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Breast cancer stem cells

Introduction

Breast cancer is relatively common and remains a lethal diagnosis for many people today. Based on 2010-2012 data, 12.3% of women will develop breast cancer during their lifetime. In 2015, it will cause an estimated 40,290 female deaths in the United States. Furthermore, breast cancer accounts for 14% percent of all new cancer cases in the United States and 6.8% of total deaths caused by cancer. After diagnosis, an estimated 89.4% will still be living in five years (15).

Although increasingly effective treatment options exist for the majority of breast cancer patients, there are perpetual risks associated with the disease, such as drug resistance, cancer relapse, and metastasis. When cancer spreads from the primary site to other parts of the body, a small subset of cells can repopulate the phenotypically heterogeneous cell population found in the original tumor. When cancer recurs, a small population of cells can give rise to many of the cellular subtypes present in the original tumor. In accordance with the cancer stem cell model of disease, the cells responsible for tumor initiation, maintenance, and progression are known as breast cancer stem cells (BCSCs) (6).

Cancer is a caricature of normal human development. Both normal stem cells and BCSCs have with the ability to self-renew and differentiate (6). Thus, it is necessary to

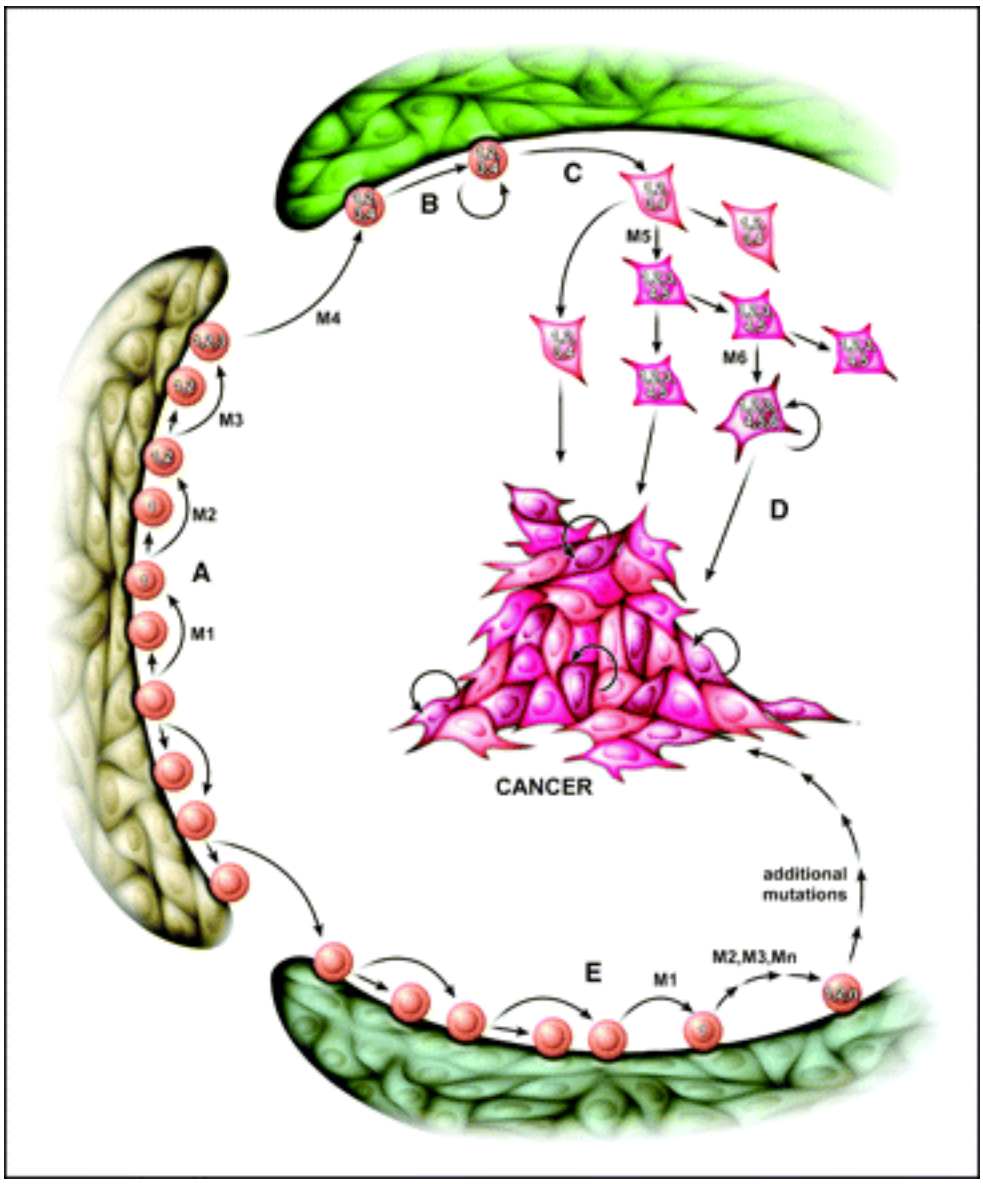
understand how normal stem cells function in the mammary gland as well as which cells in the normal breast tissue are likely to contribute to oncogenesis. As stem cell pathways in cancer biology are elucidated, novel therapeutic targets can be identified and innovative treatment strategies can be developed. In conjunction with the options today, therapies that target cells predisposed to tumorigenesis and drug resistance may reduce cancer metastasis, recurrence, and death in patients.

