HIF-1 regulates CD47 expression in breast cancer cells to promote evasion of phagocytosis and maintenance of cancer stem cells

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Increased expression of CD47 has been reported to enable cancer cells to evade phagocytosis by macrophages and to promote the cancer stem cell phenotype, but the molecular mechanisms regulating CD47 expression have not been determined. Here we report that hypoxia-inducible factor 1 (HIF-1) directly activates transcription of the CD47 gene in hypoxic breast cancer cells. Knockdown of HIF activity or CD47 expression increased the phagocytosis of breast cancer cells by bone marrow-derived macrophages. CD47 expression was increased in mammosphere cultures, which are enriched for cancer stem cells, and CD47 deficiency led to cancer stem cell depletion. Analysis of datasets derived from thousands of patients with breast cancer revealed that CD47 expression was correlated with HIF target gene expression and with patient mortality. Thus, CD47 expression contributes to the lethal breast cancer phenotype that is mediated by HIF-1.

antitumor immunity  | immune evasion  | tumor-initiating cells  | tumor microenvironment  | ‘don’t eat me’ signal

The pathogenesis of breast cancer reflects not only the consequence of somatic mutations that dysregulate oncogenes and tumor suppressor genes, but also the effect of the changing tumor microenvironment, particularly the development of intratumoral hypoxia. The median pO2 within primary breast cancers is 10 mmHg (1.4% O2) compared with 65 mmHg (9.3% O2) in normal breast tissue (1). Exposure of breast cancer cells to reduced O2 availability induces the activity of hypoxia-inducible factors (HIFs), which are heterodimeric transcriptional activators, consisting of an O2-regulated HIF-1α, HIF-2α, or HIF-3α subunit and a constitutively expressed HIF-1β subunit, that control the expression of hundreds of target genes (2). Increased expression of the HIF-1α subunit, detected by immunohistochemistry in primary breast cancer biopsies, is associated with a significantly increased risk of metastasis and mortality (2).

Preclinical studies in mouse models have demonstrated that loss of HIF-1α or HIF-2α expression impairs the metastasis of breast cancer cells to axillary lymph nodes (3), lungs (4, 5), and bone (6, 7). Specific HIF target genes have been identified that are induced by hypoxia in breast cancer cells and promote critical steps in the metastatic process, including stromal cell recruitment (8, 9), cancer cell migration (10), invasion and intravasation (11–15), margination and extravasation (5), and premetastatic niche formation (12, 16). Increased expression of HIF target genes in primary breast cancers is associated with increased patient mortality (17, 18).

To give rise to a primary tumor, a tumor relapse, or a metastatic tumor, a breast cancer cell must possess two important characteristics: first, the cell must avoid destruction by the immune system and, second, the cell must possess stem-cell–like properties. Hypoxia induces the breast cancer stem cell (CSC) phenotype (19, 20) through functional and physical interactions of HIF-1 with the coactivator TAZ (20, 21) and by HIF-dependent expression of pluriptency factors (22). Hypoxia also induces immune evasion by several HIF-dependent mechanisms (23, 24). A major mechanism by which cancer cells evade the innate immune system is by expression of CD47, which is a cell-surface protein that interacts with signal regulatory protein α (SIRPα) on the surface of macrophages to block phagocytosis (25, 26).

Expression of calreticulin (CRT) on the surface of cancer cells is the primary trigger for phagocytosis by binding to low-density lipoprotein-related protein (LRP) on the surface of macrophages (27). The phagocytic signal triggered by CRT–LRP ligation is counterbalanced by the anti-phagocytic signal triggered by CD47–SIRPα ligation (28). Analysis of circulating tumor cells isolated from the blood of patients with breast cancer revealed that CD47 expression identified a subpopulation of cells with the capability to generate tumor xenografts in mice (29). Here, we report that CD47 expression is induced in a HIF-dependent manner when human breast cancer cells are exposed to hypoxia. Modest inhibition of CD47 expression was sufficient to increase the phagocytosis of breast cancer cells and decrease the number of breast CSCs. Human breast cancer database analysis revealed that high CD47 expression is correlated with increased HIF target gene expression and decreased patient survival.

