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Cancer Stem Cells: An Overview

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Abstract

Cancer Stem Cells (CSCs) are drug-refractory subgroup of cells within a tumor having proliferative autonomy, differentiation potential, and can recapitulate the primary tumor heterogeneity after transplanting into an immunocompromised host. The intra-tumor heterogeneity caused by these immortal cells poses a challenge for cancer therapy. Understanding the genetic and epigenetic anomalies, and multifaceted mechanisms involved in immune evasion, cellular plasticity, role of extracellular vesicle cargo in the crosstalk between tumor niche and CSCs, would facilitate designing novel therapeutic strategies that exclusively target this quiescent and metabolically distinct subgroup from the heterogeneous tumors. This review summarizes models for tumor heterogeneity, effectors for cellular plasticity, CSC characteristics responsible for therapeutic refractoriness, isolation and novel approaches for targeting this subgroup from rest of the tumor for improving patient's survival.

Keywords: Cancer stem cells; Cellular plasticity; Therapeutic refractoriness; Tumor heterogeneity

Introduction

Cancer is a disease characterized by abnormal proliferation of cells with malignant behavior and is one of the leading causes of death globally. Since the last few decades, there have been outstanding developments in anticancer drug research, novel drug delivery approaches and treatment modalities to cure cancer. Despite these, cancer relapses due to therapeutic refractoriness and hence continues to be a major obstacle for cancer cure. The therapeutic resistance of tumors owes to the existence of cellular heterogeneity within them. Tumors, though start as single clone, subsequently develop into a heterogeneous group of cells composed of multiple clones with varied genetic mutations and epigenetic alterations including distinct promoter methylation patterns leading to genotypic heterogeneity within them [1].

Cancer Stem Cells (CSCs) are suggested to be one of the decisive factors accountable for tumor heterogeneity and relapse [2]. CSCs possess stemness properties such as self-renewability and differentiation characteristics similar to normal stem cells/ progenitor cells. They can differentiate into different lineages of tumor cells through symmetrical or asymmetrical divisions [3,4]. The daughter cells from asymmetric division possess varied fates due to the differences in its morphology, gene expression patterns and succeeding cell divisions endured by them [5]. The hierarchical nature of tumor cell mass is orchestrated via CSCs and is responsible for cancer progression, relapse and poor prognosis [6]. These cells are distinguished from the rest of the tumor mass in which they can stimulate tumor growth and promote metastasis [7]. The quiescent nature of CSCs confers resistance to conventional therapies and cause cancer relapse [8]. Understanding the properties of CSCs, molecular mechanisms responsible for conferring therapeutic resistance to CSCs, and enrichment methods for this population of cells, we believe would pave way for the development of effective therapeutic strategies and identification of novel drug targets to eliminate CSCs, without affecting normal cells. This review presents an overview of the concept of tumor heterogeneity, CSC properties, comprehensive mechanisms by which CSCs attain resistance to chemo- and radiotherapy, enrichment methods used and therapeutic modalities for targeting the CSC subgroup.

Origin of CSCs

Accumulating data obtained from previous studies propose that depending on the tumor type, CSCs might originate from a) bone marrow stem cells/tissue-specific adult stem cells; b) trans-differentiation processes of somatic cells; c) dedifferentiation of progenitor cells; d) reprogramming of differentiated cancer cells e) horizontal gene transfer or cell fusion of cancer cells and normal stem cells [9-13]. These molecular variations are elicited either through activation of oncogenes, inactivation of tumor suppressor genes or as a consequence of the accumulated additional genetic and epigenetic anomalies. Additional factors that regulate CSCs maintenance for tumor initiation, growth and progression are the components of the microenvironment termed as stem cell niche and CSC niche such as stromal cells, hypoxic niche, inflammatory cells etc. Cytokine loops also play a critical role in reprogramming specified cells into CSCs [14].

Two models, the stochastic model (clonal evolution model) and the hierarchical model (CSC model) were proposed to describe the tumor origin, progression and heterogeneity [15,16].

