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Review

Cancer stem cells in breast cancer

Jürgen Dittmer[1] and Achim Rody[2]
[1]Klinik für Gynäkologie, Universität Halle, Halle/Saale, Germany and [2]Klinik für Frauenheilkunde und Geburtshilfe des Universitätsklinikum Schleswig-Holstein, Lübeck, Germany

Summary. There is increasing evidence that cancer stem cells (CSCs) play a critical role in breast cancer initiation, progression, metastasis and drug resistance. It is thought that they are either generated from normal mammary stem/progenitor cells or from mammary epithelial cells by epithelial-mesenchymal transition. Breast CSCs are characterized by the activation of stemness-related pathways, such as the Notch and Wnt pathways, and by the expression of certain stem cell markers, such as CD44, EpCAM and ALDH1. CSCs form a minor population, whose proportion depends on various factors, including environmental conditions. Since CSCs are highly resistant to chemotherapy, additional treatment of breast cancer patients with CSC-specific drugs, such as salinomycin and gamma-secretase inhibitors which target the Wnt or Notch pathway, respectively, will be required. Interestingly, an equilibrium seems to exist between CSCs and non-stem cancer cells, and there are indications that CSCs can be recruited from non-stem cancer cells. As a consequence, it may be necessary to combine a therapy targeting CSCs with common chemotherapy that targets the bulk tumor to avoid the regeneration of CSCs.

Key words: Cancer stem cells, Breast cancer, EMT, Notch

Introduction

The concept of cancer stem cells is connected with the name of Julius Cohnheim, a student of the German pathologist Rudolf Virchow. Cohnheim stated in his lectures on “General pathology” about cancer development: "It just seems to be one left over, namely the innate disposition" (Cohnheim, 1882). Early on, the question of the origin of cancer was brought into association with a disposition already verifiable at the time of birth. Theories on the development of malignant diseases are also closely linked to the physiological organogenesis of an organ. This observation was summarized and published by Van R. Potter in the British Journal of Cancer in 1978 in the paradigmatic statement: "Oncogenesis is a partially blocked ontogenesis" (Potter, 1978). The female mammary gland is a highly dynamic organ, which can be documented by the dramatic structural changes during pregnancy and lactation (Russo and Russo, 1998). For this reason the existence of a "mammary stem cell" is one of the cornerstones in the explanation of specific physiological and pathophysiological changes. This concept, which is also proposed in different forms for many other organ systems, is not only able to explain the process of tumour initiation, development and metastasis, but also the limitations of conventional anti-cancer treatment approaches. The properties of a stem cell are characterized by its longevity, self-renewal and multi-lineage differentiation (Reya et al., 2001), as well as tumor formation and phenotypic plasticity of the cells. Self-renewal can occur symmetrically leading to the generation of two stem cells or asymmetrically leading to the generation of a stem cell and a progenitor cell which is able to differentiate to a specialized cell. Normal stem cells are principally responsible for growth, homeostasis and repair in various tissues within the human body. The stem cell concept has many implications regarding the definition of pathological-anatomical compartments, the initiation and progression of breast cancer, as well as the interaction between epithelium, stroma and auto-/paracrine effects (the stem cell niche). The classification of breast cancer based on stem cell-like properties also allows the identification of

Offprint requests to: Jürgen Dittmer, Klinik für Gynäkologie, Universität Halle, Ernst-Grube-Str. 40, 06120 Halle/Saale, Germany. e-mail: juergen.dittmer@medizin.uni-halle.de

new predictive and prognostic markers (Rody et al., 2008).

The concept of cancer stem cells (CSCs)

Model of carcinogenesis as a multistep process

Empirical evidence of the course of breast cancer disease after surgical treatment has radically changed our understanding of disease in recent decades. The Halstedian doctrine that breast cancer is a loco-regional disease does imply that it can be treated successfully by radical surgery alone. However, this theory was replaced by the so-called Fischer-doctrine, presuming that breast cancer is a systemic disease with a local component. Thus it was possible to withdraw surgical radicality, so that breast conserving surgery has become the standard in care of the surgical treatment of breast cancer. Moreover, the introduction of systemic therapy, encompassing not only chemotherapy, but endocrine treatment and molecular therapy, is another important milestone leading to eradication of the tumor or at least to a significant prolongation of the overall survival for patients. Vogelstein et al. proposed the model of the so-called "multistep carcinogenesis", which profoundly influenced our understanding on cancer development (Vogelstein et al., 1988). This model proposes that about 50-100 mutations must accumulate in (proto-) oncogenes and tumor suppressor genes to induce the transformation of a normal cell into a tumor cell and may explain different morphological (premalignant) intermediates in a carcinoma. This sequential, continuous process was initially formulated for the development of colorectal cancer, but was also quickly adopted for other tumor entities. For the development of breast cancer, it was hypothesized that the benign lesion of usual ductal hyperplasia (UDH) is a precursor lesion of atypical hyperplasia (ADH) and ductal carcinoma in situ (DCIS). However, by using comparative genomic hybridization, Böcker et al. were able to show that genetic alterations are not detectable in benign lesions, but in DCIS (Boecker et al., 2001). Furthermore, immunohistochemical studies on the expression of cytokeratins (CK) 5, 6 8, 18 and 19 revealed that benign UDH and premalignant ADH and DCIS lesions express a different set of CKs suggesting that UDH and ADH/DCIS originated from different cell types (Boecker et al., 2002; Rakha et al., 2006). Thus mutation(s) in different cell types may lead to different types of lesions by triggering the reprogamming of differentiated somatic cells to specific tumor stem cells (Martinez-Climent et al., 2006). In terms of therapy this means that a cure for breast cancer can only be achieved if all tumor cells, including tumor stem cells, are eliminated by an appropriate (systemic) treatment.