Significance

Uncontrolled cell proliferation and abnormal blood vessel formation result in regions of breast cancers that are hypoxic (deprived of oxygen). Hypoxia-inducible factors (HIFs) stimulate the expression of genes that enable cancer cells to invade and metastasize, leading to patient mortality. In this paper, we report that HIFs stimulate the production of CD47, a protein on the cell surface that enables cancer cells to avoid destruction by macrophages. CD47 is also important for maintaining cancer stem cells, which are a small population of cells that are required for the formation of primary tumors and metastases. Reduction of HIF activity or CD47 levels in breast cancer cells led to increased killing by macrophages and depletion of cancer stem cells.

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Results

Hypoxia Induces Increased CD47 Expression in a HIF-Dependent Manner.

Breast cancers are classified based on their expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2); they are also classified according to expression of a 50-mRNA signature (PAM50) into luminal A, luminal B, HER2-enriched, basal-like, and normal-like subgroups (30). We analyzed the effect of hypoxia (1% O2 for 24 h) on CD47 mRNA levels in six different human breast cell lines: MCF10A is an immortalized but nontumorigenic mammary epithelial line; MCF7 is ER+PR+ and tumorigenic but nonmetastatic; HCC1954 is HER2+ and tumorigenic but nonmetastatic; and MDA-MB-231, MDA-MB-435, and SUM159 are ER−PR−HER2− (i.e., triple negative), tumorigenic and metastatic. Reverse transcription (RT) and quantitative real-time PCR (qPCR) assays revealed that expression of CD47 mRNA was significantly induced by hypoxia in MCF7, HCC1954, MDA-MB-435, and SUM159 cells but not in MCF10A or MDA-MB-231 cells (Fig. 1 A).

Fig. 1. Hypoxia induces CD47 expression in a HIF-dependent manner. (A) Reverse transcription (RT) and quantitative real-time PCR (qPCR) were performed to determine CD47 mRNA levels in human breast cell lines following exposure to 20% or 1% O2 for 24 h. For each sample, the expression of CD47 mRNA was quantified relative to 18S rRNA and then normalized to the result obtained from MCF-10A cells at 20% O2 (mean ± SEM; n = 3). Significant increase at 1% O2 compared with 20% O2:*P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test). (B and C) CD47 mRNA expression was analyzed by RT-qPCR in MCF7 (B) and HCC1954 (C) subclones expressing shRNA targeting HIF-1α (sh1α), HIF-2α (sh2α), or HIF-1α and HIF-2α (DKD), or expressing a nontargeting control shRNA (NTC), which were exposed to 20% or 1% O2 for 24 h. Data were normalized to NTC at 20% O2 (mean ± SEM; n = 3). **P < 0.01, ***P < 0.001 vs. NTC at 20% O2; ##P < 0.01, ###P < 0.001 vs. NTC at 1% O2 (two-way ANOVA with Bonferroni posttest). (D) SUM159 cells were treated with vehicle (Con) or 2.5 μM acriflavine (ACF), exposed to 20% or 1% O2 for 48 h, and expression of CD47 mRNA was assayed by RT-qPCR. ***P < 0.001 vs. Con at 20% O2; **P < 0.01 vs. Con at 1% O2 (two-way ANOVA with Bonferroni posttest). (E) Immunoblot assays were performed to analyze HIF-2α, HIF-1α, CD47, and Actin protein expression in whole cell lysates prepared from MCF7 (Left) and HCC1954 (Middle) subclones exposed to 20% O2 (N) or 1% O2 (H) for 24 h, and lysates from SUM159 cells (Right), which were treated with vehicle (Con) or 2.5 μM acriflavine (ACF) and exposed to 20% or 1% O2 for 48 h. Data were normalized to NTC at 20% O2 (mean ± SEM; n = 3). **P < 0.01, ***P < 0.001 vs. NTC at 20% O2; **P < 0.01, ***P < 0.001 vs. NTC at 1% O2 (two-way ANOVA with Bonferroni posttest). (F–H) MCF7 (F), HCC1954 (G), and SUM159 (H) cells were exposed to 20% or 1% O2 for 24 h and CD47 expression on the cell surface was determined by flow cytometry using anti-CD47 antibody or isotype control antibody (ISO) (Upper). The anti-CD47 median fluorescence intensity (MFI) was determined and normalized to lane 1 in each bar graph (Lower; mean ± SEM; n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. NTC at 20% O2; **P < 0.01, ***P < 0.001 vs. NTC at 1% O2 (two-way ANOVA with Bonferroni posttest for MCF7 and HCC1954 subclones; Student’s t test for SUM159 cells).
To determine whether HIF-1α or HIF-2α was required for CD47 expression under hypoxic conditions, we analyzed MCF7 and HCC1954 subclones, which were stably transfected with an expression vector encoding short hairpin RNA (shRNA) targeting HIF-1α (sh1α) or HIF-2α (sh2α) or both HIF-1α and HIF-2α (double knockdown, DKD) or a nontargeting control shRNA (NTC). Hypoxia-induced CD47 mRNA expression was lost in MCF7 sh1α and DKD subclones (Fig. 1B) and in HCC1954 sh1α, sh2α, and DKD subclones (Fig. 1C). Acriflavine inhibits the dimerization of HIF-1α or HIF-2α with HIF-1β (31), leading to the degradation of undimerized HIF-1α or HIF-2α subunits during prolonged (>24 h) incubation (18). Treatment of SUM159 cells with acriflavine blocked hypoxia-induced CD47 mRNA expression (Fig. 1D). Hypoxic induction of CD47 protein expression in whole cell lysates was also abrogated in MCF7-DKD and HCC1954-DKD subclones and in acriflavine-treated SUM159 cells (Fig. 1E).

