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REVIEW ARTICLE

Cancer stem cell theory: Are we moving past the mist?

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Abstract: Cancer stem cells (CSC) are a subpopulation of tumor cells that have superior capacities of self-renewal, metastatic dissemination, and chemoresistance. These characteristics resemble, to some extent, the outcome of certain biological processes, including epithelial–mesenchymal transition (EMT), autophagy, and cellular stress response. Indeed, it has been shown that the stimuli that induce these processes and CSC are overlapping, and CSC and tumor cells that underwent EMT or autophagy are much alike. However, as the cross talk between CSC, EMT, autophagy, and cellular stress is further explored, these processes are also found to have an opposing role in CSC, depending on the condition and status of cells. This contextual effect is likely due to overwhelming reliance on CSC markers for their identification, and/or discrepancies in recognition of CSC as a particular cell population or cellular state. In this review, we summarize how EMT, autophagy, and cellular stress response are tied or unwound with CSC. We also discuss the current view of CSC theory evolved from the emphasis of heterogenicity and plasticity of CSC.

Keywords: Autophagy; Cancer stem cells; Cellular stress; Epithelial–mesenchymal transition; Heterogenicity; Plasticity

1. INTRODUCTION: HISTORICAL VIEW OF CANCER STEM CELLS

Increasing evidence over the past decades suggests that cancer cells are hierarchically organized within tumor, in which a subpopulation of cancer stem cells (CSC) is responsible for sustaining tumor growth.[1–3] These CSC share important characteristics with normal stem cells, including self-renewal,[4] differentiation capacity,[5] and quiescence, which, collectively, are referred to as the "stemness" properties. The CSC hypothesis was initially proposed in the nineteenth century on the basis of observation of morphologic heterogeneity in cancers. The first solid evidence supporting the existence of CSC was found in acute myeloid leukemia (AML) in 1994 by fractionating AML cells based on their cell surface markers $CD34^+$ $CD38^-$ using flow cytometry.[6] Later, CSC were also identified in other types of tumors including breast cancer, colon cancer, glioblastoma, and head and neck squamous cell carcinoma.[7–9] The principle of CSC model describes that CSC divide asymmetrically, generating undifferentiated daughter cells that remain as CSC, as well as differentiated daughter cells that compose the bulk of tumor, whereas non-CSC do not have these capacities.[10] As such, it was believed that CSC are epigenetically (or genetically) and metabolically distinct from non-CSC. Because CSC subpopulation was defined as cells with cancer-initiating ability, regardless of whether or not they derive from normal stem cells, the term "tumor-initiating cells (TICs)" is sometimes preferred by researchers.

Given that the CSC subpopulation is closely associated with poor prognosis,[11] their identification is of particular importance. Most CSC in a variety of malignancies have been identified by the detection of specific surface marker, such as CD133, CD44, and Oct-4, either alone or in combination.[8,12,13] The intracellular proteins (such as aldehyde dehydrogenase 1),[14] capability of efflux of Hoechst 33352 dye (known as side population),[15] and reduced reactive oxygen species (ROS),[16] have also been employed to mark the CSC. However, even though a list of markers has been linked to CSC, it is not uncommon that the putative CSC only express a few of them. Meanwhile, it remains to be determined how reliably these markers reflect the stemness of cells.

In addition to their identification, the investigation of the biological process closely associated with CSC is equally important. Among various biological processes, epithelial–mesenchymal transition (EMT), autophagy, and cellular stress response have been associated with CSC, although their effects on CSC are not in consensus. In the following sections, we review both sides of the evidence that couples/uncouples these processes with CSC and discuss the current view that regards CSC as a cellular state of a heterogeneous population of cancer cells.

