UNIVERSITÀ DEGLI STUDI DI PALERMO

INTERNATIONAL PhD IN IMMUNOPHARMACOLOGY

Department of Biopathology and Medical Biotechnologies (DiBIMED)

SSD BIO/13

STEM CELLS IN BREAST CANCER DEVELOPMENT AND MALIGNANT PROGRESSION

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XXV CYCLE
ACADEMIC YEAR 2015

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Summary

Breast cancer is the leading worldwide type of cancer among women and its lethality is strictly dependent on its high risk of metastatic spread. The concept that tumors are hierarchically organized and harbor cells with distinct tumorigenic potential and successful outgrowth at metastatic sites, referred to as CSCs, has long been observed in a broad range of tumors and it is now well-recognized by the scientific community.

In the past few years, the protein Sam68 (Src associated in mitosis of 68kDa) has been linked to the onset and progression of cancer. In the first chapter of this thesis (**Chapter 1**), I evaluate Sam68 aberrant expression in breast cancer specimens as well as its function in modulating the invasiveness and self-renewal capability of breast CSCs (BCSCs). Sam68 also contributes to tumorigenesis in preclinical settings.

The prominent role of CSCs in tumor relapse explains disease maintenance and the frequent failure of current therapies. In this context, we focused our attention on recombinant erythropoietin (EPO) analogs (ESAs, erythropoiesis-stimulating agents), which are used as drugs to treat anemia, including in cancer patients receiving chemotherapy. We provide evidence that EPO enhances BCSCs refractoriness to conventional chemotherapy and promotes *in vivo* tumor progression (**Chapter 2**).

Tumor dissemination is dependent on a permissive microenvironment that can favour an epithelial to mesenchymal transition (EMT) and hypoxia, contributing to chemoresistance by inducing in cancer cells a stem like-phenotype. Understanding the relationship between cancer cells and microenvironment may be fundamental in developing innovative therapeutic strategies for a better and definitive response to treatments. All these aspects are discussed in **Chapter 3**.

Thus, targeting molecular events affecting CSCs peculiarities, as self-renewal and an innate chemoresistance, could improve the ineffectiveness of current therapies which, in most cases, are not designed in a specific way such as to respond to intratumoral and intertumoral heterogeneity. Data shown in **Chapter 4** seek to emphasize that the development of new powerful tools for targeting CSCs will possibly overcome the current disappointing results and lead to the improvement of therapies' outcomes.

Chapter 1

Sam68 sustains self-renewal and invasiveness of breast cancer stem cells

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Manuscript in preparation

Abstract

Sam68, the major CD44 splicing regulator, is a multifunctional RNA-binding protein involved in multiple steps of RNA metabolism and its expression is aberrant in breast cancer. Herein, we highlight novel implications of Sam68 in the mammary tumor onset and progression. In a cohort of screened breast cancer patients, breast CSCs express higher Sam68 protein levels than tumor bulk, highlighting that Sam68 expression is likely restricted to cells with self-renewal activity. Stable knockdown of Sam68 in breast CSCs significantly curtails proliferation rate and is coupled with an increase in proteins inducing cell cycle arrest and a reduction of pro-survival signaling. Compared to control cells, downregulation of Sam68 is correlated with an attenuation of cell motility capability and Twist, Snail, CD44v6 and Met levels. Moreover, Sam68 modulates the expression of an oncogenic alternative splice variant of Met (Met-SM), probably through the ASF/SF2 splicing factor. Sam68 silencing ablates breast cancer xenograft formation in immunocompromised mice. Finally, univariate and multivariate analysis of TMA show that invasive breast carcinoma over-expressing Sam68 are associated with a significantly higher incidence of distant relapse. Based on these results, we deem that Sam68 may promote pro-invasive signals by inducing and modulating the alternative splicing of oncogenic variants of several genes. Thus, further investigations on signaling pathways affecting CSCs self-renewal and invasiveness could represent a crucial key to improve selective cancer treatment.

1.1 Introduction

Despite the outstanding advances obtained in curing localized malignancies, metastatic disease still lacks effective therapeutic approaches and represents the main cause of cancer related mortality in women worldwide (1). Breast cancer is an high heterogeneous disease that is commonly classified using *i*) histology, into ductal and lobular cancers, *ii*) ER, PR and HER2 receptors expression, and *iii*) their differentiation state and gene expression profiles, distinguishing luminal-A, luminal-B, HER2 enriched, basal-like, claudin-low and normal like. Triple negative breast cancers (ER-, PR- and HER2-) can often be classified in basal-like tumors and have the least favorable prognosis among all the breast cancer subtypes. These patients currently do respond to chemotherapy but recurrence and disease progression is still a major issue (2). Moreover, the absence of targeted treatment alternatives renders appealing the introduction of new molecular therapies.

Mammary gland tissue's homeostasis, remodeling and regeneration are finely tuned by adult stem cells, which retain self-renewal and multi-lineage differentiation ability. As a consequence of epigenetic and/or genetic alterations, those cells may acquire a malignant behavior and be in charge of tumor seeding (3). In primary breast xenografts, cells with these peculiarities are also endowed with the capability to form tumors in immuocompromised mice by limiting dilution transplantation assay (4) and to recapitulate the original heterogeneity observed in the primary tissue they are derived from. CSCs are likely to be resistant to conventional chemotherapies and for this purpose, recent advances in stem cell biology are revealing that characteristics of normal stem cells are retained by their malignant counterparts, including dormancy (quiescence), active DNA repair machinery, the expression of several ABC drugs transporters, an intrinsic resistance to apoptosis and lower concentration of reactive oxygen species (5). Recently, it has been pointed out that CSCs display a cellular plasticity that allows them to transit between EMT and MET states (6). Indeed, the EMT process plays a fundament role in embryonic development as well as in several cancer progression steps. In this context, recent finding from our group underlined that cytokines, such as OPN, HGF and SDF-1 secreted by tumor microenvironment, increase the expression of CD44v6, an alternative spliced variant of CD44 and co-receptor for MET (7). Engagement of HGF on CD44v6/MET complex activates EMT program, promoting colon cancer cell motility and invasiveness (7). Interestingly, CD44 was the first Sam68 (Src-associated protein during mitosis, of 68 kDa) target to be identified. Sam68 belongs to the heteronuclear ribonucleoprotein particle K (hnRNP K) homology (KH) domain family of RNA-binding proteins and is a member of the signal transduction and activation of RNA (Star) family (8). Sam68

interacts with the splicing activator SRm160 promoting inclusion of v5 and v6 exons in CD44 (9). EGF and HGF/MET stimulation, by modulating the non-RTK breast tumor kinase/protein tyrosine kinase 6 (Brk/PTK6), induces Sam68 phosphorylation, promoting both cell migration and cell cycle progression (10). Moreover, Sam68 has been proposed as a multifunctional effector in human cancers (11) and its up regulation is associated with tumorigenicity in breast cancer (12).

1.2 Material and methods

The immunohistochemistry analysis were performed on 3 μm or 5 μm paraffinated sections of breast tumoral tissues and xenografts. Sections were deparaffinized and rehydrated. Antigen retrieval was carried out by boiling slides with a sodium citrate solution 10mM (pH 6.0 or 9.9). Incubation with TBS 10% TritonX-100 on ice for 10 min was required to permeabilize cells. Primary antibodies used were anti-Sam68 (SC-333, Santa Cruz Biotechnology), anti-ALDH (BD biosciences clone 44 cat n. 611194) and anti-Ki67 (DAKO #M7240 clone MIB-1). The slides were stored at 4°C overnight. The staining was detected by using the chromogen AEC (3-amino-9-ethylcarbazole). Nuclei were stained with hematoxylin. Tissue microarrays (TMA) were constructed by removing 2-mm diameter cores of histologically confirmed invasive breast carcinoma (T1-T2, N0, M0; n = 155) areas from each original paraffin block and re-embedding these cores into gridded paraffin blocks, using a precision instrument (MTA, Beecher Instuments, WI). After antigen retrieval (microwave treatment at 750 W for 10 min in 10 mM sodium citrate buffer, pH 6.0), five-micrometer sections were incubated overnight at 4°C with the anti Sam68 rabbit polyclonal antibody (C-20, S. Cruz Biotechnology, CA) at 1:150 dilution. The anti-rabbit EnVision kit (Dako, Glostrup, Denmark) was used for signal amplification. In control sections the specific primary antibody was replaced with non-immune serum. Slides were evaluated by pathologist without knowledge of the clinicopathological data. Pathologic tumor size and tumor grade, as well as estrogen receptor (ER), progesterone receptor (PR) and Ki-67 expression were dichotomized according to the St. Gallen criteria (2013). HER-2 membranous staining was scored according to Herceptest (Dako) and classified as positive if the intensity was scored 3+, with more than 30% of cells showing complete membrane staining, or if the intensity was scored 2+ in presents of an amplification of the HER-2 gene as assessed by fluorescent in situ hybridization. Based on immunohistochemistry of ER, PR, Ki-67 and HER-2, we also studied the Nectin-4 distribution in breast cancer molecular subtypes: Luminal-A (n = 70), Luminal-B/HER-2 negative (n = 36), Luminal-B/HER-2 positive (n = 14), HER-2 (n = 10), and Triple Negative (n = 25).

For the immunofluorescence assay, citospins were fixed with 2% paraformaldehyde and permeabilized with TBS 10% TritonX-100 on ice for 10 min. Primary antibodies were: CD44v6 (R&D Systems) and Met (Santa Cruz). TUNEL reaction was performed with the *In Situ* AP Cell Death Detection Kit (Roche). Nuclei were counterstained with TOTO-3 (Life technologies).

For western blot analysis detail's see material and methods section in chapter 2. Antibodies used were: Sam68 (SC333, Santa Cruz Biotechnology), p27kip1 (2552, rabbit polyclonal, CST), Bcl-XL (H-5, mouse IgG1, Santa Cruz Biotechnology), β-actin (Ab-1 mouse IgM, Calbiochem), anti-mouse HRP-conjugated (Pierce #185413), and anti-rabbit HRP-conjugated (Pierce #32460).

For RNA Extraction and Real-Time PCR, RNA was isolated by using the RNeasy Plus Mini Kit (Qiagen) and retrotranscribed by using the High-Capacity c-DNA Archive kit (Applied Biosystem). The Taq Man Primer used were: Hs00153310_m1 (CD44v6), Hs00195591_m1 (Snail), Hs00173141_m1 (Sam68) and Hs01675818_s1 (Twist). qRT-PCR was performed in triplicates. The relative quantity of expression of each gene was evaluated through the comparative Ct method (ΔΔCt) in comparison to the expression of the housekeeping gene (GAPDH). Gene expression profiles were carried out with the Epithelial to Mesenchymal Transition RT^2 Profiler PCR Array (Qiagen).

Breast CICs were obtained from mechanically and enzimatically digestion of human breast tissues as described in Chapter 2 (material and methods section). Stable Sam68 knockdown was produced by lentiviral transduction of the PLK0.1 vector with the shSam68 insert and of a scrambled vector and subsequent puromicin (Sigma-Aldrich) selection. Invasion assay was performed in 8μm pore size transwell coated with GF-depleted matrigel 1:3 in serum free medium. $3x10^4$ cells were plated and 10% human AB serum medium was used as chemoattractant.

BCSCs (3×10^5) were suspended in 1:6 matrigel (BD Matrigel Matrix Growth Factor Reduced) and orthotopically injected in 6-week-old NOD/SCID mice (Charles River, Italy). Tumors were measured with a caliper each week and volume was calculated by the formula: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. At the end of the experiment mice were sacrificed and tissues were collected for histological analysis.

1.3 Results and Discussion

Sam68 expression was examined in five breast cancer tissues and the adjacent normal counterparts using immunohistochemistry. As a result, Sam68 expression was higher in cancerous specimens compared to the non-cancerous ones (Figure 1A). To support the results of protein expression we explored the mRNA level of Sam68 in seven paired tumor and normal tissues of the same patients (Figure 1B). The qRT-PCR data partially confirmed the protein analysis, indicating that Sam68 expression may vary between different molecular subtypes of breast cancer and that discrepancy between the transcriptional and translational levels could occur. For a better understanding of the role of Sam68 in breast cancer onset, we analyzed its expression in freshly isolated Cancer Stem Cells (CSCs) from tissues of breast cancer patients undergoing surgical removal, in accordance with the ethical committee at the University of Palermo. Breast CSCs (BCSCs) expressed higher Sam68 protein levels than tumor bulk and the normal counterpart, highlighting that its expression was likely confined to cells with self-renewal activity (Figure 1 C). To investigate Sam68 role among the molecular subtypes of breast cancer, its expression was examined in basal-like and luminal BCSCs. Sam68 was markedly elevated in cells with basal-like features in comparison to luminal BCSCs. The results showed the same trend between qRT-PCR and Western blotting (Figure 1D-E).

Given their high content in Sam68, basal BCSCs were selected for performing knockdown experiments. We silenced Sam68 by transducing BCSCs with a lentiviral vector carrying a short hairpin RNA specific for Sam68 (shSam68) or a scramble vector (Scr) as control (Supplementary Figure S1A). The efficiency of the procedure was verified by qRT-PCR and Western Blot (Supplementary Figures S1 B-C). The downregulation of Sam68 impaired BCSCs ability to form colonies in a 3D culture (Figure 2A) and curtailed their viability and proliferation rates (Figures 2 B-C). These findings are coupled with an increase in p27kip1 and reduction of Bcl-xL (Figures 2D-E).

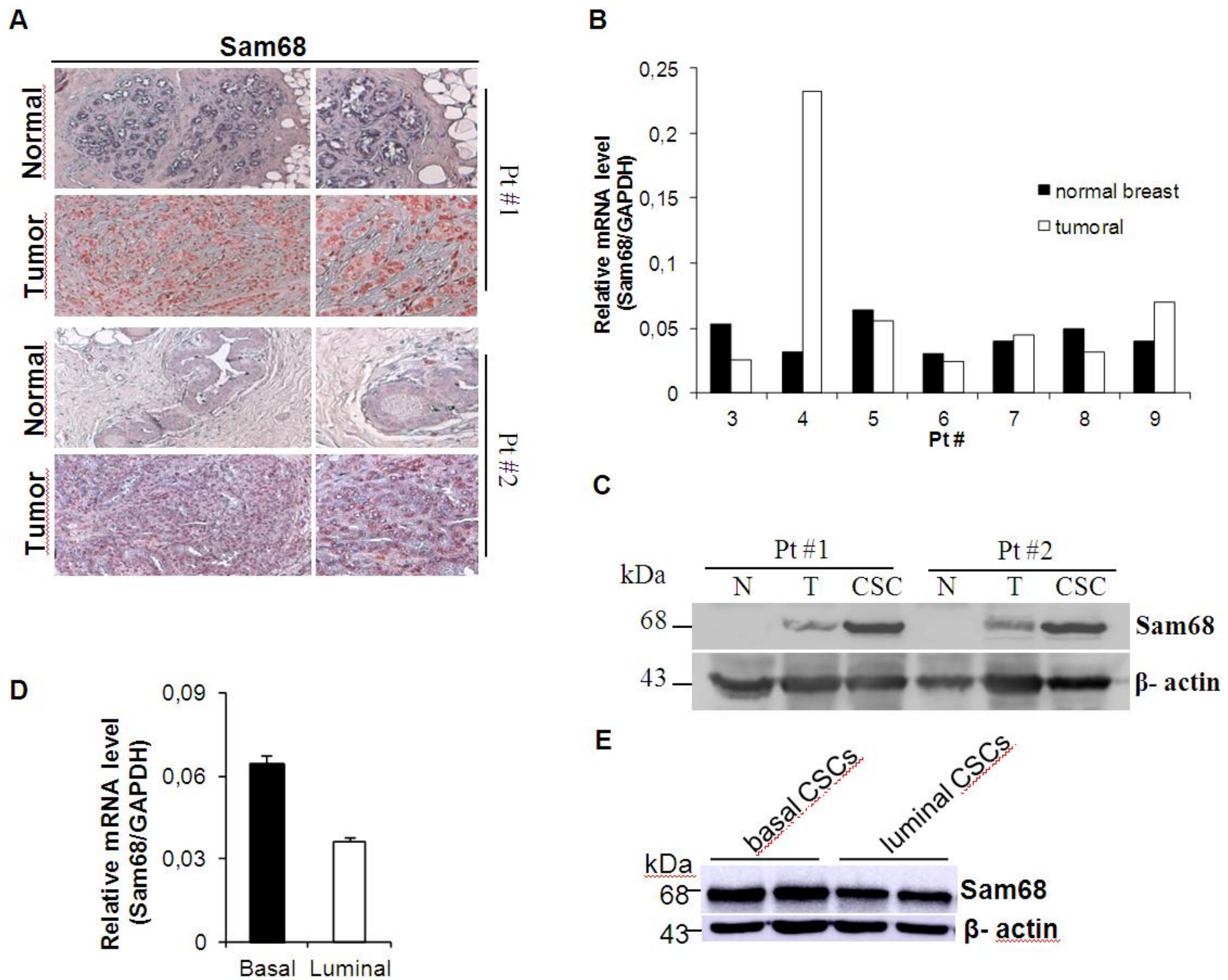


Figure 1. Sam68 is upregulated in Breast Cancer Stem Cells (BCSCs) in comparison to tumor bulk and normal tissue of the same patient. A, immunohistochemical staining with antibodies against Sam68 of paraffin-embedded sections derived from breast carcinomas (Tumor) and the paired adjacent normal tissues (Normal). Representative pictures of two samples. B, Sam68 expression level in tumors and normal breasts by qRT-PCR. C, Western blotting analysis of Sam68 in BCSCs, tumor and normal tissue of the same patient. D, Sam68 expression in basal and luminal CSCs by qRT-PCR, and E, Western blotting.

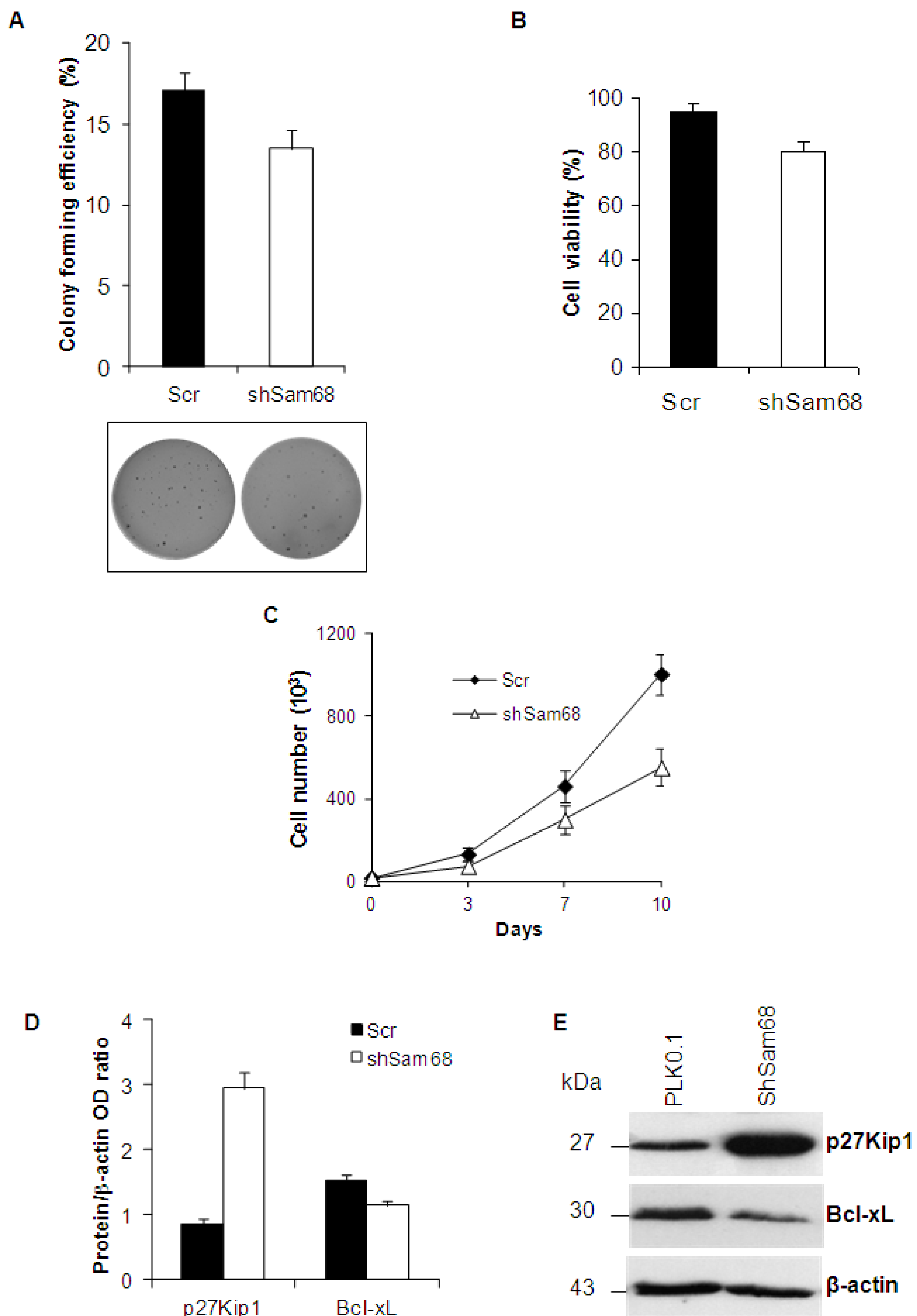


Figure 2. Sam68 interfers with BCSCs viability and proliferation. A, number of colonies generated in a soft agar assay for scr and shSam68 BCSCs. B, cells viability measured at 72 hours. C, number of cells in culture up to 10 days. D, Western blotting analysis of p27Kip1 and Bcl-xL, quantitative measurement, and E, representative images. All the experiments were carried out in triplicate with three different basal BCSCs lines.

CSCs are endowed with extraordinary migratory and invasive capabilities and for this reason they may power metastatic dissemination. Sam68 seems to be a critical input required for invasion, as shown by the invasion assay in Figure 3A. The process called Epithelial to Mesenchymal Transition (EMT) favors the migration of CSCs from the primary tumor at metastatic sites. These migratory traits are usually conferred by a network of factors, such as those included in the array in Figure 3B. Genes expression profiles revealed that the downregulation of Sam68 restrains the expression of EMT-related genes (Figure 3B). CD44 is an adhesion molecule with several functions in EMT and tumor propagation and, in combination with other antigens or functional assays, it can also enrich for breast CSCs (4). It was recently pointed out that an alternative splicing isoform of *CD44*, called CD44v6, sustains colon cancer metastasis and demarcates a subpopulation of colorectal CSCs (CR-CSCs) with metastagenic properties (7). Due to its ability to regulate alternative splicing, Sam68 is a valuable candidate as inductor of CD44 splicing. qRT-PCR data in Figure 3C show that the total CD44 transcript level is not affected by Sam68 deprivation, conversely the CD44 variant isoforms v4, v5, v7, v9, v10 (Figure 3D) and v6 (Figure 3E) are massively reduced. ShSam68 BCSCs display also a reduction in transcriptional factors involved in motility (Twist and Snail) (Figure 3E) and cell adhesion molecules such as E-cadherin (Figure 3F and Supplementary Figure S1E) levels. CD44v6 acts as a co receptor for the HGF-receptor MET, thus enhancing the HGF-MET signaling (13) (Orian-Rousseau V. 2002; Ponta, H.). Sam68 modulates also the expression of MET (Supplementary Figure S1) and of an its oncogenic variant MET-SM that lacks the ubiquitin ligase docking site (Figure 3G). Among the splicing factors analyzed in Figure 3H, ASF seems to be the only one able to force MET-SM expression. Since, as in Figure 3I, Sam68 knock-down attenuates ASF expression, it is highly conceivable that Sam68 indirectly controls MET alternative splicing.

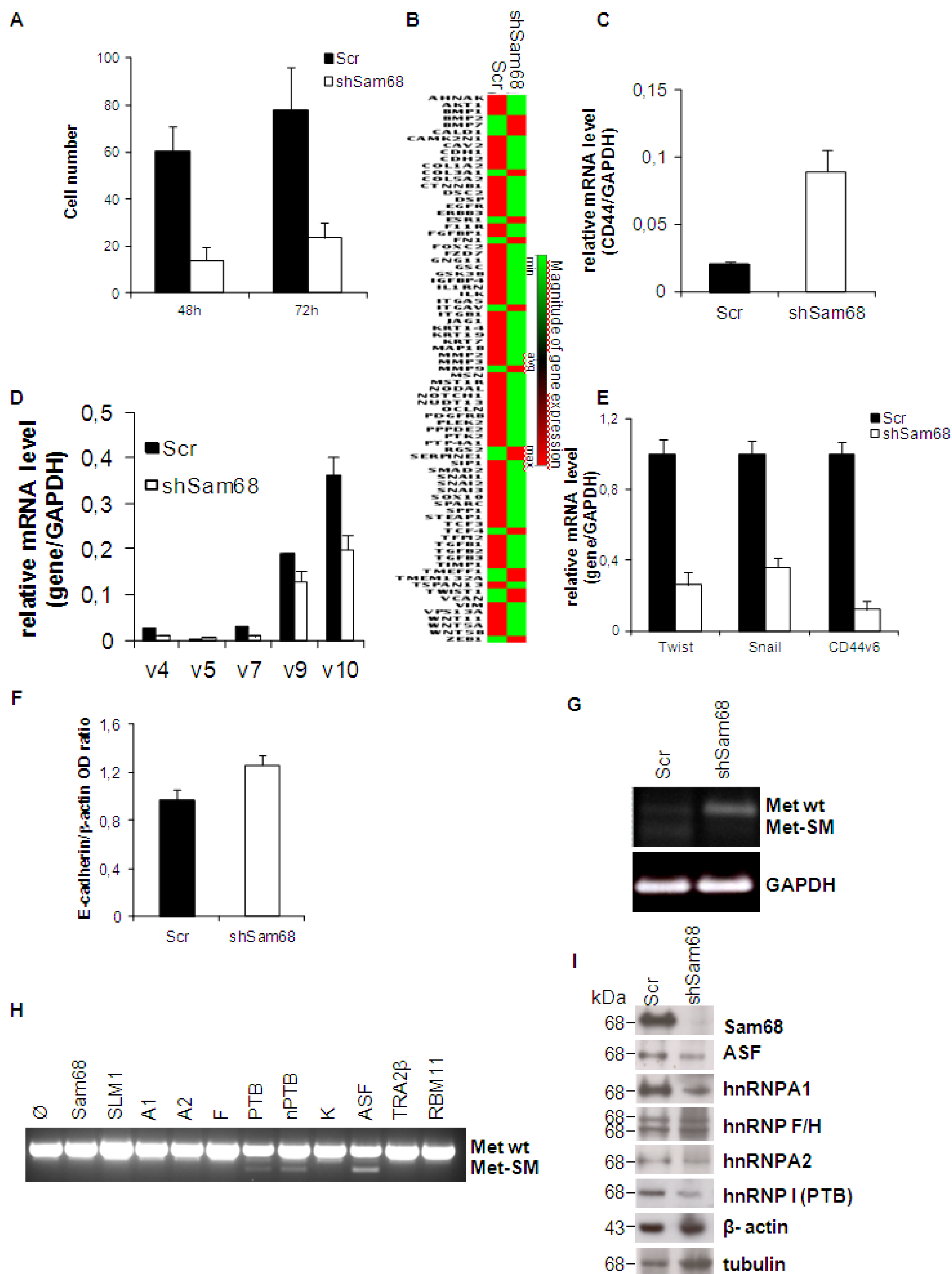


Figure 3. Sam68 hampers BCSCs invasive capabilities. A, number of invasive cells at 48 and 72 hours. B, gene expression profiles of scr and shSam68 BCSCs. qRT-PCR for CD44 (C), CD44v (D), Twist, Snail and CD44v6 (E), and E-cadherin expression levels. G, RT-PCR for Met. RT-PCR (H) and western blot analysis for several splicing factors (I). All the experiments were carried out in triplicate with three different basal BCSCs lines.

