Circulating cancer stem cells: the importance to select

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Abstract: It has been demonstrated that even localized tumors without clinically apparent metastasis give rise to circulating tumor cells (CTCs). A growing number of technically diverse platforms are being developed for detecting/isolating CTCs in the circulating blood. Despite the technical challenges of isolating rare CTCs from blood, recent studies have already shown the predictive value of CTCs enumeration. Thus, it is becoming increasingly accepted that CTC numbers are linked to patients’ outcome and may also be used to monitor treatment response and disease relapse, respectively. Further CTCs provide a non-invasive source for tumor material, ‘liquid biopsy’, which is particularly important for patients, where no biopsy material can be obtained or where serial biopsies of the tumor, e.g., following treatment, are practically impossible. On the other hand the molecular and biological characterization of CTCs has still remained at a rather experimental stage. Future studies are necessary to define CTC heterogeneity to establish the crucial role of circulating cancer stem cells for driving metastasis, which represent a distinct subpopulation of CTCs that bear metastasis-initiating capabilities based on their stemness properties and invasiveness and thus are critical for the patients’ clinical outcome. As compared to non-tumorigenic/metastatic bulk CTCs, circulating cancer stem cells may not only be capable of evading from the primary tumor, but also escape from immune surveillance, survive in the circulating blood and subsequently form metastases in distant organs. Thus, circulating cancer stem cells represent a subset of exclusively tumorigenic cancer stem cells characterized by their invasive characteristics and are potential therapeutic targets for preventing disease progression. To date, only a few original reports and reviews have been published focusing on circulating cancer stem cells. This review discusses the potential importance of isolating and characterizing these circulating cancer stem cells, but also highlights current technological limitations.

Keywords: Cancer stem cells; circulating tumor cells (CTCs); drug resistance

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Introduction

There is growing interest in the cancer field concerning two rather recently identified cell types, cancer stem cells and circulating tumor cells (CTCs), because of their fundamental biological and clinical implications. Both cancer stem cells and CTCs bear fascinating biological features that have major impact not only on the phenotype of the tumor, but also determine prognosis and therapy of individual cancers. The concept of cancer stem cells and their importance in tumor development, maintenance and metastasis has been extensively validated over the past decade in leukemia, breast, colon, brain, prostate and pancreas cancer (1-8). It is now well recognized that cancer stem cells are essential for tumorigenicity, metastasis and resistance to current therapeutic regimens, eventually leading to relapse of disease. Increasing evidence now also suggests that CTCs harbor a subset of cells that is essential for metastatic spread, hence there is great need to further understand and dissect this heterogeneous population of.
CTCs in the context of tumorigenicity and metastatic activity, respectively.

Tumor progression leading to metastasis is a complex multistage process that includes several fundamental biological processes. Metastatic cells have to successfully undergo epithelial-to-mesenchymal transition (EMT), detach from the primary tumor mass and invade the extracellular matrix, intravasate, survive in the circulating blood and disseminate into distant organs, extravasate, undergo reverse mesenchymal-epithelial transition (MET), colonize and eventually form micrometastases and, in some instances, outgrowth of clinically apparent secondary cancer lesions (9) (Figure 1). EMT is a process by which epithelial cells gain mesenchymal properties and is also involved in the efflux of CTCs from the primary tumor by means of invasion of the tumor microenvironment and evasion of cancer cells into blood stream (intravasation). Apparently, even a partial EMT process is capable of establishing such invasive phenotype giving rise to metastatic cancer cells (10). Also the reversible trait of this process, i.e., MET, implies that CTC features may be phenotypically dynamic, converting from one state to the other and vice versa.

