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Mini-review: **Cancer stem cells: the root of tumor recurrence and metastasis**

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**Abstract**

**Keywords:** Cancer stem cells, Circulating tumor cells, Epithelial-mesenchymal transition, Metastatic niche, Metastasis-initiating cells, Liquid biopsy, Biomarkers

**Acronyms**

CSC - Cancer stem cells

CTC - Circulating tumor cells

EMT - Epithelial-mesenchymal transition

ECM - Extracellular matrix

MET – Mesenchymal – epithelial transition

MIC - Metastasis-initiating cells

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**Introduction**

Despite the progress in the prevention, diagnostics and treatment, cancer remains a major cause of morbidity and mortality around the world. According to the world health organization (WHO), more than 14 million newly diagnosed cases and more than 8 million deaths were reported in 2012, and increase in new cases of cancer by 70% and cancer related deaths by 45% is expected over the next 2 decades [1-3].

The idea that tumors are heterogeneous and contain cells with different differentiation and tumorigenic properties is almost one century old [4]. Leroy Stevens was the first who introduced the term "pluripotent" in relation to cancerous teratoma cells in 1953 [5]. The cancer stem cell concept of tumor development, which has been experimentally proven by John Dick and coworkers more than 20 years ago, suggests that cancer cells differ in their tumor initiating properties and population of tumor cells called cancer stem cells (CSC) resides on top of the tumor cell hierarchy and maintains tumor heterogeneity and tumorigenicity [6]. Although considerable controversy still remains regarding the presence of hierarchy for certain types of tumors as well as for best approaches of CSC analysis, this hypothesis has been supported by a large body of experimental data, and CSC were identified and characterized in many types of solid tumor and leukemia [7]. A growing body of evidence demonstrated that CSC might serve as biomarker for early tumor detection, prognostication, and prediction of therapy response whereas different CSC targeted therapies are emerging as potentially curative approach to cancer [8, 9].

Recent studies demonstrated that tumor heterogeneity depends on the number of cells with tumorigenic potential (CSC) which contributes to tumor growth [10, 11]. These CSC give rise to the various functionally different although genetically related subclones which compete to each other and evolve during tumor progression and treatment. In addition to the genetic component, two other factors, tumor microenvironment and epigenetic mechanisms might influence tumorigenic features of cancer cells and therefore contribute to tumor heterogeneity [10, 12]. Intratumoral heterogeneity which arises during tumor expansion and associates with variable microenvironmental selection forces and random mutations is a result of cancer cell evolution but also a source of Darwinian selection and enhanced tumor progression and metastasis [12, 13].

Metastatic tumors are the cause of more than 90% of cancer related deaths due to the fact that current therapies frequently fail to provide durable curative response if tumor is spread [14]. Metastases are the formation of secondary tumors in distant organs and tissues of the body by cancer cells spread from the primary site where tumor was first developed. Metastatic spread is a complex process which includes a few sequential steps such as (i) escaping the primary

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tumor by invading of cancer cells the surrounding tissues, passing through the basement membrane and entering the blood or lymphatic vessels (intravasation), [15] transit of circulating tumor cells (CTC) and acquisition of the survival mechanisms to escape anoikis, immune response and shear stress, (iii) escaping of CTC from the bloodstream (extravasation) and adaptations to new microenvironmental conditions at distant site, which define the ability of these disseminated tumor cells (DTC) to become metastasis initiating cells [13] [13] and form a metastatic lesion [16-18]. Metastatic spread is a multistage and low efficient process of cell selection and adaptation in which less than 0,02 % of DTC are able to develop metastases at the distant sites [19-21]. As a result, the process of tumor dissemination takes considerable amount of time and clinical manifestation of metastases can occur many years after diagnosis of a primary tumor although the presence of CTC in patients' peripheral blood and bone marrow can be detected at the early stages of cancer development [20, 22-26]. The detection, enumeration and molecular characterization of CTC in the blood of cancer patients which is called "liquid biopsy" can be used for early cancer diagnostics and metastasis prevention and therefore might substantially reduce cancer associated mortality [27].

