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Cancer stem cells as new therapeutic target to prevent tumour progression and metastasis

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1. ABSTRACT

Over the past decade, increasing evidence suggested that stem cells play a crucial role not only in the generation of complex multicellular organisms, but also in the development and progression of malignant diseases. For the most abundant tumours, it has been shown that they contain a subset of distinct cancer cells that is exclusively responsible for tumour initiation and propagation These cells are termed cancer stem cells or tumour-initiating cells and they are also highly resistant to chemotherapeutic agents. Because CSC are preferentially endowed with the self-renewal capacity, it has further been hypothesized that they are also exclusively responsible for metastasis. Indeed, we were able to show that pancreatic caner stem cells contain a subpopulation of migrating cancer stem cells characterized by CXCR4 co-expression. Only these cells are capable of evading the primary tumour and metastasizing. Laboratories around the world are now aiming to further characterize these cells to eventually identify novel treatment modalities to fight cancer. Thus, cancer stem cells are promising new targets to counteract the growth-promoting and metastatic potential of solid tumours.

2. INTRODUCTION

Carcinomas represent the most prevalent malignancies in humans and currently one in four deaths is related to cancer (1). Over the past few years, increasing evidence suggests that stem cell-like cells may play a crucial role in the development and perpetuation of various human cancers. Only a subset of the tumour cells, varying in size, bear stem cell properties and are highly capable of initiating tumour growth in immunodeficient mice. Hence, the stochastic cancer model, assuming that every cell within a tumour bears equal tumour-initiation potential, while only the entry into cell cycle is governed by a low probability of stochastic events, has taken a back seat. In contrast, according to the current cancer stem cell (CSC) definition, these cells are able of self-renew, bear exclusive tumourigenicity, and produce the heterogeneous lineages of cancer cells that comprise the tumour. Based on this concept, different terms including “cancer stem cell”, “tumourigenic cell”, “tumour-initiating cell”, and “tumour-promoting cell” have been used for the description of the CSC phenotype in the literature (2-6). CSC were first identified in the hematopoietic system (7) and have subsequently also been described for solid tumours of

various origin, including breast (2), colon (4, 5), brain (6), head and neck (8), and pancreas (9). The cell of origin for CSC still remains to be determined for most tumours though. Either they arise from adult tissue-resident stem cells or from derivative progenitors that acquired stem cell properties through accumulation of key deletions, mutations, or amplifications. As adult stem cells are by far the most long-lived cells of the body, they are more likely than any other cell to be capable of acquiring the multiple mutations needed to transform into a CSC. Clinically most important, however, CSC are highly resistant to standard chemo- and radiotherapy and the increasing appreciation of this feature will certainly reshape our approaches for developing novel, more effective therapies for cancer.

3. RESISTANCE OF CANCER STEM CELLS TO DAMAGING AGENTS

Why tumours relapse despite initial clinical evidence for treatment response remains one of the unanswered key questions cancer cell biology, but could be well rationalized by the cancer stem cell concept. Currently, the assessment of tumour burden is the most widely used parameter to evaluate the efficiency of cancer therapy. However, tumours often shrink in response to standard treatment only to relapse again. Apparently, conventional radiation treatment or chemotherapy predominantly affects the bulk of a tumour, the more differentiated, rapidly proliferating cells while sparing at least the quiescent if not all cancer stem cells. Consequently, if therapeutic drugs fail to eliminate the cancer stem cells as the exclusively tumourigenic population, these cells can later repopulate the tumour including its more differentiated progeny.

Several studies (10-12), including our own (13) demonstrated that conventional therapy has limited or no significant effect on CSC numbers, and even leads to their relative enrichment due to elimination of their more differentiated progenies. These data suggest that successful targeting of this small subpopulation of cells could significantly improve cancer treatment. In fresh as well as *in vivo* expanded patient-derived pancreatic cancer cells, treatment with the first-line chemotherapeutic agent gemcitabine had virtually no effect on the $CD133^+$ subpopulation, in which the tumourigenic CSC fraction is contained (13, 14). Instead, gemcitabine treatment resulted in a marked relative enrichment in $CD133^+$ cells indicating its preferential effect on the more differentiated tumour cells. Although gemcitabine treatment of mice bearing orthotopic human tumour xenografts resulted in extended survival, this treatment effect is only related to local control of tumour growth but does not lead to the elimination of the root of the tumour, namely the cancer stem cell population. Consequently, withdrawal of gemcitabine will only result in rapid relapse of tumour growth and may even induce a more aggressive growth pattern.

Similar observations have now also been made for CSC in other tumours (10, 15). In glioblastoma, radiation therapy eliminated most of the bulk tumour cells but subsequently resulted in an increase of the tumour's aggressiveness following serial transplantation (10). The authors showed that $CD133^+$ cells, previously identified as the exclusively tumourigenic population in primary glioblastoma multiforme specimens (6), were enriched two- to four-fold following ionizing radiation both in primary tumours and xenografts. Similarly, breast cancer cells with a $CD24^{-/low}/CD44^+$ stem cell phenotype (2), were also found to be unaffected by radiotherapy compared to the remainder of breast cancer cells (16).

