"To Be or Not to Be…" Cancer Stem Cells or Not Cancer Stem Cells..

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Abstract

Cancer stem cells (CSCs) are defined as cancer cells with self-renewal capacity. These cells represent a small subpopulation endowed with the ability to form new tumors when injected in mice. Membrane markers and cancer stem cell assays have been used to identify and characterize these cells. The self-renewal ability of cancer stem cells has suggested that this population could be responsible for new tumor formation and cancer relapse. The identification of the cells responsible for the initiation and maintenance of a tumor is a main goal of research as such identification would present opportunities to design more specific therapies. In this article we will critically review these points with emphasis on the assays that the scientific community has in its arsenal to characterize and identify breast cancer stem cells, exposing the divergent literature.

Introduction

Despite advances in the diagnosis and treatment of human malignancy, cancer remains among the leading causes of morbidity and mortality worldwide, with 7.5 million deaths attributed to cancer annually (1). Breast cancer is now the most frequently diagnosed form of cancer and the second leading global cause of death from cancer in women, accounting for 23% of cancer diagnoses (1.38 million women) and 14% of fatalities due to cancer (458,000 women) (1). The combination of better screening and treatment programs, however, has moderately improved the survival rate. Nevertheless, there is still much to be done if the many women who are refractory to current therapies are to have a better chance of survival. In the last decade, the scientific community has invested a lot of effort in searching for the origin of cancer. An attractive alternative has been the cancer stem cell theory, which outlines self-renewing stem-like cancer cells that are slowly cycling and consequently, difficult to attack with chemotherapy.

Breast and Stem Cells

Breast tissue, like the tissue of many other organs, is hierarchically-organized and maintained by a series of stem and progenitor cells that possess decreasing potency as they differentiate toward terminally-committed epithelial cells (2, 3).

The breast is composed of a bilayered epithelium comprising two main epithelial cell types: luminal epithelial cells and myoepithelial cells (sometimes referred to as "basal" epithelial cells) (2) (Figure 1). The luminal cells are specialized epithelium that line the ductal structures and produce milk during lactation. The myoepithelial cells form the basal layer surrounding the luminal cells and provide contractility to push milk through the ducts. These cells are in contact with the surrounding basement membrane that separates the parenchyma from the stromal component of the tissue.

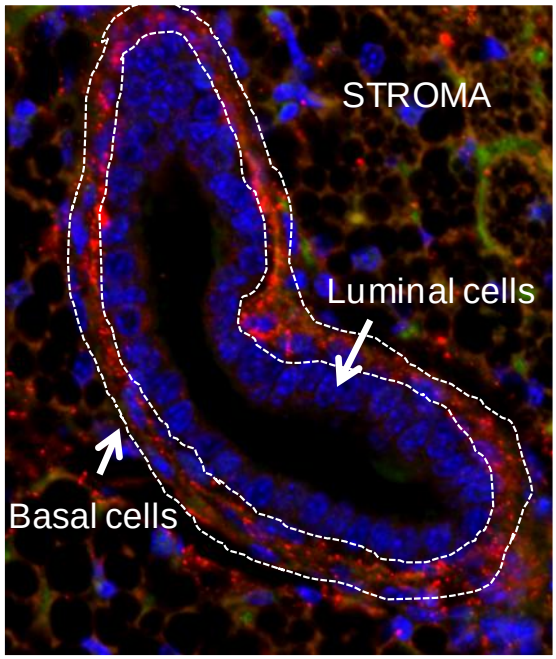

Figure 1. Mouse breast architecture. The breast is composed of a bilayered epithelium comprising two main epithelial cell types; luminal and myoepithelial cells. The myoepithelial cells surround the luminal cells and are in contact with the surrounding basement membrane that separates the parenchyma from the stromal component of the tissue.

