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## Breast Cancer Stem Cells

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### 1. Introduction

Over 150 years ago, Cohnheim and Durante formalized the concept that cancers might arise from a small subset of cells with stem cell properties<sup>1-3</sup>, and in 1961, Till and McCulloch demonstrated for the first time that the existence of hematopoietic stem cells (HSC) in the bone marrow, which was postulated that stem-like cells might be the origin of cancer<sup>4</sup>. However, only recently did an increased interest in cancer stem cells (CSC) occur, thus spurring great advances in cancer stem cell biology. The CSC model was first developed in 1994 when malignant initiating cells were discerned in human acute myeloid leukemia (AML)<sup>5</sup>. Afterwards, similar CSC model was extended to some solid tumors that originated in the breast, brain, lung, prostate, colon, head and neck, and pancreas<sup>6-12</sup>. Most importantly, the development of CSC hypothesis has fundamental implications in terms of understanding the biology of multi-step tumorigenesis, the prevention of cancer, and the creation of novel effective strategies for cancer therapy.

#### 1.1 The definition of cancer stem cells

It is well documented that tumors contain cancer cells with heterogeneous phenotypes reflecting aspects of their apparent state of differentiation. In a tumor, the mutable expression of normal differentiation markers by cancer cells implies that some of the heterogeneity arises as a result of this altered manifestation. Also, cancer is known to be the product of the accumulation of multiple genetic mutations and epigenetic alterations in a single target cell, the occurrences of which can sometimes take place over many decades. Furthermore, chemotherapy and radiation therapy for cancers have limited effectiveness in long-term scenarios, and the possible recurrence of tumors after years of disease-free survival exists in great majority of cancers. All these observations provide persuasive evidence that tumors are not mere monoclonal expansions of cells but might contain a subset of long-lived tumor-initiating cells with the ability to self-renew indefinitely and to regenerate the phenotypic diversity of original tumor<sup>13</sup>. This subpopulation is now widely termed as cancer stem cells (CSCs), also named tumor-initiating cells (T-IC). The exist of CSCs within a tumor was also supported by *in vitro* "clonogenic assays" that showed subpopulations of tumor cells (with increased proliferative capacity) using cells isolated from tumor specimens, as well as by *in vivo* self-renewal assays that indicated only a small specific subset of cancer cell population had tumorigenic potential when injected into immunodeficient mice<sup>13,14</sup>.

The definition of CSCs is defined by two main properties: 1) self-renewal that drives tumorigenesis: the ability to form new CSCs with potential for proliferation, expansion, and differentiation; 2) multipotent differentiation, which contributes to the cellular heterogeneity of a tumor: the ability to give rise to a heterogeneous progeny of tumor cells, which diversify in a hierarchical manner.

When distinguished from the majority of differentiated cancer cells, CSCs are resistant to many current cancer treatments, including chemo- and radiation therapy<sup>15-20</sup>. This suggests that lots of cancer treatments, while targeting the majority of tumor cells, may fail in the end due to not eliminating CSCs, which survive by developing new tumors. However, this would open avenues for developing novel effective drugs targeting CSCs. Although CSCs share several properties (i.e. the ability to self-renew and to differentiate, increased membrane transporter activity, the capacity for migration and metastasis, the same intrinsic signaling pathways (Notch, Wnt, Hedgehog etc) for regulation of self-renewal etc) with the normal stem cells<sup>21</sup>, they are found to have some particular characteristics. For instance, the proliferation and self-renewal of CSCs are uncontrolled and unlimited (sometimes referred to as “immortality”), and the CSCs always differentiate into abnormal cancer cells, thus they cannot give rise to mature somatic cells<sup>22</sup>. This reveals that therapies targeted at extrinsic signals generated in the microenvironment (such as CXCR1, endothelial cell-initiated signaling, IL-6 and CXCL7)<sup>23-25</sup> or microRNAs (see Part 3 of this chapter)<sup>26-29</sup>, which are found to specifically regulate self-renewal and/or differentiation of CSCs, might achieve clinical success with little adverse effects in cancer treatment.

### 1.2 Leukemia stem cells: The first cancer stem cells identified

In the early 1990s, Dick and his colleagues started a series of groundbreaking investigations to understand whether the functional hierarchy observed in normal hematopoiesis was conserved in leukemia<sup>5,30</sup>. They used magnetic separation techniques and purified cells from AML patients into several groups according to different surface markers. These groups of cells were then implanted into immunocompromised mice and assessed for the ability to produce leukemic colony forming units. Interestingly, only the CD34<sup>+</sup> CD38<sup>-</sup> subpopulation of leukemic cells had the ability to generate substantially more leukemic colonies *in vivo*. As well, they found that CD34<sup>+</sup> CD38<sup>-</sup> leukemic stem cells retained differentiative capacity, giving rise to CD38<sup>+</sup> and Lin<sup>+</sup> populations. These observations provided the first compelling evidence that in a human cancer, there was a small population of self-renewing, tumorigenic stem cells.

