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Review

Stem cells in human breast cancer

Lucinei Roberto Oliveira, Stefanie S. Jeffrey and Alfredo Ribeiro-Silva

[1]Department of Pathology, Ribeirão Preto Medical School, University of São Paulo, Campus Universitário Monte Alegre, Ribeirão Preto, SP, Brazil and [2]Department of Surgery, Stanford University School of Medicine, Stanford, USA

Summary. Increasing data support cancer as a stem cell-based disease. Cancer stem cells (CSCs) have been found in different human cancers, and recent evidence indicates that breast cancer originates from and is maintained by its own CSCs, as well as the normal mammary gland. Mammary stem cells and breast CSCs have been identified and purified in *in vitro* culture systems, transplantation assays and/or by cell surface antigen identification. Cell surface markers enable the functional isolation of stem cells that can initiate and propagate tumorigenesis in mammary gland. These observations have dramatic biological and clinical significance due to increasing evidence suggesting that the recurrence of human cancer and treatment failure may reflect the intrinsic quiescence and drug resistance of CSCs. Thus, the CSC hypothesis provides fundamental implications for understanding breast carcinogenesis and for developing new strategies for breast cancer prevention and therapy for advanced disease. Further strategies to isolate breast CSCs, to find additional trustworthy surface markers, and to compare gene expression pathways profiles with their normal stem cells counterparts are necessary to more accurately define putative breast cell-lineage markers for the different cell types present in the mature mammary gland and to identify potential therapeutical targets in breast cancer. This review discusses the current knowledge about stem cells and CSCs, focusing on mammary stem cells and breast CSCs, and their consequences for breast tumorigenesis and implications for breast cancer susceptibility, prognosis, and treatment.

Key words: Breast, Cancer, Stem cell, CD44, CD24

Offprint requests to: Alfredo Ribeiro-Silva. Department of Pathology, Ribeirão Preto Medical School, University of São Paulo, Avenida Bandeirantes 3900, Campus Universitário Monte Alegre, 14049-900, Ribeirão Preto, SP, Brazil. e-mail: arsilva@fmrp.usp.br

Introduction

All tissues in the human adult organism are derived from organ-specific stem cells with specific properties that maintain tissue integrity and are defined mainly by their capacity to undergo self-renewal, as well as differentiation into the cell types that comprise each organ (Charafe-Jauffret et al., 2008; Shipitsin and Polyak, 2008).

The malignant neoplasias are believed to result from sequential mutations that can occur as a consequence of progressive genetic instability and/or environmental factors. Experimental and clinical data has recently been accumulating which supports the hypothesis that cancer may arise from mutations in stem cell populations (Reya et al., 2001).

The cancer stem cell (CSC) hypothesis states that normal stem cells may be the cells of cancer origin, and that a specific subset of cancer cells with stem cell characteristics can lead to tumor initiation, progression, and recurrence (Campbell and Polyak, 2007).

One property that all cancers have in common is a striking variability among the cancer cells within a single tumor. These cells differ in characteristics such as morphology, size, membrane composition, and antigen expression, as well as behaviors such as proliferation rate, metastatic potential, and sensitivity to therapeutic agents (Campbell and Polyak, 2007). In diverse solid tumor types, like breast cancer, the existence of a hierarchical distribution of cancer cells is widely accepted. It is theorized that only a small subpopulation of replenishing stem-like cells can give rise to the diversity of differentiated cells that comprise the bulk of the tumor, and some reports have identified a small sub-population of highly tumorigenic cells within primary and metastatic breast tumors, as well as in some breast cancer cell lines (Al-Hajj et al., 2003; Abraham et al., 2005; Collins et al., 2005; Li et al., 2007; O'Brien et al., 2007).

The recent research interest in CSCs arises from

experiments suggesting that cells with stem-like properties can be sorted from solid tumors based on the expression of specific surface markers. The consolidation of CSCs knowledge into our current view of multistep cancer development has important implications for defining the target population for transformation and the specific events required for realization of malignant potential.

The human mammary gland

The human mammary gland is a specialized organ that undergoes most of its development after birth and follows hormonal events during its entire life (Charafe-Jauffret et al., 2008). Mammary gland evolution is regulated at three critical periods during life: embryogenesis, puberty, and pregnancy (Kakarala and Wicha, 2008).

After birth, the mammary gland develops from a small number of invading cells derived from the ectoderm, which form a lobulo-alveolar structure and are composed of three cell lineages when adult: myoepithelial cells, which are contractile cells that form the basal layer of ducts and alveoli; ductal epithelial cells, which line the lumen of ducts; and alveolar epithelial cells, which synthesize milk proteins (Hennighausen and Robinson, 2001; Kakarala and Wicha, 2008). The functional structures produced in the mature mammary gland are composed of a continuous epithelium, consisting of an outer basal layer of contractile myoepithelial cells and an inner layer of luminal cells (Stingl et al., 2006).

The breast is a structurally dynamic and specific organ. Hormonal changes during life induce episodes of extensive proliferation, remodeling and differentiation in the mammary epithelium according to age, menstrual cycle and reproductive status (Navarrete et al., 2005). To achieve this great plasticity and the large cell number expansion associated with it, there is substantial evidence of a differentiating cell hierarchy in the adult mammary gland that includes developmentally distinct stem and progenitor cell types, driving the proliferative process within the normal mammary epithelium in a hierarchical and organized way (Stingl et al., 2006; Cariati and Purushotham, 2008).

Breast cancer

Breast cancer is a major cause of death in women. The majority of breast cancer deaths occur as a result of recurrent or metastatic disease rather than from the effects of the primary tumor (Croker et al., 2009). In the past decade, earlier detection and improved treatments have reduced breast cancer mortality, but the recurrence and metastases rates for the disease remain high. Moreover, current breast cancer therapy is not optimally individualized, and is often associated with undesirable side effects (Thornton et al., 2005).

Breast tumors may be classified by dissemination patterns such as in situ, invasive, or metastatic lesions (Simpson et al., 2005). However, breast cancer is not a disease determined by a single tumorigenesis pathway, but rather a heterogeneous group of diseases at both the molecular and clinical level, and each subtype has its own stable phenotype maintained during tumor evolution (Sorlie et al., 2001; Korsching et al., 2002; Dontu et al., 2004; Polyak, 2006). Distinct gene expression profiling of a large set of breast tumors performed by independent groups have demonstrated five main molecular subtypes of breast cancer: basal subtype, luminal A and B (also known as highly proliferating luminal) (Langerød et al., 2007), HER-2^+/ER^-, and normal breast-like. This classification correlates with clinical outcome, with distinct responses to treatment and prognostics, and there is increasing evidence that risk factors can be different for each tumor subtype (Hu et al., 2006; Polyak, 2006; Sorlie et al., 2006).

