Navigating the Intricacies of Tumor Heterogeneity: An Insight into Potential Prognostic Breast Cancer Biomarkers

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ABSTRACT: Breast cancer is a heterogeneous disease with diverse histological and molecular subtypes. Luminal breast tumors are the most diagnosed subtype. In luminal breast cancer, hormone receptors (including ER, PR, HER2) play a diagnostic and prognostic role. Despite the effectiveness of endocrine therapy in luminal breast tumors, tumor recurrence and resistance occur, and this may highlight evolutionary strategies for survival driven by stemness. In this review we thus consider the association between estrogen signaling and stemness in mediating tumor processes. Many studies report stemness as one of the factors promoting tumor progression. Its association with estrogen signaling warrants further investigation and provides an opportunity for the identification of novel biomarkers which may be used for diagnostic, prognostic, and therapeutic purposes. Breast cancer stem cells have been characterized ($CD44^+$ $CD24^-$) and their role in promoting treatment resistance and tumor recurrence widely studied; however, the complexity of tumor progression which also involve microenvironmental factors suggests the existence of more varied cell phenotypes which mediate stemness and its role in tumor progression.

KEYWORDS: Breast cancer, evolution, stemness, intratumoral heterogeneity, estrogen

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Introduction

Breast cancer remains a formidable global health concern, with its incidence rate predicted to rise by over 40% by the year 2040.[1] Female breast cancer accounted for 685 000 deaths globally in 2020 making it the fifth leading cause of cancer mortality worldwide.[2] Although it is the most commonly diagnosed cancer in women globally, the burden of the disease is disproportionately felt in developing countries.[3,4] In 2020, over half of the estimated 2.26 million breast cancer diagnoses were recorded in low- to moderate-income countries.[3,4] A rapid increase in breast cancer diagnosis across Africa has been observed. South Africa experienced a 3% to 4% increase in breast cancer per year between the mid-1990s and mid-2010s.[2] According to the national cancer registry breast cancer has a lifetime risk of 1 in 30 in South Africa (NICD, 2023). In South Africa, breast cancer has a 5-year survival rate of less than 50% and it is the second leading cause of cancer-related deaths in women.[3,4] This indicates that, despite the ongoing advances in detection and treatment of breast cancer, further research into novel diagnostic and prognostic biomarkers is necessary to improve therapeutic strategies and better disease outcome, particularly in developing countries.[5]

Breast Cancer Complexity

Classification

Breast cancer is a highly heterogeneous disease that encompasses a multitude of tumors with distinct morphological and molecular manifestations.[6,7] Breast cancer affects various areas in the breast including the lobules, ducts, and connective tissue and shows distinct physiological properties and clinical outcomes.[8] The mammary gland mainly consists of a lobular region and ducts, the mammary epithelium is composed of luminal cells, responsible for milk production and basal cells which facilitate milk secretion through muscular contraction.[9] Protein expression varies between luminal and basal cells, with luminal cells expressing estrogen receptor (ER), progesterone receptor (PR) or prolactin receptors, cytokeratins (CK) CK7,[10] CK8, CK18,[11,12] and epithelial cell adhesion molecule (EpCAM).[13,14] Basal cells express CK5 and CK14[11] and CK6,[10] P-cadherin, desmosomal cadherins, and smooth muscle markers.[9] These protein biomarkers are characteristic of distinct tumor phenotypes that affect their biology and tumor progression. As such, the classification of the breast tumors into various subtypes aims in guiding treatment strategies to improve disease outcome.[15]

Traditionally, breast cancer has been classified into two broad types according to its histopathological presentation: carcinomas and sarcomas.[7,15] The majority of breast tumors are carcinomas that arise from the ductal or lobular epithelium of the breast which, depending on the proliferative capacity of the neoplasia, can further be subdivided into carcinoma *in situ* or invasive carcinoma.[15,16] Ductal carcinoma is diagnosed in ~75% of patients, making it the most common type of breast cancer. Lobular carcinoma is prevalent in 10% to 15% of patients and a small percentage of cases present with mixed ductal/lobular carcinoma.[9] Invasive breast cancer consists of specific and non-specific subtypes, which are graded by means of various histopathological parameters.[15,16] Although effective in providing a

broad categorization of breast cancer, tumors within the same histological presentation can have vast differences in their biological behavior.[15,17] Thus, assessment of these parameters alone may be insufficient in predicting the true pathophysiology of the breast tumor and many studies have shifted their focus toward assessing the molecular patterns of the disease.[15,16]

Currently, the classification of breast cancer into distinct molecular subtypes is determined by the expression of ER, PR, human epidermal growth factor receptor 2 (HER2), and the cell proliferation regulator Ki67.[18,19] The immunohistochemical analysis of these four biomarkers have proven to be efficient in the stratification of the different types of breast tumors, particularly when used with other pathological and clinical parameters, and has aided in the development of targeted treatment strategies; however, drug resistance and tumor recurrence remain prevalent.[18,19]