The cancer stem cell hypothesis

Although normal stem cells are necessary for survival, the cancer stem cell (CSC) hypothesis suggests that mutant stem cells are the root of cancer pathology. It postulates that tumor maintenance is attributed to a small population of long-lived CSCs with regenerative capacity. The CSC hypothesis states that tumors contain a hierarchy in which only the CSCs give rise to cells that make up the tumor bulk and the majority of cells do not propagate tumors (1). Essentially, a CSC is characterized by the ability to self-renew and to differentiate into all cell types found in a heterogeneous tumor (4).

The cancer stem cell hypothesis does not specify the origin of CSCs. The relationship between potential cells of origin and various molecular subtypes of breast cancer is being investigated using lineage tracing studies (20). There is evidence that normal stem cells may acquire mutations and become cancerous due to their longevity. However, there is also data to suggest that progenitor cell populations may reacquire the property to self-renew and become CSCs after multiple mutations. For example, the ß-catenin pathway confers self-renewal on granulocyte-macrophage progenitors in chronic myeloid leukemia (8).

Figure 1: Potential cancer stem cell generation mechanisms (4)



Possible mechanisms for the generation of cancer stem cells within tissues are illustrated in Figure 1. **(A)** Normal cells in a niche acquire mutations as they divide. **(B)** Normal stem cells accumulate mutations that cause the cells to thrive in a novel microenvironment, which may lead to inhabitation of an alternative niche, or that allow the cells to induce expansion of the niche cell population, which may lead to mutant stem cell population expansion. **(C)** Stem cells acquire mutations that permit survival outside

of the niche and subsequent mutations that confer self-renewal**. (D)** Mutated stem cells differentiate to progenitor cells but maintain the ability to proliferate and subsequently reacquire self-renewal capacities. **(E)** Normal stem cells are in the presence of an aberrant niche that selects for mutations in stem cells that are precursors for CSCs (4).

Overall, the CSC proposes that there are small populations of CSCs in tumors that are vital for cancer initiation, progression, treatment resistance, metastasis, and relapse. A CSC is able to self-renew and give rise to all cells within the heterogeneous population of a tumor. Hence, selectively targeting the small subset of CSCs in addition to the tumor bulk rather than solely eliminating the majority of cells in the tumor is necessary to revolutionize cancer treatment.