Because cell-surface expression of CD47 is required for its antiphagocytic capacity, flow cytometry was performed. MCF7-DKD and HCC1954-DKD subclones showed decreased CD47 cell-surface expression compared with the respective NTC subclones under both hypoxic and nonhypoxic (20% O2) conditions (Fig. 1F and G). In HCC1954-NTC and SUM159 cells, hypoxia increased CD47 cell-surface expression (Fig. 1H). It was not possible to perform flow cytometry on acriflavine-treated cells due to the inherent fluorescence of the drug. Taken together, the data in Fig. 1 demonstrate that CD47 mRNA and protein expression are induced by hypoxia in three breast cancer cell lines in a HIF-dependent manner, leading to increased cell-surface expression of CD47 in HCC1954 and SUM159 cells.

**CD47 Is a Direct HIF-1 Target Gene.** To determine whether HIF-1 directly regulates CD47 gene transcription, the human genome sequence was searched for matches to the consensus HIF binding site 5'-A(G/C)CGTG-3' (32) that were located within DNase I-hypersensitive domains at the CD47 locus. Chromatin immunoprecipitation (ChIP) assays revealed that a DNA sequence, which encompassed 5'-GGCTG-3' at −239 bp (site 1) and 5'-CACGC-3' (5'-GGCTG-3' on the antisense strand) at −200 bp (site 2), relative to the CD47 transcription start site (Fig. 2A, Top), was enriched by immunoprecipitation of chromatin from hypoxic MCF7, HCC1954, and SUM159 cells with HIF-1α or HIF-1β antibodies (Fig. 2B), indicating that hypoxia induces direct binding of HIF-1 to the CD47 promoter.

To determine whether DNA sequences encompassing either site alone functioned as a hypoxia response element (HRE), a 55-bp oligonucleotide spanning HIF binding site 1 or site 2 (Fig. 2A) was inserted into pGL2-promoter, which contains a basal SV40 promoter driving expression of firefly luciferase, to generate reporters pGL2-HRE1 and pGL2-HRE2, respectively. As a negative control, we used pSV-Renilla, which encodes Renilla luciferase driven by the SV40 promoter alone. Hypoxia significantly increased the ratio of firefly:Renilla luciferase in HCC1954 and SUM159 cells that were cotransfected with either pGL2-HRE1 or pGL2-HRE2 and pSV-Renilla (Fig. 2C), demonstrating that each of the 55-bp oligonucleotides functions as an HRE. Taken together, the data presented in Fig. 2 indicate that HIF-1 binds directly to the CD47 promoter to activate gene transcription.

![Figure 2](image-url)