Stem cells

Stem cells represent only a minuscule fraction of the cells that constitute each tissue but they are the only cells with self-renewal capacity. Stem cell divisions occur in an asymmetric way, in which a stem cell is able to produce an exact copy of itself, as well as a daughter cell that leaves the stem cell niche to differentiate and generate multipotent progenitors, which in turn can give rise to committed progenitors and differentiated cells (Dontu et al., 2003; Ginestier et al., 2007). The deregulation of this self-renewal process leading to stem cell expansion may be a key event in carcinogenesis, and while self-renewal can drive tumorigenesis, the differentiation process can contribute to tumor phenotypic heterogeneity (Kakarala and Wicha, 2008) (Fig. 1). According to Charafe-Jauffret et al. (2008), genetic and epigenetic mechanisms in the progenitor cell type and environmental influences in the niche where these cells grow may contribute to the cellular heterogeneity found in the malignant neoplasms.

The epithelial tissues are subject to continuous remodeling and renewal. According to Smalley and Ashworth (2003), this tissue renewal involves a hierarchy of cells, including slowly proliferating stem cells, rapidly proliferating transit-amplifying cells and various terminally differentiated cells with specialized functions. The slowly proliferating stem cells are long-lived, and for this reason are more exposed to damaging agents than the more differentiated cells, and can thus accumulate mutations that are then transmitted to the rapidly proliferating progeny (Dontu et al., 2003).

Mammary epithelial stem cells

The existence of mammary epithelial stem cells was first suggested by DeOme et al. (1959), who originally devised the technique of tissue fragment transplantation into mammary fat pads cleared of mammary epithelium.

It was shown through mouse transplantation studies that epithelium isolated from different regions of the mammary gland at various stages of postnatal development could recapitulate the entire glandular structure upon transplantation into a cleared mammary fat pad, generating complete differentiated and functional mammary epithelial branching ducts, lobules and myoepithelial cells. Later, Hoshino (1967) also demonstrated and confirmed that a fragment could be serially transplanted from this regenerating gland to another cleared mammary fat pad.

More recently, considerable progress has been made towards identification of human mammary stem cells, but the exact phenotype of these cells still remains indefinite. Using immunofluorescent monoclonal antibodies for basal cytokeratin 5, glandular cytokeratins 8/18 or myoepithelial smooth muscle actin, Nagle et al. (1986) identified progenitor cells of luminal and myoepithelial cell lineages and described heterogeneous populations in the normal human adult mammary gland. Dairkee et al. (1988) also showed that normal, benign, and malignant breast epithelium expressed complementary patterns of reactivity against basal and luminal cell-specific antibodies. They went so far as to suggest that some basal tumors may have originated in "undifferentiated basally located precursor cells often referred to as 'stem cells'". Further support for the pluripotent epithelial stem cell existence in the mammary gland comes from transplantation studies, and this *in vivo* assay remains the gold standard for testing functional stem cell properties (Chepko and Smith, 1997).

In 1998, the existence of stem cells in rodent mammary glands was corroborated by Kordon and Smith (1998). They established the existence of mammary stem cells through experiments with mouse mammary stem cells marked with the mouse mammary tumor virus (MMTV), which were serially transplanted in random fragments of mammary epithelium into cleared mammary fat pads, and they observed then its capacity to regenerate a new gland tissue in vivo and display self-renewal activity upon transplantation. However, Kordon and Smith (1998) highlighted that any mutation in the mammary epithelial stem cell population could be highly relevant for the entire mammary epithelial population because it will be conserved and repeatedly inherited in all the subsequent progeny of the stem cell, as well as during self-renewal.

Several other studies of clonality and implantation performed *in vitro* and *in vivo* have shown that a subset of pluripotent human adult mammary epithelial cells, called mammary epithelial stem cells, are capable of forming colonies in vitro and give rise to both luminal epithelial and myoepithelial cells, appearing to represent bipotential mammary epithelial progenitors (Böcker et al., 2002; Asselin-Labat et al., 2006; Shackleton et al., 2006).

Stingl et al. (2006) reported the use of multiparameter cell sorting to purify a subset of adult mouse mammary stem cells that are capable individually of regenerating an entire mammary gland within six weeks *in vivo*. These cells were designated as mammary repopulating unit and expressed markers like cytokeratin 5 and 14, smooth muscle actin, vimentin, and smooth muscle myosin, which are associated with basal/myoepithelial cells.

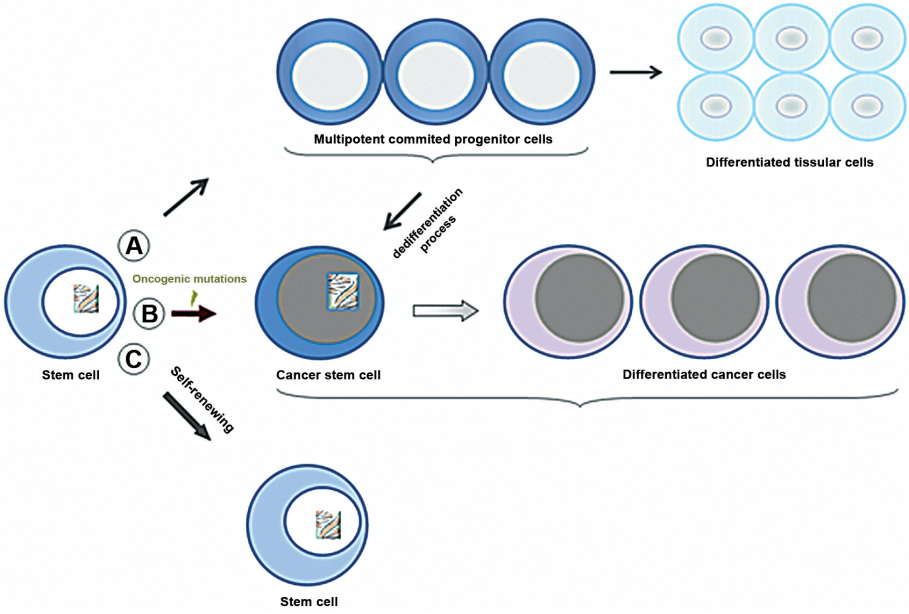

Fig. 1. Hypothetical ways of normal stem cells. **A.** Stem cells can originate committed progenitor cells that later arise differentiated tissular cells. **B.** Oncogenic mutations can transform normal stem cells in cancer stem cells. The cancer stem cells can accumulate genetic alterations that can drive tumor initiation, progression and heterogeneity. **C.** Stem cells can generate an idential cell to itself through its self-renewal capacity.

Stem cells and cancer

The stem cell origin of cancer hypothesis considers that stem cells or cells that acquired the self-renewal ability tend to accumulate genetic alterations over long periods of time, evading the strict control of their microenvironment, and giving rise to tumoral evolution (Shipitsin and Polyak, 2008).

