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REVIEW ARTICLE

Cancer Stem Cells and Their Mechanism of Chemo-Radiation Resistance

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The advent of cancer stem cell (CSC) hypothesis has revolutionized the cancer biology community's thinking in explaining the notorious resistance of cancer to conventional chemo- and radiotherapies. The hypothesis states that the CSCs are a subpopulation within the tumor endowed with superior resistance and with the exclusive ability to self-renew, differentiate into diverse type of progeny cancer cells, and initiate tumor. Here, we review recent literature that seek out to explain such resistance of CSCs. Signaling pathways involved in the regulation of proliferation and differentiation of stem cells (e.g. Notch, Hh, and Wnt) and efficient ABC transporter systems and DNA damage response machineries are starting to be identified as the means by which CSCs out-survive their non-CSC neighbors after conventional anti-cancer treatments. Direct links between receptor tyrosine kinase pathways and CSCs are also starting to emerge as well. Lastly, a promising relationship between epithelial-mesenchymal transition and CSCs is discussed. Though the precise resistance pathway of CSCs is not yet fully elucidated, the various mechanisms highlighted here provide promise for better fundamental understanding of CSCs and the subsequent development of a more effective CSC-targeting therapeutic in the foreseeable future.

Keywords: Cancer stem cells, Self-renewal pathways, Receptor tyrosine kinase pathways, ABC transporters, DNA damage response, Epithelial-mesenchymal transition

Introduction

Cancer is broadly defined as a group of diseases characterized by uncontrolled growth and spread of abnormal cells having genetic or epigenetic alterations in the cells. Since the declaration of "war on cancer" about 50 years ago, significant strides have been made in battling the diseases thanks to the worldwide scientific community's concerted effort to better understand cancer biology. In the past ten years alone, over 830,000 research papers have been indexed with Pubmed that addresses cancer. The treatment of cancer varies depending on its type and the situation of the individual patient, but in general, it has become a common practice to prescribe a combination of surgery, chemotherapy and radiotherapy. Yet despite these rigorous treatment modalities, cancer still plagues us as a largely incurable disease, especially for the patients who detect the malignant neoplasm at a late stage. In addition, frequent metastasis and recurrence further frustrates even our best treatments currently available.

In recent years, however, there has been an exciting and promising development in cancer research with the introduction of the cancer stem cell (CSC) hypothesis. CSCs (sometimes also called cancer/tumor-initiating cells) are defined as a subpopulation of cells within a tumor mass with the ability to self-renew, differentiate into a diverse type of progeny cells that make up the tumor, and reproduce the original tumor after xenotransplantation (1, 2). The hypothesis states that only the CSCs possess tu-

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mor-initiating potential whereas non-CSCs do not. Thus, the CSC hypothesis elegantly accounts for the means by which the cancer cells survive the current treatment modalities, as well as the even more complex issue of how they recur or metastasize to distal locations in the body.

However, the CSC field is still relatively nascent, and it is yet to be seen what particular molecular mechanisms purportedly endow CSCs their superior ability to resist and survive chemoradiotherapy. What remains to be seen is if some of the canonical pathways elucidated with general cancer biology also apply to CSCs. Therefore in this review, we summarize some of these key pathways involved in chemo-radiation resistance and discuss their possible relationship with CSCs.

Controversies with identifying specific markers of cancer stem cells

The first hurdle to overcome when studying CSCs is to isolate them from the heterogeneous tumor mass. In some definition of CSCs, it is proposed that the subpopulation that possess tumor-initiating potential is a small minority, sometimes as low as $<1\%$ (2). Isolation of such a small subset of cells was a near impossible task for some time, but recent breakthroughs in the development of efficient cell sorting systems (e.g. magnetic- and fluorescent-activated cell sorting) has enabled researchers to prospectively isolate CSCs by utilizing distinct cell surface markers that they possess. Each type of tumor has distinct combinations of surface markers that demark the CSCs. Commonly utilized surface markers include CD44, CD24, and CD133 (see (1) for a summary). These markers are considered CSC markers based on the observation that the xenotransplantation of such fractionated cells recapitulate the original tumor in immunocompromised mice.

