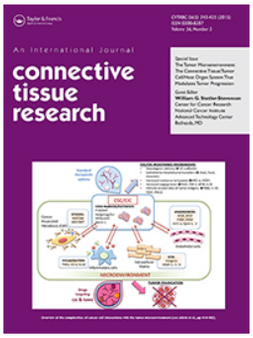

Connective Tissue Research

ISSN: 0300-8207 (Print) 1607-8438 (Online) Journal homepage: https://www.tandfonline.com/loi/icts20

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To cite this article: Adriana Albini, Antonino Bruno, Cristina Gallo, Giorgio Pajardi, Douglas M. Noonan & Katiuscia Dallaglio (2015) Cancer stem cells and the tumor microenvironment: interplay in tumor heterogeneity, Connective Tissue Research, 56:5, 414-425, DOI: 10.3109/03008207.2015.1066780

To link to this article: https://doi.org/10.3109/03008207.2015.1066780

connective tissue research

http://www.tandfonline.com/icts
ISSN: 0300-8207 (print), 1607-8438 (electronic)

Connect Tissue Res, 2015; 56(5): 414–425
Published with license by Taylor & Francis Group, LLC. DOI: 10.3109/03008207.2015.1066780

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REVIEW ARTICLE

Cancer stem cells and the tumor microenvironment: interplay in tumor heterogeneity

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Abstract

Tumor cells able to recapitulate tumor heterogeneity have been tracked, isolated and characterized in different tumor types, and are commonly named Cancer Stem Cells or Cancer Initiating Cells (CSC/CIC). CSC/CIC are disseminated in the tumor mass and are resistant to anti-cancer therapies and adverse conditions. They are able to divide into another stem cell and a ''proliferating'' cancer cell. They appear to be responsible for disease recurrence and metastatic dissemination even after apparent eradication of the primary tumor. The modulation of CSC/CIC activities by the tumor microenvironment (TUMIC) is still poorly known. CSC/CIC may mutually interact with the TUMIC in a special and unique manner depending on the TUMIC cells or proteins encountered. The TUMIC consists of extracellular matrix components as well as cellular players among which endothelial, stromal and immune cells, providing and responding to signals to/from the CSC/CIC. This interplay can contribute to the mechanisms through which CSC/CIC may reside in a dormant state in a tissue for years, later giving rise to tumor recurrence or metastasis in patients. Different TUMIC components, including the connective tissue, can differentially activate CIC/CSC in different areas of a tumor and contribute to the generation of cancer heterogeneity. Here, we review possible networking activities between the different components of the tumor microenvironment and CSC/CIC, with a focus on its role in tumor heterogeneity and progression. We also summarize novel therapeutic options that could target both CSC/CIC and the microenvironment to elude resistance mechanisms activated by CSC/CIC, responsible for disease recurrence and metastases.

Keywords

Cancer initiating cells, cancer stem cells, connective tissue, heterogeneity, tumor microenvironment

History

Received 13 March 2015
Revised 11 May 2015
Accepted 16 June 2015
Published online 19 August 2015

Introduction

Although cancer-related deaths decreased in the last decades, cancer still remains one of the leading causes of mortality worldwide, as a significant proportion of cancer patients develop disease recurrence and metastatic spread. This, coupled with the fact that many targeted anti-cancer therapies extend disease-free survival for just a few months, have pushed researchers to consider tumor heterogeneity and complexity as a new target. Tumor heterogeneity is the result of both intrinsic and extrinsic features. Intrinsic heterogeneity includes genetic, epigenetic and biological properties of cancer cells contributing to its oncogenic activity, whereas extrinsic traits are related to the cancer-surrounding environment that mutually interacts with cancer cells to influence the development and progression of the neoplastic disease. In this complex scenario, the role of Cancer Stem Cells (CSC) or Cancer Initiating Cells (CIC) still represents a debated and hot topic. First identified in 1997 by the group of Dick and colleagues in acute myeloid leukemia (1), CSC have been found in many solid tumors. CSC are transformed cells that can be rare or relatively abundant, depending on the tumor type. CSC/CIC are able to preserve tumor heterogeneity by retaining self-renewal and differentiation capacities. In addition, CSC/CIC display an innate resistance to therapies, which in turn associates their persistence in a tissue with disease recurrence and eventually metastatic spread (2). Resistance mechanisms activated by CSC/CIC include low levels of replication, expression of drug

