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RESEARCH ARTICLE

Emerging strategies for the identification and targeting of cancer stem cells

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Abstract The hypothesis of cancer stem cells (CSCs) is receiving increasing interest and has become the subject of considerable debate among cancer researchers. Recent rapid progress in CSC research has encountered increasing difficulties and challenges. Understanding the biologic characteristic of CSCs is crucial to start with better identification and diagnosis based on CSC markers and eventually targeting to CSCs will undoubtedly result in improved prevention and treatment of many types of CSCs. We discuss here some of the approaching strategies that include establishing special methods of identifying CSCs and targeting therapies of CSCs.

Keywords Cancer stem cells · Challenge · Strategy

Introduction

Researchers have gradually changed their classical view on tumors as studies have identified subpopulations of cells within tumors that drive tumor growth and recurrence. These subpopulations are called cancer stem cells (CSCs) or tumor stem cells (TSCs), or cancer-initiating cells (CICs) [1–3]. There has been evidence that malignant tumors are caused by postembryonic differentiations superimposed upon the process of tissue maintenance and renewal. Malignant stem cells are derived from normal stem cells [4]. CSCs are operationally defined by three distinct properties: (1) a selective capacity to initiate tumors and drive neoplastic proliferation, (2) an ability to create endless copies of themselves through self-renewal, and (3) the potential to give rise to more mature non-stem cell cancer progeny through a process of differentiation or partial differentiation, in which the most mature and differentiated cells exert positive and negative feedbacks upon the proliferation of their progenitor stem cells [5–7]. Over the past several years, accelerating scientific and technological advances have enabled scientists to generate an abundance of knowledge in the realm of CSC biology. However, scientists have not been able to distinguish the characteristics of CSCs from the characteristics of stem cells until today. Moreover, even now, researchers can only entertain a preliminary model of tumor development that CSCs are derived from the transformed stem cells. In fact, difficulties and challenges exist in understanding CSCs. For instance, it is unclear why some tumor cells are more or less tumorigenic than others. Some researchers believe "There is no doubt that there are CSCs, and this has tremendous clinical importance, and the therapies we currently use don't target this population" [8]. Others, however, disagree with these opinions as they think that the stem cell model overlooks the complexity of the interactions that occurs within a tumor and between a tumor and its environment [8]. Additional challenges are presented by such factors as limited number of CSCs in tumor tissues, technical difficulties in keeping CSCs in any culture, and their unusually strong drug resistance. Generally speaking, with the growing evidence that CSCs exist in a wide array of tumors [1–3, 9–14], studies of the hematopoietic origin and some solid tumors have provided proofs that cancers

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originate from CSCs. Therefore, cancer researchers all over the world now need to focus on exploring and developing methods to study the mechanisms that regulate the survival, self-renewal, and differentiation of normal and transformed stem cells. Such worldwide coordinated research effort will lead to tremendous advances in new strategies that isolate and target CSCs [14–16].

Developing the specific methods for isolating and identifying CSCs

Isolation and characterization of CSCs from tumor tissues or cell lines will be a great aid in cancer diagnostics, prevention, and therapeutics. To date, isolation of CSCs has largely depended on their probable phenotypic similarity to normal stem cells: demonstrating such defining characteristics as anchorage-independent formation of spheres, dye exclusion (due to over expression of efflux transporters), expression of cell surface differentiation markers, and clonogenicity [3, 17, 18]. The most often used method for identifying CSCs is the one that identifies surface molecules (markers) [19, 20].

CD133 molecule

CD133 molecule (a transmembrane pentaspan protein) is considered a universal marker of normal hematopoietic stem cells and organ-specific stem cells [21], and it has gained more prominence as a marker of CSCs in solid primary tumors such as medulloblastomas and glioblastomas [22] and subsequently of CSCs in a growing number of cancers of epithelial tissues. Other cancers had similar observations (epithelial ovarian CSCs [2], hepatocellular carcinoma [23, 24], pancreatic cancer [25], and colon CSCs [26]). In particular, O'Brien et al. showed that human colon CSCs within the $CD133^+$ population were able to maintain themselves, to differentiate, and to re-establish tumor heterogeneity upon serial transplantation in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. In their study, subcutaneous injection of colon cancer $CD133^+$cells readily reproduced the original tumor in immunodeficient mice [26–28].