### 1.3 Solid tumor stem cells

Subsequent experiments extended the leukemic stem cell model to human solid tumors. In the year 2003, Al-Hajj *et al* reported the identification of CSCs in human breast cancer, the first solid tumor that the existence of a functional hierarchy stem cell system had been demonstrated<sup>7</sup>. In their experiments, human breast cancer specimens obtained from primary or metastatic sites in nine different patients all engrafted in the NOD/SCID (non-obese diabetic/severe combined immune deficiency) mice. They observed that in most human breast cancers, only a minority subset of the tumor clones (defined as CD44<sup>+</sup>, CD24<sup>-low</sup> and representing 11%–35% of total cancer cells) is endowed with the capacity to maintain tumor growth when xenografted in NOD/SCID mice. Importantly, tumors grown from the CD44<sup>+</sup>, CD24<sup>-low</sup> cells were shown to contain mixed populations of epithelial tumor cells, recreating the phenotypic heterogeneity of the parent tumors. The small

subpopulation of cells was further enriched by sorting for those that expressed epithelial surface antigen (ESA). More interestingly, 200 of the enriched  $ESA^+CD44^+CD24^{-/low}$  cells were able to form a tumor following injection into a NOD/SCID mouse, while 20,000 of the  $CD44^+CD24^+$  cells failed to do so<sup>7</sup>. In summery, these results opened a new chapter in the understanding of the biology of CSCs in human solid tumors.

Soon after, Michael F. Clarke's group published similar data about CSCs in brain tumors<sup>8, 31</sup>. They carried out studies to enrich tumorigenic cells in glioblastoma multiforme and medulloblastoma by sorting for those that express positive / high levels of CD133, a neural cell surface stem cell antigen.  $CD133^{high}$  cells formed numerous colonies in suspension culture, and injection of as few as 1000 of these cells into an immunocompromised mouse successfully form a tumor. Conversely,  $CD133^{low}$  cells showed very limited proliferative potential *in vitro*, and as many as 10,000 of these cells failed to seed tumors in host mice<sup>8</sup>. Furthermore, tumors developing from orthotopic, intracerebral injection of the minority of  $CD133^{+/high}$  cells (about 5% - 30% of total tumor cells) reproduced the phenotypic diversity and differentiation pattern of the parent tumors<sup>31</sup>.

As mentioned earlier, comparable results have been obtained in other solid tumors, like lung, prostate, colon, head and neck, as well as pancreatic<sup>6, 9-12</sup>.

## 2. Isolation and identification of breast cancer stem cells

In most tumor tissues, including breast cancer, CSCs are rare. As we know, breast cancer is a histologically and molecularly heterogeneous disease, with six different subtypes, including luminal A, luminal B, normal breast-like, basal-like, claudin<sup>low</sup> and HER2 overexpressing, which are characterized by distinct histology, gene expression patterns, and genetic alterations<sup>32-35</sup>. The molecular heterogeneity between breast cancers has been revealed to issue from different targets of transformation. Recent studies found that basal-like breast cancers with BRCA1 mutations were more likely to arise from luminal progenitors rather than the basal stem cells<sup>36, 37</sup>. However, further studies that focus on breast CSCs and mammary stem/progenitor cells as well as their potential relationship are needed for determining the exact origin of luminal versus basal-like cancers, with the aim of developing targeted therapies for different subtypes of breast cancers. Moreover, CSCs was found to be the main culprit for the failure of chemo- and radiation therapy, as well as the seeds for the distant metastasis and relapse in breast cancers<sup>20, 32, 38-40</sup>. Taken together, in order to better understand the properties and biology of breast CSCs and eventually cure breast carcinoma, it is absolutely necessary and important to identify and separate breast CSCs prospectively.

### 2.1 Isolation of breast CSCs with cell-surface marker profiles

Since Dick, *et al* isolated a specific subpopulation of leukemia cells (that expressed surface markers similar to normal hematopoietic stem cells) which was consistently enriched for clonogenic activity in NOD/SCID immunocompromised mice from acute myeloid leukemias in the 1990s<sup>5, 30</sup>, scientists attempted to see if they could enrich CSCs in human solid tumors by sorting for different cellular markers. CD24, a ligand for P-selectin in both mouse and human cells, was identified as a significant marker for human breast carcinoma invasion and metastasis<sup>41, 42</sup>, and another adhesion molecular CD44 was found to correlate with cellular differentiation and lymph node metastasis in human breast cancers<sup>43, 44</sup>, whereas B3.8 was described as a breast / ovarian cancer-specific marker<sup>45</sup>. Based on these

observations, in 2003, Al-Hajj *et al* tried to determine whether these surface markers could distinguish tumorigenic from nontumorigenic cells, and flow cytometry was used to isolate cells that were positive or negative for each marker. They demonstrated that a small population of tumorigenic cells, isolated from human breast tumors and characterized by the expression of the cell surface markers  $CD44^+CD24^{-/low}Lineage^{-}$ , was capable of regenerating the phenotypic heterogeneity of the original tumor when injected subcutaneously into NOD/SCID mice <sup>7</sup>. They showed that as few as 100 cells with  $CD44^+CD24^{-/low}$  phenotype could form tumors in immunodeficient mice, while thousands of cells with fungible phenotypes failed to do so. Since then, CD44 and CD24 are widely accepted as surface markers for breast CSCs, and lots of studies have focused on roles of  $CD44^+CD24^{-}$  tumor cells in breast cancers. For example, Abraham *et al.* conducted immunohistochemical studies of  $CD44^+CD24^{-}$  tumor cells in human breast tumors and showed that breast tumors containing a high proportion of  $CD44^+CD24^{-}$  cells were associated with distant metastases <sup>46</sup>.

