

Review

Advances in the knowledge of breast cancer stem cells. A review

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Summary. Much effort has been made by researchers to elucidate the complex biology of breast cancer stem cells (BCSCs), a small subset of breast tumor cells that display stem cell properties, drive tumor initiation, and growth. In recent years, it has been suggested that BCSCs could be responsible for the process of metastasis and the development of drug resistance. These findings make the need to find the distinguishing blend of markers that can recognize only BCSCs of the utmost importance in order to be able to design new targeted therapies. This review will summarize BCSCs' main features as well as the cell surface markers that are currently used to identify them.

Key words: Breast cancer, Cancer stem cells, Breast cancer stem cells, Markers, Therapeutic resistance

Introduction

Despite great advances in medicine, breast cancer remains one of the leading causes of death in women worldwide (Abraham et al., 2005; Centers for Disease and Prevention, 2012). This type of cancer, like many others, is a heterogeneous disease that comprises molecular and histological subtypes, genetic and epigenetic changes, as well as high disease recurrence

and therapy resistance (Brooks and Wicha, 2015; Czerwinska and Kaminska, 2015; Mukohyama and Shimono, 2015)

In recent years, it has been postulated that a subpopulation of cancer cells with a number of stem cell features, the so called “cancer stem cells” (CSCs), (also known as cancer initiating cells (CICs), tumor propagating cells (TPCs), and tumor initiating cells (TICs) could be in part responsible for tumor relapse and treatment resistance (Curado, 2011; Servick, 2014; Ito and Matsuo, 2016).

These cells were first described in 1994 in acute myeloid leukemia with the specific combination of the surface markers CD34⁺ and CD38⁻ (resembling the hematopoietic stem cell phenotype (Lapidot et al., 1994).

CSCs have shown to have self-renewal and differentiation potential by injecting them into NOD/SCID mice; leukemic stem cells could initiate the disease, while the other tumor cell populations did not (Bonnet and Dick, 1997). These cells have been identified in several tumor types including breast, brain, prostate, endometrial, colon, pancreatic, head and neck, lung, liver, ovarian and skin cancer (Singh et al., 2004; Allegra and Trapasso, 2012; Wortham and Yan, 2012; Amini et al., 2014; Guzel et al., 2014; Jing et al., 2015; Karakasiliotis and Mavromara, 2015; Roudi et al., 2015).

The CSC hypothesis (also known as hierarchical hypothesis) suggests the existence of a hierarchically organized population, where a small subset of cells have the capacity to sustain tumor initiation and growth, instead of a homogeneous one, where all cells would have the same tumorigenic potential (Ponti et al., 2005;

Dalerba et al., 2007; Fillmore and Kuperwasser, 2008). As stem cells, CSCs are able to self-renew (through asymmetric or symmetric cell division), and to differentiate (they can generate cancer cells with a variety of phenotypes within the tumor) and they are able to initiate and maintain tumor growth (Kaur et al., 2014; Takeishi, 2015). Also, CSCs have low proliferative activity and longer telomeres and lifespan. Their resistance to radiotherapy and chemotherapy might be attributed to the fact that these cells have the ability to expel intracellular toxins and drugs since they overexpress ABC transporters, thus contributing to therapy relapse and tumor metastasis (Saeed and Iqtedar, 2013; Liu et al., 2015; Shitara and Doi, 2015; Takeishi, 2015).

Despite the fact that the origin of CSCs remains unknown, several hypotheses not mutually exclusive have been proposed. The first possibility is that they originate from adult stem cells, since their longer lifespan could help them accumulate multiple genetic mutations and epigenetic modifications needed for the carcinogenic switch, as well as the fact that they are present in several tissues and share many biological features. Second, they could derive from a more differentiated population, such as progenitor cells. The third possibility is that they could originate from mature progenitors or terminally differentiated cells that, after suffering transformation, dedifferentiate and acquire stem cell attributes (Chaffer et al., 2011; Shekhani et al., 2013; Xie et al., 2014; Chaffer and Weinberg, 2015; Grosse-Wilde et al., 2015; Zane et al., 2015). As to the last two possibilities, it has been proposed that epithelial-to-mesenchymal transition (EMT) plays an important role (Mani et al., 2008; Cufi et al., 2010; Taube et al., 2010). EMT can occur when E-cadherin and some cytokeratins (epithelial markers) are lost, while the expression of other mesenchymal markers like vimentin and N-cadherin increase (Mani et al., 2008; Taube et al., 2010). Bone morphogenetic proteins, transforming growth factor beta (TGF- β), and diverse transcription factors (Slug, Twist, FOXC1, FOXC2, Snail, Zeb1, and Zeb2) can activate EMT (Taube et al., 2010; Jia et al., 2014; Naka, 2015).