The Stochastic model

The stochastic model proposes that each cell within a tumor own equal potential to generate new homogeneous cell mass [17]. The function of an individual cell is affected by both extrinsic factors (tumor microenvironment) and intrinsic factors (transcription factors and signaling pathways) [9,18]. The tumorigenic ability of any of these cells is attained by acquiring oncogenic mutations stochastically/randomly as a consequence of genomic instability, hyperplasia and irregular proliferation [19]. These genetic and molecular changes confer clonal growth of novel cell types, different from old cells. As a result, cancer cells within the same patient have different functional and phenotypic characteristics [20,21].

The hierarchical model

The hierarchical model illustrates that the structured diverse cell subsets and only a small number of cells, positioned on the top are decisive and have the clonogenic potential *in vitro* and tumor-inducing capability *in vivo* after transplantation into immunocompromised animal models. These cells were later termed as CSCs. The hierarchical model was first elucidated in Acute Myeloid Leukemia (AML), in which a subset of cells expressing $CD34^{+}CD38^{-}$ markers on their surface-initiated leukemia upon transplantation into Severe Combined Immunodeficiency (SCID) mice. These cells showed normal stem cell properties such as self-renewing capacity and multi-lineage differentiation [13,22,23]. The occurrence of CSCs in several solid tumors such as lung [24-27], colon [26], liver [26], prostate [24,26], head & neck [26], ovarian [28], brain [26,29], bladder [30-32], melanoma [33-35], hepatocellular carcinoma [36,37] and retinoblastoma [38,39] has also been reported. Some cancer types like B cell lymphoblastic leukemias are not hierarchically organized and possess homogeneous histopathological features. These cancers gain heterogeneity in response to therapy through clonal evolution [40].

Characteristics of CSCs

CSCs and normal stem cells share similar stemness properties such as self-renewal ability and multi-lineage differentiation. Stem-like properties of CSCs are due to (epi) genetic alterations, changes in cell metabolism and dysregulated signaling pathways. The list of CSC characteristics is given in Table 1.

Characteristics of CSCs
Self –renewal capability
Differentiation ability into heterogeneous lineages of cells which makes up the original tumor.
Ability to survive for a long time - Quiescent nature (G_0 Stage of cell cycle)
Resistance to damaging agents
Active membrane transporter activity (MDR1 and ABCG2 responsible for chemo-resistance)
Anchorage –independent growth and metastasizing capacity through EMT
Active telomerase expression
Expression of cell surface proteins
Altered gene expression profile (genetic & epigenetics variations)
Altered signaling pathways (Wnt, Notch, Hedghog)
Expression of EMT gene signatures
Protective autophagy system
Immune evasion
Cancer stem cell plasticity through EMT
Sphere forming ability
Tumorigenicity
Metabostemness
Tumor invasion
Resistant to apoptosis (ROS scavenging, DNA Repair, Anti apoptosis signaling pathways

Table 1: List of CSCs Characteristics.

Self- renewability & differentiation

The prominent characteristics of CSCs is their ability to self-review and undergo differentiation into multiple cell types that are present in the primary tumor from which they are originated [41,42]. CSCs share both these properties with normal stem cells, but the proliferation of the former group is affected by genetic, epigenetic variations and stress (hypoxia, starvation, and inflammation) [13]. CSC theory postulates that cancer cells derived as a result of CSC differentiation process is accountable for tumor growth and metastasis. Dick and colleagues in 1997 demonstrated that $CD34^+/CD38^-$ cells from AML patients undergo differentiation into leukemia cells *in vivo* [22].

Genetic & epigenetic variations

Emerging literature and data asserts that cancer stemness is directed by genetic (loss of tumor suppressor function & oncogene activation) and epigenetic deviations (aberrant methylation patterns and chromatin remodeling) [43]. Genetic changes in genes encoding DNA methyltransferases also drive CSC formation. Among DNMT1, DNMT3A and DNMT3B; DNMT3A is the most commonly mutated enzyme especially in AML patients [44]. Abnormal methylation patterns have also been observed in solid tumors during the initial stages of carcinogenesis [45].