Nevertheless, besides CD24 and CD44, there are other surface marker candidates for the enrichment of breast CSCs. Ginestier *et al.* reported that they separated breast cancer stem/progenitor cells by sorting for Aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme responsible for the oxidation of intracellular aldehydes <sup>47, 48</sup>, and they found that fewer ALDH1-positive than  $CD44^+CD24^{-}$  tumor cells are required to produce tumors in immunodeficient mice <sup>49</sup>. Additionally, recent studies revealed that ALDH1-positive seemed to be a more significantly predictive marker than  $CD44^+CD24^{-}$  for the identification of breast CSCs, in terms of resistance to chemotherapy and more metastatic <sup>39, 50</sup>. Moreover, it has been reported that the surface marker CD133 could isolate a group of breast CSCs that doesn't overlap with  $CD44^+CD24^{-}$  cells <sup>51</sup>; and another recent study demonstrated that in a basal breast cancer cell line MDA-MB-231 (known as triple-negative), PROCR and ESA, instead of  $CD44^+CD24^{-/low}$  and ALDH, could be used to highly enrich breast cancer stem/progenitor cell populations which exhibited the ability to self renew and divide asymmetrically <sup>52</sup>.

## 2.2 Separation of breast CSCs by selecting for side-population (SP) cells

Advances in the separation of breast CSCs was accelerated by the identification of side population (SP) cells, due to lack of dye retention and chemotherapy efflux <sup>53</sup>. The method is based on cells incubated with Hoechst dye 33342 or rhodamine, after which the cells are analyzed by flow cytometry for dye exclusion and size, and SP cells would not retain dye. Isolation of SP cells facilitates purification of adult tissue stem cells comprising human and murine hematopoietic stem cells and a population of putative mammary epithelial stem cells <sup>54-57</sup>. Moreover, because some evidence revealed that breast CSCs and mammary epithelial stem cells represent biologically related entities <sup>58</sup>, scientists thought to apply this technique to isolate breast CSCs. In 2005, Patrawala *et al* successfully isolated SP cells from an ER-positive human breast cancer cell line MCF-7, and they demonstrated that these small subset (0.2%) SP cells preferentially express stemness-associated genes (such as Notch1 and  $\beta$ -catenin) and verapamil-sensitive ATP-binding cassette (ABC) transporter ABCG2 mRNA <sup>59</sup>. More interestingly, MCF-7 SP cells were highly tumorigenic, whereas MCF-7 non-SP cells could not give rise to tumors in mice at all<sup>59</sup>. Researchers then took advantage of similar method to separate SP cells with stem cell properties from an ER-negative human breast cancer cell line Cal-51 and an triple-negative human breast cancer cell line MDA-MB-231, respectively, and they both found the SP cells expressed high levels of ABCG2 <sup>60, 61</sup>. Previous



studies showed that SP cells take advantage of their ability to pump out the fluorescent dye Hoechst 33342 (H33342) through the ABCG2 (also known as breast cancer resistance protein-1), which was regarded as a major mediator of dye efflux in various stem cells<sup>54, 62</sup>. As the ability to efflux substrates is particularly important for the protection of CSCs, and CSCs survive after chemotherapy partially by effluxing cytotoxic drugs, ABCG2 seems to protect stem cells from toxins. This is evident in ABCG2 knockout mice that are more sensitive to compounds such as vinblastine, ivermectin, topotecan, and mitoxantrone<sup>63-65</sup>. Taken together, SP cells have the capacity to efflux toxic substances out of breast cancer stem like cells via an ABCG2-mediated cytoprotective mechanism and seem to contribute to chemotherapy-resistance. In addition, it is important to consider that identification of cancer stem like cells by selecting for SP cells is not limited to breast carcinomas. Similar observations have been made in other solid tumors (such as glioma, ovarian and pancreatic cancers) where the isolated SP cells proliferated infinitely and could regenerate heterologous NSP cells in culture<sup>59, 66-68</sup>.

### **2.3 Propagation of breast CSCs by isolating “mammospheres” from suspension cultures**

Colonial growth in nonadherent culture was used to test for self-renewal capacity in cultures of neural cell in 1996, and in the experiment, suspension culture led to formation of “neurospheres”, which consisted of 4% - 20% normal neural stem cells<sup>69</sup>. Based on this approach, Galli *et al.* succeeded in the characterization and isolation from human glioblastoma multiform of “cancer neurospheres”, which were highly enriched in long-term self-renewing, multi-lineage-differentiating, and tumor-initiating cells<sup>70</sup>. According to these successful procedures, researchers tried to extend this technology to the identification and propagation of mammary epithelial stem cells and breast CSCs. In 2003, Dontu *et al.* demonstrated that nonadherent mammospheres are enriched in human mammary epithelial progenitor/stem cells and able to differentiate along all three mammary epithelial lineages and to clonally generate complex functional structures in reconstituted 3D culture systems<sup>55</sup>. More encouragingly, two years later (2005), Ponti and colleagues reported the isolation and *in vitro* propagation of spherical clusters of self-replicating cells (“mammospheres”) with stem/progenitor cell properties in suspension cultures from three breast cancer lesions and from an established breast carcinoma cell line MCF-7<sup>71</sup>. They found that the isolated cells which overexpressed neoangiogenic and cytoprotective factors showed CD44+CD24- and Cx43-, and expressed the stem cell marker OCT-4, and could form tumors *in vivo* when as few as 10<sup>3</sup> cells were implanted. This was the first time showing that breast tumorigenic cells with stem/progenitor cell properties can be propagated *in vitro* as nonadherent mammospheres, and accordingly, this experimental system was then frequently used by researchers for isolating and studying the breast tumor-initiating cells (BT-IC)<sup>72-74</sup>.