The majority of breast cancers present with a luminal phenotype, which is characterized by the overexpression of the hormone-receptors.[15,17] The immunohistochemical profile for the luminal A subtype is typically characterized as: ER^+ ($\geqslant$1%), PR^+ ($\geqslant$20%), $HER2^-$ ($\leqslant$10%), and low expression of Ki-67 ($<$14%).[15,20] This subtype is correlated with a highly favorable response to targeted hormone therapy and a good disease prognosis.[15] The luminal B subtype is associated with approximately 20% to 30% of invasive breast cancer cases.[15] This subtype can be divided immunophenotypically into Luminal B (HER^-): ER^+ ($\geqslant$1%), $PR^{+/-}$, $HER2^-$ ($\leqslant$10%), and high levels of Ki-67 expression ($\geqslant$20%); or Luminal B ($HER2^+$): ER^+ ($\geqslant$1%), $HER2^+$ ($>$10%), and any level of PR and Ki-67 expression.[15,18,20] The prognosis for luminal B tumors is intermediate, with treatment being targeted hormone therapy and additional chemotherapy.[15] Although both luminal subtypes are characterized by the overexpression of ERs and are generally associated with a better prognosis than the HER2-enriched and Triple Negative Breast Cancer (TNBC) subtypes; luminal B tumors are more aggressive due to their increased expression of proliferation-associated genes.[6,19,21] This is indicative of the great diagnostic and prognostic value held by the analysis of gene expression profiles in breast cancer; however, this method of classification is hindered by a more complex heterogeneity observed between cells within a singular tumor mass.[17,22] Further metagene analyses have indicated that luminal tumors exhibit significant genomic instability, with individual cells portraying different mutational landscapes.[17,22,23] This intratumoral heterogeneity acts as a major limiting factor to treatment efficacy. Thus, the development of many multigenic assays such as Prosigna PAM50, Mammaprint, and Oncotype DX has provided crucial information into the differential gene expression profiles responsible for the expression of the aforementioned biomarkers.[2,22] This has led to breast cancer being classified into four distinct molecular subtypes: Luminal A (ER^+/PR^+), Luminal B ($ER^+/PR^+/HER2^{+/-}$), HER2-enriched ($HER2^+$), and Triple Negative ($ER^-/PR^-/HER2^-$); each with their own therapeutic and prognostic implications.[2,22]

Treatment

Treatment for breast cancer consists of local therapies: surgery, radiotherapy and systemic therapies including chemotherapy, hormonal therapy, and targeted therapy.[24] Of particular interest to this study is the luminal phenotype breast tumors which, because of positive status of hormone receptors, can be treated with hormone therapies including selective estrogen receptor modulators (SERM) that act to limit the signaling capacity of the ER, selective estrogen receptor degraders/downregulators (SERD) which degrade the ER via proteosomes, thereby preventing its signaling activity[25] and aromatase inhibitors (AI) which limit the availability of estrogen.[15,26] Tamoxifen is a triphenylethylene derivative SERM which acts by binding to the ER and inhibiting the proliferative action of estrogen on mammary epithelium.[26,27] It is the most commonly used hormone therapy for ER-positive breast cancers in both pre- and postmenopausal women; however, a significant number of patients either present with Tamoxifen-resistant tumors or acquire resistance to the drug during the course of treatment.[27,28] Most patients that relapse on tamoxifen treatment respond to other therapies including ER downregulators/ER antagonists or AIs.[27,28] AIs can be subdivided based on their nature and mechanism of action with type I AIs being steroidal compounds such as exemestane whereas type II inhibitors are nonsteroidal such as anastrozole and letrozole.[28] Anastrozole is the most commonly used AI and inhibits the aromatase enzyme via competitive inhibition thereby preventing the production of estrogen.[29] In postmenopausal women, aromatase inhibitors are vital in ER-positive breast cancer therapy and are effective drugs post-surgery in advanced breast cancer patients[30]; however, tumor cells are still able to adapt and alter their ability to respond to the therapy leading to relapse and recurrence.

Fulvestrant is the only clinically approved SERD and is administered intramuscularly, with novel orally administered drugs of this class being at different stages of development. Fulvestrant was associated with greater progression free survival (PFS) when clinically administered alone at 500 mg or in combination with other endocrine therapies (including anastrozole) at 250 mg.[31] Furthermore, fulvestrant improved PFS in metastatic breast cancer without prior use of endocrine therapy[32]; however, PFS was improved more so when fulvestrant was combined with other targeted therapies including cyclin dependent kinase (CDK) 4/6 inhibitor (alpelisib) and mTOR inhibitor (everolimus).[33,34]

Novel anti-estrogen therapies are designed to overcome drug resistance and address certain deficiencies of existing endocrine therapy drugs which include acquired *ESR1* mutations which is one of the factors that mediate drug resistance.[33]

A detailed analysis of these novel therapies has been reviewed by Patel et al,[35] this review briefly introduces these novel drug classes. Proteolysis targeting chimerics (PROTACS), the most developed drug being ARV-471, binds to a specific region in a protein and simultaneously to an E3 ubiquitin ligase in order to facilitate the degradation of the target protein through the ubiquitin-proteosome system. Complete estrogen receptor antagonists (CERANS), OP-1250 being the prototype, target transcriptional activators AF1 and AF2 domains in the ER, thus, preventing multiple pathways from promoting the transcriptional activity of ERs.[33] Selective estrogen receptor covalent antagonists (SERCAs), of which HRB-6545 is the first drug of this class, bind a cysteine residue unique to ER in order to mediate ER-degradation.[33] The drive to find new treatment methods highlights the impact of tumor heterogeneity on tumor cell adaptation during tumor progression. This review aims to give an overview of the potential prognostic value of tumor heterogeneity characterized by stemness in relation to estrogen signaling in mediating tumor processes in luminal breast cancer.