To confirm that Sam68 is essential for tumorigenesis and metastagenesis we settled an in vivo experiments in immunocompromised mice. ShSam68 and scr BCSCs were injected into the mammary gland of 6-week-old mice and tumors' onset was monitored up to 4 months. Sam68 knockdown abrogated tumor growth as opposed to control cells, which gave rise to xenografts (Figures 4 A-B). Particularly, shSam68 BCSCs were confined in to the site of injection and didn't damage the normal anathomy of the mammary gland. ShSam68 BCSCs were negative for ki67, ALDH (Figure 4C) and CD44V6 (Figure 4D). To confirm that both populations (ShSam68 and Scr BCSCs) were viable at the time of tumors' and normal breasts' excision, we performed a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reaction on paraffin-embedded sections of xenografts. The analysis proved that only few cells were apoptotic both in control and shSam68 cells (with arrows) (Figure 4D).

Next, we explored the clinical relevance of Sam68 in breast cancer patients. Univariate analysis, in a cohort of 155 breast cancers, indicated that tumors over-expressing Sam68 (Sam68^{High}), with a cut off of 90% positive cells, were associated with a significantly higher incidence of distant relapse (P = 0.011) (Figure 5A). Moreover, in multivariate analyses of distant relapse free survival (DRFS), adjusted for the other prognostic factors, Sam68^{High} was an independent prognostic factor influencing distant relapse (HR = 2.6: 95% CI, 1.1-6.3; P = 0.037) (Table 1). Finally we deemed to explore the relationship between high Sam68 expression and breast cancer molecular subtypes. Kaplan–Meier curves estimated an higher incidence of DRFS in HER2 positive *plus* Triple negative breast cancer (P=0.023) and in Luminal B HER2 positive *plus* HER2+ *plus* Triple negative (P=0.008). Moreover, in multivariate analyses, Sam68^{High} in Luminal-B HER2 Pos *plus* HER2 *plus* Triple Negative was an independent prognostic factor influencing distant relapse (HR=7.9: 95%CI 1.0-60.7; P=0.048) (Table 2).

Overall, our studies better define Sam68 clinical relevance as a prognostic biomarker for breast cancer patients and give new insights to improve the ineffectiveness of current therapies by targeting molecular events that affect CSCs peculiarities

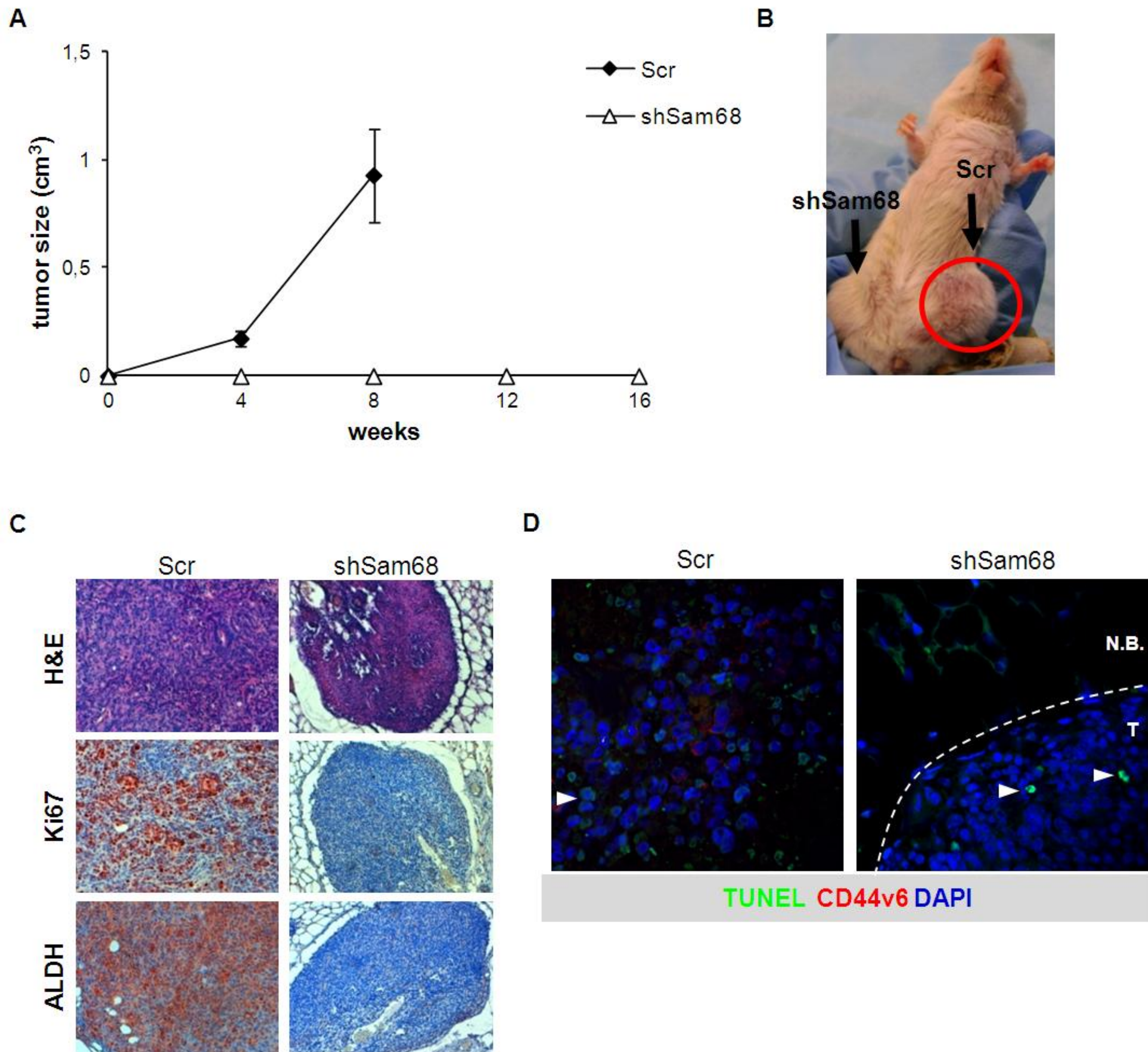


Figure 4. Sam68 impais BCSCs tumorigenic capabilities. A, shSam68 and Scr BCSCs were orthotopically injected in the mammary fat pad of immunocompromised mice (5 mice per group). This experiment was carried out with three basal BCSCs lines. B, immunohistochemical analysis for H&E, ki67 and ALDH of xenografts and normal breast glads obtained after mice were sacrificed. D, immunofluorescence on paraffin embedded tissues for CD44v6 (red), TUNEL (green) and DAPI (blues). White arrows indicate death cells.

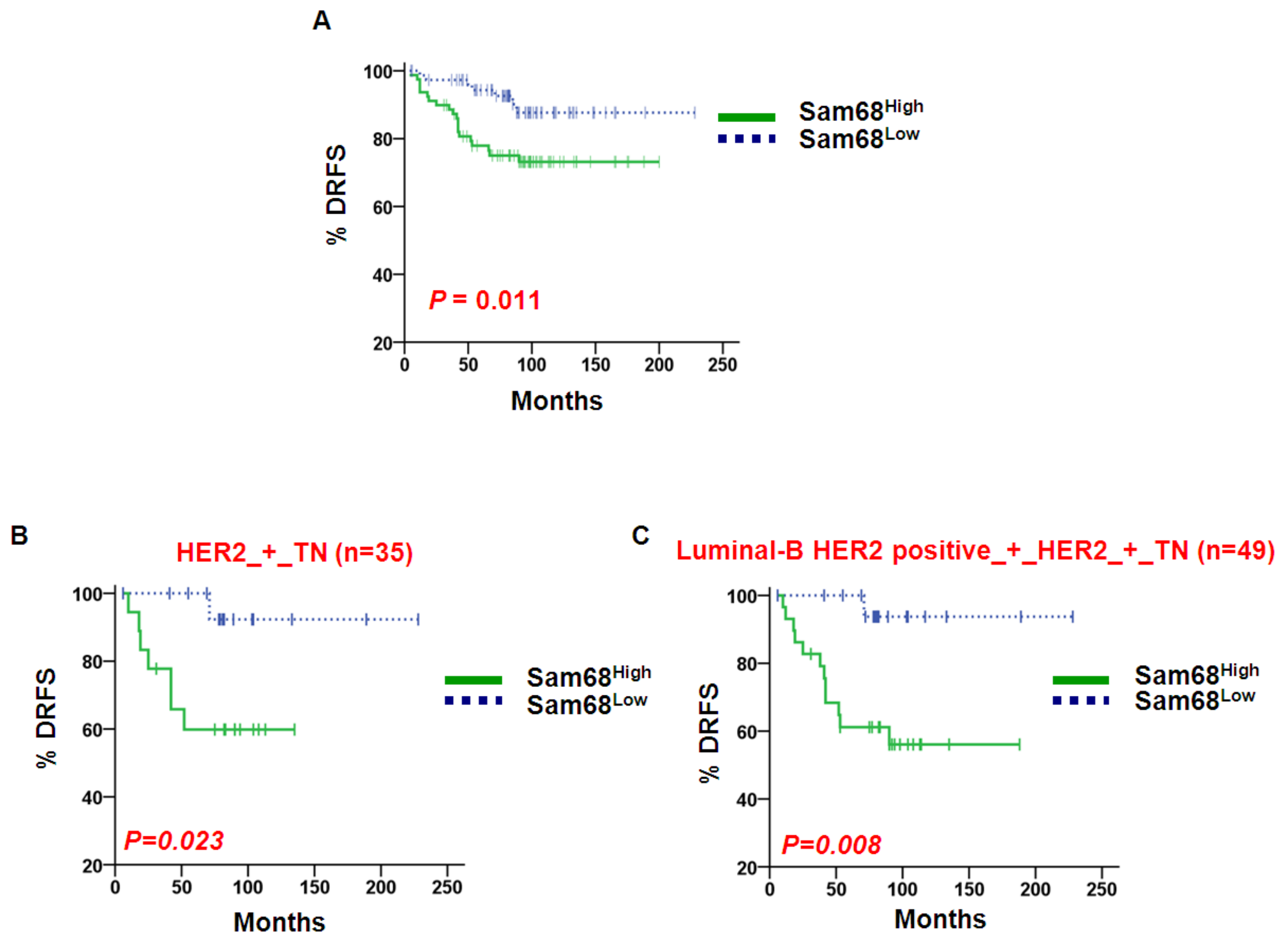
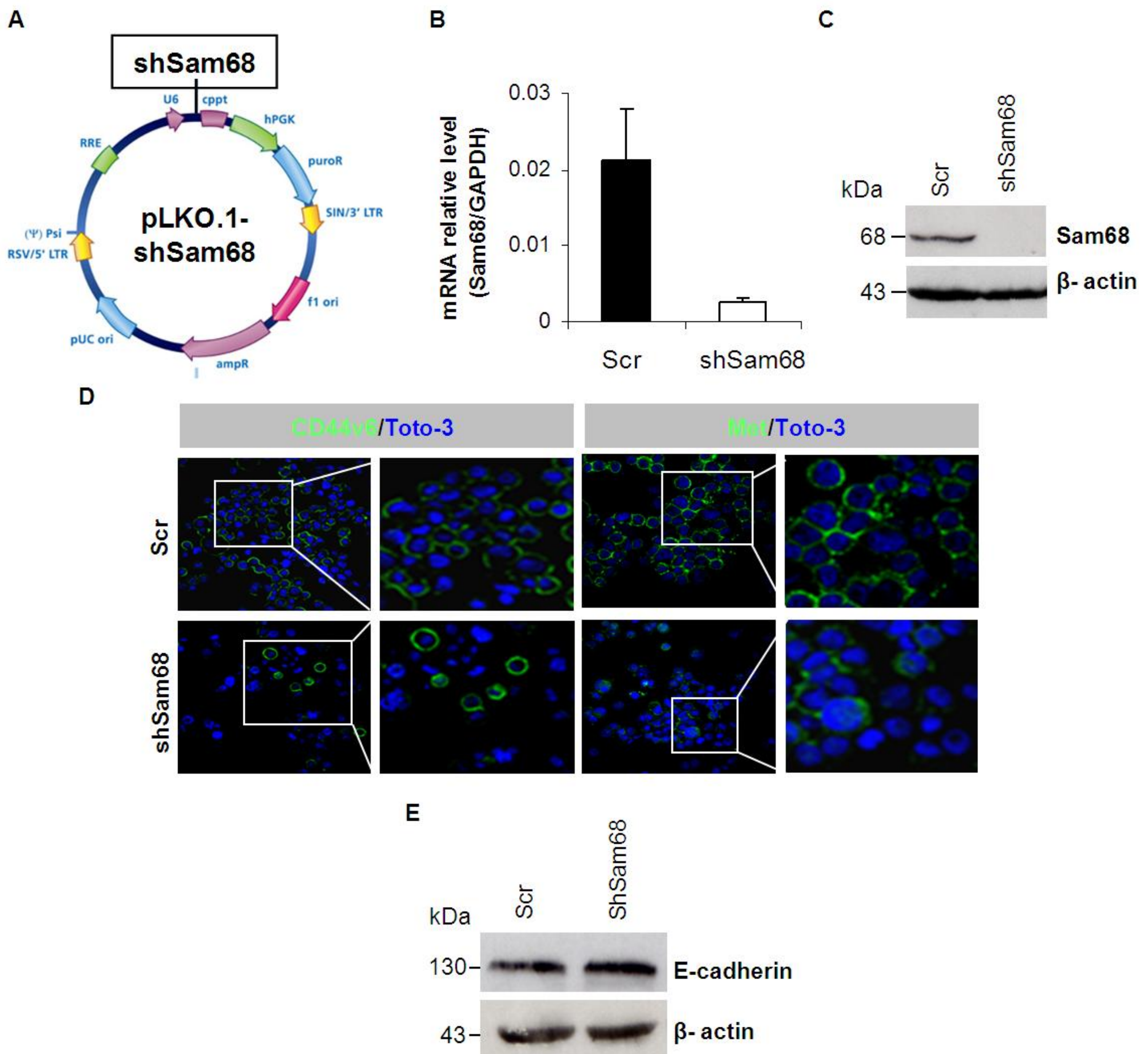


Figure 5. A, Kaplan–Meier estimates of disease-free survival (DFS), local relapse-free survival (LRFS) and distant relapse-free survival (DRFS) in all patients (n = 155) according to high (green solid line) and low (blue dashed line) expression of Sam68. B, Kaplan–Meier estimates of distant relapse-free survival (DRFS) in all patients (n = 155) according to the molecular types HER2 + TN and C, Luminal-B HER2 positive + HER2 + TN. High (green) and low (blue) expression of Sam68.



Supplementary Figure S1. A, schematic representation of the PLK0.1 vector containing the shSam68 sequence. B, qRT-PCR and, C, Western blotting analysis for Sam68 levels in scr and shSam68 BCSCs. D, Immunofluorescence images for CD44v6 and MET (both in green) in scr and shSam68 BCSCs. E, western blotting analysis for E-cadherin in Scr and shSam68 BCSCs.

Variable	HR	95% CI	P
Disease-free survival			
Tumor size, cm (≤ 2 *vs* >2)	1.4	0.7-2.9	0.305
Tumor grade (2-3 *vs* 1)	1.4	0.5-4.1	0.520
ER (negative *vs* positive)	2.3	0.9-6.0	0.095
PR (positive *vs* negative)	1.3	0.5-3.2	0.584
Ki-67 (high *vs* low)	1.1	0.5-2.1	0.847
HER-2 (positive *vs* negative)	1.1	0.5-2.6	0.753
Sam68 (high *vs* low)	1.4	0.7-2.7	0.295
Local Relapse-Free Survival			
Tumor size, cm (> 2 *vs* ≤ 2)	1.9	0.6-5.8	0.259
Tumor grade (1 *vs* 2-3)	2.3	0.7-7.6	0.183
ER (positive *vs* negative)	1.2	0.2-6.1	0.804
PR (negative *vs* positive)	1.2	0.3-5.1	0.832
Ki-67 (high *vs* low)	1.6	0.5-4.9	0.452
HER-2 (negative *vs* positive)	1.0	0.3-2.2	0.977
Sam68 (low *vs* high)	3.1	0.9-9.4	0.062
Distant Relapse-Free Survival			
Tumor size, cm (≤ 2 *vs* >2)	1.8	0.8-4.5	0.176
Tumor grade (2-3 *vs* 1)	1.0	0.3-2.9	0.975
ER (negative *vs* positive)	3.0	0.9-10.1	0.068
PR (positive *vs* negative)	1.7	0.6-5.3	0.343
Ki-67 (high *vs* low)	1.1	0.4-2.5	0.898
HER-2 (positive *vs* negative)	1.5	0.6-3.8	0.349
Sam68 (high *vs* low)	**2.6**	**1.1-6.3**	**0.037***

*Statistically significant

Table 1 Multivariate Analysis of Sam68 expression in breast tumors

Subtype	Distant Relapse Free Survival			
		HR	**95% CI**	**P**
Luminal-A (n=70)	Tumor size, cm (≤ 2 *vs* > 2)	1.0	0.2-5.4	0.972
	Tumor grade (2-3 *vs* 1)	1.0	0.1-5.5	0.977
	Sam68 (high *vs* low)	10.1	0.8-86.6	0.085
Luminal-B HER2 Neg (n=36)	Tumor size, cm (>2 *vs* ≤ 2)	1.1	0.3-4.7	0.912
	Tumor grade (2-3 *vs* 1)	1.0	0.2-5.3	0.989
	Sam68 (low *vs* high)	2.6	0.6-11.4	0.201
Luminal-B HER2 Pos (n=14)	Tumor size, cm (>2 *vs* ≤ 2)	1.3	0.1-11.6	0.825
	Tumor grade (2-3 *vs* 1)	-	-	-
	Sam68 (high *vs* low)	-	-	-
HER2 (n=10)	Tumor size, cm (>2 *vs* ≤ 2)	2.5	0.3-22.5	0.405
	Tumor grade (2-3 *vs* 1)	-	-	-
	Sam68 (high *vs* low)	5.7	0.4-80.2	0.201
Triple Negative (n=25)	Tumor size, cm (≤ 2 *vs* > 2)	2.0	0.2-22.0	0.576
	Tumor grade (2-3 *vs* 1)	-	-	-
	Sam68 (high *vs* low)	-	-	-
HER2 *plus* Triple Negative (n=35)	Tumor size, cm (≤ 2 *vs* > 2)	1.5	0.4-6.1	0.547
	Tumor grade (2-3 *vs* 1)	1.0	0.2-4.5	0.984
	Sam68 (high *vs* low)	6.4	0.8-51.8	0.084
Luminal-B HER2 Pos *plus* HER2 *plus* Triple Negative (n=49)	Tumor size, cm (≤ 2 *vs* > 2)	1.3	0.4-3.9	0.687
	Tumor grade (2-3 *vs* 1)	1.0	0.1-4.4	0.989
	Sam68 (high *vs* low)	**7.9**	**1.0-60.7**	**0.048***

*Statistically significant

Table 2. Multivariate analyses of Sam68 expression in breast cancer subtypes

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Chapter 2

Erythropoietin-mediated activation of survival pathways protects breast cancer stem cells from chemotherapy

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Published in *Cancer Research*, 2013

Abstract

Recombinant erythropoietin (EPO) analogs (ESAs, erythropoiesis-stimulating agents) are clinically used to treat anemia in cancer patients receiving chemotherapy. After clinical trials reporting increased adverse events and/or reduced survival in ESAs-treated patients, concerns have raised about the potential role of ESAs in promoting tumor progression, possibly through tumor cell stimulation. However, evidence is lacking on the ability of EPO to directly affect cancer stem cells, which are considered responsible for tumor progression and relapse. We found that breast cancer stem cells (BCSC) isolated from patient tumors express the EPO receptor and respond to EPO treatment with increased proliferation and self-renewal. Importantly, EPO stimulation increased BCSC resistance to chemotherapeutic agents and activated cellular pathways responsible for survival and drug resistance. Specifically, the Akt and ERK pathways were activated in BCSC at early time points following EPO treatment, while Bcl-xL levels increased at later times. *In vivo*, EPO administration counteracted the effects of chemotherapeutic agents on BCSC-derived orthotopic tumor xenografts and promoted metastatic progression both in the presence and in the absence of chemotherapy treatment. Altogether, these results indicate for the first time that Epo acts directly on BCSC by activating specific survival pathways, resulting in BCSC protection from chemotherapy and enhanced tumor progression.

2.1 Introduction

Erythropoiesis-stimulating agents (ESAs) have been used for two decades in the supportive therapy of cancer patients, due to their ability to increase red blood cell production and to reduce the need of transfusions (1). In 2002, ESAs were administered to approximately 45% of all cancer patients (2). However, following clinical trials reporting a shorter progression-free survival and/or overall survival in ESAs-treated patients, ESAs were suspected to increase the risks of thromboembolic events and to enhance tumor progression (3-6). Consequently, in 2008 the Food and Drug Administration (FDA) limited the indication for ESAs administration to cancer patients with hemoglobin less than 10 g/dL receiving chemotherapy for palliative intent (7). Since then, the use of ESAs in cancer patients progressively declined, and recently the FDA released new guidelines ensuring that ESAs access is strictly controlled and that patients are fully informed about ESAs-related risks (8). Despite clinical observations suggesting a possible association between ESAs and tumor progression, the effect of EPO on neoplastic cells remains a matter of debate. In particular, experimental studies on the effect of EPO on cancer cells yielded controversial results, likely due to variable methodologic approaches. Recent *in vivo* studies, however, provided important clues on tumors' response to EPO. Specifically, EPO was shown to antagonize the effect of trastuzumab on breast cancer xenografts and to decrease the effect of chemotherapy in a mouse model of metastatic breast cancer (9, 10). Such studies suggest a direct influence of EPO on breast tumors and highlight the importance of reliable *in vivo* models to elucidate the interactions between EPO and tumor cells. The existence of cancer stem cells (CSC) in solid tumors was demonstrated for the first time in breast cancer, where CSC were isolated as a $CD44^{+}/CD24^{-/low}$ population able to initiate tumors with as few as 200 cells (11). Lately, breast tumorigenic cells were identified either by distinctive phenotypes such as $ALDH^{+}$, $CD24^{high}/CD49f^{high}$/delta-like 1 $(DLL1)^{high}$, $CD24^{high}/CD49f^{high}$ /Delta-notch like epidermal growth factor repeat-containing transmembrane $(DNER)^{high}$, or through functional characteristics such as enhanced PKH26 dye-retaining capacity or low proteasome activity (reviewed in (12)). More recently, breast cancer stem cells (BCSC) were identified as a ganglioside $GD2^{+}$ population able to form tumors with as few as 10 cells (13). BCSC have been shown to increase after chemotherapy treatment (14) and to be quantitatively associated with chemotherapy resistance (15). Moreover, BCSC have been shown to mediate invasion and metastasis both *in vitro* and in mouse models (16). Elucidating the effect of EPO on BCSC is therefore crucial to fully understand the effects of ESAs treatment in breast cancer patients. As ESAs administration is reserved to patients with metastatic breast cancer receiving

chemotherapy, it is particularly important to understand whether they may influence BCSC response to anticancer drugs and metastasis progression. Here, we employed human BCSC-derived orthotopic/metastatic xenografts to show that BCSC response to EPO *in vivo* results in increased chemotherapy resistance of primary tumors and metastases, resulting in enhanced tumor progression.

2.2 Materials and Methods

2.2.1 Antibodies and reagents

Primary antibodies were: mouse monoclonal anti-EPOR MAB307 (R&D Systems; ref. 10), rabbit polyclonal anti-EPOR M20 (Santa Cruz Biotechnology; ref. 9), rabbit polyclonal anti-Akt and rabbit polyclonal anti-phospho-Akt Ser 473 (9272 and 9271; Cell Signaling Technology), mouse monoclonal anti-phospho Erk1/2 Tyr 204 (E4; Santa Cruz Biotechnology), rabbit polyclonal anti-Erk1 (K23; Santa Cruz Biotechnology), mouse monoclonal anti-Bcl-xL (H-5; Santa Cruz Biotechnology), mouse monoclonal anti-β-actin (JLA20; Calbiochem), mouse monoclonal anti-CD44 (BU75; Ancell), mouse monoclonal anti-CD24 (HIS50; BD Biosciences), mouse monoclonal anti-p63 (4A4; Santa Cruz Biotechnology), mouse monoclonal CK8-18 (5D3), mouse monoclonal CK14 (LL002), and mouse monoclonal CK5 (XM26; all from Novocastra), mouse monoclonal anti-Ki67 (MIB-1; Dako), mouse monoclonal anti-CD49f (MP4F10; R&D Systems). Secondary antibodies were: horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Pierce), HRP-conjugated anti-rabbit antibody (Thermo Scientific), mouse fluorescein isothiocyanate (FITC)- and Rhodamine red–conjugated antibodies (Invitrogen, Molecular Probes), and mouse R-phycoerythrin (PE) antibody (Sigma-Aldrich). Recombinant human EPO was purchased from R&D Systems.

2.2.2 BCSC isolation and culture

Human breast cancer tissues were obtained from patients undergoing surgery in accordance with the ethical standards of the institutional Committee on human experimentation (authorization no. CE-ISS 09/282). Tumor tissues were mechanically and enzymatically digested with collagenase (1.5 mg/mL; Gibco) and hyaluronidase (20 mg/mL; Sigma-Aldrich) in Dulbecco's Modified Eagle Medium (Gibco), shaking for 1 hour at 37°C. The resulting cell suspension was plated in ultra-low attachment flasks (Corning) in serum-free medium supplemented with basic fibroblast growth factor (bFGF; 10 ng/mL) and EGF (20 ng/mL) as previously described (17). This procedure yielded BCSC lines that were subjected to genotyping

to validate each cell line individuality and were further tested for their ability to generate tumor xenografts that replicated the histology of the parental tumor.

2.2.3 Viability, proliferation, and clonogenic assays

For viability assays, BCSCs untreated or pretreated 24 hours with 3 U/mL EPO were cultured for the indicated times in presence of doxorubicin (1 μmol/L), 5-FU (5-fluorouracil; 25 μmol/L), or Taxol (5 μmol/L). The number of viable cells was detected by the CellTiter AQueous Assay Kit (Promega). Cell death was also assessed by acridine orange (50 μg/mL)/ethidium bromide (1 μg/mL) staining and fluorescence microscopy detection. Colony-forming assays were conducted on soft agar (Seaplaque) with 0.4% base agar and 0.3% top-layer agar. After 21 days, colonies were stained with 0.01% crystal violet and visualized under a light microscope.

2.2.4 Immunoblotting

Cells were growth factor–starved for 24 hours and treated with 3 U/mL EPO for 10, 30, 120 minutes and 48 hours. Protein extracts were obtained in ice-cold T-PER buffer (Thermo Scientific) with protease inhibitors (Sigma-Aldrich). Equal amounts of proteins were loaded on SDS-PAGE gels and transferred to nitrocellulose membranes, subsequently blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20 and probed with primary and HRP-linked secondary antibodies. Immunoreactive bands were visualized with SuperSignal West Dura Substrate (Pierce). Image acquisition was conducted with a ChemiDoc Imaging system (UVP Advanced Imaging Systems).