However, the evidence is not irreproachable as recent publications question the validity of certain CSC markers (3). In glioma, for example, CD133 is a widely used CSC marker (4). $CD133^+$ cells were reported to be more tumorigenic via a higher expression of c-Myc than the $CD133^-$ cells (5). This appears to be conflicting with our (6) and others' data (7-9) that show that both $CD133^+$ and $CD133^-$ cells have similar tumor-initiating potential. Perhaps the differences can be reconciled when considering that $CD133^+$ and $CD133^-$ possess differing global gene expression patterns despite seemingly equal tumor-initiating potential. At the least, this implies that there are at least two types of CSCs in glioblastoma ($CD133^+$ and $CD133^-$ CSCs) (6), and we have since suggested SSEA-1 as a better GBM CSC marker (10). These studies therefore serve as cautious examples of the need to carefully evaluate and characterize the proposed CSC surface markers. Although CD133, CD24, CD44 and SSEA-1 have been frequently utilized to identify CSCs in various kinds of cancers, their functional implications in the CSCs remain to be determined. Therefore, the actual functional roles of the markers will also need to be evaluated to elucidate their precise contribution to the CSC phenotype and their possible role in CSCs' chemo-radiation resistance.

Developmental pathways of stem cells and their potential roles in CSC biology

In studying the mechanisms of chemo-radiation resistance of CSC, the hope is to discover a set of signaling pathways that are unique to CSCs and endow them the ability to resist and survive the current chemo-radiation therapeutic modalities. Targeting such set of pathways would ideally provide effective therapeutics that can potentially eradicate tumors entirely. Though such ideal pathway has not been found yet, developmental pathways that control survival, proliferation and differentiation of stem cells are under scrutiny of researchers. Stem cells in the various tissues are supposed to be involved in the repair process of damaged tissue. Therefore, they should survive insults and then proliferate to make functional cells. Since many evidences suggest that cancers originate from stem cells (11), it is reasonable that many of survival and proliferation pathways of stem cells have aberrant expression in cancer cells. Some of the 'classic' pathways that have been suggested to thus characterize cancer cells are Notch, Hedgehog (Hh), and Wingless/Int (Wnt) pathways (12-14). Although a direct evidence that links CSCs with these pathways are few in number, it is appealing to observe the parallels between stem cell biology and cancer biology and recapitulate the roles of these pathways to cancer cells.

When Notch is inappropriately activated, signals are transduced to up-regulate the translation of genes related to proliferation and down-regulate the translation of genes related to differentiation (12). In breast cancer, Notch was implicated to be involved in brain metastasis by endowing cancer cells increased migrative and invasive character (15). Phillips et al. (16) observed that the prospectively isolated $CD24^-/CD44^+$ breast CSCs were more resistant to radiation and that radiation induced an increased expression of Notch-1 and Jagged-1, suggesting that breast CSCs are radioresistant by utilizing the Notch pathway. Tanaka et al. (17) also showed that these breast CSCs (as

well as the Hoechst dye excluding side population) also have a high expression of Hh signaling pathway (increased expression of Shh and Gli1). Hence they were sensitive to treatment with cyclopamine but not to paclitaxel. Together, these data imply that breast CSCs are resistant to chemo-radiation through Notch and Hh signaling pathways. It is therefore tempting to speculate that a complete eradication of the breast tumor cells might be possible by first using radiotherapy to kill non-CSCs, followed by an adjuvant chemotherapy using cyclopamine or other Hh inhibiting agents to kill any remaining CSCs.

Hh and Wnt signaling pathways also trigger transcription of self renewal and proliferation genes (e.g. cyclin D, c-Myc), and continuous Hh and Wnt activation is associated with many types of human cancers by stimulating stem cell proliferation (13, 14). Ayyanan et al. (18) showed that increased Wnt signaling can lead to oncogenic transformation of mammary epithelial cells into cancer cells via an increased DNA damage response and increased Notch activation. Although their report did not specifically link their observation with breast CSCs, their data certainly corroborates with the CSC hypothesis and further suggest that CSCs may have a high activation of Wnt and Notch. Further studies will be necessary to more directly elucidate such relationship.

Taken together, these studies illustrate that it is important to study multiple pathways simultaneously to better detangle the complex network of crosstalk of various signaling pathways. It is highly likely that the Notch, Hh, and Wnt pathways would have key roles in CSC maintenance via self-renewal, and many studies have considered their roles and mechanisms in isolation from one another. It would behoove us to attempt more systemic approach offered by panoptic analyses, such as those of systems biology. Also, more direct links between CSCs and self-renewal pathways will need to be explored, again highlighting the importance of an efficient prospective isolation of CSCs.