2. EMERGING EVIDENCE UNLINKS CSC AND EMT

The EMT is a gene-reprogramming process that has been closely associated with several features of CSC. Regardless of whether it is driven by the environmental stimuli or other mechanisms, EMT enhances the migratory and invasive potentials of cancer cells.[17–19] The induction of EMT has been linked to self-renewal and tumor-initiating capacities, which is supported by the findings that EMT transcription factors, Snail and ZEB1, repress the epithelial features and subsequently initiate the dedifferentiation

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in colon, gastric, breast, and liver cancers.[20–30] Phenotypically, it has been demonstrated that cancer cells underwent EMT form at least 10-fold more tumor spheres.[31] Moreover, several studies using the *in vivo* mouse breast cancer model showed that the upregulation of Snail by the induction of EMT augments the efficiency in tumor formation[20,32] and that the $CD24^-$ subpopulation that is transitioned from $CD24^+$ breast cancer cells by TGF-β-induced EMT displays superior tumor-initiating capacity.[33,34] Accordingly, it is generally agreed that EMT promotes the acquisition of mesenchymal-like phenotype, simultaneously endowing CSC with the abilities to invade, metastasize, colonize, and self-renew.

Although previous studies favor the notion that EMT is a general feature of CSC,[20,34–37] growing evidence suggests that cancer cells undergoing EMT may not necessarily gain stemness properties, thus uncoupling the CSC and EMT. Indeed, Ye et al employed the MMTV-PyMT promoter-driven mouse breast cancer model and showed that the Slug and Sox9, two well-known EMT transcription factors, may play different roles in mammary stem cells and breast CSC-like cells.[20] Celià-Terrassa et al found that the subpopulation with mesenchymal traits is deprived in TICs and that an overexpression of Snail1 in TIC-enriched subpopulation suppresses their self-renewal and metastatic phenotype.[38,39] Conversely, knockdown of Snail1 not only enhances the epithelial features but also augments the tumor-initiating capacity of TIC. Beck et al showed that, in skin papilloma, the level of Twist1 required for the tumor maintenance by promoting proliferation has minimal effect on the repression of E-cadherin and induction of EMT, implying that the mesenchymal phenotype and tumor-initiating capability are not constantly linked.[40] Redmer et al and Polo et al even showed that the epithelial molecule E-cadherin is crucial for embryonic stem (ES) cell pluripotency and that the mesenchymal–epithelial transition initiates the cellular reprogramming of fibroblast to induced pluripotent stem cells.[41–44] These studies thus suggest that the EMT may be dispensable during the process of CSC development. Not only is the contribution of EMT to CSC formation heavily debated but also the role of EMT in metastatic phenotype of CSC is argued. As exemplified by the study from Ocaña et al, while Prrx1 is capable of inducing EMT in embryos and cancer cells, downregulation of this transcription factor actually facilitates breast stem-like cancer cells to metastasize.[45] Accordingly, it should be appropriate to consider EMT and CSC formation being two separate pathways that, nonetheless, can cross each other.

3. CONTROVERSY REMAINS BETWEEN CSC AND CELLULAR STRESS RESPONSE

Over the past decades, the majority of studies generally reach a consensus on the hypothesis of "dynamic stemness," presuming that the stemness of CSC is inducible from the subpopulation of committed cancer cells. Due to the facts that tumors are constantly challenged by various stress and that their continuous growth requires CSC, the cellular responses to stress are assumed to have a role in the reprogramming of cancer cells into CSC. Indeed, hypoxia that often occurs as tumors outgrow the normal blood supply has been demonstrated as a strong stimulus to enhance the aggressive behavior of multiple malignancies. Accumulated studies also showed that embryonic and several pluri- or multipotent stem cells reside in a relatively hypoxic environment and that the stem-like phenotype in prostate, lung, and other types of cancers is enhanced by hypoxia.[46,47] Another cellular stress that reportedly predisposes cancer cells to enhanced stem-like phenotype is the oxidative stress. Gopal et al and Saijo et al employed low dose of H_2O_2 in culture medium and showed that the induction of oxidative stress upregulates the Sox2 activity and certain stem-like phenotypes in breast (MCF7 and ZR751) and lung (ZR751) cancer cell lines.[48,49] In addition to the hypoxic and oxidative stress, high level of replication stress is also commonly found in the CSC subpopulation. The increase in replication stress is often associated with aberrant DNA replication and cell cycle progression subsequent to the oncogene overactivation. As shown in the studies of glioma and colorectal cancers, the tumor cells are heterogeneous in the amount of constitutive replication stress, and the level of which is higher in the subset of CSC than non-CSC. Consistently, a number of studies demonstrated that CSC are inherently equipped with robust replication stress and DNA damage response, allowing these cells to tolerate high level of replication stress. Taken together, these studies suggest that cellular stress and the response may directly contribute to, or at least be implicated in, the process of CSC development.

