

Review

# The Tumor Microenvironment as a Driving Force of Breast Cancer Stem Cell Plasticity

Flavia Fico  and Albert Santamaria-Martínez \* 

Swiss Institute for Experimental Cancer Research, École Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland; flaviafc@hotmail.it

\* Correspondence: albert.santamariamartinez@epfl.ch

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**Simple Summary:** Breast cancer stem cells are a subset of transformed cells that sustain tumor growth and can metastasize to secondary organs. Since metastasis accounts for most cancer deaths, it is of paramount importance to understand the cellular and molecular mechanisms that regulate this subgroup of cells. The tumor microenvironment (TME) is the habitat in which transformed cells evolve, and it is composed by many different cell types and the extracellular matrix (ECM). A body of evidence strongly indicates that microenvironmental cues modulate stemness in breast cancer, and that the coevolution of the TME and cancer stem cells determine the fate of breast tumors. In this review, we summarize the studies providing links between the TME and the breast cancer stem cell phenotype and we discuss their specific interactions with immune cell subsets, stromal cells, and the ECM.

**Abstract:** Tumor progression involves the co-evolution of transformed cells and the milieu in which they live and expand. Breast cancer stem cells (BCSCs) are a specialized subset of cells that sustain tumor growth and drive metastatic colonization. However, the cellular hierarchy in breast tumors is rather plastic, and the capacity to transition from one cell state to another depends not only on the intrinsic properties of transformed cells, but also on the interplay with their niches. It has become evident that the tumor microenvironment (TME) is a major player in regulating the BCSC phenotype and metastasis. The complexity of the TME is reflected in its number of players and in the interactions that they establish with each other. Multiple types of immune cells, stromal cells, and the extracellular matrix (ECM) form an intricate communication network with cancer cells, exert a highly selective pressure on the tumor, and provide supportive niches for BCSC expansion. A better understanding of the mechanisms regulating these interactions is crucial to develop strategies aimed at interfering with key BCSC niche factors, which may help reducing tumor heterogeneity and impair metastasis.

**Keywords:** tumor microenvironment; cancer stem cells; breast cancer; extracellular matrix; immune cells; stromal cells

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## 1. Introduction

The incidence of breast cancer (BC) has been raising over the last years, with an annual increase of 3.1% and significant variations between countries: North America, Australia, New Zealand, and Northern and Western Europe show the highest incidence compared to lower income regions, with a distribution pattern that reflects the availability of detection methods and the risk factors associated to the disease [1]. Conversely, BC mortality rate is higher in low-income countries due to the limited access to treatments and the late stage at diagnosis [2]. At present, patients with early-stage tumors, which account for 70–80% of the total cases, are successfully treated. Advanced metastatic BC (20–30% of the cases) remains incurable, and the available treatments are meant to slow its progression

and to relieve the symptoms derived from the disease [3]. Overall, the 5-year survival rate is around 99% for localized BC, 86% for regional disease, and 27% for metastatic BC (BC stage IV) [4]. Around 5–10% of BC cases are hereditary, 85% of which are related to mutations in the two high penetrance tumor suppressor genes *BRCA1* and *BRCA2* [5], whereas the majority of breast tumors are sporadic and due to somatic, genetic, and epigenetic alterations acquired during life [6]. Non-hereditary risk factors include age, body mass index, sex, density of the breast, early menarche, age at first birth, late menopause, alcohol consumption, exposure to radiation, and smoking habits.

The evolutionary dynamics in some tumor types, such as BC, are subject to hierarchical systems in which cancer stem cells (CSCs) sustain tumor growth and can colonize secondary organs. Typically, BC stem cells (BCSCs) are identified and isolated by fluorescence-activated cell sorting (FACS); the most common isolation strategies for human BC cells are the antibody staining of the cell surface markers CD24 and CD44, and the evaluation of aldehyde dehydrogenase (ALDH) activity [7], with both the  $\text{Lin}^- \text{CD24}^- / \text{low} \text{CD44}^+ / \text{high}$  and the  $\text{ALDH}^{\text{high}}$  populations in breast tumors being enriched for CSCs. Tumor initiation potential—BCSC's most distinctive feature—is tested in limiting dilution assays (primary tumors) or in metastasis assays (secondary tumors) *in vivo*. Moreover, the capacity to form spheres *in vitro* is generally used as a surrogate marker for CSC. The importance of CSC biology to the clinic is emphasized by numerous studies showing the essential role of these particular cells in metastasis, chemoresistance, and tumor relapse [8]. In the classical CSC model, a subset of cancer cells with stem cell properties can self-renew and also give rise to lineages with various degrees of differentiation. Conventionally, differentiated cells cannot revert to CSCs, since this capacity is exclusive of pluripotent cells. However, tumors are heterogeneous dynamic systems in constant evolution, and their progression is determined by the interactions between cancerous cells and the tumor microenvironment (TME), which is composed of other non-transformed cell types, such as stromal and immune cells, and the extracellular matrix (ECM). In recent years, in line with the plastic nature of tumors, the idea of a unidirectional system has been challenged by results showing that the network is strongly dependent on microenvironmental signals and on the interplay between different cell types in the tumor [8]. Thus, the complexity of the evolution of CSC-driven tumors is greater than previously thought, since in the new CSC model, non-CSCs can reacquire the CSC phenotype and CSCs can become non-CSCs depending on signals from the niche. In this manuscript, we will review the current knowledge on how components of the TME regulate the BCSC phenotype and plasticity in the BCSC niche.

## 2. Phenotypic Heterogeneity and Breast Cancer Molecular Subtypes

BC is a heterogeneous disease, and this heterogeneity is reflected in the CSC phenotypic and quantitative differences that are found between BC subtypes [9]. Likewise, it has been suggested that poorly differentiated tumors contain higher numbers of CSCs [10]. The classification of BC was originally based on histological features such as type, grade, and stage of the tumor, but the advent of microarray technology allowed researchers to classify breast tumors according to their molecular profiles [11]. Gene expression analysis led to the identification of five different subtypes of BC: luminal-like (comprising luminal subtypes A and B), HER2+, basal, and normal-like tumors. In addition, a sixth subtype was identified years later and named claudin-low, denoting the low expression of genes related to tight junctions and to cell-cell adhesion found in this group of tumors [12]. In the clinic, diagnosis and treatment decisions are still based on the immunohistochemistry markers estrogen receptor (ESR1), progesterone receptor (PGR), epidermal growth factor receptor 2 (ERBB2, also known as HER2), and the proliferation marker Ki67. However, the molecular features of tumors are known to determine their prognosis and response to therapy, and distinct gene signatures are currently used in genomic tests [13]. Luminal A tumors respond well to hormonal anti-estrogen therapy, but poorly to chemotherapy. Luminal B tumors are of a higher grade, have worse prognosis, and may benefit from combined therapies. In keeping with their more differentiated status, luminal-like tumors typically present an epithelial phenotype and contain low percentages of both  $\text{CD44}^+ / \text{CD24}^-$  CSCs

and ALDH1<sup>+</sup> CSCs. The latter represent a small fraction of CD44<sup>+</sup>/CD24<sup>-</sup> cells and are therefore considered to be enriched for a different population of CSCs [14]. Accordingly, luminal A tumors have the lowest rates of metastatic spread compared to all the other subtypes [15–17]. Phenotypically, HER2<sup>+</sup> tumors are also mainly epithelial, but they contain higher frequencies of ALDH1<sup>+</sup> BCSCs [18]. Basal-like, and particularly claudin-low tumors, are highly enriched in both CD44<sup>+</sup>/CD24<sup>-</sup> cells and ALDH1<sup>+</sup> cells [19,20]. In addition, claudin-low tumors display epithelial-to-mesenchymal transition (EMT) signatures and a mesenchymal phenotype, and are associated with poor prognosis.