The intrinsic mechanisms of drug resistance in CSC are not very well understood, but putative mechanisms include enhanced anti-apoptotic capabilities, strong DNA repair mechanisms and/or overexpression of transporter proteins that pump out administered drugs from these cells. Normal stem cells are also more resistant to DNA damaging agents than differentiated cells because of their ability to undergo asynchronous DNA synthesis and because of their enhanced capacity for DNA repair. These properties protect the stem cell population from most injuries and ensure functionality during their long lifespan. Similar to these normal stem cells, cancer stem cells have extensive self-renewal capacity and bear a number of properties protecting them from damaging agents. Consequently, at least the quiescent fraction of the stem cell compartment seems to survive traditional cancer chemo- and radiation therapy (17). Intriguingly, the ability of the CSC to resist radiation therapy in glioblastoma and breast cancer could be counteracted by a preferential activation of DNA damage response mechanisms, which apparently are mostly restricted to CSC (10, 16).

4. THE SEARCH FOR SIMILARITIES AND DIFFERENCES BETWEEN STEM CELLS AND CANCER STEM CELLS

4.1. Signalling pathways determining stemness

If indeed standard therapy fails to target the CSC population, the malignancy cannot be depleted for the suspected root of the disease, thus providing a rationale for the apparent treatment failure and subsequent tumour relapse. There is increasing evidence that the same pathways known to determine stemness in normal stem cells and are important during development may be involved in the initiation of uncontrolled self-renewal of CSC in many malignancies. Several developmental signalling pathways have been implicated in solid tumours, including the Notch, Wnt, sonic hedgehog (Shh), and PI3K/Akt/mTOR/STAT3 pathways. While different experimental studies have evaluated the significance of some of these pathways with respect to cancer, the Shh pathway has attracted the most interest yet and is already being targeted in first clinical trials enrolling patients with metastatic basal cell carcinoma.

Aberrant activation of Shh signalling could be associated with the development of different solid cancers, including pancreatic cancer (18), small-cell lung cancer (19), medulloblastoma (20) and basal cell carcinoma (21, 22). Moreover, in glioblastoma multiforme (GBM) pharmacological inhibition of hedgehog signalling with

cyclopamine resulted in a significant reduction of cells with typical stem cell features (ALDH activity, Hoechst dye exclusion) and even loss of tumourigenicity in GBM cells (15). Also in pancreatic cancer, Shh inhibition has been identified as a promising approach for targeting CSC and therewith counteracting the metastatic potential of this deadly tumour (14, 23).

In order to form metastases, cells should demonstrate similar features as observed for cells initiating the primary tumour. Indeed, recent studies provide supporting evidence that CSC represent the exclusive cell population responsible for metastatic spread (13). Feldmann and colleagues were able to demonstrate that inhibition of Shh signalling in combination with standard chemotherapy in xenografted pancreatic cancer cell lines significantly reduces cancer invasion and subsequently its metastatic spread (24).

This observation is further corroborated by findings by Li et al., who found that the SHH pathway was particularly active in the subpopulation of primary pancreatic cancer stem cells (9). The authors observed that the Shh transcript was almost 50-fold overexpressed in $CD44^+CD24^+ESA^+$ pancreatic cancer cells as compared to normal pancreatic epithelial cells. Only recently, the importance of hedgehog signalling in CSC could be further strengthened for CSC of chronic myelogenous leukaemia (CML) (25). Genetic loss- and gain-of-function experiments indicated that Shh activity regulates the maintenance and frequency of CML stem cells. Taken together, these data strongly suggest that hedgehog signalling plays a pivotal role in CSC biology and, based on emerging data from several laboratories, this seems to be particularly true for their migratory subpopulation. In addition, most recent studies suggest that Shh signalling is also of pivotal importance for the stromal fraction of human colon and pancreatic cancer cell lines (26) and in a murine model of pancreatic cancer (23).

Wnt proteins are intercellular signalling molecules that regulate developmental processes in several organisms and contribute to cancer when dysregulated. In intestinal stem cells, the canonical Wnt pathway regulates self-renewal and maintains the stem cell niche in conjunction with bone morphogenetic protein (BMP) and Notch signalling (27). Activating mutations in the Wnt cascade invariably lead to colorectal cancer. During the progression from adenoma to carcinoma, there is an increase in nuclear ß-catenin, which indicates active Wnt signalling. Consistently, another study showed that elimination of ß-catenin from either chemical- or Ras-induced skin tumours in mice resulted in the loss of $CD34^+$ cancer stem cells and thus in complete regression of tumours (28). This study provided evidence for the existence of CSC in a syngeneic mouse model and pointed out the importance of the Wnt pathway for CSC maintenance.

