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Cancer Stem Cells, Cancer Cell Plasticity and Radiation Therapy

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Summary

Since the first prospective identification of cancer stem cells in solid cancers the cancer stem cell hypothesis has reemerged as a research topic of increasing interest. It postulates that solid cancers are organized hierarchically with a small number of cancer stem cells driving tumor growth, repopulation after injury and metastasis. They give rise to differentiated progeny, which lack these features. The model predicts that for any therapy to provide cure, all cancer stem cells have to be eliminated while the survival of differentiated progeny is less critical. In this review we discuss recent reports challenging the idea of a unidirectional differentiation of cancer cells. These reports provide evidence supporting the idea that non-stem cancer cells exhibit a remarkable degree of plasticity that allows them to re-acquire cancer stem cell traits, especially in the context of radiation therapy. We summarize conditions under which differentiation is reversed and discuss the current knowledge of the underlying mechanisms.

Keywords

Cancer stem cells; reprogramming; radiation therapy

Introduction

In 2010, the estimated medical costs of cancer care in the United States exceeded $124 billion (source: National Cancer Institute). Yet, despite the enormous spending for cancer care, many cancers are still fatal and 5-year survival rates have not significantly changed over the decades. This raises the question as to whether current radiation treatment approaches can be technically further fine-tuned to improve cancer cure rates, or if cancer therapy in general needs a paradigm shift to substantially improve future outcome.

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Aside from surgery, other current standard cancer therapies, such as radiotherapy, chemotherapy, and most targeted therapies have been designed, developed and evaluated for their effectiveness based on bulk tumor responses. This approach for developing novel anti-cancer therapies continues to be widely applied despite our broad knowledge of the undisputable heterogeneity of human tumors. It has been known for over a century that tumors exhibit a remarkable phenotypical heterogeneity, which extends to their radiosensitivity, drug resistance and genetic alterations of the individual cells composing a tumor mass. Employing bulk tumor responses as the primary end point for determining the effectiveness of novel treatments is certainly a very practical approach. However, such an approach will only be successful in yielding cures if the response of the bulk tumor represents the response of the most resistant subpopulation of cells within the heterogeneous tumor and this might hold true for only some cancers like advanced melanoma.

In this review we hope to add to the ongoing discussions about clinically relevant tumor heterogeneity and its potential roots [1, 2]. We will focus on the effects of ionizing radiation on the heterogeneity of solid tumors in the context of competing models of tumor organization, the cancer stem cell hypothesis and the clonal evolution model.

The Cancer Stem Cell Hypothesis

The cancer stem cell (CSC) concept was first formulated in the 1800s, and has its roots in Rudolf Virchow's *Cellular Pathologie* [3], and a case report by Julius Cohnheim in 1875 [4]. A seminal paper by Steven Paget in 1889 first gave rise to the "*seed and soil hypothesis*" for cancer, hypothesizing that cancer cells within a tumor have the intrinsic capability to "seed" a metastasis in a distant organ that has favorable conditions for secondary tumor growth (soil) [5]. In 1961 Pierce and Speers proposed that tumors are caricatures of normal tissue development, thus formulating a hierarchical model for the development and propagation of cancer [6]. However, it took more than 100 years since Rudolf Virchow's article to validate the cancer stem cell hypothesis. In 1994, Dick and colleagues for the first time prospectively isolated leukemia stem cells [7], which not only confirmed the heterogeneity of cancer cells in this cancer, but also gave rise to the hypothesis that tumors are organized in a hierarchical manner. It took another 10 years until marker profiles for highly enriched cancer stem cell populations in solid cancers could be defined [8, 9].

According to the CSC hypothesis, cancers mirror the hierarchical organization of normal tissues with a small number of cancer stem cells at the apex of this hierarchy. In this model, the ability to initiate tumors and to give rise to the heterogeneous cell populations found in the original tumor is exclusively attributed to the CSC population with all of their differentiated progeny lacking these features [10, 11]. In analogy to normal tissue development, the idea of hierarchy and differentiating progeny suggests that epigenetic changes and signaling events regulate the structural organization of a tumor during differentiation events, which are in general believed to be unidirectional.

