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# Breast cancers, mammary stem cells, and cancer stem cells, characteristics, and hypotheses ☆,1,2

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## Abstract

The cellular heterogeneity of breast cancers still represents a major therapeutic challenge. The latest genomic studies have classified breast cancers in distinct clusters to inform the therapeutic approaches and predict clinical outcomes. The mammary epithelium is composed of luminal and basal cells, and this seemingly hierarchical organization is dependent on various stem cells and progenitors populating the mammary gland. Some cancer cells are conceptually similar to the stem cells as they can self-renew and generate bulk populations of nontumorigenic cells. Two models have been proposed to explain the cell of origin of breast cancer and involve either the reprogramming of differentiated mammary cells or the dysregulation of mammary stem cells or progenitors. Both hypotheses are not exclusive and imply the accumulation of independent mutational events. Cancer stem cells have been isolated from breast tumors and implicated in the development, metastasis, and recurrence of breast cancers. Recent advances in single-cell sequencing help deciphering the clonal evolution within each breast tumor. Still, few clinical trials have been focused on these specific cancer cell populations.

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## Introduction

The mammary gland is a highly dynamic organ that undergoes multiple phases of remodeling. Both local and systemic signals trigger the mammary epithelium's proliferation and differentiation during each estrus cycle and pregnancy [1]. These changes are choreographed by a hierarchical array of mammary stem cells (MaSCs) and progenitors typically involved in the homeostasis of the organ but also promoting the elongation and branching of the mammary ducts and development of alveoli in pregnancy [2]. Most breast cancers are not following a mendelian inheritance pattern, and are thought to originate from a single clonal lineage, after a succession of independent mutational events over a long period [3–5]. The diversity of the breast cancers has been linked to the cell of origin, while the cellular heterogeneity of the tumors originates in the nature of the mutations [6]. Two nonexclusive models have been proposed to explain clonal populations in the tumor, the first model involves the stochastic appearance of mutations and clonal selection that grant the cells stem-like properties and ability to differentiate and self-renew [7]. In the second model, the MaSC and progenitor attributes are central to the heterogeneity of the breast cancer cell populations [8]. In this review, we have attempted to provide the latest data about breast cancer incidence, risk factors, heterogeneity, and classification. We also discuss the breast's stem cell populations and their relevance to cancer stem cells (CSCs) and cancer development. Finally, we present the contribution of single-cell sequencing (SCS) in the CSCs' characterization and few therapeutic initiatives to target these small cellular populations.

## Incidence of Breast Cancers

Breast cancer is one of the leading causes of cancer for women worldwide accounting for 2088,849 (11.6% of all cancers) and 626,679 deaths (6.6% of all cancer-related deaths) in 2018 [9]. The incidence rate of breast cancer varies deeply between females and males and is nearly 100 times lower

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☆ *Abbreviations*: ALDH, aldehyde dehydrogenase, BCSC, breast cancer stem cell, CK, cytokeratin, CNA, copy numbers aberrations, CSC, cancer stem cell, CTC, circulating tumor cell, DCIS, ductal carcinomas in situ, EGFR, epidermal growth factor receptor, EMT, epithelial-mesenchymal transition, EpCAM, epithelial cell adhesion molecule, ER, estrogen receptor-α, IntClust, integrative clusters, MaSCs, mammary stem cells, MET, mesenchymal-epithelial transition, PgR, progesterone receptor, SCS, single-cell sequencing, TEB, terminal end bud, TNBC, triple-negative breast cancer.

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than women [10]. Furthermore, male breast cancers are usually diagnosed at advanced stages 3 or 4, imputable mainly to a lack of awareness [11]. The 5-year overall survival rate is also lower for men (77.6%) when compared to women (86.4%) [12].

Albeit studies remain scarce, the same discrepancy is observed for transgender male to female, where breast cancer is usually diagnosed at a younger age 51.5-year old while the incidence of breast cancer is increased by 46-fold when compared to male [13,14]. The transition from male to female relies on antiandrogens and estrogen therapies, which increase the risk of breast cancer [14]. A younger age of diagnosis at 44.5 years of age is also observed for individuals transitioning from female to male [15]. If bilateral nipple-sparing mastectomies are performed in the surgical transition to men, the risk of breast cancer is decreased with a standardized incidence ratio of 0.3 when compared to the natal females [13]. However, if any breast tissue is conserved, the risk of cancer increased and is similar to natal females for transgender men who did not undergo top surgery [15].

The incidence and mortality rates of breast cancer in women are mainly influenced by the geographical location and the socioeconomic status [16]. High-income countries in North America, Oceania, and Western Europe account for higher rates. In contrast, middle–low-income countries within South America, Eastern Africa, and South-Central Asia had the lowest number of women diagnosed [9]. Early detection programs, including regular mammography programs, have contributed to a decrease in breast cancer mortality in high-income countries [16,17]. However, the lower-incidence observed in middle-low and low-income countries is hampered with a higher cancer-related mortality rate partially due to the late diagnosis and lack of health care resources [18].

## Breast Cancer Risk Factors

Breast cancer is a complex disease arising from the association and cumulation of multiple genetic alterations and environmental factors likely to alter cellular functions. No single somatic or germline mutation or exposure to a specific agent can fully predict the developmental course of breast cancer. A prospective study, the Nordic Twin Study of Cancer, involving monozygotic and dizygotic twins in Northern Europe demonstrated that the estimated heritability of breast cancer was 31% (11–51, 95% CI), while the effect of a shared environment was estimated at 16% (0–31, 95% CI) [19]. The genetic susceptibility is driven by rare highly penetrant autosomal dominant genes such as BRCA1 and BRCA2 [20], as well as moderate-risk variants such as CHEK2 [21], ATM [22], and PALB2 [23], and common low-risk variants mostly represented by single nucleotide polymorphisms [24]. Additional risk factors have been associated with an increased risk of developing breast cancer. The incidence of most cancers increases steeply as people aged. The vast majority of breast cancers are diagnosed over the age of 50 and aging has been is associated with the accumulation of genetic, epigenetic, and hormonal alterations [25,26]. Several other factors have been associated with an increased occurrence of breast cancers including mammographic density [27], reproductive factors (early age of menarche, late menopause, low parity, late age at first birth [28,29]), anthropometric features (height [30] and body mass index for postmenopausal women [31], hormone replacement therapy [32], oral contraceptive pills [33], exposure to diethylstilbestrol [34], previous exposure to radiation therapy [35], lifestyle factors (physical activity [36], alcohol consumption [37], night shift [38]), and exposure to various chemicals [39].

## Breast Cancer Heterogeneity

### *Histological Classification*

The heterogeneity of breast cancer, as in many other cancers, constitutes the main hurdle for efficient management in the clinic. Breast cancer can be broadly categorized based on their histology into either carcinoma in situ or invasive carcinoma. The carcinoma in situ is further divided into lobular or ductal, both confined within either the milk gland (lobule) or milk duct, respectively. The carcinoma in situ represents 15% to 30% of the newly diagnosed breast cancers, and 4 out of 5 are ductal carcinomas in situ (DCIS) [40]. Both forms of carcinoma in situ, if untreated, increase the risk of developing invasive breast cancer. The coexistence of DCIS and invasive carcinoma within the same lesion suggests that DCIS is a nonobligate precursor of invasive carcinoma of no special type [41,42]. Multiple models have been proposed for the evolution of DCIS to invasive carcinoma [42]. The lack of understanding of which DCIS lesion is likely to become invasive is still a significant challenge and heavily associated with patient overtreatment [42]. Invasive breast carcinomas are also comprised of diverse histological groups of tumors that differ by their biomarkers, development patterns, and predictive patient outcomes. The World Health Organization has recently updated the morphological classification of invasive breast carcinoma in its new edition [43,44]. Invasive carcinoma of no special type lacks the features of the other subtypes and represents most of the invasive carcinoma cases (>70%); less frequent are the invasive lobular carcinoma, microinvasive carcinoma, and various rarer subtypes [43,44].