However, the precise contribution of $CD133^+$CSCs in mediating colon cancer metastasis has remained controversial. Shmelkov et al. reported that they generated a knock in *lacZ* reporter mouse ($CD133^{lacZ/+}$), in which the expression of *lacZ* was driven by the endogenous CD133 promoters. Using this model and immunostaining, researchers discovered that CD133 expression in the colon is not restricted to stem cells; on the contrary and that CD133 was ubiquitously expressed on differentiated colonic epithelium in both adult mice and humans. Both $CD133^+$ and $CD133^-$ metastatic tumor subpopulations formed colonosphere in vitro cultures and were capable of long-term tumorigenesis in a NOD/SCID serial xenotransplantation model [29]. Furthermore, CD133 expressive variation in stem cells and CSCs were not limited to colon tissues. HSC, neural stem cells, and their corresponding CSCs also have been reported with conflicting findings in the literature. In light of these new findings, the popular notion that CD133 is a marker of colon CSCs may need to be revised [29]. One explanation for this inconsistency in the research findings could be hidden in the potential difference between the presence of mRNA and the CD133 protein in epithelial cells or in that CD133 expression on stem cells may vary between species (mouse vs. humans). Alternatively, this inconsistency may be due to the antibody affinity and different glycosylation and/or splice variants of CD133. The half-life of the beta-galactosidase relative to CD133 may also affect the readout in the knock in situation. The utilization of destabilized forms of *lacZ* or GFP is to more accurately determine expression patterns [29, 30]. In addition, in the previous studies, detection of CD133 was done using commercially available antibodies, which may not recognize the full gamut of CD133 expression pattern. The antibody from the different commercial companies may also identify different epitopes. The use of such commercial antibodies could be the main reason that inconsistent findings were yielded in those experiments of the previous studies.

There is increasing evidence that evidence have indicated that the CD133 molecule is one of the specific markers in CSCs isolation and identification for cancers that include prostate cancer ($CD44^+/\alpha_2\beta_1{}^{hi}/CD133^+$) [31], murine melanoma($CD133^+/CD44^+/CD24^+$) [11, 12], childhood acute lymphoblastic leukemia ($CD133^+/CD19^-/CD38^-$) [32], ameloblastic tumors ($CD133^+$/Bmi-1/ABCG2) [33], hepatocellular carcinoma ($CD133^+/CD44^+$) [34], and ovarian cancer [35], etc. If CD133 expression is elevated, it may serve a predictive marker of a distant recurrence and a poor survival after preoperative chemoradiotherapy in the residual rectal cancer [36]. These data suggest that the CSCs are heterogeneous in their CD133 expression, and considerable overlap exists between different organ stem cells and CSCs in their repertoire of gene expression [37]. Thus, the identification of $CD133^+$ cells may thus be a potentially powerful tool for investigating the tumorigenic process. Caution, however, should be exercised when using the expression of CD133 as the primary means to identify CSCs. This is due to the fact that the CD133 molecule may change during a culture process and in any particular organ, such as the brain in which only CSCs express CD133 [38].

SP cells

SP cells usually represent only a small fraction of the whole cell population that is based on the technique initially

described in 1996 by Goodell et al. [37]. They studied the cell cycle distribution of whole bone marrow cells using Hoechst 33342 vital dye staining and discovered that the display of Hoechst fluorescence simultaneously at two emission wavelengths (blue 450 nm and red 675 nm) localizes a small, yet distinct nonstained cell population that expresses stem cell markers ($Sca1^+$ $Lin^{neg/low}$). SP cells are localized in the left lower quadrant of a fluorescence-activated cell sorter (FACS) profile. The degree of efflux is correlated with the maturation state, such that cells exhibiting the highest efflux are the most primitive or least restricted with regard to their differentiation potential. In several investigations, however, SP cell properties have shown to possess some stem cell characteristics. The studies of the hematopoietic origin and some solid tumors have provided evidence that SP cells not only have been identified in cancer cell lines (e.g., neuroblastoma [39], melanoma [40], ovarian [41], and nasopharyngeal cancer cell lines [42]), but also have been used for identification of CSCs (e.g., hepatocellular cancer [43], glioma [44], myeloma [45], melanoma [16], prostate cancer [32, 46], and murine mantle cell lymphoma [47]). On the other hand, there has also been a heightened controversy over the expression of SP markers in tumor tissue samples compared with cell lines. Burker et al. demonstrated that the SP phenotype as a universal marker for stem cells should not be applied to gastrointestinal cancer cell lines [48]. They tested four gastrointestinal cancer cell lines (HT29, HGT101, Caco2, and HRA19a1.1) for detailed phenotypic and behavioral analyses of stem cell characteristics. Sorted SP and non-SP cells were similarly clonogenic in vitro and tumorigenic in vivo, and both displayed similar multipotential differentiation potential in vitro and in vivo. The SP and non-SP cell fractions, albeit phenotypically distinct populations, did not differ with respect to the number of the stem cell-like cells or their behavior. Besides, because Hoechst 33342 dye itself induces SP cell apoptosis, the SP cells isolated by such method have limited uses. For accurate assessment, SP cells must be isolated without Hoechst 33342 [49].