The mammary stem cell concept

The female mammary gland consists of two epithelial cell compartments, the luminal and myoepithelial, which can be distinguished by immunohistochemistry.

The stem cell model differs from the classical model of tumor formation in two key aspects. First, tumorigenesis is caused by dysregulation of the tightly controlled self-renewal ability of stem and/or progenitor cells (Graziano et al., 2008). Second, tumors harbor cell populations, the cancer stem cells, which show stem cell properties, such as self-renewal and multilineage differentiation. Thus, tumor heterogeneity can be explained by a mixed population of tumor cells showing different degrees of differentiation and with it different biological activities, such as metastatic potential, proliferation and sensitivity to drugs. On the other hand, a blockage in the differentiation cascade could favor a specific phenotype, leading to a more homogenous tumor cell population. The question remains if tumor-initiating cells in breast cancer are always cancer stem cells and whether we will be able to track down and characterize the tumor-initiating cells in a given tumor. This would be important for a successful therapeutic intervention.

How are CSCs defined (different tumor entities vs. breast tumor)?

To characterize a mammary stem cell, it must be distinguished between a normal stem cell and a tumor stem cell. The characterization can be performed by analyzing functional, as well as molecular properties.

Functional characterization

Functional tests in *in vitro* cultures have become the standard procedure for the identification of stem cells. Three-dimensional (3D) cell cultivation allows the isolation of a subpopulation of cells with stem-like characteristics from a heterogeneous cell population, such as a human mammary carcinoma. Stem cells can be enriched by repetitive disintegration and re-growth of spheroids in 3D cultures. These cells, which account for approximately 8% of the cells in the healthy human mammary gland (Dontu et al., 2003), are detectable by light-microscopy as small and light cells (SLC) and have the ability to maintain DNA staining (using ^{3}H-thymidine or bromo-deoxyuridine = BrdU) due to their low proliferative activity (Molyneux et al., 2007). However, it was also shown that only 15% of [^{3}H] thymidine-positive cells are also positive for one of the two stem cell markers $p21^{CIP1}$ or Musashi-1 (Msi-1) (Clarke et al., 2005). Thus, "label retention" does not seem to be sufficient to identify a cell population with stem cell characteristics. Cairns hypothesized that stem cells are able to maintain an immortal strand of DNA during assymetrical cell division and only pass the newly replicated strand on to the progenitor daughter cell. This is regarded as a protective mechanism for stem cells to prevent an accumulation of genetic defects. Experiments

in mice have demonstrated that approximately 80% of [^{3}H] thymidine-positive cells divide asymmetrically (Smith, 2005). The gold standard for identifying stem or progenitor cells is the ability of cells (ideally of each cell) to regenerate the tissue of origin. This can be done in an *in vivo* setting using the so-called "cleared fat pad transplantation" of human cells to syngeneic or immunodeficient recipients (mice). Transplanted stem cells then form ductal sprouts, which resemble the normal epithelium and have appropriate functional activity, such as forming alveolar structures during pregnancy. By flow cytometry of cells incubated with the flourescent dye Hoechst 33342, a subpopulation (so-called "side-population") can be identified that shows stem cell characteristics, for example SCA-1 expression (Welm et al., 2002).

Molecular phenotypical characterization

Gudjonsson et al. identified progenitor cells in human *in vitro* colony forming assays that are characterized by a CK19$^+$ CK14$^+$ EpCAM^{high} CD49f$^+$ MUC1- SSEA-4^{high} phenotype (Gudjonsson et al., 2002). In recent works, so-called "mammary repopulating units" have been formed by the isolation of Lin-CD29^{hi} CD24$^+$ or CD49f^{hi} CD24$^+$ cells (Shackleton et al., 2006; Stingl et al., 2006). Moreover, it could be demonstrated that CD29^{hi} CD24$^+$ cells are negative for the expression of ERα, indicating that the early stem cell population is not responsive to endocrine treatment (Asselin-Labat et al., 2006). A subpopulation of CD24^{high} Sca-1$^+$ ER$^+$ cells appear to be a progenitor cell compartment which is characterized by colony-forming properties and proliferative activity (Sleeman et al., 2007). This is in contrast to the majority of ER+ cells, which are not dividing cells (Clarke et al., 1997). CD133 (Prominin1) was first identified in glioblastoma and neural tumors as a putative stem cell marker (Singh et al., 2003) and was later successfully used for immunohistochemical staining in breast cancer (Wright et al., 2008). Important signaling pathways involved in maintenance of stemness are the Notch, Hedgehog and Wnt pathways (Kalirai and Clarke, 2006; Kakarala and Wicha, 2008).