**Fig. 2.** CD47 is a direct HIF-1 target gene. (A) Two candidate HIF-1 binding sites in the 5'-flanking region of the human CD47 gene were identified: 5'-GGCTG-3' (site 1, located 239 bp 5' to the transcription start site) and 5'-CACGC-3' (site 2, located 200 bp 5' to the transcription start site), which is the complementary sequence of 5'-GGCTG-3' on the antisense strand, are shown in red. (B) MCF7 (Left), HCC1954 (Middle), and SUM159 (Right) cells were exposed to 20% or 1% O2 for 16 h and chromatin immunoprecipitation (ChIP) assays were performed using IgG or antibodies against HIF-1α or HIF-1β. Primers flanking the entire sequence shown in A were used for qPCR and results were normalized to IgG at 20% O2 (mean ± SEM; n = 3). *P < 0.05. **P < 0.01 vs. 20% O2 (Student's t test). (C) Cells were cotransfected with pSV-Renilla and firefly luciferase reporter pGL2-HRE1 or pGL2-HRE2, containing an oligonucleotide encompassing HIF binding site 1 or site 2, respectively, and exposed to 20% or 1% O2 for 24 h. Luciferase activity (firefly:Renilla luciferase ratio) was determined and normalized to 20% O2 (mean ± SEM; n = 3). **P < 0.01 vs. 20% O2 (Student's t test).
HIF Deficiency Increases the Phagocytosis of Breast Cancer Cells. We observed decreased expression of CD47 on the surface of HIF-deficient human breast cancer cells (Fig. 1F and G), leading us to hypothesize that HIF inhibition may promote phagocytosis of breast cancer cells by macrophages. To test this hypothesis, we performed in vitro phagocytosis assays on HCC1954 and MCF7 subclones. The breast cancer cells were labeled with the fluorescent dye CFSE, exposed to 20% or 1% O2 for 24 h, cocultured with bone marrow-derived macrophages for 2 h, stained with anti-CD11b antibody, and analyzed by flow cytometry. Phagocytosis assays were performed with HCC1954 (B) and MCF7 (C) subclones as described above and the percentage of CFSE“APC” phagocytosed cancer cells was normalized to NTC at 20% O2 (mean ± SEM; n = 3). **p < 0.01, ***p < 0.001 vs. NTC at 20% O2; ***p < 0.001 vs. NTC at 1% O2 (two-way ANOVA with Bonferroni posttest).

CD47 Deficiency Increases the Phagocytosis of Breast Cancer Cells. To determine whether CD47 mediates protection of breast cancer cells against phagocytosis, we transfected SUM159 cells with an expression vector encoding one of five different shRNAs targeting CD47. Remarkably, we were not able to establish stable subclones that expressed three of the five shRNAs and expression of the other two shRNAs (shCD47-2 and shCD47-4) caused relatively modest (50–75%) inhibition of CD47 mRNA (Fig. 4A) and cell-surface protein (Fig. 4B) expression. However, phagocytosis was significantly increased in the CD47-knockdown subclones (Fig. 4C) and CD47 levels were inversely correlated with the extent of phagocytosis. Taken together, the data presented in Fig. 4 demonstrate that CD47 expression protects breast cancer cells against phagocytosis by bone marrow-derived macrophages.

CD47 Promotes the Breast CSC Phenotype. CD47 is preferentially expressed on bladder, liver, and pancreatic CSCs compared with the bulk cancer cells (non-CSCs) (33–36). To investigate whether CD47 plays a role in breast CSCs, we first analyzed CD47 mRNA levels in SUM159 cells, which were cultured as standard adherent monolayers or as nonadherent spheroids (mammospheres), which are highly enriched for CSCs (37). CD47 mRNA levels were twofold higher in mammosphere cultures (Fig. 5A). Exposure of breast cancer cells to hypoxia for 3 d induces increased mammosphere formation in a HIF-1α-dependent manner (20). Knockdown of CD47 expression significantly decreased mammosphere formation, with the greatest reduction observed in the shCD47-4 subclone (Fig. 5B), which had the greatest inhibition of CD47 expression (Fig. 4A and B). Analysis of aldehyde dehydrogenase (ALDH) activity in breast cancer cells also identifies a subpopulation that is enriched for CSCs (38) and the percentage of ALDH+ cells increases in response to hypoxia (19, 20). Hypoxia markedly increased the percentage of ALDH+ cells in the NTC subclone, whereas knockdown of CD47 expression significantly decreased the percentage of ALDH+ CSCs at both 20% and 1% O2 (Fig. 5C). Taken together, the data presented in Fig. 5 demonstrate that CD47 expression plays an important role in promoting the breast CSC phenotype. We and others have previously reported that knockdown of HIF-1α blocks hypoxia-induced enrichment of breast CSCs, as determined by ALDH expression or mammosphere formation assay (19, 20). Thus, CD47 loss-of-function phenocopies HIF-1α loss of function with respect to CSC maintenance.