In another study performed on human glioblastoma, bone morphogenetic protein 4 (BMP4) exposure deleted the $CD133^+$ CSC in vitro, leading to a more differentiated phenotype (29) and reducing their tumour-forming ability. Pre-treatment of the cells with BMP and subsequent transplantation of $CD133^+$ tumour cells showed reduced tumour growth and increased survival of xenografted animals. Similar results were presented at the 2009 ASCO Symposium for GI Cancer from Georgio Strassi for the treatment of colon cancer initiating cells with BMP4, showing an additional chemosensitising effect for the cytotoxic agent oxaliplatin. As an important role for BMP4 is also demonstrated in the regulation of pancreatic progenitor cell expansion (30), BMP4 might also represent an interesting new target for the elimination of pancreatic cancer stem cells.

One of the major challenges in the cancer stem cell field is, to unravel the mechanism how the CSC subpopulations functionally differ from normal stem cells. The fact that many pathways known to promote tumourigenesis are intimately implicated in normal stem cell self-renewal suggests that therapeutic agents targeting such pathways may also affect resident stem cells. Ideally, a therapy should target pathways unique for CSC. Yilmaz et al. were the first to give new insights on this important issue as they showed that the tumour suppressor protein Pten distinguishes normal haematopoietic stem cells from leukaemia-initiating cells (31). The deletion of the signalling molecule Pten, which is localized upstream of mTOR, resulted in generation of leukemic stem cells, but also depletion of normal haematopoietic stem cells. These effects were mostly mediated through mTOR and inhibition of this pathway by rapamycin, the naturally occurring inhibitor of mTOR, not only led to a depletion of leukaemia-initiating cells, but also resulted in a recovery of normal haematopoietic stem cell function.

Thus, the impact of Pten loss on the self-renewal capacity of haematopoietic stem cells is independent of the role of Pten in leukaemogenesis. Notably, mTOR signalling was also confirmed to be critical for breast cancer stem cell survival and proliferation by pathway specific inhibitors, selected gene knockdown, and *in vivo* tumourigenicity assay (32). The PTEN/mTOR/STAT3 pathway seems to play a decisive role in cancer stem cell survival whereby drugs like rapamycin and its analogs may help in targeting cancer stem cells. The work by Yilmaz et al. strongly suggests that it is possible to identify and therapeutically target pathways that affect only the self-renewal of cancer stem cells through mechanisms that are distinct from those of normal stem cells within the same tissue (31). These results are supported by the finding that parthenolide can also induce death of human leukaemia stem cells *in vitro* while sparing normal hematopoietic stem cells (33). In summary, it may eventually become possible to develop new potential anti-cancer therapies that have minimal effects on the normal stem cell population.

5. MIGRATING CANCER STEM CELLS AND EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

The predominant cause of cancer-related lethality is metastasis, the most advanced stage of any