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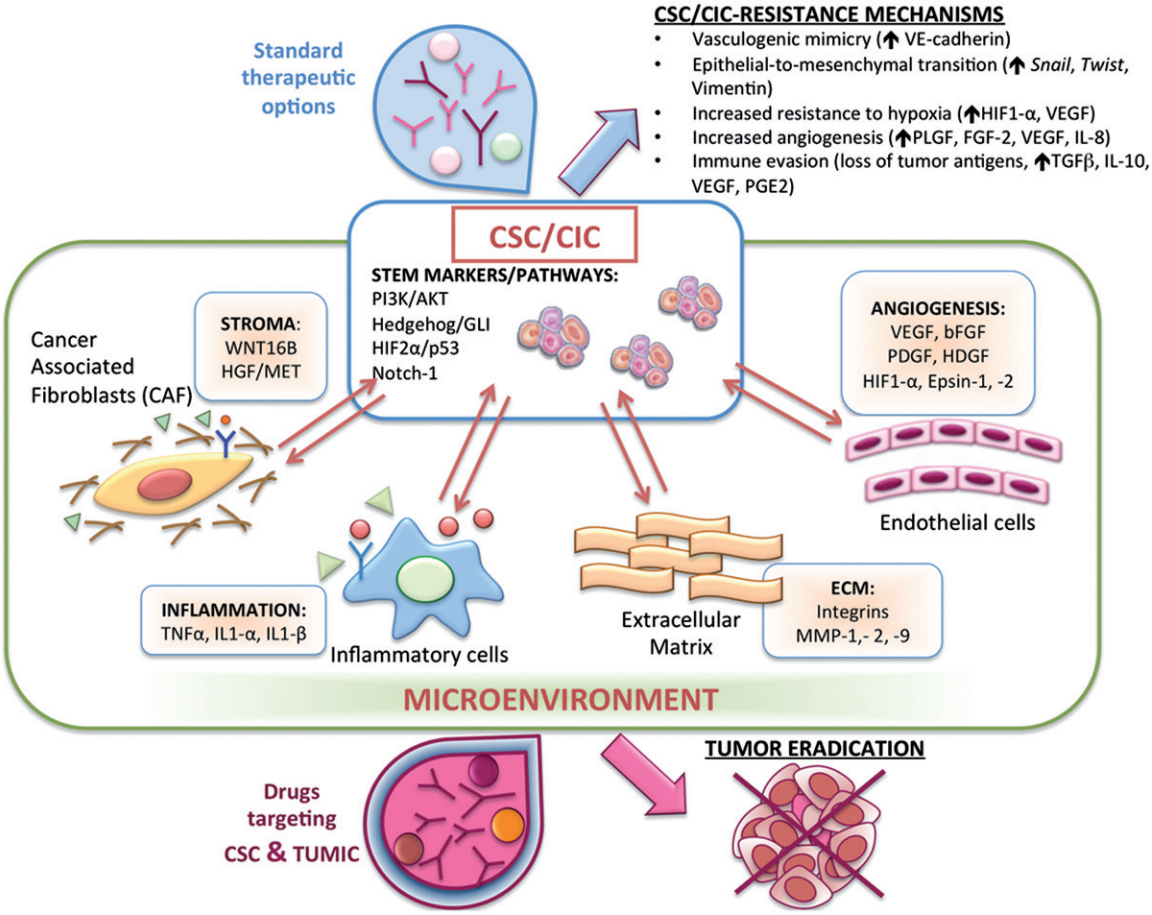

Figure 1. Cancer Stem Cells or Cancer Initiating Cells (CSC/CIC) interact with the surrounding tumor microenvironment (TUMIC) by activating stem cell- and self renewal-associated pathways, such as Notch-1, PI3 Kinase and Hedgehog. Canonical anti-proliferative therapies mainly target bulk tumor cells, sparing aggressive CSC/CIC that are responsible for disease recurrence by activating resistance mechanisms including Vasculogenic Mimicry and Epithelial to Mesenchymal Transition. In addition, these cells may be able to: (1) survive hypoxia by increasing the production HIF1-α, VEGF; (2) increase angiogenesis by producing higher amount of pro-angiogenic factors; (3) induce immuno-tolerance by producing anti-inflammatory cytokines. Novel therapeutic treatments targeting both the CSC/CIC compartment and the TUMIC with the potential to eradicate the tumor, minimizing the risk of disease recurrence, are warranted.

export systems, vasculogenic mimicry, epithelial-to-mesenchymal transition (EMT), increased resistance to hypoxia with induction of angiogenesis, and immune escape by decreasing tumor specific antigens, while increasing anti-inflammatory cytokines and growth factors (Figure 1). Interestingly, new data on cancer cell plasticity have emerged showing that cancer cells may be able to re-acquire stem cell traits, reversing their differentiation status. These new data put into question the existence of CSC/CIC as an ''entity'' in a tumor. The ''stemness trait'' might be more a state of the tumor cell, modulated by signals reflecting specific needs for tumor sustenance, such as maintenance, renewal, growth and invasion. The contribution of the microenvironment in this picture is crucial: it is now accepted that the ''cancer'' scenario is not simply composed of transformed cells working together in an isolated and strictly autonomous machinery. Neoplastic cells, including the CSC, reside in specific niches composed by stromal, immune, endothelial cells as well as connective tissue components, growth factors and cytokines, sustaining their status and modulating their activities. The tumor microenvironment actively collaborates with neoplastic cells at different levels: promoting proliferation while evading growth suppression and immune-surveillance, overcoming cell death, modulating cell metabolism, activating angiogenesis and invasion/metastasis programs (3). In addition, the interactions between CSC/CIC and the microenvironment help these cells to survive common anti-cancer therapies thus being partly responsible for disease recurrence.

In this review, we outline the recent emerging results contributing to the definition of CSC status, describe the current state of the art on the features of CSC-microenvironment interactions and discuss recently discovered therapeutic strategies directed at the microenvironmental components influencing CSC activities. We believe that a deeper knowledge of the interplay between CSC/CIC and the surrounding microenvironment, including the mechanisms responsible for cancer cell switch from a non-CSC status to CSC (and vice versa) is a key point for the introduction of new effective therapies against cancer and that targeting both stem-like cancer cells and CSC-microenvironment cellular and molecular features may prevent both tumor recurrence and metastasis.

Evolution of the definition of cancer stem/cancer initiating cells

In the last decades, with emerging evidence regarding CSC markers, properties and behavior, the meaning of the term CSC has evolved and has subsequently been revised. The