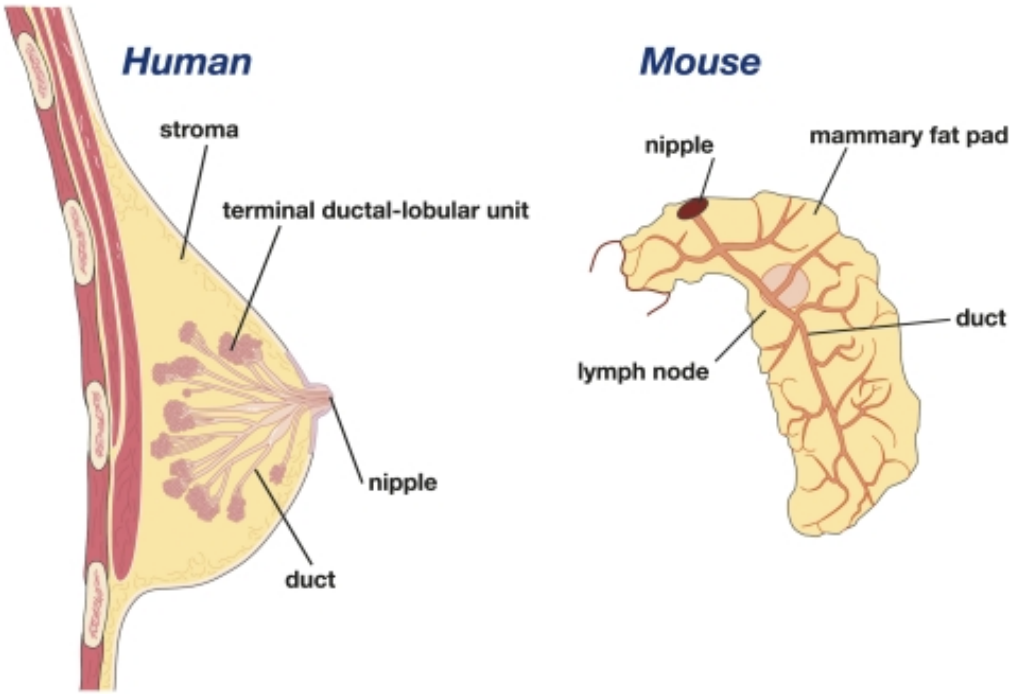
Mammary stem cells

The mammary gland undergoes drastic changes during puberty, pregnancy, lactation, and regression. Thus, its epithelium is ideal for the study of normal stem cells, breast morphogenesis, and tissue hierarchy. It is important to understand how normal mammary stem cells (MaSCs) function in order to draw comparisons about their pathological counterparts, CSCs. However, the isolation of MaSCs and the development of a MaSC assay are difficult processes because epithelial cells are tightly packed and depend on their microenvironment for normal function.

Nevertheless, single MaSCs have been isolated from mouse mammary glands that self-renew and are able to regenerate fully functional mammary gland components. The mouse MaSCs, or mammary repopulating units, also differentiate into mammary epithelial progenitor cells, or mammary colony-forming cells (14). MaSCs have also been