Additional factors could promote CSC development, such as dysregulation of stem cell self-renewal pathways like those involving Wnt, Notch, and Sonic Hedgehog (Kubo et al., 2004; Grudzien et al., 2010; Singh et al., 2011; Islam et al., 2015; Zoni et al., 2015). Later on, the CSC microenvironment, or the so called stem "niche" (composed of immune cells, adipocytes, mesenchymal stem cells, endothelial cells, and tumor associated fibroblasts) will help them regulate their growth, differentiation, survival, and self-renewal processes (Korkaya et al., 2011; Kunisaki, 2015; Mesa et al., 2015; Plaks et al., 2015).

CSCs express different surface and genomic markers, some of them common to normal adult stem cells, such as: ALDH, PROCR, Nanog, ABCG2, Oct3/4, Sox2, Nestin, Fgfr1, CD133, CD24, CD29, CD34 and

Musashi-1, which differ according to the type of cancer (Sahlberg et al., 2014; Skvortsov et al., 2014; Xia, 2014; Yu et al., 2014). This review will focus only on the breast cancer stem cell markers proposed to date.

Breast cancer stem cells

In 2003, Al-Hajj and collaborators announced for the first time the existence of BCSCs. They isolated CD44⁺CD24^{-/low} lin⁻ cells from primary human breast cancers and metastatic sites using fluorescence activated cell sorting (FACS) and injected them into NOD/SCID mice. As few as 200 cells with this phenotype generated tumors, whereas thousands of cells with different combination of surface markers did not. This property was kept even after serial passages, thus displaying the cells' self-renewal and differentiation capabilities (Al-Hajj et al., 2003).

Ever since this study, the gold standard method for evaluating BCSC activity has been the use of xenografts, where the ability to initiate tumor growth directly correlates with the number of BCSCs injected into immuno-compromised mice (Gonzalez et al., 2014; Jovanovic et al., 2014; Ke et al., 2014).

Since mammary CSCs are a small subpopulation of breast cancer cells (0.1-1%), several techniques have been used to detect, obtain and enrich BCSCs, either from breast cancer cell lines, breast cancer tissue samples, or metastatic specimens. The most common method used today relies on the immuno-phenotype profile (surface markers) present, which is detected using cytometry or immuno-labeled beads, described below (Liang et al., 2013; Saadin and White, 2013; Moghbeli et al., 2014; Wang et al., 2015c).

In addition to the main immune-methods commonly employed, there are three other assays used; side population, label retention, and spheroids/mammosphere cultures. The use of side population (SP) enrichment is useful to isolate BCSCs due to the presence of high ABC transporter expression (specifically BCRP1 and ABCG2). These transmembrane pumps are able to expel from the cell not only drugs, but also fluorescent lipophilic dyes like Hoechst 33342 and Rhodamine 123, making it possible to sort the negative fractions for these dyes (Britton et al., 2012; Christgen et al., 2012; Jin et al., 2015). The ALDEFLUOR assay, also used for this purpose, takes advantage of the ALDH enzymatic activity overexpressed by the BCSCs (Tsukabe et al., 2013; Kai et al., 2015). An additional technique used to separate BCSCs is the label retention assay, which relies on DNA labeling using Bromodeoxyuridine (BrdU), a thymidine analogue that is incorporated into newly synthesized DNA (Clarke et al., 2006; Fillmore and Kuperwasser, 2008; Wang et al., 2015a). The Fluorescent Nanodiamonds labeling technique provides an effective new tool for tracking and finding slow-proliferating/quiescent CSCs in cancer research (Lin et al., 2015).

As BCSCs have the capacity for anchorage-

independent growth, a common method used for evaluating this characteristic is the soft agar colony formation assay. This technique uses a semisolid agar medium in which cells are cultivated; after several days, colonies are formed and are either quantified manually or using a fluorometric dye (Hwang-Versluis et al., 2009; Tudoran et al., 2015). Qin, et al reported a microfluidics method that enriches cancer stem cells (CSCs) on the basis that the less adhesive phenotype is associated with a higher percentage of CSCs. Two heterogeneous breast cancer cell lines (SUM-149 and SUM-159) were successfully separated into enriched subpopulations according to their adhesive capacity (Zhang et al., 2015).