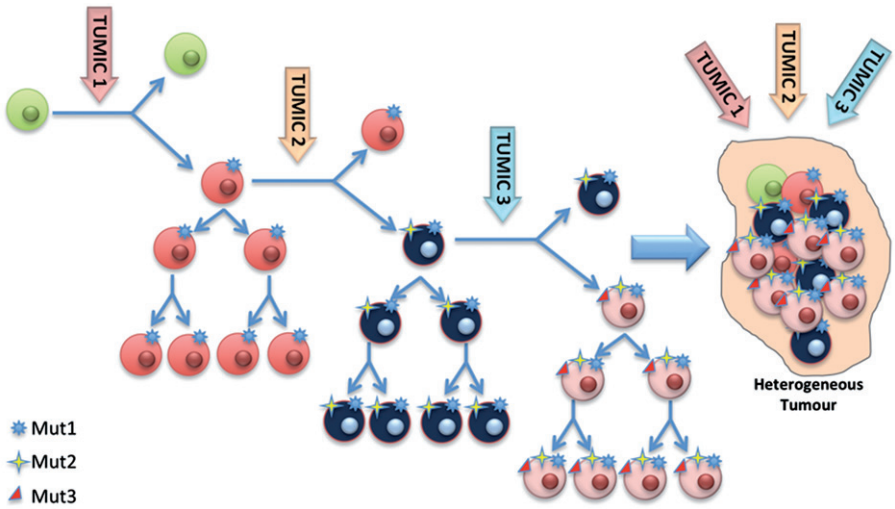

Figure 2. Mutated cells might be able to survive specific microenvironmental conditions, while the tumor microenvironments (TUMICs) perform clonal selection by releasing peculiar growth factors and cytokines. TUMIC1 may select transformed cells harboring only Mut1. The evolving microenvironment in which tumor cells live (TUMIC2) may induce a second favorable mutation, Mut2. Only cells harboring both Mut1 and 2 will survive and generate daughter cells through symmetric division. Finally, a third mutation (Mut3) induced by TUMIC3 will generate more aggressive cells harboring Mut1, Mut2 and Mut3. The process of clonal selection gives rise to tumor heterogeneity.

most established definition of CSC refers to the hypothesis that within a tumor mass, a population of cancer cells with stem cell features retains the ability to initiate and propagate the tumor *in vivo* (4). The majority of tumors are composed of a mixture of self-replicating tumorigenic cells (CSC), non-replicating tumorigenic cells (2,5) as well as cells of an intermediate state, supporting the concept of tumor heterogeneity. CSC are mostly rare populations, however, this is not a feature of all tumor types. In melanoma, for instance, about 25% of patient-derived melanoma cells are tumorigenic when implanted into immune-compromised mouse models (6). In lymphoma and leukemias of mouse origin more than 10% of neoplastic cells generate tumors *in vivo* recapitulating tumor heterogeneity (7). This might be explained by the phenotypic plasticity of cancer cells, which is consistent with the reversible changes in the expression of stem cell markers *in vivo* (6). However, clonal heterogeneity of tumors may also be the result of the interactions between different populations with specific selective proliferative advantages. It has been shown that tumor growth is the result of a balance between the driving force of a minor subpopulation of cells with lower than average fitness, and clonal interference (higher fitness clones competing each other, slowing down clonal evolution (8)). Clonal heterogeneity of tumors is in accordance with the evidence that several phenotypic markers can be used to characterize and isolate transformed cells with tumorigenic ability in the same tumor. In breast cancer, for example, selection of the $CD44^{+}CD24^{low/-}$ cell population, mammosphere formation and positivity to Aldefluor all successfully enrich tumorigenic cells with self-renewal properties (9–11). In glioblastoma multiforme (GBM), one of the most morphologically heterogeneous neoplasms, each tumor mass contains different clones with specific proliferative and differentiation capacities; single tumor cells from GBM patients display different transcriptional programs (12) and single cell-derived clones have specific drug responsiveness features, with some of them being resistant to conventional GBM treatments (13). It is likely that in highly heterogeneous tumors, each tumor-derived clone has its own stem cell of origin and that tumor heterogeneity derives from genetically distinct tumor-initiating cell subclones with a different growth advantage. In this scenario, the set of conditions characterizing the environment in which a cancer cell may evolve acquiring new mutations and/or invasive features is of paramount importance (14). The specific features of an environment may push the tumor cell to take one road or the other, thus developing one mutation instead of another [(14), Figure 2]. However, distinct mutations may occur independently in genetically distinct subclones deriving from the same cell of origin. In this respect, clonal evolution studies performed in leukemia patients have shown that a single clone of origin gives rise to several clonal lineages with diverse genetic aberrations, thus suggesting that CSC at the origin of a tumor evolve to generate heterogeneity with a multi-clonal evolution model (15). This means that although the microenvironment is a key to push the cancer cell towards defined evolutionary paths, a clear dependence on the development of specific mutational events is needed in order to maintain neoplastic growth and progression (16).

Every tumor is different and a unique definition of CSC/CIC applicable to all tumor types has not yet been established. CSC have been tracked in brain, gut and skin tumors using genetic approaches, without altering their environment, thus giving new information on CSC crosstalk with microenvironmental effectors (17–19). There is also another conflicting element concerning the definition of CSC, deriving also from the alternative names given to cancer stem cells, for instance, cancer initiating cells. Cancer initiation is the process starting from a healthy cell that accumulates DNA damage, finally undergoing transformation. The cell that originates a tumor (also named cancer cell of origin) is likely different from that responsible for tumor propagation and recurrence (CSC). However, the confounding factor is the shared stem phenotype between the cancer cell of origin and CSC. The rising question is, therefore, is the cancer cell of origin a normal stem cell or a differentiated progeny undergoing de-differentiation? In prostate, for example, basal cells from normal tissue can initiate prostate cancer (20). The normal stem cell as the cancer cell of origin has been found in skin, intestine and brain cancers (reviewed in (21)). However, according to the emerging concept of cancer cell plasticity, cells can convert bi-directionally between CSC and non-CSC: tumorigenic CSC can therefore also be generated *in vivo* by de-differentiation of non-tumorigenic cells. In this context, both

intrinsic (genetic and cell-type associated) and extrinsic (environmental) factors may modulate the cancer cell of origin activity or differently influence normal cells (either normal stem cell or differentiated progenies) to become a CSC. Recent data have suggested that tumor incidence is related to replication of stem cells (22), however, this could also be related to stem cell content within the tissues (23).

All together, we can state that the classical definition of CSC cannot be applied to every tumor type and that, although sharing features with stem cells, CSC are likely tumor-type-dependent and reflect changes in both intrinsic and extrinsic signals. Tumors are tissues that are formed by cancer and microenvironment cells. In this respect, the niche in which CSC reside finely regulates CSC identity, state and activities.