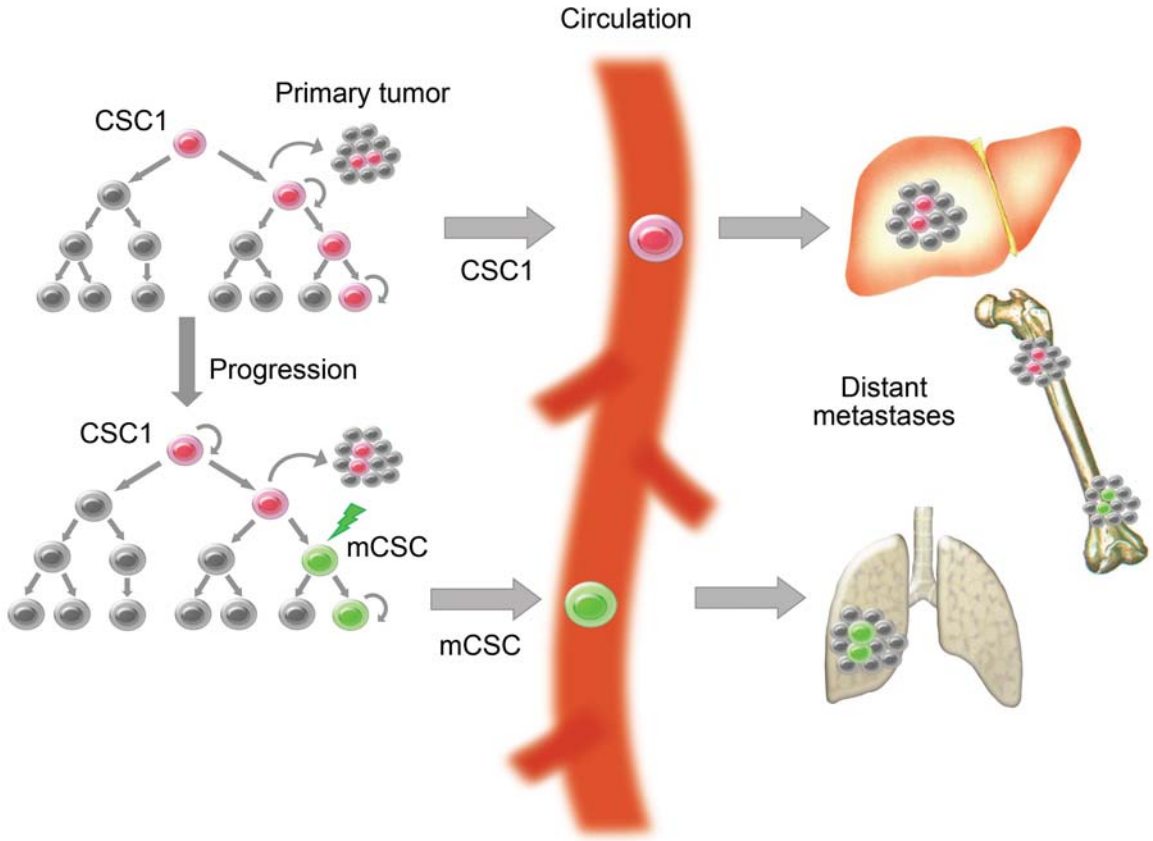

Figure 1. Cancer stem cells and metastasis. Metastasis might be carried out by the original tumour-initiating cells (CSC1) or, more likely, additional genetic and epigenetic mechanisms result in the formation of a self-renewing metastatic CSC (mCSC). This advanced cancer stem cell population expresses different cell surface markers from the CSC1 (e.g. CXCR4) giving them a more invasive phenotype. These mCSC enter the blood and/or lymphatic vessels and seed a secondary tumour in a distinct organ.

malignancy. Despite extensive research activities to improve our understanding in tumour biology and some promising advances in therapeutic options including targeting of HER-2 in breast cancer (34), targeting of vascular endothelial growth factor in colorectal (35) and non-small-cell lung cancer (36), there has hardly been any substantial progress in a metastatic setting. Therefore, an essential need for a better understanding of the complex developmental processes concerning metastasis has emerged in order to eventually be able to develop novel protocols for more successful cancer treatment. Metastasis is a succession of individual processes including surmounting physical boundaries, intravasation, dissemination via the blood or lymphatic system, extravasation into a secondary site and the recapitulation of the hierarchical organization of the primary tumours. Several studies have pointed out that the metastatic process is quite inefficient, such that very few cells that leave the tumour of origin are actually successfully forming macroscopic metastases at secondary sites (37, 38).

The current notion that only very few cells are actually "successful" metastatic cells because they are equipped with the necessary armament to promote and survive the evasion process is indeed supported by clinical observations: Despite the fact that in some cancer patients hundreds of disseminated cancer cells are detectable in the bloodstream (39), only a very small subset seems to eventually induce overt macro-metastases. Taken into account that cells require similar features as the cells initiating the primary tumour in order to be able to establish secondary lesions, cancer stem cells were proposed to represent the only cells capable of spreading and giving rise to metastases. While several studies now have provided compelling evidence for the existence of CSC not only in leukaemia (7), but also in solid tumours (2-6), the role of CSC in the metastatic process still remains unclear.

Recently, we have identified a distinct population of migrating and highly metastatic cancer stem cells in the pancreas. For the first time, were able to distinguish two phenotypes of CSC: stationary, tumour growth promoting CSC versus their migratory, highly metastatic counterparts (Figure 1). These so-called migratory CSC are characterized by co-expression of the CXCR4 receptor and responsiveness to chemotactic gradients of its specific ligand SDF-1 (13). These data suggest a strong ability for chemokines to modulate stem cell behaviour including stem cell-niche interactions.

Chemokines are defined by their capability to induce directed migration of cells towards a gradient of the chemokine. They exert significant influence on several physiologic and pathologic processes through interaction with their specific receptors. Coordination of cell trafficking and homing, mediated through homeostatic chemokines like SDF-1 is essential during developmental processes and for normal function of the immune system (40, 41). The chemokine receptor CXCR4 and its specific ligand Stromal Derived Factor 1 (SDF-1, now designated CXCL12 (42)), have originally been found to be responsible for leukocyte homing, but they also play a pivotal role in other cell types including hematopoietic progenitor cells (43).

The precise regulation of migration and homing is not only critical during embryonic development and tissue regeneration, but is also of great importance during the metastatic spread of cancers (44). Indeed, an involvement of the SDF-1/CXCR4 axis in the metastatic process had already been demonstrated for a variety of cancers (45-47). CXCR4 was first identified in lymphocytes and dendritic cells, and is required for their migration to lymph nodes (48). More recently, associations between the expression of the chemokine receptor CXCR4 in tumour cells and lymph node metastasis have been shown for different cancers, including colorectal (49), gastric (50), oesophageal (51), hepatocellular (52), and thyroid cancer (53), malignant melanoma (54), breast cancer (55), and cervical cancer (56, 57). The expression of CXCR4 allows cancer cells to respond to SDF-1 gradients and seed secondary tumours at remote sites. Such distant organs commonly involve lung, liver, bone marrow or lymph nodes, areas of constitutively high SDF-1 expression (58).