Chemo-radiation resistance through ABC transporter systems and DNA damage repair machineries

There are several possibilities by which CSCs possess therapeutic resistance, and we here focus on ABC transporter systems and DNA damage repair mechanisms. Recent studies with hematopoietic stem cells (HSCs) led to the observation that stem cells possess higher levels of ABC transporters, such as MDR1 and ABCG2 (19, 20). The parallels between stem cells and cancer cells have thus encouraged the hypothesis that cancer might have similar mechanisms of chemoresistance. Consequently, it is postulated that CSCs have a particularly efficient drug efflux pump systems compared to non-CSCs, and encouraging evidence is starting to emerge towards that end. Hirschmann-Jax et al. (21) provided a key contribution by identifying ABC transporters as the means by which side population (SP) have an increased capability to efflux chemotoxic drugs in various tumor cells. Although not all SP cells are necessarily CSCs, it certainly should possess an enriched CSC population. A recent work also showed that breast cancer $CD44^{+}/CD24^{-}$ CSCs and SP cells have comparable resistance to paclitaxel and an analogous sensitivity to cyclopamine, illustrating that SP and CSC populations are overlapping populations with similar anti-drug mechanisms (17). In hepatocellular carcinoma, SP from four different cell lines all exhibited higher resistance to chemotherapeutic drugs 5-FU, MMC, and cisplatin than main population cells (22). This was observed with a correlating increased expression (two- to six-fold) of ABCG2, a member of the ABC transporter family. Furthermore, MDR protein expression itself could be used as a discriminating marker for CSCs, at least in melanoma (23). Provocatively, it was shown that MDR^{+} cells also have increased expression of hTERT, nanog, and ABCB5. Taken together, these data seem to suggest that signaling pathways such as Hh pathway regulate the expression of ABC transporters which in turn feedback to further regulate cell survival and self renewal. It would be interesting to evaluate whether transporter proteins could be used as CSC markers in other types of cancers as well.

Since CSCs are hypothesized to have higher radioresistance, and since radiotherapy (RT) kills cells by inducing DNA damage, it is not farfetched to speculate that CSCs resist RT by a superior DNA damage repair than non-CSCs akin to normal stem cells. Such was implicated in a recent study by Bao et al. (24). Radiotherapy was shown to enrich $CD133^{+}$ glioma CSCs two- to four-fold, and these enriched cells had lower rates of apoptosis and higher activation of DNA damage repair (i.e. ATM, Rad17, Chk1, and Chk2). Furthermore, there also seems to be a link between the self-renewal pathways and DNA damage repair. One of the Wnt pathway effectors is survivin, a protein that helps cell survival in apoptosis-inducing conditions (25, 26). Also, a recent work suggests stem cells can have lower levels of reactive oxygen species (ROS) than the non-cancer stem cells (27). These findings provide another significant link between CSCs and normal stem cells since earlier reports indicated that lower ROS levels is characteristic of the parental pluripotent normal

stem cells than their more mature progeny. In short, these studies provide encouraging explanations of how CSCs resist cytotoxic drug and radiation therapies, but are still shy of providing precise mechanism of resistance. In addition, it would be interesting if future studies would address CSC survival when both chemo- and radiation treatments are concurrently applied, and thus provide a more clinically relevant mechanism of CSCs' resistance.

Implications of receptor tyrosine kinase pathways in the chemo-radiation resistance

Classic cancer biology research (i.e. not necessarily related with CSC theory) yielded many cancer-related pathways and their possible roles in treatment resistance. For example, a recent comprehensive genetic analysis revealed that in majority of cases in GBMs (86% of 206 samples tested), there is an overexpression and/or mutations of key receptor tyrosine kinases (RTKs), such as EGFR, ERBB2, PDGFRA, and Met, and their effectors (28). It is well known that EGFR activates various downstream pathways (e.g. Ras/MAPK, phospholipase C, PI3K/Akt, STAT, and SRC/FAK) and their role in treatment resistance have been well-studied (29). Therefore, a naturally occurring question is whether these pathways and their constituents have increased expression or activity in CSC subpopulation.

A key report that correlates RTK to CSC has been implicated in breast cancer. Korkaya et al. (30) showed that ERBB2 (also known as Her2, human epidermal growth factor receptor 2) overexpressed population in breast cancer is the main player that mediate carcinogenesis, tumor growth, and invasion. More poignantly, they showed that increasing ERBB2 expression can lead to increased CSC population (probed as $ALDH^+$ cells) and suggested that trastuzumab's remarkable clinical efficacy may be due to its specific targeting of breast CSC. However, it is well-known that trastuzumab is only efficacious against Her2-expressing breast cancer (31), and recently, trastuzumab-resistant Her2-expressing breast cancer has also been documented (32). So in order to accommodate Korkaya et al. (30)'s speculation, at least two types of breast CSCs (trastuzumab-sensitive and non-sensitive CSCs) must then be considered, further complicating the possible mechanism of breast CSCs chemo-radiation resistance.