It is also of interest to know if the SP cells isolated from cell lines would bear a relationship to CSCs. In principle, in any permanent cell line, there must be a self-renewing cell population. If the characterization of the SP cells in cell lines could be applied to CSCs, this would greatly advance our understanding of CSCs. [36, 50]. Despite some limitations, the advantage of SP cells is that they entail no specific markers. SP cells isolated from tumors have proven to be an attractive alternative strategy to the study of CSCs, especially in cases where specific surface marker associations with normal stem cells of the organ of origin have not been identified. This stems from the proposition that since SP cells isolated from normal tissues are enriched in normal stem cells, then the same population from tumors may also be enriched in CSCs. For example, Grichnik et al. isolated SP cells from human melanoma samples and showed that SP cells had overlapping properties that were common with normal stem cells and included smallness, possess the capacity to become larger cells, and have the greatest ability to expand in culture [51]. Thus, the SP cells have been well documented as an enriched source of stem cells in specific settings and have remained a valid and promising tool, at least in part, for the identification, isolation, and characterization of stem cells, particularly when this approach is combined with other cell markers such as ABCG2 marker [52].

ABCG2 and ABCG5 drug efflux transporters

Many putative stem cells have acquired the ability to withstand cytotoxic insults through either efficient enzyme-based detoxification systems or with their ability to rapidly export potentially harmful xenobiotics. The resistant tumor cells often over-express one of several ATP binding cassette (ABC) transporters that are capable of mediating the efflux of several classes of anticancer drugs, including MDR1 (ABCB1 or P-glycoprotein), MRP1 (the multidrug resistance protein 1, ABCC1), ABCB5, and ABCG2/BCRP1 (breast cancer resistance protein 1), whose expression is associated with multidrug resistance (MDR). Technically, ABCG2 and ABCG5 act to enable a cancer to escape the cytotoxic effects of chemotherapy [53–56]. Intriguingly, CSCs are known to be characterized by multidrug chemoresistance including intrinsic resistance and acquired resistance. Given that the $ABCG2^+$ subset of tumor cells is often enriched with cancer stem-like phenotypes, it is proposed that ABCG2 activity may enable cancer cells to regenerate post-chemotherapy [56]. Because the SP phenotype has also often been correlated with ABC expression, ABCG2 may be CSC markers, and the expression of ABCG2 has been analyzed in various cancer stem-like cells [57]. For example, breast cancer SP cells that have been recently isolated from the MCF-7 and Cal-51cell lines are found to possess ABCG2 transporter properties and may represent stem cell-like cancer cells. The level of ABCG2 mRNA and protein was also reported to be increased in purified MCF7 SP cells relative to non-SP cells, and the purified MCF7 SP cells had an increased ability to colonize the mouse mammary gland [58]. The study from Christgen et al. indicated that the genuine nature of Cal-51 SP cells was unambiguously verified by showing the 30-fold increased ABCG2-expression in isolated Cal-51 SP cells, and by showing that Cal-51 SP cells generated heterologous non-SP cells, and the $ABCG2^-$ expression declined dramatically. In contrast, non-SP cells failed to sustain proliferation [59]. However, Patrawala's study demonstrated that the highly purified $ABCG2^+$ cancer

cells had very similar tumorigenicity to the ABCG2$^-$ cancer cells, and that ABCG2$^-$ cancer cells can also generate ABCG2$^+$ cells. Furthermore, the ABCG2$^-$ population preferentially expressed several "stemness" genes [60, 61]. A study by Zhang also reported that the ABCG2$^+$ cells did not exhibit its obvious tumorigenic capability compared with the ABCG2$^-$ cells in Balb/c null mice. On the proliferative capacity and clonal formative capacity in vitro, the ABCG2$^+$ cells and the ABCG2$^-$ cells shared similar characteristics, but a few of the SP cells with high expression of ABCG2 molecule had cancer stem cell-like characteristics in human ovarian A2780 cell line [52].

Recently, the studies of Frank and Schatton indicated that ABCB5 was a novel molecular marker for a distinct subset of chemoresistant, stem cell phenotype-expressing tumor cells among melanoma bulk populations [57, 62]. In serial human-to-mouse xenotransplantation experiments, ABCB5$^+$ melanoma cells were found to possess greater tumorigenic capacity than ABCB5$^-$ bulk populations and re-establish clinical tumor heterogeneity. In vivo genetic lineage tracking demonstrated a specific capacity of ABCB5$^+$ subpopulations for self-renewal and differentiation, because ABCB5$^+$ cancer cells were able to generate both ABCB5$^+$ and ABCB5$^-$ progeny, whereas ABCB5$^-$ tumor populations could only, exclusively to ABCB5$^-$ cells and at lower rates. Thus, ABCB5 is potentially a robust CSC marker in melanoma [62]. More experiments, however, are still needed to verify this finding.