### *Molecular Classification*

#### *Immunohistochemical Profiling*

The treatment decision of breast cancer is increasingly personalized and guided by established criteria such as the tumor morphology, grade, and stage, the expression of estrogen receptor-$\alpha$ (ER), progesterone receptor (PgR), and epidermal growth factor receptor-2 (HER2 or ERBB2) as well as a complex and unique profile of genes and pathway alterations [45]. The assessment of the expression of ER, PR, and HER2 is a routine procedure to guide hormonal and anti-Her2 treatments as well as to predict patient outcome. ER-positive tumor accounts for 75% of all breast cancer cases, while PgR-positive tumors represent 55% to 65% of all breast cancers [46]. PgR exists as 2 isoforms, A or B, and PgRB being the full-length protein [47]. A high PgRA over PgRB ratio predicts a poor response to endocrine therapy and patient prognosis [48]. Tumors are interpreted as ER or PgR positive if more than 1% of the tumor nuclei are stained [49]. The amplification of HER2 gene and/or the overexpression of HER2 protein are observed in 15% to 20% of breast cancers as measured by either fluorescence in situ hybridization or immunohistochemistry, respectively [50]. The 2018 guidelines from the American Society of Clinical Oncology for the testing of HER2 established 5 groups to guide treatment decision based on the HER2 gene copy number of 4 or a HER2/CEP17 ratio of 2 and immunohistochemistry score of 2+ (membrane staining observed in more than 10% of the tumor cells) [51].

#### *Hierarchical Clustering Of Intrinsic Subtypes*

A hierarchical clustering approach categorized breast cancers into several intrinsic subgroups, including luminal A, luminal B, HER2-enriched, and basal-like [52,53]. These subgroups diverge by the etiology of cancer, onset age, and patient predictive prognosis.

Luminal A and B subtypes are the most frequent and are characterized by a higher expression ER, ER-related genes, PgR, and other genes [53]. Luminal A breast cancer patients have a better prognosis, are generally responsive to endocrine therapy, and a lower risk of recurrence [54]. However, nearly 40% of ER-positive tumors are PgR negative, a profile associated with increased resistance to endocrine therapy and a worsen patient prognosis [55]. Luminal A and B breast cancers account for the highest number of genes mutated despite a low mutation rate per tumor (Table 1). The distinction between luminal A and B relies on the lower level of expression of PgR in luminal B while ER expression remains similar, the expression of HER2 in nearly 20% of luminal B tumors, and the upregulation or amplification of genes associated with cell differentiation and adhesion in luminal A or genes associated

**Table 1**

**Breast cancer classification and main genetic alterations.**

| Subtypes (Frequency) | ER-α, PR, HER2 Status | Ki67 | Somatic Mutations and Molecular Alterations | Morphology | DNA Ploidy and Chromosomal Instability (CIN) | Treatment | METABRIC IntClust | Reference |
|---|---|---|---|---|---|---|---|---|
| Luminal A (~40–45%) | ER (+) and/or PgR (+) (high expression) HER2 (-) | Less than 20% | Frequently mutated (>10%): GATA3, MAP3K1, PIK3CA, and p53. Other less frequent mutations: AFF2, AKT1, CBFB, CCND3, CDH1, CDKN1B, CTCF, DGKG, FOXA1, GPS2, GPR32, HIST1H3B, HIST2H2BE, KRAS, MAP2K4, MED23, MLL3, NCOR1, NF1, NKAIN4, PI3KR1, PTEN, PTPN22, PTPRD, RB1, RUNX1, SF3B1, SHD, SMCHD1, TBL1XR1, TBX3, WNT7A. Increased copy number: 1q and 16p. Decreased copy number: 16q. High-level amplifications: 8p11-12, 8p13, 8q22, 8q24, 11q13-14, 12q13-14, 17q11-12, 17q21-24, and 20q13. | Well differentiated carcinomas of no special type. Low grade | High frequency of diploid/CIN (65%). Moderate aneuploid/CIN+ status (28%) | Endocrine therapy sensitive, poor response to chemotherapy. Recurrence risk is low. Metastasis | 2, 3, 4, 5, 6, 7, and 8 | [54,57–63] |
| Luminal B (~20–25%) | ER (+) and/or PgR (+) (low expression) HER2 (-) or ER (+) and/or PgR (+) HER2 (+) (20% of luminal B) | Ki67 more than 20% | Frequently mutated (>10%) PIK3CA, p53, and GATA3. Other less frequent mutations: AFF2, AKT1, CBFB, CDH1, CDKN1B, CTCF, FOXA1, HIST1H3B, KCNB2, MLL3, NCOR1, NF1, OR2L2, PI3KR1, PRRX1, PTEN, PTPN22, PTPRD, RB1, RUNX1, TBX3. Increased copy number: 1q, 8q, 17q, and 20q. Decreased copy number: 1p, 3q, 8p, 13q, 16q, 17p, and 22q. High-level amplifications: 7p22, 8p11-12, 8q11-14, 8q22, 8q24, 11q13-14, 17q23, 19q13, and 20q13. | Higher grade than luminal A. | High frequency of diploid/CIN in 41% of HER2 (-) subtype, and 12% of HER (+) subtype. Moderate aneuploid/CIN+ status in (49.5%) in HER2 (-) subtype, and 81% in HER2 (+) subtype. | Endocrine therapy combined with chemotherapy and anti-HER2 therapy for HER2(+) luminal subgroup. Relatively endocrine resistant. Recurrence risk high in the first 5 y after diagnosis. Metastasis to the bone and to the lung to a lesser extent. | 1, 2, 5, 6, and 9 | [54,57,58,60–63] |

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**Table 1 (*continued*)**

| Subtypes (Frequency) | ER-α, PR, HER2 Status | Ki67 | Somatic Mutations and Molecular Alterations | Morphology | DNA Ploidy and Chromosomal Instability (CIN) | Treatment | METABRIC IntClust | Reference |
|---|---|---|---|---|---|---|---|---|
| HER2-enriched (∼10–15%) | ER (-) and/or PgR (-) HER2 (+) HER2 is either overexpressed or gene amplified. | Ki67 more than 20% | Frequently mutated (>10%) PIK3CA, p53. Other less frequent mutations: AFF2, AKT1, CBFB, CDH1, CDKN1B, CTCF, FOXA1, GATA3, MAP3K1, MAP2K4, MLL3, NF1, PI3KR1, PTEN, PTPN22, PTPRD, RB1, RUNX1, SF3B1, SRPR. Increased copy number: 1p36.33-p36.32, 1q, 4q13.3, 5p15-p12, 7p, 8q23.3-q24.21, 11q13.5-q14.1, 14q11.1-q11.2, 17q23-q24, and 19q1216p, and 20q. Decreased copy number: 1p39, 1p36, 1p35, 1p32, 4p16.3, 7q21-q22, 7p22.3, 7q34, 7q36.1-q36.3, 8p23.3-p23.2, 8p11.23-p11.22, 9p21.3, 9q34.3, 10q26.3, 1q13.5, 11p15.5, 13q, 14q32.33, 15q11.2, 16p13.3, 18q and 19p13.31p. High-level amplifications: 4q13.3, 8q23.3-q24.21, 11q13.5-q14.1, 14q11.1-q11.2, 16q22, 17q12-q21, and 19q12. | High histological and nuclear grade. | Low frequency of diploid/CIN (5%). High aneuploid/CIN+ status (90%). | Chemotherapy and anti-HER2 treatment. | 5 | [54,58,60–63] |
| Basal-like (∼15–20%) | ER (-) and/or PgR (-) HER2 (-) | Ki67 more than 20% | Frequently mutated (>10%) PIK3CA, p53. Other less frequent mutations: AFF2, C11orf85, CTCF, HLF, GATA3, MLL3, NCOR1, NF1, PTEN, PNPLA3, PTPRD, RB1, SF3B1, TBX3. Increased copy number: 1q12-q41, 3q, 6p12-p25, 7p12, 7q22-q36, 8q23.2-24.3, 10p12-p15, 12p, 17q25, and 21q22. Decreased copy number: 3p, 3q12, 4p15-p32, 4q31-q35, 5q11-q31, 8p, 12q14-23, 13q, 14q22-q23, and 15q. High-level amplification: Rare. | High grade. High mitotic index. Chemotherapy sensitive. | Moderate frequency of diploid/CIN (17%). High aneuploid/CIN+ status (77%). | Chemotherapy High incidence of recurrence | 4 and 10 | [54,58–64] |