The resemblance between stem cells and cancer was observed a long time ago. The first register concerning the hypothesis of cancer origin from a rare population of normal cells with stem cell properties was proposed almost 150 years ago (Durante, 1874; Wicha et al., 2006). At that time, Cohnheim (1875) also proposed the hypothesis that stem cells could be misplaced during embryonic development, being the source of tumors that would be formed later in life.

The studies about this subject returned over 40 years ago, when some investigations confirmed the CSC hypothesis showing that a single tumor cell could generate a heterogeneous progeny and give rise to a new tumor, through investigations performed in tumors derived from ascites fluid in rats, and teratocarcinomas and leukemias in mice (Makino, 1956; Bruce and Van Der Gaag, 1963, Kleinsmith and Pierce, 1964).

In this way, Park et al. (1971) observed through a primary cell culture assay some myeloma tumor stem cells in mouse, and Hamburger and Salmon (1977) corroborated the hypothesis that some cancers could contain a small subpopulation of cells similar to normal stem cells, because they observed in primary bioassays that the expansive growth of malignant lesions could suggest the presence of a CSC population with stem cell properties, including indefinite proliferation.

In animal models, the ability of a small population of cells to originate a new malignant neoplasia was demonstrated in a classic experiment through transplantation of cells from human acute myeloid leukemia that expressed some cell surface markers associated with normal hematopoietic stem cells (Lapidot et al., 1994). Lapidot et al. (1994) showed that these transplanted cells could initiate leukemia in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice while other isolated cells could not. Since then, this assay has become the standard method for determining whether cell populations isolated from solid tumors are CSCs.

Based on the ability of diverse purified populations to form leukemia in NOD/SCID mice, investigations started to search for stem-like cells in leukemias. Bonnet and Dick (1997) showed that the injection of leukemic cells with a primitive hematopoietic progenitor phenotype resulted in leukemias that could be transplanted into secondary recipients, and also observed its ability to perpetually self-renew. Since then, putative CSCs have been isolated from many other tumors including brain, breast, colon, pancreas, prostate, lung, and head and neck cancer (Collins et al., 2005; Kim et al., 2005; Dalerba et al., 2007; Li et al., 2007; Prince et al., 2007).

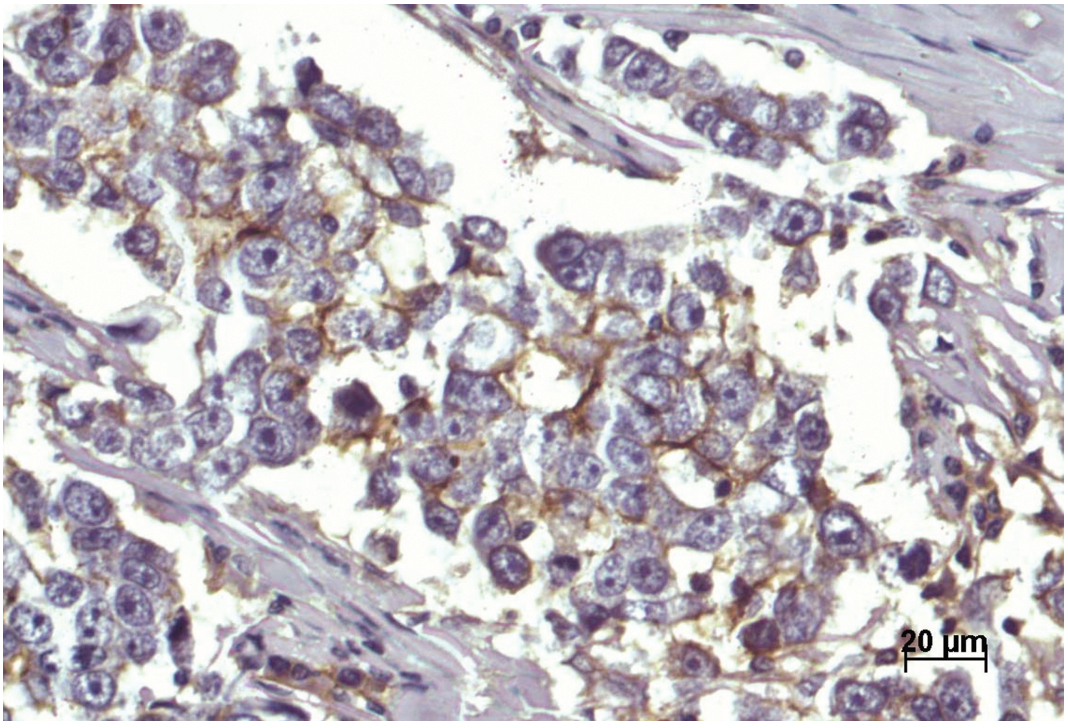

Fig. 2. Neoplastic cells from an invasive ductal carcinoma of the breast positive for CD44 (immunohistochemistry, membrane staining). The CD44 cell-surface marker has been used to identify putative cancer stem cells in breast carcinomas.

The main hallmarks of CSCs are their properties of self-renewal, their ability to generate tumors from very few cells, their slow cell division, their ability to give rise to a phenotypically diverse progeny, and their selective resistence to radio- and chemotherapy (Reya et al., 2001). The self-renewal and differentiation characteristics lead to the production of all cell types in a tumor, thereby generating tumor heterogeneity (Campbell and Polyak, 2007). The differentiated cells constitute the bulk of the tumor, but are not usually tumorigenic, due to their lack of self-renewal capacity and limited proliferation potential (Ginestier et al., 2007). However, it has been shown that the switch to carcinogenesis can occur in either the stem cells or one of their progeny, which acquires the ability to self-renew (Dontu et al., 2003). Some studies involving both transforming viruses and the direct introduction of oncogenes derived from human tumors suggested that the majority of cultured cells are susceptible to malignant transformation and that the clonality of tumors could be the end result of continuous selection for cells harboring favorable combinations of transforming gene mutations (Weinberg, 1995; Vogelstein and Kinzler, 2004). Furthermore, in several tissue systems, it has been proposed that some committed progenitor cells might become a CSC through a dedifferentiation process, which would occur by the acquisition of stem cell properties (Ponti et al., 2005; Krivtsov et al., 2006; Cobaleda et al., 2007).

Additional confirmation that stem cells can play a role in carcinogenesis are the homologies found between normal stem cells and cancer cells. In addition to self-renewal capacity, these characteristics include the production of differentiated cells, activation of antiapoptotic pathways, induction of angiogenesis, resistance to apoptosis and drugs (due to active telomerase expression and elevated membrane transporter activity), and the ability to migrate and spread in metastasis (Wicha et al., 2006).

