Bachelor thesis **REVIEW**

The role of cancer stem cells in metastasis of breast cancer

Does targeting epithelial-mesenchymal transductions of cancer stem cells improve treatment?

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July 2, 2009
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Abstract

Metastasis, the multistep process of the migration of cancer cells to secondary sites, is the major cause of death in breast cancer patients. The cancer stem cell hypothesis indicates that cancer stem cells share many properties with normal stem cells and that dysregulating the stem cell self-renewal pathways leads to tumorigenesis. It is now determined that induction of epithelial-mesenchymal transitions (EMTs) can occur in stationary cancer stem cells, subsequently leading to a more motile cancer stem cell phenotype, the migrating cancer stem cell. This process, known as the migrating cancer stem cell concept, may be the main cause of metastasis because migrating stem cells can disseminate and form metastases at secondary sites. Therefore, EMT is seen to be crucial in carcinogenesis. Cancer stem cells have many properties which make them drug resistant, pretending why current therapies fail in curing metastasized tumors. TGF-β, Wnt, Notch, Hedgehog and PTEN signaling pathways are now found to be important in EMT initiation and even in stem cell self-renewal. Therefore, it is suggested new therapies should target the cancer stem cell population by altering these pathways. Though, more research is needed because the body's normal stem cells need to left unharmed.

Keywords: breast cancer metastasis - EMT - cancer stem cell hypothesis - migratory stem cell concept - drug resistance – CSC targeted therapy

Introduction

Breast cancer is a leading cause of cancer mortality in women worldwide. It is, in the age group of 35–50 years, the major cause of death. In The Netherlands about 12,000 women are diagnosed each year [1]. In most breast cancer patients, death is caused by metastasis, mainly due to the ineffectiveness of current therapies once the tumor has been metastasized to secondary tissues [2].

Recently, two novel concepts have been found in the field of cancer research: the role of "cancer stem cells" (CSCs) in tumor initiation and the involvement of an epithelial-mesenchymal transition (EMT) in the metastatic dissemination of epithelial cancer cells [3]. Only a small subset of all the cells within a tumor seems to be capable for both tumor initiation and sustaining tumor growth. According to the cancer stem cell hypothesis, these tumor-initiating cells are called "cancer stem cells" (CSCs) and show many stem and tumorigenic characteristics. It is now known that acquisition of these characteristics is driven by the induction of EMT [3] and it is suggested that EMT may even contribute to the metastatic process [2].
Current therapy, usually chemo- and radiotherapy, can be successfully in early stage cancers, however resistance to therapy is the main problem in treating metastatic breast tumors and even relapse occurs frequently. Targeting the stem cell component might improve breast cancer therapies [2].

In this review we want to investigate if there is a link between the CSC hypothesis and induction of EMT during the metastatic process in breast carcinogenesis. Is initiation of metastasis to secondary tissues caused by EMT-induced alterations of the CSC subpopulation, or are other factors involved? We want to determine which signaling pathways are involved in the phenomenon of EMT and even are affected in stem cell self-renewal and maintenance. Furthermore we suggest, after this review, how targeting CSCs by altering these EMT- and CSC specific signaling pathways might improve treatment of breast cancer.

Breast cancer

Carcinogenesis

The carcinogenesis of cancer in general is a multistep process. Carcinomas arise from normal epithelial tissues in a multistep progression from benign precursor lesions. Proliferations combined with malignant cellular traits are called in situ carcinomas. There is no infiltration and two forms can be noticed; ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). DCIS revers to the majority of carcinomas (about 85%) which are involved in the cells lining the milk ducts. High-grade DCIS carries the greatest risk of developing into an invasive cancer. The remaining 15% is referred as lobular carcinomas, which start growing in the lobules. Invasive carcinomas develop when in situ carcinomas invade surrounding breast tissue and form metastases [4].

Metastasis

Metastasis is the final step in the tumor progression and the main cause of death for cancer patients because ineffectiveness of current therapies, it accounts for over 90% of lethality in cancer patients. It is a multistep process (fig. 1), cells have to break away from the primary tumor sites and intravasate into circulation or lymphatic system. Subsequently, they migrate to distant sites, adhere at secondary sites, extravasate into secondary tissues and vessels and form micrometastases. In presence of angiogenesis, finally macroscopic, clinically relevant metastases can be formed [2,5]. Malignant cancer cells need to survive and proliferate in an alien environment. For that reason, the formation of metastases is known as an inefficient process, only a subset of all the tumor cells is capable to successfully go through the entire metastasis cascade. Failure of disseminated cells to initiate the formation of micrometastasis in secondary sites (about 2% succeeds) and failure of these micrometastasis to form macrometastasis and acquire angiogenetic capacity (only about 0,02% of the cells succeeds) are the uppermost limiting steps. Many cancers show organic-specific metastasis, breast cancer favors metastasis to regional lymph nodes, bone marrow, liver, brain and lungs, for example. The “seed and soil theory” proposes that cancer cells can only survive in “soil” producing specific growth factors and according to the “homing theory” do secondary organs produce chemotactic factors which can attract specific tumor cell-types. These cells can home and form metastases in that particular organ [2].