The "stem cell niche"

Besides the characterization of stem cells, their localization within the tumor and their interaction with the environment have to be analyzed to understand their impact on tumor progression. The anatomical niche for stem cells is composed of different compartments, which interact via different signaling pathways and molecules with the surface of stem or progenitor cells (Jones and Wagers, 2008). The anatomical niche is formed by specialized stromal cells, a specific type of fibroblast, which interact with the stem/progenitor cells via surface receptors, gap junctions, soluble factors (cytokines, growth factors, hormones), and extracellular matrix proteins (ECM). Specific signals can control the process of self-renewal, survival and maintenance of stemness. In addition, a specific spatial configuration of the niche can induce cell polarization, which may favor asymmetric stem cell division. The interactions of stem cell, stromal cell and/or the ECM with each other are necessary for proper positioning of the stem cells in the niche environment to allow appropriate regulation of stem cell renewal and survival. The exact localization of putative stem cells within the mammary gland is still a matter of dispute. There are studies suggesting that the mammary stem cells in mice are located in the distal end of the terminal ductal lobular unit ("cap cells") (Woodward et al., 2005). In contrast, other authors could identify immature progenitor cells at the branching points of the ductuli. Still other studies failed to show a clear localization pattern. The influence of specific soluble factors is also largely unclear. Since the mammary gland is an endocrine-responsive organ, hormonal factors, such as estradiol (E2), progesterone (P), follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin and growth hormone (GH), are of tremendous importance. In addition, the impact of hormones on stem/progenitor cells in different stages of life (embryonic/fetal life, puberty, pregnancy, menopause) must also be considered. The biological influence of E2 and P on the compartment of stem and progenitor cells is largely unknown. However, it is assumed that the stem cell is estrogen receptor (ER)-negative, whereas the progenitor cell is ER-positive (Stumpf et al., 1980; Brisken et al., 2000). Experiments with mice have shown that during puberty sprouting of ducts can be stopped by the removal of the ovaries and restored by adding E2 (Daniel et al., 1987). The lack of budding could also be observed in ERα knock-out mice (Daniel et al., 1987) and only a rudimentary ductal system developed in these mice. After transplantation of mammary tissue in wild-type (wt) mice sprouting of ducts was still not observable, suggesting that ERα provides a critical signal. However, in chimeric epithelia (wt and ERα-deficient cells), it could be demonstrated that ERα-negative cells are also involved in sprouting. This suggests that ERα-deficient mammary epithelial contain stem cells that can only be active if ERα positive cells are also present. Savarese et al. reported that *in utero* mitogens are able to expand the stem cell pool, which had consequences for mammary gland development (Savarese et al., 2007). This is consistent with *in vitro* data showing that stem cell-dependent mammosphere formation increased when cells were stimulated with epithelial growth factor (EGF) and basic fibroblast growth factor (bFGF) (Dontu et al., 2003). Ahlgren et al. showed that clinical characteristics associated with increased levels of GH, such as high birth weight, low age at "peak growth", high body length, lower body mass index at the age of 14 years and high infant growth rate are independent risk factors for the development of breast cancer. The expression of intra-mammary GH, which can be induced by

progestins, also might induce abnormal morphogenesis as well as increased tumorigenesis (Ahlgren et al., 2004). There is increasing evidence that the interaction of stromal and immune cells (e.g. tumor-infiltrating leukocytes) with normal or malignant cells may also play an important role in the development and progression of epithelial tumors (de Visser et al., 2006). Innate immune cells, such as tumor-infiltrating macrophages, mast cells and granulocytes, contribute to carcinogenesis possibly by an increased release of free radicals, as well as by paracrine mechanisms. In addition, they may stimulate angiogenesis and tissue remodelling by the secretion of cytokines, growth factors and matrix metalloproteases. Furthermore, innate immune cells can inhibit tumor-suppressing activities of the so-called adaptive immune cells. Chronic inflammatory responses through humoral immune reactions mediated within the cellular microenvironment can also contribute to tumorigenesis. The lymphocytic infiltration (LI) in breast cancer is a frequently observed phenomenon and is widely regarded as a host response against the tumor. Kohrt et al. have shown that LI detectable in rapidly proliferating breast cancer is a favorable prognostic factor that is associated with node negativity, smaller tumor size and lower grading (Kohrt et al., 2005). Menard et al. also showed that LI is a favorable prognostic factor in breast cancer patients under the age of 40 years, but not in older patients, which might be associated with the ER-status or specific subtypes (Menard et al., 1997). Recently, we published an analysis of 1781 breast cancers with known gene expression profiles identifying an immune cell-associated cluster of metagenes (Rody et al., 2009). It was shown that an IgG-cluster had no prognostic relevance, whereas a T-cell cluster had a strong prognostic relevance in both the ER-negative, as well as ER-positive/Her-2 positive patients. Interestingly, tumors with expression of the T-and B-cell cluster showed a high percentage of pathological complete remissions after neoadjuvant therapy.