Breast CSCs

Most breast cancers arise within a relatively short segment of terminal ductules that might be the location of normal stem cells (Villadsen, 2005). The demonstrations that normal mammary stem cells do exist indicate that stem cells could play an influence in breast cancer. The first proposal of breast CSC existence was made by Pierce et al. (1977) through an ultrastructural comparison of breast CSCs and their normal mammary stem cell counterparts. This proposal was reinforced by the observation that early first full-term pregnancy is associated with lower lifetime breast cancer risk. This might be explained if transformation preferentially occurs in normal mammary stem cells, and these may be present in fewer numbers once the mammary gland differentiation associated with pregnancy has occurred (Russo et al., 2005).

MMTV-induced breast carcinogenesis models have provided further evidence that stem/progenitor cells may be targets for malignant transformation. The MMTV infects mammary epithelial cells and randomly inserts its proviral DNA into somatic cell DNA during its replicative cycle (Ringold et al., 1979). According to

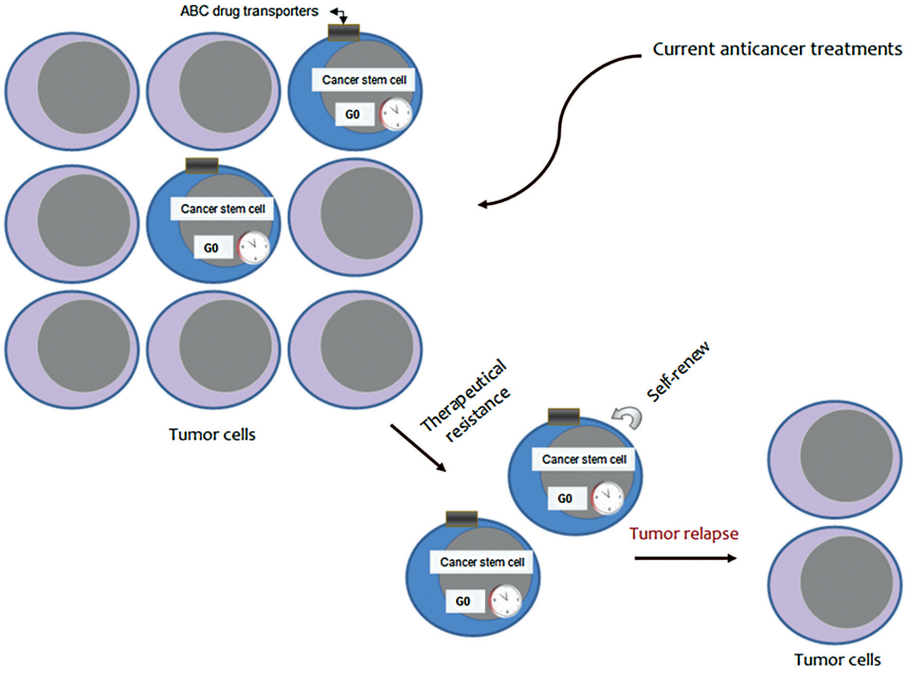

Figure 3. Cancer stem cell hypothesis implications for breast cancer treatment. Although cancer stem cells comprise a small amount of the cells within a tumor, they can be resistant to radiotherapy and chemotherapeutical agents, probably because of their quiescency. This therapeutical resistance combined with the stem cells property of self-renewal might be the cause of tumor relapse years after the clinical remission.

Callahan (1996), some of these random DNA insertions have been demonstrated to cause deregulation of specific stem cell genes leading to premalignant transformation and later to tumor progression. Nowadays, similar mammary fat pad transplantation models have been used to prospectively identify mouse mammary cells with breast CSC properties (Shackleton et al., 2006).

Mammary epithelial stem cells and breast cancer susceptibility

The long lifetime of stem cells makes them more susceptible than other cells to acquire multiple mutations required for carcinogenesis (Wicha et al., 2006). In the mammary gland, the lifetime accumulated hormonal exposure is one of the most important risk factors for breast cancer. Mammary epithelial stem cells can be the potential targets of these malignant transforming events because hormonal changes during a specific developmental period may determine the size of the breast stem cell pool and thereby influence carcinogenesis (Dontu et al., 2003; Kakarala and Wicha, 2008). Epidemiological investigations in populations submitted to radiation, like those from Hiroshima and Nagasaki, indicate that breast cancer develops years or decades after the oncogenic initiation (Tokunaga et al., 1979) and the normal mammary stem cells are the only specific population of cells that could survive enough to accumulate all the transforming events necessary for the attainment of the cancer phenotype (Cobaleda et al., 2008).

Furthermore, the terminal differentiation of mammary stem cells following pregnancy decreases the number of target cells susceptible to oncogenic events. Russo et al. (2005) demonstrated in animal studies that pregnancy or short-term treatment of virgin rats with pregnancy-associated hormones protects against chemically induced mammary carcinogenesis.

According to Ahlgren et al. (2004), the rate of increase in height during adolescence, largely regulated by growth hormone, is also strongly associated with subsequent risk of breast cancer, and the production of growth hormone was already previously associated as a paracrine regulator of mammary stem cells (van Garderen and Schalken, 2002).

The hormone susceptibility of putative mammary epithelial stem cells has been extensively investigated. Notwithstanding, there have been conflicting reports regarding the hormone receptor status of these cells in both mouse and human experiments. In mouse, based on pulse-chase methods, Booth and Smith (2006) predicted that the putative mouse mammary epithelial stem cells were ER+, while Asselin-Labat et al. (2006) found that stem cells identified by specific cell surface markers were negative for both HER-2 and hormone receptors (triple negative). Similarly, other divergent results have been described for human mammary epithelial stem cells, shown by Clarke et al. (2005) as enriched for steroid receptors, and cited as hormone receptor negative cells by Clayton et al. (2004).

Isolation and Purification of CSCs

Although the concept that cancers arise from stem cells was first proposed more than 150 years ago, it is only recently that advances in stem cell biology have allowed for more direct testing and validation of the CSC hypothesis.

It is well settled that CSCs share some properties expressed by normal stem cells. Current methods for determining whether cell populations isolated from solid tumors are CSCs, consist of purification of these cells from tumor samples based on the properties of normal stem cells, such as their ability to form spheres in culture (Dontu et al., 2003), membrane efflux activity (Goodell, 2002), specific cell surface molecule expression (Al-Hajj et al., 2003), and enzymatic activity detection of aldehyde dehydrogenase 1 (ALDH1) (Nagano et al., 2007). Purified cells are then tested for the capacity to originate tumors when injected into immunodeficient mice.

In vitro culture of spheres cells

Characterization of stem cells in both human and rodent systems has been facilitated by the development of *in vitro* culture systems that allow for propagation of stem cells in an undifferentiated state. In the *in vitro* culture system, cells grow in unattached conditions into round balls called “spheres”. This system can be used for enrichment and propagation of stem cells (Jensen and Parmar, 2006).