CD47 Expression Is Associated with HIF Target Gene Expression and Patient Mortality. To test whether the results obtained from the analysis of breast cancer cell lines are relevant to patients with breast cancer, we analyzed gene expression data from 1,040 primary human breast cancer samples that are publicly available in The Cancer Genome Atlas (TCGA) database (39). To determine whether HIFs regulate CD47 gene expression in human breast cancers, we compared CD47 mRNA levels with the levels of 41 HIF target genes in 1,040 breast cancers (Fig. 6A). Statistical analysis revealed that CD47 mRNA levels were significantly correlated with the levels of 8 of 10 mRNAs encoded by HIF target genes (Fig. 6B), which is comparable to correlations between members of this group (10, 15, 20). Expression of CD44, which is a marker of breast CSCs (40) and a HIF target gene (41), was also significantly correlated with CD47 expression in the 1,040 breast cancers (Fig. 6B). HIF target gene expression in human breast cancers is significantly associated with patient mortality (18). To determine if CD47 mRNA expression was also a prognostic factor in human breast cancer, we used two published datasets that contain both gene expression and patient survival data (42, 43). Patients were...
CD47 deficiency increases the phagocytosis of breast cancer cells. (A) CD47 mRNA levels were analyzed by RT-qPCR in SUM159 subclones expressing either of two different shRNAs targeting CD47 (shCD47) or a nontargeting control shRNA (NTC). Results were normalized to NTC (mean ± SEM; n = 3). ***P < 0.001 vs. NTC (one-way ANOVA with Bonferroni posttest). (B) CD47 protein expression on the cell surface was determined by flow cytometry of SUM159 subclones exposed to 20% or 1% O₂ for 24 h (Upper). The anti-CD47 median fluorescence intensity (MFI) was determined and normalized to NTC at 20% O₂. (Lower; mean ± SEM; n = 3). ***P < 0.01, ****P < 0.001 vs. NTC at 20% O₂; ****P < 0.001 vs. NTC at 1% O₂ (two-way ANOVA with Bonferroni posttest). (C) SUM159 subclones were exposed to 20% or 1% O₂ for 24 h, stained with CFSE, incubated with bone marrow-derived macrophages for 2 h, stained with F4/80-APC antibody, subjected to flow cytometry, and the percentage of CFSE⁻/F4/80⁺ phagocytosed cancer cells was determined and normalized to the NTC subclone at 20% O₂ (mean ± SEM; n = 3). **P < 0.01 vs. NTC at 20% O₂; *P < 0.05, ##P < 0.01 vs. NTC at 1% O₂ (two-way ANOVA with Bonferroni posttest).

Fig. 4. CD47 deficiency increases the phagocytosis of breast cancer cells. A, B, and C are panels of a figure showing flow cytometry data. A) Expression of CD47 mRNA in SUM159 subclones exposed to 20% or 1% O₂. B) CD47 protein expression on the cell surface. C) Phagocytosis of SUM159 subclones by bone marrow-derived macrophages.

Discussion
Growing evidence indicates that in various human cancers, CD47 expression is required to avoid innate immune surveillance and elimination by phagocytosis. Blocking the interaction of CD47 with its receptor, SIRPα, on macrophages enables phagocytosis and inhibits tumor growth (35, 36, 44–46). However, the molecular mechanisms regulating the expression of CD47 by cancer cells have not been delineated. In this study, we demonstrate that hypoxia, which is a critical microenvironmental stimulus in advanced breast cancers, induced HIF-dependent expression of CD47, leading to decreased phagocytosis of cancer cells by macrophages and induction of the breast CSC phenotype, which promote cancer progression and patient mortality (Fig. 6C).

Despite the recent interest in targeting CD47 for cancer therapy (46), remarkably little is known about the transcriptional regulation of the CD47 gene. The results presented here represent to our knowledge the first identification of a transcriptional regulator of CD47 expression in breast cancer cells and further studies are required to determine whether HIF-1 cooperates with other transcription factors that are induced by the tumor microenvironment, such as CREB, NF-κB, SMAD2, or STAT3. Increased NF-κB activity was observed in hepatocellular carcinoma cells that developed sorafenib resistance (36), which is of interest because the antiangiogenic effects of sorafenib induce intratumoral hypoxia that causes HIF-1-dependent induction of breast CSCs (19). Immunosuppressive functional interactions of HIF-1 and CREB have been proposed in T cells (47), but may also occur in cancer cells.