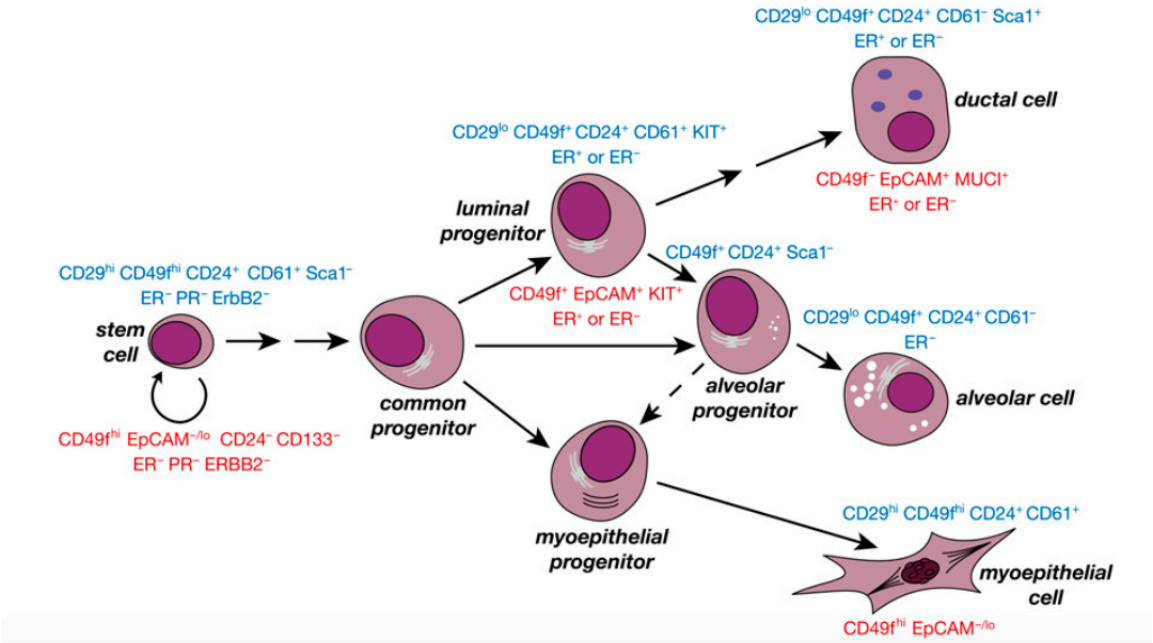
identified and isolated from human breast tissue (19). Figure 2 is a representation of both the human and mouse mammary gland.

Figure 2: Human and mouse mammary glands (20)



The gold standard of MaSC isolation and identification is a variation on the theme of mouse mammary fat pad transplantation *in vivo*. The process involves removing the epithelial parts of a pre-pubertal mammary gland and transplanting donor cells into the fat pad that only contains stromal elements. The stromal, or nonepithelial, cells of the mammary gland include fibroblasts, endothelial cells, macrophages, and adipocytes. MaSCs are able to regenerate ductal-lobular epithelial outgrowths when placed in a cleared fat pad. Furthermore, MaSCs can be transplanted into secondary mice to form secondary mammary outgrowths and continue to give rise to progenitor cells (13). Figure 3 narrates the differentiation hierarchy in mammary epithelium and the differential cell surface markers used to carry out isolation experiments. Red and blue markers indicate human and mouse markers of MaSC enriched cell populations, respectively (20).

Figure 3: The hierarchy of the mammary epithelium with characteristic cell surface marker expression (20)



Isolation of cancer stem cells

Similarly to MaSCs, a BCSC can be isolated and subsequently give rise to the heterogeneous cell population of the tissue that it previously occupied. Figure 4 illustrates the process of CSC isolation and identification using a technique known as fluorescence-activated cell sorting (FACS). Ideally, primary human tumor cells are donated from patients to be analyzed. If the cells are being isolated from a solid tumor, enzymes that degrade intercellular junctions and bounds are added to the tissue to allow cell dissociation (7).

Following incubation with antibodies for a particular surface antigen conjugated to fluorescent dye, antibodies bound to magnetic beads, or Hoechst 33342, the cells are sorted. Single cells flow through a narrow tunnel into a nozzle that creates droplets around each cell. The droplet then travels through a laser beam, which differentiates cells based on optical characteristics and administers electrostatic charges accordingly. In the electrostatic field, the charged cell flow is bent via electrostatic deflection and specific cells, namely CSCs, are collected in a vessel, whereas the excess cells continue to flow into a separate container (7).

Prospective CSCs are later xenotransplanted into immunodeficient animals, which are commonly NOD-SCID mice for BCSCs. If the injected cell suspension of candidate CSCs forms a tumor in the animal, the sorting process is repeated multiple times. It is extremely likely that cells are CSCs if they propagate secondary or tertiary tumors that are made of the heterogeneous cell population of the primary tumor, as shown in Figure 5. The capacity to initiate said tumors is known as serial transplantability (7).