In order to form metastases, cells should require similar key features as the cells initiating the primary tumour. Therefore, it is considered that CSC are the only subpopulation within a tumour that is exclusively capable of metastatic dissemination (59). Indeed, we were able to shed light on this intriguing research subject, using human pancreatic cancer as a model system (13). We identified a subpopulation of $CD133^+$ cells from fresh human primary tumour tissue that is exclusively tumourigenic in immunocompromised athymic mice. We could then conclusively demonstrate that this CSC population can be divided into two subsets of cells based on the expression of the CXCR4 receptor ($CD133^+CXCR4^-$ and $CD133^+CXCR4^+$). Intriguingly, both populations were capable of inducing an orthotopic primary tumour. However, only the $CD133^+/CXCR4^+$ cell population induced metastatic spread of the primary tumour, suggesting the crucial role of the SDF-1/CXCR4 axis in metastasis. Depletion of the primary cells for $CD133^+/CXCR4^+$ cells virtually completely abrogated the metastatic capacity of these tumours.

Consequently, pharmacological inhibition of the CXCR4 receptor by AMD3100 also prevented the metastatic activity of purified cancer stem cells (13) as well as of unselected murine pancreatic cancer cells (60). These data suggest that the metastatic process is not random, but guided by the expression of chemokine receptors and adhesion molecules expressed on specific subsets of tumour cells, and their respective ligands in the target organs providing the permissive environment for metastatic spread. Most previous studies have focused on metastatic spread through the blood stream. However, most cancers initially spread to local lymph nodes long before solid organ colonization becomes clinically apparent. Thus, the lymphatic system and lymph node metastases also need to be examined concerning the presence and contribution of migrating cancer stem cells.

Indeed, we found a close correlation between CXCR4 expression on cancer stem cells in the resected tumour and lymph node metastasis in pancreatic cancer (13). Patients with clear histological evidence for lymph node metastasis showed significantly higher numbers of $CD133^+CXCR4^+$ migrating cancer stem cells in the resected tumours. Similarly, a study by Nakata et al. also suggested that CCR7 (also know as BLR2 or CD197) expression is correlated with lymph node metastasis in pancreatic cancer and serves as an independent prognostic factor (hazard ratio of 2.0) by multivariate survival analysis (61). Apart from these observational studies and a role of this receptor in this process is conceivable, the functional significance of chemokine receptors on CSC for lymphatic metastasis still needs to be determined. Consequently, there may well be cancer stem cells that predominantly disseminate via the blood flow as well as cancer stem cells that prefer lymphatic dissemination. It still remains to be elucidated if these subpopulations are identical or if they acquire additional and/or different genetic and epigenetic alterations or environmental stimuli resulting in a change in their surface marker expression profile during metastatic progression.

Of note, other chemokine receptors may also prove to identify and functionally characterize putative metastatic CSC. In our experiments, SDF-1 as the specific ligand for the CXCR4 receptor was the most potent inducer of migration for $CD133^+$ CSC whereas c-Met – a receptor tyrosine kinase binding Hepatocyte Growth Factor (HGF) – did not seem to play a functionally relevant role in pancreatic cancer stem cells (13). However, the chemokine receptor c-Met and its ligand HGF have spurred scientific interest because they have been implicated in the epithelial-mesenchymal transition (EMT) process, which is frequently observed at the invasive edge of solid tumours. EMT and the reverse process, the mesenchymal-epithelial-transition (MET) seem to play a key role during various stages of embryogenesis as well as during numerous pathologic conditions, such as tissue fibrosis and presumably also cancer progression. EMT is characterized by loss of cell-cell contact, reduced E-cadherin expression, and increased cell mobility, traits that are needed for metastatic initiation.

Reasoning on how cancer stem cells may participate in metastasis, one possibility is that the original cancer stem cell reactivated the embryonic EMT program through additional genetic and epigenetic alterations. Thereby EMT-associated transcription factors can confer

malignant traits, such as motility, invasiveness, and resistance to apoptosis, on cancer stem cells changing them into more invasive and thus metastatic cancer stem cells. Indeed, multiple studies revealed that several signal-transduction pathways that have been identified for EMT, including the activation of several receptor tyrosine kinases and Transforming Growth Factor-β receptors play key roles in the initiation and regulation of metastasis (see (62) for review)

HGF is a multifunctional growth factor that is involved in the proliferation, migration, differentiation and survival of cells (63) and activates c-Met. C-Met has been associated with the development and progression of a number of cancers including colorectal, renal, and breast tumours. Animal studies confirmed the oncogenic potential of HGF signalling (64). In a murine model of breast cancer, treatment of tumour-bearing mice with a small molecule inhibitor for c-Met significantly inhibited primary tumour growth and metastatic dissemination (65). Similarly, small interfering RNA for reducing c-Met expression in mammary tumour cells reduced their metastatic spread, suggesting a potential role of c-Met on the motility of neoplastic cells.