### **2.4 Novel strategies for enrichment of breast CSCs**

As we mentioned in the first part of this chapter, the cancer stem cell hypothesis suggests that many cancers are maintained in a hierarchical organization of rare, slowly dividing CSCs (or T-IC), rapidly dividing amplifying cells (early precursor cells, EPC) and post-mitotic differentiated tumor cells<sup>22</sup>. Thus, the complex scheme which operates in most tumor tissues seems to be that the slowly dividing CSCs give birth to EPC, which then undertake a program of exponential growth for a limited period of time before the descendant cells differentiate and become post-mitotic (Figure 1). Although the above three

classical methods are widely used for the isolation and identification of breast CSCs, these methods purify both T-IC and some EPC<sup>7, 59, 71</sup>. To study the breast CSCs more accurately, our group was trying to search for new strategies to enrich more purified breast CSCs. We found that breast carcinomas from chemo-treated patients were highly enriched for cells with the properties of BT-IC. We then sequentially passaged tumor cells in epirubicin-treated NOD/SCID mice to get a highly malignant breast cancer cell line (SK-3rd) using the chemo-therapeutic resistance of BT-IC. Our SK-3rd cell line showed all the tentatively defined properties of BT-IC, including enhanced mammosphere formation, multipotent differentiation, chemo-therapy resistance, as well as BT-IC phenotype (OCT4+CD44+CD24<sup>-</sup>lin<sup>-</sup>)<sup>76</sup> (Figure 2). We assess that about 16% of SK-3rd cells were T-IC, while the rest cells (also CD44+CD24<sup>-</sup>) were mostly EPC, and mammospheric SK-3rd cells were ~100-fold more tumorigenic *in vivo* than the parent cell line, metastasize, and can be serial xenotransplanted<sup>26</sup>. Additionally, SK-3rd cells was capable of providing unlimited numbers of cells for BT-IC studies. This method of *in vivo* chemotherapy may provide researchers a novel approach of selecting CSCs from other breast cancer lines or possibly for other cancers.

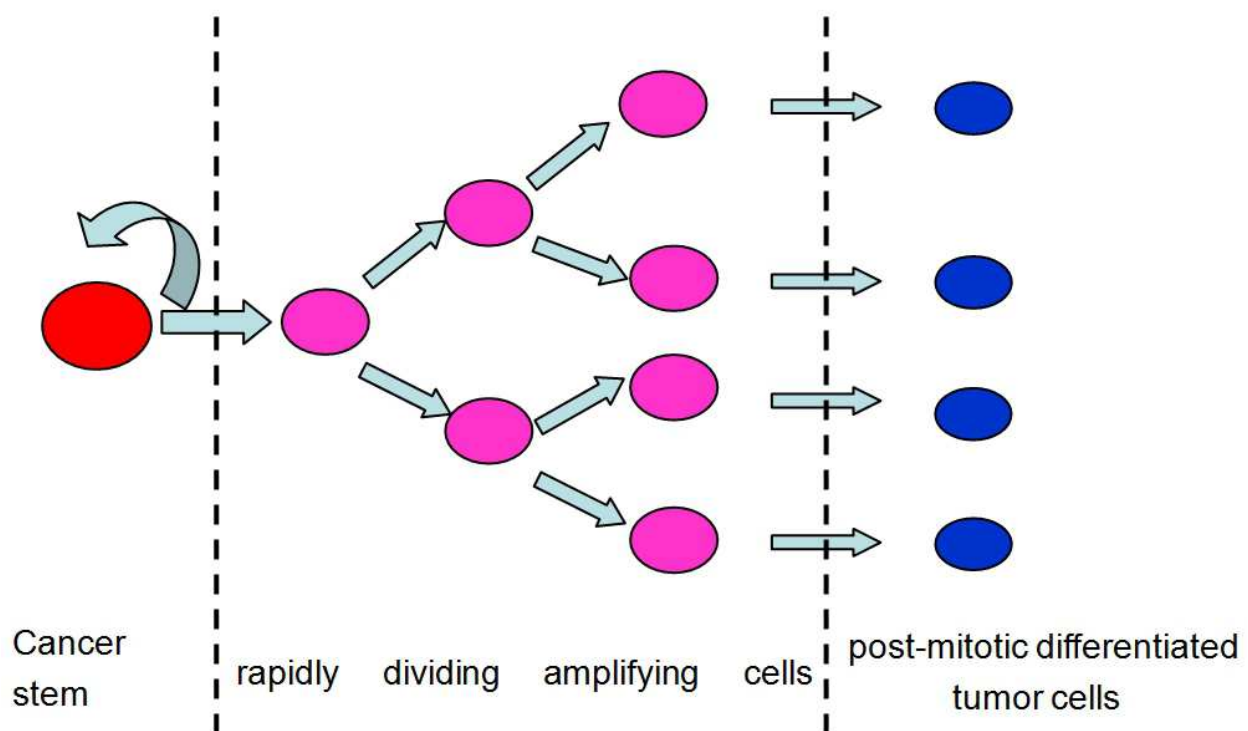


Fig. 1. A Model of the Cellular Hierarchies that May Exist in Human Cancers.