The role of chromatin in inducing CSC formation through attaining self-renewal ability was evident by genome sequencing data in which gain-of-function mutations in H3F3A gene encoding histone H3 and K27M substitution was observed in pediatric Glioblastoma (GBM) patients [46]. Several other studies have reported that mutated epigenetic regulators such as enzymes involved in chromatin-remodeling, transfer of histone acetyl and methyl groups stimulate the gain of CSCs stemness properties and initiate the tumorigenic process [43]. For instance in leukemias (AML and Acute Lymphoblastic Leukemia (ALL), Leukemia Stem Cell (LSC)) formation is activated by oncogenic Mixed-Lineage Leukemia (MLL) fusion proteins generated by translocations [47]. Notably, MLL fusion proteins can initiate the tumorigenic process in progenitor cells along with hematopoietic stem cells, which clues that committed cells can reprogram to attain stemness property (self- renewability) [48,49].

The process of CSCs development can also be initiated by interactions between non-coding RNAs, specifically microRNA (miRNA) and other epigenetic machinery by targeting its components or reciprocally regulated by epigenetic mechanisms [50].

CSC specific cell surface and functional markers

Cell surface and functional markers such as Epithelial Cell Adhesion Molecule (EpCAM), CD133, CD44, CD24, Thy-1 cell surface antigen (THY1), ATP binding cassette subfamily B member 5 (ABCB5) and ATP-Binding Cassette Super-Family G Member 2 (ABCG2) specific for CSCs were identified for numerous solid tumors and leukemias [39,51]. Combinations of two or three markers are employed to isolate CSC fraction from tumors. They were first identified and isolated from breast cancer based on $CD44^+CD24^{-/low}$ marker expression, side population and aldehyde dehydrogenase$^+$ ($ALDH^+$) subpopulations detection. The enriched fraction exhibited higher tumor-initiating capacity after transplanting into immunodeficient host [52]. In AML, a subset of cells expressing $CD34^+CD38^-$ markers showed tumorigenic potential [23,53]. CSCs from brain cancers were identified based on the expression of CD133, Epidermal Growth Factor Receptor (EGFR), Stage-Specific Embryonic Antigen 1 (SSEA-1) and CD44 [54]. Various studies reported that cells expressing alternate phenotypes of CD133 marker show varied tumorigenic potential *in vitro* and *in vivo*. Both $CD133^+$ and $CD133^-$ cells have the tumorigenic potential in several gliomas, whereas $CD133^+$ brain tumor cells does not induce tumors [55]. In metastatic colon cancer, both $CD133^+$ and $CD133^-$ cells can generate tumors and $CD133^-$ cells could form more aggressive tumors [56]. In another study on Rb primary tumors, $CD133^{lo}$ (FSC^{lo}/SSC^{lo}) population sorted by Fluorescence-Activated Cell Sorting (FACS) exhibited CSC properties, which was additionally proven by their ability to form better and larger colony, augmented invasive potential and showed resistance to therapeutic drug carboplatin when compared to $CD133^{hi}$ cells (non-CSCs) [8,39,57]. Rb CSCs were identified in both mouse and human Rb and they share normal stem cell-like characteristics [38,58,59]. Tumor specific cell surface markers are represented in Table 2.

Cancer type	Markers	Reference
AML	$CD34^+/CD38^-$	[22]
Breast	$CD44^+/CD24^{-/low}/EpCAM^+$	[52]
	$CD44^+/CD24^-/ESA^+$	[153]
	$ALDH1^+/CD44^+/CD24^{-/low}$	[154]
Pancreatic cancer	$CD44^+/CD24^+/ESA^+$	[155]
	$CD133^+/CD44^+/CD24^+/ESA^+$	[156]
Ovarian	$CD133^+$, $CD44^+$, $ALDH1^+$	[157,158]
Lung	$CD133^+$, $ALDH^+$	[159,160]
Colon cancer	$EpCAM^+/CD44^+/CD166^+$	[161]
	$CD133^+/EpCAM^+/CD44^+/CD166^+$	[162]
Liver	$CD133^+$, $CD49f^+$, $CD90^+$	[163,164]
	$CD133^+$, $CD49f^+$, $CD90^+$	