Thus, there is increasing evidence that EMT gives rise to the dissemination of single tumour cells from the sites of the primary tumours. However, during the process of tumour metastasis disseminated cancer cells need to be equipped with self-renewal capabilities, similar to that exhibited by stem cells, in order to be able to eventually generate macroscopic metastases. That raises two mechanistic scenarios. On the one hand, the EMT process may impart self-renewal capability to epithelial cancer cells that originally did not have a stem cell phenotype. Indeed, a recent study showed that EMT endows epithelial cells with cancer stem cell properties and that EMT markers are expressed by breast cancer stem cells (66). Inducing EMT in non-tumourigenic mammary epithelial cells led to the expression of proposed cancer stem cell antigenic markers $CD44^{high}CD24^{low}$ and acquisition of self-renewal and differentiation capacities. The authors demonstrated that the number of tumour-initiating cells could be increased by at least two orders of magnitude if transformed cells were forced to constitutively express either a Twist or Snail EMT-inducing transcription factor. On the other hand, the EMT process may change the phenotype of cancer stem cells pre-existing in the tumour and that already bear tumour-promoting capabilities, to an invasive phenotype thereby generating migrating cancer stem cells.

Recently, Georgio Strassi further strengthened the link between EMT and cancer stem cells by investigating this subject in another tumour entity, namely colon cancer. He found that colon CSC contain a subset of cells which co-express c-Met and are exclusively able to spread from the primary tumour and form metastatic lesions (presented at the ASCO GI Cancer Symposium 2009, San Francisco, California). Specifically, his group was able to show that the $CD133^+$ CSC population can be divided into two subsets based on the expression of the c-Met receptor ($CD133^+$ c-Met^+ and $CD133^+$ c-Met^-) and that both populations are able to induce orthotopic primary tumour formation and promote tumour growth. Intriguingly, only the $CD133^+$ c-Met^+ population was able to form tumours that bear the capacity to induce metastatic lesions at secondary sites. Consistently, the liver, which is mostly affected by metastases of colon cancer, exhibits a high content of HGF. These data suggest that overexpression and/or activation of c-Met are implicated in the progression and metastasis of human colorectal carcinoma and that only a subset of cells with advanced stem cell characteristics play a pivotal role in metastasis.

6. THE TUMOUR MICROENVIRONMENT

It is well established, that cancer cells from different primary tumours have their own "favourite" metastatic sites. In 1989, Stephen Paget proposed the "seed and soil" theory of metastasis (67). Thus, metastasis depends on interactions between selected metastatic cells (the "seeds") and specific organ microenvironments (the "soil"). Beside the relevance of the blood and lymph flow, micro-environmental factors are most likely to have significant influence on the survival of CSC through suppression of immune mechanisms, promotion of angiogenesis, and alteration of growth-related pathways. Directly targeting pathways notably upregulated in CSC as described above should be the most obvious and, at least in theory, ideal approach to eliminate CSC while avoiding potential side effects as well.

However, destroying that supportive microenvironment of the CSC may be a supplemental or even synergistic modality to completely eliminate these cells – either by directly killing them in the process or by driving them into differentiation. Normal stem cells require a specific microenvironment in order to grow and survive, the stem cell niche. Metastatic cells also seem to need such a defined and interactive space as demonstrated by Kaplan et al. (68). The authors showed that VEGFR1-positive haematopoietic bone marrow progenitors are directed to the future sites of metastasis prior to cancer cell arrival initiating a so-called pre-metastatic niche. In addition, treatment with a $VEGFR1^+$ neutralizing antibody largely eliminated cancer metastasis indicating the importance of the pre-metastatic cell niche. Moreover, for brain cancer it was recently shown that the tumour microvasculature forms a niche that is critical for the maintenance of CSC (69). Calabrese and co-workers provided compelling evidence that endothelial cells supply secreted factors *in vitro* that maintain brain CSC in a self-renewing and undifferentiated state. Furthermore, increasing the number of endothelial cells or blood vessels in xenografts resulted in a subsequent expansion of the CSC population. Consistently, anti-angiogenic therapy with bevacizumab, a recombinant humanised monoclonal antibody to vascular endothelial growth factor (VEGF) resulted in an ablation of self-renewing CSC.

Blocking VEGF has not only effects regarding tumour angiogenesis but also lymphangiogenesis. Whereas tumour angiogenesis has been extensively characterized

based on clinical and biological significance, the importance of establishing lymph vessel supply in the context of solid organ metastasis remains relatively unexplored. Nevertheless, metastasis in sentinel lymph nodes indicates the initial spread of tumours from a primary site. A study by Hirakawa et al. showed that primary tumours induce new lymphatic vessel growth in draining lymph nodes before metastasis. VEGF-A induced tumour and sentinel lymph node lymphangiogenesis and enhanced lymphatic metastasis in the carcinogenesis model (62). Furthermore, overexpression of VEGF-C, a potent lymphangiogenesis stimulator has been correlated not only with accelerated lymph node metastasis but also with lung metastasis, thus metastasis to distant sites (62). Regarding the structural similarity between the vascular niche in the bone marrow and the lymphatic niche in the lymph nodes, these data suggest that the lymphatic niche contributes to the migration, residence and/or survival of metastatic cancer stem cells.