Tumor heterogeneity

Tumor heterogeneity defines the vast landscape of cells with varying genomic characteristics within the tumor. It is characterized by various subpopulations with different genotypes and phenotypes. It is present in several tumor types including lung, breast, ovarian, and prostate cancer.[36] There are two types of heterogeneity, identified as intertumoral heterogeneity and intratumoral heterogeneity.[37] Intertumoral heterogeneity defines differences amongst tumors in different patients or within the same patient with diverse tumor deposits.[37] Intratumoral heterogeneity is the presence of distinct cellular populations with specific genetic, epigenetic, and phenotypic features within the same tumor.[36] In addition, intratumoral heterogeneity can lead to poor prognosis of the disease as the prescribed treatment may not effectively target all cell populations within the tumor, thus leading to disease relapse.[38,39] Tumor heterogeneity is seen as a challenge in characterizing cancer as it does not accurately characterize the full genomic landscape of a patient's cancer.[38,39] Clonal variation and microenvironmental factors promote intratumoral heterogeneity which suggests that tumor heterogeneity is influenced by a myriad of attributes.[40] Intratumoral heterogeneity has been identified as a key factor mediating cell plasticity, it equips cancer cells to be more effective in reprogramming gene expression and thus regulates the ability of cancer cells to adapt and modify their behavior in response to microenvironmental cues.[9] Next generation sequencing which has been widely used to detect mutations in tumors, has paved the way for the development of single cell ribonucleic acid sequencing (scRNA-seq) technologies.[41,42] scRNA-seq studies have enabled better characterization of intratumoral heterogeneity by identifying mutations in single cells within a tumor mass which allows for characterization of transcriptional and functional molecular signatures in single cells and rare cell populations.[41,42] This application has been used in disease management for diagnosis, prognosis, and treatment strategies (especially precision-based medicines).[41,42]

Tumor evolution and stemness

Intratumoral heterogeneity is underwritten by Darwinian evolution. Cell populations present genotypic and epigenetic variation that provide a foundation upon which natural selection may act, enhancing fitness strategies that are themselves "fit for purpose." In this review, we suggest that cell populations may employ evolutionary strategies to facilitate tumor progression at different stages.[43-45] Transformed epithelial cells facilitate loss of cellular apposition via downregulation of adhesion molecules (E-cadherins) and upregulation of genes associated with enhanced migratory profiles; vimentin (*VIM*), N-cadherin (*CDH2*), and fibronectin 1 (*FN1).*[46] For example, in luminal phenotype breast cancer, the "switch" from ductal carcinoma *in situ* to infiltrating ductal carcinoma, is predicated on the acquisition of a more aggressive characteristics, but subject to selective pressures of the surrounding microenvironment.[47-50] This includes pressures at the tumor-stromal interface exerted by non-tumor cells including fibroblasts, immune cells, and adipocytes that ultimately enhance cellular processes of migration and proliferation in tumor cells.[51,52] While this permits the establishment of tumor masses, heterogeneity remains. This can be described by current dogma which includes the concept of "cancer stem cells" where the stem-phenotype is retained by a small proportion of tumor cells, with the remainder of the mass considered as transit amplifying cells and their progeny. However, it is also posited that tumor cells may show a continuum of stemness, with subpopulations reflecting different fitness strategies.[43,53] These strategies could further be described in the setting of the tumor microenvironment (Figure 1). Cells at the migratory pole of tumor masses upregulate genes associated with migration and epithelial-to-mesenchymal transition (EMT); vimentin (*VIM*), N-cadherin (*CDH2*), epidermal growth factor receptor (EGFR) (*ERbB2*) and downregulate genes associated with cellular adhesion (E-cadherin, Ig-CAM, selectins), in contrast to other cells in the mass which rather may be primed for proliferation and maintenance of the mass.[54,55] Another example reflects the selective pressure of hypoxia, wherein cells in the center of a larger tumor mass undergo programmed necrosis that together facilitate the upregulation of genes associated with angiogenesis; vascular endothelial growth factor (*VEGF)*, angiopoietin-1 (*Ang-1*), and angiopoietin-2 (*Ang-2*).[56] Within this changing environment, it cannot be forgotten that tumor cells are also undergoing immunoediting, with a changing profile that permits the induction of immune tolerance.[57,58] Moreover, while the