Intratumoral hypoxia is a common finding in breast cancer and HIFs activate the transcription of a large battery of genes encoding proteins that promote multiple steps in tumor progression, including tumor growth and vascularization, stromal cell recruitment, extracellular matrix remodeling, premetastatic niche formation, cell motility, margination and extravasation of circulating tumor cells, and CSC specification/maintenance (2). Here, we find that HIFs also play an important role in immune evasion by activating the expression of CD47, an antiphagocytic signal. In three different breast cancer cell lines, which represent luminal/ER⁺ (MCF7), HER2-enriched (HCC1954), and basal-like/triple-negative (SUM159) subtypes of breast cancer, the expression of CD47 mRNA and protein was induced by hypoxia in a HIF-dependent manner. A recent study reported that CD47 protein was detected in 5% of hormone receptor-positive, HER2⁺ breast cancers (48). Our bioinformatic analysis suggests that CD47 expression is likely to be higher among triple-negative breast cancers, most of which fall into the basal-like molecular subtype that is characterized by increased expression of the HIF⁺ transcriptome (18, 39).

Hypoxia did not induce increased CD47 expression in MCF10A or MDA-MB-231 cells. This heterogeneous transcriptional response of breast cell lines to hypoxia is commonly observed. For example, analysis of mRNAs encoding lysyl oxidase (LOX) and LOX-like proteins (LOXL1–4) revealed that hypoxia induced
increased expression of LOX and LOXL4 in MDA-MB-231, whereas LOXL2 expression was induced in MDA-MB-435 cells (12). We also observed heterogeneity with respect to requirements for HIF-α subunits, as CD47 induction required only HIF-1α in MCF7 cells, but both HIF-1α and HIF-2α in HCC1954 cells. Similarly, we previously reported that hypoxia-induced LOXL2 expression required only HIF-1α in MDA-MB-435 cells, whereas expression of LOX and LOXL4 in MDA-MB-231 cells required HIF-1α and HIF-2α (12). Heterogeneity at the level of gene expression is recognized as a major obstacle to successful cancer therapy. However, in the cases described above, treatment with a HIF inhibitor, such as acriflavine, successfully blocked all hypoxia-induced gene expression (Fig. 1D).

Cells express varying levels of prophagocytic and antiphagocytic signaling proteins, and it is the integration of both signals that determines whether the target cell will be phagocytosed. The relatively modest changes in CD47 cell surface levels that resulted from expression of shRNA targeting HIF-1α and HIF-2α or CD47 led to significant changes in phagocytosis, indicating a fine balance between pro- and antiphagocytic signaling, such that a hypoxic tumor microenvironment may significantly tip the balance toward immune evasion. Several prophagocytic signals have been identified, including cell-surface expression of phosphatidylserine and CRT (49, 50). CRT is required for the phagocytosis of cancer cells when CD47–SIRPα interaction is blocked (28). CRT expression was induced by exposure of cardiomyocytes to hypoxia and reoxygenation (51), suggesting that the increased phagocytosis of NTC and DKD subclones after hypoxic exposure may be due to HIF-independent CRT expression, but further studies are required to test this hypothesis. A recent report demonstrated that CD47 blockade also promotes T-cell–mediated elimination of immunogenic tumors (52) and HIF-1 is known to inhibit effector T cells through the production of adenosine (23, 47), suggesting that HIF inhibitors may improve the response to CD47 blockade both by inhibiting CD47 expression and by dis-inhibiting T-cell–mediated antitumor immunity.

Two findings in our study suggested that hypoxia-induced expression of CD47 plays an important role that is independent of its antiphagocytic function. First, although CD47 mRNA and protein expression were induced by hypoxia in all three breast cancer cell lines analyzed, increased CD47 cell-surface expression and decreased phagocytosis were only induced by hypoxia in SUM159 cells. These data suggest that hypoxia-induced CD47 may fulfill another function that does not require cell-surface expression of the protein. Second, attempts at generating CD47-deficient cells were only successful in establishing subclones with modest knockdown of CD47, suggesting that expression of the protein was required for clonal expansion. Using two established assays for breast CSCs, we demonstrated that CD47 expression was increased in breast CSCs (relative to bulk cancer cells) and was required for breast CSC maintenance, as decreased CD47 expression led to significantly reduced numbers of CSCs in a
dose-dependent manner. Further studies are required to determine whether CD47 must be expressed at the cell surface to contribute to the maintenance of breast CSCs.