Therefore, a novel and previously unrecognized mechanism of anti-angiogenic therapy may indeed represent the targeting of the vascular microenvironment and the associated CSC. As anti-angiogenic drugs like bevacizumab have already been tested in clinical trials (35), they may present one of the few CSC-targeting therapies that are already transferrable to our patients with solid cancers. Of note, it remains an open question whether only CSC are responsible for orchestrating the formation of the pre-metastasis niche but a further and thorough characterization will be absolutely mandatory in order to achieve a better understanding of the signals that actually determine the self-renewal capacity of cancer stem cells. In this regard, the search for factors that support and maintain the respective niche of normal stem cells may indeed provide important clues. The dependence upon the microenvironment to maintain a quiescent and undifferentiated state is a well-known feature of normal stem cells (70). Also considering the significance of the tumour niche in the metastatic process, it could be well rationalized that the microenvironment of CSC may represent a promising new therapeutic perspective to prevent metastasis.

7. IMPLICATIONS FOR ANTI-METASTATIC THERAPY

When evaluating the promises and caveats of novel targeted anti-stem cell treatment regimens to prevent systematic spread of the disease, particular attention should be paid to putative side effects. Some organ systems (e.g. the liver) are more likely to tolerate a treatment that could also affect the normal stem cell population because of the high regenerative potential of their differentiated cells. However, more severe side effects can be expected in organs with a high cellular turnover rate that is more depending on a stem cell-based regeneration, including skin and intestine. Of note, potentially toxic effects on the bone marrow may even necessitate autologous bone marrow transplantation comparable to the treatment of patients with leukaemia.

One clinically most apparent property of stem cells is their ability to pump drugs out of the cell through the use of specific drug transporters. Therapies designed to block these ABC transporters may chemo-sensitize CSC, but may also sensitize normal stem cells to co-administered anti-cancer drugs and therefore may lead to their premature death. Besides, ABC transporter blockers also endanger the blood-brain barrier as they play an important role in the maintenance of the blood-brain-barrier. (71) Thus, this promising new concept of targeting cancer stem cells comes with a trade off and needs to be taken with a cautionary grain of salt.

Most promising for treating metastatic disease is to target the homing process of CSC. Several clinical trials antagonizing chemoattraction of CSC through inhibition of CXCR4 are ongoing. In general, there are four different scenarios that can be envisioned for blockade of this important signalling pathway: small peptide CXCR4 antagonists (T140 and its analogs), non-peptide CXCR4 antagonists (AMD3100), neutralizing antibodies directed against CXCR4, and modifying SDF-1 as the specific ligand of this receptor. At least in animal models blocking of CXCR4 has already been shown to effectively prevent metastasis suggesting that all metastatic CSC independent of their route of systemic spread, via the blood or the lymphatic flow, were sufficiently inhibited in their invasive activity (13, 46).

As metastatic cancer stem cells are a highly invasive subpopulation of CSC and most likely enter the circulation at a very early stage of tumour development, counteracting the metastatic potential of solid tumours can only be achieved if therapies targeting the homing or seeding of mCSC are applied significantly earlier than current practice. However, for several highly metastatic cancers such as pancreatic cancer, a treatment regimen purely focussing on metastatic spread of CSC is unlikely to succeed. Furthermore, any CSC bears the potential of becoming a migrating CSC. Therefore, the entire CSC population should be depleted to prevent tumour relapse and metastasis. It is reasonable to assume that the best results will be obtained by targeting different traits of CSC requiring a cocktail of targeted drugs that eventually should allow the elimination of every single cancer stem cell.

8. CURRENT AND FUTURE CHALLENGES FOR THE CANCER STEM CELL MODEL

Although there is increasing evidence for the existence of cancer stem cells in both mouse and human carcinomas, the CSC hypothesis should not be used as an universal model. The CSC model has been challenged by showing that non-CSC subpopulations of tumor cells can also recapitulate the diversity of the primary cancer. Contradictory to previous results (4, 5), Shmelkov et al. report that CD133 might not be a suitable marker for metastatic colon cancer stem cells bearing exclusive tumourigenicity as the $CD133^{-}$ population in metastatic lesions was shown to not only bear tumourigenic cells but to even form more aggressive tumors as compared to their $CD133^{+}$ counterparts (72). Of course, the nature of these

cells remains illusive since all EpCAM positive cancer cells reportedly expressed CD133 in these experiments.

These data are very surprising as for several primary pancreatic cancers, CD133 expression was reproducibly restricted to a discrete population of epithelial cancer cells ranging from 0.5 to 5% with the majority of the cells being negative for markers of epithelial differentiation and bearing exclusive tumorigenicity (4, 5, 13). Therefore, these results not only suggest that the utilized murine model has a limited analogy to human cancer tissue. Moreover, the isolation of cancer stem cells from solid tumor requires the use of proteolytic enzymes that may destroy or modulate some surface antigens. Regarding CD133 many different antibodies are commercially available, which vary considerably with respect to targeted epitopes and binding characteristics.