2.2.5 Immunohistochemistry

Apoptotic cells on paraffin-embedded breast cancer xenograft sections were visualized by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reaction with the *In Situ* AP Cell Death Detection Kit (Roche). Immunohistochemical analyses were conducted on 5 μm-thick paraffin-embedded sections of breast cancer tissue and xenografts. Tissues were heated for antigen retrieval and stained with specific antibodies against Bcl-xL, CK 8-18, CK 14, CK 5, p63, Ki67, EPO receptor (EPOR), or isotype-matched controls overnight at 4°C. Sections were incubated with biotinylated anti-mouse or anti-rabbit immunoglobulins and subjected to streptavidin-peroxidase (Dako). Stainings were revealed using 3-amino-9-ethylcarbazole substrate (AEC; Dako) substrate and cells counterstained with aqueous hematoxylin. Slides were mounted with synthetic resin.

2.2.5 Immunofluorescence and flow cytometry

Immunofluorescence was conducted on cytospins of cultured BCSC fixed with 2% paraformaldehyde for 20 minutes at 37°C, blocked with 0.5% bovine serum albumin for 30 minutes and exposed overnight at 4°C to antibodies against EPOR, CD44, and CD24. Stained slides were treated with Rhodamine Red- or FITC-conjugated anti-mouse antibodies with the addition of 200 ng/mL RNAse (Sigma-Aldrich). Nuclei were counterstained with TOTO-3 iodide (Invitrogen-Molecular Probes) and images were acquired using an Olympus FV1000 confocal microscope. For fluorescence-activated cell sorting (FACS) staining, BCSC were fixed with 2% paraformaldehyde and stained with primary antibodies against CD44, CD24, EPOR, or isotype-matched controls and then with fluorochrome-conjugated secondary antibodies. Samples were analyzed with a FACSCalibur equipped with CellQuest Software (BD Biosciences).

2.2.6 Mice treatment

Animal studies were carried out according to the institutional guidelines under the Italian Ministry of Health authorization (DM 23/2011-B). BCSCs (3×10^5) were suspended in 100 µl of 1:6 Matrigel (BD Biosciences) and orthotopically injected in 5-week-old nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Charles River Laboratories). Tumor size was measured weekly with an electronic caliper and volume was calculated using the formula: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. After 4 weeks, mice were treated intraperitoneally either with doxorubicin (2 mg/kg, on day 2 and 5 every week for 4 weeks) or 5-FU (150 mg/kg, on day 1 every week for 4 weeks), alone or in combination with EPO (150 U/kg, on day 1 and 4 every week for 4 weeks). PBS was used as control. At the end of the treatment, mice were sacrificed and tumors were collected for histologic analyses. To determine the *in vivo* effects of EPO on a metastatic breast cancer model, NOD/SCID mice were orthotopically injected with 4.5×10^5 BCSCs carrying a Tween Luciferase–GFP lentiviral vector. After cell inoculation, mice received a subcutaneous injection of d-luciferin (150 mg/kg; Promega) and were analyzed by *in vivo* imaging (Biospace Laboratories). Five weeks later, primary tumors of mice showing lung metastases (as measured by luciferase intensity) were removed and mice ($n = 4$ per group) received respectively intraperitoneal injections of PBS, paclitaxel (10 mg/kg, on day 1 every week for 3 weeks), EPO (300 U/kg on day 2 every week for 3 weeks), or paclitaxel + EPO. Three weeks later, mice were euthanized and lungs were analyzed for luciferase expression. Data were quantified with Biospace Lab M3 Vision software.

2.2.7 Statistical analysis

Data were expressed as mean ± SD. The statistical significance of results was determined by Bonferroni multiple comparison tests. Results were considered significant when P values were less than 0.05 (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

2.3 Results and Discussion

BCSCs can be isolated from tumor specimens by selective culture in medium containing EGF and bFGF (18), resulting in a majority of $CD44^{+}/CD24^{-/low}$ cells that form progressively expanding tumor spheres (Figure 1A and Supplementary Figure S1A). According to this method, five BCSC lines were isolated from infiltrating breast tumors (Supplementary Table S1). Cells isolated in such conditions fulfilled the functional characteristics of CSCs, as they were able to produce tumors in immunocompromised mice that replicate the original patient tumor in terms of histologic structure and marker expression (Figure 1B). As a first step to investigate the potential BCSC sensitivity to EPO, we assessed whether the EPO receptor was detectable on cultured CSC and on tissue sections of different breast tumor subtypes, whose BCSC content was reportedly related to increasing malignancy (19). Because of previous controversies about the specificity of anti-EPO receptor antibodies, we used only antibodies that were validated by recent authoritative studies (see Materials and Methods). The specificity of the anti-EPO receptor antibody used for immunofluorescence and flow cytometry was further validated by assessing its ability to detect EPO receptor increase in leukemic UT-7 EPO cells upon growth factor starvation (Supplementary Figure S1B; ref. 20). Staining of intact BCSC with anti-EPOR antibody revealed substantial (31–99%) EPOR expression on all the BCSC lines examined (Figure 1C), indicating a potential role of EPO in the regulation of BCSC proliferation and survival. EPO receptor was also detected on cultured BCSCs stained with CD49f antibodies (Supplementary Figure S1C), indicating its presence on BCSC populations identified with different stem cell–associated markers.

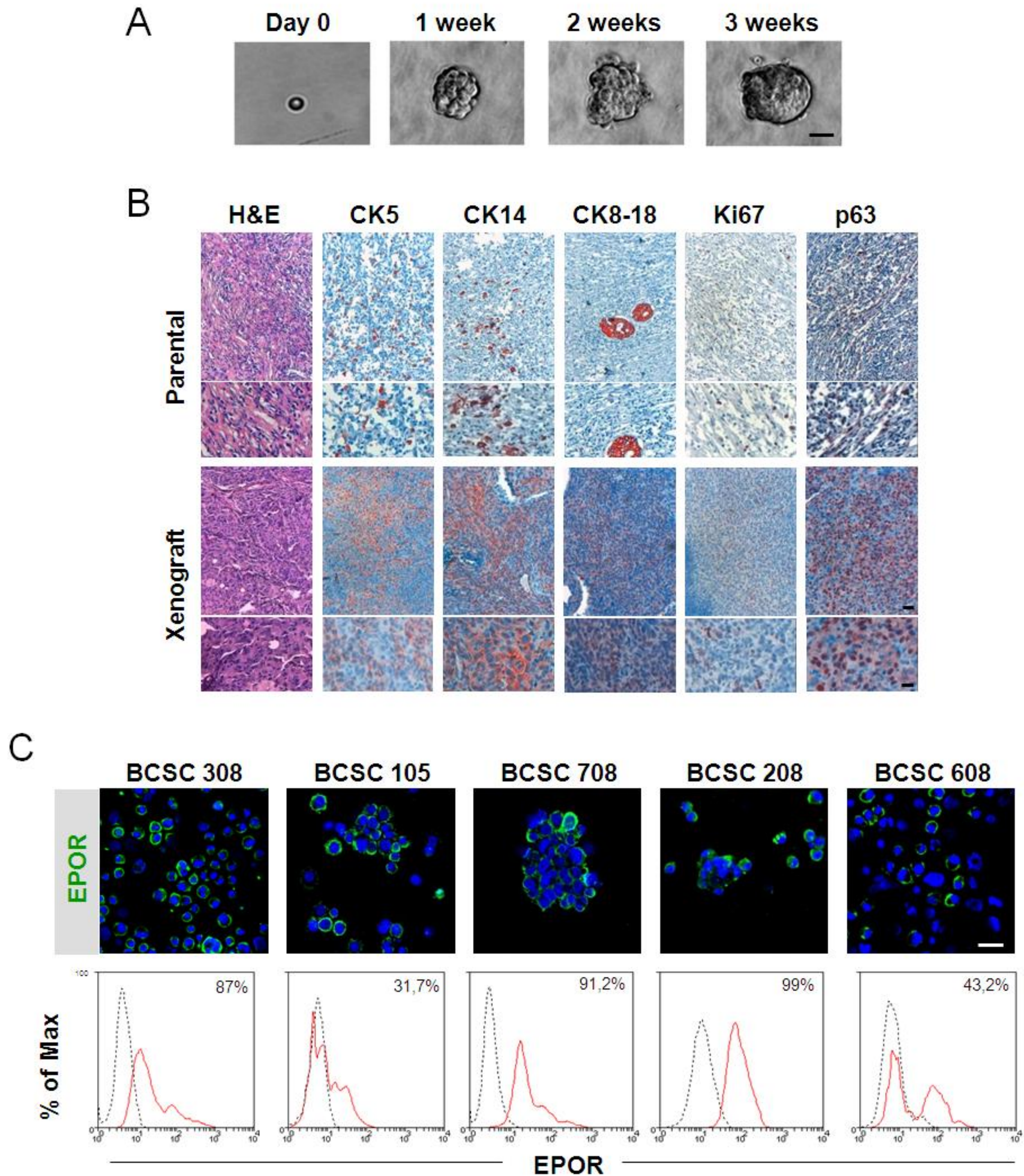


Figure 1. Characterization of BCSC lines and BCSC-derived xenografts. A, microscopic imaging of progressive sphere formation by a single BCSC (line 308). Bar, 25 µm. B, immunohistochemical staining with hematoxylin and eosin (H&E) or antibodies against cytokeratins 5 (CK5), 14 (CK14), 8-18 (CK8-18), Ki67, and p63 of sections derived from breast carcinoma (Parental) and from a mouse xenograft (Xenograft) generated with BCSC derived from the same tumor (BCSC line 308). Bar, 40 µm (inset, 30 µm). C, EPO receptor expression on BCSC lines detected by fluorescence microscopy (top; bar, 25 µm) or flow cytometry (bottom).

In tissue sections, strong EPO receptor expression was detected on the positive control (placenta) and on basal-like tumors. EPO receptor was also present, at a lesser extent, on HER2^{+} and luminal B tumors, whereas a faint expression was detectable on luminal A tumors and on normal breast, the latter showing a positivity at the limit of detection (Figure 2A and Supplementary Table S2). Treatment of BCSC cultures with recombinant human EPO resulted in increased cell proliferation, indicating that EPOR expressed on BCSC surface is functional and delivers signals that modulate cell growth (Figure 2B). To determine whether EPO was able to affect BCSC self-renewal, cells derived from dissociated mammospheres were plated in soft agar in the presence or in the absence of EPO. Colony scoring after 3 weeks showed a significantly higher number of colonies in EPO-treated samples in 3 of 5 cases, indicating that EPO can increase BCSC self-renewal *in vitro* (Figure 2C).
BCSCs were previously shown to be more resistant than bulk tumor cells to chemotherapeutic drugs (14). Because EPO is used almost exclusively in patients with cancer undergoing chemotherapy, it is essential to clarify whether EPO can further enhance BCSC resistance to chemotherapy, therefore favoring drug resistance and tumor relapse. Upon treatment of mammospheres with EPO and with chemotherapeutic agents commonly used for breast cancer therapy, we found that the presence of EPO resulted in increased BCSC survival in the presence of cytotoxic drugs (Figure 3A and B). This observation indicates that EPO activates survival signals in BCSCs that are responsible for chemoresistance. To identify pathways downstream of EPOR that may be responsible for apoptosis resistance in BCSC, we analyzed levels of phospho-Akt, phospho-Erk, and Bcl-xL at different time points upon EPO stimulation (Figure 3C and Supplementary Fig. S2). Phosphorylation/activation of Erk and Akt was maximal respectively 10 minutes and 2 hours after EPO stimulation in 5 of 5 and 4 of 5 BCSC lines (Figure 3C). Differently, an increase in Bcl-xL levels was not apparent at early time points of EPO stimulation (Supplementary Figure S2), but became clear in 5 of 5 BCSC lines after 48 hours of treatment (Figure 3C). Increase in pErk, pAkt, and Bcl-xL upon EPO stimulation was apparent also in intact spheres (Figure 3D).

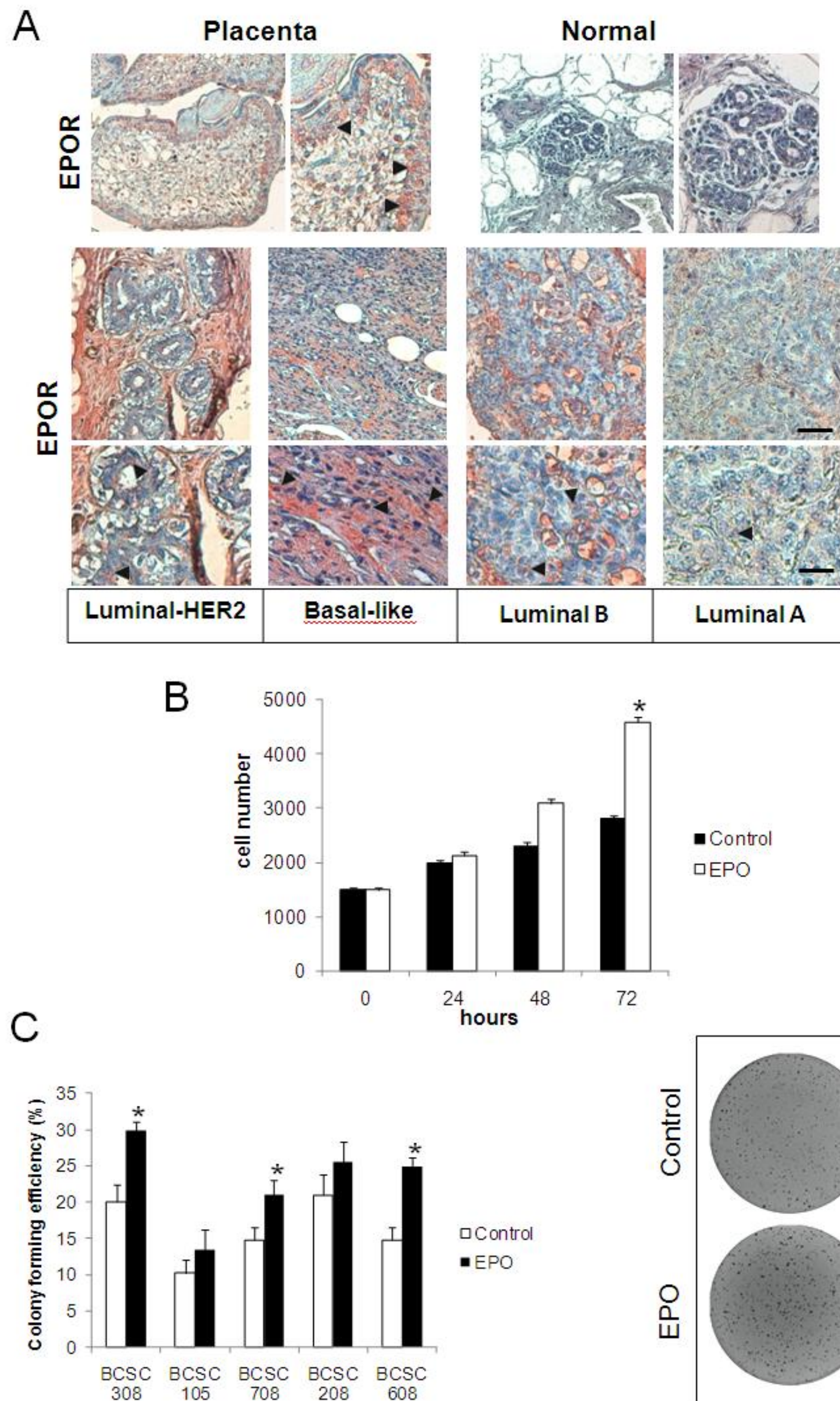


Figure 2. EPOR expression in breast cancer subtypes and EPO response of cultured BCSC. A, EPOR staining of tissue sections derived from human placenta, normal breast (top), and breast tumors of different subtypes (bottom). Black arrows indicate EPOR-positive cells. Bar, 45 µm (inset, 25 µm). B, number of cells obtained after 72 hours of culture in the absence (control) or in the presence of EPO 3 U/mL (EPO). Results shown are the mean ± SD of experiments carried out in triplicate with five BCSC lines. *, $P < 0.05$. C, number of colonies generated in semisolid culture conditions by BCSC lines in the absence (Control) or in the presence (EPO) of EPO 3 U/mL (left). Representative picture of the plates (BCSC line 308; right). *, $P < 0.05$.

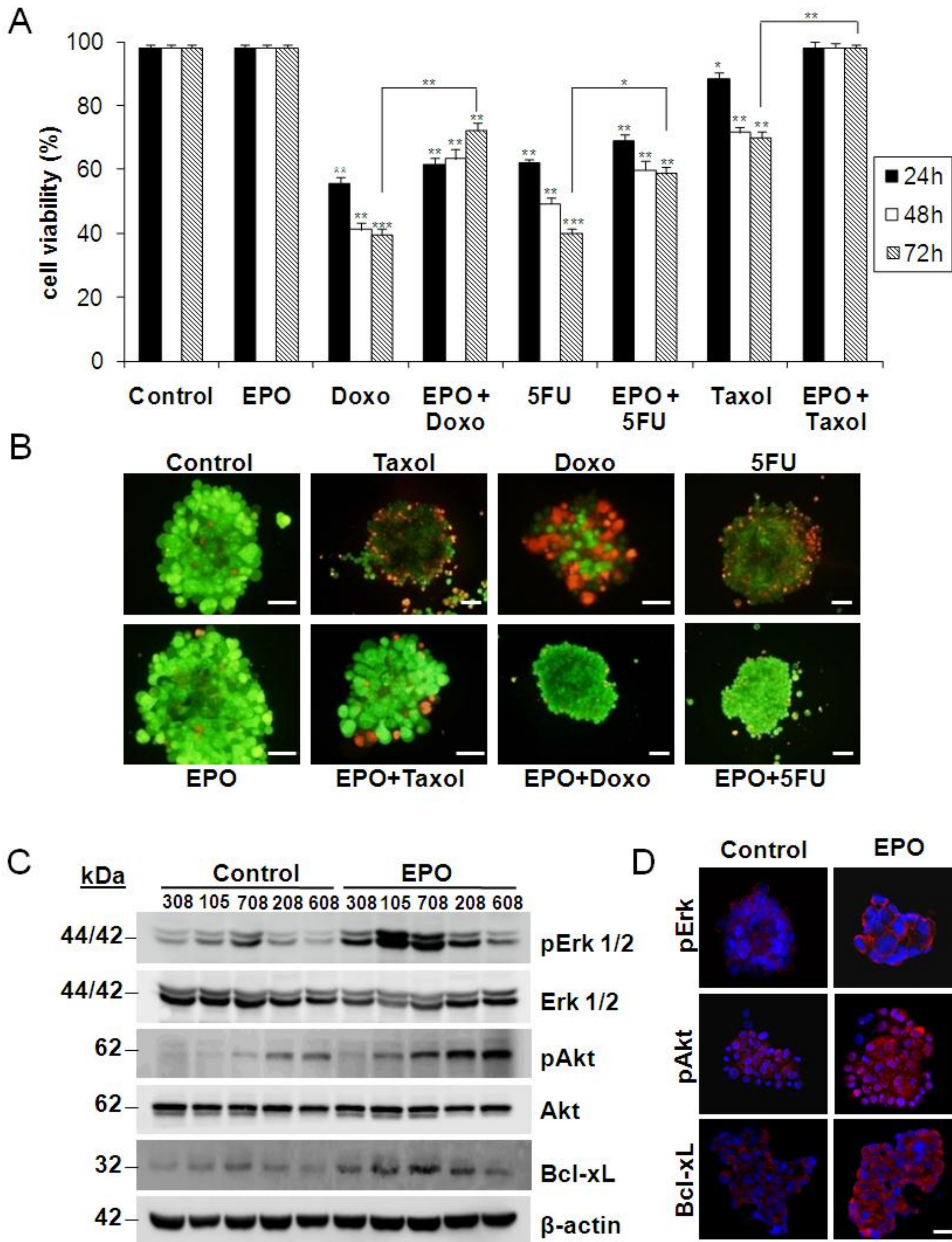


Figure 3. EPO increases BCSC resistance to chemotherapy *in vitro* by stimulating cell survival pathways. A, BCSC untreated (Control), treated with EPO 3 U/mL (EPO), with chemotherapeutic agents doxorubicin (Doxo; 1 μmol/L), 5-FU (25 μmol/L), or Taxol (5 μmol/L) and with the combination EPO-chemotherapy were assessed for cell viability after 24, 48, and 72 hours. Results shown are the mean ± SD of experiments carried out in triplicate with five BCSC lines. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. B, ethidium

bromide/acridine orange staining of tumor spheres (BCSC line 308) treated as above. C, levels of phospho-Erk (pErk), phospho-Akt (pAkt), and Bcl-xL in BCSC untreated (Control) or growth factor–starved and stimulated with 3 U/mL EPO, respectively, for 10 minutes, 2, and 48 hours. D, cells treated as in C (BCSC line 208) were stained with the indicated antibodies and visualized by fluorescence microscopy. Bar, 25 μm.

To investigate whether EPO treatment affected tumor response to chemotherapy *in vivo*, we produced orthotopic breast tumors by injecting BCSC in the mammary fat pad of NOD/SCID mice. Tumors were allowed to grow until they reached the size of 50 mm^3, then mice were treated with EPO and/or chemotherapeutic agents doxorubicin (Figure 4A and B) or 5-FU (Supplementary Figure S3A and S3B) for 4 weeks, during which tumor volume was constantly monitored with an electronic caliper. Although vehicle-treated tumors grew exponentially, chemotherapy-treated tumors were significantly inhibited. In contrast, the growth of tumors treated with EPO + chemotherapy was similar to controls, indicating a chemoprotective effect of EPO *in vivo* (Figure 4A and B and Supplementary Figure S3A). Staining of xenograft sections at the end of the treatment revealed an increased rate of apoptosis and lower levels of Bcl-xL expression in chemotherapy-treated tumors but not in tumors treated with chemotherapy and EPO in combination (Figure 4B and Supplementary Figure S3B). These results indicate that EPO reduces the efficacy of chemotherapy *in vivo* by promoting BCSC apoptosis resistance. To investigate whether EPO could influence the growth of metastatic tumors, we injected luciferase-transduced BCSC in the mammary fat pad of NOD/SCID mice and awaited the formation of spontaneous lung metastases. Five weeks after BCSC injection, when metastases started to be detectable, the primary tumor was removed to observe the effect of subsequent treatments solely on metastatic sites. Mice were then treated for 3 weeks with EPO alone, with paclitaxel, or with the EPO + paclitaxel combination. At the end of the treatment, mice were sacrificed and lungs were analyzed for luciferase expression. Tumor burden in lungs of mice treated with EPO alone was strongly increased as compared with that of control mice (Figure 4C and D). Enhanced metastatic progression was also found in the lungs of mice treated with the paclitaxel + EPO combination as compared with mice treated with paclitaxel alone, indicating that EPO exerted a chemoprotective effect on metastatic tumors (Figure 4C and D).

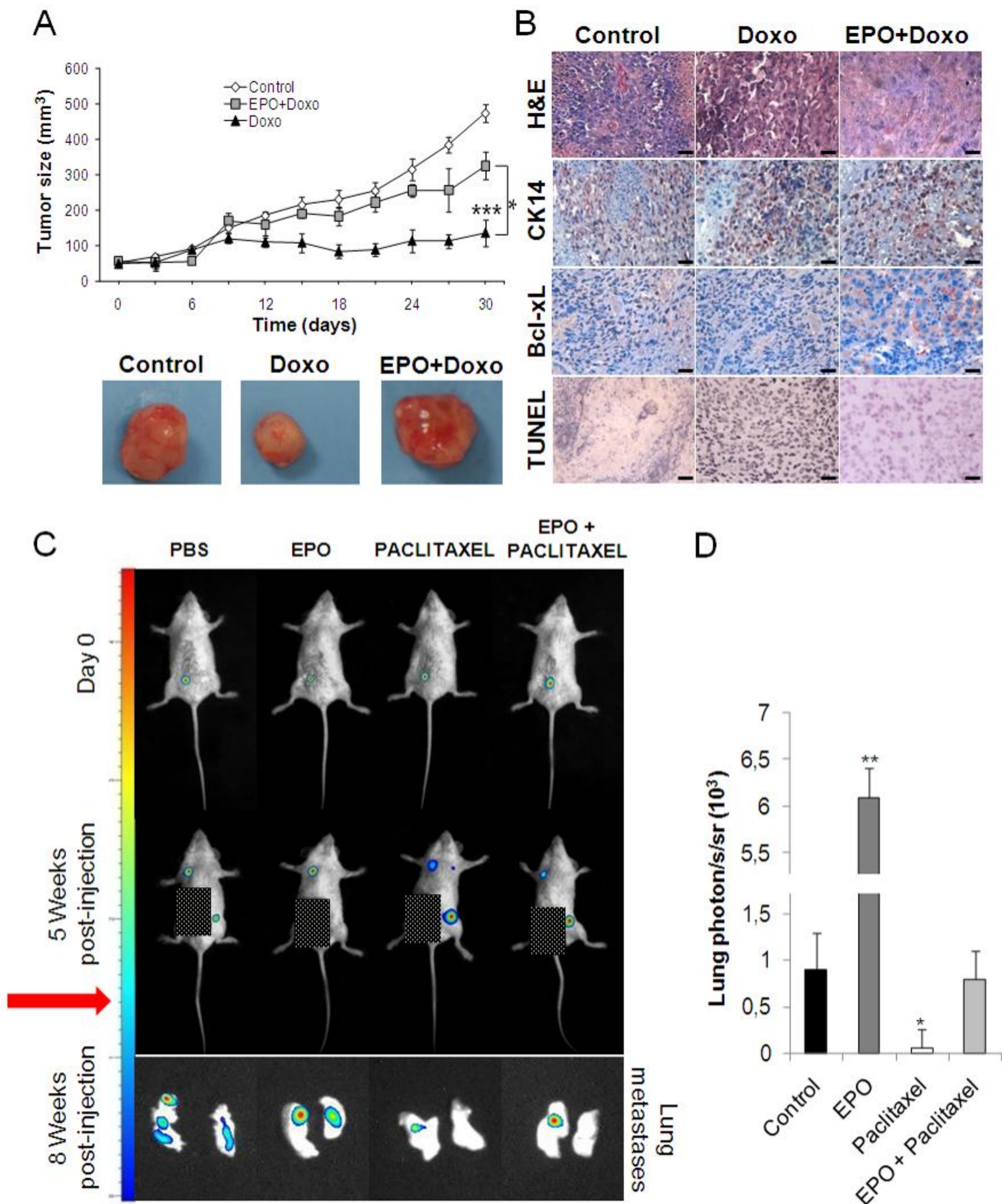
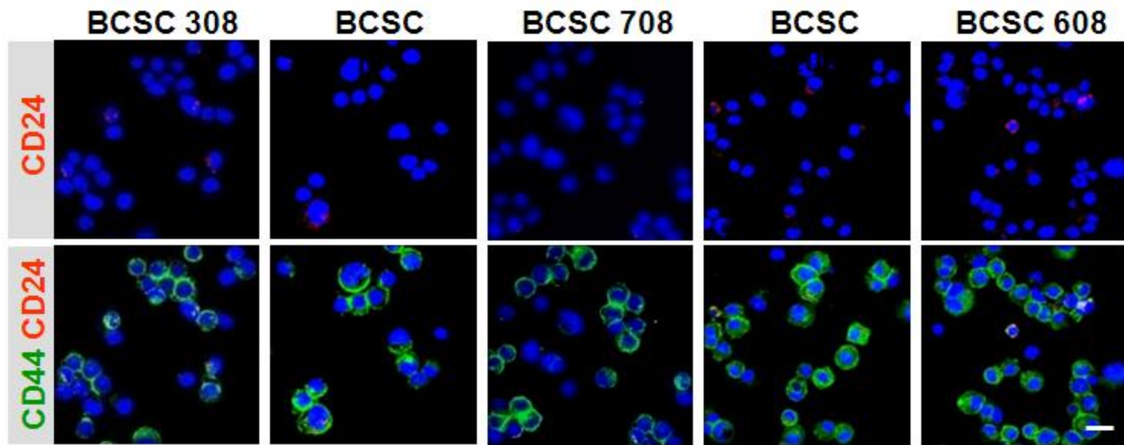


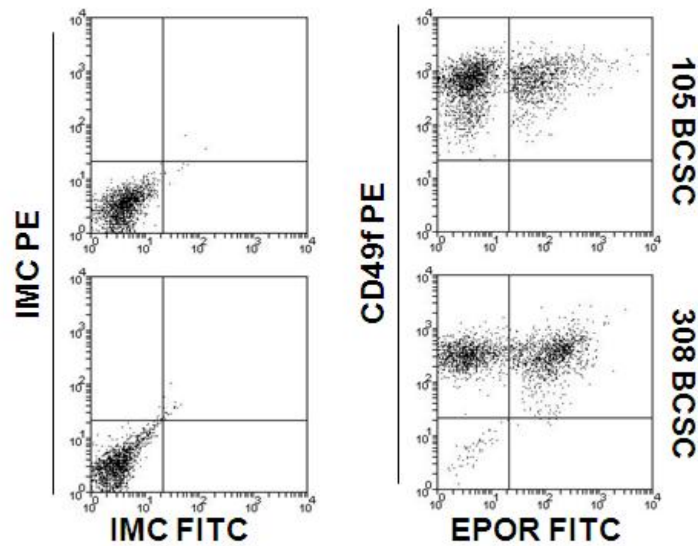
Figure 4. EPO protects primary and metastatic tumors from chemotherapy *in vivo*. A, growth of BCSC-derived tumor xenografts (BCSC line 308) vehicle-treated (Control), treated with doxorubicin (Doxo) alone, or in combination with EPO (Doxo+EPO) as described in Materials and Methods (top). Results shown are the mean ± SD of three experiments carried out with groups of 3 mice each. *, $P < 0.05$; ***, $P < 0.001$. Representative pictures of the tumors (bottom). B, immunohistochemical staining of xenograft sections obtained at the end of the experiment shown in A and stained with hematoxylin/eosin (H&E), with anti-cytokeratin 14 (CK14), anti-Bcl-xL (Bcl-xL), or

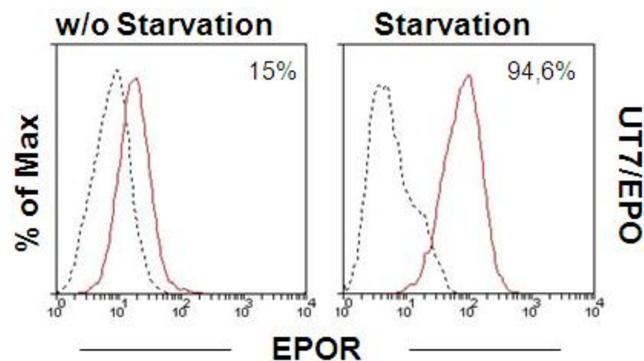
TUNEL. Bar, 30 μm. C, whole-body imaging of tumors at different time points after injection (Day 0) of 5×10^5 BCSCs transduced with Tween-LUC GFP in the mammary fat pad of NOD/SCID mice, as described in Materials and Methods. Five weeks after injection, when lung metastases (and in some cases peritoneal metastases) were visible, the primary tumor was removed and the treatment with EPO, paclitaxel, or paclitaxel + EPO was started (red arrow). After 3 weeks of treatment (8 weeks postinjection), mice were sacrificed and lungs were subjected to bioimaging to detect metastatic tumors (Lung metastases). The black square on the left side of the mice was positioned to shield luciferase signals emitted from residual cells that remained after primary-tumor removal. One representative experiment of 4 mice per group is shown. D, photon counts emitted from mice lungs derived in the experiment described in C. Photon/s/sr, photons per second per steradian. *, $P < 0.05$ and **, $P < 0.01$.