BCSC plasticity is exemplified by the capacity to bidirectionally switch between epithelial and mesenchymal states [21]. This conversion is achieved through the EMT, which is a bidirectional, reversible process. For instance, upon metastatic colonization, CSCs undergo mesenchymal-to-epithelial transition (MET) in order to regain epithelial features and higher proliferative rates [22]. EMT is now widely regarded as a key mechanism that produces cancer cells with stem cell traits and enhanced metastatic ability [23], although the degree of complexity of this process is higher than originally thought [24–28]. In addition, it is worth noting that epithelial-like CSCs and mesenchymal-like CSCs may possess different abilities to initiate tumors in the primary or the secondary site [29], and further data indicate that the expression of EMT-related genes does not guarantee an enrichment in tumor-initiating cells [30].

### 3. The Tumor Microenvironment and the Breast Cancer Stem Cell Phenotype

The phenotypic plasticity of breast tumors is tightly regulated by cues from the TME, a complex milieu where transformed cells evolve among a myriad of signals that impact their behavior and ultimately their ecological fitness. It consists of transformed cells and the surrounding normal tissue, as well as immune cells, cancer-associated fibroblasts (CAFs), endothelial cells, and the ECM [31]. As tumors evolve, the role of each component of the TME changes, sometimes with opposing effects. These alterations are often the consequence of an adaptive process, during which cancer cells take advantage of and hijack certain elements of the TME in order to grow.

#### 3.1. Myeloid Cells

Myeloid cells arise from a common hematopoietic progenitor and comprise monocytes and macrophages, myeloid dendritic cells, neutrophils, eosinophils, basophils, mast cells, erythrocytes, and megakaryocytes (which give rise to platelets). A number of groups have shown that myeloid cells play an important role in regulating the BCSC phenotype. For instance, myeloid-derived suppressor cells, a heterogeneous group of immune cells with immune inhibitory functions, sustain BCSCs through the secretion of interleukin-6 (IL-6) and nitric oxide (NO), and activation of the STAT3 and Notch pathways [32]. However, most studies have focused on the role of tumor-associated macrophages (TAMs), since it is one of the most abundant immune cell populations in breast tumors. The importance of this subset of cells in BC progression is underlined by a number of studies showing that the degree of TAM infiltration in mammary tumors is strongly associated with tumor progression, overall survival, and relapse-free survival [33–42].

##### 3.1.1. Macrophages

Macrophages are classified into non-activated, classically activated (M1), and alternatively activated (M2) macrophages. M1 macrophages are proinflammatory, while M2 macrophages, which have been subclassified into different subgroups depending on the activation mechanism (M2a, M2b, M2c, and M2d), promote tumor progression and angiogenesis, and possess immunosuppressive features [43]. However, it is worth noting that current data suggest that this classification may not reflect the biological heterogeneity of macrophages, which defies the classical M1-M2 binary classification [44].

The role of macrophages in the mammary gland stem cell niche was first suggested by experiments showing that their ablation led to a significant decrease in the repopulating ability of mammary gland stem cells (MaSCs) [45]. Further work has shown that there is a cross-talk between macrophages and

MaSCs, in which the MaSCs-dependent activation of Notch signaling in macrophages triggers the expression of Wnt ligands that in turn act on MaSCs [46]. In BC, the depletion of macrophages was also shown to reduce side population (SP) CSCs and to decrease tumorigenicity and metastasis in a mouse model of mammary gland tumorigenesis [47]. The authors found that TAM-secreted epidermal growth factor (EGF) activated the expression of the BCSC promoting factor Sox2 through a STAT3-dependent mechanism, driving CSC expansion [47,48]. Consistent with the results showing that macrophage ablation leads to CSC depletion, mixing TAMs with CSCs increased tumor initiation, sphere formation, the metastatic index, and tumor-free survival, which further supports the role of macrophages in CSC biology [49]. The coculture of monocytes and CD90<sup>hi</sup>CD24<sup>-</sup> HMLE-Ras (HMLER90hi) CSCs resulted in a rapid induction of IL-6 and IL-8, which promoted CSC self-renewal in both an autocrine and a paracrine manner [49–51]. It is worth noting that cancer cells can express factors such as multiple copies in T-cell malignancy 1 (MCT-1) or yes-associated protein 1 (YAP), which activate IL-6 expression, thus promoting CSC expansion and increasing their frequency independently of macrophages [52,53]. Likewise, a recent study reported that while the coculture of M2-polarised THP-1 macrophages with MCF-7 cells stimulated the transition to a CSC state (as defined by higher expression of CD44), the addition of recombinant IL-6 did not affect the proportion of these cells [54]. These results reinforce the notion that macrophages are strong modulators of CSC plasticity, while suggesting that the role of IL-6 might be context-dependent. C-X-C motif chemokine ligand 1 (CXCL1) is a chemokine secreted by macrophages whose expression in the tumor stroma is associated with poor prognosis and BC metastasis [55,56]. Interestingly, pharmacological inhibition of TAM-derived CXCL1 suppresses BCSCs [57]. Two complementary studies indicate that other cytokines such as interleukin-1beta (IL-1 $\beta$ ) may be involved in the cross-talk between macrophages and BCSCs. For instance, *Trp53*<sup>-/-</sup> cancer cells secrete Wnt ligands that stimulate IL-1 $\beta$  production by macrophages, which in turn activates an inflammatory program that leads to metastasis [58]. Interestingly, IL-1 $\beta$  also triggers Wnt signaling in tumor cells, thus enhancing BCSC features and promoting metastatic colonization to the bone [59]. It is therefore likely that macrophages can promote cancer stemness and metastasis also through the secretion of IL-1 $\beta$ . TAMs may also potentiate BC stemness via cell-cell interactions with CSCs: they express LSECtin, a transmembrane protein that interacts with the butyrophilin family molecule BTN3A3 in tumor cells, enhancing their stemness features both in vitro and in vivo [60].

While metastatic dissemination can be an early event in the pathology of BC [61,62], cancer cell dormancy, a process at least partly controlled by TME signals, may prevent the development of clinically detectable metastases for years or decades [63]. Dormant cells have been shown to share some features with CSCs [64]. Interestingly, M2 macrophages can secrete exosomes that maintain the dormancy of CSC-like BC cells, while M1 macrophages trigger the cell cycle via NF- $\kappa$ B [65]. Indeed, mammary epithelial cell NF- $\kappa$ B has also been reported to maintain stemness and to control macrophage recruitment in murine MMTV-ErbB2 tumors [66].

The effects of TAMs on BCSCs can also be indirect. For instance, hyaluronan synthase 2 (HAS2), an enzyme involved in hyaluronan polymerization in the ECM, is highly expressed by CSCs isolated from human BC cell lines [67]. The binding of hyaluronan to CD44 expressed on TAMs triggers the secretion of platelet-derived growth factor-BB (PDGF-BB), which in turn induces the expression of fibroblast growth factor 7 and 9 (FGF7 and FGF9) in stromal cells, and ultimately stimulates the proliferation of CSCs [67]. Similar to what happens with IL-6, HAS2 overproduction by tumor cells has also been shown to induce BC stemness independently of macrophages through the induction of a transforming growth factor-beta (TGF- $\beta$ )/Twist-driven EMT program [68]. The interplay between macrophages and CSCs may also change due to microenvironmental conditions. For instance, tumor hypoxia is known to upregulate genes such as IL-6 in macrophages, which, as we have seen, regulates BCSCs [69].