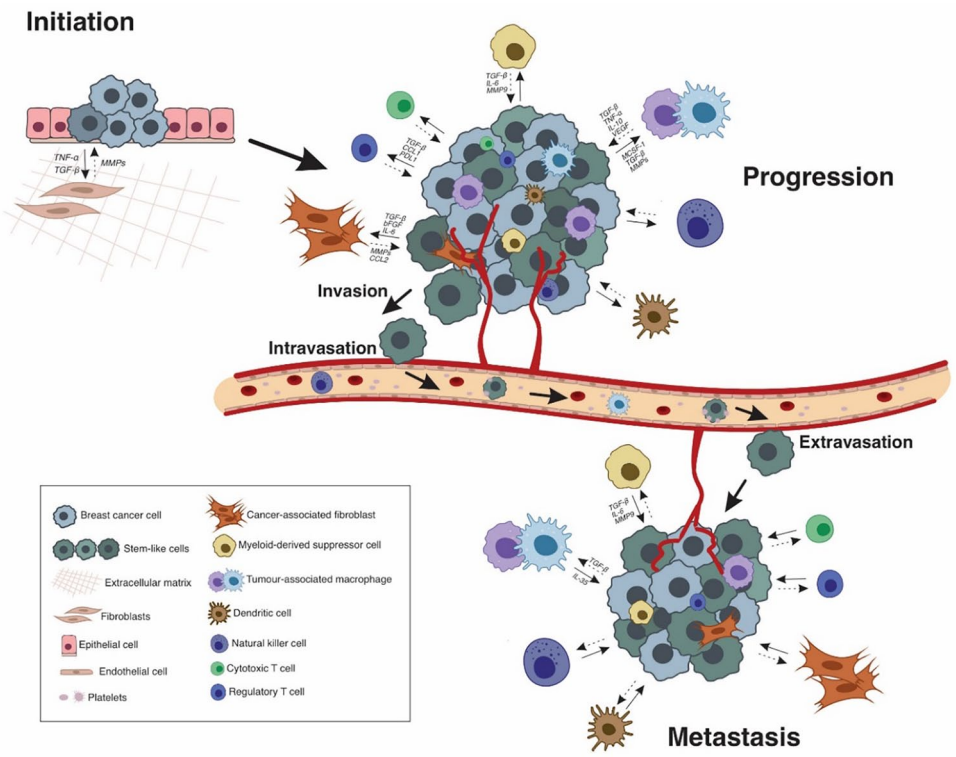

Figure 1. Schematic representation of the role of stemness in breast tumor evolution. Tumor evolution relies on interactions between transformed cells and cells of the TME. From the onset, the heterogeneous population of transformed cells are exposed to a number of selective pressures exerted by non-tumor cells within the TME. Initially, macrophages, dendritic cells, natural killer cells, and cytotoxic T lymphocytes elicit an immune response against transformed cells in an attempt to inhibit tumor potentiation. Tumor cells that present with stem-like characteristics have a higher capacity to withstand anti-tumorigenic signals exerted by these immune cells. Through their secretion of various cytokines (TGF-β, IL-6), chemokines (CCL1), and their overexpression of PDL1, tumor cells facilitate the recruitment of myeloid-derived suppressor cells and regulatory T cells that inhibit anti-tumorigenic responses by cytotoxic T cells and natural killer cells thereby aiding in immune evasion. Using cytokines, stem-like tumor cells promote the polarization of macrophages into a pro-tumorigenic phenotype (TGF-β, MCSF-1) that stimulates angiogenesis (VEGF) and epithelial-to-mesenchymal transition (TGF-β, IL-10). The re-education of fibroblasts by tumor cells (TGF-β, bFGF, IL-6) to secrete pro-tumorigenic chemokines (CCL2) and matrix metalloproteinases that disrupt the ECM further aid in increasing the migratory capacity of tumor cells and the release of ECM-sequestered growth factors. Stem-like tumor cells display enhanced migratory capabilities. Upon the initiation of the invasion-metastasis cascade, tumor cells prepare the pre-metastatic niche and enter the lumen of surrounding vessels where they interact with endothelial cells and platelets to evade circulating immune cells. These circulating tumor cells extravasate at the secondary tumor site where they are able to adapt to the new microenvironment and colonize the tissue.

immunogenicity of hormone-dependent breast cancers, may be regarded as low in comparison to the triple-negative breast cancers,[59] that tumors are able to subvert immune cells to use secreted factors for tumor progression is of interest.[57] Tumors can recruit a multitude of immune cells including myeloid-derived suppressor cells (MDSCs), which retain the capacity to differentiate into macrophages, granulocytes, and dendritic cells for example.[60] Secreted factors from MDSCs including interleukin (IL)-6, transforming growth factor beta (TGF-β), and matrix metalloproteinase 9 are implicated in promoting tumor cell growth, invasion, and angiogenesis.[60,61] MDSCs also induce immune suppression by facilitating tolerance in cytotoxic T cells via nitration or nitrosylation of the T cell receptor or secreted nitric oxide, which prevents the natural killer (NK) cell-FcR mediated activity.[60,61] Cytotoxic function by T cells and NK cells are also controlled by T regulatory cells, which can be subverted by tumor cells to elicit pro-tumor responses.[62] Tumor cells also use additional strategies to ensure evasion from cells of the innate and adaptive immune system; for example, downregulation of major histocompatibility complex (MHC) molecules, blockade of activating receptors or shedding of NK activating receptor ligands, inducing T cell anergy, and promoting macrophage differentiation into a pro-tumorigenic M2 phenotype.[57,63,64]

These immune cells, while dysfunctional in the classical sense, use cytokine-mediated crosstalk with tumor cells to facilitate tumor progression,[65] with underlying stemness a factor for consideration. For example, MDSCs are emerging as major players in both the induction and maintenance of tumor stem-like phenotypes via IL-6 and nitric oxide engagement of signal transducer and activator of transcription 3 (STAT3) and Notch signaling pathways.[61] Similarly, an *in vitro* study has shown that Notch signaling enhances stemness in breast tumor cells, as induced by direct interaction with endothelial cells, particularly under starvation.[66] This highlights the plasticity of tumor cells, in changing their response to environmental cues.

Tumor cells themselves, in addition to circulating platelets, at the interface with the vasculature secrete factors to increase endothelial cell permeability, while simultaneously undergoing EMT to permit a more invasive phenotype.[63] This ultimately leads to the acquisition of a metastatic profile that includes the capacity to prepare the pre-metastatic niche for colonization.[67,68] In this, tumor cells that intravasate need to be markedly different to withstand the shear forces in the hostile environment of the blood stream. As such, subpopulations within the tumor mass are postulated to show phenotypic variance respective to their spatiotemporal role in maintaining and progressing the tumor as a whole. It is postulated that understanding intratumoral heterogeneity may thus lead to an understanding of how tumors evolve and respond to environment pressures, ultimately impacting treatment strategies.

Breast cancer stem cells

Cancer stem cells (CSCs) are thought to contain most of a cancer's tumor-initiating and metastatic ability.[38] In breast cancer, specific cancer cell subpopulations have been found in individual cancers that are characterized by variations in tumorigenicity, induction of senescence, activation of signaling pathways, migration, angiogenesis capacity, and response to anticancer drugs.[69] It was observed that a small subpopulation of breast cancer cells, had the capacity to develop tumors when transplanted into a mouse model, showing plasticity reminiscent of stem cells.[70] Breast cancer stem cells (BCSCs) constitute a small percentage of the tumor and have been associated with resistance to various breast cancer therapies, including endocrine therapy, chemotherapy, and targeted therapy.[71] BCSCs are identified by the expression of cluster of differentiation (CD) surface markers, CD44 and CD133, and low or no expression of surface marker CD24. In addition, they have high aldehyde dehydrogenase I activity.[72,73]