Cancer stem cell markers and their proposed functions

The establishment and maintenance of stemness requires certain signaling pathways, such as the Wnt or Notch pathway, to be active (Table 1). The Notch pathway has been shown to play a particular role in mammary stem cell (MaSC) expansion (Bouras et al., 2008) and promotes breast cancer progression, by supporting epithelial-to-mesenchymal transition (EMT) (Sethi and Kang, 2011). Consequently, overexpression of components of the Notch pathway have been linked to decreased survival of breast cancer patients (Han et al., 2011). By inhibiting the Notch pathway, the CSC population can be reduced and along with it responses to chemotherapy be improved (Qiu et al., 2013). The Notch pathway is different from other signaling pathways as it requires the proteolytic cleavage of the receptor protein (Shi and Harris, 2006) (Fig. 1). The Notch receptors, Notch 1-4, are transmembrane proteins with an intracellular and extracellular domain. They can interact with five ligands (Delta-like 1-3 and Jagged 1,2), also membrane-bound that only expose an extracellular domain. If the extracellular domain of a Notch receptor on one cell tethers to the extracellular domain of a ligand on the surface of a neighboring cell, then the Notch pathway is activated. Activation involves two proteolytic cleavage steps, one mediated by TACE, the other by γ-secretase, leading to the release of the Notch intracellular domain (NICD). NICD acts as a transcriptional activator of genes, such as Hes, which is a transcriptional repressor engaged in blocking cellular differentiation. There is evidence that Notch determines cell fate. Activation of the Notch pathway in murine MaSCs leads to an increased generation of cells of the luminal lineage and an underrepresentation of cells of the basal lineage (Bouras et al., 2008). Moreover, a constitutively activated Notch pathway in luminal cells endows these cells with the capacity of self-renewal, which is intriguing since most breast cancers derive from cells of the luminal lineage (Visvader, 2009). Consequently, it has been shown that inhibition of the Notch signaling pathway by γ-secretase inhibitors (GSI) reduced the pool of breast cancer stem cells (Bouras et al., 2008). GSI and other drugs that interfere with the Notch pathway are currently being discussed as new options to treat breast cancer (Han et al., 2011). Interestingly, there is a communication between the Notch and the Her2-dependent pathways (Korkaya and Wicha, 2009). Blockage of either one was found to affect cancer stem cell survival. Hence, Her2 inhibitors, such as trastuzumab, may be an additional drug suitable for targeting cancer stem cells (Liu and Wicha, 2010).

In 2003, two proteins, CD44 and CD24, were found to be useful markers to distinguish tumor-initiating cells (TICs) from non-tumorigenic cells in breast cancer (Al-

Table 1. Selected stem cell markers in normal breast and in breast cancer.

Stem cell marker	Species
ALDH1	Human
BMI-1	Human
$CD29^{high}/CD24^{+}$	Mouse
$CD44^{+}/CD24^{low/-}$	Human
$CD49f^{high}/CD24^{med}$	Human, Mouse
Cytokeratin 5/6	Human
EpCAM	Human
ESA-/Muc1-/CALLA-	Human
Hedgehog pathway	Human, Mouse
Label retention	Human, Mouse
Mammosphere formation	Human, Mouse
Musashi	Human
Notch pathway	Human, Mouse
P21	Human
Prominin1 (CD133)	Human
Sca-1	Mouse
Wnt pathway	Human, Mouse

Hajj et al., 2003). Human breast cancer cells that express CD44 and are deficient of CD24 showed a high potential to induce tumors in nude mice. The concept of $CD44^+/CD24^-$ breast cancer cells as being tumor-initiating cells with stem cell-like features and metastatic capacity was confirmed by others (Sheridan et al., 2006; Shipitsin et al., 2007). Furthermore, the gene expression profile associated with $CD44^+/CD24^-$ cells was demonstrated to correlate with a worse prognosis in breast cancer (Shipitsin et al., 2007). Also, this cell population was shown to express components of the receptor tyrosine receptor/phosphatidylinositol-3-kinases (PI3K) signaling pathway at higher levels than the remaining bulk tumor (Hardt et al., 2012). The same cascade is involved in drug resistance (Miller et al., 2011). In addition, approximately one third of all circulating breast cancer cells in the blood of breast cancer patients are rich in CD44 and low in CD24 (Bednarz-Knoll et al., 2011). The protein CD44 is well studied and known to be a hyaluronan (HA)-binding transmembrane protein which is expressed as different isoforms and can have different glycosylation patterns (Zoller, 2011). Its smallest (standard) form (CD44s) is expressed in many cells, whereas its variant forms (CD44v) are particularly found in cancer cells. CD44v is involved in EMT, cellular migration, transendothelial migration and extravasation (Zoller, 2011). Furthermore, by interacting with receptor tyrosine kinases, such as Her2 and EGFR, it stimulates the expression of cyclooxygenase-2 (Cox-2), a key enzyme in prostaglandin E2 production. In addition, CD44 contributes to drug resistance. Thus, CD44v supports a number of cellular activities required for cancer cells to initiate tumor growth and metastasis. Downregulation of CD24, also a heavily glycosylated membrane protein, may be required to prevent its interference with CD44-dependent invasiveness (Meyer et al., 2009), though the underlying mechanism is not clear since CD24 also has tumor-promoting effects (Gires, 2011).