The clinical course of metastatic tumor progression including probability to form distant metastasis and latency period between primary tumor diagnosis and metastatic spread has a high variability between different types of tumors as well as across tumors even within one type of cancer (15). Molecular comparison of primary tumors and their metastasis by using different approaches including high-resolution genome sequencing, single cell gene expression analysis and lineage tracing provide evidence of close genetic relationship between metastatic clones and their ancestor clones in the primary tumors [25, 28-31]. These findings suggest that establishment of systemic disease by MIC depends on the driver mutations which underlie primary tumor development and present in their CSC counterparts in the primary tumor [25, 28]. Remarkably, no recurrent metastasis-driven mutation were found so far which were not present in primary tumors suggesting that clonal evolution of metastasis relies more on the epigenetic mechanisms. Therefore, metastasis formation can be considered as a culmination of the Darwinian evolutionary process within the tumor when competing of multiple subclones results in the development of the cell inherent traits that favor tumor dissemination. However, in addition to the driver mutations inherited from their primary tumor counterparts, MIC acquire additional capabilities, which provide them with a chance to persist in the circulation, extravasate, survive in distant organs and form metastasis. This evolutionary process is driven by epigenetic changes and subsequent selection under the pressure of harsh microenvironmental conditions.

The supportive sites which maintain survival of MIC are unique locations within the distant organs defined by specific cellular and molecular components and called metastatic

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niche [20, 32-34]. Thus, cancer cells which have the capacity to colonize distant organs have the features of CSC but also exert their tumor-initiating capacity under adverse microenvironmental conditions [7, 20, 35-37]. Therefore metastases can be driven by the evolved and selected subpopulations of CSC at their worst [20, 31]. In support of this, analysis of the gene expression profiling of single breast cancer cells confirmed that early metastatic cells possess a stem cell-like signatures suggesting that similarly to the primary tumors, metastases are initiated and maintained by the cells with CSC properties [31]. In this review, we summarize the main common hallmarks and evolving concept of CSC and MIC, their dynamic interaction with microenvironmental factors, discuss perspectives of using CSC, DTC and CTC as prognostic and predictive tumor biomarkers as well as possible strategies for their targeting in the clinical setting.

## 1. **Cancer as a stem cell disease**

The concept of cancer stem cells emerges from the discovery of tumor heterogeneity. Tumor heterogeneity refers to the existence of different cellular types bearing different properties within the tumor. One of the first evidences that tumors are heterogeneous emerged from the works of Rudolf Virchow [38]. Intratumoral heterogeneity within individual cancer tissues has been documented at the different levels including tumor histology, cytogenetic markers, gene and protein expression, genetic mutations, growth rate and therapy response [39-46]. Technological advances in DNA sequencing made possible the whole genome sequencing with a great resolution and enabled comparative analysis of mutational changes across thousands of tumors and within the individual tumor tissues [11, 47-49]. These analyses revealed that each individual tumor contains a high level of mutations which are associated with the phenotypes of individual clones. The existence of considerable genetic and phenotypic variations within the same individual tumor suggested that even if some of the tumors are derived from a single cell and originally are monoclonal, it does not rule out the possibility of the subsequent acquisition of additional genetic alterations and multi-clonal evolving as the tumor grows.

The ideas of developmental and population biology were applied for tumor biology in the clonal evolution model [4]. The proposed model suggests that despite the vast majority of neoplasms arise from a single cell or a very few cells of origin, tumor cell population undergo the developmental evolution associated with acquisition of genetic instability and sequential selection of favorable cell subpopulations which are produced as a results of acquired somatic mutations [4]. This model of tumor heterogeneity described the tumors as complex ecosystems following the rules of Darwinian evolution where the individual tumor clones with heritable

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advantageous mutations have a higher growth rates and become more dominant over other tumor clones which do not have such heritable survival preferences [4, 46].

While genetic mechanisms are required for tumor initiation and progression, genetic alterations represent only one of many dimensions that govern tumor cell's fitness. The functional heterogeneity within tumor cells can occur even in the absence of genetic differences. Analyses of the cell repopulation and chemotherapy response demonstrated that individual cells within a uniform genetic clone are functionally heterogeneous [11]. These studies demonstrated that in addition to the genetic components, other mechanisms might contribute to the development of heterogeneity within a single genetic lineage such as epigenetic regulation of gene expression and microenvironmental variability [10, 11]

Epigenetic regulation comprises the changes in the chromatin's structure and function and includes posttranslational histone modification and DNA methylation. These alterations occur without changes in DNA sequences and lead to the perturbation in gene expression and cellular functions. These changes are heritable and thus, in addition to DNA mutations, provide a substrate for the evolution processes during tumor development. Epigenetic modifications are the basic mechanisms governing embryonic development and maintaining a stem cells hierarchy in the adult tissues [50, 51].