Our current knowledge about surface markers for normal tissue stem cells is indeed still rather limited and even more so for cancer stem cells. Tumor cells accumulate multiple mutations during transformation so that surface markers widely used in normal tissue studies may not reflect their biological relevance in cancer specimens. Thus, CD133 as a single marker certainly bears some limitations due to lack of cancer stem cell specificity as it is also expressed on some epithelial cells resulting in relatively high number of $CD133^+$ cells that need to be implanted for generating tumour formation. The analyzed CSC populations are still highly impure and merely enriched for cancer stem cells. But at least in pancreatic cancer tissue, several investigators in numerous tumour entities find CD133 positive cells to be exclusively tumourigenic, particularly if they are combined with other markers such as CXCR4 and CD44, respectively.

Indeed, a recent study by Zhu et al. further supports the role of CD133 in the small intestine as a suitable CSC marker by using a knock-in mouse model (73). The authors showed that CD133 expression is restricted to cells located at the base of the crypt. These $CD133^+$ cells predominantly overlap with the $Lgr5^+$ cell population, the putative intestinal stem cell population (74). Lineage-tracing experiments provide compelling evidence that CD133 specifically marks stem cells capable of generating all of the differentiated cell types of the small intestine. The study further confirmed the CSC model by showing that CD133 marks an adult solid tissue stem cell that is susceptible to neoplastic transformation, forming a model of human tumour that contains $CD133^+$ cancer stem cells. Nevertheless, the ideal CSC marker panel still remains to be defined but lineage-tracing experiments can bypass the limitations and variability of the transplantation assay and additional markers may arise during gene expression analyses that also incorporate normal stem cells as reference material.

In addition to these controversies regarding the optimal markers for CSC, the CSC model has also been challenged by a recent study in malignant melanoma. It was shown that, depending on the xenograft model used, almost any freshly isolated patient-derived melanoma cell is capable of tumour initiation in the utilized setting (75). Although the authors were able to reproduce initial findings for melanoma in NodScid mice suggesting a frequency of tumour-initiating cells in the range of 1 out of 50,000 cells, they obtained vastly different results when altering various aspects of the original *in vivo* protocol (76). These changes included prolongation of the observation period, injection of the cells in combination with Matrigel^{TM}, and use of even more severely immunocompromised strains of mice as host organs that lack T cells, B cells, and NK cells eliminating virtually any immune response to the implantation of the cells and providing the most permissive environment.

However, it should be emphasized that it is presently still unknown, which of these cells are actually tumourigenic in our patients. Indeed, major qualitative differences regarding *in vivo* tumourigenicity still stand for malignant melanoma and the majority of cells are tumourigenic only in a model of severe immunodeficiency where virtually the complete host immune response against the cancer cells has been eliminated. Of note, the cancer stem cell hierarchy may also depend on tumour stage and may become diluted during later stages as the pool of highly tumourigenic cells drastically expands through symmetric division or blockage of differentiation. As this controversy is unlikely to be solved in any xenograft model, investigators are now increasingly using genetically modified mouse models. Indeed, most investigators were now able to confirm the cancer stem cell hypothesis in a syngeneic setting for an increasing number of tumours including leukaemia (31), brain tumours (77, 78), breast cancer (79), and intestinal cancer (73).

Most importantly, however, cells that are capable of exclusively forming tumours in athymic mice and NodScid mice, respectively, are highly resistant to standard chemotherapy and therefore still represent an intriguing new target for the development of advanced treatment modalities. On the other hand, as the issue concerning the origin of the CSC has not been solved definitively for most tumours yet, any cell may bear the capacity of converting into a cancer stem cell resulting in subsequent metastatic spread. Therefore, all cancer cells should be eliminated during the course of treatment. This will likely require a multimodal treatment modality designed to target CSC in combination with standard therapies. Ideally, this combination treatment would result in a reduction of the tumour mass by eliminating more differentiated tumour cells through standard chemotherapy, and additionally in the extinction of highly resistant CSC by treatment modalities directed against specific cancer stem cell pathways.

9. CONCLUSIONS

The ongoing controversy regarding the true existence of cancer stem cells, while not really surprising, actually seems to represent more of a misunderstanding than a true controversy. Functionally defined cancer stem cells certainly do exist in many forms of cancer, irrespective of their relative frequency or the stability of

respective cancer stem cells phenotype during cancer progression. It appears much more relevant at present time to approach the real issues as to whether or not understanding the properties of cancer stem cells what will guide us towards creating improved therapeutic strategies for our patients. Therefore, the evidence that support the cancer stem cell concept should arise as a consequence of successfully targeting cancer stem cells. Indeed, a growing wave of studies is now emerging based on the cancer stem cell hypothesis. By addressing the aforementioned key issues, it should be possible to improve our understanding of the role of cancer stem cells in tumour biology and to unambiguously determine their potential with regard to achieving better therapeutic outcomes.

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