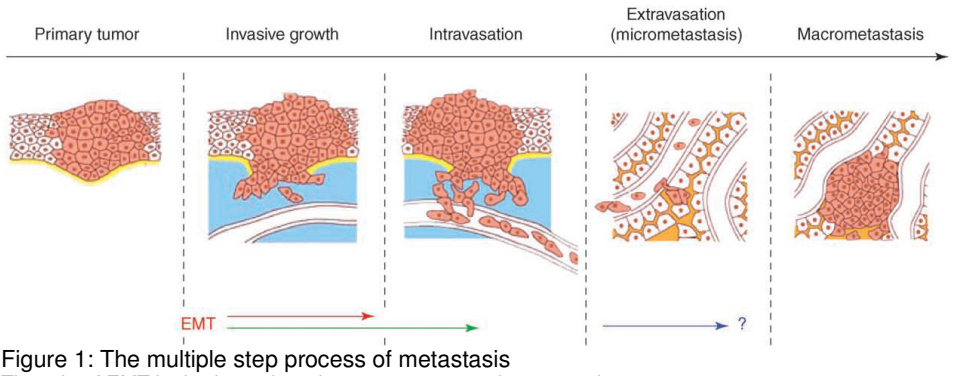

Figure 1: The multiple step process of metastasis
The role of EMT in the formation of macrometastases is not yet clear
(Obtained from Huber *et al.* 2005 [10]).

Treatment

Current therapy (surgery, chemotherapy, hormone therapy and radiation) can be successful in treating early stage cancers. However, therapy often fails when tumor cells are spread and already formed metastases. Metastatic cells have shown to be highly resistant to therapy, reflected by the high mortality rate once the tumor has been metastasized to second tissues [2].

Epithelial-mesenchymal transitions

During embryogenesis and tumorigenesis, changes in cell phenotype between epithelial and mesenchymal states play central roles. These changes are defined as epithelial-mesenchymal (EMT) and mesenchymal-epithelial (MET) transitions [6]. During embryonic development, EMT creates cells that act as progenitors of many different tissues. For example, the mesoderm is generated during embryogenesis by EMTs and develops into multiple tissue types such as bone, nerve and connective tissues. In adults functions the EMT to facilitate organ development as well as tissue regeneration during wound healing [7]. In many cases the EMT is followed by the reverse transition (MET) at the objective, to result in structures like the segmental plates, kidney, GI tract, lung and skin [8].

EMT is the complex molecular and cellular program by which epithelial cells loose their differentiated characteristics and acquire a migratory phenotype. The epithelial cells acquire mesenchymal (fibroblast-like) features instead of the differentiated traits like cell-cell adhesion, planar and apical-basal polarity and lack of motility (fig. 2).
EMT is suggested to be crucial in carcinogenesis, the program is often activated in the metastatic state of cancer since the EMT phenotype is associated with a decrease in tumor growth, increased resistance to apoptosis, increased motility, invasiveness and enhanced metastatic ability. The revered transitions, METs, include proliferation and differentiation and seem to occur following dissemination and the subsequent formation of distant metastases through new interactions with the microenvironment of the tumor [6,7,9].

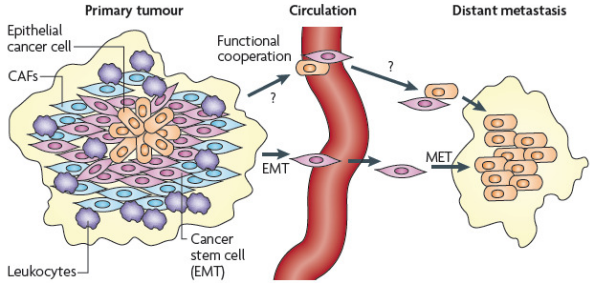

Figure 2: EMT and MET in carcinogenesis
EMTs and METs contribute in the primary tumor to intratumoural heterogeneity. Interaction with stromal cells may induce EMTs and may promote growth and survival of mesenchymal cancer cells. These cells (CSCs) are able to metastasize and disseminate into circulation. Macrometastases are more frequently composed of more differentiated epithelial cancer cells, explained by induction of METs (Obtained from Polyak *et al.* 2009 [6]).

Signaling pathways
It has been shown that tumor cells often reactivate latent developmental programs to control multiple steps during tumorigenesis [9]. Several molecular events that are essential for stem cell self-renewal and early development have a major role in initiating the EMT program during carcinogenesis, for example Wnt/β-catenin, Notch and Hedgehog. These events can be induced by genes from the TGF-β pathway or by transcription factors involved in E-cadherin (CDH1 gene) repression [2,6].

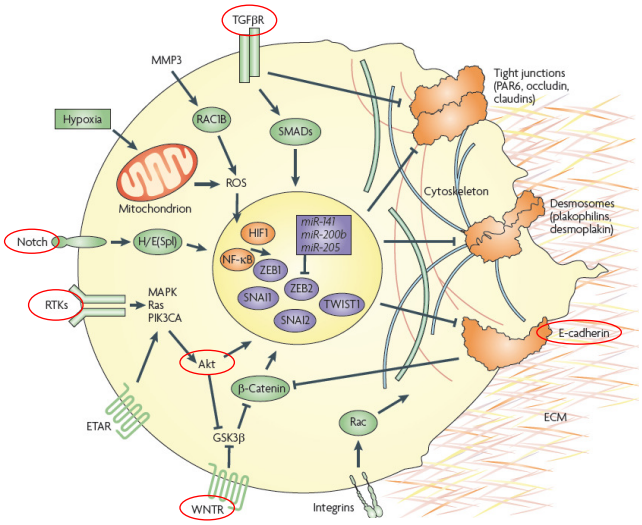

Figure 3: An overview of signaling pathways involved in E-cadherin downregulation and thus inducing EMT
TGF-β, Wnt, Notch, Hedgehog and Akt are highlighted in this section because they are the major relevant signaling pathways also important in similar stem cell self-renewal (Adapted from Polyak *et al.* 2009 [6]).