Finally, the process of sphere formation, which was first used to detect neural stem cells and subsequently developed for breast stem cells and BCSC identification by Dontu et al. and Ponti and colleagues, is used to enrich stem cells in culture on low adherence plates using serum-free medium. In these conditions, stem cells are able to form a three-dimensional cell cluster termed mammosphere. The process is repeated several times to further enrich the stem cell population (Dontu and Wicha, 2005; Ponti et al., 2005)

BCSC markers

Breast cancer is not only composed of several histological subtypes, ductal carcinoma *in situ*, lobular carcinoma *in situ*, invasive ductal carcinoma, invasive lobular carcinoma, tubular carcinoma, and mucinous carcinoma among others; it is also comprised of four molecular subtypes, luminal A and B, HER2⁺, basal-like, and normal-like, first revealed in 2000 after a global gene expression profiling of breast cancer samples (Perou et al., 2000; Sorlie et al., 2003; Gathani et al., 2005; Park et al., 2010; Prat et al., 2010; Tsang et al., 2012).

This histological and molecular heterogeneity represents a major issue not only for the development of efficient therapies but also for discovering a universal BCSC marker. Here we describe the main phenotypic markers currently used (Table 1).

CD44⁺CD24⁻

Al-Hajj and colleagues were the first to demonstrate BCSC isolation with CD44⁺CD24^{-/low}, and greater enrichment of this population was obtained when ESA, an epithelial cell adhesion molecule (known also as EpCAM or CD326) was added to the previous combination of markers (Al-Hajj et al., 2003). Ponti showed for the first time that CD44⁺CD24⁻ breast cancer cells were able to form mammospheres and to proliferate extensively (Ponti et al., 2005).

CD44 is a type I transmembrane glycoprotein expressed in some mesenchymal and neuroectodermal cell types (Baltuch et al., 1995; Portmann-Lanz et al., 2006). This glycoprotein binds to hyaluronic acid,

collagen, laminin, fibronectin (all extracellular matrix components), acts as an adhesion molecule and participates in cell-cell interactions, invasion, cell proliferation, and migration (Orian-Rousseau, 2015). In normal breast and tumoral tissue, it has been localized in the cell membranes of basal myoepithelial cells and in some luminal epithelial cells (Louderbough et al., 2011; Fitzpatrick et al., 2013), and in many malignancies it has been related to drug resistance and poor prognosis. Additionally, CD44 expression may gradually decline during breast tumor progression (Park et al., 2010).

CD24 or heat stable antigen (HSA), is a glycoprotein that anchors to the cell surface (Lee et al., 2009). In normal breast tissue it has been localized in the apical membranes of luminal cells while breast cancer samples stained mainly in the cytoplasm (Honeth et al., 2008). Several studies have investigated CD24 overexpression in a wide variety of human cancers, and it has been associated with a more aggressive stage of the disease (Ghebeh et al., 2013; Rostoker et al., 2015).

The CD44⁺CD24⁻ phenotype is more frequently observed in basal-like breast tumors just as in cell lines that had undergone EMT, and is less frequent or absent in the HER2⁺ subtype (Ricardo et al., 2011; Choi et al., 2013). In normal mammary tissue, CD44⁺CD24⁻ cells are limited to the basal layer (Park et al., 2010). Since then, using CD44⁺CD24⁻, either alone or in combination with more markers for the identification of BCSCs, has been a controversial issue since many authors believe that the use of these markers enrich the breast cancer population with stem cell like features, while others disagree. In 2005 Abraham et al., reported that CD44⁺CD24^{-/low} breast cancer cells did not associate with tumor progression, survival, and clinical outcome. Instead, they observed a significant amount of this population in primary tumors of patients with distant metastasis (especially osseous), proposing that these markers could favor distant metastasis (Abraham et al., 2005).

Shipitsin and collaborators observed that normal and tumor CD44⁺ breast cells are more similar to each other than to CD24⁺ cells (from the same tissue). CD44⁺ and CD24⁺ breast cancer cells are clonally related but not identical (CD24⁺ cells had a gain in 1q21.3 that was not present in CD44⁺ cells) CD44⁺ cells had higher expression of genes involved in cell motility, chemotaxis, hemostasis, and angiogenesis, while CD24⁺ cells presented higher expression of genes involved in RNA splicing and carbohydrate metabolism. They also found that the TGF- β pathway (involved in tumorigenesis, EMT, and in regulating pluripotency in embryonic stem cells) was activated in CD44⁺ breast cancer cells. Moreover, they determined that the CD24⁺ population was enriched in distant metastases regardless of the metastatic site and/or type of the primary tumor, consistent with previous reports that associated CD24 expression with tumor progression (Shipitsin et al., 2007).

Wright, in 2008, observed that Brca1-deficient mice

Table 1. Breast cancer stem cells markers.