Figure 4: CSC isolation by FACS (7)

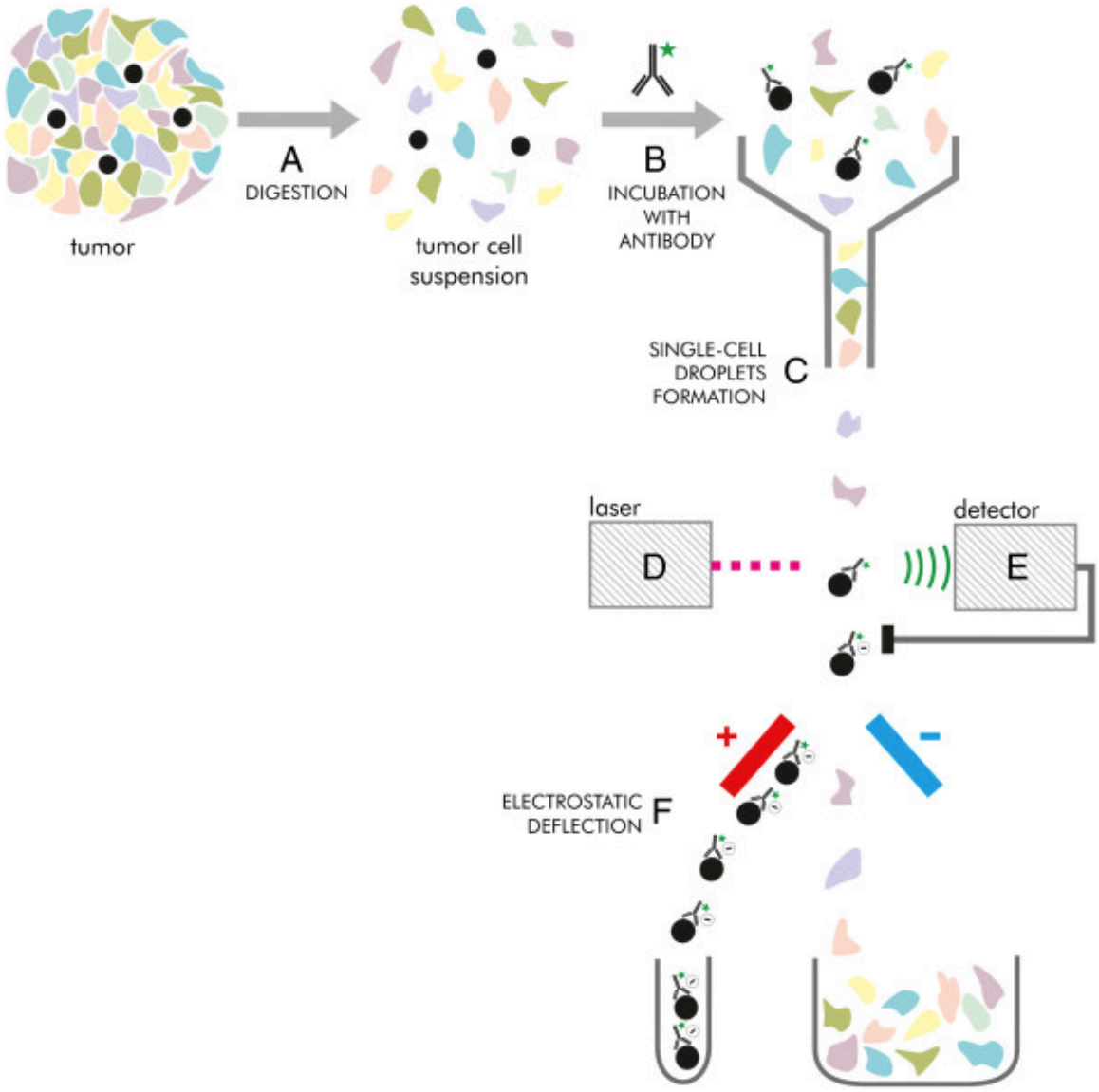
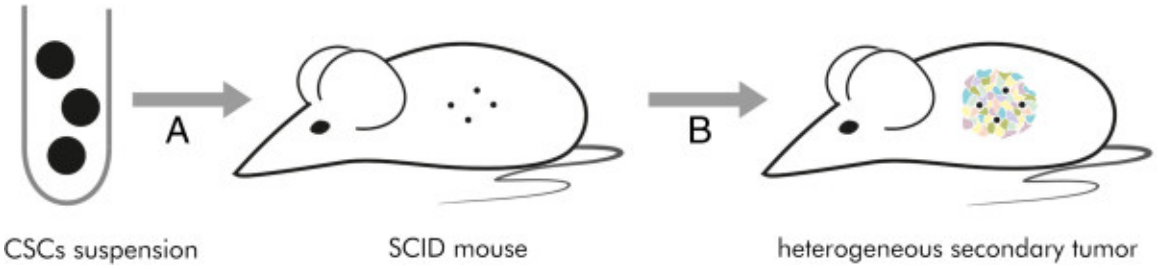