Mouse mammary stem cells (MaSCs) share cell surface and expression profiles consistent with basal cells and are thus thought to reside within the basal compartment of the gland, although they are very rare and constitute a very small percentage of the basal layer (the MaSCs are thought to be suprabasal) (4). Isolated several years ago through the use of cell surface expression markers, cell populations greatly enriched for MaSCs have been shown to be capable of reconstituting an entire mammary gland when transplanted into a mammary fat pad previously cleared of endogenous epithelium (5, 6). Furthermore, serial transplant experiments have demonstrated that the mouse MaSCs can self-renew as well as give rise to the other cell types that make up the mammary gland (6, 7).

These primitive cells, called stem cells, have been favored candidates for targets of transformation because of their inherent capacity for self-renewal and their longevity, which would ostensibly allow for the sequential accumulation of genetic or epigenetic mutations required for oncogenesis. With such versatile functions, it is easy to see why the existence of a cancer cell with stem-like properties is an attractive explanation for cancer growth and recurrence. To better understand the cancer stem cell theory, specifically in regards to breast cancer, it is important to look at the discovery of breast cancer stem cells.

Discovery of Breast Cancer Stem Cells

The idea that stem cells are tumor "cells of origin" is enticing as they possess the lifespan required to accumulate sufficient genetic lesions to produce a tumor (8, 9), and the inherent self-renewal capacity that renders the reawakening of self-renewal programs during neoplastic transformation unnecessary. By definition, cancer stem cells refer to the cells that have stem cell properties, i.e., self-renewal and differentiation, in addition to potent tumor-driving capability. Cumulative evidence suggests that breast cancers are initiated and maintained by a subpopulation of tumor cells with stem cell features (termed cancer stem cells). The first evidence was provided by Al-Hajj M et al. in 2003. Using cell surface markers, Al-Hajj and colleagues found that $CD44^{+}/CD24^{-/low}$ Lin^{-} cells from breast cancer patients were significantly enriched for tumor forming ability in NOD/SCID mice compared with $CD44^{+}/CD24^{+}$ Lin^{-} cells. Moreover, the tumors formed by $CD44^{+}/CD24^{-/low}$

Lin^- cells could be serially passaged (self-renewal) and could also reproduce the tumor's cellular heterogeneity observed in the initial tumor (differentiation) (10). The idea that tumors arise from stem cells is an attractive idea; an equally plausible explanation, however, is that tumors arise from differentiated cells that acquire self-renewal and differentiation capacity through genetic and/or epigenetic mechanisms (11, 12, 13), as well as via paracrine interactions with their environment (14, 15).

Methods to Identify Cancer Stem Cells

A variety of methods have been developed to identify and characterize mammary cancer stem cells. Inspired by the research from other systems, scientists in the mammary stem cell field have worked out various assays that help enrich the stem cell preparations and improve their purity, increasing the feasibility of studying these cells. The following discussion summarizes the major approaches established so far.

A. The side population staining technique

The technique of isolating "side population" cells is based on the observation that stem/progenitor cells take up much lower levels of vital dyes because of the overexpression of transmembrane transporters that actively pump out the dye from these cells, as compared with differentiated cells (16). Using this technique, stem cells appear on the "side" of the bulk population when cells are analyzed by flow cytometry, thus earning the title of "side population". Studies using the fluorescent blue Hoechst 33342 dye, which binds preferentially to adenine-thymine (A-T) regions of DNA, have shown that there is a subpopulation of cells with transmembrane channels, such as breast cancer resistance protein (BCRP1/ABCG2), that can pump out the dye from the cells. Patrawala and colleagues isolated cancer stem cells from the breast cancer cell line MCF-7 based on this technology, showing that only 0.2% of the population is negative for the dye (side population). This side population of cells has higher tumorigenicity and a shorter latency period when compared with the non-side population (17). Some controversy surrounds this technique, however, since Hoechst is toxic to cells, causing some false phenomena such as lower self-renewal, and less tumorigenicity of the non-side population (18), as well as differentiation of the cells unable to efflux the fluorescent dye (19, 20).