CD44 a common marker for stem cells, is a glycoprotein found on the cell surface and binds to hyaluronic acid thereby mediating communication between cells and the extracellular matrix (ECM) and facilitates interaction with ECM proteins such as matrix metalloprotease (MMP) and osteopontin (OPN).[74,75] It also plays a role in many cellular functions such as cellular adhesion, proliferation, survival, and differentiation, with a prime role in tumor cell migration and extravasation (Figure 2).[76] BCSCs exhibit a high expression of CD44 which maintains the multipotency of the BCSC population, thus increased levels of CD44 is a primary target of breast cancer stem cell therapies.[76] CD133, a cell surface glycoprotein that localizes to microvilli and the apical surface of some epithelial cells,[76] is vital for cellular processes such as glucose and transferrin uptake, autophagy, membrane-membrane interaction, and matrix metalloproteinase functions.[77] CD133+ cells are associated with stemness properties, and are linked to tumor metastasis, chemotherapy-, or- radiotherapy resistance.[77,78] Conversely, low expression of CD24, a sialoprotein facilitating cellular adhesion, is associated with stem cells.[76] CD24 is expressed on the cell surface and antagonizes the function of chemokine receptor 4 (CXCR4), thereby mediating metastasis and proliferation in BCSCs.[79]

An additional marker of stemness, aldehyde dehydrogenase (ALDH), is vital in the biosynthesis of molecules that regulate cellular homeostasis including retinoic acid (RA), γ-aminobutyric acid, and betaine.[80] ALDH superfamily consists of various enzymes including ALDH1A1, it is associated with alcohol metabolism, retinoic acid (RA) metabolism, and protection from reactive oxygen species by reducing metabolic stress.[80] Studies have revealed that small subpopulations of cells that overexpress CD44 and ALDH (CD44high/CD24–/low/ALDHbr) may be strong initiators of breast tumorigenesis.[81] ALDH1A1 is highly active in cancer stem cells, therefore, it is used as one of the markers for stemness.[82] ALDH1A1 isoenzymes converts retinaldehyde to retinoic acid (RA) which is a transcription regulator which has been shown to regulate tumor growth and metastasis in breast cancer cell lines.[83] In MCF7 cells higher levels of ALDH1A1 mRNA were detected, and when ALDH1A1 was overexpressed, this was associated with an increase in CD133 and Krüppel-like factor 4 (KLF4) in tumor spheres suggesting a role in increasing stemness.[82] Furthermore, mRNA and protein levels of VEGF were high in MCF7 cells which had high expression of ALDH1A1,[82] highlighting the association between stemness and angiogenesis. When MCF7 cells with high ALDH1A1 activity were transplanted in mice, they were associated with tumor formation and a high Ki-67 index (~70%), increased mRNA levels of Sox 2 and octamer 4 (OCT4), and protein levels of CD133, SOX2, KLF4 were high.[82] These findings highlight the role of stemness in promoting tumor progression in mice.

BCSCs also express EpCAM (also known as CD326), epithelial-specific antigen (ESA), and E-cadherin.[84,85] EpCAM promotes cell adhesion, proliferation, and invasion in BCSCs through diverse signaling pathways.[86] Other breast cancer stem cell markers identified include CD47, CD166, CD61, ATP-binding cassette super-family G member 2 (ABCG2), and leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5).[87] This shows the heterogeneous landscape in breast cancer and thus attributing the complex changes seen to a distinct breast cancer population ($CD44^{+}CD133^{+}CD24^{-}$) becomes questionable, but rather suggests the existence of a vast array of cells displaying heterogeneous features of stemness. These cells may describe the heterogeneity seen within the tumor microenvironment and the association with the various signaling pathways is involved in mediating tumorigenesis, tumor growth, and survival of breast cancer stem cells, including wnt/β-catenin, NFkB, BMP2, Notch, STAT3, Hedgehog.[8] Some studies have shown that CD44 may not be a suitable marker for characterizing BCSCs in luminal cancers, but this may be a factor of the vast stemness landscape.[81]

Malignant cells exhibit characteristics of stemness defined as the ability of a cell to expand its lineage, give rise to differentiated