The model of tumor cell hierarchy comes from concept of somatic stem cells and based on the understanding of tumorigenesis as caricature of normal tissue regeneration with cancer stem cells on the top of the hierarchical tree [52, 53]. The hierarchical model suggests that only a small population of tumor initiating cells or cancer stem cells (CSC) as they were named by analogy with somatic stem cells can extensively proliferate and sustain the clonogenic growth within the individual tumors. In other words, tumor heterogeneity depends on the heterogeneity of the CSC cells contributing to the tumor growth.

Of particular importance for the understanding of tumor hierarchy as fundamental cause of tumor heterogeneity were transplantation experiments in murine models. These studies demonstrated that only a certain portion of murine leukemia, myeloma or sarcoma tumor cells is capable to initiate the growth of new tumor in the recipient mice and thus, not every tumor cell is tumorigenic [54-56]. The first direct experimental evidence for the existence of the leukemia stem cells was provided by the work of Lapidot, Bonnet and coauthors [6, 57]. These cells with $CD34^+CD38^-$ phenotype were identified in peripheral blood of acute myeloid leukemia (AML) patients and exhibited the ability to engraft SCID mice and to give rise to the same CSC population and to more differentiated leukemic blasts in vivo. Later, tumor-initiating cells have been identified and characterized in breast cancer and then have been found in brain tumors

and other tumor entities [58-60]. CSC are now identified for most human tumors and many marker-defined phenotypes of CSC are described for the same tumor entities (Table 1).

Human cancer stem cells were defined by their ability to self-renew (give rise to identical descent stem cells with significant proliferative potential which can be activated if necessary) and to divide asymmetrically (give rise to more differentiated tumor cells with restricted proliferative lifespan) [52]. The differentiation potential of stem cells can be experimentally demonstrated by their capability to generate xenograft tumors which histologically resembling the human tumors from which these CSC were isolated, and the self-renewal potential of CSCs population can be validated by serial transplantation assays which is now a standard method to identify the tumor cell-of-origin [61, 62].

The self-renewal potential and extensive clonogenic properties of CSC, which assuring the heritability of the adaptive genotype, coupled with genomic instability of tumor cells including CSC, provide a strong argument for CSC being the unit of cancer evolution [63]. This definition of CSC is not controversial to the fact that in contrast to the somatic stem cells, CSC is not a constitutive entity, but rather a transient state. A growing body of evidence suggests that environment where the tumor cells reside might induce stem cell state in tumor cells by the processes of epigenetic reprogramming [9, 64-67]. In turn, the distinct regions of the tumor microenvironment where CSC reside and which called "niche" protect CSC from immune cells and therapeutic insults, maintain CSC properties, govern CSC plasticity and induce their metastatic potential.

## 2. CSC niche as a determinant of tumor evolution

In order to maintain the above described specific features of CSCs such as self-renewal, tumor initiating or long-term survival capacity it is believed that CSCs, comparable to normal stem cells, reside in specialized niches within the tumor microenvironment (TME). The TME consists of non-cancerous cells including stromal cells, immune cells or endothelial cells, components of the extracellular matrix (ECM) as physical scaffold and soluble signaling molecules like growth factors and chemokines [68]. For example, cancer-associated fibroblasts (CAFs) or myofibroblasts ($\alpha SMA^+CAV1^{hi}$ cells), one component of the stromal TME compartment, can derive from multiple precursors, such as normal fibroblasts, mesenchymal stem cells or myoepithelial cells in response to paracrine signaling (TGF-β, CXCL14, HH). They secrete mitogenic growth factors like epithelial growth factor (EGF) family members (e.g. hepatocyte growth factor/HGF, fibroblast growth factor/FGF, insulin-like growth factor 1/IGF1), migration-stimulating chemokines (CXCL12/SDF1) or immune suppressive factors (TGF-β). Tumor cells themselves secrete factors (IL-1, TNFα) to instruct CAFs for niche formation [69].
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This was shown for example in lung cancer were the stem cell-like properties of Oct3/4^{+}nanog^{+} CSCs are regulated by CD90^{+} CAFs mainly via the IGF1R paracrine signaling [70].In addition, other cells like mesenchymal stem cells (MSCs) within the TME are influenced by the tumor cells and support the malignant cells growth.