B. Expression of cell surface markers

Certain stem cell-enriched populations have also been isolated based on the expression of cell surface markers via fluorescence-activated cell sorting (FACS), a common approach used to isolate distinct subsets of blood cells in the hematopoietic system. Successful applications of FACS depend on well-characterized cell-surface and/or intracellular markers. One of the pending issues to be addressed in stem cell biology is related to the identification of cancer stem cells using well-validated markers. In neural stem cells, the CD133 marker has been considered a marker of cancer stem cells (21). Indeed, CD133 has been found in many cancers, including hematopoietic diseases (22), liver tumors (23), breast cancer (24), prostate cancers (25, 26), glioblastoma, and other brain tumors (27, 28, 29). However, its utility in some cancers has been disputed (30). Another example is the combination of CD44 and CD24. In humans, the markers $CD44^+/CD24^{-/lo}$ are frequently used to enrich for tumor initiation among several subtypes of breast tumors (31). Nonetheless, caution must be exercised, since controversial results were found in some of the cell lines being studied. For example, Croker et al. found sub-populations of cells demonstrating stem-cell characteristics in MDA-MB-435, MDA-MB-231, MDA-MB-468, but not in MCF-7, a cell line that was reported to have a side (cancer stem cell) population (32, 17). Also, Sarrio et al. showed that within epithelial populations, $CD44^{high}/CD24^-$ stained mesenchymal-like cells that formed mammospheres and had an invasive phenotype, but the cells lacked the capacity to produce the heterogeneity of the parental cell line (33).

Therefore, these cells did not meet all the criteria of *bona fide* CSCs. In this same direction, Chaffer et al., 2013, have shown that CSCs expressing $CD44^{hi}$ are ablated for tumor initiating ability if the expression of the transcription factor ZEB1 is inhibited (13). The above results reveal that these cell surface markers are only surrogates that help define stem cell populations, but might not have anything to do with their function as stem cells, highlighting the importance of testing "stemness" functionally rather than assuming that a particular combination of cell surface markers is indicative of a phenotype.

C. ALDH activity

Another important assay used to characterize mammary stem cells is to assess the activity of an intracellular enzyme called aldehyde dehydrogenase (ALDH) 1, which is responsible for the oxidation of intracellular aldehydes and involved in early differentiation of stem cells (34). ALDH1 activity can be easily detected by the ALDEFLUOR assay, in which ALDH catalyzes a substrate to a product that is brightly fluorescent. This assay has been successfully applied to isolate cells with stem-like properties in the hematopoietic system, neural system, and mammary gland (35, 36). In the mammary gland, high ALDH activity identifies not only normal cells with stem/progenitor properties but also tumor cells that are capable of self-renewal and of generating tumors that recapitulate the heterogeneity of the parental tumor. In addition, immunostaining analysis significantly correlates the activity of ALDH with poor prognosis of breast cancer patients (36). Controversy also surrounds this assay, however, and it has been reported recently that ALDH expression does not correlate with tumorogenicity *in vivo* (37).

D. Mammospheres assay

Neural stem cells (NSCs), when cultured in suspension (on a low-attachment surface), form clusters of cells called neurospheres. Analogously, Wicha and co-workers have developed the mammosphere assay, an *in vitro* method for isolating and propagating mammary stem cells under anchorage-independent conditions (38) (Figure 2). The mammospheres can be serially passaged without reducing the mammosphere number produced, implying a self-renewal potential. In composition, the mammospheres contain a heterogeneous population of cells, as indicated by the positive immunostaining of markers for potential stem/progenitor cells, differentiated luminal epithelial cells, and myoepithelial cells. In a 3-dimensional culture, mammosphere-derived cells grow into colonies of ductal, myoepithelial, or both cell lineages. When compared with other techniques for isolating mammary stem cells, these mammospheres also present a 30-fold higher enrichment for side population cells (38). Nonetheless, caution must be exercised, as some inconsistent results have been observed when applied to the use of tumor cells (termed a tumorsphere assay in general, and mammosphere in the breast cancer stem cell field). For example, MDA-MB-231, a highly tumorigenic and metastatic cell line *in vivo* (39), produces loosely adhered clumps of cells when it is assessed in the mammosphere assay, which in a rigorous way should not be considered as mammospheres (40) (Figure 2; Castano Z., unpublished observation).