Marker	Name	Description	Reference
CD44 ⁺ CD24 ⁻	CD44: Receptor for hyaluronic acid.	Related to tumor recurrence and distant metastasis.	Abraham et al., 2005
		High level of pro-invasive genes.	Sheridan et al., 2006
	CD24: Heat stable antigen.	Associated with the basal-like subgroup, particularly in BRCA1 hereditary tumors and with low levels in luminal and HER2 ⁺ tumors.	Honeth et al., 2008; Hwang-Verslues et al., 2009; Park et al., 2010; Bernardi et al., 2012
		CSC features in Brea1-deficient tumors.	Wright et al., 2008
Additionally ESA ⁺ (CD44 ⁺ CD24 ⁻ ESA ⁺)	Also known as EpCAM: epithelial cell adhesion molecule	Found in normal and malignant mammary cells that have undergone EMT.	Mani et al., 2008
		Positively associated with luminal B subtype.	Tsang et al., 2012
		Negatively associated with luminal A subtype.	Wei et al., 2012
ALDH ⁺	Aldehyde Dehydrogenase	Was added to this double combination of markers to achieve a higher enrichment of BCSCs.	Al-Hajj et al., 2003; Filmore and Kuperwasser, 2008
		Normal and neoplastic human mammary epithelial cells with this phenotype have stem/progenitor properties. In breast cancer it is correlated with poor prognosis.	Ginestier et al., 2007
	Enzyme that catalyzes the oxidation of intracellular aldehydes.	Associated with high expression of CXCR1/IL-8RA.	Charafe-Jauffret et al., 2009
		Significant role in chemotherapy resistance.	Tanei et al., 2009
Converts retinol to retinoic acid		More frequent in basal like and HER2 ⁺ subtypes.	Park et al., 2010; Tsang et al., 2012
		Cold mediate invasion and metastasis in IBC.	Charafe-Jauffret et al., 2010
		Associated with ER breast tumors.	Ginestier et al., 2007; Park et al., 2010
CD133 ⁺	Also known as Prominin-1: membrane glycoprotein with five transmembrane domains	Overlap of 1% with the previous BCSC phenotype reported (CD44 ⁺ CD24 ⁻ lin)	Ginestier et al., 2007
		Overexpressed protein in a cell subpopulation of breast tumors BRCA1-deficient. CD133 ⁺ cells were resistant to antineoplastic, ability to form mammospheres and stemness gene overexpression.	Wright et al., 2008
		Strong expression in MARY-X spheroids.	Xiao et al., 2008
		Related to positive lymph node status.	Hwang-Verslues et al., 2009
PROCR ⁺	Protein C Receptor or EPCR: Type I transmembrane glycoprotein.	Correlation with overall survival in triple-negative breast carcinoma	Zhao et al., 2011
		Breast cancer and normal breast cells with this phenotype are enriched with genes involved in cell motility, chemotaxis and angiogenesis.	Shipitsin et al., 2007; Hwang-Verslues et al., 2009
		PROCR ⁺ ESA ⁺ cells had many cancer stem features and expressed EMT markers.	Hwang-Verslues et al., 2009
Integrins	CD29: β1-integrin	Frequent in basal-like tumors.	Park et al., 2010
		EpCAM ⁺ CD49f ⁺ population had stem-like activity in normal breast tissue.	Villadsen et al., 2007
	CD49f: α6-integrin	CD24 ⁺ TD29 ⁺ cells from BRCA1 deficient tumors were enriched with cancer stem cells.	Vassiopoulou et al., 2008.
		A subpopulation in MCF-7 breast cancer line with α6-integrin overexpression was capable of forming mammospheres and tumors in immunodeficient mice and displayed resistance to proapoptotic agents.	Cariati et al., 2008
Claudins	Transmembranal proteins involved in the formation of tight junctions.	CD49f ⁺ EpCAM population from normal breast tissue showed mammary regenerating capacity in NOD-SCID 112rg mice.	Lim et al., 2009
		They play a mayor role in defining the four cellular states found in normal breast tissues were also seen in invasive ductal carcinomas (luminal 1, luminal 2, basal and mesenchymal cells).	Keller et al., 2010
		Claudin-low tumors have cancer stem cells and EMT features.	Creighton et al., 2009; Prat et al., 2010; Taube et al., 2010
Claudins		Claudin-low breast tumors are related with poor prognosis, triple negative tumors (ER ⁻ /PR ⁻ /HER2 ⁻) and invasive ductal carcinomas with a high rate of metaplastic and medullary differentiation.	Prat et al., 2010
		Claudin-71 positive populations are associated with IIER2 ⁺ tumors and to shorter time before cancer recurrence. Decreased claudin-7 expression was related with metastasis and a higher tumor grade.	Prat et al., 2010

mammary tumors contained CD44⁺CD24^{-/low} cells with cancer stem cell features, and that only 50 of these cells were needed to initiate tumor formation (Wright et al., 2008). Honeth et al., reported a high association between basal-like tumors (specifically in BRCA1 hereditary breast cancer) and CD44⁺CD24⁻ cells and a lower association of the same phenotype with luminal type and HER2⁺ tumors (Honeth et al., 2008). Hwang-Verslues and coworkers results were consistent with previous data where they found a negative association between CD44⁺CD24^{-/low} and HER2. They also suggested that CD44⁺CD24^{-/low} could not serve as a universal marker for CSC isolation (Hwang-Verslues et al., 2009). Additionally, Bernardi did not observe any association of CD44⁺CD24⁻ tumors with clinicopathological parameters of prognosis, progression, recurrence, and/or with high frequency of metastasis, supporting Abraham and Hwang-Verslues' previous suggestions (Bernardi et al., 2012).