Fortunately, the process of metastasis is very inefficient and only a small subset of CTCs is capable of successful metastasis and thus should bear cancer stem cell features including high invasiveness, therefore these cells are termed circulating cancer stem cells (11,12). To date, very little is known about these cells, therefore it is imperative to increase our research activities aiming for the prospective isolation and characterization of such circulating cancer stem cells. Current CTC enrichment and isolation techniques are designed for CTCs based on one or two common phenotypic characteristics amongst them and therefore may not necessarily be capable of capturing the entire heterogeneity of this dynamic population. Moreover, these common phenotypic characteristics of CTC cells are...
very few and may well vary between different cancer types, adding more complexity to the CTC isolation techniques. Thus, there is an urgent need for comprehensive studies of CTC heterogeneity and development of more robust CTC-detection methods that allow us to distinguish, select and study specific CTC sub-populations.

### Stem cells and cancer

Stem cells are defined by their capacity to undergo unlimited cell division while retaining their stem cell identity (self-renewal) and to give rise to more specialized cells with limited proliferative capacity (differentiation) (Figure 2). Stem cells constitute a population of cells that maintain daily turnover in tissue homeostasis as well as the regenerative response upon tissue injury (13). Beside their key role in tissue homeostasis and regeneration, cells with stem cell features, thus termed cancer stem cells, have also been shown to promote cancer and possibly invasion into distant organ sites (metastasis). Evidence for the

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**Figure 2** The hierarchical organization of cancer and metastasis. Cancer stem cells are capable of undergoing unlimited cell division while retaining their stem cell identity (self-renewal) and giving rise to progenies with limited proliferative capacity (differentiation). Cancer stem cells evolve during tumor progression by acquiring further genetic or epigenetic changes, but may also advance through interactions with the tumor microenvironment. Both cancer stem cells and non-cancer stem cells may be found at the invasive front of primary tumors with similar invasive/migratory features, a process frequently linked to the process of EMT. However, only cells with cancer stem cell features will be able to give rise to metastasis. Such circulating cancer stem cells may arise via two non-exclusive mechanisms: (I) circulating cancer stem cells may already exist in the primary tumor as cancer stem cells with additional features rendering them capable of surviving in the blood stream and subsequently initiating metastatic spread; (II) disseminated tumor cells, after a varying period of dormancy, may convert into circulating cancer stem cells through processes that are yet to be elucidated. Such circulating cancer stem cells derived from disseminated (originally non-metastatic) tumor cells must have acquired additional features in order to not only survive the hostile environment of the blood stream, evade immune surveillance, and extravasate at a distant location, but to also be able to form secondary lesions (tumorigenicity). Of note, circulating cancer stem cells may also originate from metastatic lesions and subsequently recolonize the tumor of origin, a process called “tumor reseeding”.

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existence and functional relevance of cancer stem cells was first convincingly documented in leukemia and multiple myeloma. Based on these early studies, only a relatively small subset of cancer cells was capable of unlimited self-renewal and represented the source for disease relapse. Specifically, in murine myeloma cells derived from ascites and depleted for normal hematopoietic cells, only a small fraction of these cells (0.01%) was able to form clonal colonies in vitro (14). Most leukemia cells were unable to proliferate extensively and only a small subset of cells was consistently clonogenic. Such tumor cells with stem cell-like characteristics were first prospectively isolated and characterized by John Dick and his colleagues in 1994 (15). The investigators studied different classes of leukemia cells and identified human AML stem cells in patient samples as CD34+CD38− cells, which represented only a small but variable proportion of AML cells capable of reproducibly transferring AML from human patients to NOD/SCID mice. These data for the first time conclusively demonstrated that a small and prospectively identifiable subset of leukemia cells is capable to self-renew and transfer disease (3). In 2003, Al-Hajj et al. studied primary breast cancer samples and determined CD44+CD24dim/− cells as functional cancer stem cells. Thus, this study suggested that cancer stem cells exist in solid cancer as well (4). Subsequently, cancer stem cells were identified in other solid cancers, e.g., glioblastoma, colorectal cancer, prostate cancer, as well as pancreatic cancer (6,16–18). It is important to note, however, that cancer stem cells do not necessarily represent bona fide stem cells nor do they necessarily arise from tissue stem cells, but rather cancer stem cells have acquired certain traits of stem cells allowing them to indefinitely self-renew and give rise to their respective differentiated progenies. While cancer stem cells share several signaling pathways that are regularly operative in normal stem cells (10), they are obviously distinct from normal stem cells in terms of their in vivo tumorigenicity defined as the generation of malignant lesions upon transplantation into secondary hosts (19). Still, while it has been shown conclusively that cancer stem cells bear cell-intrinsic stemness features, they are also a product of their relationship with the tumor microenvironment affecting their aggressiveness, metastatic activity and drug resistance (20,21). Thus, in order to advance our understanding of cancer stem cell biology and to develop meaningful cancer stem cell-centered treatment strategies, these cells need to be studied in the context of their niche. Clinically it is of utmost importance that cancer stem cells have been proven to be highly resistant to current standard of care such as chemotherapy and radiotherapy, which makes them a probable cause of tumor recurrences after treatment (22). Consistently, primary tumors with a more prominent stem cell signature are associated with adverse outcome including higher rates of metastasis (23–25).