To generate a primary, recurrent, or metastatic tumor, a breast cancer cell must avoid immune destruction and give rise to both CSCs and differentiated breast cancer cells. A recent study found that among circulating tumor cells in the blood of women with metastatic breast cancer, the cells that were capable of initiating tumors when injected into mice expressed the cell surface proteins CD47, CD44, and MET (29). Remarkably, as in the case of CD47, the expression of CD44 and MET is induced by hypoxia in a HIF-dependent manner (41, 53). Furthermore, our analysis of gene expression data from over 1,000 human breast cancers revealed that CD47 expression was significantly correlated with the expression of CD44, MET, and other HIF target genes (Fig. 6B). We also found that CD47 mRNA expression in primary human breast cancers was significantly associated with patient mortality in two large and independent datasets (Fig. 6C). Similar results were recently reported regarding the immunohistochemical detection of CD47 protein expression in breast cancer biopsies (48).

We have recently demonstrated that exposure of breast cancer cells to hypoxia or chemotherapy induces the breast CSC phenotype through multiple HIF-dependent molecular pathways (18, 20, 54). Induction of CD47 expression provides another mechanism by which hypoxia induces the CSC phenotype. In addition to interacting with SIRPα, CD47 engages in several other functional interactions (55) and further studies are required to determine the molecular mechanism by which the expression of CD47, localized to the cell surface or perhaps an intracellular compartment, promotes the specification and/or maintenance of CSCs.

**Fig. 6.** CD47 expression in primary human breast cancers is associated with HIF target gene expression and decreased patient survival. (A) The relative expression levels of 10 mRNAs encoded by HIF target genes, as well as CD44 and CD47 mRNAs, are shown for 1,040 primary breast cancer tissue samples from The Cancer Genome Atlas (TCGA), which were grouped according to the expression levels of 50 mRNAs (PAM50) that define the luminal A, luminal B, HER2+, basal-like, and normal-like breast cancer molecular subtypes (57). Color code for mRNA expression levels are as follows: red, greater than median; green, less than median. (B) CD47 mRNA expression in each primary breast cancer specimen was compared with the expression of CD44 mRNA and HIF-regulated mRNAs using Pearson’s correlation test. *P < 0.05, ***P < 0.001; n.s., not significant. (C) Kaplan–Meier curves were constructed to analyze the association of CD47 mRNA expression in the primary tumor with overall survival (OS) of patients with breast cancer using two independent datasets (42, 43). Statistical analysis was performed using log-rank tests. (D) Intratumoral hypoxia induces HIF-1–dependent expression of CD47 in breast cancer cells, leading to decreased phagocytosis of cancer cells by macrophages and induction of the cancer stem cell (CSC) phenotype, which promote cancer progression and patient mortality.
maintenance of CSCs. Genetic or pharmacologic inhibition of HIF may affect some of the percentage of CSCs (18–20, 56), which may be due in part to the inhibition of CD47 expression. Increased expression of HIF-1α mRNA or protein, or HIF target gene mRNAs, in primary breast cancer biopsies is associated with increased patient mortality (2, 18). Our data suggest that addition of HIF inhibitors to current treatment regimens may improve outcome in such high-risk patients in part by attacking CSCs and diminishing innate immunity.

Materials and Methods

Cell Lines and Culture

Human breast cell lines MCF10A, MCF7, HCC1954, SUM159, MDA-MB-231, and MDA-MB-435 were cultured as previously described (18, 20, 21, 54). All cells were maintained at 37 °C in 5% CO2 and 95.5% air (20% O2) incubator. For hypoxia exposure, cells were placed in a modular incubator chamber (Billups-Rothenberg) that was flushed with a gas mixture containing 1% O2, 5% CO2, and 94% N2.

shRNA, Lentiviruses, and Transduction.

All lentiviral vectors contained a puromycin resistance gene. Vectors encoding shRNA targeting HIF-1α and HIF-2α were described previously (55). Vectors encoding CD47 shRNAs were purchased from Sigma-Aldrich; the clone ID and nucleotide sequences coding for shRNA are as follows: shCD47-7: NM_001777.2-102461t; 5′-CCG GCC TTC GTA CCA TCA GAA GAC TAT ACT GGA TGA TAG TCT GAT TCG AAC GGT TT-3′; shCD47-4: NM_001777.2-9881t; 5′-CCG GCC ACA ATT ACT TGG ACT ACG CTA GGA CTA GCA GTA ATT GTG CCT TT-3′. Lentiviruses were packaged in 293T cells by cotransfection with plasmid pCMV-D8.91 and plasmid encoding vesicular stomatitis virus G protein using Polyeject (SignaGen). Supernatant containing viral particles was collected 48 h posttransfection, filtered (0.45-μm pore size) and transduced into MCF7, HCC1954, or SUM159 cells in the presence of 8 μg/mL of Polybrene (Sigma-Aldrich). After 24 h, cells were maintained in medium containing 0.5 μg/mL puromycin (Sigma-Aldrich).