More recently, high numbers of CD44⁺CD24⁻ cells were associated with lymph node metastases; these cells were more frequent in ductal carcinoma *in situ* than in invasive tumors. It has been reported that this cell subpopulation is positively associated with an extensive *in situ* component, as well as with the luminal B subtype; they are negatively correlated with luminal A subtype (Tsang et al., 2012; Wei et al., 2012).

ALDH

Aldehyde dehydrogenase is an enzyme that catalyzes the oxidation of intracellular aldehydes; it may play a role in early differentiation of stem cells, and converts retinol (vitamin A) to retinoic acid (Ma and Allan, 2011).

Ginestier et al., in 2007, reported ALDH1⁺ as a marker of stem cells of normal human breast and breast carcinomas. This subpopulation was able to self-renew and to differentiate, recreating the heterogeneity from the original tumor. A flow cytometry analysis of xenografted tumors revealed that the ALDEFLUOR-positive cell population had an overlap of 1% or less of the total cancer cell population with the previous BCSC phenotype reported by Al-Hajj and colleagues (CD44⁺CD24^{-/low} lin⁻). They showed that ALDH1 expression is a powerful predictor of poor clinical outcome and one that has a direct or inverse correlation with histoclinical parameters (tumor grade, ERBB2 overexpression, ER/PR (estrogen/progesterone) status, and the presence of cytokeratins 5/6 and 14) (Ginestier et al., 2007).

The same group identified, through a gene expression profile analysis that used breast cancer cell lines, a breast fibrocystic disease cell line, and a normal mammary tissue cell line, a BCSC signature that involved 413 genes. This signature contained genes with known functions in stem cell biology, cell signaling, DNA repair, chromatin remodeling, membrane protein, and apoptosis, among other categories. They demonstrated that CXCR1/IL-8RA was highly

overexpressed in this subpopulation (this axis may regulate mammary stem cell proliferation and self-renewal); finally, they reported that IL-8 promoted invasion and chemotaxis of BCSCs (Charafe-Jauffret et al., 2009). Tanei and collaborators observed that an ALDH1⁺ population was increased in breast tumors from patients following neoadjuvant chemotherapy, proposing that ALDH1⁺ could be a better predictive marker for chemotherapy resistance than CD44⁺CD24⁻ (Tanei et al., 2009).

In 2009, Hwang-Verslues and colleagues did not observe an association between ALDH⁺ breast cancer cells and an ER status (as reported by Ginestier and coworkers), and suggested (according to their results) that CD44⁺CD24⁻ and ALDH may not be universal markers for identifying highly tumorigenic stem cells from breast cancers (Hwang-Verslues et al., 2009).

Park and colleagues observed that ALDH1⁺ was expressed heterogeneously in luminal and basal cells from normal breast tissue samples, suggesting that ALDH could be a marker of mammary epithelial stem cells and luminal-lineage committed progenitors. They also reported that ALDH1 was more common in ER⁻ tumors (in invasive ductal carcinomas alone or those associated with ductal carcinoma *in situ*), confirming Ginestier's previous results. Park and colleagues also observed that ALDH1⁺ cells were frequently seen in the basal-like and HER2⁺ subtypes, rather than in luminal subtypes (Ginestier et al., 2007; Park et al., 2010). Likewise, Charafe-Jauffret et al., demonstrated that ALDH1⁺ cells could mediate invasion and metastasis in inflammatory breast cancer (IBC) (Charafe-Jauffret et al., 2010).

The ALDH1 family is composed of six enzymes; Bai et al. demonstrated that ALDH1A1 was the only ALDH11 isoenzyme able to predict poor survival in a cohort of 3455 BC patients. ALDH1A1 messenger RNA (mRNA) high expression was found to be correlated to worse overall survival (OS) for all BC patients (Wu et al., 2015).

CD133

CD133, also known as prominin-1 (for its location on the protrusion of cell membranes), is a known marker of cancer stem cells from several tissues like brain, blood, colon, liver, skin, and prostate; it is also expressed in several solid tumors, including triple-negative invasive ductal breast carcinoma (Liu et al., 2013).