Contrary to the paradigm that epithelial cell survival is anchorage-dependent, Soule and McGrath (1986) showed for the first time the ability of undifferentiated human mammary epithelial cells to survive in suspension. After that, Dontu et al. (2003) showed that the *in vitro* cultivation and propagation of these undifferentiated human mammary epithelial cells generates floating spherical colonies with anchorage-independent growth, which were named mammospheres. They also observed that these mammospheres could reconstitute an entire mammary ductal tree, because they were enriched with cells with functional properties of stem/progenitor cells. These cells were suitable for self-renewal testing and were capable of differentiating into all three lineages present in the mammary gland when cultured under differentiating conditions. Moreover, Dontu et al. (2003) confirmed that the *in vitro* culture systems and gene expression profiles of human breast stem cells correlated with the genetic programs of other tissue types of stem cells.

The pattern of immunostaining found within mammospheres was consistent with previously reported data of mammary epithelial progenitor cells in normal adult human breast tissue (Stingl et al., 2001; Gudjonsson et al., 2002), expressing CD10, α6 integrin, and cytokeratin 5 in earlier progenitors, and epithelial-

specific antigen (ESA) and cytokeratin 14 in later but still multipotent progenitors (Dontu et al., 2003). Recently, Ginestier et al. (2007) showed that mammosphere-initiating cells expressed ALDH1, a detoxifying enzyme, and were capable of generating human mammary structures when transplanted into the humanized fat pad of NOD/SCID mice.

Membrane efflux activity

The Hoechst 33342 efflux property is a discriminating feature of quiescent stem cells that is lost when these cells are activated into cycle, and this allows identification of a small stem-like cell population called side population (SP), through flow cytometric analysis (Uchida et al., 2004). It has been postulated that the SP main characteristic is a universal stem cell phenotype (Zhou et al., 2001). Although heterogeneous, SP cells are observed in cardiac and primitive retinal cells (Hierlihy et al., 2002; Bhattacharya et al., 2003), in hematopoietic, epidermal and mammary stem cells (Bunting, 2002; Alvi et al., 2003), in normal kidney and renal cell carcinoma (Addla et al., 2008), and some embryonic stem cells (Zhou et al., 2001).

Alvi et al. (2003) demonstrated that human and mouse breast epithelial cells located within the SP retain the potential to differentiate into typical mammary clones *in vitro* and can also regenerate the organ upon experimental transplantation in the mouse mammary gland, because SP cells constitute an undifferentiated subpopulation able to differentiate into myoepithelial and luminal cell types, as well as into ductal and lobular structures. Furthermore, the morphology of the structures derived from these mammary SP cells resembled those previously described by Kordon and Smith (1998) for mammary epithelial clone types in vivo.

The SP cells are also associated with resistance to drugs and toxins, and this property is a result of increased expression of membrane transporter proteins (ABC drug transporters), such as P-glycoproteins or BCRP (breast cancer resistance proteins). In addition to acting as functional regulators of stem cells, they provide defense against damaging agents (Zhou et al., 2001; Bunting, 2002). Therefore, tumors might have a population of drug-resistant pluripotent cells that can survive radio- and chemotherapy and then repopulate the tumor (Charafe-Jauffret et al., 2008).

Stem cell markers

Expression of some specific cell surface markers has been investigated to facilitate the identification and purification of normal stem cells and CSCs, and several stem cell markers may be shared by CSCs in multiple human tumor types.

The standard procedures for the isolation of CSCs have been similar in many investigations. Among the most used *in vivo* models is the fractionation of tumor cells using cell-surface markers with stem cell characteristics, followed by their implantation into NOD-SCID mice to assess xenograft growth and celular composition (Shipitsin and Polyak, 2008).

The main surface marker phenotypes associated with stem cell characteristics include CD133, CD44, and CD24 (Al-Hajj et al., 2003; Singh et al., 2004; Lim and Oh, 2005; Hermann et al., 2007; O'Brien et al., 2007). The CD133 cell-surface marker, also called prominin 1 (PROM1), was discovered as a marker of normal hematopoietic stem cells and was later used to purify putative CSCs in several tumor types (Singh et al., 2004; Mizrak et al., 2008). In brain tumors, Singh et al. (2004) found that $CD133^+$ cells could successfully grow under unattached conditions, with neurosphere-like formations, whereas $CD133^-$ cells could not. Only the $CD133^+$ cell fraction, isolated from human medulloblastomas and glioblastomas and injected into the brains of NOD SCID mice, contained cells capable of initiating tumors, with phenotypic similarity between engrafted and original tumor. According to other studies, CD133 has been shown to play a role in migration and asymmetric division of stem cells (Balic et al., 2006; Beckmann et al., 2007).

The CD44 glycoprotein is a cell surface receptor for hyaluronic acid, and is involved in cell adhesion, migration, and metastasis of cancer cells (Shipitsin et al., 2007). The CD44 cell-surface marker has been used to identify putative CSCs in breast tumors (Fig. 2) (Shipitsin et al., 2007), as well as in other tumor types, such as prostate (Collins et al., 2005), pancreatic (Li et al., 2007), and head and neck carcinomas (Prince et al., 2007). Shipitsin et al. (2007) found that $CD44^+$ tumoral mammary cells were associated with more invasive, proliferative, and angiogenic status, predicting an aggressive tumoral cell behavior. Moreover, there was a correlation between $CD44^+$ tumoral cells and decreased patient survival (Shipitsin et al., 2007).

CD24 is a mucin-like adhesion molecule expressed by neutrophils, pre B lymphocytes and a large variety of solid tumors. Functionally, CD24 enhances the metastatic potential of malignant-cells, because it has been identified as a ligand of P-selectin, an adhesion receptor on activated endothelial cells and platelets (Lim and Oh, 2005). In the mouse mammary gland, cytokeratin expression and PCR have revealed that $CD24^-$, $CD24^{low}$, and $CD24^{hi}$ populations correspond to non-epithelial, basal/myoepithelial, and luminal epithelial cells, respectively (Sleeman et al., 2006). Lim and Oh (2005) investigated the role of CD24 in various human epithelial neoplasias, and demonstrated that intracytoplasmic CD24 expression was found to be highly associated with adenocarcinoma of the colon, stomach, gallbladder, and ovary. Positive or negative CD24 expression has been used in set with other markers to identify putative CSCs in tumors, and some studies defined the phenotype of pancreatic CSCs as $CD24^+/CD44^+$ (Li et al., 2007; Zou, 2008). However, in breast and prostate cancer, putative CSCs were found

with a $CD24^{-}/CD44^{+}$ phenotype (Al-Hajj et al., 2003; Hurt et al., 2008).