with cell proliferation and immune response in luminal B which roughly define each subtypes [54,56]. Luminal B is also characterized by a higher proliferation rate with a Ki67 expression cut off established at 20% [45]. Also, the luminal subtypes differ by the frequency of the gene mutated, such as PI3KCA and MAP3K1, more frequently mutated in luminal A subtype, while p53 mutation frequency was higher in luminal B or by unique somatic gene mutations (Table 1) [54].

The diversity of the mutation landscape may explain the difference in tumor physiology, clinical behavior, and patient outcome. The copy number aberrations (CNAs) are also a driving feature of breast cancer subtypes. The telomeric amplicon 8p11-12 has been reported in 10% to 15% of breast cancers and essentially observed in luminal subtypes [62]. Amplicon can consist of several megabases and contain multiple genes and frequently observed in cancers. The amplicon 8p11-12 encompasses over 50 known genes such as the oncogene ZNF703, a specific driver for luminal B subtype [65,66] as well as FGFR1, ADAM9, and IKBKB which are amplified mainly in luminal A and to a lesser extent luminal B subtypes [62]. Additional CNAs, including increased copy number, decreased copy number, and high-level amplifications, have been reported in luminal subtypes, as shown in Table 1. The differential gene expression between luminal A and B is also associated with a distinct metastasis homing pattern. Luminal A subtype metastases are mainly observed in the bones and less frequently in the lung and liver, while luminal B metastases are observed in the bones but also frequently in the lungs and lesser in the liver and brain [67].

The HER2-enriched subtype is characterized by the amplification of the ERBB2 gene, a strong oncogenic driver located on the large amplicon 17q12-21 [68]. The overexpression of genes located on the same amplicon such as GRB7, MED1, CDC6, TOP2A, and MAPT are also likely to influence the tumor development and response to therapy when associated with HER2 [62,69,70]. The dependency of these tumors on HER2 expression has raised interest in actionable therapeutic targets. The humanized anti-HER2 monoclonal antibody, Herceptin (trastuzumab), was the first approved by the Food and Drug Administration in 1998 and gave a significant 10-year disease-free survival advantage to patients when combined to chemotherapy [71]. In addition to the amplicon 17 q, HER2-enriched subtype is characterized by numerous CNAs and mutations (Table 1). PIK3CA gain of function mutations and p53 tumor suppressor inactivation mutations are the most common, observed 39% and 72%, respectively [72]. The diagnosis of PIK3CA mutations could guide treatment decision [73], while p53 mutations are associated with a worsen patient prognosis in the Her-enriched subtype [74].

The basal-like subtype is a heterogeneous group of tumors broadly defined by the high expression of basal-like markers such as cytokeratin (CK) 5/6, 14, 17, and/or epidermal growth factor receptor (EGFR) and the lack of expression of ER and PR, and absence of overexpression of HER2; although 15% to 45% of basal-like tumors expressed either ER, PR, or HER2 [75,76]. This subtype accounts for 15% to 20% of invasive cancers and regroups tumors morphologically, genetically, and clinically diverse [57]. Also, within the basal-like subtype, several subgroups were identified and categorized based on their gene expression profile, each implementing a distinctive patient prognosis pattern [77]. Generally, the basal-like breast cancer patients have a worse patient prognosis and outcome, also initially responsive to chemotherapy, relapse is frequently observed [78].

Basal-like subtype diagnosis is also more frequent in young premenopausal women, and its incidence varies across ethnicities as more frequently observed with African American women [79,80]. The basal-like subtype is characterized by a rapid onset and by tumor cells with a high mitotic index and high Ki67 expression [75,81]. Basal-like tumors are also described by a higher rate of allelic imbalance, including high copy number variations but rare high-level amplification (Table 1) [75]. The locus 5q11, which encompassed many tumor suppressor genes MSH3, RAD17, APC, RAD50, and XRCC4, is lost in 100% of basal-like tumors [75]. The mutation rate is also higher in basal-like subtype when compared to others. Frameshift or nonsense mutations in the p53 gene were observed in 80% of basal-like subtype tumors while activating PIK3CA mutations were the second most frequent only observed in ∼9% of this subtype [82]. Furthermore, breast cancer harboring germline mutations in BRCA1/2 genes shared molecular characteristics with triple-negative breast cancer (TNBC), a subgroup of the basal-like subtype [83]. TNBC, deprived of ER, PR, and HER2 expression, represents 80% of the basal-like subtype, and 71% of TNBC are basal-like [60]. BRCA1/2 mutated genes, observed in approximately 10% of TNBC, increase the risk of developing breast cancer by 4- to 6-fold by the age of 70 [84,85]. Besides the BRCA1/2 mutations, TNBC lacks hallmark biomarkers and are further divided based on their gene expression profiles to guide treatment decisions and predict patient outcome. TNBC treatment is limited to chemotherapy, surgery, and/or radiation therapy. TNBC was initially divided into 6 groups as basal-like-1 and -2, mesenchymal, mesenchymal stem-like, immunomodulatory, and luminal androgen receptor, which was later refined to 4 groups, including basal-like 1 and 2, mesenchymal, and luminal androgen [86].

Additional breast cancer subtypes have been identified, including the controversial normal-like subtype observed in 5% to 10% of breast cancers. This subtype is characterized by the expression of a set of genes expressed in adipocytes, a lack of ER, PR, and HER2, but also K5 and EGFR [87]. Studies have also suggested that this subtype may be an artifact due to the high content of normal breast tissue in some tumors (for review, see [88]).

Additional, rare subtypes include claudin-low associated with the low expression of claudin 3, 4, and 7, E-cadherin and CD24 [87], the apocrine subtype characterized by the high expression of androgen receptor and lack of ER [89], the luminal-like subtype regrouped many unclassified tumors [90].

*Integrative Clusters*

In recent years, the integration of genomic and transcriptomic profiles of breast cancers has led to the definition of subtypes with distinctive clinical outcomes and therapeutic approaches. The Molecular Taxonomy of Breast Cancer International Consortium identified 10 subtypes termed integrative clusters (IntClust), each exhibiting nearly similar CNAs and changes in gene expression [91]. The clusters 1, 2, 3, 6, 7, and 8 are expressing ER and associated with the luminal intrinsic subtypes; the IntClust-1 includes the 17q23 amplification and is only observed in luminal B (Table 1) [91]. The IntClusts 4, 5, and 9 are luminal and nonluminal. IntClust-4 is a "CNA-devoid" group, IntClust-5 is characterized by the amplification of the 17q12 amplification including ERBB2 gene, and IntClust-9 characterized by the alterations of the regions 8q and 20q [91]. The IntClust-10 regroups the majority of the basal-like subtype (Table 1) [91].