In 2008, Wright and coworkers found, in breast cancer cell lines derived from mice breast tumors with a BRCA1 mutation and lacking p53 expression, a new BCSC subpopulation characterized by CD133⁺ cells that did not overlap with the CD44⁺CD24^{-/low} BCSCs previously reported. They demonstrated that CD133⁺ cells were drug resistant, had the ability to form mammospheres and tumors in NOD/SCID mice (Wright et al., 2008).

Xiao et al. reported that MARY-X spheroids, derived

from MARY-X cell line (established from a patient with inflammatory breast carcinoma, a lethal form of breast cancer characterized by increased lymphovascular invasion) expressed not only the BCSC phenotype CD44⁺CD24^{-low} and ALDH1⁺, but also showed high expression of CD133 in >90% of the MARY-X spheroid cells (Xiao et al., 2008). And Hwang-Verslues and colleagues observed a possible association between the prevalence of CD133⁺ breast cancer cells and lymph node status (Hwang-Verslues et al., 2009).

PROCR

PROCR is a type I transmembrane glycoprotein, also called EPCR, capable of binding and activating protein C, involved in the blood coagulation pathway; it is a known marker of hematopoietic, neural, and embryonic stem cells (Wang et al., 2015a,b,c).

When performing a gene expression profile of normal and breast cancer cells, Shipitsin and collaborators observed that PROCR was present in 100% of CD44⁺ cancer cells and that it was localized (in normal breast tissue) in the basal layer of ducts and alveoli. They also demonstrated, after using a SAGE (serial analysis of gene expression) library construction of CD24⁺ and CD44⁺ cells from normal breast tissue and ascites, pleural effusion, and primary invasive breast cancer samples, that normal and cancer PROCR⁺ subpopulations resemble the CD44⁺ population more than the CD24⁺ cells from the same tissue. The functional annotation analysis showed that normal and breast cancer cells PROCR⁺ had a higher number of genes involved in cell motility, chemotaxis, and angiogenesis (Shipitsin et al., 2007).

Hwang-Verslues et al., showed that PROCR⁺ESA⁺ cells from the basal breast cancer cell line MDA-MB-231 had greater colony formation efficiency in soft agar as well as a higher tumorigenic potential in NOD/SCID mice, compared with the rest of the bulk and PROCR-ESA⁻ cells, suggesting that this subpopulation had a higher number of cancer stem and progenitor cells. They also demonstrated that PROCR⁺ESA⁺ cells were able to divide asymmetrically, expressed higher levels of NUMB (an asymmetric division regulator of several cell types), vimentin, SLUG, FOXC2, and lower levels of E-cadherin (the last four are EMT markers) (Hwang-Verslues et al., 2009). And Park also observed an enrichment in basal-like tumors of EPCR⁺ cells (Park et al., 2010).

Integrins

Integrins are cell-surface glycoproteins and receptors for extracellular matrix proteins; they are bound to the membrane and act as counter-receptors on other cells (Lyu et al., 2015; Seguin et al., 2015). Villadsen et al. found in human mammary epithelial cells two luminal populations, a lobular (EpCAM^{hi} CD49f⁺) and a ductal

oriented (EpCAM^{hi} CD49f⁺) cell population, as well as a lobular and ductal myoepithelial population with an EpCAM^{low/-}CD49f⁺ phenotype. They observed that EpCAM^{hi}CD49f⁺ cells had stem-like activity, while the others did not (Villadsen et al., 2007; Keller et al., 2010).

Integrin CD29 (β1) and CD49f (α6), in combination with CD24, have been used for the isolation of BCSCs. For example, Vassilopoulos and colleagues observed that CD24⁺CD29⁺ cells were enriched with BCSCs in BRCA1 deficient tumors (Vassilopoulos et al., 2008; Sharma et al., 2013). Cariati identified a subpopulation in MCF-7 (a luminal breast cancer cell line) with stem cell features, such as resistance to pro-apoptotic agents, the ability to self-renew, and the ability to form mammospheres and tumors in immunodeficient mice; these cells also over-expressed α6-integrin, which was necessary for their survival and growth (Cariati et al., 2008).

Lim and coworkers reported, after performing an immunohistochemical assay and microarray profiling, high CD49f expression and mammary stem cell features in normal breast basal cells, which are characteristics concordant with the claudin-low and normal-like molecular breast tumor subtype (Lim et al., 2009). Using flow cytometry, immunohistochemistry, and tumor xenografts, Keller et al., found four cellular states in normal breast tissues, the luminal 1 (EpCAM^{hi} CD24⁺CD49f⁻), luminal 2 (EpCAM^{hi}CD24⁺CD49f⁺), basal cells (EpCAM⁺/low CD24⁻CD49f⁺) and mesenchymal cells (EpCAM⁻CD24⁻CD49f⁺). These states were also found, in different proportions, in invasive ductal carcinomas (Keller et al., 2010).