Through the secretion of EMT-inducing factors, such as IL-6, TAMs can play an important role in EMT [70], a process which is thought to contribute to the generation of CSCs [23]. For instance, mesenchymal-like BC cells secrete a granulocyte-macrophage colony-stimulating factor (GM-CSF),

which stimulates the secretion of C-C motif chemokine ligand 18 (CCL18) in macrophages, promoting EMT and metastasis [71]. Furthermore, experimentally induced EMT triggers the expression of THY1 (CD90) in HMLE cells, and the CD90<sup>high</sup> population is enriched in stem-like cells [49]. The interactions between macrophages and CSCs mediated through THY1 and EPH receptor A4 (EPHA4) activate NF- $\kappa$ B and sustain cancer stemness [49]. Likewise, the coculture of MCF-7 (luminal A), MDA-MB-231 (TNBC), or T47D (luminal A) cells with M1-polarized THP-1 or peripheral blood mononuclear cell (PBMC)-derived macrophages also promotes the CSC phenotype in a paracrine manner and triggers EMT [72]. The authors found IL-6, together with tumor necrosis factor-alpha (TNF- $\alpha$ ) and IL-1 $\beta$ , to be involved in the activation of the EMT program through STAT3/NF- $\kappa$ B, transcriptional upregulation of Lin28B/HMGA2, and downregulation of let-7. Interestingly, this study found M1 macrophages to be stronger inducers of the CSC phenotype than M2 macrophages, and suggests that, as proinflammatory M1 macrophages transdifferentiate into M2 macrophages during tumor progression, the CSC phenotype is maintained—but not expanded [72]. Macrophages have also been involved in the reversibility of the EMT process during the last steps of the metastatic cascade. Indeed, macrophages in the primary and the secondary site are known to possess different traits [73,74]. It was recently shown that in the secondary site, macrophages promote metastatic colonization [75], a process that we previously showed to be led by CSCs [76]. The authors found that whereas in the primary site TAMs mediate EMT through the secretion of TNF- $\alpha$ , in the secondary site metastatic TAMs secrete interleukin-35 (IL-35), which drives MET through the JAK-STAT6-mediated transcription of GATA binding protein 3 (GATA3) [75], a transcription factor known to inhibit metastasis and EMT [77,78].

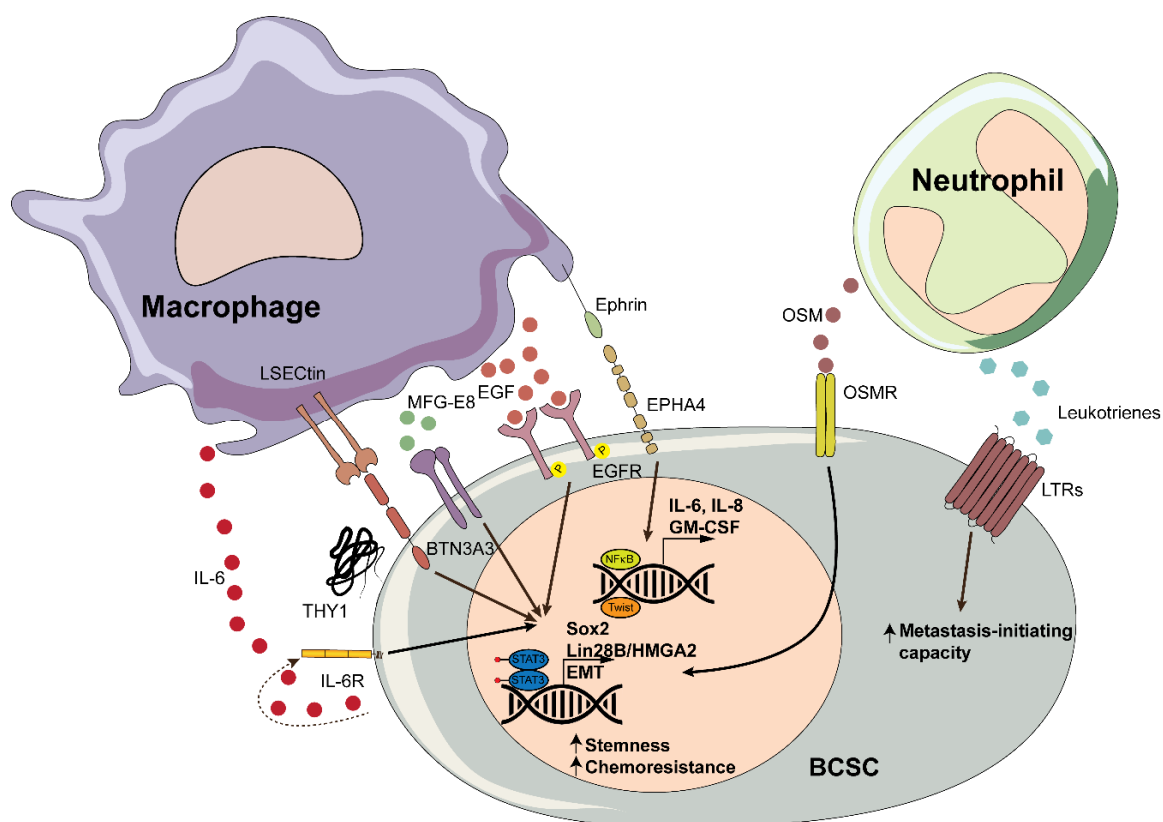
Myeloid cells can also induce other CSC hallmarks, including chemoresistance. For instance, TAMs and other myeloid cells, such as dendritic cells, secrete milk-fat globule EGF-8 (MFG-E8), which promotes specific CSC chemoresistance in BC cells [79]. In a somewhat reciprocal manner, CSCs isolated from chemoresistant MDA-MB-231-derived cells express interferon regulatory factor 5 (IRF5) and M-CSF, which polarize macrophages to an M2 phenotype and help them acquire tumor-promoting features [80]. Moreover, macrophages can induce the expression of multidrug resistance transporters such as ABCG2 (also known as the breast cancer resistance protein 1, BCRP1) in BCSCs [47], thus potentially enhancing their chemoresistance [81,82].

In summary, macrophages may promote the CSC phenotype in breast tumors through different mechanisms (Figure 1), including secretion of chemokines, macrophage-BCSC interactions, and involvement of other cell types such as stromal cells. In addition, proangiogenic macrophages contribute to aberrant tumor vasculature, which typically leads to hypoxia, a strong modulator of the BCSC phenotype [83–86].

### 3.1.2. Neutrophils

Neutrophils account for most of the granulocytes. They are a phenotypically heterogeneous population that plays a role during infection, chronic disease, and cancer. Neutrophils have also been linked to the CSC phenotype in BC. Their prognostic value is evaluated using the neutrophil-to-lymphocyte ratio (NLR): a high NLR is associated with worse disease-free survival and overall survival [87]. Similar to what happens with TAMs, tumor-associated neutrophils (TANs) secrete cytokines that modulate the plasticity of cancer cells. For instance, BC cells induce the production of oncostatin M by TANs [88], which in turn promotes EMT and the BCSC phenotype [89]. TANs also promote EMT and metastasis by secreting the cytokine tissue inhibitor matrix metalloproteinase 1 (TIMP-1) [90]. Similar to the positive feedback loops observed between TAMs and BC cells, the upregulation of THY1 in BC cells undergoing EMT boosts the secretion of TIMP1 by neutrophils. In addition, a different study found that neutrophil-secreted leukotrienes increased the frequency of metastasis-initiating cells, and that the pharmacological or genetic blockade of leukotriene synthesis reduced the metastatic colonization potential in models of mammary gland tumorigenesis [91]. The authors also observed that leukotriene receptors were expressed at higher levels in metastasis-initiating cells.

Overall, the secretion of cytokines and leukotrienes by TANs promotes the BCSC phenotype and the generation of metastasis-initiating cells in BC (Figure 1).