Besides our strategy, there might be other new approaches for generating breast CSCs. The epithelial-mesenchymal transition (EMT) is a key developmental program that is often activated during cancer progression, invasion and metastasis. Associations between the breast CSCs and EMT hypothesis of cancer were established recently as similarities in these two ideas were noted (will be discussed in Part 4 of this chapter). Several very recent studies have found that the EMT could generate mammary epithelial stem cells and breast CSCs<sup>77-79</sup>. This may provide potential novel methods to generate and enrich relatively unlimited numbers of breast CSCs, whose biology may then be studied with far greater facility.

The complex scheme which operates in most tumor tissues seems to be that the slowly dividing CSCs give birth to the rapidly dividing amplifying cells (early precursor cells, EPC), which then differentiate into post-mitotic tumor cells after a small number of cell divisions.

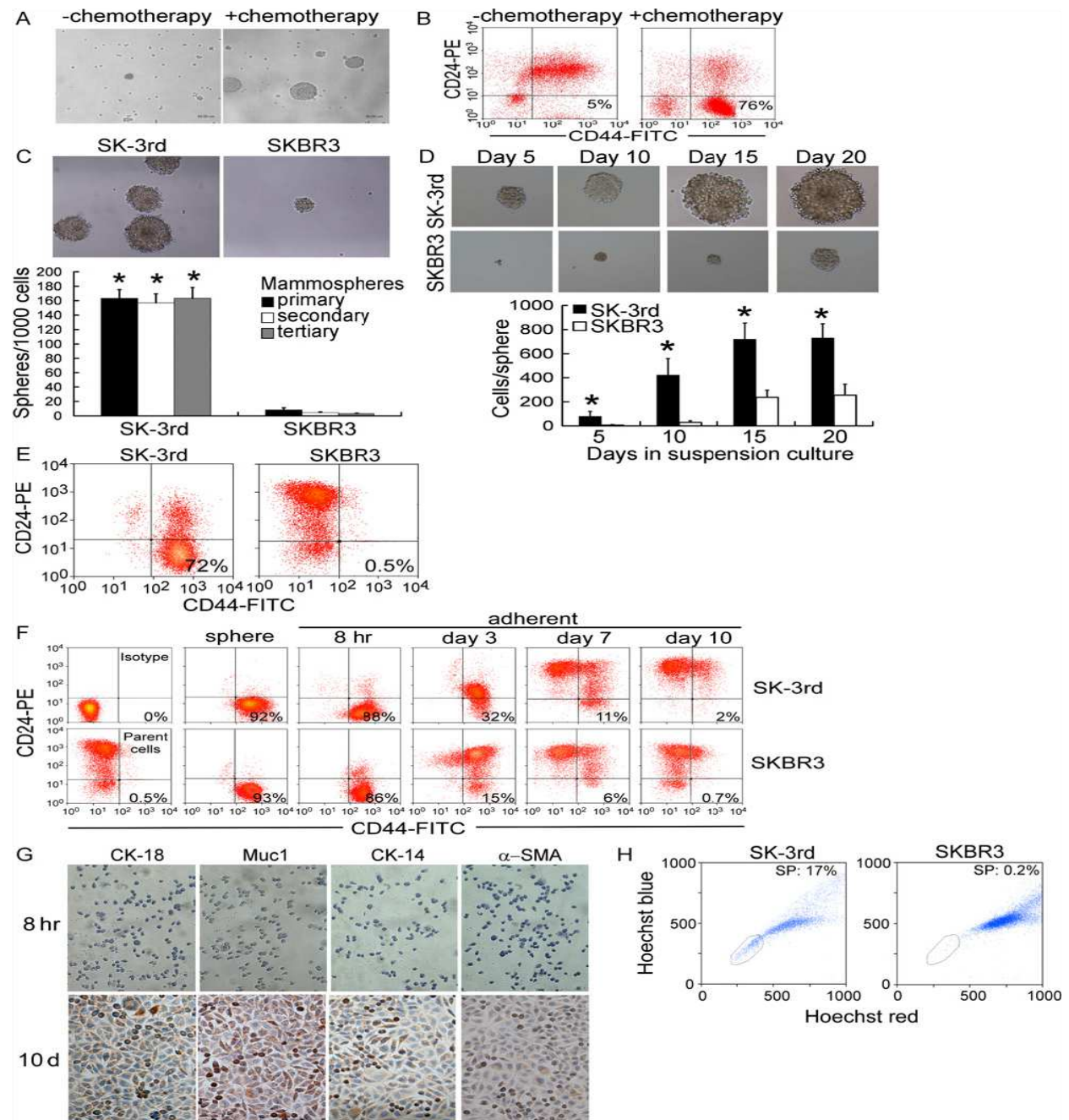


Fig. 2. Breast Cancer Cells under Pressure of Chemotherapy Are Enriched for BT-IC.