Depending on the localization and the environmental conditions of the niche, several anatomical distinct CSC niches can be distinguished (e.g. the hypoxic, perivascular, invasive niche) [71, 72]. Within the primary tumor it is hypothesized that quiescent CSCs preferably reside within the hypoxic niche and migrate after activation of the epithelial-to-mesenchymal (EMT) program to the tumor edges, the invasive front, to disseminate from the primary tumor and metastatic seeding [71]. Tumor regions are different in oxygen supply: normoxic, hypoxic and anoxic areas may exist within one tumor due to the chaotic structure of tumor vasculature [73-75]. Hypoxia (pO$_2$ < 5mm Hg; < 0.5 % O$_2$) develops due to oxygen diffusion limitations within areas with high malignant cell proliferation and impaired vascularization. In addition to the lack of oxygen and nutrients, these environmental conditions have increased acidification due to the anabolic switch in the tumor cell metabolism and increased lactate production. This microenvironment  is selective for tumor cells which are better able to withstand these harsh conditions and possess  favorable mutations and activation of the molecular mechanisms suppressing apoptosis, inducing autophagy, enhancing receptor tyrosine kinase-mediated signaling and tumor angiogenesis [76]. Novel findings suggest that CSC themselves are highly glycolytically active compared to the bulk tumor cells, which show an increased aerobic glycolysis (Warbug effect). This was first demonstrated in basal-like breast cancer were the loss of the gluconeogenic enzyme fructose-1,6-bisphosphatase 1 (FBP1), which antagonizes the glycolytic flux, is involved in the process of metabolic reprogramming to induce an epithelial-to-mesenchymal (EMT) phenotype, increases CSC-like properties and tumorigenicity [77]. Based on these findings Luo and Wicha proposes a model of metabolic plasticity of CSCs depending on the access of glucose in the niche environment. While high proliferative CSCs under glucose-rich conditions preferably utilize aerobic glycolysis, slow cycling CSCs are shifting their bioenergetics under glucose-deprivation to mitochondrial oxidative phosphorylation (OXPHOS) [78]. Data from another study support these findings, the EMT-associated oncometabolite signature (e.g. including glutamine, glutamate and alanine) have been demonstrated to correlate with poor clinical outcome of breast cancer patients [79] and with metastatic spread in colon and prostate cancer models. This is in agreement with the accepted phenomenon that normal stem cells rely on anaerobic glycolysis (2 ATP molecules per glucose) rather than on OXPHOS (38 ATP molecules) for their energy production. Moreover, the accumulation of reactive oxygen species [30] and the downregulation of DNA repair pathways under hypoxic conditions lead to

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increased genomic instability, accumulation of mutations and acceleration of clonal selection within the CSC pool. These intracellular mechanisms are regulated by the transcription factor HIF-1α and altered proteins in the unfolded protein response (UPR) signaling pathway leading to the inhibition of the DNA repair mechanisms by homologous recombination (HR), non-homologous end-joining (NHEJ) and mismatch repair (MMR) [80].

Recent evidences suggest that hypoxia may play an important role in CSC survival and development of unique stem cells characteristics. Transcriptional factors from HIF family are the key players in regulation of cellular response to hypoxia. They either can be regulated by hypoxic conditions or not, and can themselves alter the expression of a plenty of pro-survival genes [81]. Exposure of different cancer cells e.g. ovarian cancer cell line ES-2 or glioblastoma cell line U87 to hypoxic conditionsincreases their stem cell properties: maintainance of non-differentiated state, increasing colony and sphere forming efficiency and expression of stem cell markers [82, 83]. Hypoxia can activate HIF1α, which, in turn, can upregulate Notch1 signaling pathway which is responsible for maintaining cells in undifferentiated state [84]; however, it still remains unknown whether the observations reflect de-differentiation of already differentiated tumor cells or hypoxia stimulates the proliferation of existing stem-like population. Similar effect was described for pancreatic cancer cells expressing CSC marker CD133: they were more tolerant to hypoxia, and their elevated epithelial-mesenchymal transition (EMT) was the result of the modulation of HIF-1α expression by CD133 [85-87]. CXCL12, a ligand for CXCR4, is directly regulated by HIF1, promoting CXCR4+ cells migration to hypoxic areas [88]. The number of ALDH-positive breast cancer CSCs increased after treatment with antiangiogenic agents such as sunitinib and bevacizumab, and the same was observed for lung CSCs, revealing the important role of tumor hypoxia for the increase of aggressiveness and invasive potential of tumors [73, 89]. The stiffness of surrounding tissue may also have an impact on CSCs [90]. The elevated stiffness (the physical property) of ECM and hypoxic microenvironment stimulate the development of CSC in breast cancer  and this effect is mediated by integrin-like kinase ILK and PI3K/Akt signaling pathway [90]. Interestingly, that proteolysis of ECM has been shown as a process necessary for CSC spreading [91].