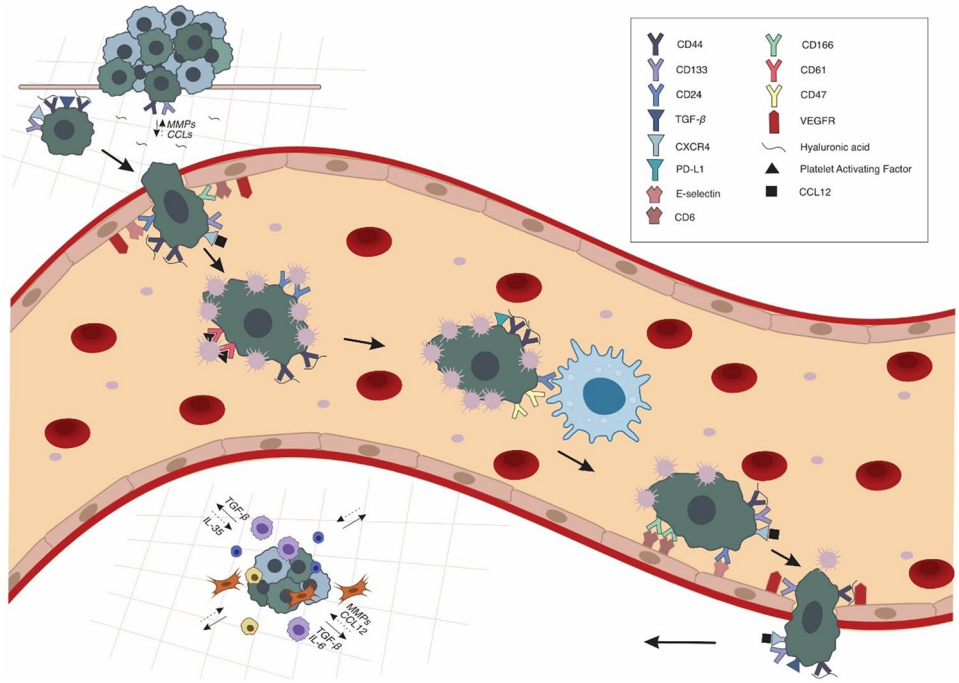

Figure 2. Diagram illustrating the transient expression of cluster of differentiation (CD) surface markers during stem-like tumor cell migration. The metastatic capacity of stem-like tumor cells is dependent on their molecular and phenotypic plasticity. The adaptive response of these cells to signals from the tumor microenvironment is a key mechanism in promoting breast tumor progression. As the primary tumor outgrows its blood supply, the activation of HIF-1 directly upregulates the expression of CD44 and CD133 surface markers in stem-like tumor cells. Activation of CD44 through its binding to hyaluronic acid (HA) is a key mediator of breast tumor metastasis. Initially, the CD44-HA complex facilitates EMT in tumor cells by promoting the upregulation of TGF-β signaling. This results in the acquisition of a mesenchymal-like morphology. Thereafter, CD133 works in concert with CD44-HA to promote the secretion of MMPs to enable migration through the ECM. Alongside the increased expression of CD44 and CD133, stem-like cells present with low levels of CD24. This exposes CXCR4 on tumor cells which enables its binding to CCL12. This activation of CXCR4 is further increased via its interaction with CD133 which aids in guiding migratory tumor cells through the vascular system. At the sites of intra- and extravasation, tumor cells attach to endothelial cells through the binding of CD24 to E-selectin. This adhesion is strengthened by the increased expression of CD166, which binds CD6 on endothelial cells. Entry to and exit from the blood vessel lumen is facilitated by the interactions of CD44-HA and CD133 with VEGFR on endothelial cells. Circulating tumor cells continuously adapt to evade immune attack. The upregulation of CD61 expression promotes rapid platelet activation. Activated platelets express P-selectin, which binds CD24 on tumor cells thereby shielding them from immune attack. Additionally, the interaction of CD24 with Siglec-10 on macrophages acts to inhibit tumor cell phagocytosis. Other molecular mechanisms of immune evasion include the upregulated expression of CD47 and PD-L1, which is facilitated by CD44-HA. These stem-like tumor cells are guided to secondary sites in response to the chemoattractant gradient generated by stromal cells at the pre-metastatic niche.

cells, and interact with its environment to sustain a balance between quiescence, proliferation, and regeneration.[88] Cells displaying stemness have an aberrant cell cycle which is prolonged, and cells divide asymmetrically.[89] Cells displaying stemness also show an ability to seed into new tumors as shown by their growth properties which form tumor spheres/spheroids and the ability of stem cells seeded in mice to form tumors.[81] These findings point to the role of stemness in mediating tumorigenesis, in addition to mediating progression, metastasis, and recurrence.

Stemness as a Driver for Novel Biomarkers in luminal breast Cancer

Estrogen signaling

Estrogen signaling plays an essential role in the growth and progression of luminal breast cancers, the most common breast cancer subtype constituting over 75% of all diagnosed breast cancers.[90-92] Estradiol (E2) binding to its receptors which include ERα, ERβ, and the membrane G-protein coupled estrogen receptors (GPER, also called GPR30) mediates signaling through genomic and non-genomic pathways.[90-92] Genomic signaling is initiated via ligand binding to the estrogen receptors, subsequent translocation of the complex to the nucleus, and direct interaction with estrogen response elements (EREs) within the promoter region of target genes. Alternatively in genes which lack ERE, activated ERα binds to activator protein 1 (AP1) and specific protein 1 (SP1) through serum response elements (SREs).[9,92] ERβ has a similar structure to ERα and is activated by the same ligands but it has a different binding affinity for E2.[9] ERβ shows opposing functions to ERα such as proliferation inhibition and apoptosis induction depending on the tissue and cell type, transcriptional coactivators, and ERα coexpression.[9] Non-genomic signaling involves the indirect regulation of gene expression through interaction with various intracellular signaling pathways such as PI3K/AKT or Ras/MAPK pathway and amplification of cAMP production.[90-92] Estrogen binding to GPER increases the concentration of cAMP and intracellular calcium levels, thus activating PI3K/AKT and Ras/MAPK signaling pathway, thereby regulating the transcription of c-fos, cyclins (D, B, and A) involved

in the cell cycle, and proliferation.[9,93] Nongenomic actions of ERα were associated with endocrine therapy resistance and poor prognosis.[94] A monomeric state of ERα was shown to promote its non-genomic signaling.[95] Therefore, tumor progression may continue independent of genomic signaling and thus suggesting the role of stemness in mediating these changes.