Nevertheless, Korkaya et al. (30)'s work is welcomed for connecting the 'classical' RTK cancer pathways to CSCs. Provided that RTKs can indeed be directly linked with CSCs, one possible straightforward explanation for CSC's chemoresistance may be their utilization of alternative RTK (e.g. Met) for continued tumorigenicity. This is possible since the above mentioned RTKs share similar effectors (e.g. PI3K/AKT pathway). In addition, to our knowledge, such a relationship between RTKs and CSCs has not been reported yet in other solid tumors. In GBMs for example, we recently showed that Met is a good prognostic marker for GBM (33), and we are currently collecting promising evidence that it can also be used for enriching glioblastoma CSC [K.M.J., J.J., Y.K., and D.H.N., unpublished data, 2009]. Taken together, we speculate that increased activity of the RTK pathways and their constituents either specifically demark CSC subpopulation or provide CSC their chemoresistance.

Correlation between epithelial-mesenchymal transition (EMT) and resistance to the conventional anti-cancer treatments

Epithelial-mesenchymal transition (EMT), a process characterized by morphological change from epithelium to mesenchyme, decreased intercellular contact, and increased cellular motility, has also been recently been implicated to provide cancer cells their resistant and metastatic capabilities (34). It was further suggested that CSCs possess higher propensity to go through EMT, suggesting that EMT-markers may also help distinguish CSCs from non-CSCs, and such a connection was recently made in breast cancer cells. Addition of EMT-inducer TGFβ was shown to switch breast non-CSCs to breast CSCs (observed as a transition from $CD44^-/CD24^+$ to $CD44^+/CD24^-$ population) (35, 36). Mani et al. (35) showed that cells that transitioned had higher tumorigenicity and stemness (tested by soft agar and sphere forming assays), and in converse, primary dissociated mammary CSCs expressed higher levels of EMT markers (e.g. Twist, Snail, Vimentin). Morel et al. (36) further showed that activation of Ras-MAPK also accelerates the EMT-dependent generation of CSCs, providing a fresh prospective to link the above mentioned RTK pathways (e.g. EGFR, Met) with CSC generation. A more recent study by DiMeo et al. (37) further showed that Wnt is involved in regulating EMT in lung cancer metastasis breast cancer via LRP6, providing further molecular justification for connecting self-renewal pathway with EMT and CSC.

Apart from breast cancer, similar connection of CSCs and EMT has been implicated in ovarian cancers (38). Although not yet uncovered in glioblastoma, one recent study connected EMT-inducer TGFβ with increased self-renewal (39), showing potential for GBM to have an EMT-CSC correlation as well. These studies also provide

enticing basis for generating CSCs in large quantities via EMT for future studies, circumventing the limitations associated with having to isolate the small subpopulation for CSC research. The benefit of EMT-induced CSC generation would be especially realized for tumors for which prospective isolation is currently imprecise.

Concluding perspective

It is our opinion that effective anti-cancer therapies (more specifically, anti-cancer stem cell therapies) would target the pathways and inhibit the prospective mechanisms of chemo-radiation resistance outlined in this review. There are a host of novel anti-cancer agents that are being developed (see a comprehensive review by Ma and Adjei (40)). As of yet, there is no known single "magic-bullet" that will eradicate all cancer cells, let alone cancer stem cells. It is also important to point out that there is no known biomarker or signaling pathway that is specific only to cancer stem cells and not to normal stem cells. In addition, intertumoral differences should be considered. Although not specifically addressed in this review, there are noticeable mechanistic differences that vary from cancer to cancer. It would be interesting to compare tissue-specific pathways that protect the CSCs from chemo-radiation therapies. Further, interpersonal differences even for the same cancer should also be accounted. It is an oft-observed phenomenon that patients diagnosed with the same cancer do not all respond to the same treatment, suggesting that CSCs from one patient may have different means of chemo-radiation resistance than the CSCs from another patient (e.g. in glioblastoma, only patients with O^6-methylguanine-DNA-methyltransferase (MGMT)- negative CSC lines respond to temozolomide (7)).

All these suggest that the field is still ripe for novel CSC biomarker discovery. To that end, employing various high-throughput systems biology '-omic' tools can help elucidate the similarities and differences between somatic and cancer stem cells, as well as help sort out the intertumoral and interpsonal differences. The systems biology approach has not been widely employed yet, but possess the potential to help piece together the very complex but integrated dynamics of the mechanisms reviewed here (i.e. Notch, Hh, and Wnt pathways, DNA damage repair pathway, RTK pathways, and EMT). Systems biology is often said to be hypotheses-generating tool and thus may help elucidate novel regulator that are not part of the "usual suspects" reviewed here and suggest an even better therapeutic than what is currently available.

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Potential conflict of interest

The authors have no conflicting financial interest.

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