TGF-β pathway
Members of the transforming growth factor-β (TGF- β) family of cytokines may cause EMTs and metastasis through several signaling mechanisms, it cooperates with oncogenic Ras or RTKs. These mechanisms include the direct phosphorylation of SMAD transcription factors by ligand-activated

receptors and by certain cytoplasmatic proteins which regulate cell polarity and tight junction formation. In mammary epithelial cells, the TGF-β type II receptor can directly phophorylate both SMAD2, SMAD3 and PAR6A protein, for example. This leads to loss of apical-basal polarity and decomposition of existing tight junctions between adjacent epithelial cells. TGF-β also influences the activities of multiple other EMT-inducing signal transduction pathways including Notch, Hedgehog, PTEN and Wnt/β-catenin, signaling [6].

Notch signaling pathway
A role for the tumor suppressor gene Notch in EMT is demonstrated during embryogenesis and development of cancer. Notch can induce EMT by altering the TGF-β activity and thereby upregulating transcription factors which repress target genes like CDH1 [10]. Notch signaling is identified as a pathway involved in the development of the breast and is often dysregulated in invasive breast cancer. Altered Notch signaling is identified as an early event in breast cancer and amplification of Notch receptors and the presence of ligands is correlated with a more aggressive phenotype. Additionally, high expression of Notch 1 intracellular domain in ductal carcinomas predicts a reduced time to recurrence after surgery [11].

Hedgehog signaling pathway
In several carcinomas, a mutated Hedgehog pathway is observed and has been shown to activate Snail family members through Gli-dependent transcriptional activation [10].

PTEN
Deletion of the *PTEN* gene (phosphatase and tensin homolog deleted on chr 10) is found in approximately 40% of human breast cancers. PTEN is downregulated by PRL-3, a metastasis-associated phosphatase of which upregulation is associated with and increase in cell motility and invasiveness. Downregulation of PTEN causes upregulation of the PI3K/Akt pathway. GSK-3B is phosphorylated and thereby the Wnt/B-catenin pathway is activated which subsequently results in the downregulation of E-cadherin [22, 25].

Wnt signaling pathway
It has been shown that alterations in epithelial cadherin (E-cadherin) expression or function play essential roles in EMT and thus carcinogenesis, especially in breast cancer. The general role of E-cadherin is to suppress invasion, thus decreased E-cadherin expression is associated with a more aggressive behavior of breast cancer [12].
E-cadherin is a Ca^{2+}-dependent, transmembrane glycoprotein which plays a critical role in establishing adherens-type junctions. It contains an extracellular domain that mediates protein-protein interactions and the intracellular domains of cadherins interact with catenins (β-catenins, for example).
A critical switch in EMT is the downregulation of membranous E-cadherin in the junctions, in several cases attributed to transcriptional dysregulation. Due to the loss of cell-to-cell junctions during EMT, loss of E-cadherin often results in the liberation of β-catenin because the catenin is normally bound to the cytoplasmatic tail of E-cadherin. The resulting liberated β-catenin molecules may then translocate to the nucleus (which indicates an active Wnt-pathway) where they can induce expression of EMT-inducing transcription factors (SNAI1 and Slug, for example).

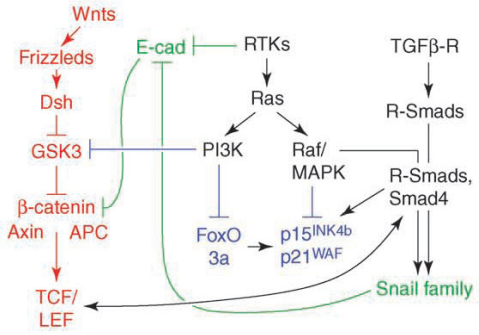

Figure 4: Basic Wnt signaling- associated molecular processes initiating EMT
In EMT initiation, E-cadherin is downregulated whereby β-catenin increases and thereby EMT-inducing transcription factors are expressed. GSK3 suppression by RTK/Ras/PI3K signaling also enhances β-catenin (Obtained from Fendrich *et al.* 2005 [9]).

Additionally, β-catenin is also enhanced when glycogen synthase kinase 3B (GSK-3B) activity is suppressed by RTK (receptor tyrosine kinases)/Ras-dependent PI3K signaling. This interferes with TGF-β /SMAD dependent upregulation of proliferation-suppressing genes (fig. 4) [6,10].

Genetics vs. epigenetics

Multiple studies described differences between the mutational and epigenetic inactivation of E-cadherin in human breast cancer carcinomas. In the study of Lombaert (2006) for example, two main clusters are identified by cluster analysis of microarray data from breast cell lines. The 'fibroblastic' cluster included only cell lines with either partial or complete CDH1 promoter methylation, the 'epithelial' cluster included cell lines with wild-type as well as cell lines with mutant CDH1 status [13].

Mutational inactivation of the CDH1 gene has been found in 56% of the lobular breast carcinomas, this is accompanied by loss of the wild-type allele. Complete loss of E-cadherin protein expression has been found in 84% of lobular breast carcinomas [14]. A role of mutational alterations is suggested in early carcinogenesis, the reason for this purpose is that mutational inactivation is especially identified in pre-invasive lobular carcinomas [16].