After the initial description of brain cancer stem cells in 2001 [10, 11], cancer stem cells were identified in breast cancer [12], and melanoma [13] a few years later. Today, cancer stem cell populations continue to be identified and isolated from a growing list of solid

cancers. The presence of CSCs in these different tumor models is generally confirmed by operational means, demonstrating increased tumorigenicity and pluripotency of a subset of cancer cells prospectively isolated from the bulk of the tumor. The gold standard of operational assays for demonstrating a CSC phenotype is the *in vivo* limiting dilution assay using immune-compromised animals [10, 11]. In 2008, the concept of CSCs in solid cancers was challenged when the Morrison lab demonstrated that in advanced melanoma CSC frequencies ranged from 1 in 2 to 1 in 8 cells if NOD/SCID interleukin-2 receptor gamma chain null (*Il2rg*$^{-/-}$) (NSG) mice were used for the *in vivo* limiting dilution assays and Matrigel was mixed with the implanted cancer cells [14]. These results were interpreted to suggest that no CSCs exist in melanoma. Recognizing the possibility that some metastatic melanomas may have very high frequencies of tumorigenic cells, a follow-up study by the Weissman lab, characterized CD271^{+} as an alternative CSC marker in melanoma. The authors prospectively isolated melanoma stem cells as a population in CD271^{+} melanoma cells occurring at a frequency of ~ 16% of the total cell population [15]. While cancer stem cells may be a common occurrence in advanced and metastatic melanoma cases, a more recent report by Ishizawa et al. confirmed the low frequency of CSCs in a panel of human pancreatic, non-small cell lung and head and neck carcinomas. This study also confirmed the increased tumorigenicity of CSCs derived from these tumors in both NOD/SCID and NSG immune-deficient mouse models [16]. Taken together with the Weissman report on melanoma, the Ishizawa study suggested that advanced melanomas should not be used as *pars pro toto* for all solid cancers, as an example against the CSC hypothesis.

It is noteworthy to point out that no population of cells exhibiting all the agreed-upon properties of CSCs has yet been isolated, therefore we will discuss below an alternative model for initiation and propagation of cancer, the “clonal evolution model”.

The Clonal Evolution Model

The clonal evolution model of cancer is an alternative model for the organizational structure of tumors initially described by Peter Nowell in 1976 [17]. Similar to the cancer stem cell hypothesis, the model assumes a clonal origin of cancers with the important distinction that it does not propose a hierarchical organization for tumors. The clonal evolution model postulates that the genetic instability of cancer cells leads to different clones of cells that contribute to the cellular heterogeneity of cancers; in turn, subsequent acquisition of additional mutations that favor cellular proliferation generate cells that outcompete other cell populations and become the driving cell population in a tumor [2, 17]. Taking into account the stochastic nature of acquiring additional genetic mutations, this model predicts that every cell in a tumor can acquire cancer stem cell traits through genetic changes, rather than epigenetic modifications. There is indisputable evidence supporting the genetically unstable nature of solid cancers and its contribution to the genetic heterogeneity of solid tumors, even if tumors originate from specific cell clones [18–20]. What is less clear is whether stem cell traits are shifting from one clone to another in a stochastic manner. There is evidence that the clonal evolution model may hold true for some cancers however, a growing body of scientific evidence supports a hierarchical model for the majority of solid tumors [21]. For example, a recent study by Penny et al. looked at Gleason grade progression and found that even though PSA screening leads to a significant decrease of advanced prostate cancers, the

Gleason grade did not follow this trend very closely, suggesting that in the vast majority of prostate cancers the most aggressive cell population arises early during cancer development [22].

Cancer Stem Cell Markers

The CSC hypothesis and the clonal evolution model are not necessarily mutually exclusive. Both models agree on the existence of a subpopulation of cells with increased tumorigenicity in solid cancers. However, a disagreement arises from these models on whether the tumorigenic population of cells within a tumor is static and rare and exhibits stem cell traits, or whether increased tumorigenicity is a transient feature of competing cell populations that shifts from one cell population to another during the malignant progression of a cancer. Such a distinction is crucial in determining whether novel therapeutics specifically targeting CSCs would ultimately lead to cures.