Colony-forming in soft agar and serum-free medium

One property of cancer cells in solid tumor, like stem cells, is their ability to grow in soft agar and serum-free medium cultures. It has been found that only a fraction of cells in a tumor cell culture can form a colony in soft agar. Are the cells forming soft agar colonies CSCs? This would be a logical conclusion from the information at hand [61, 63]. Matsui et al. demonstrated that the human multiple myeloma (MM) cell line contained small (<5%) subpopulations that lacked CD138 expression and had greater clonogenic potential in vitro than corresponding CD138$^+$ plasma cells. CD138$^-$ cells from clinical MM samples were similarly clonogenic both in vitro and in NOD/SCID mice, whereas CD138$^+$ cells were not [64]. The study by Dou et al. also found that a small subset of murine myeloma cell line (SP2/0 cells) could generate colonies in soft agar growth media and had high frequencies of clone forming. Dou et al. seeded 100 SP2/0 cells into the soft agar growth media for 1-week culture, and that resulted in the final formation of 24 colonies. A few cells in SP2/0 cells contains bromodeoxyuridine (BrdU)-label DNA immortal strand and possess the characteristics of tumor stem-like cells [45]. In murine melanoma cell line, Dou et al. likewise demonstrated that the clone-forming rates of CD133$^+$ cells, CD44$^+$ cells and CD44$^+$CD133$^+$ cells in soft agar media were higher than those of CD133$^-$cells, CD44$^-$cells and CD44$^+$CD133$^-$ cells respectively in vitro. CD133$^+$, CD44$^+$, and CD44$^+$CD133$^+$cells also had stronger tumorigenicity than CD133$^-$, CD44$^-$ and CD44$^+$ CD133$^-$ cells, respectively, in C57BL/6 mice [15]. These findings suggest that the clonogenicity of tumor cells in soft agar is one of the important methods used in identification of cancer stem-like cells or CSCs.

Pancreatic cancer is among the most aggressive solid malignancies. The identification of the CSCs may be based on the techniques of clone formation assay in serum-free medium and tumor formation assay used by Gou et al. [65]. The study by Gou et al. demonstrated that a subpopulation of pancreatic cancer cell line (PANC-1) could propagate to form spheres, and the dissociated single cells of primary spheres in serum-free medium could form filial spheres again. The propagation capacity of PANC-1 spheres was higher than that of the cells cultured in serum-containing medium both in vitro and in vivo [64]. Another study indicated that the sorted SP cells from the B16F10 cells, after 7 days of culture in serum-free medium, grew up as spherical clone cells, proliferated and formed free-floating sphere-like tumor cells. In contrast, the non-SP cells shrank, the cellular growth vigor declined, and some non-SP cells showed apoptosis after 7 days of culture in the serum-free medium [16]. Therefore, this bioassay may be suitable for clinical studies of effects of anticancer drugs or irradiation on human CSCs [63].

The aforementioned strategies are commonly used methods that have both merits and shortcomings for isolating and identifying CSCs. Stem cells are known to be immature, self-renewing multipotent cells that can differentiate into specialized cell types. Due to their immature phenotype, some stem cells as well as some CSCs are a little hard to be characterized and isolated. Thus, tumor bulk is not a problem, but the identification of CSCs and the factors that regulate their behavior are likely to have an enormous bearing on how we will treat neoplastic disease in the future [66]. The established cancer cell lines may be useful for the validation of biological properties of tumorigenic subpopulations, they are, on their own, unlike primary tumor material, not sufficient for the identification of CSCs. Nevertheless, using the experimental approach, tumorigenic populations that were capable of transferring human disease into immunodeficient murine hosts and that recapitulated the phenotype and morphology of the original patient tumors characterized in diverse human malignancies; therefore, this still is gold standard for tests of 'stemness'. Importantly, when xenograft models are used in identifying CSC's tumorigenicity, it was

recommended to consider using the classical NOD/SCID mouse rather than NOG mouse, which has a higher degree of immunosuppression than the classical NOD/SCID mouse [67].