Multiple myeloma CD138 - Multiple myeloma	$CD138^{-}$	[165]
Retinoblastoma (Y79 cells)	FSC^{lo}/ SSC^{lo}/$CD133^{lo}$/ $CD44^{hi}$	[8,57]
	$CD133^{+}$, $Nestin^{+}$ and $OCT4^{+}$	[166]
	$ABCG2^{+}$	[58]

Table 2: Tumor specific cell surface markers.

Altered signaling pathways in CSCs

Common signaling pathways involved in CSCs are Janus kinase (JAK), Signal Transducer and Activator of Transcription (STAT) proteins, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), wingless-related integration site (Wnt) β-catenin. Among all, Hedgehog and Notch are considered as important regulators of CSCs. Normal stem cells and CSCs share almost similar type of signaling pathways, however because of the irregularities in the signaling cascade of the latter group they acquire survival properties such as self-renewability, differentiation through abnormal regulation of transcriptional factors and proapoptotic genes [60-62] resulting in therapeutic resistance towards cancer therapies. Notch signaling is an evolutionary conserved and the most activated signaling pathways in almost all cancer types and play a key role in cell differentiation and cell cycle progression [63]. Canonical and noncanonical pathways of Wnt signaling cascade regulate cell polarity, survival, proliferation and migration [64]. Hedgehog pathway helps in gaining stemness properties through EMT [60]. By understanding the molecular mechanisms involved in deregulated pathways would pave way for designing novel therapeutic strategies for targeting CSCs.

Therapeutic resistance of CSCs

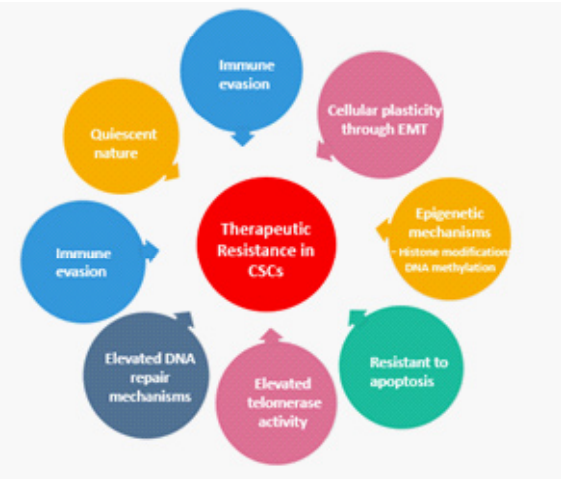

Figure 1: Molecular mechanisms that contribute to therapeutic resistance in CSCs.

The quiescent nature of CSCs

A small subset of tissue-specific adult stem cells persists in quiescent state (G_0) to compensate tissue loss, to survive under metabolic stress and to maintain genomic integrity throughout the lifespan of an organism [65]. Similarly, for CSCs, quiescent state is necessary for maintaining their self-renewal ability and is a critical factor for these cells to develop therapeutic resistance [66]. In several malignancies like breast and colon, the tumor can relapse after many years due to the survival of quiescent CSCs [18]. Stem cell quiescence is regulated by various intrinsic molecules like reactive oxygen species (FoxO group), transcription factors (HIF-1α & NFATc1 poietin-1) and extrinsic molecules such as TGF-β, thrombopoietin, osteopontin, adhesion proteins (N-cadherin & β1-integrins) that are found within the niche which are critical for the long-term maintenance of stem cells in quiescence. Understanding the regulatory mechanisms (ATM, mTOR and Wnt/β-catenin signaling) involved in quiescence in stem cells is important for the development of novel therapeutic strategies to target quiescent CSCs [67].