The complex system composed by CSC/CIC and the microenvironment

Another aspect influencing CSC frequency and abundance in a tumor is the permissiveness of the experimental model used to evaluate cancer cell ability to grow and recapitulate tumor phenotypes *in vivo*. Because continuous culture of tumor-derived cells *in vitro* selects only those that adapted to propagate in plastic dishes and specific nutrient media, thus reducing tumor heterogeneity and likely CSC population, the introduction of *in vivo* xenograft models recapitulating tumor complexity, including the presence of microenvironmental factors, represents a necessity. Immune-deficient mouse models currently in use for the analysis of cancer cells ability to grow *in vivo* include NOD-SCID, SCID, nude and the highly immune-compromised NOD-SCID/IL2γ -/- (NSG) mice. These mice differ for the presence of residual immune cells, in particular, B-lymphocytes and Natural Killer (NK) cells, and differently allow the growth of CSC/CIC, with the less immune-compromised models being less permissive and underestimating their frequency (6,24–26). The presence or absence of residual immune cells in the recipient animal positively or negatively influences cancer cells ability to grow (27) also evidencing the presence of distinct populations of CIC in the same tumor (28). For these reasons, in the last few years, the use of congenic or syngeneic animal models is preferred and strongly suggested whenever possible.

Since *in vitro* experimental conditions differ from the natural environment found *in vivo*, including the absence of specific microenvironmental players supportive for CSC growth and maintenance, it is obvious that CSC behave differently in the two conditions. As discussed above, one of the most reliable tools to address the presence of CSC in tumors is the serial transplantation of single cancer cells into immune-compromised mice/rats. However, often the sole injection of cancer cells is not sufficient to ensure tumor growth even when the cells retain intrinsic tumorigenic ability. This is probably due to the growth conditions offered by the new environment in which CSC are placed, which is often different from that where they usually reside and/or are generated. The major limitations of the *in vivo* tumorigenesis assay will be further discussed in detail, however, some *in vivo* models used for these purposes have therefore been improved in order to obtain tumor growth, by injecting tumor cells together with supporting cells of the microenvironment, or specifically enriched matrices, such as matrigel. Squamous cell carcinoma (SCC) of the skin is an example of a slow-growing tumor whose complexity is difficult to be fully recapitulated in *in vivo* xenograft models. A suitable ''stromal bed'', generated by pre-conditioning an artificial matrix with human fibroblasts before xenografting tumor tissue, allows to successfully generate human SCC with precise recapitulation of tumor histology and phenotype (29). Interestingly, these models also provide the optimal conditions to identify and estimate the frequency of CSC (30), thus indicating that the model chosen for CSC studies is of relevant importance to overcome methodological biases.

The stroma also plays a major role in reverting differentiated cells towards a de-differentiated phenotype. This has been proposed to be one of the mechanisms generating cancer stem cells (31). Consistently, it has been shown that the tumor microenvironment contributes to cell-plasticity during tumor development, initiating stem-like programs in non-CSC or normal cells and involving key signals, such as Wnt pathway (32) or inflammation-associated signatures (33). Microenvironmental cells can induce metabolic changes, including hypoxia, variations in growth factors/cytokines concentrations, pH changes as well as many other factors. Cancer Associated Fibroblasts (CAF) or adipocytes are able to secrete a wide variety of cytokines associated with tumor progression, such as Platelet-Derived Growth Factor, Vascular Endothelial Growth Factor (VEGF) and Hepatocyte Growth Factor (34). Acidity and hypoxia are two strongly associated conditions often characterizing the tumor micro-environment. Hypoxia-inducible factors, HIF1α and HIF2α, two of the main tissue controllers of oxygen homeostasis, are sensitive to cellular pH conditions (35). Both hypoxia and changes in pH can regulate stem cell behavior by modulating their metabolic status and promoting metabolic reconfiguration of cancer cells towards glycolysis, induction of the Epithelial-to-Mesenchymal Transition (EMT) phenotype (including C-X-C-chemokine receptor 4 (CXCR4), Snail and Twist gene expression) as well as increases in the number and renewal-potential of CSC and induction of pluripotency-associated transcription factors, such as Oct-3/4, Nanog and Sox-2 (36,37). Hypoxia pushes tumor cells to undergo an aerobic glycolysis in order to survive the oxygen-free environment, and this phenomenon promotes tumor growth and metastasis (31). This scenario indicates that ''stemness'' is more a cellular state, than a cancer cell quality, that can be modulated by the microenvironment.

Within the concept of cell plasticity, it has been recently proposed that beside genetic and epigenetic regulators of cell fate, metabolic reprogramming, which includes variation of metabolic parameters both at the cancer cell site and systemically, may be a new cancer hallmark redirecting cancer cell state from non-CSC to CSC (38). Cell metabolism regulates cell proliferation and differentiation (39) and several recent reports show that CSC and their differentiated progeny are in different metabolic states. In breast cancer, CSC are more prone to undergo oxidative phosphorylation, while the non-CSC counterparts preferentially perform aerobic glycolysis (40). However, tumors are complex tissues where cancer and microenvironmental cells communicate through a metabolic flux, a bidirectional relationship where one cellular

component influences the other and vice versa, in a mutual metabolic reprogramming. In this respect, CAF exert a metabolic reprogramming of cancer cells by inducing a reverse Warburg phenotype (41).

Metastatic dissemination of tumor cells often occurs in the absence of symptoms, given the ability of disseminated cells to enter a dormant state that reflects the refractoriness of advanced stage tumors to therapies. Since dormant cells may cause tumor recurrence, and quiescence or slow-growing ability is a prerogative of tissue-residing stem cells, a rising question is whether CSC may be at the origin of metastatic dissemination. The presence of metastasis initiating cells (MIC) has been only recently demonstrated in breast cancer (42), colon cancer (43) and lung cancer (44). Some lines of evidence suggest that MIC might be found in CSC subpopulations (44,45). Tumor spread to local or distant sites needs a supportive accommodating environment for disseminated cancer cells. The so-called ''metastatic niche'' may also be a native stem cell niche of the distant organ, enhancing stem cell properties while repressing differentiation (46). Overall, the CSC niche is an active environment governed by developmental signaling pathways, such as Wnt, Notch and the chemokine CXCL12 (47); endothelial-mediated paracrine stimulation; extra-cellular matrix components, such as tenascin and periostin; secreted enzymes associated with local stiffness, such as Lysyl oxidase (LOX) (48). Moreover, primary tumors prepare a ''pre-metastatic niche'' in distant organs by systemically releasing inflammatory cytokines and enzymes, which modify the recipient microenvironment (49,50). The microenvironment is also a regulator of tumor dormancy and dormant cell fate, although both dormancy-permissive and -restrictive microenvironments have been observed and characterized (51). Interestingly, tumor dormancy is not only the result of cancer cells undergoing cellular quiescence, but may also be caused and accompanied by reduced vascularization (angiogenic dormancy) and high cytotoxic activity by the immune system (immune mediated-dormancy). Finally, tumor cells may grow, arrest or die depending on the presence of defined growth factors and cytokines in the surrounding environment. The ability to survive distinct sets of environmental conditions is likely a result of the mutations harbored by these cells. It is therefore conceivable that the environment might be able to force genetic evolution towards some mutations that favor cancer cell survival, while less-favorable aberrations lead to cancer cell death and are not positively selected by the microenvironment. The microenvironment is therefore a promoter and executor, among other factors, of a ''clonal'' choice that selects those cells with the ability to sustain tumor growth and maintenance.