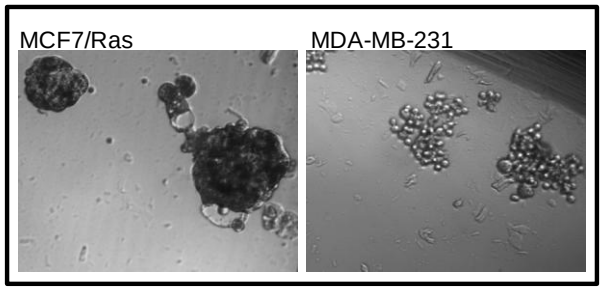

Figure 2. Mammosphere assay. Example of mammospheres formed after 7 days in culture by MCF7/Ras and MDA-MB-231 human tumor cell lines after 100 cells were plated under anchorage-independent conditions (protocol described by Dontu G et al., 2003 (38)).

In summary, although various methods are available to isolate and characterize mammary cancer stem cells based on the intrinsic features of these cells, no single method has proven

sufficiently robust to identify the *bona fide* cancer stem cells, and these assays always require functional validation *in vivo* in order to confirm their capacity to form a tumor.

E. Limited dilution assay *in vivo*

Several studies have demonstrated that some cancer cell lines contain a small fraction of cells that can give rise to tumors *in vivo* when injected in limiting dilution. In theory, one single cancer stem cell should be able to give rise to an entire tumor. However, given the technical limitations of isolating and injecting a single cell, researchers consider limiting dilution analysis as an indicator of CSC function. Some examples in the literature show that tumors are formed when 10^2 and 10^7 tumor cells, sorted by FACS for CSC expression markers or ALDH1 activity, are xenotransplanted in mice (10, 40, 36). These studies demonstrated that as little as a couple hundred cancer cells gave rise to a tumor, suggesting an enrichment for CSCs. In these studies, the resulting tumors could be serially passaged to give rise to new tumors with similar heterogeneity observed in the initial tumor (10). These studies provide functional *in vivo* validation of the CSC theory.

Unfortunately, discrepancies have been reported and some groups have recently shown that the *in vivo* tumor-initiating capacity of different breast cancer cell lines derived from primary tumors do not correlate with the expression of the CSCs makers (CD44/CD24, CD133, or ALDH1) (41, 37), calling into question the legitimacy of the *in vitro* assays in predicting the tumorigenicity of tumor cells *in vivo*.

Conclusions and future directions

Different alterations are involved in breast cancer initiation and progression, including genetic mutations, epigenetic changes, or surrounding stromal stimuli (44). Studies on the characterization of those properties could be the key to controlling the neoplastic process, drug resistance, relapse, and even metastasis. A major aspect that requires further clarification is the identification of the cancer stem cell subpopulation in human derived cell lines, in order to understand what fraction of cancers follow the stem-cell model. Also, there are facets of the cancer stem cell theory that the scientific community does not yet fully understand, such as a nuanced view on exactly what unique ability these cells possess that allow them to generate a full tumor. What remains to be determined is whether these tumor cells or CSCs are inherently and genetically (by mutation) endowed with the ability to initiate and propagate the tumor, or whether these tumor cells have the ability to set up a permissive tumor microenvironment that secretes all the growth factors that will allow their propagation. If the latter premise is what defines a cancer stem cell, then all the available *in vitro* assays should be re-conceptualized, and we should start to focus our efforts on developing assays that classify tumor cells as passive or active in mobilizing and activating components of the tumor microenvironment. In this context, the correct development of more specific and accurate assays to characterize them is required.

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