ABC transporters and Drug efflux pathways in the maintenance of drug resistance in CSCs

ATP Binding Cassette (ABC) transporters are highly conserved transmembrane proteins, which are classified into seven gene subfamilies, designated ABCA–G. Their normal physiological functions are detoxification system, defense against oxidative stress, xenobiotics and lipid metabolism [68]. High expression of ABC transporters has been described in numerous cancers and the chemo resistance for CSCs is attributed to members of this group of proteins called Multi Drug Resistant Proteins (MDR), which are responsible for MDR phenomenon, in which cancers become resistant to numerous drugs that have little structural and functional similarity. These proteins transport cytotoxic drugs as well as signaling molecules out of the cells and promote tumorigenesis [69,70]. Among all, ABCB1 (MDR1 or P-glycoprotein), ABCC1 (multidrug resistance-associated protein 1, MRP1) and ABCG2 (breast cancer resistance protein, BCRP) are the most studied proteins in the MDR family as these transporters are expressed in majority of drug resistant tumors. As compared to normal cells and cancer cells, some MDR transporters are expressed more highly in CSCs. For instance, ABCG2 is overexpressed in breast CSCs [71], ABCB1 in ovarian CSCs [72] and ABCB5 in malignant melanoma initiating cells (MMIC) [73].

The efficacy of anticancer drugs can be elevated by inhibiting members of ABC transporter family of proteins that are regulated by various signaling pathways which are involved in stem cell renewal and differentiation. For instance, Lapatinib inhibits receptor tyrosine kinase 2 (ERBB2) and doxorubicin sensitizes breast CSCs by inhibiting ABCB1 and ABCG2 [74].

The ALDH1 mediated drug detoxification systems

Aldehyde Dehydrogenase (ALDH) is a superfamily of phase I oxidizing and $NAD(P)^+$-dependent enzymes that protect living organisms against oxidative stress and lipid peroxidation [75] and are essential for the biosynthesis of Retinoic Acid (RA), γ-aminobutyric acid, and betaine [76,77]. Other than these functions, ALDH members such as ALDH1A1 and ALDH1A3 are studied as metabolic stem cell markers as they regulate cellular function in both normal stem cells and CSCs [78-80]. The higher activity by ALDH members has been implicated in cellular resistance against radiation in breast cancer cells [79,81] and in prostate cancer progenitor cells [76,82]. Several studies have reported that, within a tumor cell population $ALDH^{br}$ cells are more clonogenic and tumorigenic than $ALDH^{dim}$ cells [83] and the presence of $ALDH^{br}$ cells has been associated with poor outcomes in a number of cancers [80].

Immune evasion

The first line of defense against growth of tumor cells under normal physiological conditions is the activation of innate immunity of the organism. Fundamental molecules involved in this mechanism are interferon-gamma, Interleukin-12 (IL-12), perforin, TNF-Related Apoptosis-Inducing Ligand (TRAIL), Death Receptors (DR4 and DR5) and the Recombination Activating Genes (RAG1, and RAG-2) which are also required for cell development [84]. The capability of cells to escape from the immune system is thus recognized as a hallmark of cancer metastasis and is one of the distinct properties of CSCs. This phenomenon is caused by three different mechanisms: a) by down regulating the components of the antigen processing and presentation pathways such as transporter (TAP)1, TAP2, Major Histocompatibility (MHC)I and/or MHC-II, which prevent the exposure of neo-antigens [85]; b) either by low expression of T-cell activation co-stimulatory molecules or by elevated expression of T-cell inhibitory molecules, as well as Programmed Death-Lignad1 (PD-L1) [86]; c) by promoting the expansion of pro-tumorigenic immune phenotypes [87,88]. This phenomenon of immune evasion was detected in CSCs of head and neck squamous cell carcinoma, melanoma, glioblastoma and colorectal cancer, where they have decreased expression of MHC and/or TAP molecules [88]. These properties make CSCs poor targets for the cell-mediated immune response of T cells and contribute to the immune privileged status of CSCs within the tumor microenvironment.