It is evident that both the presence and features of CSC and the microenvironment influence each other, through a signaling crosstalk that includes among other factors, matrix modulation and growth factors exchanges. In this fine interplay, different cellular players are involved.

Tumor microenvironment cellular players

Cancer Associated Fibroblasts and Epithelial to Mesenchymal Transition

CAF represent one of the cellular components of the tumor microenvironment in the so termed ''active'' stroma, contributing to drive tumor progression either by secreting soluble factors, interacting with other cell types or modulating the composition of the extracellular matrix (52). These cells can orchestrate tumor cell behavior by secreting exosomes that stimulate cell migration, invasion and metastasis formation (53). In prostate cancer, CAF contribute to enhance the growth potential of CSC by increasing spheroid formation and cancer cell proliferation index through paracrine signals (54,55). Moreover, co-injection of CSC from prostate cancer and CAF into immune-compromised mice increases the number of neoplastic lesions with a representative histology, as compared to normal fibroblasts, thus supporting the significance of CAF in cancer biology (55). The paracrine contribution of CAF to cancer stemness has been recently elucidated also in lung cancer, where CAF induce a de-differentiation program mediated by Nanog, through the release of paracrine factors and activation of insulin-like growth factor 1 receptor (IGF1R) signaling (56). CAF exert tumor sustenance also through direct contact with cancer cells. When exposed to hypoxic or low nutrient environments (the tumor inner mass is an example of such an environment), CAF express higher levels of CD44, a glycoprotein stem cell marker regulating tumor cell migration and cell–cell contacts, which in turn promotes cancer stem-phenotype including CSC refractoriness to therapies (57).

Epithelial to Mesenchymal Transition (EMT) is the process through which epithelial cells change their morphology/behavior from epithelial-like to fibroblast-mesenchymal one, accompanied by increased motility, invasiveness and extracellular matrix component turnover. EMT confers to cancer cells the ability to invade the basement membrane, migrate towards distant sites, finally forming secondary tumors (58). Genes responsible for EMT, such as *twist1* and *snail* have been associated to the maintenance of stemness properties (59,60). EMT induction in normal epithelial cells of mammary origin increases the $CD44^{high}CD24^{low}$ pattern expression, a profile specific for CSC detention, and mammosphere formation, through *snail* or *twist* expression (59). Several reports have shown the ability of tumor-derived CAF or CAF conditioned media to induce the EMT phenotype in cancer cells (61–63). Mesenchymal stromal cells trigger EMT in tumor cells through membrane-bound (64) and secreted (61) TGFβ. In colorectal cancer cells, the CAF-derived chemokine CCL2 induces the expression of fibroblast growth factor receptor 4 that activates β-catenin, which in turn promotes EMT (62). In prostate cancer, CAF secrete CXCL12 that converts cancer cells towards the EMT phenotype and increase expression of its receptor, CXCR4, which facilitates cancer cell migration and the formation of metastasis *in vivo* (65). The contribution of CAF to metastasis through the enhancement of stem cell features in cancer cells has been also recently demonstrated in breast cancer, where Tissue Inhibitor of Metalloproteinases (TIMP) loss by CAF enhances the formation of distant metastasis (66)). This phenomenon is associated with activation of Notch signaling followed by up-regulation of typical CSC markers, such as aldehyde dehydrogenase (ALDH) 1A3 and integrin α6 (66). In accordance with the influence of the microenvironment on

cancer cell survival and propagation, in triple negative breast cancers, a stroma-driven selection for cell clones able to generate distant metastasis has been recently shown. Mesenchymal stromal cells select cancer cells with increased Src activity able to adapt to a CXCL12/IFG1-rich environment found in bone metastatic sites (67). Interestingly, while the majority of reports show a pro-tumor activity exerted by CAF, recent evidence demonstrates that myofibroblasts and myofibroblast/collagen I-associated fibrosis is protective against the development and progression of pancreatic cancer (68), thus suggesting a protective role for CAF in this specific tumor context. Myofibroblast and fibrosis depletion in these mouse models is also associated with the acquisition of EMT features, increased hypoxia and an increased number of $CD44^+/CD133^+$ cancer stem cells as well as impaired immune response. Lastly, upon myofibroblast depletion, mice developed less differentiated, more invasive tumors, features associated with specific and unfavorable genetic aberrations (68).

Native immunity: a role for natural killer cells

Immune cells recruited at the tumor site often show altered phenotypes and behavior as compared to those observed in healthy tissues. This scenario is related with functional alterations associated with the induction of a tolerogenic state and a polarized phenotype that promotes tumor progression. The attenuation of anti-tumor activity by immune cells is induced by several TUMIC components that are crucial in the orchestration of immune cells plasticity, including the dormant component, as a consequence of tumor intrinsic and extrinsic heterogeneity. CSC/CIC ability to escape immune response mainly includes the induction of T cell-anergy, the generation of regulatory T cells, crucial for immunological tolerance, the low expression of tumor-associated antigens on the surface of cancer cells and the modulation of the microenvironment towards an anti-inflammatory phenotype.