These investigations suggest that diverse stem cell markers can be expressed in different tumors by the CSCs, and the significance of these observations in most human cancers remains to be determined. Therefore, it is possible that each tumor could have a prevalent and specific CSC phenotype (Table 1).

Stem cell markers in mammary stem cells and breast CSCs

Identification of normal and malignant stem/progenitor cells by the same marker can support the concept that these cells are primary targets of transformation. Research has applied knowledge obtained in the fields of hematopoietic, neural and epidermal stem cells, and investigated markers borrowed from these areas to identify prospective stem/progenitor cells in the mammary gland (Cariati and Purushotham, 2008).

In the last years, specific populations of breast cancer cells with stem cell-like features and tumorigenic characteristics were identified and investigated in some *in vivo* models. Al-Hajj et al. (2003) isolated the first CSCs in a solid tumor and identified human tumorigenic breast CSCs with an enriched $CD44^{+}/CD24^{-/low}/ESA^{+}$ antigenic phenotype. As few as 200 of these cells in a primary invasive breast tumor, which comprised between 1% and 10% of the total cell population, were capable of forming new tumors when implanted in the mammary fat pad of female NOD/SCID mice. Conversely, 20,000 cells isolated from the same tumor that did not display this phenotype were unable to form tumors. Moreover, the $CD44^{+}/CD24^{-/low}/ESA^{+}$ cells isolated from tumors initiated by these cells were able to transfer the tumor to secondary and subsequent hosts, demonstrating the capacity for maintenance of self-renewal and tumorigenic properties. The generated tumors also reproduced the phenotypic heterogeneity of the original tumors.

The CSC hypothesis suggests that breast cancer initiation may take place preferentially in a normal mammary stem or progenitor cell expressing the CD44 marker (Fig. 2) (Abraham et al., 2005). A hypothetical model of tumor progression to metastatic disease in breast cancer considers that metastasis can be initiated by invasive $CD44^{+}$ breast cancer cells and that tumors rich in $CD44^{+}$ cells have a significantly worse clinical outcome (Shipitsin et al., 2007; Shipitsin and Polyak, 2008). In agreement with this assessment, it has been postulated that breast cancers of basal-like phenotype, which carry a poor outcome, are enriched with $CD44^{+}$ cells (Sorlie et al., 2001; Shipitsin et al., 2007). Honeth et al. (2008) recently demonstrated an association between basal-like phenotype and $CD44^{+}/CD24^{-}$ cells. They found, however, that not all basal-like tumors contain $CD44^{+}/CD24^{-}$ cells, emphasizing that a putative tumorigenic ability may not be confined to cells of this phenotype and that other breast CSC markers remain to be identified.

In agreement with the stem cell property of anchorage-independent growth in sphere cells, Dontu et al. (2003) and Ponti et al. (2005) found that isolated $CD44^{+}/CD24^{-}$ human breast cancer cells can also form tumor mammospheres and propagate i*n vitro*. Moreover, Dontu et al. (2003) demonstrated that these cells produce vascular endothelial growth factor (VEGF) and are highly angiogenic. Subsequent experimental studies have also isolated $CD44^{+}/CD24^{-}$ breast cancer cells and demonstrated increased *in vitro* expression of stem cell markers and enhanced capacity for mammosphere formation, invasion, and resistance to radiation (Phillips et al., 2006; Sheridan et al., 2006). Furthermore, clinical studies indicate that $CD44^{+}/CD24^{-}$ tumor-initiating cells express an invasive gene signature and may be associated with distant metastases (Abraham et al., 2005; Balic et al., 2006; Liu et al., 2007).

There are some studies implicating CSCs in breast metastasis. To investigate the association of the stem cell phenotype to metastasis, Balic et al. (2006) examined the expression of stem cell markers in disseminated metastatic cancer cells detected in bone marrow sites of breast cancer patients and found an increased number of $CD44^{+}/CD24^{-}$ expressing cells. Similarly, Sheridan et al. (2006) demonstrated that $CD44^{+}/CD24^{-}$ breast cancer cells have enhanced invasive characteristics.

There are also other sets of markers associated with breast stem cells and CSC phenotype. Stingl et al. (2006) found that cells expressing CD29 and/or CD49F, as well as CD24, displayed the stem cell properties of self-renewal and multilineage differentiation, while Shackleton et al. (2006) demonstrated that a single cell from the $CD29^{high}/CD24^{+}$ or $CD49F^{high}/CD24^{+}$

Table 1. Cancer stem cell phenotypes according to stem cell markers in different organs.

Stem cell marker	Organ	Cancer stem cell phenotype	Reference
CD44	Breast	$CD44^{+}/CD24^{-}$	Al-Hajj et al., 2003
	Pancreas	$CD44^{+}/CD24^{+/}ESA^{+}$	Li et al., 2007
	Head and Neck	$CD44^{+}$	Prince et al., 2007
	Prostate	$CD44^{+}/CD24^{-}$	Hurt et al., 2008
CD133	Brain	$CD133^{+}$	Sinh et al., 2004
	Prostate	CD133/CD44/α1ß2	Collins et al., 2005
	Colon	$CD133^{+}$	O'Brien et al., 2004
CD24	Colon, Stomach, Gallbladder and Ovary	$CD24^{+}$	Lim and Oh, 2005
	Pancreas	$CD24^{+}/CD44^{+/}ESA$	Li et al., 2007
ALDH1	Hematopoietic	$ALDH1^{+}$	Matsui et al., 2004
	Breast	$ALDH1^{+/}CD44^{+/}CD24^{-/}lin^{-}$	Ginestier et al., 2007

population was capable of reconstituting a functional mammary gland when this cell was transplanted into a cleared mouse mammary fat pad. In a recent investigation, Croker et al. (2009) found that $ALDH^{high}/CD44^{+}/CD24^{-}$ and $ALDH^{high}/CD44^{+}/CD133^{+}$ stem-like cancer cells isolated from some human breast cancer cell lines (MDA-MB-231 and MDA-MB-468) demonstrate enhanced malignant/metastatic behavior in both *in vitro* and *in vivo* experiments.

Enzymatic activity detection of ALDH1

The ALDEFLUOR assay is a simple method for identifying CSCs, and is based on enzymatic activity detection of ALDH1, a detoxifying enzyme responsible for the intracellular oxidation of aldehydes. According to Sophos and Vasiliou (2003), ALDH1 may have a role in early differentiation of stem cells through its function in oxidizing retinol to retinoic acid. Retinoic acid signalling is linked to cellular differentiation during development and plays a role in stem cell self-protection throughout an organism's lifespan (Croker et al., 2009).