Therapeutic strategies to target CSCs

Tumor ablation, hormonal therapy, radiotherapy, anti-angiogenic, and chemotherapy, individually or in combination, are currently the mostly applied therapies for treating patients diagnosed with cancers including leukemias and malignant solid tumors. Although these therapies are effective in an initial phase of treatment, the progression of cancer to locally invasive and metastatic disease is often associated with intrinsic or acquired resistance to treatment and disease relapse [68, 69]. The hypothesis of CSCs has dramatically changed the past view of cancer therapy as a majority of current treatment modalities target the terminally differentiated cancer cells instead of the CSCs [70]. The strategy to target CSCs has begun to revolutionize approaches to cancer therapy and drug design nowadays.

CSC targeting therapy

CSCs have two potential subtypes: primary tumor CSCs (pCSCs) and migrating CSCs (mCSCs). pCSCs constitute the original population of tumorigenic cells which initiate formation of the hematopoietic and solid tumors and are the center of most CSC research. mCSCs represent a distinct population of cells with the intrinsic properties to disseminate from the primary site and generate the distant metastases, strikingly, metastases of most carcinomas recapitulate the organization of their primary tumors and lead to cause of most cancer mortality. Although pCSCs explain distinct and important aspects of carcinogenesis, each alone cannot explain the sum of the cellular changes apparent in human cancer progression. mCSCs that are an integrated concept of malignant tumor progression is consistent with all aspects of human tumor [71, 72]. The blockade of multiple oncogenic cascades activated in CSCs may be essential for improving the current clinical treatments against cancers. It is conceivable that if CSCs truly are the root of malignancy and solely responsible for tumor growth, only therapies that deplete CSCs will ultimately be successful as an effective cure [73, 74].

Use of oncolytic adenoviruses presents an attractive antitumor approach for eradication of CSCs. The $CD44^{+}CD24^{-/low}$ cells that were identified as CSCs isolated from breast tumor patients could be effectively killed by oncolytic adenoviruses Ad5/3-Delta24 and Ad5. pk7-Delta24. In mice, $CD44^{+}CD24^{-/low}$ cells formed orthotopic breast tumors but Ad5/3-Delta24 and Ad5. pk7-Delta24 were effective against advanced orthotopic $CD44^{+}CD24^{-/low}$-derived tumors. This suggested that Ad5/3-Delta24 and Ad5.pk7-Delta24 could kill $CD44^{+}CD24^{-/low}$ cells as well as committed breast cancer cells, making them promising agents against CSCs [75]. Jiang et al. [76] adopted a similar approach against brain CSC therapy. In their study, four brain tumor stem cell lines from surgical glioblastoma specimens expressed high levels of adenoviral receptors and allowed for efficient viral infection and replication. An oncolytic adenovirus Delta-24-RGD induced autophagic cell death. The treatment of xenografts derived from brain CSCs with Delta-24-RGD could improve the survival of glioma-bearing mice. The results showed that brain CSCs were susceptible to adenovirus-mediated cell death via autophagy in vitro and in vivo [76].

Given the similarities between CSCs and stem cells, CSC-targeted therapeutic agents could exert adverse effects on the renewal and maintenance of physiologic tissues due to potential toxic effects on a tumor host's stem cell compartment. Therefore, preferred CSC targets would comprise those molecules or pathways that are preferentially induced or operative in malignancy as opposed to stem cells [5]. The molecular targeting of developmental pathways include hedgehog (Hh), Wnt/β-catenin, Notch, etc. [77]. Because Hh signaling regulates the progenitor cell fate in normal development and homeostasis, aberrant pathway activation might be involved in the maintenance of such CSCs in cancer. Craig's study indicated that the subset of MM that manifested Hh pathway activity was markedly concentrated within the CSCs compartment. The Hh ligand promoted expansion of MM stem cells without differentiation, whereas the Hh pathway blockade markedly inhibited clonal expansion accompanied by terminal differentiation of purified MM stem cells [78].

Wnt/β-Catenin is another essential component of both intercellular junctions and the canonical Wnt signaling pathway [79].The aberrant activation of Wnt signaling is involved in tumor development and progression. The study by Pu et al. found that Wnt2, Wnt5a, frizzled2, and β-catenin were overexpressed in gliomas. Knockdown of Wnt2 and its key mediator β-catenin in the canonical Wnt pathway by siRNA in human U251 glioma cells inhibited cell proliferation and invasive ability, and induced apoptotic cell death. Furthermore, treating the nude mice carrying established subcutaneous U251 gliomas with siRNA targeting Wnt2 and β-catenin intratumorally also delayed the tumor growth [80]. This study suggested that the Wnt/β-catenin pathway might provide a new therapeutic approach against CSCs in the malignant gliomas. Similarly, β-catenin signaling is essential in sustaining the epidermal tumor CSC phenotype. Ablation of the β-catenin gene resulted in the loss of CSCs and complete epithelial tumor regression [81].