The role of NK cells in both host immunity against cancer development and tumor progression has recently emerged. NK cells are innate effector lymphocytes primarily involved in the immune-surveillance against tumors. The ability of NK cells to spare or kill depends on their expression of activatory (mostly stress-induced proteins) and inhibitory (in particular MHC class I molecules) ligands on the surface of target cells. The tissue environment significantly influences their killing or tolerogenic activities. Several NK cell subsets have been described; among these, the major subset, approximately 95% of peripheral blood NK cells, is $CD56^{dim}CD16^+$ and exerts strong cytotoxic activity. Approximately 5% of peripheral blood NK cells are $CD56^{bright}CD16^-$ and show cytotoxicity through strong cytokine production. We were the first to identify a novel NK subset in non-small cell lung cancer (NSCLC), able to promote tumor angiogenesis (69) that were termed tumor infiltrating NK cells (TINKs) and peripheral NK cells as tumor associated NK cells (TANKs) (70). The pro-angiogenic switch in normal NK cells is induced by TGFβ, abundantly present in the TUMIC and a necessary mediator for EMT. CD133 positive glioblastoma stem cells, that are able to express low levels of MHC-class I molecules and high levels of the activating DNAM-1 ligands PVR and Nectin-2, have been reported to be poorly recognized and lysed by NK cells (71). Their cytotoxic activity was restored following IL-2 or IL-15 activation (71). Breast cancer CSCs have also been reported to fail to express detectable levels of NK ligands, consistent with metastatic spread (72). In addition, in tumor tissues, the number of recruited NK cells is strongly reduced as compared to normal counterparts (69,70). However, increasing evidence supports also a microenvironmental modulation of NK activity, as NK cells are able to target the resistant CSC population in different tumor settings. In colorectal cancer (CRC), CIC express lower MHC-class I and higher levels of NK-activating ligands, including NKp30L and Nkp44L as compared to differentiated cells, which are responsible for the CIC susceptibility to NK cell killing (73). However, the preferential killing of CSC by NK cells is not the rule, as melanoma and GBM CIC are highly resistant to allogeneic NK cells, and become susceptible to NK cytotoxicity only following stimulation of freshly isolated NK cells with IL-2 (71).

Another mechanism through which cancer cells may elude the immune response by NK cells is the induction of apoptosis in microenvironmental immune cells through the interaction of CD95 (Apo1/Fas) with its ligand (CD95L). CD95 or CD95L expression by cancer cells protects them from apoptosis and $CD95^+$ lymphocytes are killed upon engagement by cancer cells bearing membrane-bound CD95L. CD95L can also be released following cleavage from the cell surface by matrix metalloproteinases. Interestingly, CD95R/L regulate CSC plasticity, its blockade reduces CSC in different tumor cell models, while CD95R/L stimulation increases the number of CSC and is responsible for CSC reduced sensitivity to CD95-mediated apoptosis (74). Finally, NK cells are able to modulate metastatic dissemination in different tumor types, including breast cancer (72), lung cancer (75) and prostate cancer (76). In this scenario, it becomes clear that the compromised cytotoxic activity of NK cells is implicated in the lack of CSC/CIC killing in cancer patients.

Endothelial cells and CSC/CIC

The fine crosstalk between cancer cells and their microenvironment involves also the endothelial compartment, with strong evidence suggesting a key role for endothelial cells in supporting the cancer stem cell-phenotype. In this scenario, Notch signaling, a promoter of self-renewal in normal stem cells and a key mediator of angiogenesis, is crucial. In GBM, Notch ablation induces a reduction in the number of $CD133^+$ CSC, CD133 expression and self-renewal potential. *In vivo,* treatment with DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester), a γ−secretase inhibitor that prevents Notch cleavage, also decreases CD105 expressing cells and the expression of endothelial cell markers, such as CD31, von Willebrand factor and CD146. Interestingly, the reduction of the stem cell compartment is caused by the effect of Notch inhibition in endothelial cells as endothelial cell depletion *in vivo* directly decreases cancer cell neurosphere-forming ability to 50% of that of controls (77,78). In gliomas, the platelet-derived growth factor-nitric oxide synthase and

Jagged-1-Nocth signaling pathways are specifically expressed by glioma initiating cells (GIC). The platelet-derived growth factor-nitric oxide synthase axis activates Jagged-1-Notch signaling in both GIC and endothelial cells influencing the crosstalk between stem cells and the microenvironment (79). Similarly, in CRC, co-culture of endothelial cells with tumor cells increases the fraction of ALDH+ and $CD133^+$ cells, two populations containing CSC. In $CD133^+$ cells, which reside in proximity of endothelial cells in tumor sections, Notch signaling is still a key player, being activated by the soluble form of Jagged-1, released in the surrounding environment by endothelial cells (80). The migration of GBM CSC towards endothelial cells, which justifies their perivascular localization, appears to be mediated by IL-8 (CXCL8), a pro-angiogenic chemokine supporting cancer cell invasion and CSC self renewal as well as tumor vascularization (81,82). Consistently, IL-6 secreted by endothelial cells promotes sphere formation and self-renewal of head and neck SCC stem cells (83). The dependence of CSC by vascular niches is therefore evident in neural tumors (77,84) as well as in head and neck SCC, where CSC reside in close proximity to blood vessels. Conditioned media from endothelial cells induces Bmi-1 expression (a known CSC marker) and promotes self-renewal and proliferative potential of ALDH+/CD44+ head and neck SCC CSC (85). In addition, selective ablation of endothelial cells *in vivo* significantly reduces the proportion of $ALDH^+CD44^+Lin^-$ cells in the tumors (85). It is therefore clear that endothelial cells maintain stem-like cells and their activities in tumors. Endothelial cells exert their functions also by secreting growth factors, such as epidermal growth factor. In human head and neck SCC, epidermal growth factor secretion by endothelial cells induces EMT and stem cell features in tumor cells; when EGF secretion is inhibited in endothelial cells, *in vivo* xenograft-derived tumors were less invasive and contained a lower proportion of $ALDH^+CD44^+$ CSC (86).