ERα acts as a transcription factor which regulates cell cycle, proliferation, and apoptosis genes.[9] The expression of MYC, cyclin D1, forkhead box protein M1 (FOXM1), growth regulation by estrogen in breast cancer 1 (GREB1), B-cell lymphoma 2 (Bcl2), or amphiregulin, IGF1 and CXCL12 is induced by ERα activation, these factors promote cell proliferation and elevate DNA damage risk, thereby increasing genomic instability and providing a basis for natural selection. The activity of ERα is also regulated by post-translational modifications (PTMs), independent of ligand binding.[9] These PTMs influence ERα stability, dimerization, subcellular localization, deoxyribonucleic acid (DNA) binding, and interaction with cofactors.[96] The activation of certain intracellular kinases following growth factor stimulation, can lead to phosphorylation of ERα,[97,98] similarly when serine residues 118 (S118), S167, S305, and tyrosine 537 (Tyr 537) were phosphorylated, ERα activity was increased through coactivators.[99-102] Acetylation also leads to ligand independent activation of ERα, acetylation of lysine 266/268 activates transcription whereas acetylation of lysine 302/303 inhibits ERα activity.[102] Further PTMs, palmitoylation of cysteine 447 in ERα was shown to increase its hydrophobicity and anchored it to caveolae in the plasma membrane.[103] Methylation of arginine 260 on ERα by protein arginine methyltransferase (PRMT1) is required for Src and P85 pathways.[94,96,104]

Estrogen plays a diverse role in mediating stemness.[73,105] Estrogen was shown to promote CSC properties in ER+ breast cancer cell lines through inducing transcription of Gli1 despite the downregulation of ER,[73,105,106] suggesting non-ER mediated effects. Estrogen was also shown to increase the expression of vimentin, a mesenchymal cell marker and decreased the expression of E-cadherin thus possibly mediating EMT and promoting stemness.[107] BCSCs have also been shown to be enriched following administration of endocrine therapy and are implicated in mediating resistance.[108] Endocrine resistance occurs in at least two thirds of patients with ER+ breast cancer patients. Tamoxifen-resistance and poor prognosis was indicated by P21-activated kinase 4 (PAK4) in breast cancer tumors, inhibition of PAK4 reduced cancer stem cell activity and restored sensitivity to endocrine treatment.[109] Further highlighting the role of stemness in facilitating endocrine resistance.

Reciprocal interactions between estrogen signaling and stemness

The cyclin-dependent kinase (CDK) 4/6 pathway was targeted for therapy in a clinical trial (PALOMA-1/TRIO-18) of advanced luminal breast cancer using a CDK 4/6 inhibitor (Palbociclib) combined with an aromatase inhibitor (Letrozole),[110,111] due to the role of CDK 4/6 in facilitating cell proliferation, migration, and angiogenesis.[112] This study (PALOMA-1/TRIO) found that the drug combination improved disease-free survival.[110] An *in vitro* study showed that a luminal breast cancer cell line (MCF7) acquired stemness features shown by elevated gene and protein expression of ALDH1, OCT4, $CD24^-$, and $CD44^+$ phenotype.[113] This stemness facilitated resistance to CDK4/6 inhibitors (Palbociclib). However, improved sensitivity to Palbociclib could be accomplished by silencing 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (*PFKB4)* which mediates metabolic rewiring by regulating enzymes involved in glycolysis and glucose metabolic reprogramming.[113] Metabolic rewiring is a hallmark of cancer that facilitates how cells use energy.[114] Despite the clinical benefit of targeting CDK 4/6 pathway in luminal tumors, presentation of stemness as the tumor progresses could limit the efficacy of CDK 4/6 inhibitors. These findings suggest that stemness may mediate resistance to various targets within the tumor and highlights the utility of a multi-target approach in treatment strategies.

Estrogen was shown to promote the development of stem cells indirectly, mediated through paracrine signaling.[115,116] 17-β-estradiol treatment of MCF7 and T47D cell lines increased the proportion of cancer-stem like cells which expressed $CD44^+CD24^-ESA^+$.[73] In this subpopulation in MCF7 cells, ~70% of cells expressed ERα receptors with 20% to 25% showing nuclear ERα staining; however, reduced receptor expression levels were noted compared to parent population.[73] Conditioned media from MCF7 cells was shown to increase the proportion of stem cells expressing $CD44^+CD24^-ESA^+$ in ER^- breast cancer cell lines (SUM149, SUM159, BT-20).[73] This conditioned media contained elevated levels of fibroblast growth factors (FGF2/bFGF, FGF4, FGF6, FGF7, FGF9).[73] Estrogen and FGF were thus shown to have a synergistic effect. This was further highlighted by inhibition of FGF signaling using PD173074 which impeded the ability of MCF7 cells pre-treated with 17-β-Estradiol to initiate tumors when injected into mice.[73] Similar findings were shown in primary cultures from breast cancer patients whereby sphere formation was reduced following inhibition of FGF.[73] Tbx3, which mediates FGF and Wnt signaling, increased in response to estrogen treatment in MCF7 cells and reduced in response to FGF-inhibition. Silencing of *Tbx3* in MCF7 cells resulted in a decrease in sphere formation and stem cells expressing $CD44^+CD24^-$ and epithelial specific antigen (ESA), suggesting that *Tbx3* is involved in maintaining stemness. Corresponding clinical data showed that *Tbx3* was upregulated in breast cancer compared to normal tissue, and associated with ER expression. This association was further identified with metastasis occurrence at 3 and 5 years.[117,118] These findings highlight the relationship between mediators of stemness and estrogen signaling.

ERα signaling also indirectly activates integrin β4 signaling,[119,120] which mediates various signaling pathways involved

in cancer progression.[121] The binding of integrin β4 to laminin activates (PI3K) AKT pathway which leads to cell proliferation, survival, and invasiveness.[121] Integrin β4 transcription can be mediated by delta Np63,[121] the expression of which is high in breast cancer and correlated with poor prognosis.[122,123] In addition to promoting tumor progression, delta Np63 mediates Notch signaling, a commonly upregulated pathway in stem cells.[121] The impact of estrogen signaling on these parameters has been demonstrated *in vitro*, where estrogen treatment in MCF7 increased the delta Np63 mRNA and protein, this was associated with cell viability and motility.[121] Subsequent knockdown of delta Np63 decreased cell migration suggesting it may play a role in facilitating tumor dissemination. These findings also suggest that delta Np63 may be involved in promoting endocrine therapy resistance, which was shown to occur without the loss or changes in the expression of ER.[124]