As outlined earlier, the presence of stem cell markers on a subpopulation of cancer cells does not confer a CSC phenotype to that population. Instead, operational assays are necessary to confirm the functional CSC phenotype of the putative population. However, the detection of a variety of cell surface proteins has been successfully applied as surrogate markers for prospective identification of cell populations highly enriched for cells that fulfill the functional definition of CSCs. The first marker used for enriching CSCs in human solid tumors was CD133 (prominin-1), a five-transmembrane glycoprotein with incompletely understood biology [23]. Detection of two glycosylated epitopes, AC133 and AC141 could enrich for CSCs in brain tumors [8, 9, 11]. However, CD133 has been questioned as a reliable CSC marker for a variety of reasons, ranging from the specificity of anti-CD133 magnetic beads [24], to reports claiming that CD133 is solely a marker for bioenergetic stress [25], and others reporting that in some gliomas both, $CD133^{+}$ and $CD133^{-}$ cells fulfill the definition of CSCs [26]. Furthermore, in embryonic stem cells, colon cancer and melanoma, AC133 fluctuated with progression through the cell cycle, with cells in the G_0/G_1 phase of the cell cycle expressing the lowest levels of this epitope [27]. Nonetheless, despite the above-listed caveats, high level of CD133 expression has been used to identify and isolate a highly radioresistant and highly tumorigenic subpopulation of glioma cells from fresh human specimens. In these insightful mechanistic studies, the radioresistance was attributed to hyper-phosphorylation of cell cycle checkpoint kinases and increased DNA repair in glioblastoma CSCs [28]. Importantly, the expression of CD133 in clinical tumor specimen predicted adverse clinical outcome for glioma patients [29, 30]. Aside from glioma, CD133 expression has been used to enrich for CSCs in other solid cancers including hepatocellular carcinoma cell lines [31], human prostate cancers [32], human melanoma [22], and many others. However, the evidence in other tumor entities is less compelling and more controversial than in glioma.

In breast cancer, a marker combination of $CD24^{-/low}/CD44^{high}/ESA^{+}/Lin^{-}$ enriched for CSCs in luminal breast cancers [12]. $CD24^{-/low}/CD44^{high}$ breast CSCs were shown to be highly radioresistant due to efficient free-radical scavenging in breast CSCs [33]. Using a panel of established breast cancer cell lines, Fillmore and Kuperwasser demonstrated that breast cancer cell lines do contain CSCs. However, they also found that the $CD24^{-/low}$/

$CD44^{high}$ marker combination identified cells with a CSC phenotype in only a subset of breast cancers, and did not correlate with tumorigenicity in basal breast cancer cell lines [34].

$CD44^{high}$ has been reported to identify a cell population enriched for CSCs in head and neck squamous cell carcinomas (HNSCC) [35], however due to the ubiquitous expression of CD44 in this tumor entity these results are discussed controversially [36, 37].

The expression and activity of aldehyde dehydroxygenase (ALDH) appears to be a more robust marker for CSCs. ALDHs are a family of 18 isoenzymes expressed in humans. ALDH1 is one of the four vertebrate retinaldehyde dehydrogenases and oxidizes retinal to retinoic acid. Initially, ALDH1 was reported to be elevated in $CD34^{+}$ hematopoietic progenitor cells [38]. Ginestier and colleagues identified ALDH1 activity and expression as a CSC marker for breast cancer and demonstrated that $CD24^{-/low}/CD44^{high}/ALDH1^{+}$ breast cancer cells could form tumors from as few as 20 cells. More importantly, high ALDH1 expression was associated with a poor clinical prognosis [39]. A follow-up study could not confirm these results and suggested that ALDH1 expression is associated with a basal and Her2-positive phenotype and that ALDH1 expression in cancer cells does not correlate with outcome. Interestingly, the latter study reported that high ALDH1 expression in the stromal cells correlated with superior outcome in triple negative patients [40]. A more recent report demonstrated that the ALDH1A3 isoenzyme is the major source of ALDH1 activity in breast cancer stem cells [41] bringing into question the specificity of the antibodies used for the different isoforms of ALDH1. This study may explain some of the conflicting results from different laboratories. Aside from breast cancer, ALDH1 activity is widely used as a marker for CSCs in other solid tumors including head and neck squamous cell carcinoma (reviewed in [42]), colorectal cancer [43], and lung cancer [44].