2.4 Conclusions

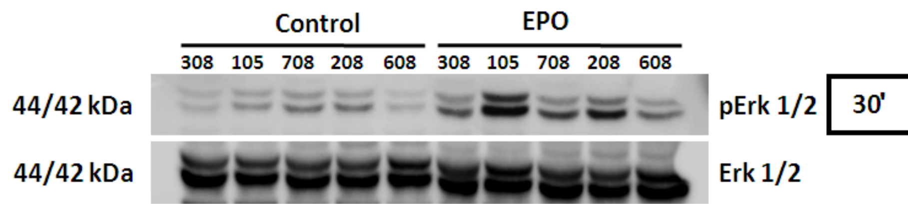
Because the disclosure of clinical trials showing that ESAs treatment had an adverse influence on patient survival, the effect of EPO on tumor cells has been the subject of an intense debate. Although ESAs likely influence patient survival through multiple mechanisms, few *in vivo* studies have specifically addressed the question of whether EPO modifies tumor response to therapy. We have shown for the first time that EPO can bind and stimulate BCSCs, resulting in increased tumor growth and chemoresistance. These results confirm and expand previous observations by Hedley and colleagues on xenografts obtained with breast cancer cell lines (10). In patients with breast cancer, EPO-mediated BCSC stimulation may not result in immediate effects on tumor growth or response to chemotherapy, as BCSCs represent a minority of cells, but may favor subsequent tumor relapse. Further clinical studies that evaluate rates of relapse in ESA-treated patients would be required to clarify this issue.

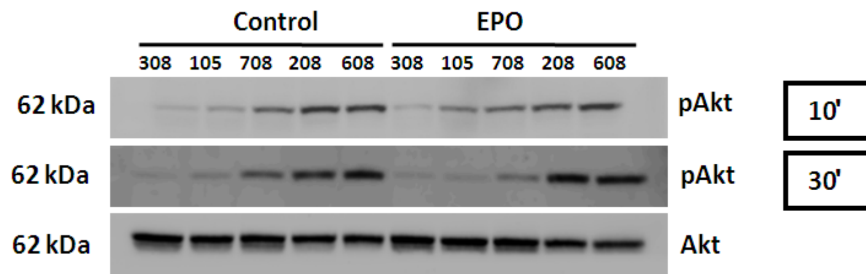


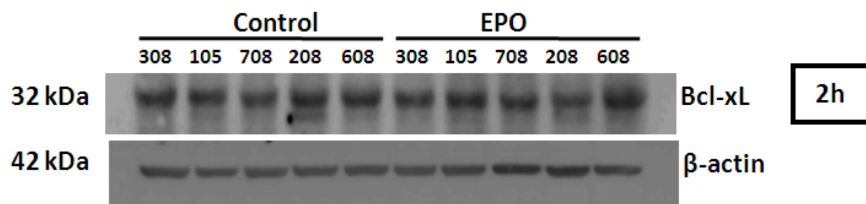




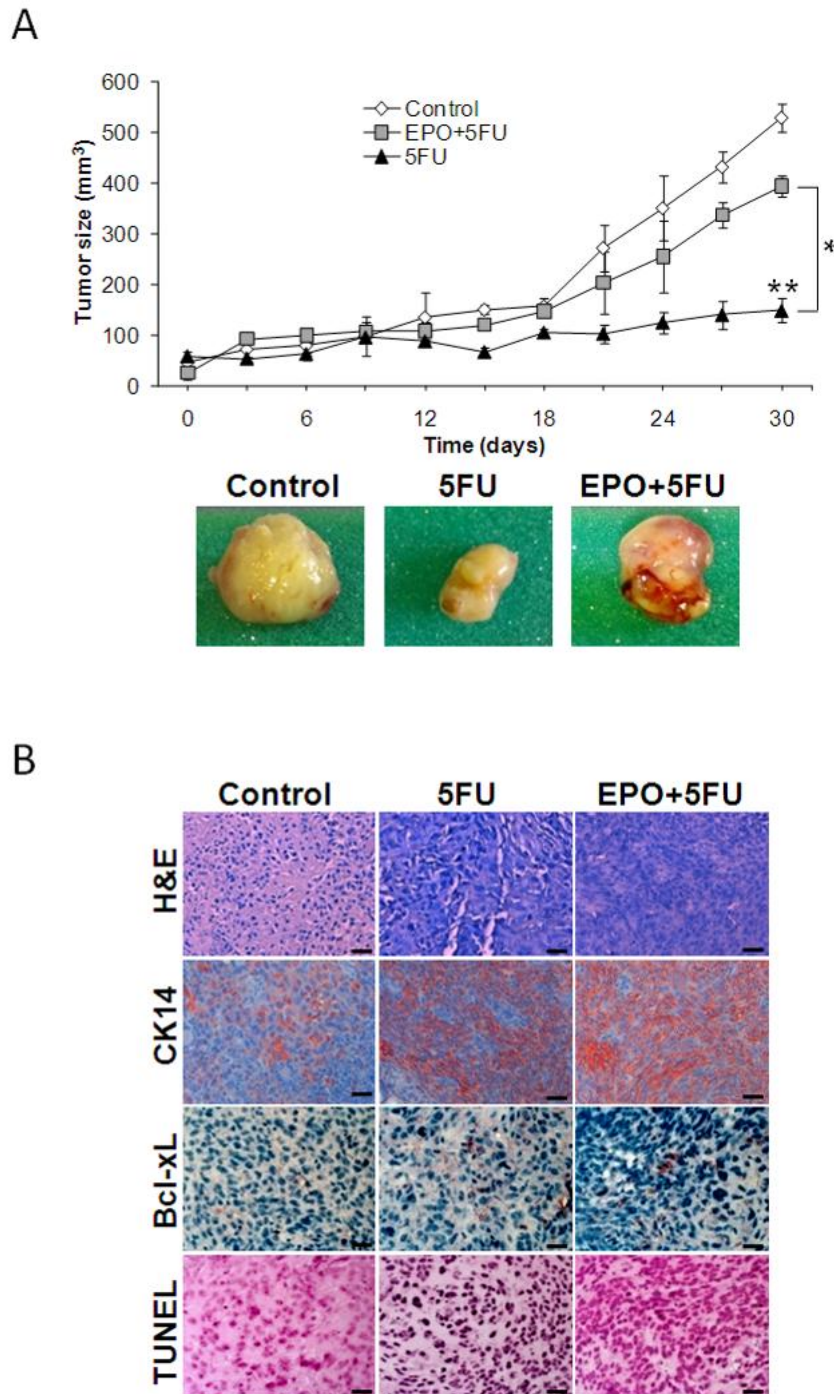
Supplementary Figure S1. A, Immunofluorescence staining of CD44 and CD24 on BCSC derived from five different tumors. Bar, 25 µm. B, Flow cytometry staining of EPO receptor (EPOR) on UT-7 EPO cells kept in EPO-containing growth medium (w/o starvation) or upon 24 hours of EPO deprivation (Starvation). C, flow cytometry staining of the indicated BCSC lines with antibodies against CD49f and EPO receptor (EPOR) IMC isotype matched control.







Supplementary Figure S2. Levels of phospho-Erk (pErk), phospho-Akt (pAkt) and Bcl-xL in BCSC lines untreated (Control) or growth factor-starved for 24 hours and subsequently stimulated with 3U/ml EPO (EPO) for 10 minutes, 30 minutes and 2 hours.



Supplementary Figure S3. A, Upper panel: growth of BCSC-derived tumor xenografts (BCSC line 208) vehicle-treated (Control), treated with 5-fluorouracil (5FU) alone or in combination with EPO (5FU+EPO) as described in Materials and Methods. Results shown are the mean ± SD of three experiments performed with groups of three mice each. *, $P < 0.05$; **, $P < 0.01$. Lower panel: representative images of tumors at the end of the treatment. B, immunohistochemical staining of xenograft sections obtained at the end of the experiment shown in and stained with hematoxylin/eosin (H&E), with anticytokeratin 14 (CK14), anti-Bcl-xL or TUNEL. Bar, 30 µm.

BCSC LINE	Patient age	Tumor type	Grading	ER (%)	PR (%)	c-ERB	Ki67(%)
105	69	IDC	G2	90	60	+	25
208	55	IDC	G2	90	60	+++	>10
308	85	ILC	G2	-	-	+	>10
608	59	IDC	G2	90	30	-	>10
708	74	IDC	G2	80	80	+++	>10

Supplementary Table S1. Tumor type, patient age and grading of breast tumors that were used to derive the indicated BCSC lines. Expression of estrogen receptor (ER), progesterone receptor (PR), c-ErbB2 (c-ERB) and Ki67 of the respective BCSC lines is shown. IDC, infiltrating ductal carcinoma; ILC, infiltrating luminal carcinoma.

	Luminal-HER2	Basal-like	Luminal B	Luminal A
EPOR	30% ± 10%	40% ± 2%	22% ± 3%	8 % ± 1%

Patient no.	ER (%)	PR (%)	HER2	Ki67 (%)
1	90	0	2+	>20
2	0	0	0	>10
3	90	50	1+	>30
4	80	15	1+	<10
5	0	0	0	>60
6	0	0	0	>40
7	95	0	2+	15
8	70	80	1+	>20
9	60	0	1+	>25
10	80	90	0	<10
11	0	0	0	70
12	55	0	2+	25

Supplementary Table S2. A, Evaluation of cells positive for EPO receptor (EPOR) in 12 breast cancer patients of different subtypes, as specified in B. B, breast cancer diagnostic markers ER (estrogen receptor), PR (progesterone receptor), HER2/c-ErbB2 (HER2) and Ki67 in tumor tissue samples analyzed for EPO receptor expression

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Chapter 3

Tumor and its microenvironment: a synergistic interplay

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Published in *Seminars in Cancer Biology*, 2013

Abstract

The mutual and interdependent interaction between tumor and its microenvironment is a crucial topic in cancer research. Recently, it was reported that targeting stromal events could improve efficacies of current therapeutics and prevent metastatic spreading. Tumor microenvironment is a “complex network” of different cell types, soluble factors, signaling molecules and extracellular matrix components, which orchestrate the fate of tumor progression. As by definition, Cancer Stem Cells (CSCs) are proposed to be the unique cell type able to maintain tumor mass and survive outside the primary tumor at metastatic sites. Being exposed to environmental stressors, including reactive oxygen species (ROS), CSCs have developed a GSH-dependent antioxidant system to improve ROS defense capability and acquire a malignant phenotype. Nevertheless, tumor progression is dependent on extracellular matrix remodeling, fibroblasts and macrophages activation in response to oxidative stress, as well as Epithelial Mesenchymal Transition (EMT)-inducing signals and endothelial and perivascular cells recruitment. Besides providing a survival advantage by inducing *de novo* angiogenesis, tumor-associated vessels contribute to successful dissemination by facilitating tumor cells entry into the circulatory system and driving the formation of pre-metastatic niche. In this review, we focus on the synergistic effect of Hypoxia Inducible Factors (HIFs) and Vascular Endothelial Growth Factors (VEGFs) in the successful outgrowth of metastasis, integrating therefore many of the emerging models and theories in the field.

3.1 Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world and one of the major causes of death worldwide (1). The prevention and the early diagnosis are surely the most important approaches for reducing the burden of CRC, given the symptoms of early disease occur just in 5% of cases. A significant portion of patients who receive surgery and adjuvant therapy still develop recurrences and metastasis and this phenomenon seems to be driven in some cell subsets by the acquisition of resistance to conventional therapy, such as chemo- and radio-therapy (2).

Growing evidence indicates that a cellular subpopulation with stem cell like features, commonly referred to as Cancer Stem Cells (CSCs), is critical for tumor generation and maintenance.

A recent study showed that within the tumor population it is possible to identify a heterogeneous population of cells with different biological roles (3). Recent advances in stem cell biology are revealing that this cellular fraction shares many properties with normal adult stem cells, including dormancy (quiescence), active DNA repair machinery, the expression of several ABC drugs transporters and an intrinsic resistance to apoptosis (4). As their normal counterpart, also the colon CSCs reside in a specialised microarchitectonic structures or niches that respond to both local and systemic conditions providing also protection against conventional therapies (5).

Moreover, microenvironmental stimuli, such as those involved in the epithelial-mesenchymal transition (EMT) and hypoxia, indirectly contribute to chemoresistance by inducing in cancer cells a stem like-phenotype. Understanding the driving force of tumor progression and the relationship between cancer cells and microenvironment could be fundamental in developing innovative therapeutic strategies for a better and definitive response on patient treatments.

3.2 CRC, Stem Cell Niche and Colon CSCs

It is widely accepted that CRC progression is driven by the acquisition of 4-5 progressive mutations in oncogenes or tumor suppressor genes (6). Some driver mutations frequently occur in the same gene sequences and are shared by most of the people affected by this cancer, whereas some mutations are different and responsible of the final cancer phenotype in individual patients (7). Most of the information about CRC derives from the study of familial adenomatous polyposis (FAP), an autosomal dominant colon cancer syndrome caused by *APC* gene mutation (8). APC is involved in the regulation of Wnt pathway that, as we will discuss later in this review, can

regulate cell proliferation, differentiation, migration and apoptosis (9). Tumor progression is also achieved by other mutations such as *KRAS, SMAD2/4, TP53* and deletion of chromosome 18q (10).
It was recently demonstrated that despite the great heterogeneity and biological diversity of CRC it is possible to distinguish three different subtypes. De Sousa E Melo *et al.* indeed showed that two of these subtypes have already been identified for chromosomal-instable and microsatellite-instable cancer. A third one, prognostically unfavourable, is characterized by microsatellite stability and relatively more CpG island methylator phenotype-positive, thus rendering it impossible to be identified on the basis of characteristic mutations (11).
The presence of a distinct population with stem cell characteristics among disseminated and circulating cancer cells may be of clinical relevance, not only for their putative role in metastasis formation and recurrence, but also for their role in resistance to conventional therapy. CSCs are likely to share many properties of normal stem cells as mentioned above, which may underlie their capacity to survive therapeutic protocols based on genotoxic agents targeting actively proliferating cells (12).
First invoked by Paget, the "seed and soil" hypothesis suggests that the successful growth of metastatic cells depends on the interactions and properties of cancer cells (seeds) and their potential target organs (soil). Additionally, new concepts include: (i) the role of cancer stem-like cells as putative cells of metastatic origin (the "seeds"); (ii) the mechanism of EMT in driving epithelial cell into the blood stream to avoid *anoikis*, or anchorage independent cell death; and (iii) the reverse process of EMT, or mesenchymal to epithelial transition (MET), which promotes conversion back to the parent cell morphology and growth of macrometastasis in the target organ, open a new broad of aspect on this issue (13).
The microenvironment plays a crucial role in maintaining the pluripotency of colon SCs at the base of colon crypts influenced by fibroblast, endothelium and inflammatory cells and the cytokines and growth factors secreted by these cells (in particular HGF) finely regulate the balance between self-renewal and differentiation of the staminal population (14-16). The most characterized pathway involved in the maintenance of colon stem cells is Wnt (17-19), and it is clearly highlighted by the different expression of Wnt members along the colon crypt (20), even if the maintaining of stemness and the differentiation pattern is actually the result of the fine collaboration between Wnt with other important pathways such as PTEN-PI3K-Akt (21,22), BMP (23), Notch (24) and Sonic hedgehog (Shh) (25).

3.3 EMT, Pre-metastatic niche and Metastasis Formation

Metastasis formation is considered a complex multi-step process that includes sequential molecular and cellular events that permit transformed cells to gain access to the blood stream (intravasation), survive their journey through the blood stream, and ultimately traverse through the microvasculature of target organs (extravasation) to deposit, survive, and grow in a foreign tissue environment. The EMT represents the first step of this highly regulated cascade and it is an important biological process initially studied in normal tissues during the organogenesis and then extended in the pathogenesis of cancer diseases, particularly referred to the acquisition of migratory phenotype in CRC cells (26). After they extravasate from the circulation into the target organ, aberrant cells must implant, proliferate, and induce angiogenesis in order to survive and grow in a foreign and presumably "hostile" environment. These phenomena are driven not only by genetic and/or epigenetic alteration of cancer cells, but also by the non-neoplastic stromal cells (27).

The EMT is characterized by the loss of epithelial properties, including the apico-basal polarity and cell adhesion, the E-cadherin, occluding and cytokeratins expression, and at the same time the acquisition of N-cadherin, vimentin, fibronectin, Twist1, zinc-finger proteins (SNAIL, SLUG, ZEB) and matrix metalloproteinases (MMPs) expression, all events that lead to an increased cell mobility (28). Moreover, EMT-inducing factors released by the surrounding microenvironment (29) can affect the invasive phenotype in epithelial malignancies initiation. Key regulators of this process are TGF-β (by the activation of Twist, SLUG and ZEB2), PI3K/Akt (increasing the mTOR kinase expression), Shh and Wnt (30,31).

Currently, dissemination and spread of cancer cells during the tumor progression are elective events underling the invasion through the tissue extracellular matrix (ECM). It was recently shown that tumor cells have two different modes of motility: (1) the acquisition of a mesenchymal phenotype, as previously described that identify a mesenchymal motility mode and the amoeboid migration (32). The mesenchymal mode is characterized by the acquisition of an elongated morphology and activation of the small GTPase Rac (33); (2) the amoeboid motility is defined by a rounded or ellipsoid cell morphology and weak interactions with the surrounding matrix, driven by Rho expression, which induce membrane blebbing through Rho-associated protein kinase (ROCK)-dependent myosin II phosphorylation and consequent actomyosin contractility (34). These two migration modes are interconvertible and regulated by microenvironmental influences. The possibility to switch from one mode to the other one highlights the cell plasticity that accomplishes movement from the primary tumor, establishment in an ectopic site, and survival therein (35).

The balance between high levels of activated Rac and Rho proteins regulates finely the motility mode. Moreover, Rac signalling inhibits amoeboid movement through its effector WASP-family verprolin-homologous protein 2 (WAVE2), and in the same way Rho/ROCK suppresses Rac by the activation of ARHGAP22, a GTPase-activating protein (GAP) (36).
Although *RHO* gene mutations are extremely rare, their altered expression has been assessed in many human cancers, including CRC. In particular, RhoA is frequently overexpressed and its induction is rapidly mediated by TGF-β (37), while depletion of Rac1 strongly correlates with the inhibition of lamellipodia formation, cell migration and invasion in carcinoma cells (38).
Furthermore, recent study established the independent contribution of *KRAS* and *BRAF* mutations, which rarely coexist in human tumors, to migration and invasion of CRC cells through Rho GTPases signalling. Although KRAS and BRAF are common members of the same pathway, Makrodouli *et al.* showed that *BRAF* mutation enhances cell migration through RhoA activation, and its effect is more pronounced compared to KRAS. These findings are expected to eventually result in tailor-made therapies against Rho pathway components, since it depends on the genetic background of the cancer patient (39).

3.4 Status redox and Hypoxia: two sides of the same coin

In the absence of an aberrant microenvironmental stimuli, genetic and epigenetic alterations in tumor cells are insufficient to induce primary tumor progression (27). Either through structure and function-based mechanisms, including ECM remodelling, release of cytokines and growth factors , metabolic changes, or activation of stromal components, microenvironment enables tumor cells to achieve an aggressive phenotype (32).
As observed, reactive oxygen species (ROS) has emerged as an important factor affecting several cancer hallmarks. ROS are involved in the acquisition of self-sufficiency in proliferation signals by a ligand-independent receptor tyrosine kinase transactivation as well as loss of contact inhibition and anchorage-dependence cell growth. The development of a more aggressive phenotype is also promoted by ROS through MMPs secretion, EMT program activation, Met overexpression and regulation of cellular plasticity induced by the Rac1/RhoA antagonism (40,41). Moreover, ROS sustain *de novo* angiogenesis by inducing the recruitment of perivascular cells and the activation of endothelial progenitors through the Vascular Endothelial Growth Factor (VEGF) and angiopoietin (Ang) release. Besides being involved in evading apoptosis by the activation of survival pathways, specifically

PI3K/AKT, NF-kB, and *anoikis* resistance, ROS increase the sensibility to mutagenic agents and help escape from the immune surveillance system (42).
Oxidative stress can derive from either extrinsic or intrinsic source (Figure 1).

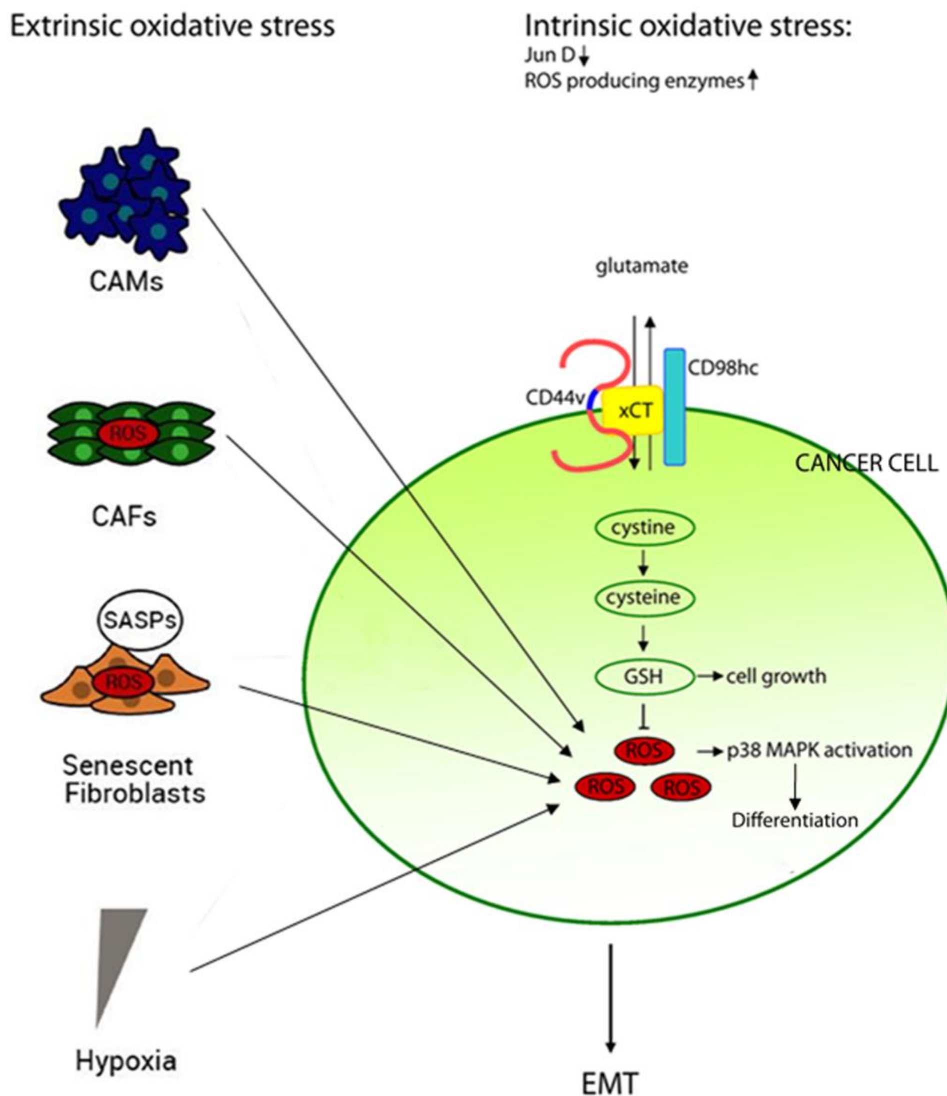

Figure 1. Extrinsic and intrinsic production of oxidative stress. CAMs and hypoxia induce a pro-oxidant environment, mandatory for CAF activation and senescent fibroblasts conversion into pro-inflammatory cells, affecting in turn EMT of cancer cells. Due to Jun D downregulation and increased activity of ROS-producing enzymes, cancer cells exacerbate the production of oxygen radicals. CD44v stabilizes the subunit xCT at the plasma membrane by promoting GSH synthesis and tumor growth. Cancer-associated macrophages (CAMs), Cancer-associated fibroblasts (CAFs), Senescent Activated Secretory Pathways (SASPs), Reactive oxygen species (ROS), Reduced glutathione (GSH), CD44 variant (CD44v), xCT (the light-chain subunit of cystine-glutamate antiporter system xc(-)), EMT (Epithelial Mesenchymal Transition).