Cellular plasticity through Epithelial to Mesenchymal Transition (EMT)

One of the possible mechanisms responsible for attaining chemo résistance by CSCs is cellular plasticity through EMT, which is a complex and reversible phenotypic process. In this process epithelial cells lose their differentiated features and acquire mesenchymal characteristics and contribute to invasiveness, metastatic propagation and acquisition of high resistance to apoptosis resulting in therapeutic resistance of cancer cells towards conventional therapies [3,89]. This switch might be induced by the tumor microenvironment that secretes EMT-inducing growth factors (Transforming Growth Factor Beta (TGFβ) family of cytokines), activation of signaling pathways (Wnt, Notch, Receptor Tyrosine Kinases (RTKs), NF-κB and Hedgehog) and the interaction with the extracellular matrix and is closely associated with the acquisition of stem cell-like characteristics in tumors [90-92] . Some of the well-known EMT markers are E-cadherin, β-catenin, N-Cadherin, vimentin, fibronectin, snail, slug, twist and ZEB1. The reversal of EMT, i.e MET (Mesenchymal to epithelial) transition seem to happen through subsequent dissemination and leads to the consequent development of distant metastases. Recent reports have suggested that a new concept of a hybrid Epithelial/Mesenchymal (E/M) state exists, which has higher tumor - initiating potential than their individual states “E” and “M” and its expression was correlated with tumor aggressiveness in triple negative breast cancer patients [93-95]. This hybrid state was also observed in ovarian, lung and renal cell carcinoma cell lines, where both epithelial and mesenchymal markers were co-expressed at the same time [96,97]. The process of EMT, factors responsible for EMT and MET transitions, and epithelial and mesenchymal cell properties are elucidated in figure 2.

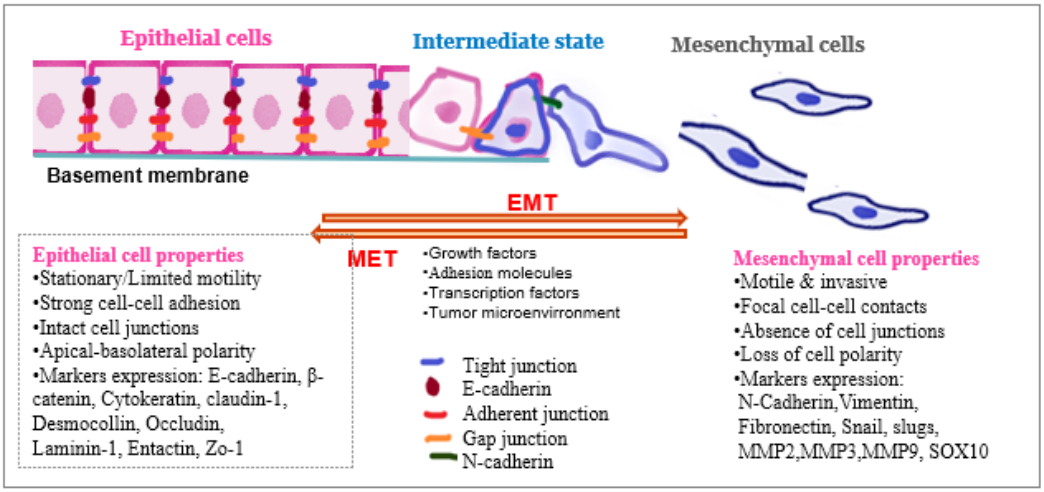

Figure 2: Epithelial-Mesenchymal Transition. During metastasis "CSCs undergo EMT", which involves functional transition of stationary epithelial cells into motile mesenchymal cells through an intermediate state where the cells exhibit both the properties of epithelial and mesenchymal cells under the influence of growth and transcription factors, adhesion molecules and tumor microenvironment. The mesenchymal cells migrate to adjacent stromal tissues and invade blood or lymph vessels. The properties of epithelial and mesenchymal cells are cited here.