Cancer stem cell populations bear characteristic cell surface expression profiles, which allows for their prospective isolation from other cells in the tumor. Several of the most commonly used cancer stem cell markers are CD44, CD24, CD133, CD166, and ALDH1. ATP-Binding Cassette Transporters (ABCG2, ABCB5), EPCAM, CXCR4, Nestin and LRCs have also been utilized for the identification of cancer stem cells (26). As these can already be conveyed from this rather large and diverse panel of markers, the development of reliable cancer stem cells biomarker profiles for accurately and prospectively isolating viable cells at high purity represents a daunting task. While numerous cell surface proteins have each been positively evaluated in certain settings, the expression levels of many of these markers can drastically change based on environmental conditions (e.g., tumor digestion, cultivation in different conditions, xenografting), in response to treatment, and their expression is neither exclusively nor reproducibly linked to a functional cancer stem cell phenotype (2). Thus, alternative detection and isolation methods based on functional properties of cancer stem cells would not only avoid the use of such artifact-prone surface markers but should also provide novel insights into cancer stem cell biology. Towards this end, an intrinsic autofluorescent phenotype has been identified in cancer stem cells and was subsequently established as a novel and functionally relevant tool to isolate and characterize these cells down to single cell level (27). This distinct inherent cancer stem cell property represents a novel biological feature that is traceable in real time and provides unprecedented robustness and power for the identification and purification of cancer stem cells without the use of antibodies nor any kind of manipulation, thus drastically reducing experimental errors and artifacts. While surface marker panels are regularly validated for only certain cancer types, this novel marker has already been shown to reproducibility identify cancer stem cells across many tumor types including pancreatic, breast, lung, liver and colorectal cancer (27). Thus, it has now become possible to more accurately capture the dynamic complexity of cancer stem cells.
CTCs and circulating cancer stem cells

Increasing evidence suggests that a presumably small subset of CTCs also bears cancer stem cell characteristics based on their ability to give rise to tumors (28-30) and thus could be considered blood-born functional cancer stem cells or circulating cancer stem cells. These circulating cancer stem cells may represent cancer stem cells (31) with specific features allowing them to survive in the circulation and to give rise to metastatic lesions. Recent data also indicate an interesting link between cancer stem cells and CTCs that appears to show different functional states of the same pathogenically relevant subpopulation of cancer cells (32-36). Circulating cancer stem cells are likely to represent a small subset of CTCs as that only blood samples from patients with rather high numbers of CTCs were capable of giving rise to tumors in secondary recipients (37,38).

Most importantly, the origin of circulating cancer stem cells has not been established to date. Mostly two non-exclusive hypotheses have been put forward (Figure 2). First, circulating and thus metastatic cancer stem cells already arise in the primary tumor as cancer stem cells with additional features rendering them capable of evading the primary tumor, surviving in the blood stream and subsequently initiating metastatic spread (39). Second, circulating cancer stem cells may actually arise post hoc from disseminated tumor cells, e.g., out of a state of dormancy at a distant site after they already evaded from the primary tumor (40). Importantly, such disseminated tumor cells giving rise to later circulating cancer cells need to survive the hostile environment of the blood stream, evade immune surveillance and extravasate at a distant location, features that most certainly are not present in all CTCs that can be tracked in the blood stream. Of course, while both hypotheses are reasonable, none of them has been validated conclusively to date (41).