In addition to CD133, $CD24^{-/low}/CD44^{high}$, and ALDH1 marker systems, additional surface CSC markers have been identified in melanoma [13] and prostate cancer [45]. Our own laboratory discovered that subpopulations of cells in glioma, breast cancer, and HNSCC with reduced activity of the 26S proteasome are enriched for CSCs [46, 47]. The lack of activity is based on a concerted down-regulation of proteasome subunit mRNAs caused by binding of Musashi1 to the 3'-UTR of the mRNA coding for the NF-YA subunit of the tetrameric transcription factor NF-Y [48], the master regulator of basal proteasome subunit expression in mammalian cells [49]. We have exploited this discovery to establish an imaging system for CSCs based on a fusion protein between a reporter protein and the C-terminal degron of murine ornithine decarboxylase (cODC). This unique reporter system for CSCs allows for identification, tracking and isolation of CSC populations over time without further manipulation of cells, *in vitro* and *in vivo* [46]. Other laboratories have employed our reporter system to identify CSC in lung cancer [50], pancreatic cancer [51] and hepatocellular carcinoma [52]. In breast cancer, cells with low proteasome activity partially overlap with $ALDH1^{+}$ and $CD24^{low/-}/CD44^{high}$ cells [53]. In glioma, cells with low proteasome activity express Sox2, Nestin, and Musashi-1 [46]. Most importantly, lack of proteasome subunit expression correlated with inferior survival in glioblastoma [54], HNSCC [47] and breast cancer patients [55].

It is important to emphasize at this point that none of the marker combinations reported in the literature to date are sufficiently specific to prospectively identify individual CSCs. Instead, they only enrich for CSCs populations. Therefore, *in vivo* limiting dilutions assays remain the gold standard for estimating CSCs frequencies.

Stem cells factors and plasticity

The stem cell state of pluripotent normal stem cells is governed by stem cell factors, which become silenced during differentiation as a consequence of DNA methylation and chromatin remodeling. This process is reversible and transfection of somatic cells with the four Yamanaka factors Sox2, Oct4, Klf4, and c-Myc [56] generates induced pluripotent stem (iPS) cells. All four transcription factors are wired in an incompletely understood network with Nanog, a unique set of transcription factors, Poly-comb complexes, microRNAs and histone modification enzymes to allow for a unique permissive chromatin state for reprogramming events to occur (reviewed in [57]). It is noteworthy that the Oct4 [58], Sox2 [59], Klf4 [60], c-Myc [61], and Nanog [62] are all substrates of the 26S proteasome and thus, spared from degradation in putative CSCs that lack proteasome activity.

Oct4 (also known as POU5F) is a homeodomain transcription factor involved in early embryonic development and pluripotency [63] through stabilization of higher-order chromatin structure of the Nanog locus [64]. The expression of Oct4 in cancer cells, outside the context of teratoma, and in differentiated cells has been discussed controversially due to the existence of several transcribed pseudogenes of Oct4 with unknown function. These pseudogenes can lead to false-positive RT-PCR results [65], and unreliable Western blotting and flow cytometry [66]. While these are valid concerns, a growing number of publications report Oct4 expression (and therefore, most likely expression of its pseudogenes) as predictors of adverse clinical outcome in cancer, thus suggesting a functional role for Oct4 (and/or its pseudogenes) in solid cancers. For example, de Resende and coworkers reported shortened biochemical progression-free survival in prostate cancer patients with $Oct4A^{+}$/$Oct4B^{-}$ tumors using a monoclonal antibody considered to specifically recognize the first 134 amino acids of Oct4A [67]. A study by Koukourakis et al., found cytoplasmic expression of Oct4 to be correlated with local control in head and neck cancer patients after chemo-radiotherapy. In the latter study, the antibody used was raised against the C-terminal epitope of Oct4, and, if the cytoplasmic staining pattern is taken into consideration, this antibody most likely only recognized Oct4B [68]. Using the same specific antibody for Oct4A as de Resende et al., Zhang and colleagues found the expression of Oct4A to be inversely correlated with the survival of patients with adenocarcinoma of the lung [69]. In another study on rectal cancer, Salgusa et al. used a non-specific antibody against Oct4 and found cytoplasmic staining and thus, most likely Oct4B expression to be correlated with distant failure [70]. In summary, a growing body of literature points to a possible role of Oct4 expression in solid tumors. The function of Oct4, and its expression patterns of splicing isoforms and pseudogenes remain to be studied in more detail.