Recent data suggest that CD44 and CD24 may not be sufficient to distinguish the cancer cell subpopulation with CSC/TIC activity. Other proteins, such ALDH1 (aldehyde dehydrogenase 1) and EpCAM (epithelial cell adhesion molecule), may also be required for cancer cells to develop tumor-initiating potential (Fillmore and Kuperwasser, 2008; Charafe-Jauffret et al., 2009; Gupta et al., 2011). ALDH1-positive breast cancer cells, which can be identified by the ALDEFLUOR assay, have been

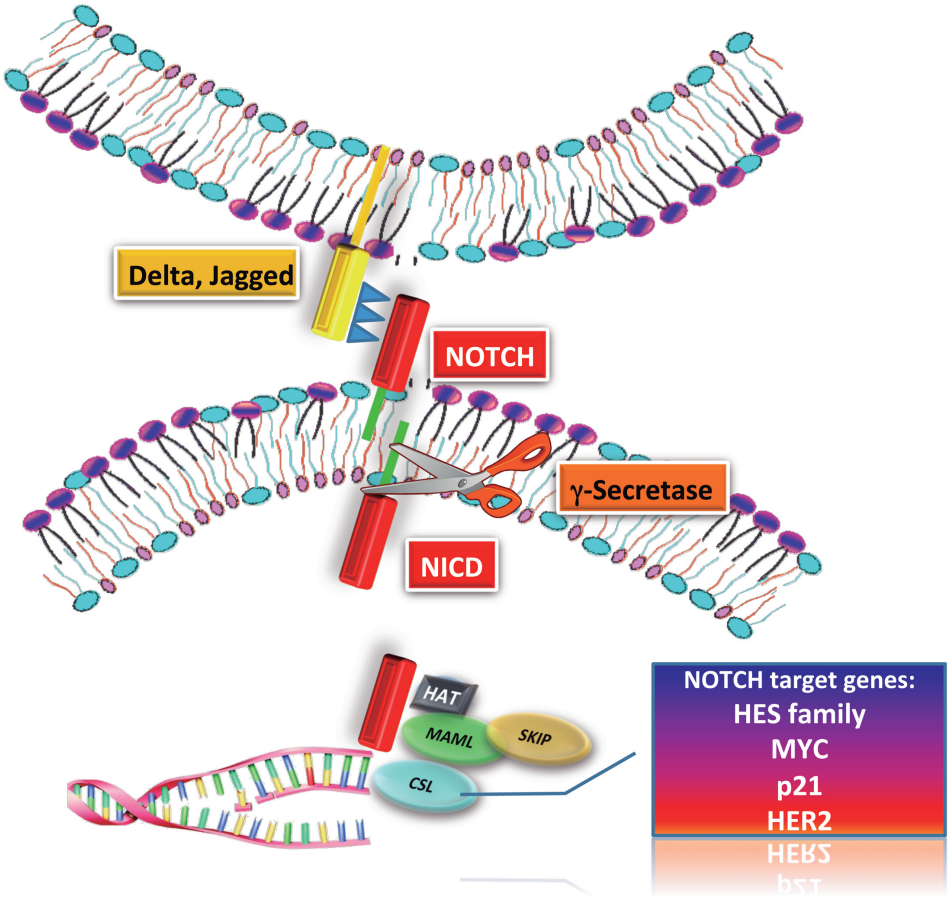

Fig. 1. The Notch signaling pathway. Interaction of Delta or Jagged with Notch leads to its proteolytic cleavage by γ-secretase releasing NCID (Notch intracellular domain) to the cytoplasm. After entering the nucleus, NCID interacts with the transcription factor CSL (CBF1/Su (H)/Lag-1) to activate a number of genes, such as Hes (Hairy/Enhancer of split), through the recruitment of co-activators, like MAML (mastermind-like), SKIP (c-Ski interacting protein) and HAT (histone acetyltransferase).

shown to have stem-like and tumor-initiating activities (Ginestier et al., 2007). Expression of this enzyme has been linked to an unfavorable outcome (Ginestier et al., 2007) and is more frequently found in breast cancer cell lines of basal A and Her2-enriched subtypes (Ricardo et al., 2011). Of the different members of the ALDH1 family (Ma and Allan, 2011), ALDH1A1 and ALDH1A3 are thought to be the most important ones in contributing to ALDEFLUOR positivity and hence to stem cell activity in cancer cells (Marcato et al., 2011). The function of ALDH1 in cancer stem cells remains unknown. Its detoxifying effect against various aldehydes may induce resistance to certain drugs, while its regulating effect on retinoic acid synthesis may play a role in differentiation (Ma and Allan, 2011). Recently, ALDH1 expression has been linked to the expression of RhoC (Rosenthal et al., 2012), a GTPase known to be involved in metastasis (Clark et al., 2000). Like CD44, EpCAM is a transmembrane protein which was first thought to be a cellular adhesion molecule until it was discovered that this protein was able to activate c-myc, a proto-oncogene, involved in maintenance of stemness (Gires, 2011). EpCAM is now accepted as a critical factor in maintaining stemness in embryonal stem cells where it is highly expressed. The proteolytic release of its intracellular domain Ep-ICD and its nuclear translocation, often seen in breast cancer cells (Ralhan et al., 2011), may be important for its stem cell-promoting effect. Thus, the level of EpCAM expression may be critical for defining stem cells. A recent report demonstrated that cancer stem cell activity was associated with low EpCAM expression, whereas luminal or basal cells showed either high or no expression of EpCAM, respectively (Gupta et al., 2011). It is possible that the maintenance of the cancer stem cell phenotype is dependent on the proper mixture of several stemness-related factors, such as CD44, ALDH1, Her2 and EpCAM.

