY, Nguyen AV, Russell RG, Pollard JW**.** (2001) Colony-stimulating Factor 1 Promotes Progression of Mammary Tumors to Malignancy. *Journal of Experimental medicine*, **193**.

35. Aharinejad S, Paulus P, Sioud M, Hofmann M, Zins K, Schafer R,Stanley ER, Abraham D. (2004). Colony-stimulating factor-1 blockade by antisense oligonucleotides and small interfering RNAs supprsses growth of human mammary tumor xenografts in mice. *Cancer research*, **64**.

36. Wanga H, Shaoa Q, Suna J, Maa C, Gaoa W, Wanga Q, Zhaoa L, and Qua X. (2016). Interactions between colon cancer cells and tumor-infiltrated macrophages depending on cancer cell-derived colony stimulating factor 1. *Oncoimmunology*, **5**(4).

37. Murdoch C, Giannoudis A, and Lewis CE (2004) Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood*, **104**(8).

38. Allavena P, Sica A, Solinas G, Porta C, Mantovani A**.** (2008). The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Critical Reviews in Oncology/Hematology*, **66**, 1-9.

39. Sica A, Mantovani A. (2012)**.** Macrophage plasticity and polarization: in vivo veritas. *The Journal of Clinical Investigation*, **122**(3).

40. Yang L and Zhang Y. Tumor-associated macrophages: from basic research to clinical application (2017), *Journal of Hematolgy & Oncology*,**10**(58).

41. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, Qian H, Xue X and Pollard JW (2006). Macrophages Regulate the Angiogenic Switch in a Mouse Model of Breast Cancer. *Cancer research*, **2**(66).

42. Qian BZ and Pollard J W (2010. Macrophage diversity enhances tumor progression and metastasis. *Cell*, **141**.

43. Jiang WG, Sanders AJ, Katoh M, Ungefroren H ,Gieseler F, Prince M, Thompson SK, Zollof M, Spano D, Dhawan P, Sliva D, Subbarayan PR, Sarkar M, Honoki K, Fujii H, Georgakilas AG, Amedei A, Niccolai E, Amin A,Ashraf SS, Ye L, Helferich WG,... Santini D. (2015) Tissue invasion and metastasis:

Molecular, biological and clinical perspectives. *Seminars in cancer biology*, **35**.

44. Gocheva V, Wang HW, Gadea BB, Shree T, Hunter KE, Garfall AL, Berman T and Joyce JA.(2010). IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes & Development*, **24,** 241–255.

45. Wyckoff JB **(**2007), Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Research*, **67**, 2649-2656.

46. Shigeoka M, Urakawa N, Nakamura T, Nishio M, Watajima T, Kuroda D, Komori T, Kakeii Y, Semba S, Yokozaki H. (2013). Tumor associated macrophages expressing CD204 is associated with tumor aggressiveness of esophageal squamous cell carcinoma. *Cancer Science.* **104**, 1112-1119.

47. Yang L, Wang F, Wang L, Huang L, Wang J, Zhang B, Zhang Y (2015). CD163+ tumor-associated macrophage is a prognostic biomarker and is associated with therapeutic effect on malignant pleural effusion of lung cancer patients *Oncotarget*, **6**, 10592–10603.

48. Brana I, Calles A, LoRusso PM, Yee LK, Puchalski TA, Seetharam S, Zhong B, de Boer C J, Tabernero J, Calvo E. (2015). Carlumab, an anti-C-C chemokine ligand 2 monoclonal antibody , in combination with four chemotherapy regimens for the treatment of patients with solid tumors: an open-label, multicenter phase 1b study. *Targeted Oncology*, **10**, 111-123.

49. Pyonteck SM, Gadea BB, Wang HW, Gocheva V, Hunter KE, Tang LH, Joyce JA. (2012). Deficiency of the macrophage growth factor CSF-1 disrupts pancreatic neuroendocrine tumor development. *Oncogene*, **31**,1459-67.