Understanding the complex physiological processes involved in EMT and MET transitions and subsequent cellular plasticity to acquire stemness properties by CSCs will help to design targeted drugs against these cells [93].

Resistance to Apoptosis

One of the important characteristic of CSCs that contribute to therapeutic resistance is their reduced apoptosis potential [98,99]. CSCs acquire this property by multiple mechanisms such as up regulation of antiapoptotic Bcl-2 family proteins, drug resistance proteins like Interleukin (IL)-4, Inhibitors of Apoptosis Proteins (IAPs), activation of Phosphoinositide 3-Kinsae (PI3K) or serine-threonine protein kinase (AKT) signaling cascade, down regulation of death receptors such as Fas and TRAIL and paucity in mitochondrial- mediated apoptosis contribute to enhanced resistance to apoptosis initiation in numerous cancers [100,101]. Novel approaches that can be used to target CSCs by inducing apoptosis are through a) activating cell death pathways using synthetic Fas ligands like Apo010 (a hexameric FAS-L), b) triggering the extrinsic apoptosis pathways using proteasome inhibitor bortezomib, which will increase surface expression of DR5, c) using unique delivery methodologies of DR ligands, d) inhibiting Bcl-2 and Inhibitor Apoptotic (IAP) family of proteins with small RNA molecules and e) disrupting mitochondrial energy metabolism [102].

Elevated telomerase activity

Telomerase is a RNA-dependent DNA polymerase enzyme that uses catalytic subunit, Telomerase Reverse Transcriptase (TERT) and plays a key role in adding 5′ - TTAGGG - 3′ repeated sequence to the terminal ends of the human chromosomes as they lose telomeric repeats with each cell division. Expression of telomerase is observed in 90% of human cancers, but is inactive in normal somatic cells [103-105]. Numerous studies have reported that, the expression of telomerase is higher in CSCs compared to non-CSCs, and its expression is critical for the self-renewal, progression and immortalization of CSCs [106-108].

CSCs & Tumor microenvironment

Tumor Microenvironment (TME) is composed of two components: cellular and non-cellular. Along with malignant cells, the cellular portion constitutes mesenchymal stem cells, endothelial cells, Cancer Associated Fibroblasts (CAFs), immune cells, adipocytes, myeloid cells, Natural Killer (NK) & NKT cells and pericytes [109]. The non-cellular component of TME includes Extracellular Matrix (ECM), which constitutes of collagen, glycoproteins, proteoglycans and integrins [110]. Number of studies have reported that stromal cells such as CAFs support the stemness of CSCs by releasing cancer cell derived secretory molecules, which further activates Wnt/β-catenin and Notch signaling pathways [111] and cancer progression is mediated via a feedback mechanism, where CSC influences CAF activity through activation of Hedgehog signaling cascade [112]. In addition, the metastatic latency of CSCs is attributed to TME, as it provides the signals that regulates self-renewal, EMT, inflammation, hypoxia and angiogenesis which will either regulate entry of CSCs into

quiescent state or stimulate the reactivation of CSCs that initiate metastasis and modulates the ability of CSCs to escape the innate immune response and survive in the metastatic niche [9].

The observed interactions between CSCs and TME provides critical insights into the mechanism of cancer drug resistance and recurrence.

CSCs & Hypoxia

Impaired vascularization within a primary tumor results in hypoxic condition, which is an important characteristic feature of locally advanced solid tumors. In cancer patients, tumor hypoxia is positively correlated with poor prognosis, aggressive tumor behavior and resistance to therapy [113,114]. Heddleston, et al. demonstrated that between the two transcriptional hypoxic signatures hypoxia inducible factor (HIF)-1α and HIF2α, the former is known to express in normal neural progenitors and cancer cells. Its expression is critical for the proliferation and survival of these two cell types, whereas HIF2α is expressed only in cancer stem cells and not expressed by normal neural progenitors indicating its role as a specific target for CSCs [7].