**Figure 1.** Schematic representation of interactions between myeloid cells and breast cancer stem cells (BCSCs).

### 3.2. T Cells

T lymphocytes are a main component of the adaptive immune system and are usually classified into cytotoxic CD8<sup>+</sup> T cells, helper CD4<sup>+</sup> T cells, regulatory CD4<sup>+</sup> T cells, and  $\gamma\delta$  T cells. Cytotoxic CD8<sup>+</sup> T cells account for most of the tumor infiltrating lymphocytes (TILs), and they typically play a key role in anti-tumor immunity [92]. Indeed, high CD8<sup>+</sup> infiltration correlates with good prognosis in BC [93–95]. However, as cancer progresses, T cells fail to eradicate the tumor, and other components of the immune system such as myeloid cells are reeducated to promote tumor growth. The dual role of the immune system in promoting or suppressing tumor growth is explained by the immunoeediting theory, which consists of three phases: elimination, equilibrium, and escape [96]. Although little is known about the particular immunological properties of BCSCs, they may play an important role in tumor immune escape [97]. For instance, BCSCs are known to secrete higher levels of TGF- $\beta$  [98], a strong suppressor of T cell cytotoxicity [99]. They can also establish immunological tolerance due to the following mechanisms: decreased expression of tumor antigens, downregulation of major histocompatibility complex class-I (MHC I), and inhibition of antigen-presenting cells (APCs) [100]. It is known that cell-cell interactions between BCSCs and CD8<sup>+</sup> T lymphocytes can impact the killing ability of the latter. One of the main reported immunosuppressive feature of BCSCs consists in programmed death-ligand 1 (PD-L1) expression [101,102], a T-cell inhibitory molecule that binds the cell surface protein PD-1 on effector T cells, causing their exhaustion [103]. Interestingly, human TNBCs expressing high levels of PD-L1 show higher expression of stemness markers, increased capability to form mammospheres, and more tumorigenic potential compared to PD-L1<sup>low</sup> TNBCs [104]. Additionally, PD-L1<sup>high</sup> cells were found to be in close contact with infiltrating T lymphocytes, suggesting that they

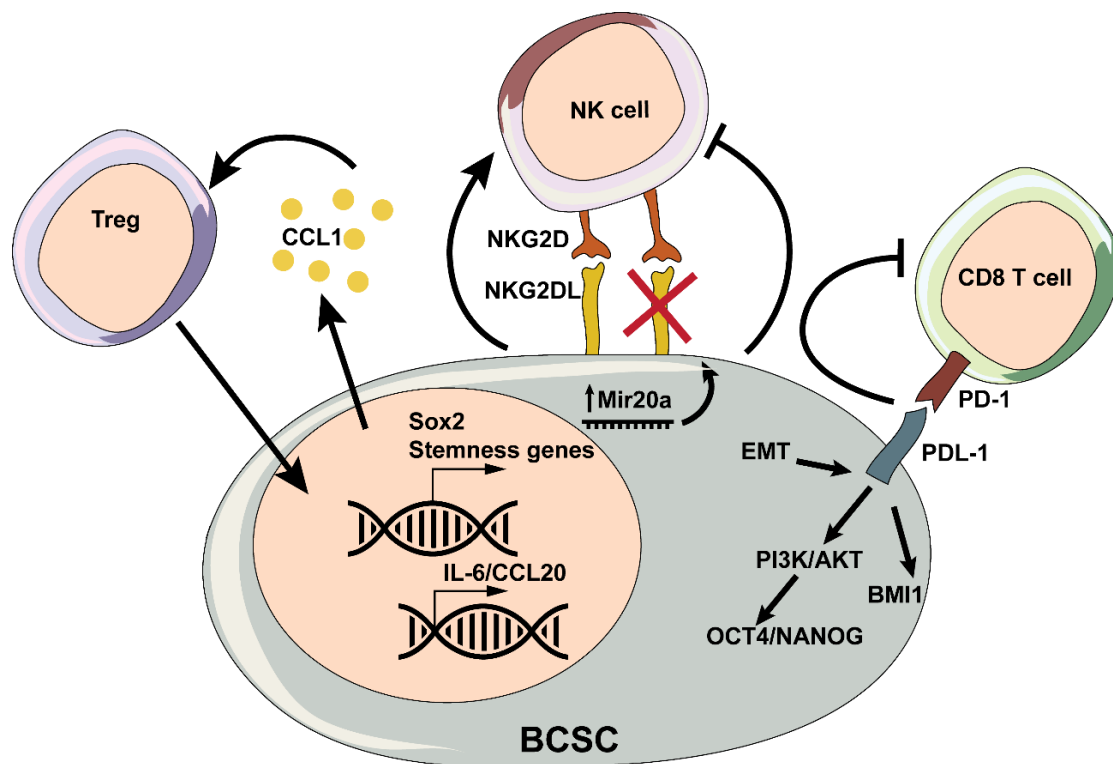
might be able to evade T cell-mediated anti-tumor immunity [104]. Furthermore, Alsuliman et al. reported that PD-L1 expression is induced upon EMT in BC, especially in the claudin-low subtype [105]. Likewise, PD-L1 is important for the expression of the stemness markers OCT4 and Nanog, likely via PI3K/AKT, and concomitantly sustains the expression of the stemness-controlling factor BMI1, independently from the AKT signaling pathway [106]. BCSC activities can in turn be modulated by the T cell compartment. For instance, cognate interactions between killing-deficient CD8<sup>+</sup> cells and MCF-7 BC cells were found to increase the CD44<sup>high</sup>CD24<sup>low</sup> stem-like cell population [107]. Moreover, when injected in vivo, the immunologically challenged tumor cells showed a higher capability to form tumors and increased metastatic potential [107].

The pro- or anti-tumor functions of CD4<sup>+</sup> T lymphocytes largely depend on their polarization towards CD4<sup>+</sup> T helper (Th1, Th2, Th17) or CD4<sup>+</sup> regulatory T cells (T<sub>reg</sub>). Not much is known about the role of CD4<sup>+</sup> T cells on BCSCs, but an immunohistochemical analysis of 47 tissue samples from BC patients revealed that CD4<sup>+</sup> T cells were positively associated to the presence of CD44<sup>+</sup>CD24<sup>-</sup> BCSCs [108].

Immunosuppressive CD4<sup>+</sup> T<sub>reg</sub> lymphocytes play a role in inhibiting several immune cell types, including CD8<sup>+</sup> and some CD4<sup>+</sup> subsets [109]. Contrary to what happens with CD8<sup>+</sup> T cells, high T<sub>reg</sub> infiltration in breast tumors correlates with worse outcomes [110]. While T<sub>regs</sub> have been recently shown to regulate CSCs in a number of tumor types [111], little is known regarding their role in BC stemness. Nevertheless, T<sub>regs</sub> have at least been shown to increase the SP and the ALDH<sup>high</sup> fraction of 4T1 and EO771 BC cells, their capacity to form mammospheres, and their tumorigenic and metastatic potential [112]. Moreover, the overexpression of the stemness factor SOX2 in the tumor cells leads to the recruitment of T<sub>regs</sub> through CCL1, thus highlighting the bidirectional communication between T<sub>regs</sub> and BCSCs [112]. However, a recent work showed that the depletion of T<sub>regs</sub> in early stage lesions in the MMTV-PyMT model led to an unexpected expansion of CD24<sup>-/low</sup>CD44<sup>+</sup>, CD24<sup>+</sup>CD49f<sup>+</sup>, and CD24<sup>+</sup>CD29<sup>high</sup> stem-like cell subsets and to a progression toward bigger and more invasive tumors [113]. These results were accompanied by a concomitant increase in Th2-derived cytokines and M2 macrophage numbers, suggesting that at earlier stages of tumorigenesis, T<sub>regs</sub> may play a rather anti-tumorigenic role by modulating the immune landscape and controlling BCSC expansion.

$\gamma\delta$  T cells are a small subgroup of T cells that express heterodimeric T-cell receptors (TCR) formed by  $\gamma$  and  $\delta$  chains. These lymphocytes contribute to the immune response against cancer, and there is an increasing interest in translating their singular properties into novel cancer immunotherapies [114]. In the context of BC, it was shown that HMLER-derived CSC-like cells were resistant to  $\gamma\delta$  T cell-mediated killing; however, the inhibition of the farnesyl pyrophosphate synthase induced the upregulation of both MHC class I and CD54/ICAM-1, and consequently increased the susceptibility to  $\gamma\delta$  T and CD8<sup>+</sup> T cell-mediated lysis [115].