Generation of breast cancer stem cells

The luminal lineage

Mammary gland development takes place in three phases, during embryogenesis, adolescence and adulthood (Sternlicht et al., 2006). Before adolescence, the immature breast displays an epithelial structure of lightly or unbranched ducts containing terminal end buds (TEB) or terminal ductal-lobular units (TDLU) at their tips (Hinck and Silberstein, 2005; Petersen and Polyak, 2010). At this stage, two epithelial layers, the inner luminal and the outer basal layer, forming the ducts and buds can already be distinguished. During adolescence the tips branch out to form a tube system followed by the formation of secretory alveoli during pregnancy (Hinck and Silberstein, 2005). As discussed above, mammary gland development is driven by stem cells (Luo et al., 2010; Petersen and Polyak, 2010). It is thought that a multipotent mammary stem cell (MaSC) generates a common progenitor cell which divides into two unipotent precursor cells restricted to either the basal (myoepithelial) or luminal lineage (Visvader, 2009). Recent data obtained in transgenic mice showed that multipotent MaSCs are rare in adults and that branching morphogenesis after birth depends almost exclusively on existing lineage-restricted basal and luminal precursor cells (Van Keymeulen et al., 2011). Cells of the basal and luminal lineages can be distinguished by their expression of certain keratins. Cells of the basal lineage are cytokeratin CK14-positive and CK19-negative, whereas the luminal trait consists typically of CK19-positive and CK14-negative cells (Petersen and Polyak, 2010). Strikingly, nearly all breast cancer cells are CK19-positive suggesting that they derived from cells of the luminal lineage (Bartek et al., 1985). This hypothesis is supported by more recent data showing that not only the luminal A and B breast cancer subtypes originated from cells of the luminal lineage, but also the basal A and Her2-enriched subtypes (Visvader, 2009). Only the basal B (claudin-low) subtype is considered not to be generated by cells of the luminal lineage, but by transformation of the multipotent MaSC. Recent data show that normal MaSCs and CSCs have different effects on breast development. CSCs induce a branching pattern with a higher number of branches and branching points (Parashurama et al., 2012). This abnormal remodelling of the breast epithelium by CSCs may contribute to breast cancer initiation.

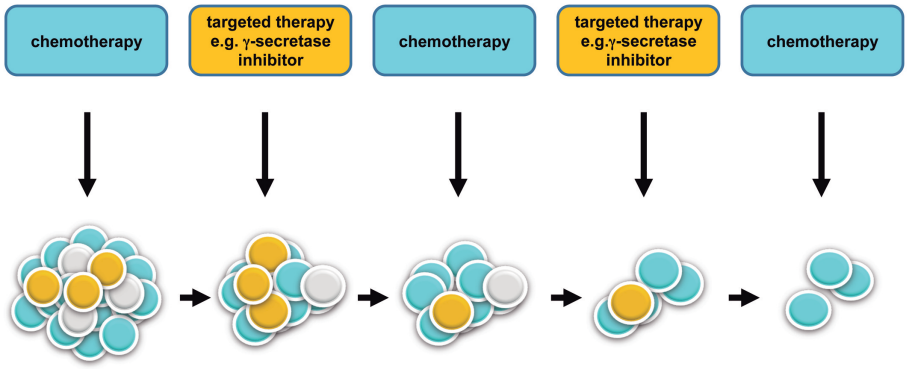

Fig. 2. Treatment options to target tumor stem cells in bulk tumors. Since recent data suggest that cancer stem cells can be recruited from non-stem cancer cells, both the cancer stem cells and the bulk tumor need to be targeted. This could be done in an alternate fashion as indicated.