Sox2 (SRY [sex-determining region Y)-box 2] is a HMG-box transcription factor that hetero-dimerizes with Oct4. Similar to Oct4, Sox2 is essential for pluripotency in ES cells [71] and for acquired pluripotency in iPS cells [72]. Sox2 is highly expressed in neural stem

cells in the subventricular zone and the dentate gyrus of the central nervous system. In mice it is a marker for stem cells in the stomach, duodenum, cervix, anus, testes, lens, and multiple glands. Sox2 seems to be the only stem cell factor commonly expressed in ES cells, fetal progenitor cells and adult stem cells [73]. In neural stem cells, Sox2 regulates self-renewal through expression of the intermediate filament nestin [74]. Deregulated Sox2 expression in tumor cells has been described by several groups and in different tumor types including gastric cancer [75, 76], breast cancer [77], pancreatic cancer [78], brain tumors [79] and lung cancer [80].

Krüppel-like factor 4 (Klf4) is a zinc-finger-containing transcription factor first described in the gut [81] and in the skin [82]. The downstream events of Klf4 signaling are context-dependent allowing Klf4 to act as a tumor suppressor in the colon [81] and gastric epithelium [83], and as an oncogene leading to breast cancer [84] and squamous cell carcinoma of the skin [85]. When used to reprogram somatic cells into iPS cells, the role of Klf4 is incompletely understood. However, its anti-apoptotic effects have been suggested to contribute to reprograming. It is noteworthy that Klf4 is dispensable for reprogramming somatic cells into pluripotency, and together with c-Myc it can be replaced by Nanog and LIN28 [86]. Many of the target genes of Klf4 are involved in cell cycle control [87]. Klf4 inhibits cell proliferation by inhibiting cells to transition from the G1- to the S-phase of the cell cycle [88]. In response to DNA damage, Klf4 facilitates arrest of the cells in the G1/S checkpoint in a p53-dependent manner and maintains the G_2/M checkpoint of the cell cycle.

The c-Myc oncogene is a member of the family of basic helix-loop-helix leucine zipper transcription factors. In pluripotent stem cells c-Myc is downstream of LIF/STAT3 signaling and acts as a universal amplifier of gene expression. While c-Myc seems to be dispensable for the reprograming of somatic cells it greatly enhances the efficiency of the remaining Yamanaka factors to induce a pluripotent state [72].

The ability of CSCs to self-renew and give rise to more differentiated cell types found in a tumor defines the CSC state. However, it is not clear which molecular pathways govern the CSC state and signaling requirements most likely vary between different tumor entities and even within subpopulations of cells of the same tumor type. For example, Suva et al. recently reported that expression of the neurodevelopmental transcription factors POU3F2, SOX2, SALL2, and OLIG2 was sufficient for the generation of a glioma stem cell phenotype [89]. The functional contribution of many of the markers used to identify CSCs is only incompletely understood, and a number of them, such as CD133, may only be surrogate markers without a functional link to the CSC state.