## Origin of Breast Cancers

The implication of stem cells in the origin and development of breast cancer has been debated for many years. Analogies between stem cells and cancer cells can be drawn on many levels. Both can self-renew and share signaling pathways commonly associated with the replication of stem cells such as Wnt, Bcl-2, sonic hedgehog (shh), and Notch (for review see [92]). SCS has also identified phenotypically diverse clonal populations of cancer cells characterized by stem cell-like profiles [93]. The mutagenesis observed in cancer cells contributes to some extent to their diversity, but it is also likely that this heterogeneity is the result of incomplete or aberrant hierarchical cellular differentiation. These similarities suggested the stem cell origin of the cancer cells, long before the discovery of CSCs in the breast [94,95]. It is also important to underline the cancer cells' heterogeneity as not all cancer cells are stem cells or exhibit properties similar to stem cells.

## *Stem Cell Population of the Breast*

### *Formation of The Mammary Gland*

The epithelium of the mammary gland is composed of apically oriented luminal cells and basally oriented elongated myoepithelial cells in contact with the basement membrane of the ducts and alveoli. The luminal lineage can be further divided into either ER-positive ($ER^+$) or ER-negative ($ER^{neg}$) ductal cells, and $ER^{neg}$ secretory alveolar cells [96].

The mammary glands are highly dynamic tissues and undergo several phases of development during their lifetime. The early stage of development of the mammary glands involved the migration of epidermal cells to form the mammary anlage (for review see [97]). The murine primitive mammary epithelium is observed at embryonic day 11.5 (E 11.5) as a skin placode; at E 15.5, the invagination in the mesenchyme leads to the formation of tubular structures developing in the fat pad by E18.5 before birth at day 21 [97]. In humans, the primary mammary anlage is observed in the 7-week-old embryo and develops in waves to form the fetal mammary gland near the term of pregnancy [98].

In early childhood, mammary ducts are still developing in both sexes, with the formation of several branching terminated by lobules, followed by a period of quiescence lasting until the age of puberty [98]. Although there are many levels of discrepancies between the murine and human mammary gland development, the MaSCs/progenitors share physiological and functional similarities.

The puberty is orchestrated by the sequential secretion of hormones from the hypothalamus, the pituitary, and the ovaries involving gonadotropin-releasing hormones, gonadotropins, and estrogens, respectively, as well as transcription regulators, such as FoxP1, awakening the dormant stem cells to direct the ductal morphogenesis [99,100]. These events stimulate a profound remodeling and proliferation of both mammary cell types leading to the ductal invasion of the female mammary stroma and formation of terminal end buds (TEBs) at the tips of the mammary ducts [99]. At least a few hundred luminal and basal unipotent MaSCs/progenitors are driving the development of a mammary gland during puberty [101]. The hormonal fluctuations during each estrus cycle induce waves of proliferation, leading to the appearance of small alveolar buds, followed by resorption [102,103].

During the pregnancy, a profound rearrangement involving branching and alveologenesis is observed; the mammary gland expansion is orchestrated by hormones such as progesterone, prolactin, and other factors leading to the differentiation of the alveolar epithelium as milk-producing cells [99]. The regenerative potential and expansion of the mammary epithelium have suggested the presence of heterogeneous populations of stem cells and progenitors. Different subpopulations of alveolar cells were characterized, including the lobuloalveolar cells contributing to most of the alveoli [101]. In each alveolus, several MaSCs/progenitors contribute to the luminal and basal clonal populations [101]. These MaSCs/progenitors are also preserved during the involution of the mammary gland to promote the next cycle [101].

### *Mammary Stem Cells*

The existence of MaSCs and progenitors has been inferred by the early experiment of DeOme and colleagues who demonstrated the reconstitution of the entire mammary gland when transplanting diluted mammary cells into cleared murine mammary fat pads [104]. Using a similar approach, a single stem cell population reconstructed the murine mammary epithelium [105]. These cells were characterized as lineage negative ($Lin^{neg}$) ($CD31^{neg}$ (endothelial marker)/$CD45^{neg}$/$TER119^{neg}$ (hematopoietic markers)), integrin-$\beta$1 high ($CD29^{hi}$), integrin-$\alpha6^{hi}$ ($CD49f^{hi}$), and heat stable antigen positive ($CD24^+$) [105]. These cells gave rise to basal and luminal cells and also self-renew; 2 fundamental characteristics of the stem cells [106,107].

Subsequently, many studies have used different cell surface markers to isolate MaSC/progenitor populations in adult mice and human mammary tissue including CD10, CD24, CD29, CD49f, CD49b, c-Kit, THY1, Lrp5, Axin2, CK5, CK8, CK14, CK18, CK19, Lgr5, Lgr6, CD1d, epithelial cell adhesion molecule (EpCAM), Procr, sca-1, Myh11, CD61, and CD133 (Figure. 1) [106,108–116]. The combination of these markers refined the criteria for the MaSCs/progenitors and demonstrated their heterogeneity and specificity during the life cycle of the mammary gland (Figure. 1).

Bipotent MaSCs are proposed to maintain both the luminal and basal cell populations during embryogenesis and expressed markers associated with both luminal such as CK8 and myoepithelial such as CK14 cell lineages [117]. The fetal MaSCs identified in the late embryogenesis are characterized by a high level of expression of CD24 and CD49f [117]. The CD49f protein forms heterodimers with CD29, though most but not all $CD29^{hi}$ cells are also $CD49f^{hi}$ [118]. Lilja et al. identified Notch-1 as a potential double edge factor associated with the maintenance of the bipotent stem cells in the early mouse embryo; but, promoting the luminal progenitor lineage after E15.5 [109]. The Notch-1 receptor is only expressed on the surface of $ER^{neg}$ unipotent luminal progenitor in adult mice [119]. The basal-restricted lineage was also evidenced by the expression of the protein p63, sufficient to promote the switch to basal unipotent MaSC/progenitors [120].

An ongoing debate subsists on the presence of bipotent MaSCs during the puberty and adult life, as the experimental approaches currently available do not provide an unequivocal answer. The transplantation assays, in cleared mammary fat pad, create a physiological environment propitious for lineage-restricted progenitor cells to behave as multipotent entities [110,111,116,121]. Also, methodologies used to pool MaSCs may not provide a pure population and be prone to contamination by other types of cells. Although these nonhomeostatic conditions still reveal a set of cells that responds to physiological cues and adopts a level of genetic plasticity sufficient for them to reverse to a multipotent status, which may also be relevant candidates for oncogenic transformation and highlight the importance of the microenvironment. In contrast, the lineage tracing assays are based on the labeling of established murine stem cell markers, and rely on tissue specific promoters such as CK14, CK5, CK8, and others, to uncover MaSCs progeny in physiological conditions. However, this approach depends on the nature of the promoter, the toxicity of the agent inducing the recombination such as tamoxifen, as well as the nonspecific depletion of cell surface markers due to the enzymatic digestion required for the 3D-imaging preparation [122].

A high-resolution cell fate mapping in 4 different transgenic models identified bipotent MaSCs in the adult mammary gland, actively involved in the maintenance of the ductal architecture [123]. The coexistence of bipotent and unipotent MaSCs in the adult murine mammary gland was also supported by the characterization of bipotent MaSC subpopulation expressing Axin2, a Wnt transcriptional target [111]. Lineage tracing of $Axin2^+$ cells showed that the cells were maintained through multiple pregnancies and demonstrated adjacent $Axin2^+$ luminal and basal cells originating from the same $Axin2^+$ clonal cluster. More recently, the contribution of the Wnt signaling into the development of the adult mammary gland was further demonstrated by the isolation and characterization of a subpopulation of bipotent MaSCs ($CD24^+$/$CD29^{hi}$) characterized by the expression of protein C receptor (Procr), a Wnt target [112]. The $Procr^+$ cells and $Axin2^+$ were largely mutually exclusive in mammary basal cells [112].