(A and B) 1<sup>o</sup>breast cancers from patients who received neoadjuvant chemotherapy are substantially enriched for self-renewing cells with the expected properties of BT-IC. Representative images show increased numbers of mammospheres after 15 days of culture (A) and a higher percentage of CD44<sup>+</sup>CD24<sup>-</sup> cells in freshly isolated tumors (B) from a patient who received chemotherapy. (C) Similarly, passaging the human breast cancer line SKBR3 in epirubicin-treated NOD/SCID mice enriches for cells with BT-IC properties.



Shown are numbers of 1°, 2° and 3° mammospheres on day 15 from 1000 cells. (D) Mammospheres generated from single-cell cultures of SK-3rd and SKBR3, imaged on indicated day of suspension culture. (E) The majority of freshly isolated SK-3rd cells are CD44<sup>+</sup>CD24<sup>-</sup>, while cells with this phenotype are rare in SKBR3. (F) SK-3rd and SKBR3 cells cultured as spheres are CD44<sup>+</sup>CD24<sup>-</sup>. When they differentiate in adherent cultures, they gradually assume the parental SKBR3 phenotype, but somewhat more rapidly for SKBR3 mammospheres. (G) When SK-3rd spheres are removed from growth factors, and plated on collagen for 8 hr (top), they do not express luminal (Muc1 and CK-18) or myoepithelial (CK-14 and  $\alpha$ -SMA) differentiation markers, while after further differentiation (bottom), they develop into elongated cells with subpopulations staining for either differentiated subtype. (H) Freshly isolated SK-3rd cells are enriched for Hoechst<sup>low</sup> SP cells compared with SKBR3 cells<sup>26</sup>. Adapted from Yu F, et al. *Cell*, 2007: 131:1109-23.

### 3. The dysregulation of MicroRNAs in breast cancer stem cells

MicroRNAs (miRNAs) are endogenously synthesized small non-coding RNAs, 19-25 nucleotides in length that negatively regulate gene expression by repressing translation of target mRNAs or targeting them for degradation<sup>80</sup>. The active miRNA is produced by the RNase III enzyme Dicer in the cytosol from a precursor-miRNA (pre-miRNA) by removing the loop of the pre-miRNA stem-loop. The Dicer-processed miRNA is then taken up by the RNA-induced silencing complex (RISC), which becomes activated when one strand (the antisense or guide strand) is incorporated into the complex and the other strand separates and is discarded. The activated RISC complex can then seek out target mRNAs, which have partially complementary sequences to the guide strand (often in their 3'-UTR), and suppress their translation into protein<sup>81</sup>.

MiRNA expression is altered in cancer cells and can be used to predict tumor type and prognosis. Cancer-associated miRNAs are frequently deleted, mutated or associated with satellite DNA expansions in cancers, suggesting that these molecules serve as important regulators of tumor development<sup>82</sup>. Emerging evidence has made it clear that miRNAs also function as important regulators of stemness, collaborating in the maintenance of the pluripotency, control of self-renewal, and differentiation of both normal stem cells and CSCs<sup>83</sup>. Except for certain miRNAs have high level transcripts, the global downregulation of miRNAs are present in CSCs when compared to their differentiated counterparts<sup>82</sup>. Dysregulation of miRNAs may result in excessive self-renewal and survival of CSCs which is a likely cause for the chemo-resistance and relapse in tumor patients.

MiRNAs can serve as either tumor suppressors or oncogenes depend on their expression levels in CSCs. Tumor suppressor miRNAs are supposed to inhibit tumor progression while their expression is downregulated. Oncogenic miRNAs are often called oncomiRs and are upregulated in the cancer cells<sup>84</sup>.

#### 3.1 Tumor suppressors

Let-7 is the first human miRNA to be discovered and its expression has been observed to be reduced in a number of tumor cell lines including lung and breast cancer<sup>85</sup>. Recent research indicated let-7 acted as tumor repressor playing an important role in the self-renewal potential of cancer stem cells. Yu and colleagues demonstrated that let-7 family was not expressed by breast CSCs generated from cell lines or 1° patient tumors and increased with differentiation. By expressing of let-7 in breast CSCs or antagonizing let-7 in more

differentiated cells, it was found that let-7 regulated the key features of breast CSCs – self renewal in vitro, multipotent differentiation, and the ability to form tumors. Because the two targets of let-7 RAS and HMGA2 were responsible for the self renewal and multipotent differentiation, respectively, aberrant expression of let-7 in breast CSCs helps to maintain their stemness<sup>26</sup>.