It is known that a better therapeutic endpoint would involve direct sampling of CSC populations. However, it is also possible that the elimination of the CSCs within a tumor may not result in its complete regression, since non-CSCs, while less aggressive, may nonetheless be capable of maintaining an already established tumor for an extended period of time [3]. One strategy to address this concern would be to look for agents that target both the CSCs and non-CSCs within tumors. Alternatively, combined therapies that involve both CSC-directed agents as well as tumor bulk-targeted regimens would be expected to prove most effective in improving clinical treatment responses and patient outcomes [3, 5]. Therefore, preferred CSC targets would comprise those molecules or pathways that are preferentially induced or operative in malignant as opposed to physiological stem cells. These factors should be taken into account when designing CSC-directed treatment strategies [6].

Targeting CSC surface molecular markers

CD133 is considered the most important CSC-associated marker identified so far. The CD133 down-regulation in vitro and in vivo in human metastatic melanoma using two different short hairpin RNAs resulted in slower cell growth, reduced cell motility, and decreased capacity to form spheroids under stem cell-like growth conditions. Monoclonal antibodies directed against two different epitopes of the CD133 protein induced a specific, dose-dependent cytotoxic effect in FEMX-I melanoma cells. The down-regulation of CD133 severely reduced the capacity of the cells to metastasize, particularly to the spinal cord. It has been suggested that CD133, in addition to its role as a CSC marker, is an important therapeutic target for metastatic melanoma and, potentially, for other CD133-expressing cancer types [82]. In another study, human malignant melanoma CSCs were defined by the expression of the chemoresistance mediator $ABCB5^{+}$ molecule that the specific targeting of this tumorigenic minority population inhibited tumor growth. $ABCB5^{+}$ tumor cells showed a primitive molecular phenotype and correlated with clinical melanoma progression. In the serial human-to-mouse xenotransplantation experiments, $ABCB5^{+}$ melanoma cells possess greater tumorigenic capacity than $ABCB5^{-}$ bulk populations. Systemic administration of a monoclonal antibody directed at ABCB5 molecule was shown to be capable of inducing antibody-dependent cell-mediated cytotoxicity in $ABCB5^{+}$ CSCs [83]. Analogously, the resistance to vincristine in the $ABCG2^{+}$ cells (human ovarian cell line A2780) was higher than that of the $ABCG2^{-}$ cells, and after the $ABCG2^{+}$cells were incubated with the anti-ABCG2 antibody, the resistance to vincristine was markedly decreased. The study suggested that the ABCG2 molecule may be responsible for target molecule for therapy of ovarian cancer [84]. Thus, it is a feasible strategy to first use inhibitors on the drug transporters and then to target both the CSCs and the cancer non-stem cells. Meanwhile, we also understand that there may be a possibility that the antibody will have toxic side effects on normal tissues when utilizing it.

Targeting CSC survival Niche

Targeting the stem cell niche is another promising therapeutic strategy. In all tissues, stem cells are located in a specialized vascular microenvironment, the niche; intrinsic and extrinsic signals from the niche regulate self-renewal and differentiation [17]. As many properties of stem cells are shared by at least a subset of cancer cells or CSCs, targeting the CSC survival niche may disconnect intrinsic and extrinsic signals from the niche that maintains and governs CSCs in their division and differentiation.

It is known that the secretion of vascular endothelial growth factor (VEGF) by primary tumor cells recruits bone marrow-derived cells (BMDC) to what is now referred to as the pre-metastatic niche [85]. Interfering with BMDC induction by blocking secretion of VEGF virtually eliminates metastasis in these cancers. For example, a VEGF antibody drug (bevacizumab) is currently in clinical trials for its anti-angiogenic properties that have shown promising preclinical and clinical activities against metastatic colorectal cancer, particularly in combination with chemotherapy [86]. Injecting animal hosts with the media conditioned by tumor cells was sufficient to re-direct tumor tropism to the organ associated with the source of secreted factors. The mechanism of this effect is still unclear, but potential candidates for involvement are the inflammatory chemoattractants S100A8 and S100A9. While untreated animals develop metastases, inhibiting the overexpression of these inflammatory chemoattractants in lung tissues with neutralizing anti-S100A8 and anti-S100A9 antibodies reduced metastasis to lung tissue [87]. Thus, metastatic niche signaling pathways will also be interesting targets for molecular intervention.