The cell fate of the luminal progenitors into differentiated terminal ductal/alveolar luminal cells is determined by the activation of the Notch signaling and the expression of factors, such as breast cancer type-1 susceptibility protein (Brca1), E74-like factor 5 Elf-5 (Elf5), GATA-binding protein 3 (GATA3), ER, PR, leucine-rich repeat-containing G-protein coupled receptor 6 (Lgr6), B lymphocyte-induced maturation protein-1, Sox2, Sox9, and forkhead box protein M1 (FoxM1), promoting distinct

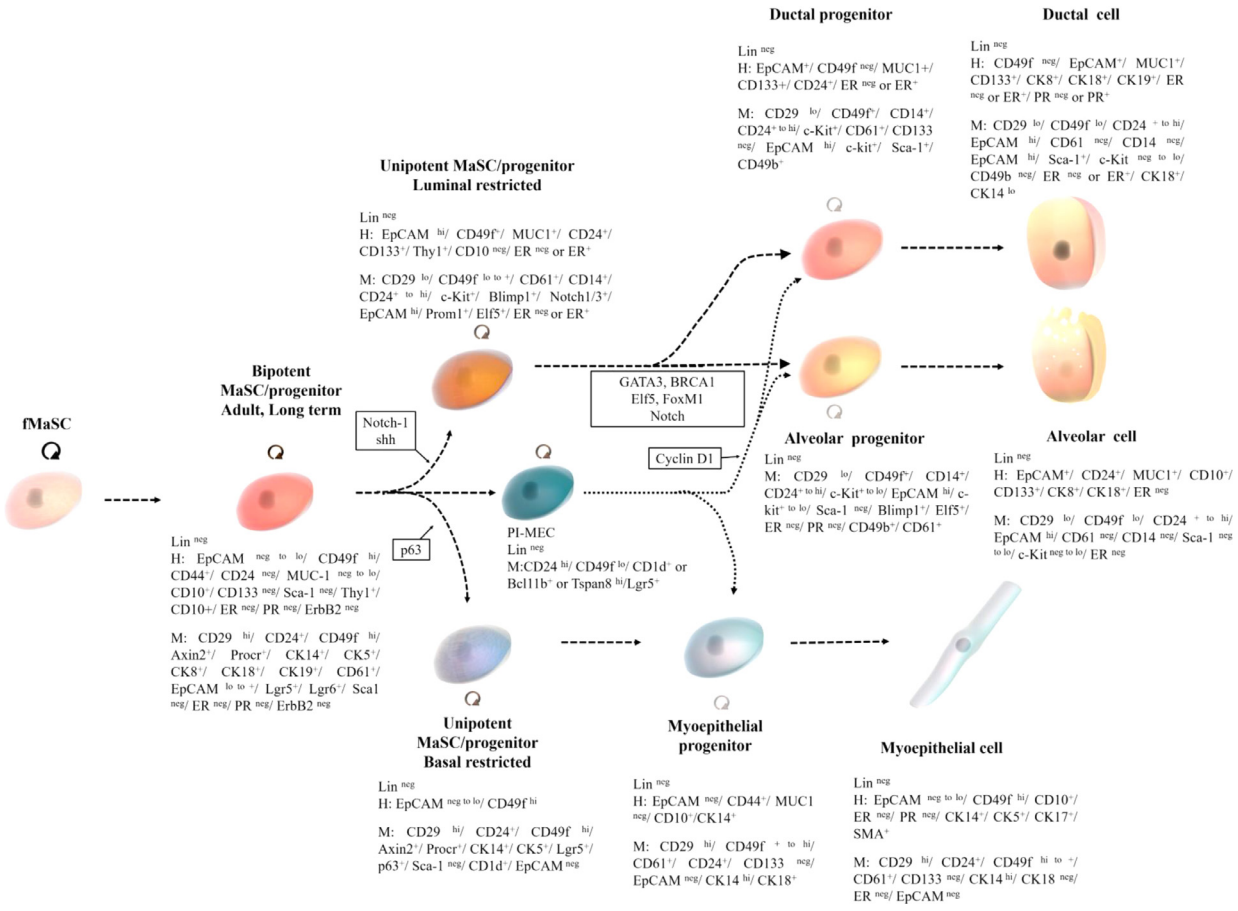


**Figure. 1.** Schematic representation of the hypothetical human and murine breast epithelial hierarchies. A bipotent stem cell gives rise to the myoepithelial and luminal epithelial cell lineage. The renewal potential of the cells is depicted by a circular arrow, which darker color indicates a higher self-renewal potential. Broken arrows hypothesize intermediate states on the path of differentiation. fMaSC, fetal mammary stem cell; LdBCs, luminal-derived basal cells; Lin, lineage; MaSC, mammary stem cell; neg, negative; PI-MEC, parity-identified mammary epithelial cells; +, positive. Adapted from [1,115,118,130,137,142,182,184,211,212].

differentiation potentials [124–131]. Rare populations of highly proliferative luminal progenitors were heterogenous for the expression of ER and PR. The existence of an $ER^+$ luminal progenitor was reported in both murine and human mammary epithelium. Although in humans, these $ER^+$ cells are restricted luminal progenitors (for review, see [108]). During pregnancy, hormone-responsive $ER^+$ and $PR^+$ luminal cells promote the proliferation of $ER^{neg}$ progenitors [1]. The $ER^{neg}$ luminal progenitors associated with the expression of Elf5, a key regulator of alveolar differentiation, are highly proliferative (Figure. 1) [119,123,125].

The basal cell committed progenitors is observed in a subpopulation of MaSCs characterized by the surface receptor $CD24^+/CD61^+/CD29^{hi}$ and enriched by the expression of the transcription factor inhibitor of differentiation 4 [132]. The basal cells are transcriptionally distinct from the luminal cells in the adult; however, the chromatin accessibility to luminal gene loci is identical to the one observed in fetal MaSCs, which may also explain the capacity of basal cells MaSCs/progenitors to regenerate both lineages in transplantation studies [128].

To add to the complexity and diversity of the likely MaSCs populations, the phases of expansion of the mammary epithelium during the reproductive life suggest the recruitment of one or several dormant long-lived MaSC populations entering the cell cycle during each estrus cycle and pregnancy. Three putative dormant stem cell subpopulation with a basis $Lin^{neg}/CD24^+/CD29^{hi}$ or $Lin^{neg}/CD24^+/CD49f^{hi}$ were identified in transplantation experiments and characterized by the expression of a unique cell surface marker either $CD1d^+$, $Bcl11b^+$, or $Lgr5^+$ $Tspan8^{hi}$ [115,133,134]. These long-lived MaSCs/progenitors named parity-induced-mammary epithelial cells can initiate alveologenesis repetitively following each pregnancy [135]. Also, a distinct bipotent MaSC population induced by pregnancy was characterized in mice; these cells called luminal-derived basal cells express basal markers and ER [136].