In contrast, because it is shown that E-cadherin somatic mutations are rare in ductal breast cancers, it is suggested that epigenetic modifications are involved in controlling the functions of E-cadherin [17]. DNA hypermethylation and chromatin rearrangements (histone H3 deacetylation, HDAC) within the regulatory regions of the CDH1 gene have been linked to the loss of E-cadherin mRNA expression in breast cancer cell lines. So, these epigenetic events may be part of a larger program directed towards EMT, thereby increasing invasive and tumorigenic capacity [13].

EMT inducers

Several EMT-inducing developmental regulators repress E-cadherin transcription during breast carcino-genesis by silencing of CDH1 via interaction with specific E-boxes of the proximal E-cadherin promoter. Most prominent EMT-inducting factors will be defined in this section. These include the Snail-related zinc-finger transcription factors (Snail and Slug), factors from the ZEB-family (SIP1 and ZEB1) and the basic helix–loop–helix (bHLH) transcription factor Twist. Furthermore, the bHLH transcription factor E12/E47 also shows the ability to repress E-cadherin [10] (fig. 5).

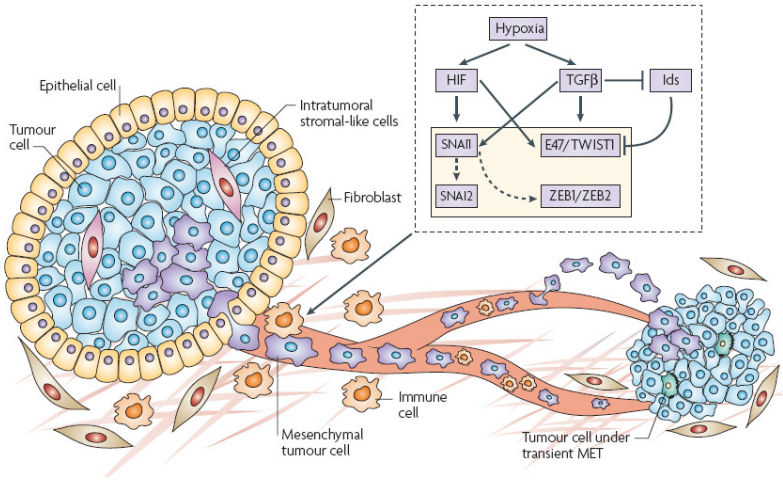

Figure 5: E-cadherin repressors and their potential interplay.
Most prominent EMT-inducing factors are Snail, Slug, ZEB1, SIP1 (ZEB2) and TWIST (Obtained from Peinado *et al.* 2007 [19]).

Factors from the Snail family

The Snail super family of transcription factors consists of several proteins that have four to six zinc finger domains. Several studies have shown a role for the Snail family in cellular events that play a role in the progression of epithelial malignancies, in mediating EMT, migration, invasion and survival. It is shown that the Snail family members are able to repress the transcription of E-cadherin. Expression of Snail correlates with that of E-cadherin in solid breast carcinoma, it is associated with higher histological grade and lymph node metastasis, for example [18].

SNAI1 is identified as a transcriptional repressor of genes whose products are involved in cell-cell adhesion, E-cadherin for example. SNAI1 is often overexpressed in cancers and it requires

histone deacetylase (HDAC) activity to repress the E-cadherin promoter and subsequently the liberation of cytoplasmic β-catenin and EMT. Interaction of SNAI1, HDAC1, HDAC2 and the co-repressor SIN3A leads to the formation of a multi-molecular repressor complex to repress E-cadherin expression. This depends on the SNAG N-terminal domain of SNAI1, indicating that the SNAI1 transcription factor mediates the repression by recruitment of chromatin-modifying activities [19]. The C-terminal binding protein (CTBP) is another co-repressor and it has been indicated that at least these two distinct co-repressor complexes, SIN3 and CtBP, can be recruited by to mediate CDH1 silencing. SNAI1 has a prominent role in the induction of EMT in primary tumors, while other factors are involved in maintaining the migratory phenotype. SNAI1 has been found in infiltrating ductal carcinomas associated with lymph node metastases and distant metastases including effusions. SNAI1 expression in breast cancer correlates with E-cadherin repression, metastasis, tumor recurrence and poor prognosis [8,19].
SLUG (SNAI2) is another transcription factor involved in CDH1 regulation and thus downregulating E-cadherin. It is a downstream target of the cKIT pathway and is significantly upregulated in the 'fibroblastic cluster'. SLUG might participate in EMT via downregulation of the components of the desmosome adhesion complex, thus, the status of SLUG expression correlates well with the loss of E-cadherin in human breast carcinomas [17]. SNAI2 can cooperate with CTBP1, CTBP2, HDAC1 and HDAC3 to repress CDH1 and SNAI2 also represses the breast cancer associated BRCA2 gene through CTBP1 and HDAC1. SNAI2 expression is associated with tumor effusions, metastasis and recurrence, but is also associated with partially differentiated phenotype in breast cancers, reflective of the role of SNAI2 in the developing breast [8,19].

Factors from the ZEB family
Smad-interacting protein1 (SIP1/ZEB2) is a DNA-binding transcriptional repressor of CDH1 and identified as a protein binding to the MH2 domain of Smad1 and later found to interact also with the signaling proteins SMAD2, SMAD3 and SMAD5. As already mentioned, SMADs play a critical role in transmitting TGF-β signaling from the cell surface to the nucleus and thus inducing EMT. Also in the ZEB family the CTBP co-repressor complex has the ability to affect the histone modification status and silences the E-cadherin transcription cells [19,20].