Recent findings suggest that CSCs have metabolic phenotype different from normal cells as well as from bulk tumor cells. For the energy production normal tissues use oxidative phosphorylation (OXPHOS) process, while cancer tissues rely on less efficient aerobic glycolysis accompanied by excessive production of lactate (Warburg effect)[92]. In case of CSCs the question which process they prefer to obtain energy remains controversial, as described in the recent review by Sancho et al [93]. For instance, ovarian CSCs prefer OXPHOS over glycolysis to obtain energy, and also had a higher rate of pentose phosphate pathway (PPP) [94].

Interestingly, that results obtained from studying CSC metabolism of the same tumor entity can be different depending on the model; it has been shown that ovarian CSCs from spheroid model obtain energy by active usage of Krebs cycle while the same cells in monolayer culture don't [15]. Lipid metabolism also plays an important role for tumors progression. De novo lipid synthesis has been described as one of the main features in cancer cells [95, 96]. Fatty acid synthase (FASN) is the enzyme which is responsible for de novo fatty acid synthesis; its expression is elevated in many cancers and inhibition of its activity is considered as a potential treatment option for many cancers [97-100]. Leukemic stem cells can enhance their fatty acid synthesis by using the lipolysis of surrounding gonadal adipose tissue [101]. Tumor cells of Lewis lung carcinoma influence surrounding myeloid cells by inducing fatty acid synthase and PPARbeta/sigma through M-CSF, which enhances their tumorigenic potential [102]. Interestingly that high-fat diet may have an impact on the properties of intestine progenitor cells making them more tumorigenic [96].

In summary, all the already described molecular and cellular mechanisms of interaction between CSCs and their niches including hypoxia, nutrients, inflammation, anti-cancer therapy, cell-cell and cell-ECM interaction, growth factors and cytokines determine the CSC fate, leading to tumor cell reprogramming, therapy resistance and metastatic spread. Despite the TME is directly modulating the CSCs on the cellular and molecular level it is thought that the initial signal coming from the CSCs themselves. They send signals to the TME to modulate their surrounding according to their needs.

Metastasis initiating cells (MICs) are a subset of CSCs and by definition this are cells capable of seeding metastatic colonies in secondary organs [103]. The key feature of these cells is the induction of the EMT program and the activation of the master transcription factor (TF) of the zinc finger E-box-binding homeobox family members SNAIL, TWIST and ZEB through various signals derived from the tumor stroma (PDGF), the ECM (e.g. collagen), secreted growth factors (TGF-β, PDGF, IL-6) and the WNT signaling. The repression of the epithelial (cell adhesion, polarity) and the induction of the mesenchymal phenotype (motility, invasion) during EMT is demonstrating the high plasticity of CSC. For example, the EMT-TFs are recruited to the promoter of E-cadherin (*CDH1* gene) to repress its transcription. Clinically relevant was the finding that CSCs with a high level of SNAIL expression and nuclear β-catenin accumulation are detected within the invasive front of colorectal cancer [104, 105] and is correlating with poor prognosis of colorectal cancer patients [106]. This proposed correlation of EMT induction with metastatic spread was questioned last year were two studies providing evidence that EMT is not necessary for metastasis formation. Instead it contributes to drug resistance. Fischer et al. used a Cre-mediated EMT lineage tracing system to monitor lung metastasis of breast cancer.