Taken together, the interactions that BCSCs establish with lymphocytes are largely meant to avoid immunosurveillance and therefore to secure BCSC survival. However, the interplay between T cells and BCSCs can also modulate their expansion and self-renewal, as well as their tumor- and metastasis-initiating potential (Figure 2).



**Figure 2.** Schematic representation of interactions between T cells, natural killer (NK) cells and BCSCs.

### 3.3. Natural Killer Cells

Natural killer (NK) cells are lymphocytes that show both innate and adaptive immune features [116]. NK cell responses depend on interactions between activating or inhibitory receptors with their ligands. Activating receptors typically recognize ligands expressed on transformed cells, while inhibitory receptors bind those expressed on healthy cells [117]. In the presence of an activating signal, NK cells kill the target through two distinct mechanisms: granule exocytosis and death receptor-mediated apoptosis [118]. Due to their cytotoxic role, a high infiltration of NK cells associates with good prognosis in BC [119]. Interestingly, NK cells were shown to preferentially target cells displaying the CSC phenotype, including in MDA-MB-231 BC cells [120]. As a matter of fact,  $CD44^+CD24^-$  BCSCs from MCF-7 cells were shown to be more susceptible to the NK-mediated lysis, as they express high levels of UL-16 binding proteins ULBP1 and ULBP2, and MHC class I polypeptide-related sequence A (MICA), ligands of the activating NK cell receptor NKG2D [121]. Moreover, Tallerico et al. provided in vitro and in vivo evidence that tumorspheres enriched in CSCs from HER2+ and TNBC cell lines were more susceptible to NK cell killing [122].

However, CSCs can escape immunosurveillance through the abrogation of NK cell functions [97]. For instance, it was recently reported that primary human  $ALDH1^+$  BCSCs become more resistant to NK-cell-mediated cytotoxicity by overexpressing miR20a, which in turn downregulates MICA and MICB, eventually resulting in increased lung metastasis [123]. Interestingly, targeting the miR20a-MICA/B axis with the differentiation agent all-*trans* retinoic acid induced the differentiation of BCSCs and prevented metastasis [123].

The capacity of BCSCs to evade NK-mediated killing may also be responsible for the resistance against trastuzumab, a common agent used in the clinic for the treatment of HER2+ tumors. In fact, a study showed that the immunoselection of human BC cell lines with trastuzumab and polyclonal NK cells promoted the preferential survival of the  $CD44^{high}CD24^{low}$  population, which expresses lower levels of HER2 [124]. Nevertheless, when the selected cells were expanded again, the proportion of CSCs decreased, and the culture was again partially sensitive to NK cytotoxicity—although after



repeated cycles of immunoselection the cells acquired a higher tumor initiating-potential and the CD44<sup>high</sup>CD24<sup>low</sup> population increased [124].

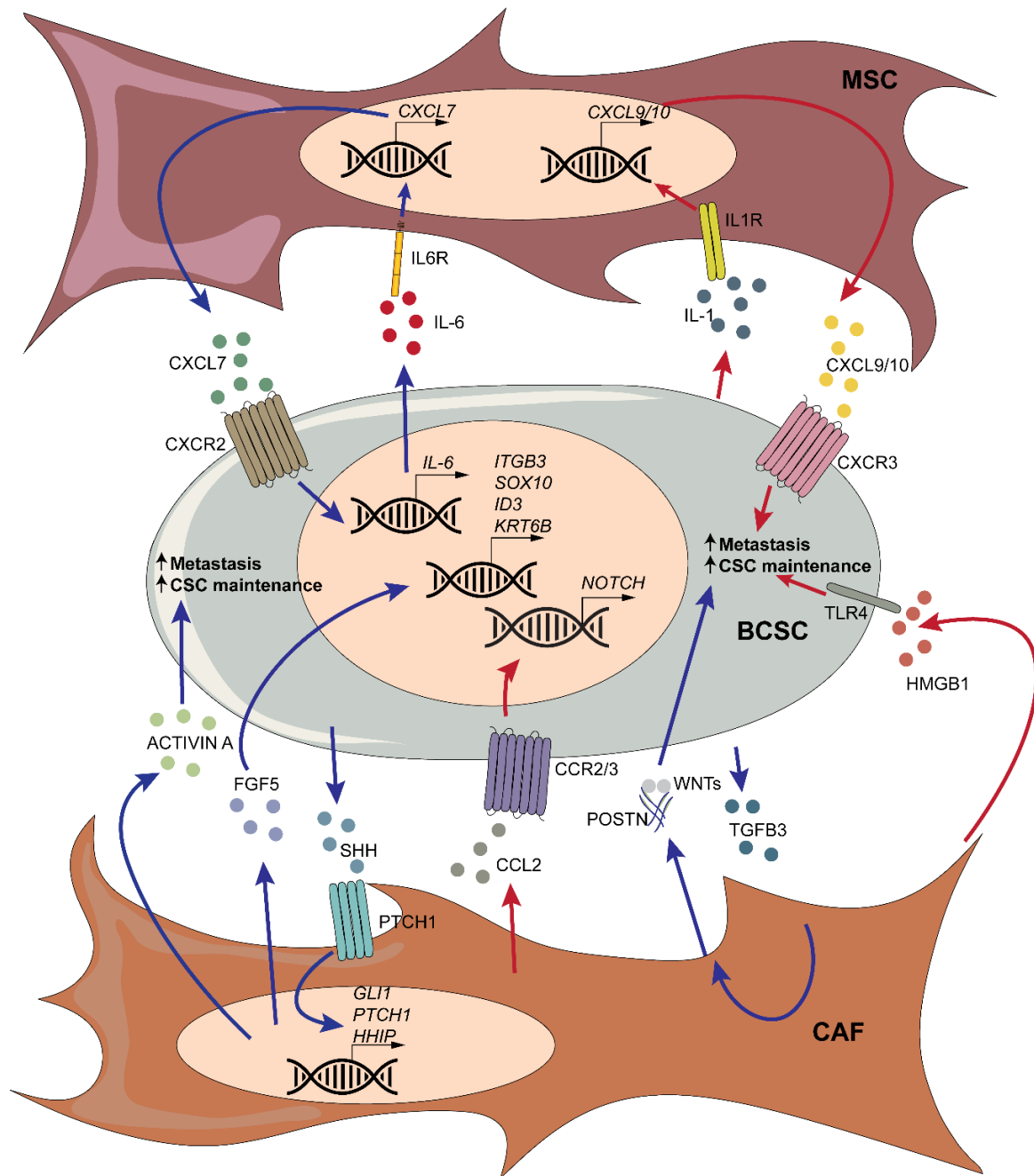
Whereas some reports suggest that BCSCs may be more sensitive to NK cells, BCSCs can also avoid NK-cell-mediated killing, which contributes to tumor relapse and metastasis [125] (Figure 2).