TWIST
The TWIST transcription factor prevents cells from *ras* oncogene-induced senescence and subsequently causes completion of EMT. It is a highly conserved basic helix-loop-helix (bHLH) transcription factor and its expression appears to be specific for ductal breast carcinoma. TWIST is associated not only with the invasive state but also the development of carcinoma angiogenesis [21].

The retinoblastoma gene
The retinoblastoma (Rb) gene product is another tumor suppressor that regulates multiple cellular processes like growth, differentiation and apoptosis and is often mutated and involved in carcinogenesis. It represses the transcription genes required of G1-S phase transition and thereby inhibits the cell cycle. Loss of Rb is critical for tumorigenesis, especially in high-grade breast carcinomas. Therefore, a relationship between Rb silencing and a high metastatic potential is suggested. Knockdown of Rb disrupts the cell-cell adhesion, which is normally controlled through positive regulation of E-cadherin transcription. A mesenchymal-like phenotype is induced, resulting in reduced expression of E-cadherin leading to EMT. As already mentioned, loss of E-cadherin alone is not sufficient for EMT induction. ZEB1 expression is also induced by depletion of Rb and also SIM2, which binds and represses the SLUG promoter, was reduced in Rb-depleted cells. All of these events are related to downregulating E-cadherin, inducing EMT and thereby metastasis [23].

Tumor protein 53

Another crucial tumor suppressor gene in cancer biology is p53. p53 protein checks cell proliferation during damage and other stressful situations and loss of p53 function is leading to deregulation of cell cycle checkpoints and apoptosis, crucial events in carcinogenesis. Recent studies have shown that p53 raised cell survival in cells with disrupted cell-cell interactions and that the tumor suppressor gene cause cell motility leading to metastasis. These effects are largely mediated through the regulation of Rho signaling, thereby controlling organization of the actin cytoskeleton [23].

During EMT, both Rb and p53 expression levels are significantly reduced and CDC42 (part of the Rho signaling pathway) was activated by silencing both factors. The combination led to a much greater alterations of the actin cytoskeleton than inactivation of Rb alone. The loss of both E-cadherin and p53 resulted in accelerated invasion and metastasis. Loss of p53 may raise cell survival in cells with disrupted cell-cell interactions and cause cell motility, whereas loss of Rb down-regulates the E-cadherin transcription leading to EMT. Thus, both well known tumor suppressor genes Rb and p53 are involved in tumor invasiveness and metastasis [23].

Cancer stem cells and their role in metastasis

Cancer stem cell hypothesis

It is not a new concept that cancer may be originated and sustained by a small set of stem-like, self-renewal cells. According to the cancer stem cell hypothesis share these so-called 'cancer stem cells' (CSCs) or 'tumor-initiating cells' several properties with normal stem cells, like unlimited capacity for self-renewal, the ability of multilineal differentiation to progenitors, require-ment for a specific niche to grow, enhanced resistance to apoptosis and an increased capacity for drug resistance. Thus, tumors develop through dysregulation of stem-cell self-renewal pathways and it is suggested that the inefficiency of the metastatic process, especially the initiation and maintenance of tumor growth in secondary organs, should be related to the cancer stem cell hypothesis. Only a rare subset of cells within the primary tumor is capable of re-initiating growth to form metastasis in distant sites. It is reasonable that these metastatic cells are stem cells because several links between stem cell and metastatic behavior, like the requirement of self-renewal capability and the necessity of a specific microenvironment, can be noticed [24,25,2].

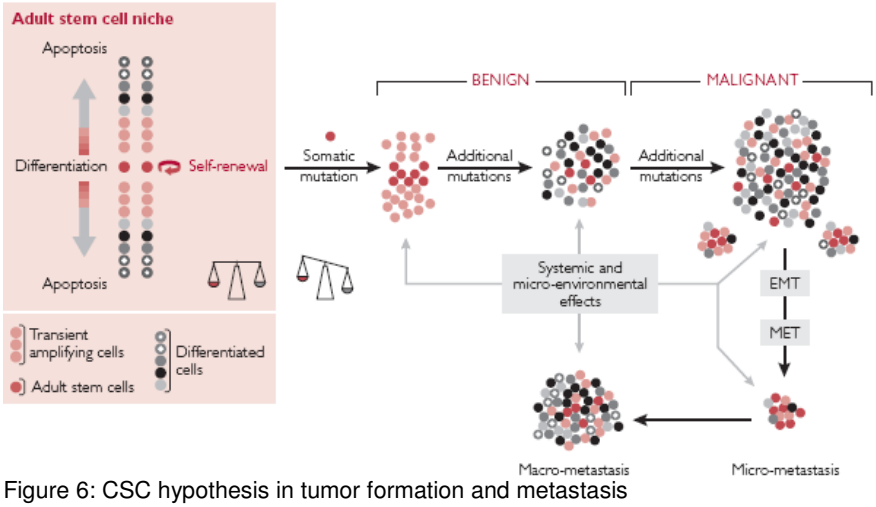

Figure 6: CSC hypothesis in tumor formation and metastasis
Gene mutations alter the equilibrium of pluripotent SCs, progenitors and fully differentiated cells in the adult stem cell niche. This results in lack of apoptosis and excessive proliferation thus leading to the formation of a heterogeneous benign tumor. Additional gene mutations lead to more malignant tumor stages. CSCs are the cells that are motile, detach from the primary tumor, invade distant organs by forming micrometastases and eventually macrometastases due to EMTs and METs (Obtained from Fodde *et al.* 2006 [30]).