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Surprisingly they found that the lung metastasis mainly consists of non-EMT tumor cells, while the lung metastases after cyclophosphamide chemotherapy were formed by EMT-positive cells [107]. The second study used a genetically modified mouse model of pancreatic ductal adenocarcinoma (PDAC) with deletion in either SNAIL or Twist and found that the suppression of EMT within the PDAC is not changing the invasiveness, dissemination and metastatic spread. Instead its contributing to enhanced sensitivity to gemcitabine treatment and prolonged overall survival of the mice [108]. The molecular mechanisms underlying the higher chemo- and radioresistance of CSCs are still not fully understood, but it was shown that CSCs exhibit a higher expression of drug efflux pumps, such as the ABC transporter and multi-drug resistance protein (MDR) ABCG2 [109]. In addition to the process of EMT, the cellular plasticity of CSCs is enabled trough a coordinated epigenetic modulation, especially by histone modifications, to achieve a highly flexible and fast genome wide change of the gene expression and stabilizes the cellular phenotypic switch. As already mentioned above the key signal is the epigenetic repression of E-cadherin by histon 3 lysin 27 trimetylation (H3K27me3) by the polycomb group complex PRC1&2 and the histone methyltransferase EZH2 [110]. A study from our group is indicating that ionizing radiation is inducing a CSC-like phenotype in prostate cancer cells trough the modulation of histone marks. Using the EZH2 inhibitor DZNeP the epigenetic reprogramming was inhibited and prostate CSCs were turned into a less tumorigenic and highly radiosensitive population [111]. In general it is thought that normal stem cells exhibit in general a open chromatin state to keep the pluripotency, while under hypoxic conditions all histone methylation marks (activating (H3K9 and repressing marks) are increased due to the fact that histone demethyltansferases are inhibited [112-114]. Malignant cells exhibit a tremendous selective pressure during the multistep process of metastatic colonization including invasion, intra- and extravasation, within the circulation, evading immune defenses, during organ infiltration and the replacement of host tissue [20]. This genetic and epigenetic evolution of the metastatic tumor traits is selective for favorable mutations. One groundbreaking study in 2012 sequenced multiple biopsies of kidney cancer patients including the primary tumor and metastasis from multiple regions and found > 100 mutations while no biopsy was the same. This demonstrates the highly intratumoral heterogeneity. In addition, they found that app. one third of the mutations were found in all samples, meaning that app. 60 mutations were acquired during the metastatic process and a relationship of all cancer cells within one patients could be plotted within a evolutionary tree [115].

The concept of metastatic CSC niche is historically going back to the 'seed and soil' hypothesis from Stephen Paget in 1889 proposing a cross-talk between the malignant cells (the seeds) and the organ microenvironment (the soil) [116-118].

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3. **Tumor metastasis as a multistep process (Klaus Pantel)**

Tumor cells are released from the primary tumor and/or metastatic sites into the bloodstream where they circulate for a short period (half-life: 1-2.4 hours) (Meng et al., Clin Cancer Res. 2004). The conditions in the bloodstream are harsh for epithelial tumor cells and CTCs undergo a strong selection process (Kang & Pantel, Cancer Cell 2013). The clearance of surviving CTCs happens through extravasation into secondary organs (e.g., the liver in colon cancer patients (Deneve et al., Clin Chem 2013). Survival of CTCs might be supported by close association with activated platelets and macrophages (Smith et all, J Mol Med 2013; Gay et al., Nat Rev Cancer 2011). Interestingly, there is some evidence that human tumor cells can re-circulate from secondary metastatic sites such as the bone marrow into the blood stream (Kim et al., Cell 2009). Whether the bone marrow is a reservoir of disseminated cancer cells from where they recirculate and colonize other organs remains still controversial. However, it should be noted that DTCs in the bone marrow of colon cancer patients are predictors for relapse in the liver and lungs (Lindemann et al., Lancet 1992).