Angiogenesis provides the necessary nutrient and oxygen supplies required for cancer cell survival, growth and dissemination (87–89). Tumor cells can make their own vasculature through different mechanisms, including formation of new vessels from pre-existing ones, simulation of vasculature through vasculogenic mimicry and recruitment of endothelial progenitor cells. CSC are promoters of tumor vascularization. In renal cell carcinomas, the CD105+ cell fraction, which retains properties of CSC, such as clonogenic ability and overexpression of pluripotency-associated genes, such as nanog and Oct-3/4, is able to uniquely secrete microvesicles containing several pro-angiogenic mRNAs (VEGF, FGF, angiopoietin1, matrix metalloproteinases) and microRNAs associated with unfavorable prognosis (90). Microvesicles and exosomes containing proteins, mRNAs, microRNAs are mediators of cell-cell communications and sustain tumor progression. In renal cell carcinoma, CSC microvesicles are able to create a pre-metastatic niche in the lung and support metastatic diffusion of tumor cells (90). Another mechanism through which cancer cells sustain tumor perfusion is vasculogenic mimicry, the *de novo* formation of vascular-like tubular structures, perfused by plasma and red blood cells, was first discovered in melanomas (91). The ability of cancer cells to behave as endothelial cells is induced by hypoxia, occurring both in highly proliferating-fast growing tumors or following anti-angiogenic therapies. This suggests that retention of cell plasticity is a pre-requisite for vasculogenic mimicry and consistent evidence suggests that CSC actively participate in this mechanism (92). In melanoma, for instance, $ABCB5^+$ stem cells are also $CD144^+$ vasculogenic mimicry performing cells (93,94). Consistently, a population among the $CD133^+$ fraction of brain tumor cells resembles endothelial progenitor cells, distinct but co-existing with brain tumor CSC, with pro-angiogenic potential strongly dependent on hypoxia (95). Finally, endothelial cells may promote cancer cell conversion towards the endothelial phenotype. In gliomas, VEGF secretion by endothelial cells stimulates glioma stem-like cells expressing highest levels of VEGFR-2 to undergo vasculogenic mimicry (96).

Novel therapies targeting CSC and the microenvironment

Cancer immuno-therapy is among the novel anti-cancer therapies with a recent increasing success. Immune checkpoint receptors, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1) are both inhibitory mediators that limit T-cell–mediated immune responses. CTLA4 is an inhibitory receptor expressed by T-cells, acting by inhibiting the T-cell activating receptor CD28. PD-ligand-1 [PD-L1, the ligand of the programmed cell death-1 receptor (PDCD1 or PD-1)] interaction with PD-1 receptor inhibits T-cell activation and proliferation and CTL-mediated lysis. In 2012, three different studies on melanoma, kidney cancer and lung cancer patients treated with anti-PD-1 mAb therapy resulted in high rate of tumor regression (97–99). Tumors can maintain immune tolerance against these novel drugs by expressing PD-L1 (100). Interestingly, CSC/CIC seem to have unique immune evasion features including overexpression of PD-1/PD-L1 molecules. In melanoma, the $ABCB5^+$ CSC cellular subset selectively express the B7.2 (a CTLA4 ligand) and PD-1 (PD-L1 receptor) as compared to bulk and negative populations (101). Similarly, in lung SCC, $SCA1^+NGFR1^+$ cells, displaying increased tumor-propagating activity compared with bulk cells, also show enrichment for PD-L1 expression (102). All together, these data suggest the potential of immunotherapy for the eradication of CSC in different tumor settings. The U.S.A. FDA approved the use of anti-CTLA4 anti-PD-1 and anti-PD-L1 antibodies for the treatment of metastatic melanoma patients (103,104). Whether anti-CTLA-4 antibody activated T-cells target CSC or bulk tumor cells is still unclear.

Given the importance of angiogenesis to sustain tumor growth and promote disease progression, also by interplaying with CSC, anti-angiogenic therapy represents a valid approach to target tumor microenvironment and starve CSC. However, CSC are often responsible for resistance to anti-angiogenic therapy. The use of anti-angiogenic agents in cancer therapy generates intra-tumor hypoxia, which is accompanied by an increased expression of HIF-1α and HIF-2α. We have previously reported the ability of HIF-1α and HIF-2α to sustain CSC in cancers. In breast cancer, the administration of the VEGF receptor tyrosine kinase inhibitor

sunitinib and bevacizumab (a humanized monoclonal antibody blocking VEGF-A) induces tumor hypoxia; therefore, increasing the CSC population (105). Consistently, bevacizumab often improves cancer patient outcome, however, nearly all patients progress. In GBM, the combination of bevacizumab with a CXCR4 inhibitor depleted CIC residing in the perivascular niche, while in the clinic bevacizumab alone is sufficient to prevent the neo-formation of a perivascular niche, but not to deplete GBM CIC (106). By using a human NSCLC hetero-transplant model (which contains human stromal cells), Zhao and colleagues (107) demonstrated that treatment with chemotherapy causes initial tumor shrinkage with a substantial up-regulation of stem-cell associated genes, suggesting that chemotherapeutic regimens spare CSC. The combination of chemotherapy or bevacizumab with an anti-hepatoma-derived growth factor antibody seems to impair CSC, preventing tumor relapse and progression (107). In GBM, VEGFR2 receptor is preferentially expressed by $CD133^+$ GBM CSC, sustaining their viability and tumorigenic potential. Neuropilin-1 (NRP1), an important pro-angiogenic factor, stabilizes VEGFR2 when bound to VEGF ligand, thus promoting VEGF-VEGFR2 induced pro-survival signaling. Knockdown of NRP1 decreases VEGFR2 levels with marked apoptosis and decreased survival of GBM CSC, thus indicating that targeting the VEGF–VEGFR2–NRP1 interplay is a novel attractive therapeutic strategy in GBM (108). Anti-angiogenic therapies might therefore reduce the proportion of CSC in different tumors, thus being a valuable therapeutic approach to eradicate resistant and aggressive tumor cells. However, resistance mechanisms, such as vasculogenic mimicry performed by CSC, should not be underestimated.