Myc, a transcription regulator dysregulated in 30% to 50% of advanced breast cancers shows different alterations in breast cancer subtypes,[125] it is increased in TNBC; however, in luminal breast cancer while not high, Myc nevertheless influences estrogen and progesterone signaling as shown via the KEGG database analysis.[126] Using the TCGA database, Myc was shown to play a role in mediating stemness through correlation with CD44, CD133, CD29, ALDH, and EpCAM.[126] Through interaction with cancer-associated fibroblasts within the TME, Myc also participates in tumor initiation, releasing factors such as IGF and insulin-like growth factors binding protein-6 (IGFBP-6).[127] As a transcription regulator, Myc mediates DNA synthesis and genomic instability acting on cyclin D1, cyclin A, cyclin E, and cdc25A which phosphorylate CDK2/4.[126] Furthermore, Myc was shown to increase stemness and facilitate EMT by activating *SNAIL, ZEB1, ZEB2* and promoting epigenetic regulation of lysine-7q (methylation) and H3 (acetylation) through its interaction with DOTIL and P300.[128]

Angiogenesis is essential for tumor survival, growth dissemination, and formation of the pre-metastatic niche.[82] During angiogenesis in the TME new aberrant blood vessels are formed through the growth of endothelial cells,[114] and stromal cells in response to the secretion of angiogenic factors (such as VEGF, IL8) by endothelial cells, tumor cells, and CSCs.[82] Hypoxia is associated with tumors and stemness and acts as a signal to initiate angiogenesis.[82]

Discussion

In our efforts to continually improve diagnosis and treatment strategies in cancer, it is becoming increasingly important to consider that tumors are subject to evolutionary forces which are the underlying factors that drive intratumoral heterogeneity characterized by stemness. This may help to better predict tumor behavior and response to therapy at different stages of the diseases. There is a need to identify novel biomarkers in luminal breast cancer which may improve treatment outcomes and reduce endocrine resistance. A better and in-depth understanding of the intersection and reciprocal interactions between estrogen signaling and other signaling pathways activated in cells displaying stemness will contribute to the identification of these novel markers. Thus far, studies point to the involvement of cell cycle regulators and the non-genomic signaling effects of ERα, which enables it to be involved with various signaling pathways mediating cell survival. The tumor microenvironment is also important to consider because of its role in mediating EMT, tumor progression, and stemness of subpopulations of tumor cells. In addition to the hormone receptors that define the luminal phenotype, the identification of diverse biomarkers which target different aspects of the tumor, including intrinsic cell factors (e.g., cell cycle components, transcription factors, receptors which are targets for non-genomic signaling, etc.) and microenvironmental factors may better predict tumor prognosis. Identification of such biomarkers and the tracking of clinical outcomes may lead to the development of more targeted treatment strategies. The scope of this review briefly discussed the intersection between stemness markers and tumor signaling pathways, as such, a further in-depth analysis is warranted to describe the mechanism of these interactions in more detail.

Conclusion

The studies reviewed in this paper indicate that the presentation of stemness was not associated with loss of the luminal phenotype, and that non-genomic effects of estrogen may mediate or form a bridge between the luminal phenotype and stemness induction which promotes EMT, tumor dissemination, treatment resistance, and tumor relapse. Endocrine therapy remains an important and relevant treatment strategy, but there is an opportunity to limit endocrine therapy resistance and tumor recurrence, which typically present much later in luminal subtypes than more aggressive breast cancer subtypes. Further studies are needed to investigate multi-treatment approaches involving endocrine drugs and targets of stemness.

List of abbreviations

AI: aromatase inhibitors
ALDH: aldehyde dehydrogenase
Ang-1: angiopoietin-1
Ang-2: angiopoietin-2
AP1: activator protein 1
BCSC: Breast cancer stem cells
CDK: cyclin dependent kinase
CERANS: Complete estrogen receptor antagonists
CK: cytokeratins
CSC: Cancer stem cells
CXCR4: chemokine receptor 4
E2: Estradiol
EGFR: epidermal growth factor receptor
EMT: epithelial-to-mesenchymal transition

EpCAM: epithelial cell adhesion molecule
ER: estrogen receptor
ERE: estrogen response elements
ESA: epithelial-specific antigen
FGF: fibroblast growth factors
FN1: fibronectin 1
GPER: G-protein coupled estrogen receptors
GREB1: growth regulation by estrogen in breast cancer 1
IGFBP-6: insulin-like growth factors binding protein-6
IL: interleukin
KLF4: Krüppel-like factor 4
Lgr5: leucine-rich repeat-containing G-protein coupled receptor 5
MDSC: myeloid-derived suppressor cells
MHC: major histocompatibility complex
MMP: matrix metalloprotease
NK: natural killer
OCT4: octamer 4
OPN: osteopontin
PAK4: P21-activated kinase 4
PFKB4: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4
PFS: progression free survival
PR: progesterone receptor
PRMT1: protein arginine methyltransferase 1
PTM: post-translational modifications
RA: retinoic acid
scRNA-seq: single cell ribonucleic acid sequencing
SERCA: Selective estrogen receptor covalent antagonists
SERD: selective estrogen receptor degraders/ downregulators
SP1: specific protein 1
SRE: serum response elements
TGF-β: transforming growth factor beta
TNBC: Triple Negative Breast Cancer
VEGF: vascular endothelial growth factor
VIM: vimentin

Declarations

Ethical Approval

Ethical approval was not applicable because this study is a review and is based exclusively on published literature.

Consent to Participate

Not applicable as it is a review based on published literature.

Informed Consent

Not applicable.

Consent for Publication

Not applicable as it is a review based on published literature.

Author Contributions

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Competing Interests

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Availability of Data and Materials

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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