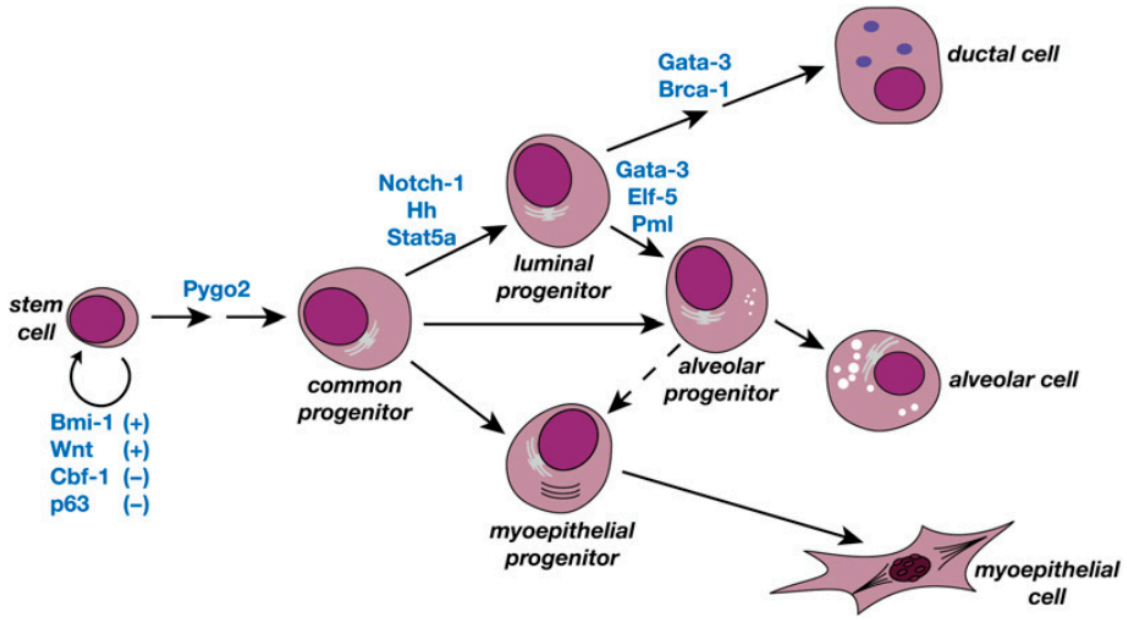
Figure 5: CSCs xenotransplantation into NOD-SCID mouse (7)



Significant signaling pathways and therapeutic implications

The molecular mechanisms that govern stem cell function in the mammary gland have been investigated but are complex and not fully understood. Figure 6 displays the transcriptional regulators and molecular pathways that play key roles in MaSC self-renewal, lineage commitment, and luminal differentiation. A (+) sign indicates positive effects on self-renewal and a (-) sign refers to inhibitory effects (20). Notch, Hedgehog, and Wnt signaling pathways all have strong relationships to BCSC maintenance and have been linked to oncogenesis.