Recently, Yu et al. found that similar to let-7, the expression of miR-30 was reduced in breast cancer stem-like cells (BT-ICs), and its target genes, Ubc9, an E2-conjugating enzyme essential for sumoylation, and integrin  $\beta 3$ (ITGB3), were upregulated at protein levels. Overexpression of miR-30 in BT-ICs inhibited their self-renewal ability by repressing Ubc9 and promoted apoptosis by inhibiting Ubc9 and ITGB3. Furthermore, ectopic expression of mir-30 or blocking the expression of Ubc9 in BT-ICs xenografts reduced their tumor-forming capacity and metastasis in NOD/SCID mice, while miR-30 inhibitor enhanced tumorigenesis and metastasis of SKBR3 breast cancer cells with low metastasis potential<sup>86</sup>. These results suggested that miR-30 could be one of the important miRNAs in regulating the stem-like features of breast cancer

MiR-15/ miR-16 are also tumor suppressors. It was first identified in B cell chronic lymphocytic leukaemia (B-CLL) that miR-15/ miR-16 was lower in their expression level while their target protein the anti-apoptosis Bcl-2 was overexpressed<sup>87</sup>. The downregulation or deletion of miR-15/miR-16 was also found in other cancer types, such as prostate cancer<sup>88</sup>, pituitary adenomas<sup>89</sup>, non-small cell lung cancer (NSCLC)<sup>90</sup>, and ovarian cancer<sup>91</sup>. Expression of these miRNAs inhibited cell proliferation, promoted apoptosis, and suppressed tumorigenicity both in vitro and in vivo by targeting multiple oncogenes, including Bcl-2, MCL1, CCND1, Wnt3A and Bmi-1. There has been growing evidence illustrated that the pivotal signaling pathways of the “stem cell genes”: Notch, Hedgehog, Wnt, HMGA2, Bcl-2 and Bmi-1 were involved in the self-renewal of CSCs<sup>92</sup>. Since the oncogenic activation of Bmi-1, Bcl-2 and Wnt3A were frequently correlated with the downregulation of miR-15/miR-16, it was strongly suggested miR-15/miR-16 played a key role in the regulation of CSCs.

MiR-34 has been implicated in cell cycle control related to p53<sup>93</sup>. In p53 deficient human gastric cancer cells, restoration of functional miR-34 inhibited the formation of tumorsphere in vitro and tumor initiation in vivo<sup>94</sup>. In parallel, miR-34 was reported to be involved in pancreatic CSCs self-renewal<sup>95</sup>. The mechanism of miR-34 mediated suppression of self-renewal of CSCs was potentially related to the direct modulation of downstream targets Bcl-2 and Notch, suggesting that miR-34 might play an important role in gastric and pancreatic CSCs' self-renewal and/or cell fate determination. However, reduced expression of miR-34a in prostate cancer stem cells facilitated tumor development and metastasis by directly regulating CD44. Accordingly, CD44 knockdown inhibited prostate cancer growth and metastasis<sup>96</sup>. These results provided a solid experimental basis for developing miR-34a as a promising therapeutic agent against prostate CSCs.

MiR-128 is also a tumor suppressor involved in CSCs. Its expression was dramatically reduced in high grade gliomas, while application of miR-128 inhibited glioma proliferation and self-renewal by targeting Bmi-1 oncogene/stem cell renewal factor<sup>97</sup>. Same result was found in neural tumor medulloblastoma that miR-128a had growth suppressive activity in medulloblastoma and this activity was partially mediated by targeting Bmi-1 and thereby increasing the steady-state levels of superoxide and promoting cellular senescence. This data has implications for the modulation of redox states in CSCs, which are thought to be resistant to therapy due to their low ROS states<sup>98</sup>.

miR-200 is an evolutionary conserved family which were found to be strongly suppressed in CD44+/CD24- lineage human breast cancer cells<sup>27</sup> and poorly differentiated pancreatic adenocarcinomas<sup>99</sup>. Recent research conducted in an inducible oncogenesis model showed that inhibition of miR-200b expression resulted in enrichment of the CSC population, and CSC or mammosphere growth was blocked by overexpression of miR-200b. Meanwhile one of its target Suz12 subunit of PRC2 was increased in CSC which in turn repress the transcription of E-cadherin. Thus, miR-200b acts as a tumor suppressor that blocks the formation and maintenance of mammospheres by targeting Suz12-E-cadherin pathway<sup>100</sup>. These results identified miR-200 microRNA family as a critical regulator for CSC growth and function.

### 3.2 Oncogenes

The miR-17-92 polycistron which is composed of 7 members is found to be overexpressed in multiple tumors, including lung<sup>101</sup>, lymphoma<sup>102</sup>, myeloid leukemias<sup>103</sup>, hepatocellular carcinomas<sup>104</sup>, medulloblastoma<sup>105</sup> and colorectal<sup>106</sup>. It's known to function as oncogenes to promotes cell proliferation and tumor progression. Introduction of miR-17-92 into hematopoietic stem cells was shown to significantly accelerated the formation of lymphoid malignancies partly by inhibiting apoptosis<sup>101</sup>. Also Wang et al found members of the miR-17 family were notably more abundant in a mouse model of MLL leukemia stem cells compared with their normal counterpart granulocyte-macrophage progenitors and myeloblast precursors. Forced expression of miR-17-19b in leukemia cells, was consistent with a higher frequency of leukemia stem cell, reduced differentiation and increased proliferation. The oncogenic effects of miR17-92 on leukemia stem cell self-renewal in MLL-associated leukemia in part due to modulating the expression of p21, a known regulator of normal stem cell function<sup>103</sup>. Taken together, these studies implicated the miR-17-92 cluster as a potential human oncogene that played a role in cancer stem cells.