50. Norton. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. (2000). *Journal of cell science*, **113**, 3897-3905.

51. Patel D, Morton DJ, Carey J, Havrda MC, Chaudhary J. (2015) Inhibitor of differentiation 4 (ID4): From development to cancer. *Biochimica et Biophysica Acta*, **1855**, 92-103.

52. Lasorella A, Benezra R and Iavarone A. (2014). The ID proteins: master regulators of cancer stem cells and tumour aggressiveness. *Nature*, **14**.

53. Perk J, Iavarone A, Benezra R. (2005) Id family of helix-loop-helix proteins in cancer. *Nature Rev. Cancer*, **5**, 603-614.

54. Mern D, Hoppe-Seyler K, Hoppe-Seyler F, Hasskarl J, Burwinkel B (2010) Targeting Id1 and Id3 by a specific peptide aptamer induces E-box promoter activity, cell cycle arrest and apoptosis in breast cancer cells. Breast Cancer Res Treat, **124**, 623-633.

55. Mern DS, Hasskarl J, Burwinkel B. (2010) Inhibition of Id proteins by a peptide aptamer induces cell-cycle arrest and apoptosis in ovarian cancer cells. *Br.J. Cancer*,**103**, 1237-1244.

56. De Candia P, Benera R & Solit D B. (2004). A role for Id proteins in mammary gland physiology and tumorigenesis, *Adv. Cancer Res*. **92**, 81–94.

57. Bedford L, Walker R, Kondo T, Van Crüchten I, King ER, Sablitzky F. (2005), Id4 is required for the correct timing of neural differentiation. *Developmental biology*, **280**, 386-395.

58. Murad JM, Place CS, Ran C, SKN H, Watson NP, Kauppinen RA. (2010) Inhibitor of DNA binding 4 (ID4) regulation of adipocyte differentiation and adipose tissue formation in mice. *Journal of biological chemistry*, **285**, 24164-73.

59. Tokuzawa Y, Yagi K, Yamashita Y, Nakachi Y, Nikaido I, Bono H. (2010) Id4, a new candidate gene for senile osteoporosis, acts as a molecular switch promoting osteoblast differentiation., *PLoS genetics*, 1-15.

60 Jeon H, Lee X, Oh SY, Sohn YW, Park HJ, Joo KM, Park WY, Nam D, DePinho R, Chin, Kim H**.** (2008). Inhibitor of differentiation 4 drives brain tumor-initiating cell genesis through cyclin E and notch signaling.*Genes and development*, **22**.

61. Vinarskaja A, Goering W, Ingenwerth M, Schulz WA**.** (2012). ID4 is frequently downregulated and partially hypermethylated in prostate cancer. *World journal of urology*, **30**, 319-325.

62. Kuzontkoski PM, Mulligan-Kehoe MJ, Harris BT, Israel MA.(2010). Inhibitor of DNA binding- 4 promotes angiogenesis and growth of glioblastoma multiforme by elevating matrix GLA levels. *Oncogene*, Vol. **29**(26), 3793-3802.

63. Baker LA, Holliday H and Swarbrick A (2016) ID4 controls luminal lineage commitment in normal mammary epithelium and inhibits BRCA1 function in basal-like breast cancer. *Endocrine related cancer*, **23**.

64. **Lim E, Wu D, Pal B, Bouras T, Asselin-Labat M-L, Vaillant F, Yagita H.** (2010) Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. *Breast cancer research*, **121**,1-14.

65. Junankar S, Baker LA, Roden DL, Nair R, Elsworth B, Gallego-Ortega D,Lacaze P, Cazet A, Nikolic I & Teo WS. (2015). ID4 controls mammary stem cells and marks breast cancers with a stem cell-like phenotype. *Nature communications*, **6**(6).

66. Prat A, Cruz C, Hoadley A, Díez O, Perou CM and Balmaña J.(2014). Molecular features of the basal-like breast cancer subtype based on BRCA1 mutation status. *Breas cancer research and treatment,* **147**(1), 185–191.