The existence of a cancer cell subpopulation inside of a tumor with functional properties of CSCs has important implications for clinical anti-cancer therapy. The demonstrated inherent chemo- and radioresistance [28, 33, 90] of CSC populations make them the prime target for any anti-cancer therapy. Importantly, we [54] and others [29, 39] have shown that the number of CSCs in breast cancers and glioma, present at the time of treatment, is inversely correlated with clinical outcome [91]. These observations imply that the existing number of CSCs in a tumor is one of the most important constraints for the curability of a patient. Indeed, initial reports have suggested that radiation preferentially kills non-tumorigenic

cells, thereby enriching for CSCs [28, 33, 92]. However, follow-up studies revealed that the absolute increase in breast CSC numbers detected after exposure to ionizing radiation could not easily be explained by preferential killing of non-tumorigenic cells [53, 93]. In earlier reports, Erenpreisa and colleagues observed up-regulation of embryonic transcription factors Sox2, Oct4, and Nanog in p53-mutated lymphoma cells in response to irradiation [94]. The up-regulation of the transcription factors coincided with radiation-induced polyploidy and the authors concluded that increased gene dose in polyploid cells was sufficient to express Sox2, Oct4, and Nanog at levels beyond the necessary threshold to induce a CSC state. Our own work in breast cancer did not show dependence of this process on p53 mutations, but did confirm radiation-induced preferential up-regulation of Sox2, Oct4, Klf4, and Nanog in polyploid cells, as well as increased expression of these transcription factors if polyploidy was induced pharmacologically [53]. More importantly, our data demonstrated that radiation-induced expression of these factors reprogrammed non-tumorigenic cancer cells and led to acquisition of functional CSC traits *in vivo* [53], offering an explanation for the significant increase in the absolute number of breast CSCs after radiation therapy. A recent study by Ghisolfi et al. confirmed radiation-induced up-regulation of Oct3/4 and Sox2 and acquisition of a CSC phenotype in two hepatocellular carcinoma cell lines [95].

Effect of tumor microenvironment on CSCs plasticity

As summarized above new evidence is emerging supporting the phenomenon of spontaneous and therapy-induced reprogramming of cancer cells into CSCs. The number of these studies is still small; nonetheless they have begun to uncover the mechanism behind the conversion of cancer cells into CSCs. In the case of radiation-induced reprogramming, the re-expression of stem cell factors correlates with the reprogramming events. Although radiation-induced reprogramming of non-stem cells into CSCs is readily observed *in vitro* [53], the *in vivo* scenario may be a more complex network of micro-environmental signals.

In the normal stem cell field extensive effort has been applied to reprogram terminally differentiated somatic cells into pluripotent stem cells, which was rewarded in 2006 when Takahashi and Yamanaka successfully reprogrammed mouse somatic cells into induced pluripotent stem cells by the transduction of only four transcription factors [72]. However, it has been known for many years that reprogramming/dedifferentiation of differentiated cells *in vivo* can happen spontaneously in the absence of exogenous reprogramming factors under the right micro-environmental conditions, as summarized in a recent review [96]. Normal tissue non-stem cells, and their malignant counterparts, have the ability to spontaneously convert into a normal tissue stem cell or CSC state [97]. These observations suggest that the process of differentiation is not unidirectional. A recent report demonstrated the conversion of differentiated broncheo-alveolar epithelial cells into epithelial stem cells upon viral infection or chemical injury *in vivo* [98]. Furthermore, Shaykhiev et al. reported an embryonic stem cell gene expression signature for lung stem/progenitor cells in healthy smokers, which suggests the possibility that acquisition of genetic mutations cooperate with a stress-induced stem cell state to initiate malignant growth [99].

The tumor microenvironment is composed of a variety of cell types, including cancer associated fibroblasts (CAFs), immune cells, endothelial cells lining the tumor vasculature

and extracellular matrix components, among others [100]. For most solid cancers, the CSC niche is currently undefined and the supporting signals for a multi-potent CSC state have not yet been unveiled. However, in brain cancers it has been shown that CSCs preferentially reside in relatively well-oxygenated perivascular niches inside the tumor [101]. Interestingly, endothelial cells express both, CXCL12/SDF1 [102] and Notch ligands [103], which cause chemotaxis towards tumor blood vessels and promote self-renewal in glioma [104], which may hold a druggable target against CSCs. A second, hypoxic niche for glioma cells was described by Jeremy Rich's laboratory [105]. Importantly, hypoxic conditions resulted in reprogramming of CD133-negative glioma cells into CD133-positive cells with a CSC phenotype. These reprogramming events coincided with increased Oct4, Nanog, and c-Myc expression [105]. The same group later reported that exposure of non-stem glioma cells to low pH, frequently found in hypoxic tumors also mediated the acquisition of a CSC phenotype [106].