Cancer-associated- fibroblasts (CAFs) or -macrophages (CAMs) synergize in the induction of a pro-oxidant environment. Due to the activation of Nitric Oxide Synthase 2 (NOX2), CAMs can directly produce ROS resulting in CAFs recruitment and MMPs activation (43). Moreover, by secreting the master pro-inflammatory cytokine TNFα, CAMs primes the NF-kB activation in both stromal and cancer cells, which in turn up-regulates *SNAI1* expression (44). In response to intrinsic and extrinsic oxidative stress, CAFs support tumor growth and promote EMT changes in cancer cells by secreting growth factors and ECM degrading proteases. Moreover, their production of extracellular matrix proteins promotes the recruitment of endothelial precursor cells from bone marrow (45). Ageing-induced oxidative stress concurs to transform fibroblasts into pro-inflammatory cells and induce an EMT program in the neighboring epithelial cells by secreting the so-called Senescent Activated Secretory Pathways (SASP) factors, which composed of pro-inflammatory cytokines and MMPs (46). Klimova *et al.* demonstrated that hypoxia also improves ROS generation by deregulation of the mitochondrial complex III resulting in ROS release into the cytosol (47).
Interestingly, TGFβ has been correlated to redox control of EMT, either directly by the activation of MAPK or indirectly by ERK-mediated Smad 2 phosphorylation. As shown by Rhyu *et al.,* in renal tubular epithelial cells, TGFβ1 stimulation induces E-cadherin loss, α-SMA and fibronectin up-regulation. These EMT-related molecular events are prevented by the inhibition of both NADPH oxidase (NOXes) and mitochondrial electron transfer chain subunit I, suggesting that NOXes and mitochondrial metabolism are important sources of TGFβ-induced cellular ROS (48). Similarly, Zhang *et al.* identified ferritin heavy chain (FHC) as a critical modulator of TGFβ-induced EMT. By repressing the synthesis of FHC, a cellular iron storage protein, TGFβ promotes iron release and subsequent increase in the intracellular labile iron pool (LIP), which is associated with redox-mediated activation of p38MAPK. Thus, FHC overexpression abrogates TGFβ-induced LIP increase resulting in ROS elimination and EMT suppression (49).
Cancer cells exacerbate the oxidant microenvironment by enhanced basal metabolic activity through aberrant growth factors and cytokines signaling as well as increased activity of ROS-producing enzymes, such as NOXes, cyclooxygenase (COXes) or lipoxygenases (LOXes) (50). Moreover, high levels of ROS may result from down-regulation of Jun D, a transcriptional activator of FHC that is known to minimize LIP-dependent ROS generation (51).
To protect themselves from oxidative stress, cancer cells develop adaptation strategies, including increased expression of scavenger anti-oxidative enzymes and

pro-survival molecules. Particularly, reduced glutathione (GSH) is the major intracellular antioxidant factor by reducing the ROS levels and suppressing ROS-dependent activation of p38MAPK. Ishimoto *et al.* demonstrated that in gastrointestinal cancer cells a CD44 variant (CD44v) maintains high levels of GSH by stabilizing the xCT expression at the plasma membrane. xCT is the light-chain subunit of cystine-glutamate antiporter system xc(-), which exchanges extracellular cystine uptake for intracellular glutamate, thereby promoting GSH synthesis (Figure 1). At first, glutamate-cysteine ligase couples glutamate and cysteine to form γ-glutamylcysteine. Glutatione synthetase then catalyzes the formation of GSH from glycine and γ-glutamylcysteine. Since cysteine availability is a rate-limiting factor for GSH synthesis, CD44-mediated stabilization of xCT plays a key role in the GSH-dependent antioxidant system, promoting the proliferation of cancer cells and the formation of lethal gastrointestinal tumors. This is supported by the observation that CD44 depletion reduces the number of proliferating tumor progenitor cells and inhibits gastric tumor development in Gan (Gastric Neoplasia) mice through the ROS-dependent p38MAPK activation and $p21^{CIP1/WAF1}$ upregulation. The antioxidant potential of gastric cancer cells confers resistance to ROS-inducing anticancer drugs, such as cisplatin and docetaxel. Consistently, in an HCT116 xenograft model, the specific xCT inhibitor sulfasalazine suppresses CD44-dependent tumor growth in parallel with the activation of p38MAPK, suggesting that the suppression of xCT by sulfasalazine might impair the ROS defense ability of CD44v-expressing CSCs and improve the efficacy of currently available treatments (52).
CD44 and its variant isoforms have already been identified as tumor metastasis-associated proteins. Ectopic expression of CD44v6 splice variant confers metastatic potential to non metastatic tumor cell lines, promoting Met activation by its ligand HGF that is mainly secreted by mesenchymal cells (53). The importance of the CD44v6 and Met multimeric signaling in cancer progression has been strengthened by the observation that adenoma growth in the $Apc^{Min/+}$ mice model was reduced by inhibiting the CD44v6 expression through short hairpin RNA/nanoparticles technology (54). Moreover, Jung *et al.* showed that CD44v6 supports tumor cell migration and apoptosis resistance since only the matrix assembled by CD44v6-competent but not -deficient cells induces metastasis formation (55). Given that disseminating cells are exposed to high levels of ROS during tumor progression, metastatic growth requires also adequate ROS defense ability to successfully colonize secondary sites. Interestingly, knockdown of the redox protein thioredoxin-like 2 has been reported to inhibit tumorigenesis and metastasis of human breast cancer cell lines upon transplantation into immunodeficient mice by enhancing ROS levels and reducing NF-κB activity (56). It has also been investigated the role of CD44v-xCT in

lung metastasis. By promoting xCT-dependent GSH synthesis, CD44 expression allows mouse 4T1 breast cancer cells to evade high levels of ROS produced by neutrophils and colonize the lung. It is not surprising that knockdown of epithelial splicing regulatory protein 1 in $CD44^+$ subpopulation induces an isoform switch from CD44v to CD44s, resulting in reduced xCT expression and lung metastasis suppression (57).

Proliferating tumor cells distance themselves from the vasculature and colonize an environment deficient in oxygen and nutrients. Therefore, tumor cells need to reprogram their metabolism by increasing glycolytic activity and decreasing aerobic respiration rate. This shift is mediated by an increase in ROS levels generated by mitochondrial complex III, which accounts for hypoxia-inducible factor-1 (HIF-1) stabilization *via* oxidation/inactivation of prolyl hydroxylases and release from Von Hipper-Lindau (VHL)-mediated degradation. When stabilized in hypoxia, HIF-1α dimerizes with HIF-1β and translocates into the nucleus. By interacting with the co-activators CBP/p300, the α/β heterodimer HIF-1, bound to hypoxia-response elements (HREs) in target genes, mediates the expression of proteins involved in the formation of new vasculature and metabolic adaptation to hypoxia (58). HIF-1α increases the transcription of glucose transporters and glycolytic enzymes as well as lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1), resulting in the diversion of pyruvate towards lactate away from mitochondrial oxidative phosphorylation (59). Additionally, mutations of tumor suppressor genes (*PTEN, VHL*) and oncogenic pathways (Ras/MAPK, PI3K-Akt) converge on HIF-1α activation through an oxygen-independent mechanism (58). Specifically, in CRC hypoxia activation of wild-type K-Ras mediates Akt phosphorylation and resistance to apoptosis (60).

Similar to HIF-1α, HIF-2α is involved in the regulation of hypoxia tumor response. Interestingly, Heddleston *et al.* reported a role of HIF2α in reprogramming non-stem cancer cells towards a stem-like phenotype by inducing the expression of key stem cell genes, like *OCT4*, *NANOG* and *MYC*. Concordantly, overexpression of HIF-2α in glioma non-stem cells increased neurospheres formation and tumorigenic capacity (61). Moreover, as shown by Xue *et al*, HIF2α activation modulates colon tumorigenesis in $Apc^{Min/+}$ mice by overexpression of intestinal iron transport. The resulting iron intake contributes to dysregulation of local iron homeostasis, which in turn affects cancer progression through increasing cell survival and proliferation (62).

Hypoxia has been reported as an important driving force for the multistep process of metastasis (Figure2).

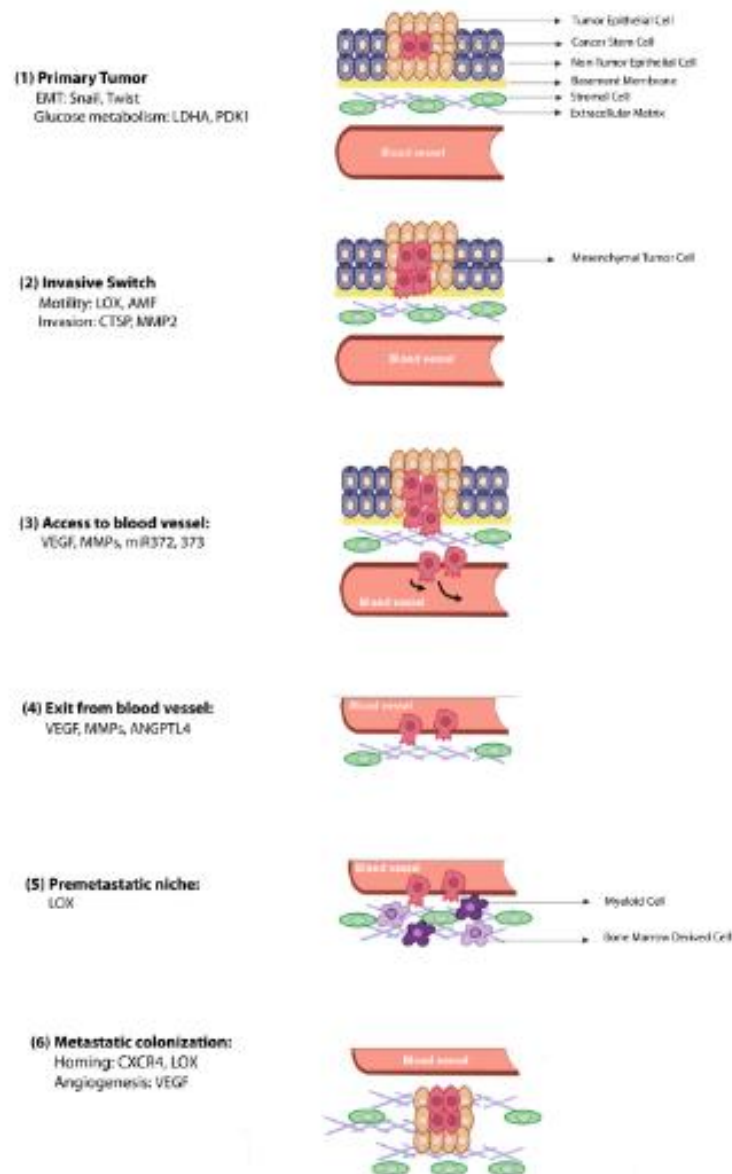

Figure 2. Regulatory functions of hypoxia in different steps of metastasis. 1) During primary tumor growth, hypoxia acts as inductor of "glycolytic" phenotype and executor of EMT. 2) Under hypoxia, tumor cells gain an improvement in motility and invasion capacity, facilitating thereby detachment and dissemination from the primary site. 3,4) Increased expression of VEGF and MMPs induced by hypoxia is critical to penetrate the vasculature and promote the subsequent exit. 5) By the recruitment of bone marrow-derived cells and $CD11b^+$ myeloid cells to secondary organs, LOX secreted by hypoxic tumor cells forms the premetastatic niche. 6) Hypoxia-dependent induction of CXCR4 and angiogenesis contribute to the successful metastatic colonization. EMT (Epithelial Mesenchymal Transition), LDHA (lactate dehydrogenase A), pyruvate dehydrogenase kinase 1 (PDK1), Lysyl Oxidase (LOX), Autocrine Motility Factor (AMF), cathepsin D (CTSD), Matrix Metalloproteinase (MMPs), Vascular endothelial growth factor (VEGF), Angiopoietin-like 4 (ANGPTL4).

The early EMT-related events induced by hypoxia support ROS-dependent GSK-3β inactivation, followed by SNAIL nuclear translocation and E-cadherin loss (63,64). In response to hypoxic conditions, Notch signaling up-regulates Snail expression by two distinct but synergistic mechanisms, involving both direct transcriptional activation of *SNAI1* (65) and an indirect mechanism operating *via* the ECM protein lysyl oxidase (LOX) (66). Moreover, Twist expression, directly induced by HIF-1α through the HRE located in its promoter, contributes to cadherin profile changes with E-cadherin down-regulation followed by N-cadherin upregulation (64). At a later stage, activation of Wnt/β-catenin pathway and increased invasiveness are sustained by HIF-1α- and VEGF-dependent events (63). Particularly, hypoxia-induced invasion is associated with basement membrane degradation and ECM remodeling by upregulation of cathepsin D (CTSD) and MMP2 (58,67). Hongo *et al*. proposed that the up-regulation of β1 integrin expression by hypoxia in CRC cells increases the ability to adhere and migrate on collagen fibers (68).

The role of HIF-1α in cell migration is related to improved LOX expression. In hypoxic cancer cells, LOX mediates the covalent cross-linking of collagen fibers and elastin, thereby increasing cell focal adhesion kinase activity that is known to induce cell motility by acting as a signal between integrins and actin cytoskeleton. These remodeled matrix events are essential for invasive cell movement and provide a metastasis freeway by which other tumor cells may walk and spread to adjacent tissues (69). Hypoxia-induced "invasive switch" is also mimicked by Met and autocrine motility factor (AMF) overexpression. Pennacchietti *et al.* demonstrated that hypoxia synergizes with HGF to affect basal cell morphology and induce cell scattering by transcriptional activation of the *MET* proto-oncogene. Consistently, increased Met expression sensitizes tumor cells to HGF produced by fibroblasts, promoting thereby the invasive growth towards tissue parenchyma and blood circulation (70). One of the most important tumor-secreted cytokines, AMF promotes resistance to apoptosis in tumor cells and angiogenesis induction *via* autocrine and paracrine mechanisms (71).

Hypoxia-selected tumor cells are able to evade the hostile milieu of primary site by promoting angiogenesis and affecting vascular integrity and permeability. Consistently, hypoxia-dependent expression of VEGF, MMP1 and MMP2 is essential to offend the vasculature and promote intravasation. MiR-372/373, upregulated in response to hypoxia through HIF-1α, contribute to increased intravasation by targeting the MMP inhibitory protein RECK, resulting in excessive activation of MMPs (72). Besides VEGF, MMP1 and MMP2, tumor cells extravasation is promoted by Angiopoietin-like 4 (ANGPTL4), a member of vascular regulators angiopoietin family upregulated in the primary tumor by both TGFβ and hypoxia

(58). As shown by Padua *et al.*, the expression of ANGPTL4 in cancer cells primes these cells to disrupt vascular endothelial tight junctions and increase the capillary permeability, thereby affecting the transendothelial passage (73).
Recent reports suggested that the metastatic seeding at distant organs is influenced by hypoxia-induced factors released from primary tumor, critical for pre-metastatic niche formation. It has been reported that in breast cancer LOX, secreted by hypoxic tumor cells into the bloodstream, modifies the collagen cross-linking in the lungs and promotes the recruitment of $CD11b^+$ myeloid cells to pre-metastatic sites. By the consequent adhesion to cross-linked matrix, $CD11b^+$ myeloid cells produce MMP-2, which supports collagen remodeling by LOX and thereby leading to increased recruitment and subsequent invasion of bone marrow-derived cells. This cell population is thought to create a favorable environment for the incoming primary tumor cells (69).
Hypoxia in primary tumor may also improve metastatic seeding of tumor cells by heightening chemokine C-X-C motif receptor 4 (CXCR4) expression. Specifically, CXCR4-mediated signal transduction can enable tumor cells to home to secondary organs where its ligand Stromal Derived Factor 1 (SDF1) is highly expressed (e.g., lymph nodes, lungs, liver, or bones). The responsiveness of $CXCR4^+$ cells to SDF-1 gradient is positively affected by several molecules produced during inflammation, specifically fibrinogen, fibronectin, C3a, hyaluronic acid, suggesting that inflammation affects the spreading of $CXCR4^+$ tumor cells (74).
Similarly to primary tumor, hypoxia response molecules facilitate tumor-stromal interactions in secondary sites to support the metastasis colonies proliferation. However, the role of hypoxia in determining the organ-specific metastasis is still unknown. Microarray profiling revealed that hypoxia promotes the expression of lung-metastasis gene signature, including *Connective tissue growth factor, Osteopontin, IL-6 and -8, ANGPTL4,* and primes ER^- breast cancer cells in promoting lung colonization by activating an effective angiogenesis. Since bone marrow vasculature is already fenestrated, facilitating the transendothelial passage of tumor cells, hypoxia-induced angiogenesis does not provide an advantage for bone metastasis seeding. Thus, it is not surprising that hypoxia activates a limited percentage of bone-metastasis genes, including *CXCR4* and *dual specificity phosphatase 1,* which functions as a stress-inducible MAPK signaling activator (58,75). Interestingly, experimental models and human cancers implicated TFGβ in promoting distal metastasis formation. After seeding the lung parenchyma, ER^- breast cancer cells take a proliferative advantage from local TGFβ through induction of the cell differentiation inhibitor ID1 (76). As shown by Kakonem *et al*, in mice inoculated by MDA-MB-231 breast cancer cells, osteolytic bone metastases require

the recruitment and activation of osteoclasts. In particular, induction of IL-11 and parathyroid hormone-related protein production by TGFβ promotes differentiation of osteoclast precursors and bone resorption, thereby increasing the osteoblastic expression of Receptor Activator for NF-κB (RANK) ligand (77). Lastly, Batlle *et al.* speculated that IL-11, a TGFβ-target gene in stromal cells, confers metastatic initiation capacity to CRC cells *via* GP130/STAT3 signaling, critical to induce a survival advantage and suppress apoptotic stimuli in metastatic sites (78).

3.5 CSCs and vasculature cells crosstalk: a mutual convenience

Tumor cell growth and nurture require several strategies to supply the oxygen and metabolic demand, all involving new vessels formation and captivation from the surrounding stroma. Tumor neovascularization can occur through *a)* sprouting from existing vessels (sprouting angiogenesis), *b)* lumen invagination and splitting of vessels (intussusceptive angiogenesis), *c)* enfolding of vessels by cancer cells (vessel co-option), *d)* simulation of endothelial features by tumor cells (vasculogenic mimicry), *e)* formation of lymphatic vessels from pre-existing ones (lymphangigogenesis) and finally *f)* endothelial progenitor cells recruitment (79).
Angiogenesis has been defined as a key process for tumor and metastasis formation and CSCs are predicted to be strong promoters of this phenomenon. For instance, Bao *et al.* demonstrated a profound interplay between CSCs and tumor vasculature. Injection of glioblastoma stem cells (GSCs) $CD133^+$ in the right frontal lobes of athymic nude mice displays strongly angiogenic and hemorrhagic tumors compared to the $CD133^-$ counterpart. The angiogenic advantage of the $CD133^+$ fraction may be supported by a 10-20 fold increase of VEGF secretion. Significantly, conditioned medium from these fractions fosters human endothelial cells migration and tube formation (80). According to these data, the concomitant presence of CSCs correlates with more angiogenic tumors in terms of enhanced resident endothelial cells function and recruitment of bone marrow-derived endothelial progenitors to the tumor site. VEGF and SDF1 are the main powering determinant of these CSCs properties (81).
On the other hand, it is likely conceivable a possible impact of endothelial cells on CSCs state. A paracrine signaling by endothelial cells may induce CRC cells to gain CSC properties with Notch pathway as the main player of this conversion. Indeed, Jagged-1, a Notch-activating ligand, is released from endothelial cells as a soluble form by ADAM17 proteolitic cleavage and its binding to Notch receptor of adjacent CRC cell triggers the onset of stem-like features. Co-culturing CRC cells either with endothelial cancer cells or with endothelial cell-conditioned medium lead to an

increase of the CD133$^+$/ALDH$^+$ subpopulation compartment and sphere forming capability as well as *in vivo* tumor growth and spreading (82).
Similarly, as showed by Calabrese *et al.*, it was demonstrated that endothelial–derived factors support self-renewing of brain tumor cells and keep them in an undifferentiated state. These stem-like cells closely interact with CD34$^+$ capillaries and are strictly dependent on microvasculature density. Co-injection of primary human endothelial cells and CD133$^+$ medulloblastoma cells accelerates initiation and promotion of brain tumor xenografts by expanding the CSCs pool. Thus, tumor microenvironment orchestrates a vascular niche formation determining the CSCs fate (83).
Furthermore, the presence of ‘mosaic’ blood vessels in which both endothelial and tumor cells are located into the lumen surface of tumor vessels has long been described (84). Consistent with these findings, glioblastoma stem cells can be induced to differentiate into endothelial cells and directly contribute to tumor vasculature architecture when injected in immunocompromised mice, as proven by the presence of CD34$^+$/CD144$^+$/VEGFR2$^+$ human-derived endothelial cells (85). Likewise, vasculogenic mimicry can occur *via* a multipotent intermediate (CD133$^+$/CD144$^+$) that can differentiate either into a tumoral or endothelial phenotype (86).
Another related possibility is that, rather than differentiation into endothelial lineage, CSCs generate vascular pericytes that mainly support endothelial cells to maintain vessels function and integrity. It was recently shown that, after GSC differentiation induction, a fraction of 4-11% cells expressed several pericyte markers such as α-SMA, NG2, CD146 and CD248. Significantly, *in vivo* cell lineage tracing with specific fluorescent reporter confirmed that the majority of pericytes had GSC origin. Of note, selective deletion of GSC-derived pericytes hampered microvessel development and tumor growth. CXCR4 expressing GSCs were recruited toward epithelial cells by an SDF-1 chemoattractant gradient and then induced to pericytes differentiation upon TGF-β release by endothelial cells (87).

3.6 Angiogenic pathways orchestrate CSCs survival and motility

Although CSCs represent a minority of tumor cells population, deregulation of pathways involved in cell self-renewal and motility contributes to cancer conversion and promotion. In addition to well established CSCs radioresistance and chemoresistance mechanisms, an increasing adaptability to antiangiogenic treatment was shown (88). These cells can elicit resistance and increase their tumorigenic and

invasive potential by exploiting an hypoxic microenvironment (89) as well as the activation of anti-apoptotic pathways (88) (Figure 3).

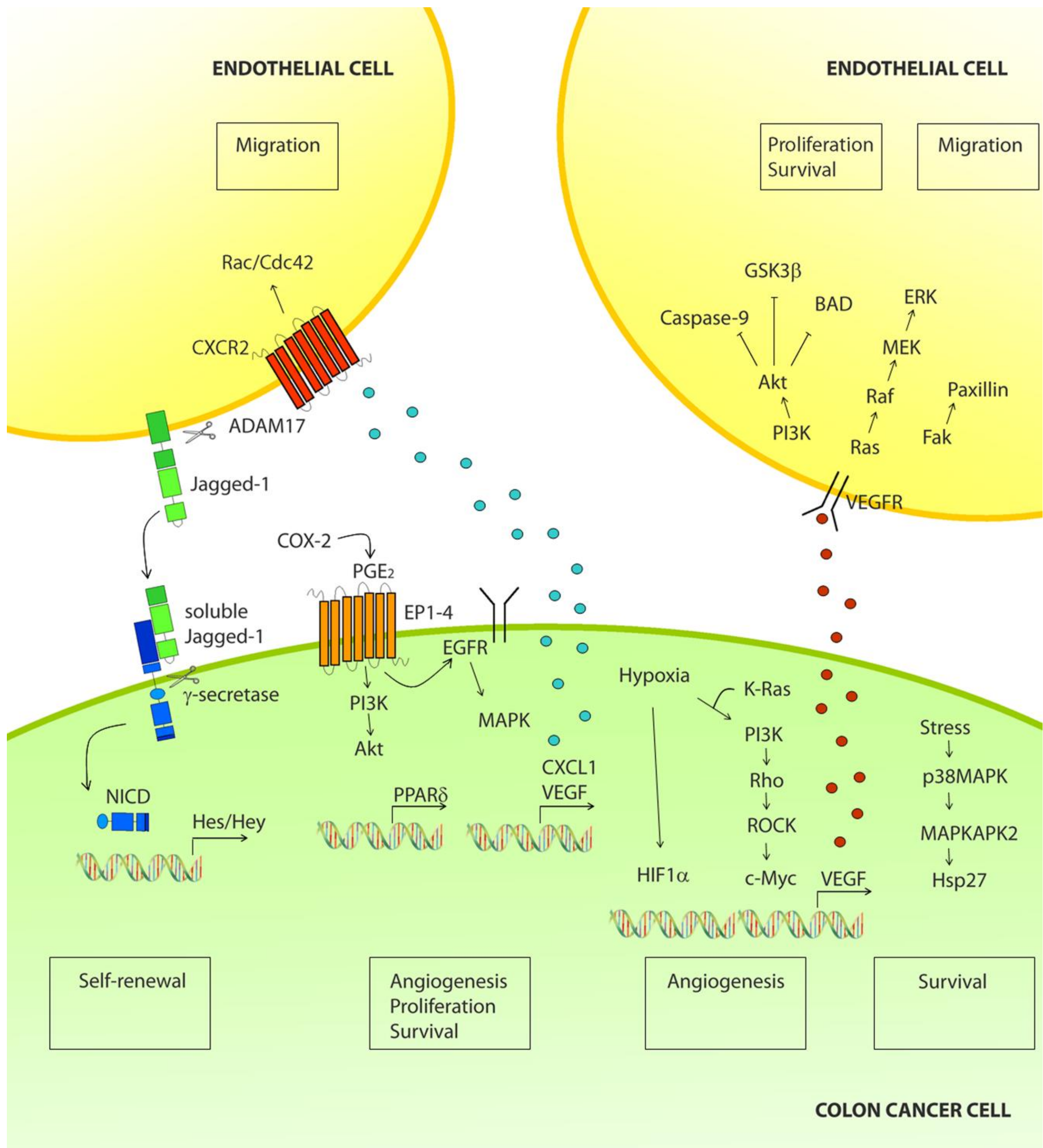

Figure 3. Tumor microenvironment is conducive to angiogenesis promotion. A truncated soluble form of Jagged-1 is released by endothelial cells and its binding to Notch receptor on nearby colon cancer cells promotes a stem-like phenotype. PGE_2 mediates the release of the angiogenic factors CXCL1 and VEGF in colon cancer cells, *via* an EP1-4/EGFR/MAPK cascade. CXCL1 secretion stimulates endothelial cell migration by CXCR2 binding and Rac/Cdc42 pathway activation. Furthermore, PGE_2 induces colon cancer cell proliferation and survival trough PI3K/Akt signaling and transcriptional activation of PPARδ. Under hypoxic conditions, induction of HIF1α and alternative K-Ras pathways results in further VEGF release from cancer cells. In endothelial cells, VEGF/VEGFR interaction promotes cell proliferation, survival and migration *via* PI3K, Ras and FAK pathways. Finally,

activation of pro-survival signals in tumoral cells is triggered by microenvironmental stress and p38MAPK, MAPKAPK2 and Hsp27 cascade. Notch intracellular domain (NICD), Prostaglandin E2 (PGE_2), Chemochine C-X-C motif ligand 1 (CXCL1), Prostaglandin E receptor 1-4 (EP1-4), Epidermal growth factor receptor (EGFR), Mitogen-activated protein kinase (MAPK), Chemochine C-X-C motif receptor 2 (CXCR2), Cell division control protein 42 (Cdc42), Perixisome proliferator-activated receptor δ (PPARδ), Rho-associated protein kinase (ROCK), MAP kinase-activated protein kinase 2 (MAPKAPK2), Heat shock protein 27 (Hsp27).