Although an association between stress response with CSC has been demonstrated, it is still puzzling that cellular stress, which is potentially cytotoxic, exerts a promotive effect on CSC development. Specifically, it is unclear why and how CSC harbor high level of replication stress, which essentially opposes DNA replication and cell proliferation. Notably, McGrail et al recently used systemic-level approaches and found that CSC are featured with gene signature defective in replication stress response and that such defects in replication stress response rewrite nonmalignant cells into a CSC-like state.[50] Parkes et al also showed that deficiency in genes associated with DNA repair in S phase promote cancer cells transitioning toward CSC.[50,51] These data, along with several lines of evidence, thus provide a solid link between replication stress response defects and cancer stemness. Likewise, Polewski et al showed that the deregulation of SLC7A11, a catalytic subunit of electroneutral transporter that functions as an ROS scavenger, increases the level of endogenous ROS and enhances the CSC phenotype.[52] Accordingly, CSC may carry certain defective cellular stress response, and their stemness phenotypes are possibly the consequence of such inherent deficiency.

4. CONTEXTUAL DEPENDENCY COMPLICATES THE RELATIONSHIP BETWEEN CSC AND AUTOPHAGY

Autophagy is a lysosomal degradation pathway originally recognized as a response to nutrient deprivation and starvation. Autophagy is increased in senescent cells, which, however, can be interpreted as either a cause or a consequence of senescence.

Since autophagy has been considered as a sign of senescent cells, the observation that autophagy is upregulated and required for the maintenance of CSC is intriguing. Indeed, as shown in the studies of CSC enriched from breast, pancreatic, liver, osteosarcoma, ovarian, and glioblastoma cancers, targeting the components of autophagy negatively impacts the self-renewal capacity and the expression of stemness markers.[53] Molecularly, the autophagy is linked to EGFR/Stat3, TGF-β/Smad, and STAT3/JAK2/IL-6 pathways, and the signaling of which is interrupted by disruption of autophagy, ultimately leading to a decrease in tumorigenicity of breast cancer-stem like cells.[54–56] Peng et al showed that Forkhead Box A2 (FOXA2) is overexpressed in ovarian cancer stem-like cells, thus serving as a CSC marker of this cancer type and that knockdown of autophagy not only decreases the level of FOXA2 but also reduces their self-renewal ability.[57] More recently, FOXO3, a mediator of the transcription of several autophagy-related genes, is shown to be required for sustaining the leukemia-initiating cells,[58] although an opposite role of FOXO3 in CSC is also demonstrated in multiple malignancies.[59–62] Sharif et al also found

that the perturbation in basal autophagy decreases the stemness and promotes the differentiation of teratocarcinoma cells, which are highly malignant totipotent stem-like cancer cells.[63] These studies suggest that the effect of autophagy on CSC is context dependent, with distinct and perhaps opposite roles in different stages of CSC development.