RT-qPCR.

Total RNA was extracted, cDNA was synthesized by RT, and qPCR analysis was performed as described (18), using PCR primers with the following sequences: CD47, 5′-CGG GGC ACA ATT ACT TGG ACT AGT TCT CGA TGA CTA GCA GTA ATT GTG CCT GC-3′ and 5′-AGA TCG AAC CCT GAT TCC CCG TC-3′; HIF-1α, 5′-TGA GGT CCC CGG TGG TGA AAC CAC AGG AGG TAT CCT GCT CCT GC-3′ and 5′-TGG AGG AGG AGG CTG ACT CCT GGT GCG CGG TCA TTC GAG TTC TT-3′; HIF-2α, 5′-GAT GCG TTC AGC CTC TGT CGG CGG CGG AGC AGC CGG ACC CAA GCC GGG GGG GGG GCC TGG GA-3′ and 5′-TGC AGA CCC GGC CCC TGG GTC GGT CGT CCG CCG CAG GAG CCT GAA CC-3′. 20% or 1% O2 for 45 min at 37 °C. As a negative control, for each sample an aliquot of cells was subjected to flow cytometry (FACScalibur, BD Biosciences). Dead cells were gated out by side-scatter and forward-scatter analysis.

Isolation of Bone Marrow-Derived Macrophages.

Bone marrow cells were isolated from mouse long bones and cultured for 7 d in RPMI-1640 supplemented with 10% (vol/vol) FBS, 1% penicillin/streptomycin, and 30 ng/mL CSF1 (R&D Systems) as previously described (9).

Phagocytosis Assay.

Macrophages were plated (5 × 104 per well) in a 24-well tissue-culture plate in complete RPMI-1640 medium, which was supplemented with 10% FCS and antibiotics, 24 h before the experiment. Breast cancer cells were stained with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE) according to the manufacturer’s protocol (Invitrogen). Macrophages were incubated in serum-free medium for 2 h before adding 2 × 104 CFSE-labeled breast cancer cells. After coculture for 2 h at 37 °C, cells were harvested, macrophages were stained with APC-conjugated F4/80 (Novus Biologicals), and flow cytometry (FACScalibur, BD Biosciences) was performed. A total of 10,000 cells in each sample were analyzed. Unstained control and single-stained cells were prepared for gating. Phagocytosis was calculated as the percentage of F4/80+CFSE− cells (Q2) among CFSE+ cells (Q1+Q2): phagocytosis (%) = (Q2/(Q2 + Q1)) × 100%.

Mammosphere Assay.

Cells were exposed to 20% or 1% O2 in monolayer adherent culture for 72 h. Single cells were plated in six-well ultra-low attachment culture plates (Corning) at a density of 5,000 cells per well in complete mammmocult medium (Stem Cell Technologies). Mammospheres (diameter ≥50 μm) were counted after 5 d. Mammosphere cultures were imaged using an Olympus phase-contrast microscope and mammosphere diameters were determined using ImageJ software (National Institutes of Health).

ALDH Assay.

Cells were exposed to 20% or 1% O2 for 72 h and harvested for Aldefluor assay (Stem Cell Technologies). Cells were suspended in assay buffer containing 1 μM ALDEFLUOR and incubated for 45 min at 37 °C. As a negative control, for each sample an aliquot of cells was treated with the ALDH inhibitor diethylaminobenzaldehyde (50 mM). Samples were subjected to flow cytometry analysis (FACScalibur, BD Biosciences).

Statistical Analysis.

Data are expressed as mean ± SEM and were analyzed using Student’s t test for two groups or ANOVA with Bonferroni posttest for multiple groups. Kaplan–Meier curves were generated using two independent datasets containing gene expression and overall survival data on patients with breast cancer (42, 43). The log rank test was performed to determine whether observed differences between groups were statistically significant. P values <0.05 were considered significant.

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