Consistent with the hypothesis that circulating cancer stem cells are already present in primary tumors, only the stem cell marker positive cells isolated from primary tumors are able to form distant metastases when transplanted into secondary hosts (39,42,43). Moreover, it has been clearly demonstrated that cancer stem cells in the primary tumor display heterogeneous characteristics, which coincided, at least in pancreatic ductal adenocarcinoma with the expression of distinct surface markers (39). As cancer stem cells also bear the functional plasticity for transitioning between mesenchymal-like and epithelial-like states, these cells are indeed most likely the most relevant source for metastasis at distant sites (44).

Compelling evidence exists that cancer cells are endowed with invasive characteristics through EMT, which is a complex process leading to loss of epithelial and gain of mesenchymal traits via cellular de-differentiation and subsequent increased motility via rearrangements of cellular contact junctions and eventually the loss of cell adhesion. During this process, cells partially or fully transition from their epithelial phenotype into a mesenchymal one (45). EMT naturally occurs during organogenesis and wound healing, but also plays a crucial role during tumor cell dissemination (46). This transition enables the tumor cells to acquire migratory and invasive abilities, which facilitates their evasion from the primary tumor site and penetration into the microenvironment and intravasation into the vasculature (47). EMT is induced by several transcription factors, such as SNAIL, TWIST, ZEB1, ZEB2, SLUG, BMI-1, and others (48). By disrupting epithelial adhesion and losing apical-basal polarity, carcinoma cells at the tumor invasive front acquire invasive capabilities allowing them to disseminate via the circulating blood (47). Importantly, EMT is thought to provide neoplastic epithelial cells not only with a mesenchymal and thus invasive phenotype, but may also induce stemness characteristics (33,49). Indeed, it has been shown that cells undergoing EMT acquire stem cell-like properties, which can be tracked in formerly differentiated epithelial cells by up-regulation of CD44 and down-regulation of CD24 as well as increased expression of other stem cell phenotypic markers (49,50). Thus, EMT may propagate or, in some instance, even generate de novo cells with exclusive tumorigenic and metastatic behavior (49). Actually, Mani et al. first demonstrated that EMT was sufficient to induce a population of cells with characteristics of stem cells bearing migratory and invasive capabilities (49). However, EMT is often transient and reversible. Re-establishment of micrometastasis in the distant sites requires a reversal process, termed mesenchymal-to-epithelial transition (MET), by which the cells re-gain their epithelial characteristics necessary for further colonization. Thus, the EMT-MET transition processes are considered as a driving force of metastasis that can occur in most if not any cancer cells (51).

On the other hand, however, it has also proposed that EMT is a dynamic process that occurs both in cancer stem cells and non-cancer stem cells, but actually only a subset, namely cancer stem cells are capable of giving rise to metastatic cancer stem cells via EMT. In this context, it is important to note that, by definition non-cancer stem
cells cannot give rise to tumors in vivo, which suggests that their potential for de novo generation of cancer stem cells via EMT (or other mechanisms) is very limited (27). Still, more studies including in vivo cell fate tracking experiments are needed to conclusively demonstrate whether non-cancer stem cells are indeed capable of replenishing the cancer stem cells pool via EMT and therefore contributing to metastasis. Finally, while the cell autonomous signaling cascade initiating or reversing the EMT process has been studied extensively, very little is known about the exogenous triggers that control the fine balance between the EMT and MET during the metastatic cascade (52).