ALDH1 activity can provide a common marker for both normal and malignant stem cells. Cells with high ALDH1 activity have been associated with several types of human hematopoietic and neural stem cells (Armstrong et al., 2004; Corti et al., 2006), and the ALDEFLUOR assay was also successfully used to isolate CSCs from leukemia and multiple myeloma (Matsui et al., 2004). Confirming these findings, Nagano et al. (2007) demonstrated that the ALDH1 enzyme can identify rapidly dividing cells that represent a progenitor cell population in human umbilical cord blood and bone marrow. Therefore, in agreement with Croker et al. (2009), the use of ALDH1 activity detection as a purification strategy allows an efficient isolation of normal and malignant human stem cells based on a developmentally conserved stem cell function.

In the mammary gland, Ginestier et al. (2007) demonstrated that ALDH1 is a marker of stem/progenitor cells of the normal human breast and breast carcinomas. The ALDEFLUOR positive cells isolated from both normal and tumoral human breast have phenotypic and functional characteristics of mammary stem cells, are capable of self-renewal and recapitulate the heterogeneity of the parental tumor. There was also partial overlap between the $CD44^{+}/CD24^{-}/lin^{-}$ and $ALDH1^{+}$ populations, with cells expressing the phenotype $CD44^{+}/CD24^{-}/lin^{-}/ALDH1^{+}$ able to form tumors from as few as 20 cells. Furthermore, the ALDEFLUOR positive population isolated from human breast tumors had the ability to generate tumors in NOD/SCID mice. Its expression was also associated with aggressiveness and poor clinical outcome in a series of 477 breast carcinoma patients (Ginestier et al., 2007). Detection of expanded stem cell clusters using markers such as ALDH1 in breast biopsy tissues may identify women with increased risk for subsequent breast cancer development (Liu et al., 2008).

Pathways regulation in stem cell maintenance

Different mutations associated with cancer occur in pathways that govern stem cell maintenance, suggesting that deregulation of normal mechanisms of stem cell functionality may be involved in carcinogenesis (Reya et al., 2001). Similar to normal stem cells, when a CSC divides, one daughter is an exact copy of the original and retains the ability to divide and initiate additional tumors, whereas the other daughter cell differentiates to produce nontumorigenic cells. This asymmetric division is strictly regulated by various pathways that govern the normal stem cell self-renewal and differentiation (Charafe-Jauffret et al., 2008). Human cancer mutations have been identified that can cause dysregulation of important pathways involving Hedgehog (Hh), Bmi-1, Wnt, NOTCH, HER-2, p53 and PTEN signalling (Crowe et al., 2004; Woodward et al., 2005).

Hedgehog pathway

The Hh pathway is one of the main pathways that control stem cell fate, self-renewal, and maintenance. Sonic hedgehog (SHH) acts as secreted morphogen in developmental patterning. Mutations in the SHH receptor patched (PTCH), a putative tumor suppressor, with consequent mRNA upregulation (due to loss of transcriptional autoregulation) were first shown in human basal carcinomas of the skin (Unden et al., 1996). More recently, other work has shown Hh pathway dysfunction in other human malignant neoplasms, such as prostate and breast carcinomas (Karhadkar et al., 2004; Olsen et al., 2004). In human gliomas, Hh signalling represents a new therapeutic target through its essential control in the behavior of glioma CSCs (Clement et al., 2007).

In the mammary gland, strict regulation of the Hh pathway is required for normal development (Charafe-Jauffret et al., 2008). Using both in vitro culture systems and NOD/SCID mice, Liu et al. (2006) found that this pathway, together with the polycomb protein Bmi-1, play important functions in regulating self-renewal of both normal and malignant human mammary stem cells. More recently, Charafe-Jauffret et al. (2008) showed that the Hh signalling pathway is activated in human breast CSC defined as $CD44^{+}/CD24^{-/low}$ phenotype.

The capacity of angiogenesis induction can be inherent to normal stem cells as well as their transformed counterparts, and some investigations suggest a role for Hh signalling in this process. According to Byrd and Grabel (2004), Hh signalling can target endothelial stem cells directly or stimulate blood vessel support cells to produce vascular growth factors. In agreement with this assessment, Fu et al. (2006) found that Hh protein promotes bone marrow-derived endothelial progenitor cell proliferation, migration and

VEGF production. Moreover, recent data suggest Hh ligands produced by tumor cells activate signalling pathways in the stromal microenvironment (Yauch et al., 2008).

Based on these findings, the development of specific Hh inhibitors, such as cyclopamine is currently underway in breast cancer, and clinical trials utilizing these chemotherapeutical agents are in the planning stages (Liu et al., 2006; Kakarala and Wicha, 2008).

Wnt pathway

Wnt signalling is known to regulate cell fate decisions, influencing morphology, proliferation, apoptosis, differentiation, migration, and stem cell self-renewal (Turashvili et al., 2006). Wnt proteins may assist in maintaining stem cells in an undifferentiated, self-renewing state within their niche (Nusse, 2008). Defects in the Wnt pathway are seen early in colon cancer carcinogenesis (Olsen et al., 2004).

In embryonic mammary development, the Wnt pathway is implicated at several stages and has been shown to be associated in the regulation of self-renewal and differentiation of the mammary stem cells (Turashvili et al., 2006). In breast cancer, Li et al. (2003) showed that tumors induced in MMTV transgenic mice codify components of the Wnt signalling pathway and that Wnt-1-induced tumors displayed markers of both epithelial and myoepithelial lineage. The authors suggest that deregulated Wnt signalling causes excess proliferation of mammary progenitor cells, predisposing them to cancer. Corroborating these findings, Stingl et al. (2006) observed that in MMTV-Wnt transgenic mice, the number of cells displaying stem cell markers expanded more than six-fold than in the preneoplastic phase.

NOTCH signalling

NOTCH signalling has been shown to play an influence in cell fate in hematopoietic, neural, and embryonic stem cells (Brennan and Brown, 2003). Aberrant NOTCH signalling has been observed in several human cancers, such as human T-cell acute lymphoblastic leukemia, cervical cancer, and breast cancer, suggesting that inhibition of NOTCH may represent a potential therapeutic target (Dievart et al., 1999; Nickoloff et al., 2003).

In human normal mammary stem cells, Charafe-Jauffret et al. (2008) demonstrated that induction of NOTCH signalling can promote self-renewal. According to Brennan and Brown (2003), unregulated NOTCH signalling in the mouse mammary gland leads to tumour formation. In an investigation performed in human breast cancer, the high expression of NOTCH intracellular domain in ductal carcinoma in situ (DCIS) predicted a reduced time to recurrence five years after surgery (Farnie and Clarke, 2007).

HER-2

An initial event in oncogenesis of sporadic breast cancer can be the amplification and overexpression of the HER-2 (human epidermal growth factor receptor 2) gene. The HER-2 gene is amplified in about 18-25% of human breast cancers and has been implicated in mammary tumorigenesis as well as in mediating aggressive tumor growth and metastasis (Korkaya et al., 2008).