Among molecules that regulate tumor angiogenesis, such as Plateled–Derived Growth Factor (PDGF), FGF, HGF and TGF-α/β, VEGFs and their cognate receptors (VEGFRs) are the driving force of angiogenic response due to their specific expression on endothelial and tumoral cells, resulting in multiple signal pathways activation.
VEGF family is represented by five members (VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor [PGF]) coupled with three tyrosine kinase receptors (VEGFR1 [Flt1], VEGFR2 [KDR/Flk1] and VEGFR3 [Flt4]). As a soluble factor, VEGF serum concentration, in preoperative CRC, reflects the stage and correlates with disease progression. Both VEGFs and VEGFR2 are associated with a worse prognosis, tumor spreading and enhanced microvessel density. Particularly, VEGF expression increases during the colonic adenoma-adenocarcinoma pathogenesis conversion and before an invasive phenotype switch (90).
VEGFR1 is mostly expressed on endothelial cells, monocytes, macrophages, hematopoietic stem cells and some tumoral cells, including CRC cells (91). VEGFB and PGF have been identified as its exclusive ligands. VEGFR2 is not restricted to endothelial cells but it is also shared by, for example, colitis-associated colon cancer epithelial cells (92) and GSCs (93). Furthermore, VEGFR3, the first normal lymphatic endothelium marker (94), together with VEGFC is involved in cancer lymphangiogenesis (95).
VEGFA/VEGFR2 interaction is recognized as a potent proangiogenic stimulus increasing survival, proliferation, migration, and vascular permeability of endothelial cells (96). Although VEGFA has a higher binding affinity for this receptor, VEGFR2 possesses a greater tyrosine kinases activity that governs the activation of MAP-kinase, PI3K, Fak and Rac pathways. Interestingly, phosphorylation of p38MAPK, in colon CSCs, protects them from antiangiogenic treatment through the activation of Heat shock protein 27 (Hsp27) (88). Hypoxic induction of VEGF is not merely dependent on HIF-1α. It was already reported that CRC cells are forced to express

VEGF through a K-Ras/PI3K/Rho/ROCK/c-Myc axis. Indeed, a putative Myc-Max binding site was found on *VEGF* gene promoter (97).

It was extensively observed that Prostaglandin E2 (PGE_2) is abundantly secreted by both colon cancer cells and stromal cells and promotes the release of the angiogenic factors C-X-C motif ligand 1 (CXCL1) and VEGF through the Prostaglandin E receptor 1-4 (EP1-4)/Epidermal growth factor receptor (EGFR)/MAPK cascade. Tumor-derived CXCL1 stimulates endothelial cell migration and *in vivo* tumor growth and microvessels density, by CXCR2 binding and Rac/Cdc42 pathway activation. Furthermore, PGE_2, *via* PI3K/Akt signalling, enhances transcriptional activation of Perixisome proliferator-activated receptor δ (PPARδ) that is required for colorectal adenoma growth (98,99).

The angiogenic properties of VEGF may be amplified when tumoral endothelium is previously destabilized by other growth factors, such as Ang-2. Ang-1, 2 and 4, that bind the same endothelial receptor Tie2. While Ang-1 is expressed by pericytes, smooth muscle cells and tumor cells, Ang-2 is exclusive to endothelial cells. Ang1 preserves vascular integrity by reducing cell-to-cell gaps whereas Ang2 increases pericytes dissociation and vessels destabilization, rendering endothelial cells more receptive to foreign stimuli, for instance, VEGF (100).

A broad spectrum of clinical data reports that activating *KRAS* mutations could occur up to 50% of early stages CRC patients (101). Interaction of Ras with the catalytic subunit p110 of PI3K appears to be extremely relevant to the induction of *VEGF* gene expression. PI3K phosphorylates Akt which subsequently inhibits GSK-3β leading to β-catenin nuclear translocation. Mutated *KRAS* enhances the stability of β-catenin and promotes the formation of nuclear β-catenin/TCF4 complexes (102). In addition, further evidence of a cooperative interaction between K-Ras and Wnt pathway in CRC lies in the presence of a consensus TCF4 element in the *VEGF* promoter (103). At the early onset of colon neoplastic lesion, a crosstalk between Ras and the microenvironment has been described. Particularly, *RAS* oncogene can orchestrate endothelial and inflammatory cells recruitment to the tumor site in an IL-8-dependent manner (104). On the other hand, as previously mentioned, in wild type *KRAS* CRC and in presence of an hypoxic microenvironment, VEGF expression is strictly regulated by Akt and c-Src pathways (60).

Entirely conflicting with other Ras oncoprotein features, R-Ras is described as a supporter of tumor vessels normalization by counteracting VEGF angiogenic potential. Tumor vasculature differs from the normal counterpart for the presence of saccular, tortuous and high permeable vessels with fibrin-gel matrix deposition. Pericytes are poorly associated with endothelial cells supported by an irregular basement membrane. Vessel leakiness allows cancer cells to easily penetrate into the

bloodstream and thus colonize distant organs. In addition, plasma leakage from vessels, due to an higher interstitial hydrostatic pressure at the tumor site, reduces the delivery of chemotherapeutic agent (105). However, R-Ras does not affect the oxygen-sensing mechanism of vessel normalization exerted by PHD2 or HIF-2α under hypoxic condition. Conversely, it facilitates the accumulation of VE-cadherin on cell-to-cell junction, favoring the stabilization of the endothelial barrier. Indeed, it reduces phosphorylation of Ser665 in the cytoplasmic domain of VE-cadherin, suppressing its internalization on endothelial cells. Interestingly, this phenomenon antagonizes VEGF-mediated VE-cadherin phosphorylation. Furthermore, R-Ras activity in pericytes increases their interaction with endothelial cells, leading to normal vessels morphogenesis (106).
Based on this observation, antiangiogenic therapies may contribute to the normalization of tumor vasculature architecture and consequently improve their distribution and efficacy (107).
Finally, the BMPs pathway was observed aberrantly regulated in the majority of sporadic CRC and germline mutation on BMP receptors and downstream substrates were detected in juvenile polyposis (108). Furthermore, BMP signaling has been shown to be essential in human intestinal development and regeneration regulating also the number and the self-renewal state of colonic stem cells (109). To date, little is known about BMPs role in angiogenesis. Recently, BMP9 was identified as a ligand of the orphan Activin receptor-like Kinase 1 (Alk1) in endothelial cells and the resulting interaction affects several angiogenic steps. BMP9/Alk1 signaling counteracts bFGF-stimulated endothelial cells proliferation and migration as well as VEGF-induced angiogenesis. Indeed, BMP9/Alk1/BMP receptor II (BMPRII) complex abolished VEGF expression through suppression of TGFβ/Alk5/BMPRII signaling (110). Certainly further investigations are needed to identify the underlying mechanism of BMP engagement during angiogenesis promotion.

3.7 Therapeutic Advances

Quiescent cells within the stemness niche have been associated with tumor recurrence and relapse after chemotherapy. Targeting the molecular mediators and signaling pathways affecting EMT and tumor progression may provide novel therapeutic strategies to prevent CSCs-dependent distant metastasis formation.
Fighting neovascularization to counteract cancer promotion is a crucial step of the long-standing theory of J. Folkman (111). Based on this hypothesis, the first antiangiogenic compound approved by the FDA, in 2004, was Bevacizumab. It is a monoclonal antibody against VEGF recommended in first and second line settings,

either with FOLFOX (5-Fluorouracil, Leucovorin and Oxaliplatin) or FOLFIRI (5-Fluorouracil, Leucovorin and Irinotecan). As shown by preclinical data, Aflibercept is a VEGFA, VEGFB and Placental growth factor (PIGF) decoy receptor, composed of VEGFR1 and VEGFR2 extracellular domains fused to the constant portion of immunoglobulin gamma chain. In 2012, FDA approved the administration of this compound plus FOLFIRI in patients with metastatic CRC with disease progression after oxaliplatin treatment. Recently, advanced clinical trials validate the efficacy of Regorafenib as a VEGFR1/2/3 and Tie2 tyrosine kinase inhibitor (112).
Despite initial therapeutic benefits in patients with metastatic CRC, classic antiangiogenic strategies failed to improve long-term clinical outcomes (113).
Since new development of tumor vasculature implies several complex signaling, alternative angiogenic or anti-apoptotic mechanism could be devised by cancerous cells (88). Indeed, it has been recently pointed out, by Lu *et al.,* that glioblastoma multiforme treatment with Bevacizumab developed more invasive tumors, as the blockade of VEGF enhances HGF-induced MET phosphorylation (114). Another attractive approach takes into account that anti-angiogenic treatments favor a hypoxic microenvironment that gives to CSCs population a metabolic advantage and preserves their self-renewal state (89).
Given that anti-angiogenic drugs may enhance tumor invasiveness by blocking *de novo* angiogenesis and inducing hypoxia, the development of HIF-1α targeted therapies may reduce or prevent metastasis (58). There are several agents that affect directly or indirectly the HIF-1α expression or activity. The binding of HIF-1α to the co-activator p300/CBP has been attenuated by the chetomin, a small molecule that interferes with hypoxia-inducible transcription (115). In addition, the proteasome inhibitor bortezomib, approved for treatment of patients with multiple myeloma and mantle cell lymphoma, impairs the interaction with the co-activator p300/CBP by inducing the hydroxylation of Asn803 in the C-terminal transactivation domain (116). By blocking HIF-1α binding to HRE sequence, a step required for transcription induction, anthracyclines have been reported to significantly reduce the prostate tumor growth and vascularization in a mouse model (117). The topoisomerase I inhibitor topotecan, cardiac glycoside digoxin and PX-478 have also been implicated in HIF-1α expression, consistent with their remarkable antitumor activity in a variety of human tumor xenograft models (118). HIF-1α protein translation is also inhibited by the chaperone Hsp90, which induces its proteasomal degradation in a VHL-independent manner (119). Nontoxic prodrugs that generate active species in hypoxic tissue by selective bioreduction have now reached advanced clinical trials. Nitroaromatics, quinones, tertiary amine N-oxides, and transition metals are selectively reduced and activated in the absent of O_2 to release or activate toxic

effectors to eradicate surrounding hypoxic tumor cells. Similarly, the gene-directed enzyme prodrug therapy uses HRE sequence to improve the expression of reductase enzymes, including P450 reductase, HSV thymidine kinase and cytosine deaminase, which kill hypoxic tumor cells by converting a prodrug into a cytotoxin (58). Nevertheless, a robust validation of hypoxia inhibitors in clinical trials is needed to support the hypoxia-targeted therapies. Overall, these findings suggest that advanced compounds need to be developed to selectively target cancer microenvironment.

3.8 Conclusions

The reviewed data emphasize the supporting role of the microenvironment in primary tumor establishment and dissemination to distant sites. The critical event of EMT depends on the complex signals produced by stromal components ensuring the generation of CSCs phenotype with increased proliferative capacity and metastatic potential in hostile milieu. In addition, perivascular, hypoxic and premetastatic niches have been proposed to enhance the resistance of CSCs to therapy. Based on this observation, combination therapies targeting hypoxia and *de novo* angiogenesis may have enormous therapeutic implications by blocking the successful homing of cancer cells to metastatic sites. Thus, a better understanding of cancer microenvironment framework could be a crucial key to improving patient cure.

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Chapter 4

Targeting Cancer Stem Cells and the Tumor Microenvironment

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Published by *Springer (chapter of the book entitled Cancer Stem Cells: Emerging Concepts and Future Perspectives in Translational Oncology)*, In press

Abstract

Compelling evidence indicates that the survival and behavior of Cancer Stem Cells (CSCs) are positively regulated by specific stimuli received from the tumor microenvironment, which dictates the maintenance of stemness, invasiveness, and protection against drug-induced apoptotic signals. CSCs are *per se* endowed with multiple treatment resistance capabilities, thus the eradication of CSC pools offers a precious strategy in achieving a long-term cancer remission. Numerous therapies, aimed at eradicating CSCs, have been elaborated such as: i) selective targeting of CSCs, ii) modulating their stemness and iii) influencing the microenvironment. In this context, markers commonly exploited to isolate and identify CSCs are optimal targets for monoclonal antibody-based drugs. Furthermore, the molecules that inhibit detoxifying enzymes and drug-efflux pumps, are able to selectively suppress CSCs. Auspicious outcomes have also been reported either by targeting pathways selectively operating in CSCs (e.g. Hedgehog, Wnt, Notch and FAK) or by using specific CSC cytotoxic agents. Other compounds are able to attenuate the unique stemness properties of CSCs by forcing cell differentiation, and this being the case in ATRA, HDACi, BMPs and Cyclopamine, among others. Targeting the interplay between paracrine signals arising in the tumor stroma and the nearby cancerous cells *via* the inhibition of VEGF, HIF, CD44v and CXCR4, is increasingly recognized as a significant factor in cancer treatment response and holds alluring prospects for a successful elimination of CSCs. In the present chapter, we discuss the latest findings in the optimization and tailoring of novel strategies that target both CSCs and tumor bulk for the eradication of malignancies.

4.1 Introduction

The concept that tumors are hierarchically organized and harbor cells with distinct tumor-initiating capabilities and self-renewal potential, referred to as cancer stem cells (CSCs), has long been observed in a variety of hematopoietic malignancies and solid tumors and is now well-recognized by the scientific community (1). By virtue of their innate plasticity, it is worth considering that CSCs fuel and succeed in tumor growth, treatment resistance, distant metastasis formation and patient relapse. Mechanistically, CSCs share several biological properties with normal adult stem cells that endows them with a survival advantage upon chemotherapeutic intervention. These include dormancy (quiescence), active DNA repair machinery, an enhanced reactive oxygen species (ROS) defence capability, a higher expression of multiple drug resistance (MDR) membrane transporters and anti-apoptotic proteins (2, 3).

Thus, attractive emerging strategies have been developed to selectively target CSCs by using agents directed at CSC-surface markers, drug-detoxifying enzymes, drug efflux pumps or key signaling pathways sustaining the stemness properties of CSCs. Otherwise, stemness modulator drugs force CSCs to differentiate terminally, resulting in the loss of self-renewal potential and the gaining of susceptibility to cytotoxic therapies. To eventually overcome cancer resistance and relapse, a simultaneous delivery of stem cells targeting drugs or stemness modulator compounds, has been tested in combination with standard anticancer drugs to successfully eliminate CSCs, tumor bulk cells and spontaneously dedifferentiated non-CSCs (4, 5). Of note, stem cell targeting drugs eradicate CSCs but at concentrations less toxic to non-CSCs. Conversely, stemness inhibiting drugs aim at reducing the stemness of CSCs and uniquely, at high doses, they may eliminate CSCs and non-CSCs with similar potency. Finally, paracrine signals between cancer cells and stromal cells are required to trigger an epithelial-to-mesenchymal transition (EMT) program. Besides the acquisition of a mesenchymal and invasive state, EMT seems to confer stem-like properties to neoplastic epithelial cells (6), and subsequently additional autocrine signals, arising from cancerous cells themselves, appear to maintain this mesenchymal state (7). Therefore, specific molecular therapies that target CSC peculiarities and prominent tumor microenvironment signals, may be powerful determinants in tumor shrinkage and successful elimination of CSCs (Figure1).

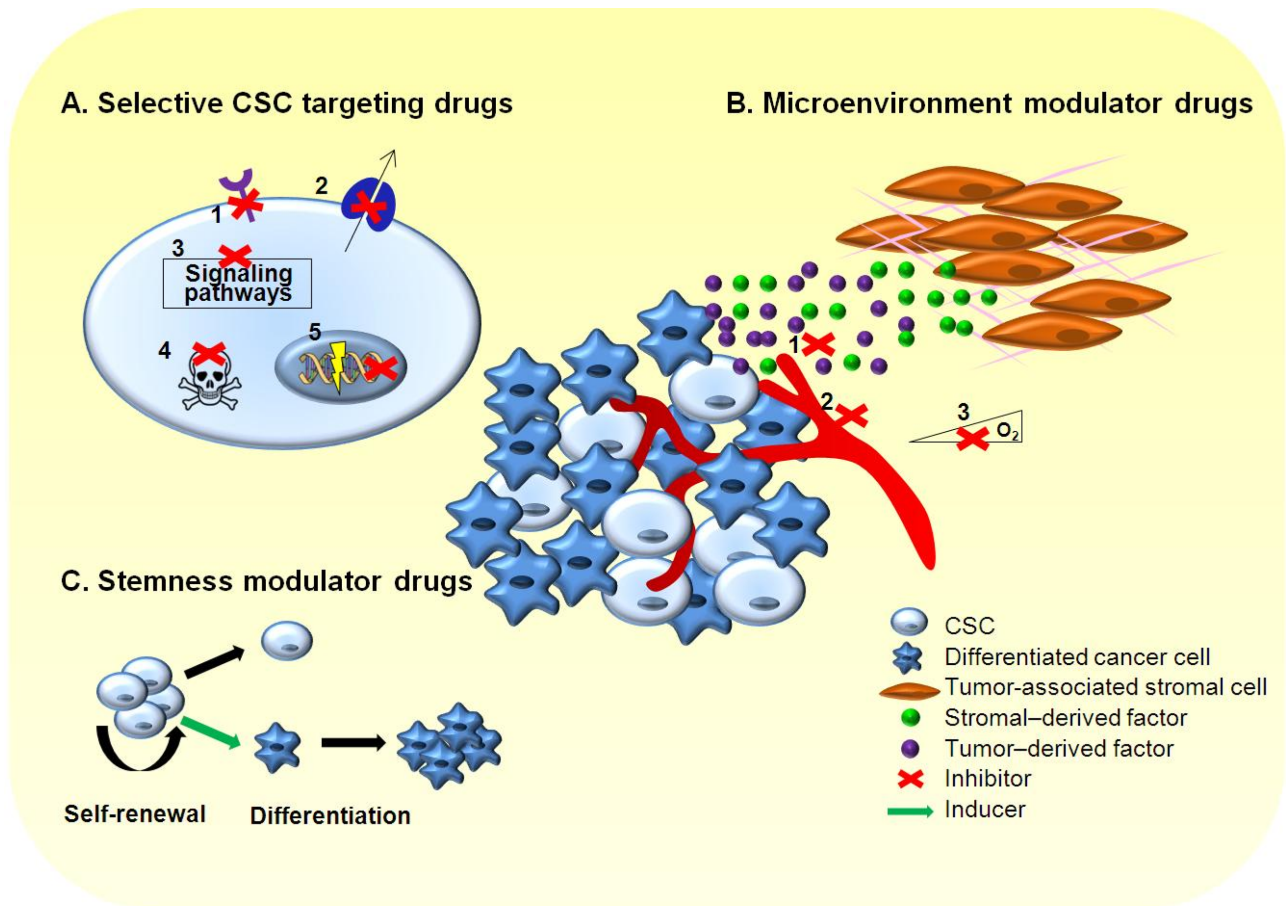

Figure 1 Targeting cancer stem cells and the tumor microenvironment. (**A**) Therapeutic approaches to selectively target CSCs use mAbs directed to CSC-surface markers (1), agents blocking drug efflux pumps (2), inhibitors of signaling pathways that take part in controlling the fate of CSCs (3), CSC-specific cytotoxic compounds (4) and inhibitors of the DNA repair machinery (5). (**B**) Microenvironment modulator drugs can impair the effect of stromal- and cancer-derived factors (1), inhibit angiogenesis (2) and counteract the pro-oxidant environment generated by tumor hypoxia (3). (**C**) Stemness modulator compounds force the differentiation of CSCs and in combination with standard chemotherapy contribute to the successful elimination of CSCs and tumor bulk. Cancer stem cell (CSC), monoclonal antibody (mAb).

4.2 Selective cancer stem cells targeting drugs

Proof of evidence that CSCs are endowed with self-renewal and differentiation capabilities is represented by the ability to engraft tumors when serially transplanted in immunocompromised mice. Further support, recently emerging from *in vivo* genetic cell fate tracking experiments, confirmed the capability of CSCs to seed a tumor and recapitulate its heterogeneity (8, 9). The criteria used to identify CSCs in solid tumors and hematopoietic disorders include certain *in vitro* properties among

which i) CSCs can be distinguished and isolated with specific cell-surface marker profiles or intracellular molecules, ii) CSCs are endowed with increased resistance to chemotherapeutic compound (CSCs are detectable for their high levels of detoxify enzymes and MDR) and iii) the activation of CSCs-dependent pathways, which could offer a functional marker for their identification (10).

4.2.1 CSC surface markers as a therapeutic target

Thus, the ability to use CSCs' peculiar surface markers has been suggested as a promising therapeutic approach. One must bear in mind that some limitations do exist such as, the existence of inter- intra- tumor heterogeneity and splicing variants, the different methodologies used for CSCs detection and the presence of some common markers shared by normal adult stem cells. For instance, CD44 is a transmembrane glycoprotein and the receptor for hyaluronic acid (HA) and osteopontin (OPN), among others. It is expressed in CSCs from distinct solid tumor types and H90, an anti-CD44 monoclonal antibody (mAb), was the first antibody that showed CSC targeting properties. *In vivo* administration of H90 interfered with acute myeloid leukemia (AML) stem cells' homing capability in the microenvironmental niche and maintained their stem cell status (11). Similarly, in a xenograft model initiated by triple negative breast cancer cells, the anti-CD44 mAb P245 inhibited tumor growth and recurrence if injected during the apparent tumor remission period achieved after treatment with doxorubicin and cyclophosphamide (12). GV5 is a recombinant human mAb that recognizes the extracellular domain of CD44's alternative splicing variant, termed CD44R1(v8-v10). In athymic mice GV5 inhibited tumor formation, after the subcutaneous transplantation of larynx and cervix cancer cells, due to the induction of antibody-dependent cellular cytotoxicity (ADCC) and internalization of CD44R1 (13). H4C4 is an anti-CD44 mouse mAb that decreased pancreatic CSC capabilities of *in vitro* tumor sphere formation and *in vivo* tumor growth. It also impaired metastasis formation and recurrence after radiotherapy *via* Nanog and STAT3 signaling pathway inhibition (14). Finally, due to its promising preclinical results, RO5429083, which is a humanized mAb directed against an extracellular epitope of human CD44, has been evaluated in a phase I clinical study on CD44-expressing metastatic and/or locally advanced solid tumors. Another phase I clinical study is still ongoing involving patients with AML (http://www.cancer.gov/clinicaltrials). MT110 is a bispecific bifunctional T-cell-engaging (BiTE) antibody that concomitantly binds to the epithelial cell adhesion molecule (EpCAM), a common CSC marker, and to the T-cell receptor complex CD3 which, leads to the activation of cytotoxic T cells against EpCAM-expressing cells and causes cell death *via* redirected lysis. MT110 reduced the capacity of colon and

pancreatic CSCs, cocoltured with peripheral blood mononuclear cells (PBMCs) as source of T cells, to form spheres *in vitro* and to generate tumors *in vivo* (15, 16). MT110, is in early stages of clinical trials for patients with locally advanced, recurrent or metastatic solid tumors, known to widely express EpCAM (http://www.cancer.gov/clinicaltrials). Catumaxomab is a bispecific trifunctional antibody (Triomabs) binding to EpCAM and the CD3 complex in T cells. In addition, it binds macrophages, natural killer (NK) and dendritic cells *via* its Fc fragment thus, synergizing the anti-tumor effects exerted by T cells. When Catumaxomab is administered to patients with advanced solid cancers and suffering from malignant ascites, it activated peritoneal T cells, stimulated the release of proinflammatory Th1 cytokines, decreased the peritoneal level of VEGF and eliminated $CD133^+/EpCAM^+$ CSCs (17). Catumaxomab has been approved in Europe for clinical use in the treatment of malignant ascites and the results, from a prospective randomized phase II/III clinical trial, have been reported by Heiss *et al.* (18). The ubiquitous expressed transmembrane antigen CD47 can trigger inhibition of phagocytosis (the so-called 'don't eat me' signal) on SIRPα-expressing phagocytic cells. CD47 blocking *via* the mouse mAb B6H12.2 favors the phagocytosis of human AML stem cells through mouse and human macrophages. Interestingly, B6H12.2 spares normal hematopoietic stem cells because they express low levels of CD47 (19). 7G3 is a mouse mAb and recognizes the human interleukin-3 (IL-3) receptor α chain (CD123), which is overexpressed on AML blasts and $CD34^+$ AML stem cells. 7G3 inhibits the engraftment and homing of AML stem cells in immunocompromised mice through ADCC (20). CSL362, a humanized anti-CD123 mAb with an increased affinity for human CD16, induces massive NK-mediated ADCC in both AML blasts and $CD34^+CD38^-CD123^+$ AML stem cells (21). CSL362 is currently in the beginning stages of clinical trials for patients with AML (http://www.cancer.gov/clinicaltrials). A more detailed list of CSC specific markers and their use as putative therapeutic targets has been reviewed recently (22, 23).

4.2.2 Targeting ABC transporters in CSCs

ATP-binding cassette (ABC) transporters have been used to identify CSCs because they are overexpressed on the membrane of both normal and cancer stem cells. ABC transporters enable the efflux of drugs and are responsible for MDR. Thus, CSCs are able to expel the Hoechst 33342 dye by adopting such machinery and thus creating a 'side population' (SP) which, can be isolated by fluorescence-activated cell sorting (FACS). ABCB1 (P-glycoprotein), ABCG2 and ABCC1 are the most extensively studied ABC transporters in stem cell biology. In order to avoid drug resistance, much effort has been devoted to the design of ABC transporter inhibitors which,

selectively eliminate CSCs but spare normal stem cells. However, several ABCB1 inhibitors, such as verapamil, tariquidar, and quinidine, have shown little efficacy in clinical settings. The elimination of CSCs has not been successful perhaps due to: clinical studies that were not designed correctly, the choice of an incorrect ABC transporter as a target and other combinations of CSC targeting drugs would have been preferable (24). Some ABCG2 inhibitors showed high toxicity both *in vitro* and *in vivo*. Novel compounds are in preclinical studies such as the ABCG2 inhibitor YHO-13351 which, sensitized the human cervical carcinoma cell line to irinotecan and reduced the CSC population (25). Xia *et al.* developed an image-based high-content screening system and identified 12 potent high drug efflux cancer cell inhibitors from 1280 screened compounds. These inhibitors sensitized lung cancer cells to chemotherapeutic drugs and possibly affected *in vivo* tumorigenic capabilities of the CSC compartment (26).