Epithelial-mesenchymal transition

While it is easy to comprehend that the transformation of MaSCs generates breast cancer stem cells, it is not as obvious in cases where the cancer derives from more differentiated cells. A likely mechanism by which epithelial cells can acquire stem-like features was offered by Weinberg's group, who demonstrated that stem-like cells can derive from normal epithelial cells by epithelial-to-mesenchymal transition (EMT) (Mani et al., 2008). EMT, which also plays an important role in development and wound healing (Kalluri and Weinberg, 2009), has been known for quite some time (Oft et al., 1996) and is linked to cancer progression (Thiery, 2002). The mechanism by which EMT is induced has been well investigated (Peinado et al., 2007). Basically, EMT is a process by which epithelial cells are converted to cells with fibroblastic morphology, which is accompanied by the loss of epithelial cell-cell adhesion, loss of polarity, the expression of mesenchymal markers instead of epithelial markers and the acquisition of motility (Moreno-Bueno et al., 2009). It is thought that EMT-induced motility is a prerequisite for carcinoma cells to leave their primary lesion and move to distant sites where they may reconvert to epithelial cells by mesenchymal-to-epithelial transition (MET) (Peinado et al., 2007). A key epithelial protein that is targeted by the induction of EMT is E-cadherin. E-cadherin is the major protein responsible for cell-cell adhesion between epithelial cells (van Roy and Berx, 2008). Loss of E-cadherin is sufficient to induce EMT and to increase the invasive and metastatic capacity of breast cancer cells (Onder et al., 2008). There are a number of known mechanisms that may lead to the loss of E-cadherin in cancer cells. Among them are silencing of the E-cadherin gene by DNA methylation (Lombaerts et al., 2006), overexpression of NF (nuclear factor)-κB (Chua et al., 2007) and the expression of transcription factors involved in maintaining the mesenchymal phenotype, such as snail (snai 1), slug (snai 2) and Twist (Cano et al., 2000; Mani et al., 2008). Of note, the expression of any of these transcription factors in breast cancer is linked to an unfavorable prognosis (Martin et al., 2005). Induction of EMT can also be induced by the cytokine TGFß (transforming growth factor ß) (Piek et al., 1999; Mani et al., 2008). TGFß is able to activate Snai 1 and 2 to block E-cadherin synthesis (Massague, 2008) and to suppress apical-basal polarity by phosphorylation of PAR6 (Ozdamar et al., 2005). Interestingly, PAR6 and other genes regulating polarity are also involved in spindle orientation (Martin-Belmonte and Perez-Moreno, 2011) suggesting that TGFß may also be important for asymmetric division, a hallmark of stem cells. Of note, TGFß is also known to play an important role in maintenance of pluripotency of embryonal stem cells (Valdimarsdottir and Mummery, 2005). Full EMT by TGFß may require hyperactive Ras (Oft et al., 1996; Janda et al., 2002). TGFß is known to have a dual function: it may act as a tumor suppressor by inhibiting cell cycle initiation or as a tumor promoter by inducing invasion (Bierie and Moses, 2006; Massague, 2008; Ikushima and Miyazono, 2010). The induction of EMT is one reason why TGFß promotes tumor progression. Another is its modulating effect on the micro-environment, such as its suppressive effect on immune cells (Barcellos-Hoff and Akhurst, 2009). The same interventions that induce EMT, such as downregulation of E-cadherin, overexpression of Twist or Snai 1 or incubation with TGFß, also generate $CD44^{high}/CD24^{low}$ breast cancer stem cells (Mani et al., 2008; Gupta et al., 2009). *Vice versa*, $CD44^{high}/CD24^{low}$ breast cancer stem cells overexpress markers, such as Twist, Snai I, vimentin, which are typically expressed in cells that underwent EMT (Gupta et al., 2009). Also, the TGFß signaling pathway is very active in isolated $CD44^{high}/CD24^{low}$ breast cancer stem cells (Shipitsin et al., 2007; Hardt et al., 2012). This led to the conclusion that EMT not only induces epithelial cells to adopt a mesenchymal phenotype, but also endows them with stem-like features (Polyak and Weinberg, 2009; Ouyang et al., 2010; Drasin et al., 2011; Floor et al., 2011; May et al., 2011). Hence, two models, one that postulates the metastasizing cells to be recruited from the pool of cancer cells that underwent EMT (Peinado et al., 2007) and one that links cancer stem cells to metastasis (Sheridan et al., 2006; Li et al., 2007; Liu et al., 2010) can now be combined. In support of this notion, approximately half of the circulating breast cancer cells were found to express stem cell markers, such as CD44 and ALDH1 (Theodoropoulos et al., 2009; Bednarz-Knoll et al., 2011), and between 35-90% of the circulating breast cancer cells were shown to express Twist and Snai 1 (Kallergi et al., 2011; Bednarz-Knoll et al., 2011). It is thought that the circulating, metastasizing cells are cancer stem cells that after extravasation and homing in a remote tissue may undergo MET to generate a metastatic lesion consisting of cells with an epithelial phenotype reminiscent of the primary tumor (Polyak and Weinberg, 2009; May et al., 2011). EMT may not be the only mechanism by which cancer stem cells may be produced. Recent data suggest that breast cancer stem cells are spontaneously generated from transformed breast epithelial cells without any treatment (Chaffer et al., 2011). Other reports support this notion by demonstrating that a dynamic phenotypic equilibrium exists in a cancer cell population allowing more differentiated cells to dedifferentiate to stem cells (Meyer et al., 2009; Gupta et al., 2011). It is therefore likely that once a breast epithelial cell is transformed, the conversion to a stem cell may be easier than previously thought. It may be either induced by intrinsic factors, by communication with other epithelial cells or by interaction with stromal cells.