The CSC's origin

It is suggested that CSCs arise as a consequence of genetic mutations in normal stem or progenitor cells and thereby cause dysregulation of the stem-cell self renewal pathway (fig. 6). The similarity between CSCs and normal stem cells is convincing and multiple mutations are necessary for a cell to become tumorigenic and metastatic. Stem cells are, because of their quiescence, around long enough to accumulate these necessary gain-of-function mutations and thereby produce a cancer stem cell phenotype [2]. Stem cells may also silently carry genotypic alterations which become phenotypical evident only in their progeny, leading the progenitor cells to act as CSCs [26]. Furthermore, normal stem cells have differentiation capacity, they can divide symmetrically or asymmetrically and thereby generating a heterogeneous population. Symmetric division gives rise to either two identical daughter stem cells, asymmetric division gives rise to one daughter stem cell and one cell that leaves the stem-cell niche to differentiate. This is also reason to suggest that CSCs originate from normal stem cells, CSCs possess the capacity to cause the heterogeneous lineages of cancer cells that form the tumor.

Breast cancer stem cells

Advances is stem-cell technology have led to the identification of stem cells. Breast cancer was the first human carcinoma for which a CSC subpopulation has been isolated. All Hajj and colleagues (2003) identified stem-like breast cells based on $CD44^+/CD24^-$ cell surface phenotype and isolated the population. CD44 is a cell-adhesion molecule, CD24 is a negative regulator of the chemokine receptor CXCR4, involved in breast cancer metastasis. This rare population was highly tumorigenic and shared classical features of normal stem cells, including capacity for self-renewal and generation of heterogeneous progenitors. The $CD44^+/CD24^-$ population was identified as the CSC population of breast cancer cells and contained several upregulated genes, including Notch and Hedgehog [27].

Stem cell niche

As already named, metastatic cells and stem cells share the property that they require a specific niche or microenvironment to grow and that they use chemokine pathways for migration. The stem cell niche serves as protection, maintains the stem cell pool and prevents its differentiation. More differentiated daughter progenitor cells direct tissue growth and renewal, they house and interact with the stem cells [2]. Altering of the microenvironment by activation of integrins and chemokines promotes attachment, survival and growth of tumor cells. Other niche cells are also involved in cancer metastasis, non-metastatic breast cancer cells increase the metastatic potency of the mesenchymal stem cells and also stimulate chemokine secretion from mesenchymal cells, enhance their motility, invasion and metastasis. It is known that the chemokine stromal cell-derived factor 1 (SDF-1) cooperates with its receptor chemokine receptor 4 (CXCR4) to induce stem cell migration. Breast cancer cells migrate, adhese, invade and metastasize especially to organs like lymph nodes, liver and bones. These organs express high levels of SDF-1 so they attract the CXCR4-expressing breast cancer cells, this explains the organ-specific metastasis. CXCR4 expression is very low in normal breast tissue, as its expression is increased during the different steps of carcinogenesis. This proves the role of CXCR4 in regulating carcinogenesis and primary tumor growth [28].

The migrating cancer stem cell concept

An important, conclusive theory has obtained from the consistency between metastatic cells and stem cells. The 'migrating cancer stem (MCS)-cell concept' explains many different traits of cancer cells and the relation between CSCs and EMT [24]. According to this MCS-cell concept, there are two forms of cancer stem cells identified in the progression of tumors. Stationary cancer stem cells (SCS cells) are cells still embedded in the epithelial tissue and present in differentiated areas throughout the whole process of carcinogenesis.

Though, they cannot disseminate and are active in tumor lesions such as benign adenomas. Migrating, mobile cancer stem cells (MCS cells) originate from SCS cells by induction of EMT, triggered by environmental factors such as increased nuclear β-catenin. The MCS cells retain stem-cell functionality and can disseminate from primary tissues to form metastatic colonies. MCS cells divide asymmetrically, one daughter cells starts proliferation and differentiation. The remaining MCS cell can migrate over a short distance before it undergoes another asymmetrical division and can contribute to the primary tumor mass. When migration occurs over long distances through blood or lymphatic vessels it forms metastasis after several asymmetric divisions at its new location [24]. Induction of MET is reason for the heterogeneity of the macroscopic distant metastasis because of the formation of more differentiated, epithelial cancer cells [6]. Both stemness and mobility are traits that are usually not combined in epithelial cells, so these traits indicate an important role for the EMT-driven MCS-cell evolvement in primary carcinomas and subsequently metastasis.

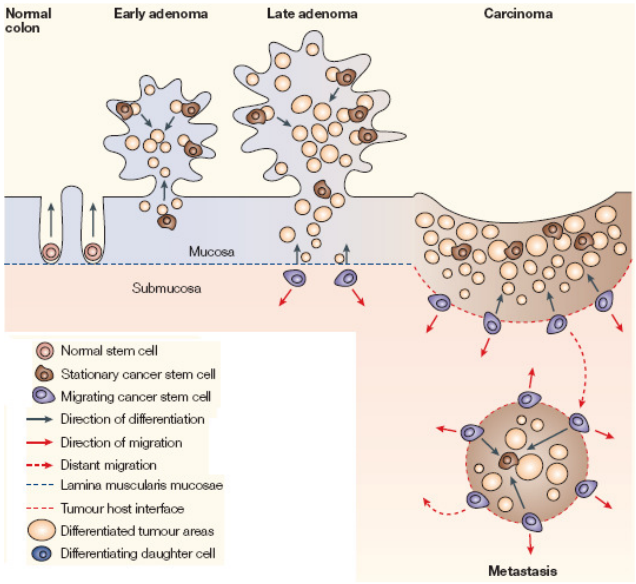

Figure 8: The migration cancer stem cell concept
Normal stem cells are located in basal crypt areas and stationary cancer stem (SCS)-cells appear in adenomas. Important is the initiation of EMT in tumor cells, including the SCS-cells which become mobile, migrating cancer stem (MCS) cells and can divide and form metastases (Obtained from Brabletz *et al.* 2005 [24]).