#### 3.4. Mesenchymal Stromal Cells and Cancer-Associated Fibroblasts

Mesenchymal stromal cells (MSCs) are multipotent progenitor cells that were first derived from the bone marrow, but ultimately identified in most adult tissues. Besides their potential to differentiate into the osteoblastic, chondrogenic, and adipogenic lineages, they are the precursors of tissue resident fibroblasts. Likewise, tissue resident fibroblasts and MSCs are two main potential cellular origins of CAFs [126]. MSCs have prominent roles in the regulation of tissue inflammation due to their immunomodulatory properties. As with immune cells, MSCs can play dual, opposing roles in BC progression, depending on the context. However, a significant body of evidence indicates that they interact with BCSCs, promoting stemness and tumor progression. Indeed, bone-marrow-derived MSCs can home to breast tumors and fuel growth and metastasis to the lungs, lymph nodes, and bone [127–129]. Analogous to what happens with immune cells, MSCs can interact and regulate BCSCs through the secretion of cytokines. Indeed, mixing MSCs and BC cells triggers BC lung metastasis through a CCL5-mediated mechanism involving increased extravasation and motility [130]. Likewise, coculturing experiments show that MSCs promote the BCSC phenotype of SUM159 cells, enhancing their self-renewal capacity [128]. The authors found that this increase was mediated mainly through MSC-secreted CXCL7, and also CXCL5 and CXCL6. CXCL7 was induced, in turn, by cancer cell production of IL-6. As a result of this cytokine cross-talk between BCSCs and MSCs, cancer cells that had previously been in contact with MSCs showed increased capacity to initiate secondary tumors [128]. In line with these results, CCL2 secreted by CAFs potentiated the sphere formation efficiency and self-renewal of BT474 and MDA-MB-361 BC cells through the activation of Notch signaling [131]. In the context of metastasis, MDA-MB-231 derivatives were found to express IL-1 via NF- $\kappa$ B signaling, which promoted the secretion of CXCL9/10 in lung fibroblasts [132]. These two cytokines were found to act on their receptor C-X-C motif chemokine receptor 3 (CXCR3) on metastatic stem cells and boost sphere formation in vitro and promote lung metastasis in vivo [132]. Similarly, a few years ago we reported that the interactions between CSCs and lung fibroblasts are crucial for metastatic colonization [76]. We found that metastasis-initiating cells induce the expression of periostin (POSTN) in lung fibroblasts, which in turn recruits Wnt ligands and presents them to the CSCs, promoting their stemness [76].

In Wnt-Met mammary gland tumors, CAFs were found to undergo Hedgehog signaling and to secrete a series of ligands, including ACTIVIN A, which support CSC expansion and self-renewal [133]. Accordingly, in vivo treatment with the Hedgehog inhibitor vismodegib reduced the number of fibroblasts and the ability of tumor cells to form mammospheres. Moreover, Hedgehog activation in CAFs through paracrine signaling with M6 TNBC mammary carcinoma cells induced the secretion of FGF5 and the remodeling of the ECM, which in turn controls BCSC plasticity and promotes chemoresistance [134]. Accordingly, smoothed inhibitors exert strong synergistic effects when combined with docetaxel chemotherapy in preclinical models, a combination therapy that has shown some promising results in an ongoing clinical trial [134]. MSCs and CAFs can also promote the BCSC phenotype through at least two alternative pathways. For instance, when cocultured with MSCs, BC cells expressed mir-199a and mir-214, which fostered CSC propagation and metastasis through the expression of forkhead box P2 (FOXP2) [135]. In a different study, autophagic CAFs were found to release high mobility group box-1 (HMGB1), which acted on toll-like receptor 4 (TLR4) to promote the stemness of luminal BC cells [136].

Briefly, the crosstalk between stromal cells and BCSCs generally promotes stemness, tumor progression, and metastasis (Figure 3).



**Figure 3.** Schematic representation of interactions between mesenchymal stromal cells (MSCs)/cancer-associated fibroblasts (CAFs) and BCSCs.

### 3.5. Adipose-Derived Mesenchymal Stem Cells and Adipocytes

About 90% of the human breast is made up of adipose tissue, which is composed mainly of fat cells, but also contains macrophages, lymphocytes, endothelial cells, pericytes, fibroblasts, ECM, and adipose precursor cells. The adipose tissue plays important roles during mammary gland development and breast carcinogenesis. Breast adipocytes can be classified into adipose-derived mesenchymal stem cells (ADSCs), preadipocytes, and mature adipocytes. ADSCs share similar properties with MSCs isolated from the bone marrow, and they can contribute to mammary tumorigenesis and metastasis through the activation of a number of pathways [137,138]. For instance, ADSCs secrete adiponin, an adipokine shown to enhance BCSC properties. In particular, adiponin is able to increase the sphere-forming capacity of BC cells and to drive tumor growth [139,140]. Likewise, ADSCs pretreated with the adipokine

visfatin and subsequently cocultured with MDA-MB-231 cells enhanced their viability, migration, and tumorsphere formation capacity, as well as tumor growth and metastasis in preclinical models of BC [141]. Interestingly, it was recently shown that ADSCs have the ability to fuse with BC cells and to form cellular hybrids which are enriched in CSCs and have higher tumorigenicity [142].

Adipocytes and cancer-associated adipocytes are important players during breast tumorigenesis, as they release a number of factors in the TME, including growth factors, adipokines, hormones, and cytokines that can impact tumor behavior [137,143–146]. For instance, adipocyte-derived leptin and IL-6 have been shown to activate CSC signaling pathways on adjacent BC cells in a paracrine manner [147,148]. Specifically, upon binding their receptors on target cells, IL-6 and leptin activate the transcription of stemness factors such as OCT4 and Sox2, thus mediating BCSC self-renewal. More recently, adipocyte-derived leptin and its receptor were linked to CSC enrichment and EMT in TNBC [149–151]. Interestingly, the leptin-mediated activation of the JAK/STAT3 pathway results in an increase of fatty acid  $\beta$ -oxidation (FAO), which is required for BCSC self-renewal and promotes chemoresistance [152]. As a consequence, targeting the FAO/leptin axis led to decreased BCSC numbers, reduced chemoresistance, and reduced tumor growth in vivo. Similarly, leptin deficiency was found to impair tumor-initiating cells and consequently tumor growth in obese mice [153]. Moreover, interactions between immature adipocytes and BC cells were shown to increase tumor-initiating cell numbers and to drive metastasis formation [154]. Finally, a fourth adipokine, resistin, was found to potentiate the BCSC phenotype and increase tumorsphere formation and EMT [155,156].

The impact of the adipose tissue on BCSC biology is exemplified in clinical conditions that involve tissue inflammation, such as obesity. Obesity is characterized by macrophage-driven, low grade, chronic inflammation of the adipose tissue, and it is associated with increased risk of BC and poor prognosis, particularly in post-menopausal women [157–159]. We recently showed that, experimentally, obesity triggers EMT and increases the number of metastasis-initiating cells in a mouse model of postmenopausal breast cancer [160]. In addition, Hao and collaborators showed that circulating adipose tissue-derived fatty acid binding protein 4 (FABP4) levels increase during obesity and can activate the axis IL-6/STAT3/ALDH1, thus driving cancer stemness [161].

Given its abundance as well as its capacity to secrete molecules, including growth factors and hormones, the adipose tissue has very relevant functions in mammary gland development, tumorigenesis, and CSC biology. Once they are released in the extracellular space, adipokines modulate the behavior of the neighboring BCSCs, including their capacity to form spheres, initiate tumors, and form metastasis.

### 3.6. The Extracellular Matrix

The ECM consists of a complex network of proteins that provides structural and functional support to the tissues. It also serves as a reservoir for many secreted growth factors and molecules, thus playing a significant role in regulating cell signaling. Likewise, the ECM is a very important element of the BCSC niche and is a crucial player during BC progression. For instance, matrix stiffening, which depends on the amount of collagen fibers and hyaluronan contained in the tumor mass, promotes malignant transformation [162], alters the vasculature [162], modulates the immune landscape [163], and drives metastasis [164]. Interestingly, ECM stiffness and hypoxia were shown to upregulate the expression of integrin-linked kinase (ILK), which signals through AKT/PI3K and promotes BCSC survival [165]. As a downstream event, ILK upregulates vascular endothelial growth factor A (VEGF-A), thus promoting angiogenesis and the dissemination of breast tumor cells.

As happens with other components of the TME, certain components of the ECM have been proposed to have prognostic value in breast cancer. These include hyaluronan [166,167], asporin [168,169], syndecans [170,171], versican [172,173], collagens [174,175], fibronectin [174,176], transforming growth factor beta-induced (TGFBI) [177], osteopontin (OPN) [178,179], POSTN [180,181], laminins [174,182,183], and tenascin C (TNC) [172,174,184].