HER-2 overexpression in normal human mammary epithelial cells, as well as in mammary carcinomas, increases the proportion of stem cells, as indicated by ALDH1 expression (Korkaya et al., 2008). Ginestier et al. (2007) also found a significant correlation between expression of the stem cell marker ALDH1 and *HER-2* overexpression. The development of *HER-2* inhibitors such as trastuzumab or lapatinib, have demonstrated clinical benefit, and the clinical efficacy of trastuzumab may relate to its ability to directly target the CSC population in HER-2-amplified tumors (Kakarala and Wicha, 2008; Korkaya et al., 2008).

Additional pathways

Tumor suppressor genes such as *PTEN* and *p53* have also been implicated in the regulation of CSC self-renewal. According to Korkaya and Wicha (2007), these genes are deregulated in CSCs, leading to uncontrolled self-renewal, which in turn can generate tumors that are resistant to conventional therapies.

Stem cell niche

Normal stem cells and cancer cells are both believed to be regulated by epigenetic mechanisms and their microenvironments. Evidence suggests that the stem cell niche is the specialized microenvironment surrounding stem cells that maintains their stemness and prevents their differentiation, controlling normal stem cell maintenance and self-renewal (Spradling et al., 2001; Wicha et al., 2006). Moreover, stem cells that replenish and repair adult tissues must be able to resist the stress events associated with episodes of tissue damage. Experimental evidence suggests that stem cells residing in adult tissues are extremely resistant to alterations in pH, temperature, and toxicant exposure (Woodward et al., 2005).

The pathophysiologic microenvironment in tumors can be very heterogeneous. It comprises stem cells, extracellular matrix properties, and signalling from surrounding cells. The niche to which normal stem cells are exposed can determine their ability to differentiate, and can also modify their biological properties, such as invasion and metastatic potential (Brisken and Duss, 2007).

In breast tumors, it has been stated that breast density is an important risk factor for breast cancer

development (Filip et al., 2006). According to Savarese et al. (2006), some growth factors produced by the breast stromal fibroblasts appear to influence breast density and also mammary stem cell behavior. In addition to mammary fibroblasts, the role of endothelial cells and adipocytes in mammary stem cell behavior is also currently being investigated (Kakarala and Wicha, 2008).

Regarding metastatic dissemination, Karnoub et al. (2007) showed that human mesenchymal stem cells derived from bone marrow mixed with human breast carcinoma cells markedly enhanced the metastatic capacity of the tumor cells. This occurred through tumor cell stimulation of chemokine CCL5 secretion by mesenchymal stem cells, which then acted in a paracrine manner on the breast cancer cells to enhance their motility, invasion, and metastatic potential. According to Karnoub et al. (2007), this metastatic capacity is reversible and depends on CCL5 signalling through the chemokine receptor CCR5.

CSC hypothesis implications for breast cancer treatment

The heterogeneity and molecular complexity of breast cancer are the great challenges for the development of effective strategies to prevent and treat this disease. Current therapeutic strategies in breast cancer (surgery, radiotherapy, hormonal therapy, and chemotherapy) have reduced recurrence rates; however, disease still recurs in a significant proportion of women after these treatments. The episodes of recurrences and lack of curative treatment in metastatic disease raise the question of whether current therapies target the correct cells (Charafe-Jauffret et al., 2008; Kakarala and Wicha, 2008).

The existence of CSCs is significant to breast cancer treatment. Beyond the limitations of current therapies in targeting the CSC component, there is also evidence that breast CSCs, as well as CSCs from other cancer types, are relatively more resistant to both radio- and chemotherapy (Sakariassen et al., 2007; Tang et al., 2007; Kakarala and Wicha, 2008). Although CSCs comprise only a very small percentage of the cells within a tumor, a postulated mechanism for this resistance is that stem cells are slowly proliferating and remain in the G0 phase of the cell cycle for extended periods of time. They also express increased levels of transporting proteins that enable efflux of chemotherapeutic drugs, making the cells tolerant to cell-cycle-based cytotoxic therapies (Kakarala and Wicha, 2008) (Fig. 3). Moreover, according to Smalley and Clarke (2005), the ALDH1 enzyme that is highly expressed in stem cells is capable of metabolizing some chemotherapeutic agents, such as cyclophosphamide. As shown by Abraham et al. (2005) and Glinsky et al. (2005), the breast tumors with elevated proportion of CSCs are associated with a higher risk of local and distant recurrences. In the breast cancer cell line MCF7, Phillips et al. (2006) observed that cells expressing the $CD44^{+}/CD24^{-/low}$ phenotype are associated with resistance to both radiotherapeutic and tamoxifen treatment at clinically relevant doses, with the size of this population increasing after short sessions of fractionated irradiation. According to these authors, these findings provide a possible mechanism for the accelerated repopulation of tumor cells observed during gaps in radiotherapy.

Different pathways may be associated with the determination of mammary stem cell fate and can be deregulated in cancer. The clarification of pathways that regulate self-renewal and maturation of mammary stem cells may provide potential targets for breast cancer prevention and development of new therapeutic strategies. Despite stem cell compartments constituting a very small target population for therapy, successful clinical treatment will require the knowledge of particular tumor cell pathways and proteins that are specifically expressed in these cells and that will be susceptible to drug targeting (Wicha et al., 2006; Campbell and Polyak, 2007). Hopes for new treatments that selectively kill these cancer cells comes from recent work showing the signalling pathways that control the CSC proliferation and their local microenvironment during cancer evolution (Korkaya et al., 2007). These new findings have crucial implications for the aiming of current therapeutic strategies and for the development of the next generation of targeted therapies.

Conclusions

Stem cells have been documented in a variety of normal tissues, and also identified in malignant neoplasms. The CSC hypothesis is changing our current understanding of breast carcinogenesis and provides a new paradigm that should impact breast cancer risk assessment, prevention, early detection, outcome, and treatment.

The presence of a rare CSC population among the heterogeneous mix of tumoral cells supports the hypothesis that CSCs can have a fundamental influence on the local regrowth of cancers following treatment.

Despite the increased number of publications in the CSC field over the past few years, further work is imperative. Strategies to isolate breast CSCs, find additional reliable cell surface markers, and perform comparative gene expression profiling of CSCs and their normal stem cell counterparts will be required to more accurately define mammary stem/progenitor cells, terminally differentiated luminal and myoepithelial cells, and breast CSCs, and to better identify putative therapeutic targets. It will be of enormous interest to determine whether $CD44^{+}/CD24^{-}/ESA^{+}$ breast CSCs can be directly targeted by specific therapies without associated toxicity to normal stem cell niches or harmful effects on wound healing. The use of breast cancer cell lines and cancer stem cell cultures and xenograft models

for drug discovery offer potential methods to identify successful new therapies that prevent self-renewal or contribute to a depletion of tumorigenic breast CSCs.

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