5. CURRENT VIEW OF CSC

5.1. CSC/non-CSC transition states: it is more than binary

Accumulated from years of studies, including those mentioned previously, the current view of CSC is updated. The concept of CSC states has evolved from a binary to a continuum, which includes intermediate CSC that exhibit partial stemness and differentiated phenotypes. This CSC/non-CSC continuum in cell phenotypes reflects the extra- and intracellular cues that simultaneously activate several dedifferentiation and differentiation-associated processes, and thus, cells can be in the middle of phenotypic transitioning due to their differential response to diverse stimuli. Indeed, as shown in the study exploiting androgen analog to enrich prostate cancer stem/progenitor population, cells that co-express stem cell-like markers CD44 and/or $\alpha_2\beta_1$-integrin, as well as basal–luminal markers p63, CK5/14, and CK8/18, are detected. These cells exhibit an intermediate phenotype between basal and secretory cells of prostate epithelium and are able to self-renew or acquire a more differentiated phenotype after injuries.[64–68] Another study analyzing the generation of leukemia stem cells by introducing MLL-Af9 fusion protein into myeloid cells also identified abnormal hybrid cells that have features of both stem cells and more differentiated cells.[69,70] Experimentally, it is not surprising to find that the expression of several CSC markers (such as CD133, CD44, and Nanog) at single-cell level as revealed by flow cytometry often shows a single population in relatively normal distribution, instead of bimodal distribution that clearly specifies CSC vs non-CSC subpopulations.[71–73] Therefore, rather than a distinguished subpopulation of cancer cells, it may be more relevant to consider CSC as a state or process by which cancer cells gain certain malignant characteristics, including enhanced tumorigenicity, chemoresistance, and metastasis.

5.2. CSC entity: it is a pool of multifunctional and interdependent cells

Another update of CSC concept, being two sides of a coin with CSC/non-CSC continuum, is that CSC refers to an entity that encompass a group of multifunctional and interdependent cells. Phenotypic heterogeneity within pluripotent ES cells, multipotent adult stem cells, and CSC of various origins has been characterized in numerous studies.[74–79] Such coexistence of multiple cellular states within stem cells and CSC population was previously presumed to be the consequence of differentiation of CSC. Notably, studies of mice ES cells showed that Nanog is heterogeneously expressed, and similar Nanog distribution would be unavoidably reestablished even after repetitive cell sorting and purification.[80,81] Likewise, combining the use of media that has been proven effective to enrich CSC subpopulation with flow cytometry–based cell sorting nonetheless fails to maintain CSC at a high ratio.[75,82] These evidences suggest that CSC is a pool of cells that are in a dynamical equilibrium of different cellular states through reversible interconversion. Perhaps, CSC in intermediate state is important to maintain the stemness of CSC pool as a whole by releasing signaling factors or forming part of the tumor microenvironment known as niche. In support of the notion that CSC is an entity that comprises cells in different states or with varying properties, Dieter et al employed molecular tracking strategy and were able to categorize TICs of human colon cancers into three different functional types, including extensively self-renewing long-term TIC (LT-TIC), tumor transient amplifying cells (T-TACs), and delayed contributing TIC (DC-TIC).[83] Importantly, different types of cells separately predominate tumor formation or metastases but collaboratively sustain the whole population of TIC. Therefore, CSC should be a pool of cells featured with functionally diverse properties that are crucial for their existence.

In conclusion, expanding the understanding of CSC population reconciles the clonal evolution "stochastic" and the CSC "hierarchical" models, allowing us to appreciate that these two models may not be mutually exclusive but actually are interrelated. Indeed, the demonstrations of heterogenicity and plasticity of CSC population derived from their ability to reprogram and revert between CSC and non-CSC refine our view of CSC. This insight rationalizes the observation that certain biological processes, including EMT and autophagy, may have dual effects on CSC. Moreover, the evidence that certain stemness-associated transcription factors, such as Nanog, Oct4, or c-Myc, fluctuate between low and high expression levels within CSC population suggests that CSC cannot be defined by a rigid phenotype.[79,84–88] These updates bring into a new perspective that the state of CSC/non-CSC is a continuum in transition and the entity of CSC may contain diverse cell types of subclones. Bearing these concepts in mind, CSC population in a given study may not necessarily be the culprit for all of cancer initiation, metastatic dissemination, chemoresistance, and disease recurrence or relapse. Future studies focus on reverting cancer cells in CSC state should hold great therapeutic implications.

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