Molecular pathways

Amplification of the HER-2 gene is seen in an early stage of sporadic breast cancer. The HER-2 gene is member of the epidermal growth factor family and about 25% of human breast cancers display amplification of the gene. Overexpression of HER-2 increased the proportion of stem cells in mammary epithelial- and tumor cells, indicated by aldehyde dehydrogenase 1 (ALDH1) which is a stem-cell marker. Thus, HER-2 may play a role in mammary carcinogenesis, (dys)regulation the stem-cell population [25].

It is clear that some molecular pathways which are often mutated in EMT and thus metastasis, may be activated stem cell self-renewal, including Wnt, Notch, Hedgehog and PTEN (fig. 7). Mutations in the BRCA1 gene predispose women to up to an 80% lifetime risk of developing breast cancer. The gene plays an important role in DNA repair, activation of cell-cycle checkpoints and maintenance of chromosome stability. Recently, functioning of BRCA1 as a stem-cell regulator is suggested. Accumulation of genetically unstable breast stem cells may be the result of loss of BRCA1. Women with BRCA1 germ line mutations develop micro deletions in PTEN. Deletions of PTEN, as already mentioned are associated with cell motility and invasiveness, dysregulated self-renewal in

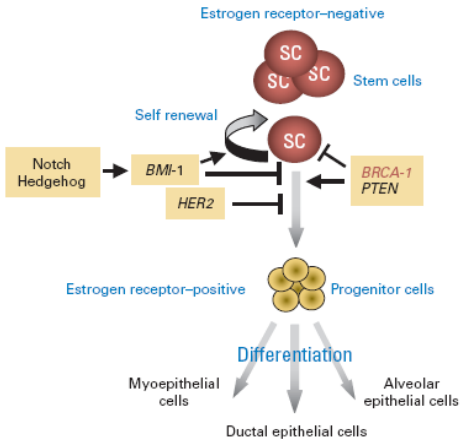

Figure 7: Self-renewal and differentiation pathways in breast stem cells.
Breast CSCs originate from breast stem cells. Carcinogenesis is initiated by dysregulating the process of stem-cell self-renewal through loss of BRCA1, PTEN or by activation Notch or Hedgehog signaling (Wnt not shown) (Obtained from Kakarala *et al.* 2008 [25]).

hematopoietic stem cells. Recently there is evidence that there are similar effects on breast CSCs. Furthermore, Notch expression is involved in self-renewal as well as the Wnt signaling pathway. The latter has been shown to be also involved in differentiation of a variety of stem cells and expressing stem-cell markers is enhanced during its activation. Additionally, there is evidence for dysregulation of the Hedgehog pathway in a subset of breast cancers. Hedgehog signaling acts through BMI-1 and leads to aberrant self-renewal and possibly delaying senescence [25,2]. EMT induction accelerates generation of stem cell properties [2] and thus may play a critical role in self-renewal. This evidence also proves the possible link between CSCs, EMT and metastasis [25].

Therapeutic implications

Drug resistance

Most current cancer therapies (surgery, hormono-chemo-and radiotherapy) fail once the tumor has been metastasized to secondary tissues. The CSCs have shown to be the cancer cells that can initiate and sustain primary tumor growth, that can be transformed into migratory cells and subsequent can form metastases and may relapse. It is suggested current therapies do not target the right cells. Therapies are based on homogeneous cancer populations, tumor regression and may target proliferating, differentiated tumor cells. Hence, it is likely that therapy fails because of its inability to target the CSC component. Recent studies demonstrate an increase in the CD44+/CD24- breast cancer stem cell population after chemotherapy, suggesting chemotherapy is indeed unable to target the CSC component [26].

CSCs may be resistant to radio- and chemotherapy because of their high expression of the ABC family of drug transporters (adenosine triphosphate-binding cassette proteins), their quiescence and their capacity for DNA repair. The CSCs can pump out nontargeted, chemotherapeutic agents like Paclitaxel and may be therefore resistant to chemotherapy [29]. In breast cancer, breast cancer-resistance proteins (ABCG2) were identified and were upregulated in breast stem cells. Additionally, CSCs are resistant to cell-cycle active chemo-therapeutic agents because they are slowly proliferating (especially in the G0 phase of cell cycle) for extended periods of time [25]. Accelerated re-population is a clinical problem after radiotherapy.

CSCs appear to be more radio resistant than other cell types because they had fewer double stranded DNA breaks. It is suggested that they can repair their DNA more efficiently and additionally the CSCs population is increased within the tumor, also associated with activation of the developmental Notch-pathway [2,25].

Implications of the CSC hypothesis for therapeutic approaches

The knowledge of the failure of targeting proliferating cells, begins to change the therapeutic approach to cancer. Given the specific stem cell features, targeting CSCs seems to be advantageous (fig. 9). There are meanwhile some CSC-targeted therapeutic strategies proposed, for example targeting the Notch pathway which needs the enzyme gamma secretase for processing. Gamma secretase inhibitors may be able to inhibit Notch signaling.