Figure 6: Transcription factors and molecular pathways involved in the mammary epithelial hierarchy (20)



1) Notch

Notch signaling is involved in cell fate determination in the mammary gland. In the Notch signaling pathway, a ligand from one cell binds a Notch receptor in a secondary cell and causes a cascade of proteolytic cleavage events. The Notch intracellular domain (NICD) is cleaved and travels to the nucleus to regulate transcription of Notch target genes in the secondary cell. NICD activates transcription with CBF1/Suppressor of Hairless/LAG-1 and Mastermind-like protein (2).

It has been reported using *in vitro* and mouse xenotransplantation assays that Notch+ breast cancer cells exhibit higher expression of BCSC markers and an increased level of sphere formation. Moreover, Notch- breast cancer cells were not able to initiate tumorigenesis at serial dilutions in mouse xenografts whereas Notch+ cells were able to propagate tumors. Utilization of a Notch blocker, γ-Secretase inhibitor, selectively targeted Notch+ cells *in vitro* and in mouse xenografts. Analysis of primary patient samples of breast cancer cells revealed that increased Notch4 and Hey1 levels correlated with poor patient survival (9).

2) Hedgehog

The Hedgehog/Patched (Hh/PTCH) signaling pathway is vital for early embryogenesis and tumorigenesis. Hh ligands reverse the inhibitory effect of PTCH receptors on Smoothened, leading to a signaling cascade in which GLI functions as a transcription factor (16). The overexpression of components of the Hedgehog (Hh) pathway, such as Sonic Hh, Patched1, and Gli1, has been demonstrated in human breast cancer samples and not in the neighboring epithelium. Furthermore, exposure to a steroidal alkaloid that

blocks the Hh pathway decreases Gli1 expression and suppresses the growth of Hh pathway-activated breast cancer cells (10).

3) Wnt

Along with Notch and Hedgehog signaling pathways, the deregulation of Wnt signaling is implicated in oncogenesis. Specifically, aberrant Wnt signaling has been linked to altered regulation of self-renewal in the mammary gland. Mouse mammary tumor virus-Wnt1 mammary tissue maintains higher numbers of mammary stem cells and a population of progenitor cells with abnormal *in vivo* regenerative capacity. Furthermore, the progenitor population exhibited increased expression of cytokeratin 14, a basal marker, which indicates reacquisition of stem-like properties (18). Wnt signaling also plays a role in epithelial mesenchymal transition, which is a central process in breast cancer metastasis. Several nonsteroidal anti-inflammatory drugs that inhibit Wnt signaling are being investigated in clinic trials for their efficacy in cancer treatment. Additionally, monoclonal antibodies that neutralize Wnt ligands or interfere with the Wnt receptors Frizzled and LRP are in the early phases of clinical investigation (16).