Factors that regulate the pool of CSCs

Given the dynamic equilibrium between cancer stem

cells and non-cancer stem cells in a cancer cell population, the question of how this equilibrium is regulated arises. This is important, because if CSCs drive tumorigenesis, then the size of the CSC pool matters, particularly when considering that CSCs may also be subject to clonal evolution (Visvader and Lindeman, 2008; Clevers, 2011). There is evidence that environmental factors play a critical role in regulating the CSC pool (Korkaya and Wicha, 2010). A study, which addressed why not all cancer cells carrying a mutation of the stemness-related Wnt pathway in a given colon cancer population would possess stem-like features, revealed that a second factor, HGF (hepatocyte growth factor) was required for generating (maintaining) stem cells (Vermeulen et al., 2010). This factor was secreted by neighboring stromal cells. Consequently, the colon cancer cells with stem-like behavior were found close to the stromal cells that secreted HGF. The importance of environmental factors was also shown for the activation of the stemness-related Notch and Hedgehog signaling pathways (Yauch et al., 2008; Indraccolo et al., 2009). There are a number of stromal cells that have been shown to communicate with cancer cells and modulate cancer progression (Tlsty and Coussens, 2006; Arendt et al., 2009). Among them are bone marrow-derived mesenchymal stem cells (MSCs) (Uccelli et al., 2008; Dittmer et al., 2011) and cancer-associated fibroblasts (CAFs) to which MSCs can differentiate (Mishra et al., 2008; Spaeth et al., 2009). MSCs may be considered as "repair cells" and are primarily attracted to wounds, but also to cancer lesions (Dittmer, 2010). The interaction of MSCs with breast cancer cells is complex and involves paracine and direct cell-cell interactions (Dittmer et al., 2011). In terms of the CSCs, MSCs have recently been reported to increase the CSC pool of basal-like SUM159 breast cancer cells (Liu et al., 2011), the same cell line that showed a phenotypic equilibrium between stem and non-stem cells (Gupta et al., 2011). This MSC effect required a paracrine crosstalk with the breast cancer cells involving the cytokines CXCL7 and interleukin-6. Other environmental factors that modulate the CSC pool are drugs. Paclitaxel and 5-fluorouracil, chemotherapeutics routinely used in breast cancer treatment, were shown to increase the pool of cancer stem cells (Gupta et al., 2009, 2011). This is likely to be mainly a consequence of the higher resistance of stem cells to drugs. The finding that the acquisition of stemness in cancer requires the presence of an appropriate microenvironment supports the idea that, like normal stem cells, cancer stem cells exist in niches (see above) (Visvader and Lindeman, 2008). Disruption of the interaction of the cancer stem cell with its niche may therefore be a promising future therapeutic intervention (Scadden, 2006). Various strategies are discussed to block the ability of the tumor microenvironment to promote tumor progression (Place et al., 2011). One of these is microenvironmental reprogramming where, for example, tumor-associated macrophages with an antitumor/pro-inflammatory phenotype are converted to macrophages with pro-tumor/proangiogenic features (Rolny et al., 2011).

Besides stromal cells, the carcinoma cells themselves may regulate the pool of stem cells. It was found that, in the presence of progesterone receptor (PR)-positive luminal epithelial cells, the proliferative activity of non-transformed mammary stem cells can be increased by progesterone (Asselin-Labat et al., 2010; Joshi et al., 2010). This effect could be mimicked by RANKL (receptor activator of NF-κB ligand) (Mukherjee et al., 2010). This led to the conclusion that progesterone first stimulates PR-positive luminal cells to secrete RANKL which then stimulates the expansion of MaSC expressing the RANKL receptor, RANK (Lydon, 2010). Strikingly, RANK also increased the incidence of breast cancer (Schramek et al., 2010; Gonzalez-Suarez et al., 2010) suggesting that RANK may affect the pool of CSCs. Hence, RANKL inhibitors, such as the RANKL antibody denosumab, originally developed to prevent bone metastasis, are now being discussed to treat primary breast cancer (Lipton and Jacobs, 2011).

Conclusions and outlook

The concept of cancer stem cells has changed the view of how cancer develops and progresses and with it, a new discussion on therapeutic strategies has started due to the resistance of the stem cells to common therapeutic drugs (Smalley et al., 2012). New drugs, such as the γ-secretase inhibitor which interfers with the NOTCH pathway, may be helpful to specifically attack the stem cell pool in breast cancers. Theoretically, eradication of these cancer stem cells should be sufficient to eliminate the whole cancer, as cancer stem cells are thought to differentiate to cancer cells that lack the ability to form new tumors. Hence, once the cancer stem cells are all removed, the tumor should not further progress. However, as discussed above, new data suggest that cancer stem cells can be regenerated from the non-stem cell pool of the cancer. Therefore, it is necessary to target both the cancer stem cells and the non-stem cancer cells. One approach could be to alternate between treatment with common drugs to target the bulk tumor and treatment with cancer stem cell-specific drugs to target the stem cell pool until the cancer is completely eliminated (Fig. 2). Whether this will be a successful strategy depends on how efficient these stem cell-specific drugs are and whether and how quickly resistance against these drugs will be developed.

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