The inhibition of the HER-2 gene even seems to be efficient. Trastuzumab and Lapatinib are agents that inhibit HER-2 and thereby reduce the CSC population, resulting in a significant increase in cell death. Treating cancers with the cytotoxic agent cyclopamine results in inhibition of the growth of tumors with activated Hedgehog signaling.

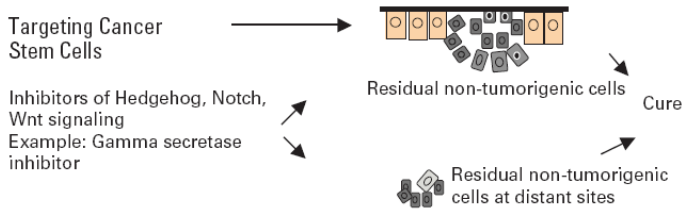

Figure 9: Targeting CSCs may lead to a cure for breast cancer. Gamma secretase inhibitors inhibit Notch signaling (Obtained from Kakarala *et al.* 2008 [25]).

Possibly this drug is able to inhibit self-renewal and thereby inhibiting the overall tumor growth [25]. Other possibilities to sensitize CSCs to therapy can be blocking the function of ABC transporters so CSCs are not able to efflux the chemotherapeutic agents, and target CSCs via targeting of Bmi-1 which would drastically shorten the lifespan of CSCs. The CSC hypothesis has exciting clinical implications. However, it is very important to keep in mind targeting CSCs is risky because of the fact that CSCs and the body's normal stem cells share so much properties. Targeting CSCs could also result in attacking normal stem cells and thereby following bad consequences because normal stem cells need their ability to self-renew. For example, blocking ABC transporters may decrease resistance to chemotherapy but may also cause normal stem cells to become sensitive to drugs which leads to prematurely death. Blocking signaling pathways like Wnt, Hedgehog and Bmi-1 is also risky because normal stem cells may than become unable to self-renew or may senesce prematurely [25].

Conclusions and future perspectives

In this review the relationship between the CSC hypothesis and induction of EMT leading to breast cancer metastasis is determined. The cancer stem cell subpopulation is identified as the main cause for metastasis. The "migrating cancer stem cell concept" indicates that EMT occurs in stationary cancer stem cells, thereby generating mobile cancer stem cells which can disseminate and form macroscopic metastasis at secondary sites. The Wnt/β-catenin, Notch, Hedgehog and PTEN signaling pathways are found to be initiated in both EMT and stem cell self-renewal. Most of the current therapies fail because they only kill proliferating cells and not the CSC subpopulation. This population is resistant against current drugs partly because of their expression of ABC transporters, their senescence and their enhanced capability to DNA-repair. Targeting CSCs and the pathways involved in EMT and stem cell self-renewal may have advantageous clinical properties but it is important to be careful killing the CSCs because of their similarities with the body's normal stem cells.

Because it is reliable that, according to the migratory stem cell concept, cancer stem cells get their migratory phenotype by EMT induction, my first suggestion is that new therapies need to be based on altering the process of EMT. Because stationary cancer stem cells do not have the ability to disseminate, restrain the cancer cells in this stage in tumorigenesis may be an important target for therapy. When the cancer cells can be hold in the stationary stage, they are unable to spread and form metastases. To keep the stem cells out of the migratory stage, EMT has to be inhibited. The pathways important for (breast cancer) stem cell self-renewal and EMT are reviewed for the reason that if the pathway is initiated in both events, it may be specific for the EMT occurring in the cancer stem cells. Many different pathways can be implicated and can inhibit the EMT process, thereby cancel the formation of migratory cancer stem cells. This seems to be a beneficial way for targeting the CSCs, but a circumstance we have to keep in mind is that also the body's normal stem cells and its functioning are dependent of undamaged signaling pathways because they need their self-renewing capacities for the body's overall homeostasis and development. Further research can lead to agents affecting the EMT and CSC self-renewal pathways without (or very less) affecting normal stem cells through the investigation of optimal conditions.
However, because normal stem cells and CSCs share many properties and CSCs may originate from normal stem cells, it is important to find the differences between these two. When different properties are elucidated, targeting specific CSCs without affecting normal stem cells, for example the CSC-specific signaling pathways, would be promising for targeted therapy against breast cancer. It is proposed that one of the differences between normal stem cells and CSCs is their dependency on the stem cell niche [31]. It is known that under normal conditions a short proliferating signal is required to support tissue regeneration, without the need for another niche.

In cancer, cancer stem-cells become self-sufficient to undergo uncontrolled proliferations or due to a change in niche signals. Thus, deregulation of the niche may result in carcinogenesis.
Exploring the mechanisms underlying breast cancer stem cells mobilization from or homing to the niche may provide insight in their role in the metastatic process and may lead to possible clinical targets. Novel pathways initiated in niche signaling need to be localized because destroying CSCs, by altering the specific breast stem cell niche signals they need for proper functioning, may lead to a targeted therapy without affecting normal stem cell self-renewal.

In this review many processes leading to metastasis are investigated. For clinical applications further research is needed but I am sure the discovery of the cancer stem cell hypothesis and the role of EMT in the metastatic process are important steps forward. It is important to keep in mind the essential role of normal stem cells, differences between these and cancer stem cells need to be studied so cancer stem cells can be specifically targeted. The targeting of CSCs, without attacking normal stem cells, is in my opinion a very important field of cancer stem cell research because it may lead to a promising cure for breast cancer.

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