Over more than a decade, epithelial markers such as EpCAM and cytokeratins have been used to distinguish carcinoma cells from the surrounding blood cells (Alix-Panabieres & Pantel, Nat. Rev. Cancer 2014). However, epithelial tumor cells can downregulate expression of epithelial markers during epithelial-to-mesenchymal transition (EMT), a process that has been connected to stem cell-like properties of cancer cells (Alix-Panabieres & Pantel, Cancer Discovery, 2016). However, the present view is that tumor cells with a partial EMT also called the "intermediate phenotype" might have the highest plasticity to adapt to the conditions present in secondary sites (Tam et al., Nat. Med. 2013). The reason is probably that experimental studies have shown that disseminating tumor cells need to undergo the reversal of EMT called MET to form an overt metastasis (Kang & Pantel, Cancer Cell, 2013). Thus, cells with a high plasticity have the advantage to disseminate easily (EMT-phenotype) but also to proliferate and form a metastasis at the secondary site (MET-phenotype). Consistent with this view, colon CTCs that grow in culture express strongly epithelial markers (EpCAM and cytokeratins) together with the EMT-inducing transcription factor Snail and the cancer stem cell markers ALDH1 and CD133 (Cayrefourcq et al., Cancer Res. 2015). Moreover, CTCs from breast cancer patients xenografted into immunodeficient mice expressed EpCAM and cytokeratins next to the stem cell marker CD44, the immune inhibitor molecule CD47 and the Met oncogene (Baccelli *et al., Nat. Biotech, 2013*).

Many solid tumors undergo an extended period of dormancy, characterized by the presence of disseminated tumor cells (DTCs) or small micrometastases over many years before

overt metastases may eventually arise. Metastatic relapse in patients with estrogen receptor-positive breast cancer can occur more than 10 years after the diagnosis and resection of the primary tumor (Goss &Chambers, Nat. Rev. Cancer 2010). Little is known about the factors that might have a role in the 'awakening' of dormant tumor cells that leads them into the dynamic phase of metastasis formation. The steady state that regulates dormancy might be disturbed by both changes in DTCs (for example, additional mutations or epigenetic modifications in genes controlling cell proliferation and apoptosis) and the surrounding microenvironment (Kang & Pantel, Cancer Cell 2013).

Cancer cells in patients with breast, lung and prostate cancer disseminate early into the bone marrow and pose a risk for subsequent relapse (Pantel et al., Lancet 1996; Braun et al., NEJM, 2005; Köllermann et al., J Clin Oncol., 2008). Despite the common belief that tumor cell "shedding" is a passive random process, there is evidence for an expression signature related to the presence of CTCs/DTCs (Wölfle et al., Cancer Res. 2002; Krämling et al, Clin Cancer Res. 2009; Werner et al. Cancer Discovery 2015). Among others retinoid-acid inducer-2 (RAI2) has been discovered as new metastasis-suppressor affecting homing and survival of DTCs in bone marrow (Werner et al, Cancer Discovery 2015). Functional analysis of the previously uncharacterized RAI2 protein revealed molecular interaction with CtBP transcriptional regulators and an overlapping function in controlling the expression of a number of key target genes involved in breast cancer including those relevant for EMT.

Metabolic adaptation of DTCs is important for their survival in bone marrow and probably also in other organs (LeBleu et al, Nature Cell Biol. 2014). Experimental studies indicate that DTCs prefer homing to the hematopoietic stem cell niches where they may find optimal conditions to survive (Shiozawa et al., J Clin Investigation, 2011). However, DTCs that want to survive under these hypoxic conditions need to activate stress response proteins such as the unfolded protein response (Bartkowiak et al., Cancer Res. 2015). Among the factors and pathways that are regulate the progression from DTCs to bone metastases VCAM1, Jagged-1 and tumor-induced osteoclast miRNAs seem to play an important role (Lu et al Cancer Cell 2011; Ell et al, Cancer Cell 2013; Bednarz-Knoll et al, Clin Chem 2016).

Metastatic spread is also supported by the formation of niches that are prepared by non-malignant cells leaving the primary tumor or the bone marrow (e.g., VEGFR1+ hematopoietic progenitors (Kaplan et al, Nature 2005)). These niches provide favorable microenvironments for homing and survival of CTCs. As recently reviewed (Plaks et al., Cell Stem Cell 2015), CSCs reside in niches, which are anatomically distinct regions within the tumor microenvironment. These niches maintain the principle properties of CSCs, preserve their phenotypic plasticity,

protect them from the immune system, and facilitate their metastatic potential. Since CSCs survive many commonly employed cancer therapies, the niche components might become preferable therapeutic targets. Recent work indicates that the primary tumors (and possibly metastases) can also release small microvesicles called exosomes, and the integrin composition of these exosomes mediate homing of metastatic cells to specific distant sites (Hoshino et al., Nature, 2015).