Treatment resistance

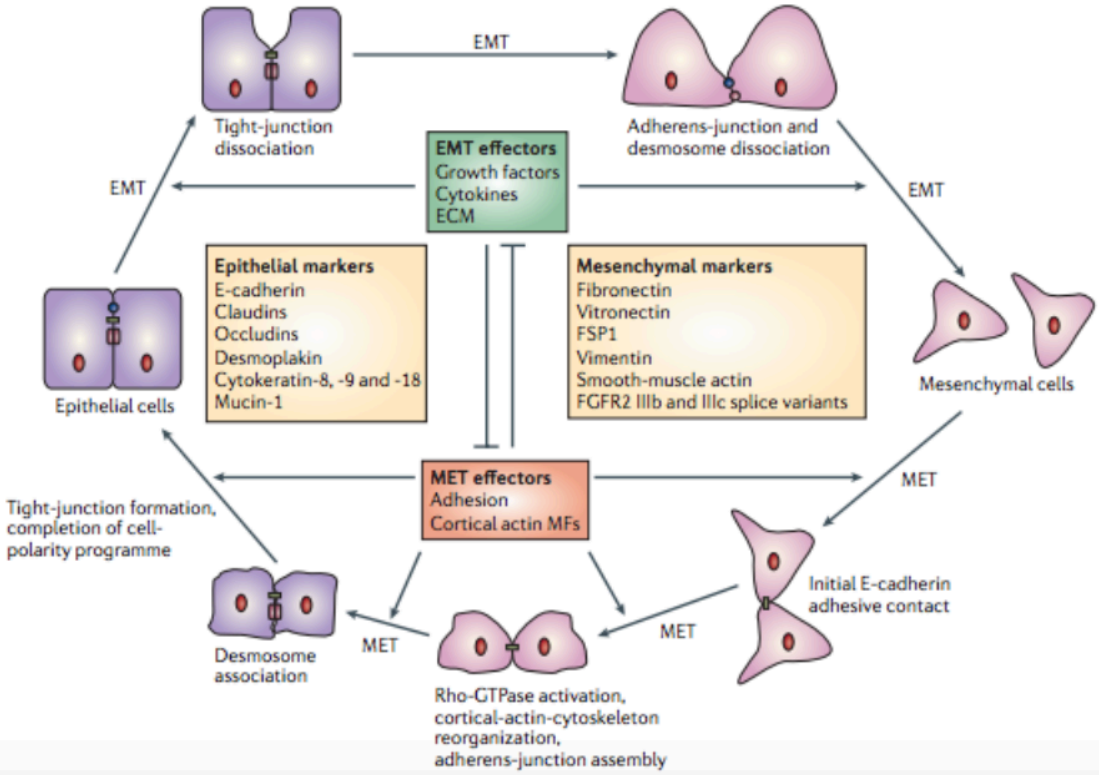
Approximately 30% of breast cancer patients develop metastatic breast cancer. Of the patients with metastatic disease, 90% will experience multi-drug resistance, which may be linked to the persistence of BCSCs (12). CSCs are typically resistant to current chemo- and radiation- therapies due to DNA damage repair mechanisms and abnormal apoptosis pathways. In particular, the population of CD133+ CSCs is enriched after radiation in glioblastoma and exhibits lower levels of apoptosis after chemotherapy (11). Moreover, residual breast cancer cells have been shown to exhibit increased expression

of CSC-marker genes following conventional cancer therapy (5). Normal stem cells are generally quiescent, which is an inherent advantage against traditional treatment methods that target rapidly cycling cells. It is suggested that CSCs are similar to normal stem cells in regards to quiescence and may evade chemo- and radiation- therapy whereas the cells that form the bulk of the tumor are eliminated (1).

Metastasis and Epithelial Mesenchymal Transition

Epithelial to mesenchymal transition (EMT) has major implications in cancer metastasis, as it is a process in which epithelial cells acquire mesenchymal properties. Epithelial cells are stationary and characterized by tight cell-cell adhesion, maintenance of cell polarity, and expression of epithelial cell surface markers. EMT results in loss of E-cadherin expression and gain of N-cadherin expression, vimentin, and fibronectin. Cells that undergo EMT have been reported to exhibit stem cell-like properties. Acquired mesenchymal properties include loss of cell polarity and tight cell junctions as well as an increase in motility and invasiveness (3). Figure 6 is a schematic representation of EMT and lists the cell surface markers that are differentially expressed between epithelial cells and mesenchymal cells. The cell junctions present in each cell type are also displayed (17). It is important to note that the reverse of EMT, known as mesenchymal to epithelial transition and referred to as MET, is possible. The induction of MET in stem cells has therapeutic potential, as it would diminish metastatic ability.

Figure 6: Epithelial to Mesenchymal Transition (17)



Conclusion

The cancer stem cell hypothesis states that CSCs contribute to tumor formation, maintenance, relapse, metastasis, and drug resistance. The xenotransplantation assay is the experimental standard for the isolation and identification of CSCs. Further research on the molecular mechanisms that regulate BCSCs in the hopes of a comprehensive cure for breast cancer is warranted. Wnt, Hedgehog, and Notch signaling pathways are attractive candidates for novel therapies because they are linked to breast oncogenesis. Epithelial to mesenchymal transition is a process that is essential for cancer metastasis and provides yet another target area for drug innovation. Therapeutic agents that target BCSCs as well as the bulk of the tumor have the potential to revolutionize breast cancer therapy by reducing cancer morbidity and mortality.

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