4.2.3 The inhibition of pathways that sustain CSCs

CSCs are dependent on activated signaling pathways different from those sustaining the bulk population. Therefore, targeting the stemness determinants could effectively conduct to the most durable remission and prevent resistance to chemotherapy and radiotherapy. Being an important player in self-renewal and maintenance of CSCs (27), the Wnt signaling pathway has been targeted by both small-molecule and biologic inhibitors. The first class of compounds includes ICG-001 which, acts as an antagonist of CREB-binding protein (CBP)/β-catenin (28) and showed to selectively eliminate drug resistant leukemic stem cells (29). Moreover, the small LGK974 (30) and IWP2 (31) molecules target the porcupine enzyme which, is responsible for palmitoylation of Wnt ligands, a required step in activating their secretion. A LGK974-based phase I clinical trial on patients with solid tumors is still ongoing (http://www.cancer.gov/clinicaltrials). The second class of compounds includes, the humanized mAb OMP-18R5 that binds to the extracellular domain of multiple Frizzled (FZD) receptors and blocks the Wnt3A-induced downstream pathway. In preclinical settings, it reduces tumorigenic capabilities of human breast, pancreatic, colon and lung cancer cells, compared to standard chemotherapy (32), and is currently in its early stages of clinical trial for patients with solid tumors (http://www.cancer.gov/clinicaltrials). The activation of the Hedgehog (Hh) pathway is mandatory for the maintenance of CSC properties in various human cancers. The molecules antagonist of smoothened (SMO), a G protein-coupled transmembrane serpentine receptor that usually acts as a signal transducer of the proximal Hh pathway, such as GDC-0449, inhibit cell growth and induce apoptosis of pancreatic CSCs (33). Interestingly, the antineoplastic compound mithramycin, showed

properties that target Sox2$^+$ medulloblastoma stem cells and bear the aberrant Sonic hedgehog (Shh) pathway activation. Specific to this context, although Sox2$^+$ cancer cells were driven by Shh signaling, they were not affected by either the Shh-targeted therapy with GDC-0449 or anti-mitotic chemotherapy. This suggests the existence of heterogeneity even within the Shh medulloblastoma subgroup and that a combination of bulk targeting drugs and CSCs targeted therapy could lead to a more notable control of the disease (34). GDC-0449 is in phase II of the clinical trial regarding the treatment of basal cell carcinoma (http://www.cancer.gov/clinicaltrials).
The Notch signaling pathway is a well-recognized positive regulator of CSCs fate (35, 36). The best way to target Notch activation, is to inhibit the proteolytic cleavage of the Notch intracellular domain (NICD) *via* the γ-secretase complex. γ-secretase inhibitors (GSIs) reduce self-renewal and tumorigenicity of GSCs and breast CSCs (37, 38). A phase I/II clinical trial that foresees the use of GSIs MK-0762 followed by docetaxel, whose purpose is killing breast cancer stem cells in advanced or metastatic breast cancer, has recently been completed (39). Antibodies targeting the Notch ligand Delta-like 4 (Dll4) such as the humanized mAb OMP-21M18, have been developed and efficiently reduced CSC frequency in solid tumors (40, 41). A comprehensive analysis of all ongoing and completed Notch clinical trials has recently been published (42). FAK activity seems to be critical for survival, migration and resistance to chemotherapy of CSCs (43, 44). Kang *et al.* demonstrated that the FAK inhibitor VS-6063 (which inhibits FAK autophosphorylation) overcomes resistance to paclitaxel in ovarian cancer by decreasing the AKT-dependent YB-1 phopshorylation which, in turn downregulates the CD44 expression (45). Others showed that the upregulation of CD44 favors breast cancer cell self-renewal, tumorspheres formation and induces paclitaxel resistance (46). Furthermore, CD44 upregulates Nanog, responsible for increased ABCB1 expression and ovarian cancer cells acquired resistance to paclitaxel (47). VS-6063 is currently in phase II of its clinical trial for *K-RAS* mutant non small cell lung cancer (NSCLC) patients. Similarly, other FAK inhibitors such as VS-4718 and PF-00562271, are in phase I of clinical evaluation (http://www.cancer.gov/clinicaltrials). Finally, the BMI-1 inhibitor PTC-209, has recently been proposed as an interesting small molecule affecting self-renewal of colorectal cancer cells with no systemic toxicity in preclinical settings (48).

4.2.4 Agents that selectively eradicate CSCs

A high-throughput screen for agents that selectively kill CSCs has been performed by Gupta et al. Among a library of 16.000 compounds tested, salinomycin induced breast CSC-specific toxicity. Breast cancer cells were initially forced to undergo an

EMT by means of an E-cadherin knockdown. Pre-treatment with salinomycin inhibited tumorsphere formation *in vitro* and reduced tumor seeding ability *in vivo* by >100-fold, compared to paclitaxel. Salinomycin treatment also decreased tumor mass and metastasis and increased epithelial differentiation of breast CSCs in an immunocompromised mouse model (49). Successively, similar results have been reached in some type of cancers, including leukemia, colorectal cancer, lung cancer, GIST and osteosarcoma. Some findings also suggested that, a combination of salinomycin and conventional cytotoxic drugs could be a much more efficient strategy than the use of a single agent to improve therapeutic outcomes (50, 51). Moreover, being that salinomycin seems to be toxic to normal stem cells at concentrations also effective in CSCs (52) it will render its clinical use as a single agent difficult. Salinomycin acts as a K^+ ionophore in biological membrane that promotes mitochondrial and cytoplasmic K^+ efflux however, the exact mechanisms underlying its toxicity against CSCs still remains unclear. It has been shown that salinomycin is a powerful inhibitor of the multidrug resistance protein 1 (MDR-1) (P-glycoprotein/ABCB1) (53). It inhibits the phosphorylation of the Wnt co-receptor LRP6, induces apoptosis in chronic lymphocytic leukemia (54) and is an antagonist of the mTORC1 signaling pathway in breast and prostate cancer cells (55). On the other hand, it encourages ROS production and inhibits oxidative phosphorylation in mitochondria (56), resulting in the possible elimination of CSCs, which rely on this metabolic process. In addition, recent studies have unveiled that salinomycin induces cell growth inhibition and apoptosis in multi drug resistant ovarian cancer cell lines, by ablating the activity of the signal transducer and activator of transcription 3 (Stat3) and thus, diminishing the expression of Stat3 target genes, such as *cyclin D1, S-phase kinase-associated protein 2 (SKP2)* and *SURVIVIN* (51). This is not surprising if we consider the most recent evidence which highlights the major role that Stat3 plays in reducing the effectiveness of drugs treatment. Specifically, the inhibition of MEK in ‘oncogene-addicted’ cancer cells, (driven by activated EGFR, HER2, ALK, MET and KRAS pathways) triggers the feedback activation of Stat3 through IL-6R and FGFR, leading to treatment resistance (57). In line with these results, Kim *et al.* showed that the constitutive activation of the IL-6/Stat3/NF κB pathway in $p53^-PTEN^-$ non-transformed MCF10A, was dependent on the proteolytic degradation of SOCS3 and generated highly metastatic and EMT-like CSCs. Thus, proteasoma inhibition restored SOCS3 protein levels and the selective IL-6R antagonist, tocilizumab, repressed the CSC compartments, hampered tumor growth and dissemination *in vivo* (58).

4.2.5 PARPi affects CSC survival

Recent breakthroughs displayed that inhibition of poly-ADP-ribose polymerase (PARP) could be a promising selective CSC-targeted therapy. Mechanistically, PARP is an abundant nuclear protein that mediates the repair of single strand breaks (SSBs) through base excision repair. The inhibition of PARP leads to the accumulation of SSBs that during replication are converted into double-strand breaks (DSBs), usually repaired by the homologous recombination (HR) pathway, mediated by BRCA1 or BRCA2. Whereas, in neoplastic cells with defective HR, the DSBs cannot be repaired and lead to cell death. It was shown that AZD2281, a PARP inhibitor (PARPi), preferentially targets glioblastoma stem cells (GSCs) and reduced their survival, expansion and tumor initiation capabilities, as well as having sensitized them to radiation therapy (59). Moreover, a PARPi, GPI 15427, was able to counteract GSC's resistance to temozolomide (60). These examples opened a new road for the use of PARPi, even in the absence of mutations of *BRCA1/2*. This changed the classical idea of 'synthetic lethality' which exists between PARP and BRCA1/2 signaling pathways. Indeed, patients affected by triple negative breast cancer (non carriers of *BRCA1/2* mutations), have shown increased therapy response and survival following PARP inhibition (BSI-201) in combination with DNA-damaging chemotherapy. The latter of which may eventually obstruct the cellular DNA repair machinery and cause cell death (61). Moreover, deletions or mutations in other genes involved in key genotoxic stress pathways such as *PTEN*, may sensitize them to PARPi administration (62). PARPi are currently under clinical evaluation in solid tumors as single agent or in combination with chemotherapy and detailed information about ongoing clinical trials has been published elsewhere (63) (http://www.cancer.gov/clinicaltrials).

4.3 Stemness modulator drugs

Notwithstanding that CSCs embody a small portion of the tumor bulk, they are responsible for the heterogeneous cell population that constitutes the tumor mass and their intrinsic resistance to chemotherapy and radiotherapy shown by aggressive tumors. Indeed, CSCs possess both self-renewing capabilities, by means of generating two identical CSCs daughter cells through symmetrical division, and the ability to differentiate through asymmetrical division, yielding the multitude of cancerous cells that account for overwhelming tumor growth (64). As previously discussed, a prominent mechanism of therapeutic resistance includes an altered kinetic cell cycle in quiescent CSCs. They are spared by chemotherapy-induced cytotoxicity because they are not actively cycling cells but are capable of activating

DNA repair mechanisms. Thus, forcing terminal differentiation of CSCs could be an extremely powerful weapon in preventing resistance and relapse. Ideally, a clinically effective response could be achieved by the simultaneous administration of anti-CSC therapy and conventional chemotherapy, in order to eliminate cytotoxic drug-susceptible non-CSCs and prevent their dedifferentiation in CSCs (5). Given that the development of clinical endpoints in this field may prove challenging, an emergent amount of stemness modulator drugs is already in clinical use and others are in preclinical or early stages of clinical evaluation. Some examples are listed below.

4.3.1 ATRA induces differentiation of CSCs

Among these, all-trans-retinoic acid (ATRA), a derivate of vitamin A, has already been demonstrated to be a potent differentiation-inducing drug and a successful treatment strategy, in combination with arsenic trioxide, for AML patients carrying the PML-RARα fusion protein (65). Campo *et al.* reported that ATRA induced differentiation and radio- and chemo-sensitization of stem-like glioma cells (66). Given that, ALDH is a common marker of breast CSCs and a detoxifying enzyme responsible for the oxidation of intracellular aldehydes as well as of retinol to retinoic acid, it was shown that DEAB-mediated ALDH inhibition increased the CSC compartment by abrogating CSC differentiation. Conversely, ATRA treatment induced differentiation of breast CSCs and decreased the stem population (67). Similarly, Hammerle *et al.* suggested that the neuroblastoma stem cells' response to 13-cis-retinoic acid (RA), could be enhanced by the proteasome inhibitor MG132 (68). Interestingly, a combination of CSC genomics with connectivity map, analyzed a database of 6100 gene expression profiles of four breast cancer cell lines, treated with different concentrations of approximately 1000 FDA approved drugs. This revealed that ATRA is negatively associated with CSC-enriched gene expression signature. ATRA induced apoptosis, hampered mammosphere formation and forced differentiation of fulvestrant-resistant cells. Intriguingly, in the same study, a MEK inhibitor, selumetinib, sensitized the *K-RAS* mutant breast cancer cell line, which was enriched with CSCs, to the ATRA treatment (69).

4.3.2 SAHA modulates differentiation and apoptosis of CSCs

Suberoylanilide hydroxamic acid (SAHA), also called vorinostat, a potent inhibitor of the histone deacetylase (HDAC) family, caused differentiation and apoptosis of several tumor type cells. In an *in vivo* prostate cancer tumor model, SAHA hampered tumor growth with low systemic toxicity (70). Additionally, HDAC inhibitors can be therapeutically exploited to specifically target slow cycling cells. For instance, SAHA, coupled with imatinib mesylate, successfully fostered apoptosis in quiescent

chronic myelogenous leukemia stem cells and offered a novel strategy to overcome chemoresistance and the difficulties in targeting dormant cells (71).

4.3.3 BMPs: an actor of balance between differentiation and stemness

It is the general understanding that the bone morphogenic protein family (BMPs) is required to inhibit the stem cell state and mesenchymal traits in a variety of normal and cancerous epithelial tissues (7, 72) and promote differentiation of adult and pluripotent stem cells (73). Mechanistically, BMPs are members of the transforming growth factor-β (TGF-β) superfamily and bind to a combination of type I receptors (anaplastic lymphoma kinase 2 (Alk2), Alk3 (or BMPR1A), and Alk6 (or BMPR1B)) and type II receptors (BMPR2)). They activate either the canonical BMP signaling pathway, through phosphorylation of smads receptors, or the PI3K/AKT-mediated non canonical BMP signaling pathway. Specifically, a BMP7 variant (BMP7v) abrogated *in vitro* proliferation of glioblastoma stem cells (GSCs) as well as the expression of stem associated markers and endothelial cord formation. In a glioblastoma orthotopic mouse model, BMP7v impaired tumor growth, invasion and angiogenesis (74). Likewise, our group demonstrated that BMP4 enhanced colorectal CSCs' differentiation and apoptosis and it their sensitized them to 5-fluorouracil and oxaliplatin treatment. However, the *SMAD4*-defective tumors carrying either mutations in *PI3K* or loss of *PTEN* are refractory to the treatment mentioned above thus, confirming the BMP4-mediated activation of both canonical and non canonical pathways (75). On the contrary, molecules such as Coco, an antagonist of TGF-β ligands, reverses the effect of BMP thereby, enhancing the self-renewal of metastasis-initiating cells (76).

4.3.4 Resveratrol affects CSC self-renewal

A number of epidemiological studies have proposed that resveratrol, a polyphenolic compound with which, many plant species are enriched with, exerts several biochemical activities associated with tumorigenesis such as, inhibition of inflammation, cell proliferation and angiogenesis as well as, sensitizing tumor cells to chemotherapy (77). Even though the influence of resveratrol on CSCs is still under evaluation, recent evidence showed that *KRAS*^{G12D} mice, which spontaneously develop aggressive pancreatic cancer, treated with resveratrol developed smaller tumors (dimension and weight). Moreover, patient-derived pancreatic cancer and mice-derived *KRAS*^{G12D} CSCs, lost their self-renewal capability in presence of resveratrol, possibly by the inhibition of Nanog, Sox-2, c-Myc and Oct4. In the same study, patient-derived CSCs underwent resveratrol-evoked apoptosis by activating caspase 3/7 and inhibiting XIAP and Bcl-2. Migration and invasion were suppressed

following the inhibition of EMT related markers such as ZEB-1, SLUG and SNAIL (78). Similarly, in Glioblastoma multiforme (GBM), resveratrol induced apoptosis and differentiation of stem-like cells and sensitized them to radiotherapy *in vitro* and *in vivo*, *via* disruption of STAT3 signaling (79). Thereafter, Sato *et al.* mechanistically explained the inhibitory effect observed after resveratrol treatment on self-renewal and the tumorigenicity of CSCs. Indeed, resveratrol promoted the phosphorylation and activation of p53, which in turn may directly favor Nanog degradation *via* proteasome machinery (80).

4.3.5 Cyclopamine limits the self-renewal of CSCs

An additional plant-derived compound, the steroidal alkaloid cyclopamine, is a potent cancer preventing compound that directly binds to the heptahelical bundle of SMO (81). As already discussed in the present chapter, Hh signaling is essential for the maintenance of stem-like traits in multiple myeloma, leukemia and gastric cancer, among others (82-84). Hh pathway inhibition through cyclopamine inhibited tumorsphere formation *in vitro* and the establishment of orthotopic glioblastoma tumors (85). The newly synthesized cyclopamine-derived inhibitor of the Hh pathway, IPI-926, ameliorated cyclopamine characteristics such as oral bioavailability, higher metabolic stability, and a better pharmacokinetic profile (86). Cyclopamine and IPI-926 limited self-renewal potential of B-cell acute lymphocytic leukemia (B-ALL) cells (87). Interestingly, delivery of conventional chemotherapy, such as gemcitabine, to the tumor site, may be potentiated by the simultaneously administration of IPI-926. Indeed, *in vivo* inhibition of the Hh pathway increased intratumoral drug absorption in a gemcitabine-resistant pancreatic ductal adenocarcinoma model thus, making IPI-926 an important therapeutic strategy for the management of pancreatic cancer chemoresistance (88). IPI-926 is undergoing early step clinical trials for solid malignancy in combination with standard chemotherapy (89) (http://www.cancer.gov/clinicaltrials).

4.3.6 Curcumin promotes CSC differentiation

Curcumin (diferuloylmethane) derives from the Indian spice plant turmeric. Extensive preclinical studies showed its therapeutic potential in a variety of human diseases, including cancer. Due to its pleiotropic activities, curcumin is able to modulate a variety of normal or aberrant biological processes, hence it has been selected as a promising anti-cancer drug in several clinical trials reported in detail by Gupta *et al.* (90). Moreover, studies have shown that curcumin displayed capability of eliminating colon CSCs either alone or in combination with standard chemotherapy, such as FOLFOX (5-fluorouracil and oxaliplatin) and dasatinib (91,

92). Furthermore, Curcumin promotes GSCs terminal differentiation, which culminated in autophagy. Whereas, in an intracranial glioblastoma xenograft model, it repressed their self-renewal capability and tumorigenicity (93). Intriguingly, breast CSCs, derived from the MCF7 cell line, displayed inhibition of tumorsphere formation and the Wnt signaling pathway (94).

4.3.7 Metformin in CSC biology

Metformin is a well established oral anti-diabetic drug of the biguanide class. It is an agonist of the adenosine monophosphate-activated protein kinase (AMPK) and an inhibitor of PI3K, mTOR and IGF. It has gained attention for its *in vitro* and *in vivo* antitumor effects and is now being tested in several advanced clinical trials (95) (http://www.cancer.gov/clinicaltrials). Metformin has also emerged as an important factor to counteract the retention of stemness and the activation of the EMT program of some cancer populations (95). Metformin was able to inhibit the expression of Oct4 in the MCF7 cell line, mediated by 17-β-estradiol treatment, and to reduce the fraction of $CD44^{high}/CD24^{low}$ cells (96). In line with these results, Vazquez-Martin *et al.* observed that metformin deprived basal-like breast cancer cells of the stem compartment and suppressed an EMT program activation through the transcriptional repression of ZEB1, TWIST1, SNAI2 and TGFβ (97). Metformin depleted the CSC pool in both gemcitabine-sensitive and -resistant pancreatic cancer cells, by decreasing the expression of CSC-specific markers such as EpCAM, Notch, Nanog, and CD44, as well as reexpressing miRNAs, (e.g. let7a, let7b, miR200b, and miR-200c) usually associated with cellular differentiation (98). The studies performed by Oliveras-Ferraros *et al.* attempted to anticipate the possible mechanisms of acquired resistance to metformin treatment. They observed that the potential of metastatic dissemination of breast stem-like cells seemed to be fueled by the chronic administration of metformin to the estrogen–dependent MCF7 cell line. Thus, the drug selected for the emergence of resistant cells, leads to a transcriptome reprogramming which, drives them towards a metastatic stem-like profile (99).

4 Microenvironment modulator drugs

4.4.1 Targeting the CSCs vasculature niche

There is proof of evidence that tumor-associated stroma and the extracellular matrix, are an extremely powerful source of herotypic signals, responsible for the activation of an EMT program on cancer cells and possibly to nurture the CSCs within their niche. Among the stromal compartment, endothelial cells play a major role in supporting the self-renewal capability of CSCs and in building up all the vasculature

architecture needed from these cells to provide nutrients and an easy route to metastatic dissemination. While the contribution of endothelial cells to tumor angiogenesis is self-evident, our understanding on CSC survival and drug resistance is still incomplete. Pioneer work from Calabrese *et al.*, showed how the formation of a vascular niche is directly involved in the function of CSCs. Interestingly, glioblastoma stem cells (GSCs) can be induced to differentiate in either endothelial cells or pericytes, as a consequence of their undifferentiated state and their strict dependence on microvasculature stimuli (100). Tumor vasculature is classically composed of a network of tortuous, saccular and extremely permeable vessels, endothelial cells that are abnormally covered by pericytes and an irregular basal membrane. As a result, cancer cells can easily penetrate into the bloodstream and colonize distant metastatic sites, and a higher interstitial hydrostatic pressure, due to plasma leakage, may impair the delivery of chemotherapeutic drugs to the tumor site (101). Vascular endothelial growth factor (VEGF) was identified as an endothelial compartment mitogen which has a prominent role in positively regulating physiological and pathological angiogenesis. The mammalian VEGF family consists of five heparin-binding homodimeric glycoprotein of 45 kDa referred to as, VEGFA (VEGF), VEGFB, VEGFC, VEGFD and Placental growth factor (PlGF). The predominant VEGF molecules are represented by several spliced variants denoted as, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148,}$ $VEGF_{165}$, $VEGF_{183,}$ $VEGF_{189}$, and $VEGF_{206}$ (102). They are commonly secreted by macrophages, neutrophils, fibroblast and several cancer cells but not by endothelial cells themselves. VEGF receptors consist of VEGFR1 (FLT1), VEGFR2 (FLK1) and VEGFR3 (FLT4). VEGFR1 is able to bind VEGF, VEGFB and PlGF. VEGFR2 is activated by VEGF, VEGFC and VEGFD. Lastly, VEGFR3 is primarily involved in lymphangiogenesis as a receptor for VEGFC and VEGFD. Although all VEGFRs are tyrosine kinase receptors, VEGFR2, in response to VEGF stimulation, has captured the most attention as the predominant effector in cancer initiation and progression. This is explained by the fact that VEGFR1 binds VEGF with a higher affinity than VEGFR2 but conversely exhibits weaker tyrosine kinase activity in response to its ligand (103). In this context, Park *et al.* also proposed that VEGFR1 could act as a ‘decoy’ receptor able to negatively regulate VEGF activity, by preventing its binding to VEGFR2 (104).

The binding of VEGFs to their cognate receptors induces dimerization and autophosphorilation of the intrinsic receptor’s tyrosine residues and consequently activates the dominant PI3K-AKT, MAPK and FAK pathways. It is now well established that VEGFs and VEGFRs are expressed in a variety of tumors (including colon, breast, lung, prostate, and ovarian cancer). VEGF signaling interferes in cancer biology and interestingly in CSC function, independently of angiogenesis and in

autocrine fashion. Conversely, it is popular belief that tumors rely on the classical paracrine VEGF-mediated sprouting angiogenesis, the increased permeability and the influence from the immune cells and the tumor microenvironment's fibroblasts (105). The realization that VEGF signaling is a crucial determinant in EMT-induced cancer stemness, is becoming an emerging theme and was recently pointed out by Fantozzi *et al.*. Indeed, VEGF-mediated angiogenesis by itself is not sufficient but required to increase tumor initiating capacity and dissemination of breast cancer cells undergoing EMT, also suggesting that additional factors from the microenvironment are required (106). For instance, a fraction of $CD133^+$ GSCs showed a 10-20 fold increase of VEGF secretion and displayed strongly angiogenic and hemorrhagic tumors through the enhancement of resident endothelial cell function and recruitment to the tumor site of bone marrow-derived endothelial progenitors (107). In murine models, GSCs may be induced to differentiate into endothelial cells and to directly contribute to tumor vasculature architecture, as proven by the positivity of those cells to VEGFR2 (108). These findings clearly establish that VEGF, secreted by tumoral cells, acts as a paracrine factor to sustain angiogenesis and as an autocrine factor to boost cancer stemness.

Judah Folkman was the first scientist to introduce the pioneer idea that solid neoplasms were always sustained by new vessel growth and envisioned angiogenesis as a new target for cancer treatment (109). In 2004, for the first time the FDA approved an anti angiogenic compound, called Bevacizumab, for clinical use in combination with standard chemotherapy. It is a humanized monoclonal antibody specific to VEGF that prevents the interaction of VEGF to its receptor. It became the standard means of treatment for metastatic HER2 negative breast cancer, metastatic colorectal cancer, glioblastoma, advanced or metastatic non-small-cell lung cancer, advanced renal-cell carcinoma and recently, for persistent, recurrent, or metastatic cervical cancer (110). Later, Aflibercept was approved as a 'decoy' receptor for VEGFA, VEGFB and PlGF (111). The inhibition of VEGFR kinase activity, is another valid approach to counteract tumor angiogenesis. Sunitinib targets multiple receptor tyrosine kinases including PlGFR and VEGFRs in unresectable, local, advanced or metastatic disease in well differentiated pancreatic neuroendocrine tumors, renal-cell carcinomas, and imatinib-resistant gastrointestinal tumors. Similarly, Sorafenib inhibits Raf kinases, VEGFRs and PlGFR in thyroid, liver and hepatocellular carcinoma (112). Since 1971, lots of studies have been published in the field and seemed promising but little efficacy has been shown yet. Besides their remarkable activity in the inhibition of primary tumor growth, anti-angiogenic drugs failed in producing lasting responses and patients' illnesses eventually progress (113). This could be partially explained by the fact that alternative adaptive resistance

mechanisms, used to overcome the drug-mediated anti angiogenic effect, can occur. This could be the case when there is: an activation of alternative angiogenic pathways, including Fibroblast growth factor 1 (FGF1) and FGF2, Ephrin A1 (EFNA1) and EFNA2 and Angiopoietin1 (ANGPT1), the recruitment of proangiogenic cells, and the increased coverage of pericytes to support vessel integrity. Interestingly, in an *in vivo* engineered model of KRAS-driven pancreatic ductal adenocarcinoma, resistant to anti-VEGF therapy, the MEK inhibitor substantially decreased the release of granulocyte–colony stimulating factor (G-CSF) by the tumor cell, which is usually responsible for the recruitment and mobilization of pro-tumorigenic and pro-metastagenic CD11b^{+} Gr1^{+} myeloid-derived suppressor cells. CD11b^{+} Gr1^{+} cells also helped the establishment of metastases by secreting matrix metalloproteinases (MMPs) as well as the Bv8 molecule, endowed with pro angiogenic features. This study revealed that a combination of MEK inhibitor and anti-VEGF therapy substantially decreased tumor burden and angiogenesis (114). Likewise, anti angiogenic therapy eradicated the brain tumor stem cell niche in an *in vivo* model of c6 rat glioma cell line and enhanced the effect of the conventional cytotoxic agent, cyclophosphamide (115).
Even upon anti-VEGF therapy, functional vessels tightly covered by pericytes have been observed. Indeed, endothelial cells can recruit perycites to protect themselves from anti angiogenic treatments and preserve their vascular structure. An attractive hypothesis suggested that CXCR4^{+} GCSs were mobilized towards the tumor site through an SDF-1 gradient and, upon TGF-β release by endothelial cells, were forced to differentiate in pericytes and contributed to tumor vasculature and growth (116). Moreover, Conley *et al.* showed that, hypoxic conditions limit the effectiveness of the antiangiogenic agents bevacizumab and sunitinib, by increasing breast CSC populations (117).