Claudins

Claudins are transmembrane proteins involved in the formation of tight junctions between epithelial cells. These proteins establish the paracellular barrier that controls the flow of molecules between the intercellular space and the cells from the epithelium (Singh and Dhawan, 2015). Two groups led by Perou and Sorlie classified breast cancer into four molecular subtypes (Perou et al., 2000). Herschkowitz and coworkers proposed the fifth subtype: the claudin-low subtype, characterized by low expression of claudins 3, 4, 7, occludin, and E-cadherin (Herschkowitz et al., 2007). In 2010, Taube reported an association between the claudin-low subtype and EMT; this study validated Creighton's work where he suggested that claudin-low tumors had features of tumor initiating cells and EMT (Taube et al., 2010).

The clinicopathological characteristics of claudin-low breast tumors were described by Prat in 2010. Prat described it as a subtype with poor prognosis and a high rate of metaplastic and medullary differentiation; they also observed that these tumors were significantly enriched in EMT and stem cell-like features (Prat et al., 2010).

Recently, Bernardi et al. using an immunohistochemical expression analysis of tissue microarrays with ductal invasive carcinoma samples, suggested an association of claudin-7 to a shorter time of recurrence. They also showed a higher frequency of relapse in claudin-7 positive tumors than in claudin-7 negative ones. Lower expression of claudin-7 correlated with higher tumor grade and metastatic disease. Although they did not observe any correlation between claudin-7 and CD44⁺CD24⁻ (BCSC phenotype), they saw an expression of 38.9% in ductal invasive breast carcinoma (Bernardi et al., 2012).

Other markers

It has been reported that CD105(+)/CD90(+) subpopulation from MDA-MB-231 breast cancer cells possesses "mesenchymal stem cell-like" characteristics, and its high migratory ability might be associated with EMT (Wang et al., 2015b).

BCSC therapeutic implications

Today, standard therapies (radio, immuno and chemotherapy) usually eliminate differentiated bulk cells, leaving BCSCs alive, allowing for tumor recurrence, proliferation, and generation of more aggressive and resistant tumor cells (Bhere and Shah, 2015; Yang et al., 2015). It has been demonstrated that BCSCs have the ability to escape chemo and radiotherapy effects (Khan et al., 2015; Lv and Shim, 2015; Takebe et al., 2015). Since Fillmore et al., showed that CD44⁺CD24⁻EpCam⁺ BCSCs were resistant to paclitaxel, many authors have observed the same effect with many other chemo-agents. These results have been explained by the presence of the transmembranal drug pumps ABCG2 or BCRP1, and ABCB1 (Fillmore and Kuperwasser, 2008).

Radio-resistance was demonstrated by Pajonk who proposed that resistance is caused by low levels of ROS (reactive oxygen species) followed by a decrease in

Table 2. Therapeutic agents that target BCSCs.