Moreover, fucosylation has also been implicated in the process of metastasis and is one of the most common glycosylation modifications, involving oligosaccharides on glycoproteins or glycolipids. Fucosylation is also one of the most important types of glycosylation in cancer. Hakomori et al. first reported the role of fucosylation in cancer in 1979 which compared the fucosylation levels of glycoproteins in hepatoma cells and normal hepatocytes (53). Increased fucosylation has been associated with invasive and metastatic properties of cancer cells (54). A recent study by Desiderio et al. investigated the role of fucosylation in cancer stem cells and found that inhibition of fucosylation affected sphere formation and invasion ability of cancer stem cells, respectively (55). Moreover, inhibition of fucosylation was found to affect E-selectin binding and cell extravasation (56), features that are of crucial importance for the metastatic process (57). Thus, fucosylation may be a novel mechanism utilized by cancer stem cells to acquire invasive and metastatic features in order to generate metastatic cancer stem cells and seems amendable for therapeutic intervention.

**Isolation and characterization of CTCs**

The isolation of CTCs from the blood of patients with cancer bears great potential as a minimally invasive approach, but it certainly is technically challenging. Methods for the separation of CTCs vary greatly with respect to the underlying technology ranging from positive immunoselection (e.g., EPCAM-based enrichment), negative immunoselection (e.g., depletion of leukocytes by CD45 antibodies), size-based filtration, and density-based isolation (e.g., via centrifugation) and thus in terms of sensitivity and specificity. Various microfluidic-based devices have been developed over the past years to enrich CTCs in peripheral blood samples (58), but it is difficult to assess, which techniques captures most if not all CTCs as no gold standard for validation currently exists and devices are rarely compared head-to-head.

Cell surface proteins have been used as a target for antibody-based enrichment methods to attach CTCs to columns, chips or magnetic beads for their subsequent detection, isolation and characterization. For example, CellSearch® and IsoFlux™ both utilize magnetic beads targeted towards antigens expressed on the cell surface. Epithelial markers are expressed on carcinomas, but are downregulated/absent on mesenchymal leukocytes and therefore are frequently used to distinguish cancer cells from normal blood cells (59). Epithelial cell adhesion molecule (EPCAM) is the cell surface marker that is most frequently utilized for positive enrichment of CTCs, and members of the family of cytokeratins have become the “gold standard” for the validation of CTCs with an epithelial phenotype in patients with carcinoma (60,61). However, carcinoma cells can undergo EMT, which may result in reduced expression of epithelial markers, and thus EPCAM-based techniques may not efficiently capture CTCs with mesenchymal characteristic following EMT (62). The addition of markers for mesenchymal CTCs that are up-regulated during EMT, such as vimentin and N-cadherin, may be needed, but bear the caveat of potentially increasing the rate of false-positive findings. Tumor or tissue-specific markers for certain tumor types can also be utilized for the isolation of CTCs. Prostate-specific antigen (PSA), mammaglobin, HER2 and epidermal growth factor receptor (EGFR) provide high specificity (63-65). However, these markers may not cover the whole spectrum of CTCs due to their heterogeneity including undifferentiated cancer stem cells.

Alternatively, CTCs can be enriched by depleting blood from leukocytes using antibodies against CD45 or lineage cocktails, which are not expressed on cancer cells. This negative immunoselection method could avoid false-negative results or loss of CTCs due to phenotypic heterogeneity, but the isolated CTC are regularly still contaminated with large numbers of remaining blood cells resulting in rather low purity. Furthermore, CTCs can be isolated using immunodensity negative selection cocktail such as RosetteSep™, which is a technique combining an antibody-mediated enrichment step with density gradient centrifugation. Importantly, this technique has been used for the isolation and generation of the first CTC-derived xenografts (CDX, see below) (38).