The miR-181 has an oncogenic role within cancers as well. MiR-181 family members were up-regulated in EpCAM(+)/AFP(+) hepatocellular carcinoma(HCCs) and in EpCAM(+) HCC cells isolated from AFP(+) tumors which have the cancer stem/progenitor cell features. Downregulation of miR-181 reduced EpCAM(+) HCC cell quantity and tumorigenesis, whereas enforced expression of miR-181 in HCC cells resulted in an enrichment of EpCAM(+) HCC cells. The mechanism underlying the regulation of miR-181 on the stemness of EpCAM(+) HCC cells was partially by negatively regulating two hepatic transcriptional regulators of differentiation and an inhibitor of Wnt/-catenin signaling (nemo-like kinase [NLK])<sup>107</sup>. Other evidence also showed miR-181 was elevated in breast cancer stem cells. Overexpression of miR-181a/b, or depletion of its target ataxia telangiectasia mutated(ATM), was sufficient to induce sphere formation in breast cancer cells and promote tumorigenesis<sup>108</sup>.

### 3.3 EMT

The epithelial-mesenchymal transition (EMT) is a vital developmental process that is often activated during cancer invasion and metastasis. During EMT, epithelial cells lose its epithelial characteristics including cell polarity and acquire mesenchymal phenotypes. On the molecular level, cells undergoing EMT down-regulated epithelial markers such as E-cadherin and up-regulated mesenchymal markers such as N-cadherin, vimentin, and fibronectin<sup>109</sup>. Mani and colleagues were the first group to demonstrated that the immortalized human mammary epithelial cells (HMLEs) undergoing EMT displayed not

only mesenchymal traits, also cancer stem cell like properties as characterized by their CD44<sup>high</sup>/CD24<sup>low</sup> phenotype and increased ability to form mammospheres. On the other hand, HMLE mammospheres expressed markers similar to those of HMLEs that have undergone an EMT<sup>77</sup>. These findings illustrated EMT cells have cancer stem cell features and CSCs exhibit mesenchymal phenotype.

MiR-200 is the most discussed family that involved in the regulation of EMT process. Several studies have demonstrated suppression of endogenous miR-200 family members was sufficient to induce EMT, whereas their ectopic expression induces MET in normal and cancer cell lines through direct targeting of ZEB1/2<sup>110</sup>. While in CSCs with EMT phenotypes, miR-200 was also detected to be aberrant or absent in breast, pancreas and prostate. Wellner et al showed ZEB1 not only promoted tumor cell dissemination, but also was necessary for the maintaining a stem cell phenotype of pancreatic and colorectal cancer cells by inversely inhibiting the stemness-inhibiting miR-200 family members<sup>111</sup>. Hence, ZEB/miR-200 feedback loop is a driving force for cancer progression towards metastasis by controlling the state of CSCs. MiR-200 and let-7 both were differentiation associated miRNAs, sometimes they work together regulating the EMT status of CSCs. It has been shown in prostate cancer cells the expression of miR-200 and/or let-7 was decreased in EMT phenotypic tumor cells which also expressed stem-like cell features as defined by increased expression of Sox2, Nanog, Oct4, Lin28B and/or Notch1. Restoration of miR-200 in prostate cancer cells inhibited the EMT process, as well as the clonogenic and sphere (prostasphere)-forming ability and tumorigenicity in mice which was consistent with the inhibition of Notch1 and Lin28B expression. Along with the decreased expression of Lin28, let-7 was increased which further repressed self-renewal capability<sup>112</sup>.

As discussed above miRNAs are critically involved in the regulation of CSCs and EMT which were considered the “root causes” of chemo-resistant and tumor relapse. Therefore, targeting specific miRNAs could be a very promising therapeutic approach for the treatment optimization aiming at restoring the sensitivity of drug-resistant cells to chemotherapy. If it was possible to introduce miRNA mimics and/or antagonists into CSCs, it could in principle result in reversal of the some of the cells’ tumorigenic properties. However, from a clinical/translational research point of view, the critical hurdle to developing this type of approach for cancer therapy is to find an efficient way to selectively deliver miRNAs into CSCs or just cancer cells, but not normal tissues. So far the effective and safe therapeutics are still to be studied.

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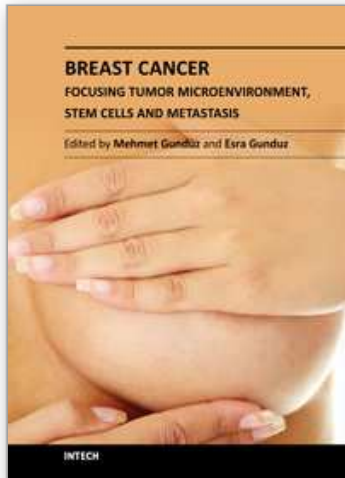
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## **Breast Cancer - Focusing Tumor Microenvironment, Stem cells and Metastasis**

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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed characteristics of breast cancer cell, role of microenvironment, stem cells and metastasis for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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