### 3.6.1. Proteoglycans

Proteoglycans (PGs) are one of the major constituents of the ECM. They are heavily glycosylated molecules that can form complexes with other PGs, hyaluronan, and fibrous proteins in the matrix. Interestingly, the G3 domain of versican was found to play a role in modulating BCSC self-renewal by activating EGFR/AKT/GSK-3 $\beta$  signaling and increasing chemoresistance [185]. A second PG, syndecan-1, plays an analogue role in modulating BCSC properties by regulating IL-6/STAT3 [171,186], as well as Notch and EGFR signaling in TNBC, and it has been recently proposed as a novel CSC marker in BC leptomeningeal metastasis [187]. Asporin, a member of the small leucine-rich proteoglycans (SLRP) family, can act as a tumor promoter or a tumor suppressor in BC [168,169,188]. Maris and collaborators found that hormone receptor-positive BC cells induced a strong expression of asporin in breast fibroblasts, whereas TNBC inhibited it through the secretion IL-1 $\beta$  [188]. Asporin was found to inhibit TGF- $\beta$ -induced EMT, as well as tumor growth and metastasis in TNBC. Accordingly, tumors expressing asporin also contained lower frequencies of ALDH<sup>high</sup> and CD44<sup>high</sup>/CD24<sup>low</sup> cells [188].

### 3.6.2. Hyaluronan

The non-proteoglycan polysaccharide hyaluronan (HA) is a very abundant ECM molecule and also a crucial CSC niche factor. It is the only glycosaminoglycan that does not associate with proteins and does not contain any sulfate. The binding of HA to its receptor CD44 triggers the expression of several stem cell markers and promotes drug resistance, cell invasion, and the reorganization of the cytoskeleton in breast cancer cells [189,190]. Moreover, it has been shown that the overproduction of HA drives BC progression through the expansion of the CD44<sup>high</sup>/CD24<sup>low</sup> cell fraction [68].  $\Delta$ Np63, a p53 family member frequently expressed in basal-like BC, is a key regulator of HA family genes, including HA synthase HAS3, hyaluronidase HYAL-1, and CD44. By modulating these pathways,  $\Delta$ Np63 contributes to creating a microenvironment enriched for HA, which in turn promotes cancer stemness [191]. Additionally, excessive HA production has been shown to activate hypoxia-inducible factor 1 (HIF-1) and consequently BCSC properties through increased hexosamine biosynthetic pathway activity [192].

### 3.6.3. Collagens

The majority of proteins in the ECM have a fibrotic and non-globular structure, and as a result, they can assemble in more complex networks. Collagens are the most abundant fibrous proteins in the ECM, and they are classified into a number of families according to their structure. Several collagens are involved in modulating the CSC phenotype in BC. For instance, collagen type XIII is a transmembrane protein which is highly expressed in BC and plays a role in enhancing stemness, cell invasion, and resistance to anoikis [193]. Furthermore, a fragment of collagen VI  $\alpha$ 3 named C5A acts as a ligand for anthrax toxin receptor 1 (ANTXR1), a putative BC stem cell marker [194]. The binding of C5A to ANTXR1 activates Wnt signaling, expands CD24<sup>-</sup>CD44<sup>+</sup> cells, and boosts metastasis [194]. Collagens are also target genes for stemness-related pathways, and they contribute to tumor initiation and metastasis [134,195]. For instance, the FZD7-WNT5b axis has been shown to modulate the expression of collagen VI, and the knockdown of either FZD7 or COL6A1 in BC cells was reported to decrease invasion and sphere formation [196].

### 3.6.4. Glycoproteins

Glycoproteins can bind to collagens and integrins in order to favor cell migration. The multifunctional protein fibronectin was shown to be the downstream target of the TGF- $\beta$ /Smad3/COX-2 pathway in TNBC, where it correlates with poor overall survival [197]. In particular, fibronectin regulates BCSC self-renewal through the expansion of ALDH<sup>+</sup> and CD24<sup>-</sup>CD44<sup>+</sup> cell populations, revealing its potential use in developing novel targeted therapies for BC [197,198]. In addition, BCSCs can secrete the matricellular protein laminin (LM)-511, which can

bind to the integrin  $\alpha 6\beta 1$  and subsequently activate tafazzin (TAZ) [199], a component of the Hippo pathway relevant for CSC maintenance [200]. In turn, TAZ modulates the expression of LM $\alpha 5$ , the  $\alpha$  subunit of LM511, highlighting the existence of a positive loop that sustains BCSC self-renewal and tumor-initiating potential. Conversely, the pretreatment of ER+ LM05-E BC cells with laminin decreases Sox2, Nanog, and Oct-4 through the modulation of the MAPK/ERK pathway, and it also induces tamoxifen resistance mediated by  $\alpha 6$  integrin [201]. CSC niches are also enriched for POSTN, a matricellular protein that promotes the initial expansion of BCSCs [76]. POSTN is produced by lung fibroblasts, binds Wnt ligands, and presents them to metastatic stem cells, thus promoting stemness, survival, and growth. The knockdown of POSTN in basal-like BC cell lines impairs mammosphere formation and tumor initiation [202]. As a matter of fact, an intact signaling between POSTN and its receptor  $\alpha v\beta 3$  integrin can activate the transcription of IL-6 and IL-8, which in turn activate STAT3, crucial for CSC maintenance [202]. POSTN can bind TNC, a glycoprotein that promotes the metastatic outgrowth of disseminated tumor cells in the secondary site. In particular, breast tumor cells that infiltrate the lungs were shown to release high levels of TNC, which drives the expression of musashi RNA binding protein 1 (*MSI1*) and leucine-rich repeat-containing G-protein-coupled receptor 5 (*LGR5*), leading to the activation of the Notch and Wnt pathways, and thus promoting stemness and the outgrowth of pulmonary micrometastases [203]. Moreover, enhanced JNK signaling in BC cells induces the expression of ECM genes, including TNC, that contribute to chemoresistant metastasis [204]. TGFBI, a POSTN paralog, is a 683 aminoacid ECM protein, which contains a secretory signal sequence, an N-terminal cysteine-rich domain (EMI), four fasciclin-1 (FAS1) domains, and an RGD (Arg-Gly-Asp) integrin binding site at the C-terminal end. We have recently identified TGFBI as a novel prognostic factor in BC and a crucial player in modulating interactions between tumor cells and the microenvironment: depletion of TGFBI reduces tumor hypoxia and normalizes the vasculature, thus dramatically decreasing CSC numbers and ultimately impairing metastasis formation [177]. The matricellular phosphoglycoprotein OPN has been shown to trigger the tumorsphere formation of ALDH<sup>high</sup>CD44<sup>+</sup>CD24<sup>-</sup> BC cells and to boost their metastatic behavior through the interaction with CD44 and RGD-dependent cell surface integrins [205]. Interestingly, OPN was also shown to play a role in controlling the effect of the fibroblast T cell lymphoma invasion and metastasis-inducing factor 1 (Tiam1) in BC, with implications for cancer stemness [206]. In particular, this study showed that Tiam1 expression in fibroblasts modulated the BCSC phenotype in BC cells in vitro and in vivo, and that this effect was dependent on fibroblast OPN. Blocking fibroblast OPN led to a decrease in CD44<sup>+</sup>/CD24<sup>-</sup>/ESA<sup>+</sup> SUM1315 cell populations, in tumorsphere formation, and prevented lung metastasis [206]. Fibulin-3, also known as EFEMP1, is known to inhibit angiogenesis and was found to be downregulated in sporadic breast carcinomas [207]. Interestingly, EFEMP1 acts as a downstream target of HIF-2 $\alpha$  and plays a role in enhancing sphere formation and BCSC self-renewal [208]. Finally, the ECM glycoprotein vitronectin has been identified as another important factor inducing BCSC differentiation and tumor formation upon binding to integrin  $\alpha v\beta 3$  [209].