*Human Mammary Stem Cell Marker and Location*

In humans, the MaSCs/progenitors were sorted based on the expression of theEpCAM, Mucin-1 (MUC1), the common acute lymphoblastic leukemia antigen (CALLA or CD10), CD133, and CD49f. EpCAM is highly expressed by the luminal cells, and less by basal cells, while CD49f expression is higher in basal cells [137]. MUC1, CD133 with CK8, CK18, and CK19 are luminal markers, while CD10 and THY1 with CK14 are markers of basal cells [138]. The $MUC1^+/CD10^{neg}/EpCAM^+$ progenitor cells similarly as the $EpCAM^+/MUC1^{neg}/CD49f^+$ cells produced luminal ductal and alveolar cells expressing CK8, CK18, and CK19 in colony-forming cell assay in vitro [138,139]. CK8 and CK14 expressions are exclusive to luminal and basal, respectively, in adult mice, though in humans, some cells expressed both markers in luminal and basal compartments [140]. The $MUC1^{neg}/CD10^+/EpCAM^{neg}$ progenitors produced only myoepithelial cells expressing CK14 [139]. The $MUC1^{neg\ to\ lo}/CD10^{lo}/EpCAM^+$ cells similarly

to the $EpCAM^{+}/CD49f^{+}$ cells were characterized as bipotent and produced both basal and luminal cells [138,139]. Cell sorting based on lineage exclusion ($CD31^{neg}/CD45^{neg}$) and the expression of CD49f and EpCAM similarly identified 4 MaSCs/progenitor cell populations when implanted in immunodeficient mice [141]. Bipotent MaSCs were characterized as $Lin^{neg}/EpCAM^{lo}/CD49f^{hi}$, luminal progenitor as $Lin^{neg}/EpCAM^{+}/CD49f^{+}$, basal progenitor as $Lin^{neg}/EpCAM^{neg\ to\ lo}/CD49f^{+}$, and differentiated luminal cells as $Lin^{neg}/EpCAM^{+}/CD49f^{neg}$ [141,142].

The clonal studies and lineage tracing in situ of the MaSCs during puberty and adulthood have identified unipotent stem cells transiently located in the TEBs at puberty and stochastically disperse in the adult mammary epithelium following the elongation of the TEBs during puberty [101,110,113,143–146]. The TEBs are structured by an outer layer of cap cells and filled with multiple layers of inner body cells [147]. In the mouse, the cap and body cells are considered the precursor of the basal and luminal cells, respectively [145]. As the TEBs elongate and bifurcate, the highly proliferative MaSCs get randomly redistributed, lose the capacity of asymmetric cell division, and undergo a limited number of mitosis creating clonal clusters [146]. In the human mammary gland, stem cell niches with quiescent MaSCs were identified in the ducts, and zones of proliferative heterogeneous stem cells/progenitors, principally with luminal lineage, were characterized in the lobules [148]. The nature and location of these stem cells are relevant for breast cancers. Human breast cancers arise from the ductal and lobular regions, though breast cancer cases of a ductal origin have a poorer prognosis [149,150].

## *Breast Cancer Stem Cells*

### *Origin of Breast Cancer Stem Cells*

The cellular origin of cancer remains the central question. During the lifetime, somatic and germline cells accumulate mutations as a result of errors made during DNA damage repair or replication (for review see [151]). Two major models were formulated to explain the cellular origin of cancer. In the stochastic model, a succession of mutational events in differentiated somatic cells promotes a stepwise ability for reprogramming and the acquisition of a malignant genotype, while the second hypothesis involved the occurrence of mutations in stem cells or progenitors [3,152,153]. It is also likely that both models coexist. The longevity, pliancy, and quiescence of stem cells and, to some extent, progenitors are essential for the cumulation of the serial mutational events [92]. The mutations of progenitor cells may also lift the restricted potential and promote reprogramming and tumor initiation when provided with a stimulatory microenvironment. These opportunistic stem cell niches abolish the conceptual stem cell hierarchical organization [154]. Along the differentiation path of the mammary epithelium, some cellular states may facilitate the appearance of subtype-specific mutations as, GATA3 mutations which are frequent in luminal A and B and less in HER2 and basal-like subtypes, or MAP3K1 which is more abundant in luminal A (see Table 1). The mosaic of genetic alterations contributes to the singularity of breast cancer stem cells (BCSCs) and, ultimately, the response of breast cancer patients to treatment.

The CSCs are defined conventionally by their ability to self-renew and differentiate into heterogeneous cell populations, some with no tumorigenic capacity. CSCs are involved in all the stages of cancer development, including the initiation and progression of the primary tumor, the development of metastasis, and recurrence [155]. The resistance to chemotherapeutic agents through the elevated expression of efflux transporters, the increase expression of antiapoptotic proteins, or resistance to ionizing radiation are potential events that increase the post-treatment risk of recurrence and poor patient outcome [155–159].

A CSC population was isolated for the first time from the breast tumors of various patients and characterized as $Lin^{neg}/CD24^{neg\ to\ lo}/CD44^{+}$ [95]. These cells, when transplanted in immunodeficient mice, generated new heterogeneous tumors containing few cells characterized as $CD24^{neg\ to\ lo}/CD44^{+}$ [95,160]. A fraction of the $CD24^{neg\ to\ lo}/CD44^{+}$ cells, characterized by expression of EpCAM on their cell surface, were more tumorigenic and displayed strong invasive properties when transplanted in immunodeficient mice [95]. Circulating breast cancer cells are also heterogeneous, and a fraction of this population contained metastasis initiating cells characterized by the expression of CD44 [161].

A distinct BCSC population was later identified and characterized by high activity of aldehyde dehydrogenase (ALDH) [162]. The $ALDH^{+}$ cells are epithelial-like stem cells that could reconstitute the tumor heterogeneity in vitro and in vivo [162]. Moreover, a fraction of the cells with a high ALDH activity and characterized as $CD24^{neg\ to\ lo}$ and $CD44^{+}$ was highly tumorigenic, also found to be enriched in the TNBCs and associated with a higher risk of metastasis and poorer patient outcome [162–164].

Based on the cell surface markers and similar to the normal MaSCs, the BCSCs exist under 2 states, either as epithelial-like such as cells with high ALDH activity, or mesenchymal-like characterized as $CD24^{neg\ to\ lo}$ and $CD44^{+}$ [158,159]. The epithelial-mesenchymal transition (EMT) and reversibly the mesenchymal-epithelial transition (MET) define the metastatic potential of the BCSCs [159]. However, breast cancer cell line such as MDA-MB-231, a basal mesenchymal cell line, is mainly characterized as $CD24^{neg}$ and $CD44^{+}$ but with high ALDH activity [160], suggesting that cell surface markers in BCSCs are not always consistent and additional ones may be required for their characterization.

Subsequent studies identified additional cell surface markers of BCSCs characteristic of MaSCs and luminal progenitors, as for example $CD133^{+}$ [165], $CD49f^{+}$ [166], $CD61^{+}$[167], $CD29^{+}$ [168], MUC1 [169], and $CK5^{+}$ [170]. Also, several factors and pathways associated with stemness, and EMT are either overexpressed, and/or activated in BCSCs as for example Sox2 and Sox9 [131], Wnt pathway [171], hedgehog pathway [172], hippo pathway [173], and notch pathway [174]. These pathways also represent potential actionable drug targets for the treatment of breast cancer. Most importantly, there is no universal marker of the BCSC populations, imputable to the diversity of the cell of origin, the heterogeneity and fluidity of the microenvironments, and a plethora of possible mutational events [175].

The gene expression patterns of the breast cancer subtypes were related to distinct normal mammary epithelial cell lineages as well as the gene expression profile observed in the murine MaSCs/progenitors [117,142,176–178]. The claudin-low subtype is not identified as an independent group by hierarchical clustering and encompasses the 5 breast cancer subtypes [179]. The claudin-low subtype is characterized by the high expression of genes associated with the EMT, and a gene expression signature similar to the MaSCs/basal epithelial cells (Figure. 2) [178]. The basal-like tumors, including BRCA1 mutants, have a gene expression signature associated with the luminal progenitors, while gene expression profiles of luminal A and B subtypes were closest to the mature luminal epithelial cells (Figure. 2) [142,176,180]. Embryonic stem cell genes such as MYC, NANOG, OCT4, and SOX2 are overexpressed in poorly differentiated basal-like subtype [181]. Luminal progenitor cells lacked the expression of ER but highly expressed CK5/6, and EGFR, markers of basal-like subtype of breast cancers [182]. The $CD24^{neg\ to\ lo}$ and $CD44^{+}$ cells were enriched in basal-like subtype and less frequent in luminal type tumors [183].