67. Roldán G, Delgado L, Musé IM**.** (2006) Tumoral expression of BRCA1, estrogen receptor alpha and ID4 protein in patients with sporadic breast cancer. *Cancer biology and therapy*, **5**, 505-10.

68. Thike AA, Tan PH, Ikeda M, Iqbal J. (2015). Increased ID4 expression, accompanied by mutant p53 accumulation and loss of BRCA1/2 proteins in triple-negative breast cancer, adversely affects survival. *Histopathology*, **68**(5).

69. Fontemaggi G, Dell'Orso S, Trisciuoglio D, Shay T, Melucci E,Fazi F, Terrenato I, Mottolese M, Muti P, Domany E, Del Bufalo D, Strano S and Blandino G.(2009). The execution of the transcriptional axis mutant p53,E2F1 and ID4 promotes tumor neo-angiogenesis. *Nature structure & molecular biology*, **16**(9).

70. Pruszko M, Milano E, Forcato M, Donzelli S, Ganci F, Di Agostino S, Bates DO, Bicciato S, Zylicz M, Żylicz A, Blandino G and Fontemaggi G.(2017). The mutant p53-ID4 complex controls VEGFA isoforms production by recruiting lncRNA MALAT1. *EMBO reports*, **18**(8).

71. Wen YH, Ho A, Patil S, Akram M, Catalano J, Eaton A, Norton L, Benezra R, Brogi E. **(**2012) Id4 protein is highly expressed in triple-negative breast carcinomas: possible implications for BRCA1 downregulation. *Breast cancer research and treatment* .**135**, 93–102.

72. Anghelina M, Krishnan P, Moldovan L, Moldovan NI. (2006) Monocytes/macrophages cooperate with progenitor cells during neovascularization and tissue repair: conversion of cell columns into fibrovascular bundles. *The American Journal of Pathology*, **68**(2), p. 529- 41.

73. Zhao X, Qu J, Sun Y, Wang J, Liu X, Wang F , Zhang H, Wang W, Ma X, Gao X and Zhang S. (2017) Prognostic significance of tumor-associated macrophages in breast cancer: a meta-analysis of the literature. *Oncotarget*, **8**(18), 30576–30586.

74. Murao S, Gemmell MA, Callaham MF, Anderson NL, Huberman E. (1983). Control of macrophage cell differentiation in human promyelocytic HL-60 leukemia cells by 1,25-

dihydroxyvitamin D3 and phorbol-12-myristate-13-acetate. *Cancer research*, **43**, 4989–96.

75. Mangelsdorf DJ, Koeffler HP, Donaldson CA, Pike JW, Haussler MR. (1984) 1,25-Dihydroxyvitamin D3-induced differentiation in a human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells. *The journal of cell biology*, **98**(2), 391-8.

76. Krock BL, Skuli N, Simon C. (2011). Hypoxia-Induced Angiogenesis: Good and Evil. *Genes & Cancers*,**2**(12) 1117-1133.

77. Gilkes DM, and Semenza GL. (2013). Role of hypoxia-inducible factors in breast cancer metastasis. *Future Oncol.*, **9**(11), 1623–1636.

78. Wang WX, Kyprianou N, Wang X, Nelson PT (2010), Dysregulation of the mitogen granulin in human cancer through the miR-15/107 microRNA gene group. *Cancer research*, **70**(22)

79. Yamakuchi M, Lotterman CD, Bao C, Hruban RH, Karim B, Mendell JT, Huso D, Lowenstein CJ. (2010). P53-induced microRNA-107 inhibits HIF-1 and tumor angiogenesis. *Proceedings of the National Academy of Sciences USA*, **107**(4), 6334-9.

80. Chen L, Li ZY, Xu SY, Zhang XJ, Zhang Y, Luo K, Li W-p. (2016) Upregulation of miR-107 Inhibits Glioma Angiogenesis and VEGF Expression. *Cell Mol Neurobiol*, **36** (1), 113–20.