Name	Description	Target	Reference
Cydopamine (plant-derived steroidal alkaloid) Vismodegib (GDC-0449, belongs to 2-arylpiperidine class)	Cyclopamine inhibits Hh through its interaction with Smo. GDC-0449 is a potent Smoothed inhibitor and has a selectivity for Sonic Hh-Gli signaling.	Hedgehog pathway	Kubo et al., 2004; Schwarz-Cruz-y-Celis and Melendez-Zajgla., 2011; Singh et al., 2011; Prud'homme, 2012
γ -Secretase inhibitors (GSIs) (LLNle, LY411, 575, MRK003, MK-0752, RO4929097)	GSIs block activation of all Notch receptors. Induce growth arrest and/or cell death in many cancer cell types. Mammosphere formation decreases after GSIs treatment. Under specific conditions γ -Secretase inhibitors could induce apoptosis in BCSCs.	Notch pathway	Grudzien et al., 2010; Al-Hussaini et al., 2011; Schwarz-Cruz-y-Celis and Melendez-Zajgla., 2011; Prud'homme, 2012
IWR-1 and XAV939 (inhibit tankyrase) Pyrvinium (CK1 α activator) Stapled Axin-derived (StAx) peptides	CK1 α activators and tankyrase inhibitors promote the activity of the β -catenin destruction complex. StAx peptides antagonizes β -catenin <i>in vitro</i> . Wnt pathway aStAx-35R inhibits the growth of Wnt-dependent cancer cells.	Wnt pathway	Grossmann et al., 2012; Prud'homme, 2012
Retinoic acid	Regulates gene transcription through the communication with retinoic receptors (RC). Can initiate cellular differentiation when acting on RC. All-trans retinoic acid (ATRA) induces constitutive activation of retinoid signaling target genes. ALDH oxidizes retinol (vitamin A) into retinoic acid, and has been linked with early differentiation of stem cells.	Induces BCSC differentiation. ATRA decreases primary and secondary tumorsphere formation.	Chute et al., 2006; Ginestier et al., 2007, 2009
Metformin (biguanide derivative, N ¹ , N ¹ dimethylbiguanide)	This well tolerated drug has been used to treat patients with type 2 diabetes, obesity, metabolic syndrome and polycystic ovarian syndrome. Metformin reduces the tumor mass and prevents relapse in xenograft mouse model. This biguanide derivative prevents the development of BCSCs by interfering with EMT transcription factors (ZEB 1, TWIST1, SNAIL2. and TGF β 3s) , Synergistically interacts with trastuzumab (Tzb) to suppress selfrenewal and proliferation of BCSCs.	Inhibits, in combination with Tzb, BCSC self renewal and proliferation, and kills them in collaboration with doxorubicin.	Hirsch et al., 2009; Cufi et al., 2009; Korkaya et al., 2011; Vazquez-Martin et al., 2011; Prud'homme, 2012
Oncolytic herpes simplex virus (oHSV) vector G47 Δ (derived from vector G207) has the IPC47 gene and US11 promoter deleted	oHSV vectors have the ability to: - transfer therapeutic transgenes; - disseminate within the tumor; - replicate <i>in situ</i> ; induce antitumor immune responses	Blocks BCSC tumor formation	Li et al., 2012
Tranilast (N-[3,4-dimethoxycinnamoyl] anthranilic acid) is an aryl hydrocarbon receptor (AHR) agonist	Initially developed for allergic diseases, nowadays it is also being used for the treatment of fibrotic diseases. This aryl hydrocarbon receptor inhibits EMT, migration, invasion, MAPK signaling, TGF- β activity, cell cycling, and has <i>in vivo</i> antimetastatic functions in breast cancer cells. Tranilast inhibits Oct4 in BCSCs.	Blocks BCSC colony and mammosphere formation.	Prud'homme et al., 2010, 2012

DNA double-strand breaks (Phillips et al., 2006; Lagadec et al., 2010).

Other therapeutic agents that target specific cancer pathways (self-renewal, inflammatory response, growth factors, among others) are currently in development or in clinical phase trials (Table 2). Some of these can, in principle, target BCSC as a side-effect. For example, three major signaling pathways are being targeted in order to block and/or induce BCSC differentiation, such therapeutic agents include cyclopamine and vismodegib against the Hedgehog (Hh) pathway, γ -secretase inhibitors against the Notch pathway, and IWR-1, XAV939, hydrocarbon-stapled peptide and pyrvinium against the Wnt pathway (Kubo et al., 2004; Grudzien et al., 2010; Singh et al., 2011; Prud'homme, 2012). For the same purpose, retinoic acid and metformin have been used to block BCSC self-renewal and to inhibit proliferation. Oncolytic virotherapy with herpes simplex virus that blocks the BCSC ability to form tumors, and tranilast, which prevents metastasis, tumor growth, colony and mammosphere formation are promising new approaches (Table 2) (Hirsch et al., 2009; Cufi et al., 2010; Vazquez-Martin et al., 2011; Prud'homme, 2012). Additional strategies have been suggested, like NF-KB, IL-6, IL-8, TGF- β , CXCR4, and CXCR1 targeting; this would regulate the BCSC niche interaction and the EMT process (Korkaya et al., 2011; Prud'homme, 2012). All these approaches need to be evaluated, proven safe, and must be relatively non-toxic for human use. Evidently, an ideal therapy will be one that kills BCSCs and differentiated cells, leaving aside normal stem and parenchymal cells (Clarke et al., 2006; Hirsch et al., 2009; Schwarz-Cruz-y-Celis and Melendez-Zajgla, 2011). This will surely require the use of combined therapy with conventional or directed therapy (Clarke et al., 2006; Hirsch et al., 2009; Tsang et al., 2012). For this reason, a clear assay that effectively identifies BCSCs is needed.

Conclusion

Different markers have been used to isolate and enrich the BCSC population, but none of them can be applied to all molecular breast cancer subtypes. The need for a universal marker remains a priority for the development of targeted therapies. Nevertheless, considerable advances in treatment strategies show great promise. The field of CSC research has increased over the past few years and many groups are trying to analyze the link between this unique population and tumor initiation, relapse, metastasis, and therapeutic resistance. Moreover, the stem cell's similarity with CSCs needs to be taken into account for better understanding of their functional and biological features, as well as for their interactions with the microenvironment. Epigenetic screening, gene expression profiles, and next generation sequencing could be an alternative approach for deciphering the complexity of CSCs and tumor biology.

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