Although not necessarily linked (109), previously reported data support a strong association between EMT and stemness phenotype in tumors (110–112). A recent study aimed at clarifying the mechanisms that render GBM resistant to anti-angiogenic therapies found that treatment of tumors with bevacizumb increased tumor vascularity at the time of tumor progression and changes tumor phenotype towards a mesenchymal phenotype (113). This suggests that anti-angiogenic therapies in heterogeneous and highly plastic tumors, such as GBM need to be delivered cautiously as it may push tumor cells towards a more invasive migratory metastasis-associated phenotype.

Targeting tumor cell quiescence represents a new and promising approach to eradicate CSC in cancers. Quiescent cells are refractory to anti-proliferative therapies, such as canonical chemotherapy. CSC are often found in a quiescent state in native tumors, although retaining the highest proliferative potential. Therefore, either CSC need to be ''awakened'' in order to be targetable, or tumor dormancy could be pursued with specific therapies in order to maintain, rather than awake, dormant CSC. In melanoma, histone deacetylase inhibitors (HDACi) reduced the metastatic-risk of melanoma cell lines, prolonging the dormancy of these cells (114).

Recently, significant attention has been given to biguanides, as drugs able to target both cancer and microenvironmental compartments (115,116). The biguanide class of compounds, which mainly includes metformin and phenformin, display chemopreventive properties, with chemoprevention indicating natural or synthetic compounds suppressing or preventing carcinogenesis. Metformin, a hypoglicemic drug is the first line treatment for type-II diabetic patients; on the other hand, the anti-diabetic phenformin was withdrawn from the market for rare but on occasion lethal induction of acidosis. Several clinical and epidemiological studies have shown that metformin is associated with a reduction in risk of developing cancers, apparently by activating AMP kinase (AMPK) and insulin/IGF-1 signaling pathways. Both metformin and phenformin appear to possess the ability to attenuate the CSC phenotype. In GBM, the chloride intracellular channel-1 (CLIC-1) modulates cell cycle progression of CSC and is necessary for GBM tumorigenesis. Interestingly, metformin targets CLIC-1 thus abrogating stem-cell features in GBM (117). Metformin inhibits inflammation by decreasing inflammation-associated genes (Lin28B, VEGF, signal transducer and activator of transcription 3 (STAT3) phosphorylation), necessary for switching normal cells towards transformation (118). In addition, the effects of metformin on inflammatory pathways result in inhibition of CSC growth (118). However, resistance to metformin and metastatic dissemination of tumor cells has been reported, and it is associated with the activation of pro-migratory, stem-like, EMT-associated signals (119). There are only few data on the effects of phenformin on CSC. However, in melanoma, phenformin selectively targets JARID1B (Lysine-specific demethylase 5B)-positive cells which have been shown to mark slow cycling, stem-like melanoma cells. Further, a combination of phenformin with vemurafenib (a BRAF inhibitor) gives a therapeutic advantage by inducing tumor regression in mouse models of melanoma, possibly by decreasing the fraction of aggressive stem-like cells in the tumor (120). Metformin and phenformin seem to exert different effects on cell metabolism depending on the stage of cellular transformation. Both drugs target mitochondrial complex I by inducing the tricarboxylic acid (TCA) cycle during transformation, while depleting nucleotide triphosphates and blocking nucleotide synthesis in CSC (121).

In the context of the immune escape by CSC, a possible strategy to overcome CSC resistance to NK-induced killing is to modulate surface activating NK receptor expression in cancer cells to elicit NK-mediated immune response against CSC. In glioblastoma, inhibition of metalloproteinases, such as ADAM10 and ADAM17, which are able to cleave extracellular domains of MICA (MHC class I chain-related proteins) and ULBP2 (UL16-binding protein-2), increase cell surface expression of ULBP2, which results in enhanced recognition and cytolysis of GBM CSC cells by NK cells (122). In GBM, activated NK cells following stimulation with IL-2 or IL-15 were able to successfully lyse GBM CSC (71). Recently, cytotoxic and matured NK cells from induced pluripotent stem cells and human embryonic stem cells able to persist in the tumor and migrate to the tumor site have been generated, and appear to be suitable for future anti-cancer therapeutic applications as well as to study NK killing efficiency against CSC (123). In addition, NK-cell-based immunotherapy of cancer patients is gaining in clinical interest and includes genetic modification of NK cells, infusion of activated autologous and allogeneic NK cells,

and *in vivo* expansion of endogenous NK cells by cytokines [reviewed in (124)]. NK cells are able to kill CSC in some tumor settings (71,73,125), thus they represent a valuable therapeutic instrument to target refractory CSC.

Conclusions

Anti-cancer therapy includes several crucial effects: (1) killing of cancer cells, (2) pushing cancer cells towards a differentiated state, (3) inducing cellular quiescence of highly proliferating cells, (4) modulating the TUMIC to suffocate tumor cells, (5) polarizing immune cells towards an anti-tumor phenotype and functions. CSC might be able to evade the mechanisms associated to these therapeutic strategies; however, if a stronger effort will be put to the comprehension of the signaling pathways, cellular and molecular mechanisms underlying the interactions between these cells and the microenvironment and how these interactions modulate the state of these cells, including proliferative and metabolic activities, new therapeutic drugs and approaches will pave the way to anti-cancer therapeutic successes. Finally, both immuno-therapy and chemoprevention are novel promising strategies in the battle against drug-resistant cancer cells, including CSC.

Acknowledgments

We thank Alessandra Panvini Rosati for assistance and Dr Paola Corradino and Dr Martina Inoretti for bibliographic searches.

Declaration of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Our studies are funded by AIRC (Associazione Italiana per la Ricerca sul Cancro, IG10228 and IG15895), Italian Ministry of Health, PRIN (Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale, 2010NECHBX_003), and a Fondazione Umberto Veronesi fellowship (to KD). AB was a FIRC (Fondazione Italiana per la Ricerca sul Cancro) fellow and is a recipient of a fellowship from the University of Insubria.

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