However, it has been challenging to attribute a single-cell lineage to the HER2-subtype, presumably derived from the luminal lineage (Figure. 2) [182]. Using mouse mammary tumor virus-Her2/neu transgenic mice model that develops tumors similar to the human HER2 subtype, tumor initiating cells characterized as $CD49f^{hi}/CD61^{hi}$ displayed tumorigenicity in vitro and in vivo and resistance to chemotherapeutic agents, both hallmark of CSCs [167]. These tumor initiating cells were suggested to derive from luminal progenitor cells [184].

The latest research using SCS are providing new understanding of the breast cancer heterogeneity and the contribution of BCSCs.

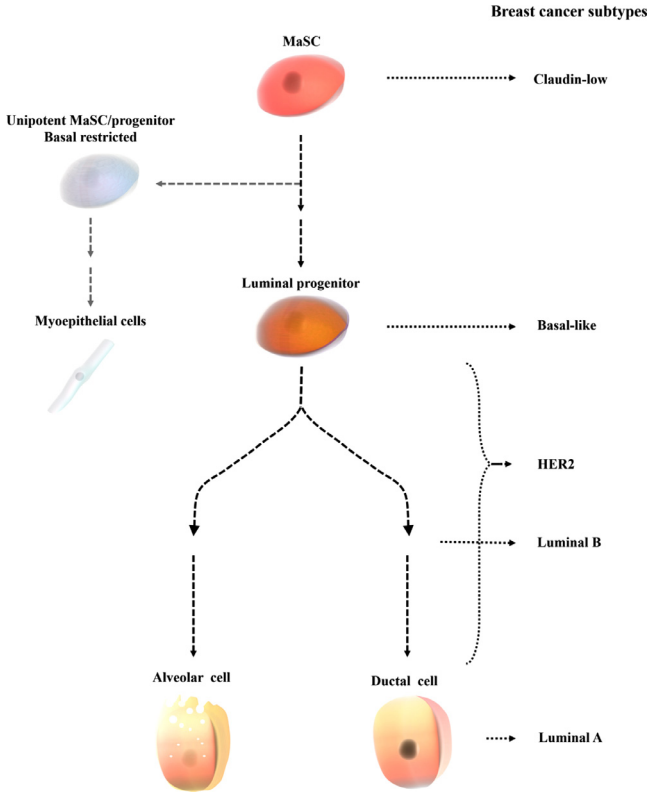


**Figure. 2.** Schematic representation of the hypothetic links between breast cancer subtypes and the mammary luminal epithelial cell hierarchy. Adapted from [182].

*Single-Cell Technology to Characterize Breast Cancer Stem Cells*

Applications of SCS include the identification of unique functional properties such as heterogeneity and plasticity, rare cell types, gene regulatory networks, and pathways of differentiation [185]. The SCS technology also provided clearer insight on the characteristics of CSCs [186–188]. Chen et al. used single-cell RNA sequencing to define the heterogeneity of established breast cancer cell lines such as SUM149, SUM159, MDA-MB-231, and GUM36 cells based on cell migration instead of cell surface markers [189]. Migratory cells had a gene expression signature of CSCs and EMT, but this approach also identify distinct migratory cell populations with epithelial and mesenchymal gene signatures [189]. Enrichment of BCSCs was represented by upregulated expression of ALDH isoforms, CD44 and CD29. Rare cells were also identified that expressed both EMT-CSC and MET-CSC markers, denoting extreme capability of metastasis and cellular plasticity. Furthermore, 3 distinct gene expression profiles were displayed by migratory cells associated with BCSCs, including enhanced antioxidant defense, low mitochondrial fragmentation, and downregulation of proteasome genes [189]. The BCSCs in TNBC were also heterogenous and associated with 3 distinct patterns of expression of stemness, including EMT, MET, and dual EMT-MET [190]. The EMT-CSC gene expression signature was associated with the expression ITGA6, EPCAM, CCND1, CD44, EGFR, CDH1, CCND1, and MKI67 [190].

Akrap et al. used single-cell gene expression to reveal the heterogeneity of $ER^+$ and $ER^{neg}$ breast cancer subtypes [191]. $ER^+$ breast cancer cell lines were characterized by a hierarchical organization, and an identifiable transition between each clusters, and a similarity in gene expression between the different cell lines; but 2 modes of differentiation were observed, in which MCF7 cells underwent a progenitor-like state and gradually differentiated whereas T47D cells differentiation operated as a switch from quiescent to differentiated state [191]. In $ER^{neg}$ cell lines, MDA-MB-231 and CAL120, the hierarchical organization was not as obvious as in $ER^+$ cells, as the gene expression signatures are not well dissociated from the one of CSCs [191]. This approach also identified a pool of quiescent BCSCs common to both $ER^+$ and $ER^{neg}$ breast cancer cell lines [191]. Moreover, Jonasson et al. identified CSC-related genes in MDA-MB-231 cells by single-cell RNA sequencing thereby detecting 14 significantly upregulated genes coding genes: LGALS3, CRTC1, ETV1, ARL6IP5, CD81, MYH9, IFITM3, HMGA2, NAB1, MBNL1, DSTN, MARCKSL1 and noncoding genes MALAT1 and NEAT1 [192]. In terms of their correlation to clinical outcome, 3 genes LGALS3, MYH9, and DSTN were found to be significantly associated with a worse prognosis [192].

The influence of surrounding stromal cells such as cancer associated fibroblasts on the BCSCs was also demonstrated by SCS. The coculture of a cancer cell line, SUM149, with CAFs increased tumorigenicity of the SUM149 [193]. The coculture also increased the number of BCSCs, including $ALDH^{hi}$ subpopulation associated with the upregulation of epithelial markers as EpCAM, cadherin-1, CK8, and CK16 and downregulation of mesenchymal markers as vimentin, hypoxia inducible factor-1$\alpha$, nicotinamide N-methyltransferase, and snail transcriptional repressor-1 [193]. SCS was also useful to characterize circulating tumor cells (CTCs) intrapatient heterogeneity and the contribution of BCSCs to this cell population [194]. BCSC regulatory genes BMI1, GATA3, SOX9, STAT3, Notch 1, Notch 2 as well as ALDH were overexpressed in CTCs demonstrating that CTCs are enriched in BCSCs [194]. A fraction of these BCSCs expressed simultaneously epithelial marker (ALDH) and mesenchymal markers (CD90) suggesting a high cellular plasticity and great metastatic potential [194].

Essentially, SCS assists our understanding of biological processes and cancer mechanisms in ways that bulk sequencing has not been able too. Continuous use of this technology will provide fundamental information that will aid in the diagnosis and therapeutics.

*Drug Targeting Breast Cancer Stem Cells*

Targeting BCSCs should hold a great promise for the prevention of metastasis, decrease the risk of drug resistance, and recurrence. However, only a few clinical trials are evaluating the effectiveness of treatments on the expression of BCSC markers, mostly measuring ALDH activity (clinical trial numbers: NCT01077453, NCT01864798, NCT01424865, NCT01372579, NCT01281163, NCT01190345).

Several pathways have been associated with the proliferation and survival of BCSCs as Notch, Wnt, hedgehog, or TGF-$\beta$ pathways (Figure. 3). As previously mentioned, these pathways are not unique to the BCSCs and are also essential to MaSCs, progenitors, and other cells populating the breast.

The inhibition of the Notch signaling by inhibitors of $\gamma$-secretase, MK-0752, or RO4929097 was shown to reduce the number of BCSCs in preclinical studies (for review see [195]). In phase I/II clinical trial (NCT00645333), the combination of MK-0752 with docetaxel reduced the regenerative capacity, and the number of $CD44^+$/ $CD24^{neg}$ and $ALDH^+$ cells obtained from the biopsies of the breast cancer patients undergoing treatment [196]. All the clinical trials involving RO4929097 were terminated as the manufacturer Roche ceased the production of the drug (for review [197]) (Figure. 3).