81. Shi Y, Zhao Y, Shao N, Ye R, Lin Y, Zhang N, Li W,Zhang Y and Wang S (2017). Overexpression of microRNA-96-5p inhibits autophagy and apoptosis and enhances theproliferation, migration and invasiveness of human breast cancer cells. *Oncol Lett*, **13**(6), 4402–12.

82. Wang WX, Wilfred BR, Madathil SK, Tang G, Hu Y, Dimayuga J, Stromberg AJ, Huang Q, Saatman KE, Nelson PT. (2010) miR-107 regulates granulin/progranulin with implications for traumatic brain injury and neurodegenerative disease. *Am J Pathol*, **177**(1), 334-45.

83. Fontemaggi G, Bellissimo T, Donzelli S, Iosue I, Benassi B, Bellotti G,Blandino G and Fazi F. (2015). Identification of post-transcriptional regulatory networks during myeloblast-to-monocyte differentiation transition. *RNA biology*, **12**(7), 690–700.

84. **Serrero G.** (2003) Autocrine growth factor revisited: PC-cell-derived growth factor (progranulin), a critical player in breast cancer tumorigenesis. *Biochemical and Biophysical Research Communications,* **308**(3).

85. Elkabets M, Gifford AM, Scheel C, Nilsson B, Reinhardt F, Bray MA, Carpenter AE, Jirström K, Magnusson K, Ebert BL, Pontén F, Weinberg RA, McAllister SS. (2011) Human tumors instigate granulin-expressing hematopoietic cells that promote malignancy by activating stromal fibroblasts in mice. *J Clin Inves*, **121**(2), 784–799.

86. Bateman A and Bennett HPJ. (2009). The granulin gene family: from cancer to dementia. *BioEssay*, **31**, 1245–1254.

87. Eguchi R, Nakano T, Wakabayashi I (2017). Progranulin and granulin-like protein as novel VEGF-independent angiogenic factors derived from human mesothelioma cells. *Oncogene*, **36**(5), p. 714-722.

88. Wei F, Zhang Y, Zhao W,Yu X, Liu C. (2014). Progranulin Facilitates Conversion and Function of Regulatory T Cells under Inflammatory Conditions. *Plos One*, **9.**

89. Jian J, Li G, Hettinghousen A, Liu C. (2016) Progranulin: A key player in autoimmune diseases. *Cytokines.*

90. Mundra JJ, Jian J, Bhagat P, Liu CJ. (2016) Progranulin inhibits expression and release of chemokines CXCL9 and CXCL10 in a TNFR1 dependent manner. *Scientfic reports* , **6**.

91. Elbarghati L, Murdoch C, Lewis CE. (2008) Effects of hypoxia on transcription factor expression in human monocytes and macrophages. *Immunology*. **213**,899–908.

92. Sotoodehnejadnematalahi F and Burke B. (2014). Human activated macrophages and hypoxia: a comprehensive review of the literature, Iran J Basic Med Sci. **17**(11), 820–830.

93. Dery MC, Michaud MD , Richard DE. (2005). Hypoxia-inducible factor 1: regulation by hypoxic and non-hypoxic activators. *The International Journal of Biochemistry & Cell Biology,* 535–540.

94. Blouin CC, Page E L, Soucy, GM and Richard DE. (2004) Hypoxic gene activation by lipopolysaccharide in macrophages:Implication of hypoxia-inducible factor 1alpha. *Blood*. **103**, 1124–1130.

95. Sawamiphak S, Seidel S, Essmann CL, Wilkinson GA, Pitulescu ME, Acker T, Acker-Palmer A (2010). Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. *Nature,* **465**, 487–91.

96. . Sulpice E, Plouët J, Bergé M, Allanic D, Tobelem G, Merkulova-Rainon T. (2008) Neuropilin-1 and neuropilin-2 act

as coreceptors, potentiating proangiogenic activity, *Blood*, **111**, 2036–45.

97. Finnerty JR1, Wang WX, Hébert SS, Wilfred BR, Mao G, Nelson PT. (2010). The miR-15/107 group of microRNA genes: evolutionary biology, cellular functions, and roles in human diseases. *J Mol Biol*, **402**(3), 491-509.