Altogether, the ECM is a crucial element of the TME that facilitates interactions between different cell types, provides physical and mechanical support for BCSCs, sustains BCSC self-renewal and expansion in the primary and the secondary sites, and promotes tumor progression (Table 1).

**Table 1.** Extracellular matrix molecules with BCSC modulatory activities.

ECM Components		Activity	References	
Proteoglycans	Versican	Versican modulates BCSC self-renewal by activating EGFR/AKT/GSK-3 $\beta$ signaling.	[185]	
	Syndecan-1	Syndecan-1 modulates IL6/STAT3, NOTCH and EGFR signaling in TNBC, and it has been proposed as a novel CSC marker in BC leptomeningeal metastasis.	[171,186,187]	
	Small leucine-rich proteoglycans (SLRP)	Asporin	Asporin inhibits TGF- $\beta$ 1-induced EMT, tumor growth, and metastasis; tumors expressing asporin also contain lower frequencies of ALDH <sup>+</sup> cells and CD44 <sup>high</sup> /CD24 <sup>low</sup> cells.	[188]
Non-proteoglycan polysaccharides	Hyaluronan (HA)	HA-CD44 interactions promote the expansion of CD44 <sup>high</sup> /CD24 <sup>low</sup> , modulate drug resistance, cytoskeleton reorganization, and cell invasion; The p53 family member $\Delta$ Np63 contributes to create a microenvironment enriched for HA, which promotes cancer stemness; excessive production of HA activates HIF-1 and BCSC properties.	[68,189–192]	
Fibrous protein	Collagens	Collagen type VI	Binding of collagen VI $\alpha$ 3 (C5A) to ANTXR1 activates Wnt signaling, expands CD24 <sup>+</sup> CD44 <sup>+</sup> cells, and boosts metastasis; The FZD7-Wnt5b axis modulates the expression of collagen VI, whose knock-out leads to decreased sphere formation ability, tumorigenesis, and metastasis.	[194,195]
		Collagen type XIII	Collagen type XIII is highly expressed in BC and it plays a role in enhancing stemness through $\beta$ 1 integrin, cell invasion, and resistance to anoikis.	[193]
Glycoproteins	Fibronectin		Fibronectin is the downstream target of the TGF- $\beta$ /Smad3/COX-2 pathway in TNBC; it modulates BCSC self-renewal through the expansion of ALDH <sup>+</sup> and CD24 <sup>+</sup> CD44 <sup>+</sup> cells.	[197,198]
	Laminin	Laminin 511	(LM)-511 binds to $\alpha$ 6 $\beta$ 1 and activates TAZ, relevant for CSC maintenance; pretreatment of ER+ LM05-E BC cells with laminin decreases Sox-2, Nanog and Oct-4 through modulation of MAPK/ERK pathway, and it induces tamoxifen resistance mediated by $\alpha$ 6 integrin.	[199,201]
	Periostin		Lung fibroblast-secreted POSTN recruits Wnt ligands and presents them to metastatic stem cells, thus promoting cell survival and growth; in basal-like BC cell lines, POSTN binds its receptor $\alpha$ v $\beta$ 3 and activates the transcription of IL6 and IL8, which in turn activates STAT3, crucial for CSC maintenance.	[76,202]
	Tenascin	Tenascin C (TNC)	Breast tumor cells in the lungs release high levels of TNC which drives the expression of MSI1 and LGR5 and promote survival and growth of pulmonary micrometastases; enhanced JNK signaling in BC cells induces the expression of ECM genes including the stem cell niche component TNC that contribute to chemoresistant metastasis.	[203,204]
	TGFBI		Depletion of TGFBI reduces tumor hypoxia and normalizes the vasculature, thus impairing CSC numbers and metastasis formation.	[177]
	Osteopontin		OPN triggers tumorsphere formation and the expansion of ALDH <sup>high</sup> CD44 <sup>+</sup> CD24 <sup>-</sup> BC cells and boost their metastatic behaviors through interaction with CD44 and RGD-dependent cell surface integrins; blocking OPN in CAFs leads to a decrease in CD44 <sup>+</sup> /CD24 <sup>-</sup> /ESA <sup>+</sup> SUM1315 cell populations and in tumorsphere formation.	[205,206]
	Fibulin	Fibulin-3	Fibulin-3 acts as a downstream target of HIF2 $\alpha$ and plays a role in enhancing sphere formation and BCSC self-renewal.	[208]
Vitronectin		Vitronectin regulates BCSC differentiation and tumor formation upon binding to integrin $\alpha$ v $\beta$ 3.	[209]	

#### 4. Conclusions

The TME is an essential player driving tumor progression and shaping the evolution and heterogeneity of breast tumors. The complex network of interactions between BCSCs and the TME is crucial to modulate their plasticity and, consequently, the tumor-initiating and metastasis-initiating capabilities of cancer cells. Microenvironmental signals can restrict or promote the acquisition of BCSC traits and modulate the transition of epithelial cancer cells to mesenchymal states, which facilitates some steps of the metastatic cascade. Likewise, in the secondary site, they can trigger the acquisition of epithelial traits and increase the proliferative potential of BCSCs.

The CSC field still suffers from some of the same long-standing problems, such as the lack of universal markers and the recurrently discussed issue of the immunological status of the hosts used for *in vivo* experiments in CSC research. *In vivo* studies involving human BC cells are commonly performed in immunocompromised mice (e.g., nude, NOD-SCID, or NSG), which precludes observing the potential interactions between BCSCs and different subsets of immune cells, and usually leads to an overestimation of the CSC content in a tumor. This poses a problem also for the interpretation of the potential pre-clinical trials aiming at disrupting the axis TME-BCSCs. As discussed elsewhere, the use of well-defined mammary gland tumorigenesis mouse models in fully immunocompetent syngeneic settings can help to at least partially overcome this limitation. In parallel, *ex vivo* organoid culture models such as the air-liquid-interface system [210], which allows for the study of the TME—including the immune compartment—might represent useful tools to explore these interactions and to test therapies targeting the TME. Achieving synergistic effects through combinatorial treatments that target both cancer cells and the TME holds great promise for treating cancer. In this respect, drug repurposing has gathered a considerable momentum over the last few years [211]. For instance, recent studies suggest that aspirin—a non-steroidal anti-inflammatory drug that has become a paradigm of drug repurposing—can act on both BCSCs and certain components of the TME in experimental settings [212–217]. Although ongoing clinical trials are still evaluating its potential benefits in BC [218], this example serves as an illustration of the possibilities of such strategies. Additionally, immunotherapy is emerging as a promising tool to manipulate the TME and target BCSCs, directly or indirectly [115,219–223].

Both intrinsic and extrinsic factors determine the BCSC phenotype. Single-cell RNA sequencing studies have revealed specific transcriptional profiles of BCSCs [224–226]. In parallel, other studies have focused on unraveling the underlying complexity of the TME. For instance, analyses at the single-cell level have allowed the identification of subgroups of breast CAFs possessing singular transcriptional profiles [227]. Likewise, single-cell RNAseq analyses have uncovered the diversity of the immune TME in breast cancer [228]. Furthermore, the heterogeneity of the TME has been recently explored as a potential prognostic factor in BC, since clustering breast tumors according to their tumor immune microenvironment identifies differences in molecular subtypes, prognosis, proliferation, and EMT features [229]. However, holistic approaches that integrate transcriptional and spatial information may be poised to overcome the drawbacks of single-cell RNA sequencing analyses (i.e., loss of a spatial context and potential underrepresentation of certain cell types due to the need for tissue dissociation) and to provide novel information on extrinsic cues impacting the BCSC phenotype. In this respect, spatial transcriptomics might represent an opportunity to shed more light on how BCSCs interact with the microenvironment *in situ*, and to study the co-evolution of the TME and BCSCs. Untangling the complexity of the BCSC niche will help design therapies aimed at perturbing the interplay with the microenvironment, restricting plasticity, reducing tumor heterogeneity, and ultimately impairing metastasis.

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