CTCs can also be positively or negatively enriched on the basis of physical properties such as size, density, deformability or electrical charges. Lymphocytes are
around 8 µm in size, and have a very compact nucleus and minimal cytoplasm. CTCs are generally much larger, although this may depend of their level of differentiation. Thus, the vast majority of lymphocytes and neutrophils in blood sample can be removed by using filters or microchips with pores (66,67). Unfortunately, owing to the variable size and deformability of CTCs, this method is limited by capturing large and thus more differentiated cancer cells, whereas undifferentiated and invasive cells, respectively, may be captured with less efficiency resulting in low sensitivity and specificity. CTCs have also been enriched by centrifugation on a density gradient owing to the relatively distinct density of CTCs (68). Furthermore, CTC-iChip (69), a novel chip-based platform, separates nucleated cells from whole blood by using size-based separation, then aligns cells in a microfluidic channel using inertial focusing, and subsequently isolates CTCs by means of negative selection (leukocytes depletion) using microfluidic magnetophoresis. Thus, this innovative platform combines size-based filtration with an immunomagnetic depletion and therefore should significantly reduce contamination of the isolated CTCs with undesired hematopoietic cells as well as include CTCs that have undergone EMT and thus lost epithelial traits.

In summary, all listed techniques bear certain advantages and disadvantages and the selection of the most suitable method may also depend on the aim of the study (e.g., preferences for viability and purity) and the type of cancer studied. The use of epithelial markers for antibody-based technologies certainly misses out on CTCs that have undergone EMT. Moreover, circulating cancer stem cells also regularly express lower levels of epithelial markers (52). Thus, combinations of markers may overcome these limitations, but they need to be tested and validated in prospective studies including functional validation for the presence of circulating cancer stem cells. Currently, the limited availability of specific markers for CTCs combined with the inherent technical limitations of most, if not all CTC isolation platforms represent major challenges for further developing broadly applicable CTC isolation techniques.

**Isolation and characterization of circulating cancer stem cells**

While capturing rare CTCs from circulating blood is rapidly evolving, the prospective and reproducible identification and characterization of viable circulating cancer stem cells within the population of CTCs has remained technically challenging due to their low numbers, poorly defined identify and harsh isolation methods. Recently, Hodgkinson et al. showed that at least a subset of CTCs isolated patients with small cell lung cancer is capable of forming tumors in immunodeficient mice with preserved morphological and genetic characteristics (38). These results, while not prospectively identifying circulating cancer stem cells, are supporting the existence and presence of such cells with tumor-initiating capabilities within the blood. These findings clearly demonstrate that clinically relevant patient-derived circulating cancer stem cell models, also known as “liquid biopsies”, can be generated, although it is also important to note that the authors succeeded only with samples that contained very high numbers of CTCs suggesting that circulating cancer stem cells are indeed a very rare population. Importantly, those CDX also recapitulated drug responses recorded for the donor patients. Thus, such CDX models may now enable us to examine mechanisms of acquired drug resistance as blood samples can be collected before and after development of drug resistance.

CTCs are believed to represent indicators of residual disease and thus pose an increased risk for disease relapse. However, as the subsets of circulating cancer stem cells is the main driver of tumor progression and metastatic spread, it may be even more important to track and eliminate such rare circulating cancer stem cells (70). Putative biomarkers for identifying circulating cancer stem cells have been proposed in recent studies (Table 1). For example, CTCs with stem cell-like characteristics have been found in primary and metastatic breast cancer. Aktas et al. found that detection of stem cell-like CTCs in peripheral blood of breast cancer patients was associated with therapy resistance (28). Most disseminated tumor cells in the bone marrow of breast cancer patients presented a CD44+/CD24−/low phenotype, which have been shown to be linked to a more aggressive phenotype including high metastatic activity (73-75). ALDH1 has also been shown to identify breast cancer stem cells in vivo and in vitro. Kasimir-Bauer et al. showed that 46% of CTC-positive primary breast cancer patients were also positive for ALDH1 (30). ANTXR1, a stem cell-enriching functional biomarker, has been associated with enhanced self-renewal capacity and metastatic ability of breast cancer stem cells (76). Finally, Krohn et al. showed that CXCR4 expression is also essential for invasiveness of breast cancer stem cells (77).