The role of the immune system as a potentially important host component for controlling metastatic progression is still under investigation (Mohme et al., Nature Rev. Clin. Oncol. 2016). The bone marrow microenvironment has special features for the maintenance of tumor dormancy and immunological T-cell memory, whereas certain subsets of macrophages can support metastatic spread by facilitating angiogenesis and extracellular matrix breakdown and remodeling. Indirect evidence that the immune system might control minimal residual disease in patients with cancer was derived from a strong positive correlation between T-cell activation and survival in patients with colon cancer (Mlecnik et al., Cancer Met Rev 2011).

## 4. Implication of CSC concept for the development of anti-metastatic therapy

The CSC hypothesis is attracting attention not only for the explanation of tumor heterogeneity, resistance to conventional anti-cancer therapies or metastatic spread, but also due to the expected high potential of CSC-targeting therapies for cancer cure. Current treatment strategies against solid tumors have limitations due to non-specificity, high toxicity and acquired resistance leading to high rates of treatment failures. A growing body of evidence is indicating that conventional chemo- and radiotherapy has the potential to kill highly proliferative bulk tumor cells and reduce significantly the tumor burden, but a small number of malignant cells survive and re-grow the tumor. As already in detail discussed above, this therapy-resistant clones are thought to be malignant cells with stem cell features. Therefore, selective inhibitor screens for CSC-specific targeting agents using high throughput methods (HTS) were started. In 2009, the group of Eric Lander published such kind of HTS-screening were they identified salinomycin (Procoxacin), a traditionally used antibiotic drug, with highly selective activity against breast CSCs [119]. The molecular action remains unknow, but it is thought that the effectiveness is due to his action as potassium ionophore. The clinical use may be limited by the high toxicity and the narrow therapeutical window

### Conclusion

Problems of the functional validation of CSC and CTCs & identification and characterization of metastasis-initiating cells

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Table 1. CSC markers

| Marker | CSC | CTC | DTC | Function | Tumor type | Reference |
|--------|-----|-----|-----|----------|-----------|-----------|
| CD133 | + | + | + | Transmembrane protein; functions remain unknown | Breast, glioblastoma, colorectal, prostate, pancreatic, melanoma | |
| CD44 | + | + | + | Cell surface family of proteins; interacts with hyaluronan | Breast, gastric, ovarian, colorectal, prostate | |
| ALDH | + | + | + | Catalyzes the oxidation of aldehydes to the corresponding carboxylic acids; esther hydrolyses; nitrate reductase activity; | Colorectal, lung, breast, melanoma, prostate, pancreatic | ( |
| ABCG2 | + | + | + | Transmembrane exporter of exogenous substances from cell, is involved in multidrug resistance in cancer | Hepatocellular carcinoma, lung, pancreatic | |
| Beta-catenin | + | + | + | Transcription factor, the component of Wnt/beta-catenin signaling pathway | Colorectal | |
| ABCB5 | + | + | ? | Transmembrane transporter of different molecules | Melanoma | |

Table 2. Running clinical trials addressing CSC-specific targeting for anti-cancer therapy
(Examples only)

| Drug name | Target molecule and molecular function | Tumor entity | Clinical trial phase and Identifier no | Primary endpoint and results | References |
|---|---|---|---|---|---|
| Metformin | Biguanides, lower circulating insulin, mTOR inhibition | Colon cancer, ovarian cancer | Phase I , randomized, interventional (NCT01440127) NCT01579812 | Recurrence-Free Survival | |
| Verapamil | calcium channel | Meningiomas | Phase II, Interconventional , Safety/Efficiancy study (NCT00706810) | | |
| Fursultiamine (thiamine tetrahydrofurfuryl disulfide, TTFD) | derivative of vitamin B | Esophageal squamous cell carcinoma | Phase II, NCT02423811 | | |
| Valspodar (*PSC-833*) | P-glycoprotein | Breast and ovarian Cancer | Phase I (NCT00001302) | Compeleted in 2002 (no results) | |
| Mithramycin | tricyclic pentaglycosidic antibiotic | Lung cancer | Phase I/II, Safety/efficiency (NCT02859415) | Maximum tolerated dose, Overall response rate | |

Figure 1. Metastases as a result of tumor evolution