The above studies demonstrate an apparent preference for localization of CSCs within the tumor microenvironment, thus defining a CSC pseudo-niche. Alternatively, these observations could suggest that the CSC state is not a cell autonomous process, but rather dictated by the tumor microenvironment. In some elegant studies Vermeulen et al. demonstrated that the CSC state of Wnt-activated colon cancer cells was dependent on activation by stromal-derived hepatocyte growth factor (HGF) present in the tumor microenvironment [107]. In a different study Giannoni et al. uncovered the importance of IL-6 in activating tumor-associated fibroblasts, which in turn induced EMT and stemness in prostate cancer cells [108]. In support of these studies, Iliopoulos et al. identified IL-6 as the mediator responsible for converting non-stem cancer cells into CSCs in patient-derived breast cancer specimen and breast and prostate cancer cell lines [109]. A dynamic equilibrium was observed between CSCs and their progeny, resulting in a constant ratio between both cell populations over many passages. Ohanna et al. confirmed these findings reporting that the secretome of melanoma cells entering senescence after chemotherapy induced a CSCs phenotype in non-stem melanoma cells [110]. Taken together, these data suggest that differentiation in normal tissues and cancers is directed, but not unidirectional. There is now clear experimental evidence strongly suggesting that non-stem cells can revert to a stem cell state to compensate for cell loss in the stem cell compartment in a normal or malignant tissue. Most importantly, these studies strongly support the idea that cancer stem cell plasticity, a phenomenon now observed by many labs to occur spontaneously, or in response to therapy, is tightly regulated by the tumor microenvironment.

Clinical radiation therapy applied in daily fractions of 2 Gy from different directions with the tumor mass at the center, effectively controls tumor growth while sparing normal tissues. Still stromal cells inside or adjacent to the tumor, such as cancer-associated fibroblasts (CAF) receive considerable amounts of radiation, which can affect their interaction with cancer cells. Although the molecular pathways remain to be elucidated, collectively, most literature highlights the pro-malignant phenotype acquired by CAFs after radiation exposure [111, 112]. Therapy-induced changes in the tumor stroma may play a crucial role in ultimately determining the degree of phenotypic plasticity of cancer cells by specific therapies. Ionizing radiation generates waves of primary and secondary reactive oxygen species in cells, with the latter persisting for weeks and months after the irradiation event.

The late responses to radiation treatment can have an effect on lipids, proteins and most importantly DNA [113, 114]. Such events give rise to a pro-inflammatory microenvironment characterized by increased expression of TNFα, pro-inflammatory interleukins and interferon-γ [113, 114] and consequently activation of downstream STAT3 signaling, which is known to promote self-renewal in embryonic stem cells [115].

Epithelial-to-mesenchymal transition (EMT) and its reverse process, mesenchymal-to epithelial transition (MET) are essential cellular transitions during embryonic development. A plethora of scientific evidence has also implicated these two processes (EMT and MET) in tumor development and metastasis. EMT is regulated by a variety of different pathways including proinflammatory signals like NF-κB and TGF-β signaling. Interestingly, these pathways are activated in response to irradiation.

The Wicha laboratory recently reported that breast cancers have two different pools of CSCs: (1) an ALDH1-positive population with an epithelial phenotype found in hypoxic areas of a tumor and (2) a $CD24^{-}/CD44^{+}$ population of cells at the invasive front of the tumor exhibiting a mesenchymal phenotype. Their study provided convincing evidence that both populations could convert into each other via EMT/MET transitions [116]. Unfortunately, the study did not address if cells from the bulk tumor population, lacking both the ALDH1-positive and the $CD24^{-}/CD44^{+}$ phenotype, could convert into a CSC and if this process was also mediated by EMT/MET.