In pancreatic cancer, c-Met is considered a marker for (metastatic) cancer stem cells and is required for metastasis (71). Moreover, CD133+CXCR4+ cancer stem cells are mostly...
found in the invasive front of pancreatic cancers and have been shown to be essential for metastasis (39). Consistently, CD133+CXCR4+ cancer cells in colorectal cancer patients also have a higher metastatic capacity as compared to CD133+CXCR4– cancer cells (78). Todaro et al. reported that all colorectal cancer stem cells express CD44v6, which was required for their migratory activity and generation of metastatic tumors (79). Moreover, CD26+ colorectal cancer stem cells have been identified in colorectal cancer patients with liver metastasis, and they generated distant metastasis upon orthotopic transplantation into mice (43). CD44+ circulating cancer stem cells could be detected more frequently in gastric cancer patients with metastasis and served as a prognostic factor (80). CD133+ osteosarcoma cancer stem cells showed high tumorigenicity in vivo (84,85) and CD117+Stro-1+ osteosarcoma cancer stem cells have strong invasive and drug-resistant properties (85,89). In glioblastoma, both CD133+ and MMP-13+ cells showed stem cell properties (6,81). Furthermore, high expression of ABCG2 has been demonstrated in cancer stem cells of lung and pancreas cancer as well as retinoblastoma (72,82,86).

Thus, as already observed for cancer stem cells residing in primary tumors, a large panel of biomarkers has been used by now in various cancers to track blood-born or circulating cancer stem cells, but more stringent and large-scale studies are still needed to define the most suitable setup and marker panel for the prospective isolation of true and viable circulating cancer stem cells.

**Conclusions**

CTCs are rare events among millions of blood cells and they are a heterogeneous population of cells, including circulating cancer stem cells, bearing different phenotypic and functional characteristics. Hence the identification and characterization of CTCs requires highly sensitive and specific technologies, which, despite major advances over the past years, still has not been achieved to complete satisfaction due to their heterogeneity and dynamics (32,90). Circulating cancer stem cells are an even smaller subpopulation of these CTCs indicating the gravity of today’s technical challenge for isolating and studying these cells. However, recent data already documented the potential for in-depth assessment of viable metastatic tumor cells from CTC populations by capturing single CTCs for next-generation sequencing analyses (91) as well as functionally their in vivo xenotransplantation into immunodeficient mice (37).

Developing new methods for efficient and reproducible isolation and subsequent comprehensive characterization of circulating cancers stem cells should provide the basis for eventually improving patient survival by specifically targeting these cells. Enumeration of CTCs bears prognostic value and is now commonly used in clinical settings for monitoring disease. An increasing number of studies is currently evaluating whether therapies directed by CTC numbers can improve the outcome of treatment and, whether reduced numbers or even eradication of CTCs in response to therapy is actually associated with improved long-term survival (92). Still, these gross CTC numbers may not provide the expected insights into tumor biology and treatment response. Similar to the regression of the bulk tumor that does not necessarily reflect successful targeting of the contained small subpopulation of cancer stem cells, a decrease in CTCs could be misinterpreted as evidence for treatment response while rare circulating cancer stem cells...
cancer stem cells have remained unaffected. Therefore, detection and characterization of circulating cancer stem cells appears to be even more important for selecting and directing therapeutic strategies (93).

Thus, to further advance the CTC research field, we must acknowledge and address the issue of CTC heterogeneity similar to that found in primary tumors, but defining specific markers for such CTC subpopulation remains a challenging issue. Numerous studies have focused on epithelial markers for selection e.g., EPCAM and cytokeratins. Consequently, a varying fraction of CTCs undergoing EMT or bearing stemness features might have been overlooked. In addition, it is just now that we come to realize that most (circulating) cancer cells actually lack the ability to form new tumors and only rare circulating cancer stem cells will lead to metastatic disease. In light of these findings, the main goal of CTC research should shift towards the identification, characterization and subsequent elimination of circulating cancer stem cells, which may be challenging based on the enhanced drug resistance that has already been reported for cancer stem cells in primary tumors. Still, their detection and characterization should serve as a real-time “liquid biopsy” to continually improve prognosis and facilitate patient tailored therapy (Figure 3).

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**Footnote**

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