4.4.2 Therapeutic implications of Neuropilins in CSC biology

VEGF receptors can functionally interact with other receptors and foster CSC-driven tumor growth and progression. Within the same context, Neuropilins (NRPs) were described earlier as neuronal receptors for the semaphoring family and also involved in axon guidance. They act as transmembrane glycoproteins with a short cytoplasmic domain that lacks intrinsic catalytic activity and function as co receptors of VEGFR1 and VEGFR2. NRP1 is commonly expressed by endothelial cells and tumor cells (118). Upon autocrine VEGF stimulation, NRP1 promotes stemness and renewal of VEGFR2^{+} squamous skin CSCs (119). Similarly, viability, self renewal and tumorigenicity of CD133^{+} GSCs rely on autocrine VEGF/VEGFR2/NRP1 signaling and are maintained by a continuous secretion of VEGF (120). Cao *et al.* showed that

VEGF and NRP1 induced a dedifferentiated phenotype *in vitro* and promoted tumor formation *in vivo* (121). α6β1 integrin is necessary for the tumorigenicity of some subpopulations of breast CSCs and GSCs (122, 123). In triple negative breast cancers, NRP2 resulted preferentially expressed in breast CSCs and associated with α6β1 integrin. Upon VEGF stimulation of the NRP2- α6β1 complex, the focal adhesion kinase (FAK) mediated the activation of MAPK signaling and the subsequent expression of GLI1, an effector of the non canonical Hedgehog pathway. GLI1 in turn, induced BMI1 and positively fed back to the NRP2 expression, thus contributing to tumor initiation (124). NRP2 is also associated with aggressive prostate cancer and its expression is forced by PTEN loss. Activation of the VEGF/NRP2 axis culminates in BMI1 expression, which represses the transcription of the insulin like growth factor 1 receptor (IGF1R), commonly responsible for tumor progression. Interestingly, single targeting of NRP2 led to compensatory IGF-1R activation (125).

Therefore, these findings offer a perfect example of how an ideal combination of conventional chemotherapy, stemness modulator drugs (in this case anti-NRP specific antibodies), and anti IGFR antibodies could reduce tumor bulk, overcome treatment resistance and prevent relapse (Figure 2).

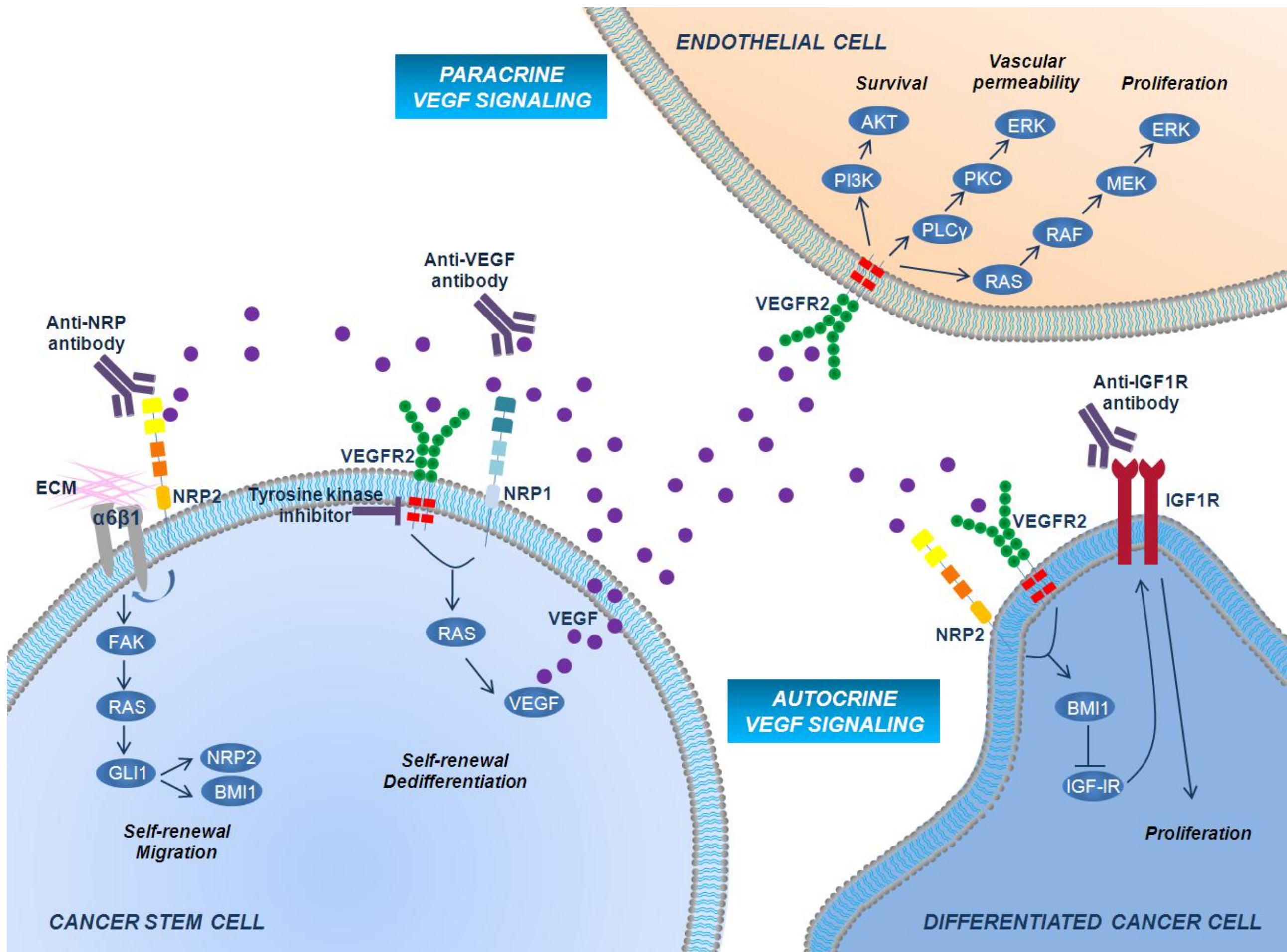

Figure 2. Therapeutic strategies to inhibit VEGF signaling in tumor cells. Besides regulating the common paracrine pathway on endothelial cells to sustain angiogenesis, VEGF signaling, when potentiated by NRPs, exerts its role in the autocrine stimulation of CSC self-renewal and migration. NRP2 can also interact with α6β1 integrin and trigger the integrin-mediated activation of FAK signaling cascade that culminates in the induction of BMI1 and NRP2. NRP1 interaction with VEGFR2 promotes the release of VEGF in the extracellular compartment, sustaining both the autocrine loop and the paracrine endothelial cell activation. Inhibition of VEGF signaling can be achieved mainly by mAb targeting VEGF and small molecules TKIs. mAbs directed against NRPs have been developed and proved to hamper self-renewal and tumorigenic capabilities of CSCs. However, inhibition of NRP2 can lead to compensatory IGF1R expression via BMI1 downregulation, supporting the importance of multiple therapy administration aimed at targeting both NRPs and IGF1R. Vascular endothelial growth factor (VEGF), Neuropilin (NRP), cancer stem cell (CSC), focal adhesion kinase (FAK), monoclonal antibody (mAb), tyrosine kinase inhibitor (TKI), insulin-like growth factor 1 receptor (IGF1R), extracellular matrix (ECM).

For instance, multiple compensatory signals could be activated when a single anti-angiogenic treatment is administrated, regardless of possible collateral stimulation of pathways involved in invasiveness or tumor cell stemness. Given that Bevacizumab does not inhibit VEGF binding to NRPs, Pan *et al.* generated two anti-NRP1 monoclonal antibodies specific to the binding site of semaphorin and VEGF on NRP1. This caused a reduction in cell proliferation as well as vascular density in a NSCLC *in vivo* model, assuming that the inhibition of NRP1, impairs vascular remodeling and thus rendering vasculature more responsive to anti VEGF treatment (126). In contrast with these findings, Snuderl *et al.* recently showed that the exclusive targeting of the PlGF/NRP1 pathway with the previously used phase I clinical trials, TB403 and 5D11D4, respectively an anti-murine PIGF antibody and an anti-human/murine PlGF antibody, reduced primary tumor burden and progression of medulloblastoma. PlGF seemed to be secreted by the tumor stroma, following tumor-derived Shh stimulation. PlGF only interacts with NRP1 rather than with VEGFR1 on medulloblastoma cells, for the enhancement of tumor spread. Authors suggested that the use of anti-NRP1 and –PlGF, in concert with standard chemotherapy, could make an additional improvement in the clinical setting (127).
Another example of multiple compensatory signaling activation was shown by Lu *et al.*. Indeed, bevacizumab treatment fostered an invasive phenotype in an *in vivo* model of GBM. The inhibition of VEGF suppressed the recruitment of the protein tyrosine phosphatase 1 B (PTP1B) from the VEGFR2/MET complex, consequently restoring hepatocyte growth factor (HGF)-mediated MET phosphorilation and tumor invasiveness. Authors suggested that in selected patients with GBM, tumor recurrence could be avoided by the combined use of anti VEGF and anti MET treatments (128).

4.4.3 Targeting microenvironment stimuli

AMD3100 is an antagonist of CXCR4. This drug, in combination with G-CSF to improve hematopoietic stem cell mobilization to peripheral blood for autologous transplantation, was approved in 2008 by the FDA for clinical use as a treatment for non-Hodgkin's lymphoma and multiple myelomas (129, 130).
Commonly used for leukemia in several clinical trials, AMD3100 prevents $CXCR4^+$ leukemia cell recruitment to the SDF-1-secreting bone marrow microenvironment, thus rendering cancerous cells more susceptible to cytotoxic drugs (131). In agreement with this, invasive $CD133^+$ pancreatic CSCs expressed CXCR4 and predominantly metastasize in the liver, being attracted by a gradient of SDF1, which is secreted by the stroma compartment (132).

Recently, CXCR4-SDF1 signaling has been identified as the driving force behind the establishment of bone metastasis in triple negative breast cancers. Particularly, CAF-rich stroma found in primary breast cancer secretes SDF-1 and IGF and selects tumor cell clones with high Src activity and thus, characterized by an activation of PI3K-AKT pathway. Src hyperactive clones were primed for bone metastasis because endowed with a greater chance of survival in the bone environment enriched with SDF-1 and IGF. Mechanistically, human mesenchymal stem cells were stimulated with a conditioned media from MDAMB231 cell line to constitutively secrete SDF-1 and IGF. Subsequently, authors cotransplanted breast cancer cell lines and stromal cells in an orthotopic mouse model. Following an *in vivo* treatment with CXCR4 inhibitor (AMD3100) and IGF1R inhibitor (BMS754807), the recovered cells were reimplanted and resulted in tumors, low in bone metastasis, compared to reimplanted cells from untreated tumors (133).

Similarly, we recently showed that in colorectal cancer, the exposure to SDF1, HGF and OPN, increased the migratory capabilities of colorectal CSCs and induced the CD44v6 expression, an alternative splicing isoform of CD44, on transiently amplifying progenitors. Interestingly, in untreated colorectal CSCs, CD44v6 was already highly expressed whereas, it was lower in sphere-derived differentiated progeny and bulk primary cells. CD44v6 acts as a coreceptor of the tyrosine kinase receptor MET, and together with its ligand, the pleyotropic cytochine HGF, cooperates to promote survival and migration through the PI3K-AKT pathway. When blocking SDF-1-CXCR4 activity with AMD3100, it reduced the invasive potential and abrogated the CD44v6 expression induced by HGF and OPN. Similarly the PI3K inhibitor, BKM120, killed CD44v6^{+} colorectal CSCs and impaired metastatic dissemination (134). It is worth considering that targeting these powerful effectors in the tumor microenvironment could have tremendous therapeutic implications. In this context, the use of compounds which, target both MET and HGF, are still under evaluation in several clinical trials (135) and only few of them were recently approved by the FDA. Although discovered as a MET tyrosine kinase inhibitor, Crizotinib was approved at the end of 2013 exclusively for the treatment of NSCLC as an ALK blocking compound (136). Similarly, Cabozantinib is a multi kinase inhibitor against VEGFR1, 2 and 3, RET, MET; TIE-2 and KIT and is currently administered uniquely for progressive medullary thyroid cancer (137). Clinical trials for prostate, brain, breast, and NSCLC are still undergoing (http://www.cancer.gov/clinicaltrials).

4.4.4 Hypoxia as a therapeutic target

Evidence that CD44 variant isoforms (CD44v) could promote survival and multidrug resistance has been shown by Ishimoto *et al.* In gastrointestinal cancer cells, CD44v enhanced the synthesis of reduced glutathione (GSH), the predominant intracellular antioxidant factor, by physically interacting with and stabilizing the cystine transporter subunit (xCT) at the plasma membrane. xCT is the light chain subunit of the cysteine-glutamate exchange transporter, which exchanges intracellular glutamate for extracellular cysteine, required for GSH synthesis. GSH protects the cell against reactive oxygen species (ROS) damages and suppresses $p38^{MAPK}$ activation, leading to cancer cell proliferation and resistance to ROS-inducing agents, such as docetaxel and cisplatin. As a result of these findings, *in vivo* exposure to sulfasalazine, a selective xCT inhibitor, induced $p38^{MAPK}$ signaling, enhanced response to chemotherapy, and avoided CD44-dependent tumor growth. Therefore, authors suggested that either sulfasalazine or CD44v-target therapy could abrogate ROS defense capabilities of CSCs and in turn sensitize to conventional cancer treatments (138).

Normal stem cells as well as CSCs, harbor low levels of ROS and possess an efficient defense mechanism against oxidative stress (139). An increase in ROS levels can occur in response to either environmental extrinsic (e.g. CAFs, CAMs, and hypoxia) or intrinsic oxidative stress (e.g. ROS producing enzyme and Jun D downregulation), along with iron chelators, nitric oxide (NO), and genetic alterations in PTEN, von Hippel-Lindau (VHL), succinate dehydrogenase (SDH), RAS-MAPK, and PI3K-AKT accounts for the hypoxia-inducible factor 1 α (HIF-1α) stabilization (140-142). As well as under normoxia, HIF-1α exerts its role in shielding tumor cells from oxygen deprivation and thus aids in meeting the metabolic requirements of the expanding tumor mass. The HIF family of transcription factors has a prominent role in a finely tuned and well characterized oxygen-sensor mechanism. They comprise a heterodimer of an oxygen dependent α–subunit (either HIF-1α, HIF-2α or HIF-3α) together with a constitutively expressed β-subunit (HIF-1β). Under normoxic conditions and in presence of iron, prolyl hydroxylases (PDH) modifies Pro402 and Pro564 of HIF-1α and promotes the interaction with VHL, leading to ubiquitination and proteasomal degradation. It prevents HIF-1α to dimerize with HIF-1β and to bind with the coactivator CBP/p300 to the hypoxia response element (HRE) in the promoters of hypoxia-target genes, regulating proliferation/apoptosis, glycolysis, angiogenesis, and invasion/metastasis (143). A high HIF-1α level is observed in many human cancers and is associated with poor prognosis in brain, breast, ovary, cervix, colorectal, prostate, bladder, and oropharynx cancers (144, 145). Particularly, HIF-1α has been reported to be hyperactivated in TNBCs and necessary for the

maintenance of the CD44^{high}CD24^{low} cell population. Chen *et al.* identified XBP1, a component of the unfolded protein response (UPR) pathway, as a major controller of HIF-1α transcriptional activity in TNBCs. It is required for tumor relapse in a murine model and directly enriches the CD44^{high}CD24^{low} population *in vitro.* XBP1 can also be associated with poor prognosis, suggesting that combinatory therapy using stem cell targeting drugs, such as inhibitors of the UPR pathway and standard chemotherapy may improve cancer therapeutic intervention (146).

A tight relationship exists between hypoxia and tumor dissemination. Low oxygen levels in a tumor microenvironment promotes the overexpression of EMT master regulators such as SNAIL, TWIST, and ZEB1, while it attenuates E-cadherin expression. Matrix remodeling requires basal membrane degradation *via* HIF-1α-dependent production of MMP2 and cathepsin D (CTSD). The so-called "invasive–switch" is guided by hypoxia and sustained by MET and lysyl oxidase (LOX) expression. Hypoxia facilitates both intravasation and extravasation of tumor cells through the increased production of VEGFA. Meanwhile, CXCR4, OPN, and Angiopoietin-like 4 (ANGPTL4) increase the chance of homing and outgrowth to secondary organs (147).

HIF-2α also contributes to the hypoxia-driven "angiogenic-switch" and is directly linked to stem cell biology as a regulator of *OCT4* (148) and *c-MYC* (149). Given that it displays a restricted tissue-specific expression pattern compared to its homologs, little attention has been given to addressing its pro angiogenic and pro tumorigenic features (149). One key study showed the preferential expression of HIF-2α on GSCs compared to the differentiated and normal counterpart and its association with poor survival in glioblastoma patients. Authors underlined that HIF-2α may support the CSCs niche by providing survival and metabolic advantages through the modulation of *OCT4*, *GLUT1*, and *SERPINB9* expression. This suggests that new therapeutic approaches should be aimed at targeting stem cell specific molecules involved in neoangiogenesis (150)

On the contrary, besides being a member of the HIF system, HIF-3α's role in the tumor hypoxia-inducible adaptive response system, is not well characterized. Indeed, it lacks the transactivation domain and likely functions as a negative regulator of HIF-1α and HIF-2α due to sequestration of HIF-1β (151).

As previously discussed, preclinical data provide evidence that hypoxic tumor cells play a pivotal role in tumor progression and resistance to therapies. Moreover, the pro metastatic effect elicited by angiogenesis-induced hypoxia can compromise clinical outcomes in patients. Thus, targeting intratumoral hypoxia, can be considered the gold standard to be exploited in neoplastic malignancy. Nevertheless, it is clear that hypoxia is heterogeneously diffused within a given tumor cell population and is

endowed with an even more differentiated extension among patient tumors. Based on this observation, an appropriate measuring of tumor hypoxia either by direct or indirect methods, will facilitate the selection of the patient's treatment as well as, the monitoring of their treatment-response (152). However, an interesting finding recently reported for the first time is that, a chemotherapeutic agent, in this case doxorubicin, can stabilize HIF-1α even in normoxic cells. Indeed, doxorubicin increased the expression of STAT1, with consequent stimulation of iNOS, intracellular synthesis of NO and HIF-1α accumulation (153).

In recent years, several drugs have been designed to selectively target chemo- and radio-resistant hypoxic cancer cells. According to the action mechanism, they could be tentatively categorized as *a)* agents targeting HIF-1α DNA binding, *b)* agents attenuating HIF-1α protein translation, *c)* agents inducing HIF-1α protein degradation, *d)* prodrugs inducing hypoxia-mediated cytotoxicity *e)* HRE-driven expression of enzymes converting prodrugs and *f)* agents targeting downstream HIF pathway effectors.

Specifically, HIF-1α function can be directly targeted *via* chetomin, a small molecule that precludes HIF-1α binding to the transcriptional coactivator p300/CBP (154). Similarly, the proteasome inhibitor bortezomib, which has been approved by the FDA for clinical use in multiple myeloma and mantle cell lymphoma patients refractory to at least one prior therapy, affects the C-terminal activation domain (CAD) of HIF-1α. It was shown that bortezomib enhanced the HIF-1α hydroxylation of Asn803 residue, by the dioxygenase factor-inhibiting hypoxia 1 (FIH-1), causing the inhibition of p300-HIF interaction (155). Intriguingly, anthracyclines, such as doxorubicin and daunorubicin, block HIF-1 binding to HRE sequence, providing new evidence in refining their use as antiangiogenic drugs (156).

HIF-1α expression can be modulated by the topoisomerase I inhibitor topotecan, one of the first hypoxia inhibitor ever tested on humans and currently approved for the treatment of small cell lung cancer and recurrent cervix carcinoma. Cardiac glycoside digoxin inhibited the translation of HIF-1α in an mTOR-independent manner. In preclinical settings, PX-478 appeared to inhibit HIF-1α mRNA expression and translation, and foster HIF-1α degradation by preventing its deubiquitination (157). Contrasting data have been generated regarding the contribution of the mTOR pathway in the modulation of hypoxia. Besides several mTOR inhibitors, such as everolimus and temsirolimus, which have been approved by the FDA for clinical use in renal cancer patients and displayed remarkable antiangiogenic activity and inhibition of HIF-1α (158). Hypoxia, especially in early stage tumors, may negatively regulate HIF-1α expression according to the intensity and duration of oxygen deprivation (159). Another indirect mechanism of HIF-1α inhibition includes the

targeting of upstream pathways (e.g. PI3K-AKT and RAS-MAPK) involved in HIF-1α protein translation (160). Interestingly, the tumor suppressor p53 mediates apoptosis under hypoxic conditions. However, cancer cells with dysregulated p53, escape programmed death and p53-mediated HIF-1α inhibition (161). p53 may either interact with HIF-1α and mediate its degradation through HDM2 (161) or compete with HIF-1α for p300 thus, blocking its transcriptional activity (162). Agents targeting p53, aim at reactivating mutant p53. This is the case of RITA (reactivation of p53 and induction of cell apoptosis), which induces DNA damage in order to stimulate p53-evoked cell apoptosis and inhibits MDM2 to prevent p53 degradation. This mechanism seems to be hypoxia-independent (163).

HIF-1α degradation may be forced by the inhibition of chaperone HSP90. In both normoxia and hypoxia, the HSP90 antagonists GA and 17-AAG mediate elimination of HIF-1α through E3 ubiquitin ligase and reduces angiogenesis *in vivo* (164). Trichostatin A is an inhibitor of HDAC and promotes a proteasome-dependent HIF-1α degradation in osteosarcoma (165). Similarly, HDAC inhibitors FK228 and LAQ824 resulted in the abrogation of HIF-1α activity (166, 167). Of note, SAHA, the potent pan HDAC inhibitor, may act together with TRAIL, in breast cancer orthotopic models and downregulate both VEGF and HIF-1α (168).

One promising approach seeks to develop prodrugs that can be reduced by hypoxia in prodrug radicals, as intermediate products. In normoxia, they can be re-oxidized and converted back by oxygen, while in hypoxic cells they can be either further reduced or fragmented so as to generate an active toxic drug. Examples of bioreactive prodrugs still in clinical development, include RH-1, mitomycin C, AQ4N, PR-104, and SR4233. Some concerns have been reported regarding the prodrugs' penetration into poorly perfused tumors and their toxicity. The activation of aerobic reductase also in normal tissues or the additional generation of DNA reactive cytotoxins, make it hard to combine bioreductive prodrugs with standard chemotherapy (152).

Moreover, in tumoral cells prodrugs can be converted into cytotoxins by an hypoxia-regulated expression vector which, encodes the enzyme responsible for this reaction. Hypoxia targeted gene therapy has been tested in a preclinical setting and consists in the expression, in tumoral cells, of plasmid vector carrying genes driven by a promoter containing HRE and encoding: thymidine kinase (TK), cytosine deaminase (CD), uracil phosphoribosyltransferase (UPRT), and flavoprotein cytochrome c P450 reductase (CPR) (169, 170). A triple suicide gene therapy has proven to enhance cytotoxicity to ganciclovir and 5 fluorocytosine and sensitize colorectal cancer cells, both *in vivo* and *in vitro*, to radiotherapy by simultaneous expression of TK, CD and UPRT (170).

Finally, multiple agents also aim at targeting the downstream component of the HIF signaling pathway such as the LOX inhibitors, β-aminoproprionitrile (βAPN) or anti-LOX antibody, which binds the LOX active site and blocks its enzymatic function (171)

4.5 Challenges and limitations of targeting cancer stem cells and their niche

Conventional anti-cancer drug development has been focused on the identification of cytotoxic chemotherapeutic agents that can target deregulated pathways and molecular markers in tumor cells. Despite all efforts, patients undergoing chemotherapy, after an apparent remission, often relapse and develop more aggressive diseases. This emphasizes that CSCs may be responsible for therapy failure due to the specific activated mechanisms which are peculiar to the undifferentitated status of these cells. In this context, novel compounds have been precisely designed to eliminate CSCs or affect their microenvironment and, administered in concert with conventional chemotherapy, can lead to tumor bulk shrinkage and ablate resistance and relapse. Of note, there is a need to refine such therapies to counteract their side effects. Indeed, such approaches could impair normal stem cell niches, have 'off target' effects on signals required for normal cells survival or, and as well as standard treatments, they should be administered at concentrations harmless to patients.

4.6 Conclusion and future perspectives

The reviewed data show only a partial portion of the existing therapies in the field. Anyhow, they seek to emphasize that despite of the efforts that have been made to develop powerful CSCs targeted therapy, multiple obstacles still need to be faced for the achievement of long lasting clinical benefits. The future use of appropriate tumor models and technologies reflecting the phenotypic, genetic and epigenetic tumor heterogeneity constantly evolving to counteract the hostile milieu, will possibly overcome the achieved disappointing results. Moreover, a multitude of new inhibitors are currently being investigated and will possibly conduct to some encouraging experimental evidence.

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Acknowledgments

It is said that if you know your enemies and know yourself, you will not be imperiled in a hundred battles; if you do not know your enemies but do know yourself, you will win and lose one; if you do not know your enemies nor yourself, you will be imperiled in every single battle (Sun Tzu). This is what I learned at the beginning of my adventure in the field of Cancer Research and what will accompany me probably forever. Every detail is important and can make the difference. Only curiosity, passion and meticulous study will not overlook any little feature that makes the cancer cells able to escape and resist to therapies.

This is the reason why I am really thankful to the Stassi's Lab that introduced me to Cancer Research and that has made possible this thesis: Tiziana, Miriam, Virginia, Antonella, Mary, Emanuela, 'the two Alessandros', Marco, Rosachiara, Laura, Tatiana, Cecilia, Annalisa, Veronica, Simone, Francesco and Giovanni. I am grateful to the Lab Heads Matilde Todaro and Giorgio Stassi who first give an example of sacrifice and dedication to Research. Every day they carry out arduous tasks with passion and firmness.

I thank the Prof. Dieli, coordinator of the International PhD program in Immunopharmacology.

A huge thanks goes out to Prof. Max Wicha and his Laboratory at the University of Michigan for welcoming me and giving me the opportunity of a unique experience, in particular: Ebrahim, Shamileh, Yad, April, Khaled, Hasan, Li, Maria, Yongyou, Ming, Leo, Sunitah, Tahra, Shawn and the flow core. My months in Ann Arbor would not have been the same without Bonnie, a colleague and a friend with whom I spent unforgettable days.

I deeply thank my grandpa Pino for conveying to me the passion for traveling and the curiosity to know more and more about people in the world with different culture.

Thank you also to my volleyball team 'Primula', for the joyful moments while playing and for the happiness during celebrations of our life events.

Thank you to my friends Roberta, Simona, Valeria, Teresa and Giusy for having grown up together and supporting me over the years.

I am grateful to all my relatives and especially to the 'Intravaia crew', 9 cousins who never get bored together.

Thank you Massimiliano for your foresight, which inspired me in any choices of my life, faith and patience.

I am so incredibly thankful to my parents. Thank you for always suggesting to me, Martina and Emanuela (two amazing sisters) to make our decisions solely in accordance with our passions.

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Participation to international meetings

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Books, papers and abstracts published during the PhD course

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Papers

- Breast cancer metastasis via DNp63 expression in cancer stem cells.
  Di Franco S., Benfante A., Colorito M.L., Bonanno M., Gaggianesi M., **Turdo A.**, Barcaroli D., Dieli F., Medema J.P., De Laurenzi V., Stassi G., Todaro M.
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- miR-205-5p mediated down-regulation of ErbB/HER receptors in Breast Cancer Stem Cells results in targeted therapy resistance.
  De Cola A., Volpe S., Budani M. C., Ferracin M., Lattanzio R., Barcaroli D.,**Turdo A.**, Stassi G., Todaro M., Di Ilio C., Sala G., Piantelli M., Negrini M.,Veronese A., and De Laurenzi V.
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Abstracts

- Sam68 sustains self-renewal and invasiveness of breast cancer initiating cells.
  Turdo A., Gaggianesi M., Benfante A., Piantelli M., Todaro M., Stassi G.
  Poster presentation. AACR, San Diego, USA 2014.
- Annexin A3 is selectively expressed in MET-like as compared to EMT-like breast cancer stem cells.
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- Role of Sam68 in modulating the tumorigenic and metastatic potential of breast Cancer Stem Cells.
  Turdo A., Gaggianesi M., Bonanno M., Todaro M., Stassi G.
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- p63 in breast cancer stem cell: a possible key role in metastagenic potential.

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