Manipulation of miRNA expression

MicroRNAs (miRNA) constitute a large family of small, approximately 21-nucleotide long, non-coding RNAs that have emerged as key post-transcriptional regulators of gene expression in metazoans and plants. miRNAs are often deregulated in various cancers and have significant therapeutic potential [87, 88]. In mammals, miRNAs are predicted to control the activity of approximately 30% of all protein-coding genes, and have been shown to participate in the regulation of almost every cellular process investigated and to play an important role in many developmental processes

[89, 90] Thus, miRNAs have spurred studies to investigate whether miRNAs play an important role in the CSC phenotype [90, 91]. Recently, a study demonstrated that certain miRNA that regulate the critical promoter of stem cell self-renewal factor BMI1 was downregulated in purified populations of normal mammary epithelial stem cells and breast tumor-initiating cells (CSCs). The data showed that the mRNA encoding BMI1 was specifically targeted by three miRNA clusters (miR-200c-141, miR-200b- 200a-429, and miR-183-96-182). The three miRNA clusters were identified that had decreased expression in freshly isolated $CD44^{+}CD24^{-/low}$ breast CSC compared to cells in the tumor bulk [88]. It is possible that miR-200c blocked stem cell self-renewal by targeting the 3′UTR of the self-renewal gene BMI1, resulting in the loss of BMI1 protein, and the attenuation of the ability of CSCs to self-renew and form tumors [88, 92]. The studies also showed that the embryonic program 'epithelial–mesenchymal transition' (EMT) is thought to promote cellular mobility and subsequent dissemination of tumor cells. The transcriptional repressor zinc-finger E-box binding homeobox 1 (ZEB1) is a crucial inducer of EMT in various human tumors by inhibiting expression of the microRNA-200 (miR-200) family, whose members are strong inducers of epithelial differentiation in pancreatic, colorectal and breast cancer cells. ZEB1 not only promotes tumor cell dissemination, but is also necessary for the CSCs of pancreatic and colorectal cancer cells. ZEB1 links EMT activation and stemness maintenance and is a promoter of mobile mCSCs. Thus, targeting the ZEB1–miR-200 feedback loop might form the basis of a promising treatment for fatal tumors, such as pancreatic cancer [93, 94]. These findings provide additional evidences for the critical regulatory roles that miRNAs play in cancer biology [89]. From the studies described above, we come to see that miRNA may control a variety of cell functions including cell proliferation, stem cell maintenance, and differentiation. These studies were to identify roles for specific miRNAs in parallel populations of stem cells and in CSCs, and it will be especially interesting to determine whether miRNA genes are directly targeted, either alone or cooperatively, during cancer initiation in normal stem cells or progenitor cells. Obviously, the therapeutic approaches of targeting CSCs through manipulation of miRNA expression may be an alternative strategy [88, 89, 91, 95].

Screening directly target drug-resistant CSCs in 3D cell culture

Tissues and organs are three dimensional (3D). Cell behavior, which includes survival, motility, and differentiation [96], mainly depends on its growth environment. However, our ability to understand organ formation, function, and pathology has often depended on two-dimensional (2D) cell culture studies or on animal model systems [97] Nevertheless, cells growing on flat 2D tissue culture substrates can differ considerably in their morphology, cell–cell, and cell–matrix interactions, and differentiation from those growing in more physiological 3D environments [31, 96]. Unlike the 2D substrates, 3D matrix interactions also display enhanced cell biological activities and promote normal cell polarity and differentiation. Cell adhesion structures can evolve towards in vivo-like adhesions with distinct biological activities in the 3D environments. In addition, signaling and other cellular functions also differ in 3D compared with 2D systems [97, 98]. In the animal models, although the end of the experimental continuum frequently provide definitive tests of specific molecules and processes, the animal models may not adequately reproduce features of, for example, human tumors, drug therapeutic responses, autoimmune diseases, and stem cell differentiation. Therefore, the in vitro 3D tissue models provide a third approach that bridges the gap between the traditional cell culture and the animal models [98, 99].

Hebner et al. have demonstrated how one feature of breast cancer biology, stromal rigidity, and its effects on morphogenesis can be modeled in 3D cultures to allow the delineation of the signaling pathways that regulate these processes [100]. The investigators mimicked the stromal rigidity of breast tumor by reducing the matrix compliance, which was sufficient to promote tumor-like morphogenesis of normal mammary epithelial cells. In a recent study of gene expression like morphology, the molecular profiles of 25 breast cancer cell lines were separately cultured in 2D and 3D conditions and were then compared. One of the results indicated that, in the 3D environment, the morphologies reflected, at least in part, the underlying gene expression profile and the protein expression patterns of the cell lines, and that those distinct morphologies were also associated with tumor cell invasiveness and with cell lines originating from mCSCs [101, 102]. Cukierman and his colleagues revealed their approach that an in vivo-like ovarian stroma 3D system would enable researchers to study ovarian stromal progression and to uncover mechanisms that promote ovarian tumor development, progression, and metastasis. Interactions between cancer cells and stroma in the 3D environment were considered critical for growth and invasiveness of epithelial tumors [103, 104].