Concluding remarks

For most cancers, survival rates have remained unchanged for decades and systemic disease is almost always fatal. Experimental and clinical data provide a growing body of evidence supporting the hierarchical organization of cancers with a small number CSCs able to self-renew, repopulate a tumor after treatment and initiate metastatic growth. The resistance of CSCs to chemotherapy and their relative resistance to radiotherapy explain why macroscopic tumor response to anti-cancer treatments is not a robust predictor for clinical outcome. Yet, most established chemotherapies, including targeted therapies, have been and continue to be developed based on their effects on bulk tumor cell populations despite the knowledge that responses of the bulk tumor cell populations are unable to predict effects on the CSC population. It is worth noting that proliferation is a prerequisite for most chemotherapeutics, as well as radiation therapy to be effective and that any senescent and quiescent (non-CSCs) cells would also be resistant to these therapies. Interestingly, we found that about one third of the CSC population in established glioma and breast cancer cell lines were in a quiescent state and were recruited into the cell cycle after irradiation [46, 93]. In contrary, non-tumorigenic cells entered a senescent state after exposure to radiation [93]. While senescent cells have a finite life span and would not contribute to tumor relapse, a rare population of quiescent cells without stem cell characteristics (i.e. self-renewal, tumorigenicity) could eventually terminally differentiate and not contribute to tumor relapse or acquire CSC traits.

The discovery of marker profiles for the prospective identification of CSCs has led to a renaissance of the CSC hypothesis, and has reinvigorated the hope of identifying novel

treatments that specifically target CSCs. However, the growing number of reports suggesting a remarkable plasticity of the non-stem cell population adds an additional layer to the already complex picture of treatment responses of cancer cells. Understanding the underlying mechanisms could hold the key for improving the efficacy of chemotherapy and radiation treatment in the future. The role of the tumor microenvironment on the reprogramming of cancer cells has not been fully established, however the existing data suggests that modifying the tumor microenvironment (i.e. IL-6 depletion) during treatment may also offer therapeutic benefit. It will be very important that future studies shed more light on the role of the tumor microenvironment on therapy-induced reprogramming and plasticity of cancer cells. Deeper insight into this phenomenon will be very important in enhancing future anti-cancer therapies.

Recent data on the prospective identification of CSCs supports the CSC hypothesis with a hierarchical organization of tumors [9, 11, 12]. However, it also highlights the phenotypical plasticity of cancer cells in support of the clonal evolution model [14]. While the classic clonal evolution model describes acquisition of CSC traits as a stochastic process, recent studies indicate that this process is not random, especially in response to cellular injury as seen after radiation therapy [53]. This has important clinical implications for radiation therapy. The CSC hypothesis predicts that a drug that targets CSCs specifically would lead to improved outcome or even cure with no real need for radiation therapy since all progeny lack the ability to repopulate the tumor and would eventually die or enter senescence. The response predicted by the clonal evolution model is more complex, as every cancer cell could acquire CSC traits over time through acquisition of a diverse set of mutations and many of these clones would most likely escape a drug with a limited scope of molecular targets thus, indicating the need for radiotherapy.

The observation that radiotherapy as well as chemotherapy are driving forces in the acquisition of an induced CSC phenotype suggest that this dedifferentiation step is less random and rather a direct response of the cancer cell population to treatment. Importantly, when the rate at which a treatment-induced, treatment-refractory CSC phenotype is acquired, exceeds the rate of cell killing, cure becomes impossible. Conversely, drugs that interfere with treatment-induced reprogramming may significantly improve the efficacy of the therapy with only few or no side effects, as opposed to classic anti-cancer agents in the past, which added little therapeutic benefit while contributing significantly to the increased toxicity of combined treatments. Spontaneous, as well as therapy-induced reprogramming of cancer cells is most likely subject to complex negative and positive feedback regulation and it remains to be investigated if the molecular mechanisms that underlie therapy-induced reprogramming are unique to cancer or if normal tissue damage repair employs the same pathways before it can be determined if this phenomenon has druggable targets with a therapeutic window.

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