Several inhibitors of the Wnt pathway were also evaluated in preclinical studies (for review, see [198]). CWP232228, an inhibitor targeting the interaction between $\beta$-catenin and T-cell factor (TCF), reduced the number of the BCSCs in vitro and tumor development in vivo using murine breast cancer models [199]. PKF118-310, an inhibitor of the TCF/ $\beta$-catenin signaling, decreased the number of BCSCs in vitro and in vivo in a murine Her2 breast cancer model [200]. Pyrvinium pamoate, an anthelmintic drug and an inhibitor of the Wnt pathway, decreases the number $CD44^+/CD24^{neg\ to\ lo}$ and $ALDH^+$ BCSCs, inhibits their self-renewal and

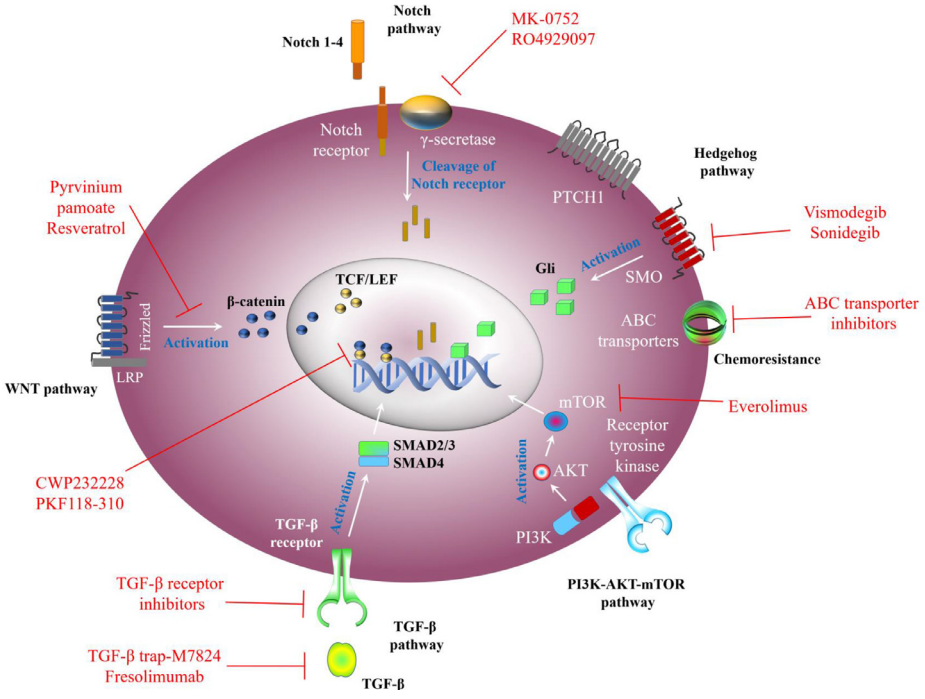


**Figure. 3.** Different strategies used to target BCSCs. Different pathways have been implicated in the development and survival of BCSCs. Several inhibitors were developed to inhibit specifically either Wnt, Notch, Hedgehog, or TGF-$\beta$ pathways. Secondary pathways such as PI3K-AKT-mTOR and ABC transporters responsible for the rapid clearance of chemotherapeutic agents are also targeted. BCSCs, breast cancer stem cell.

metastasis [201]. Resveratrol, a polyphenolic compound targeting the Wnt pathway, targeted the proliferation of BCSCs and reduced their population in xenograft tumors [202]. However, despite the demonstration of the efficacy of Wnt inhibitors in preclinical studies, no Wnt inhibitor has been approved in the clinic. Few clinical trials in phases I and II used or will test various Wnt inhibitors for the treatment of breast cancer but with no specific focus on the BCSCs (for review see [198]). The essential nature of the Wnt pathway in normal mammary cells, and other surrounding cells and the lack of consistent Wnt pathway alteration in breast cancer limit the applicability of the Wnt inhibitors at the clinical level.

Inhibitors of the hedgehog pathway, such as vismodegib and sonidegib, both smoothened homolog (Smo) antagonists demonstrated the potential for targeting BCSCs (Figure. 3). Smo is a nonclassical G-protein essential in the hedgehog pathway. Sonidegib, in combination with chemotherapeutic agent docetaxel, was assessed for the treatment of advanced TNBC in phase I clinical trial ((NCT02027376); a total of 3 patients out of 12 showed clinical benefit from the combination [203]. A future phase II study (NCT02694224) is currently recruiting and will look at the effect of a combination of vismodegib with the standard neoadjuvant chemotherapy, consisting of epirubicin, cyclophosphamide, and paclitaxel, for the treatment of TNBC patients. The inhibition of the TGF-$\beta$ signaling pathway is also regarded as critical to decrease EMT of the BCSCs and reduce the risk of metastasis (for review see [204]). Various inhibitors of the TGF-$\beta$ signaling pathway have been developed targeting either the TGF-$\beta$ receptor kinase or interfering with TGF-$\beta$ and were shown to decrease the breast cancer metastasis in preclinical studies (for review see [204]. In phase II clinical trial, fresolimumab, a monoclonal antibody directed against TGF-$\beta$, in combination with radiation therapy, were assessed for the treatment of metastatic breast cancer (NCT01401062) and demonstrated tolerance for the dose and longer overall survival for patients receiving the highest dose of fresolimumab [205]. A clinical trial using M7824, a bispecific anti-PD-L1–anti-TGF-$\beta$ trap, is recruiting patients for the treatment of breast cancer (NCT03620201). Also, none of these studies will be considering the effect on the BCSC population. The PI3K/Akt/mTOR pathway inhibitor, everolimus, in combination with docetaxel, inhibited the growth of BCSCs in vitro isolated from MDA-MB-231 and MCF7 cells, reduced the resistance of BCSCs to docetaxel and affected the growth of MDA-MB-231 xenograft tumor in vivo using a murine model of TNBC [206].

Strategies were assessed to circumvent the resistance to chemotherapeutic drugs by the BCSCs using inhibitors of the drug efflux pumps such as the ABC transporters, but the low specificity and toxicity limited their applications [156,207]. Further approaches are currently evaluated using mesenchymal stem cells, miRNA, immunotherapy, or targeting metabolic pathways.

Additional considerations for the targeting of BCSCs need to be given to the tumor microenvironment. It is known to express factors of stemness promoting self-renewal, plasticity, and resistance to chemotherapeutic agents [208,209]. BCSCs were also reported to reshape the tumor microenvironment and establish niches that promote specific and complex interaction with various cell types, including immune cells, mesenchymal cells, cancer-associated fibroblasts, endothelial cells, and other components [208,210]. The fate of the BCSCs is indissociable from its environment, and treatment strategies will need to be implemented to consider this intricate relationship.

## Conclusion

The knowledge on BCSCs improves in the last decades in part due to the progress made in microfluidic and SCS. It is now established that BCSCs are present in most, if not all, of breast cancer subtypes. Multiple studies have also suggested that BCSCs have prognostic relevance and can influence the therapeutic outcome. However, the majority of clinical studies ignore the impact of the treatments on these specific subpopulations. There is still a Brobdingnagian lack of understanding of the mechanisms of genomic

instability leading to the tumor initiation, the contribution of BCSCs to the growth of the tumor, the influence of the microenvironment, and quiescence of the BCSCs in part due to the lack of proper modelization.

## Author contributions

**Sebastien Taurin:** Conceptualization, original draft preparation, writing, graphic preparation, and editing. **Haifa Alkhalifa:** Original draft preparation, writing, and editing. Sebastien Taurin and Haifa Alkhalifa contributed to design, writing, and proof reading of the article.

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