At present, we know very little about cell-based assays used for screening target CSCs drugs in 3D cultures. In order to develop such drug screening systems, it is essential that novel methods be explored to use the 3D culture system in vitro for development of specific drugs that target chemoresistant pCSCs or mCSCs and, at the same time to

minimize unwanted side effects of the drugs to host tissues [105]. Those chosen drugs from the methods used in the 3D culture should be strongly antiproliferative to drug-resistant CSCs cells, so that these new therapies may have the potential to target CSCs directly.

Antitumor immunity and CSCs

Recent findings that suggest a negative correlation between degrees of host immunocompetence and rates of cancer development raise the possibility that only a restricted minority of CSCs, may possess the phenotypic and functional characteristics to evade host antitumor immunity. Schatton and Frank recently identified human malignant melanoma initiating cells (MMICs), a novel type of CSCs, based on selective expression of the chemoresistance mediator ABCB5[106]. $ABCB5^+$ MMICs have a relative immune privilege, suggesting refractoriness to current immunotherapeutic strategies and might be responsible for melanoma immune evasion. It was hypothesized that CSCs may foster tumor initiation and growth at least in part via attenuation of the antitumor immune response [105–107]. Natural antitumor immunity may be limited and often insufficient to destroy rapidly growing CSCs, which arises from the organism's own tissue and therefore predominantly expresses self-antigens to which host immune cells have been tolerized [108]. In view of the CSC concept, it would appear more promising to target markers specific $ABCB5^+$ MMICs [83, 106].

The susceptibility of CSCs to immune effector cell-mediated lysis has been poorly explored so far. In particular, no information is available on the ability of NK cells to recognize and lyse human melanoma CSCs. Recent study from Pietra and his colleagues showed that IL-2-activated NK cells efficiently killed malignant melanoma cell lines that were enriched in putative CSCs by the use of different selection methods of identifying CD133 expression, radioresistance, or the ability to form melanospheres in stem cell-supportive medium. NK cell-mediated recognition and lysis of melanoma cells involved different combinations of activating NK receptors. The data suggest that NK-based adoptive immunotherapy could represent a novel therapeutic approach to possibly eradicate mCSCs [109].

Recent trials, in particular, were based on the use of tumor-homogenate pulsed dendritic cells (DC) for patients with recurrent gliomas and provided encouraging results [110]. Serena's study suggests that only DC vaccination against neurospheres in GL261 glioma cell line can restrain the growth of a highly infiltrating and aggressive model of glioma and may have implications for the design of novel, more effective immunotherapy trials for malignant glioma [106]. In glioblastoma multiforme (GBM), Xu's study showed that CSCs express high levels of tumor-associated antigens as well as major histocompatibility complex molecules and that DC vaccination using CSC antigens elicited antigen-specific T cell responses against CSCs. DC vaccination induced interferon-γ production is positively correlated with the number of antigen-specific T cells generated. Understanding how immunization with CSCs generates superior antitumor immunity may accelerate development of CSC-specific immunotherapies and cancer vaccines [111].

Concluding remarks

The idea that cancer arises from stem cells was first proposed over 150 years ago as the embryonal rest theory of cancer. The theory stated that tissues resemble fetal tissues in many ways but they differ from fetal tissue in being unable to recapitulate the total program that led to an orchestrated collection of organism-serving cells that were programmed to make the organ as adaptive as possible to the range of environmental variations in which it evolved [7]. However, by the beginning of the 20th century, the embryonal rest theory of cancer was discarded, and the hypothesis that cancer arises from de-differentiation has been generally accepted. The hypothesis of CSCs that represent malignant cell subsets in hierarchically organized tumors has also been gradually accepted by both basic and clinical scientists. Regardless of whether these CSCs come from tumor-initiating cells within heterogeneous tumor populations or from transformed stem cells or from progenitor cells, it is clear that they have a role in the initial tumor formation (pCSCs) and in tumor metastasis (mCSCs). Emerging and evolving models of CSC-mediated tumor progression offer potential windows of opportunity for developing novel therapeutic strategies aiming at thwarting the menacing power of CSCs [73]. Understanding the biologic characteristic of CSCs is crucial to start with better identification and diagnosis based on CSC markers. It is hoped that, eventually targeting of pCSCs and mCSCs will undoubtedly result in improved prevention and treatment of many types of cancer [29, 72, 106].

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