Role of Extracellular Vesicles in crosstalk between tumor niche and CSCs

Extracellular Vesicles (EVs) are a diverse group of membrane-bound vesicles, first identified in plasma by Wolf, who referred to them as "Platelet dust" [115]. Three main types of EVs have been described based on their subcellular origin, type of release and size: exosomes (30–100 nm in diameter), micro vesicles (100–1,000 nm) and ectosomes or shedding vesicles (larger than 100nm) [116]. EVs are considered as a new mode of intercellular communication and tumor-derived EVs play a significant role in various cancers via transfer of cargo lipids, proteins, DNA and RNAs to the recipient tumor cells [117]. CSC derived EVs reprogram the local cells genetically and affect the immune cells of TME, which influences the tumor cell fate. Recent studies have demonstrated that, EVs particularly exosomes derived from various solid tumors such as prostate [118-120], breast [121-123], lung [124,125] and colorectal cancers [126-128] could influence the components of the microenvironment such as macrophages, mesenchymal stromal cells, CAFs, osteoclasts etc., and alter the tumor niche through a cargo of microRNAs like miR-409, miR-21, miR-378e, miR-143, miR-125a, miR-126 and by activation of AKT, IL6/STAT3 signaling pathways [117]. The released exosomes also have the ability to travel to distant organs and form a pre-metastatic niche [125]. However, other *in vitro* and *in vivo* studies have reported the anti-tumor effect of EVs derived from stem cells by obstruction of cell proliferation and induction of apoptosis in gliomas, lympho blastomas and liver carcinomas [129-131]. In addition, EVs derived from human liver stem cells improved the efficacy of Tyrosine Kinase Inhibitors (TKIs) by elevating the apoptosis of CSCs isolated from renal carcinomas [117]. By understanding the role of EVs crosstalk between tumor niche, and CSCs and characterization of EVs from both normal stem cells and CSCs could help in the identification of novel tumor biomarkers and development of new therapeutic targets.

Isolation and characterization of CSCs

Compelling evidence suggests that CSCs are the core cause of tumor initiation, progression, invasion, metastasis, therapeutic resistance and relapse which therefore makes their analysis crucial for targeting and eradication of this sub group from the tumors [13,132]. Various enrichment methodologies have been developed based on CSC properties. The isolated CSC populations are further tested for their self-renewal capacity and ability to form tumors. Two *in vitro* assays that are being widely used for demonstrating self-renewal of CSCs are clonogenic assay and sphere formation assay [133]. The tumor seeding capability of CSCs is validated by *in vivo* xenotransplantation assay using immunocompromised animal models. Other CSC isolation methods are based on cell surface and functional markers, ability to efflux Hoechst 33342 dye, quiescent nature, invasion capability and *in vivo* tumor forming ability [134].

Therapeutic strategies for targeting CSCs

Strong evidence suggests that after the standard treatments including chemo and radiotherapy, the CSC subset remains alive, and are accountable for Minimal Residual Disease (MRD) and cancer relapse [135]. In recent years multiple mechanisms, physiological processes of CSCs have been elucidated in order to eradicate CSCs to improve patient survival [125,136,137]. Numerous CSCs-specific tactics such as ATP-Binding Cassette (ABC) transporters, obstruction of self-renewal and survival of CSCs, surface marker and functional marker specific for CSC targeted drugs delivery, molecular agents for disrupting tumor microenvironment and MDR group of enzymes have already been developed against various cancers [136,138-140]. Elevated telomerase activity was observed in CSCs of breast and lung cancer cells compared to non-CSCs, targeting these cells based on this property is an effective strategy to eliminate this subgroup [141,142]. The telomerase inhibitor, imetelstat has effectively inhibited the cancer stem-like cell proliferation in glioblastoma and prostate cancer [142,143].

The prime reason for therapeutic resistance of CSCs is due to their quiescent state which can be altered by inducing them to enter into cell cycle through the inactivation of Fbw7, a component of E3 ubiquitin ligase contribute to the cell cycle arrest evident in CSCs [144,145]. Another promising approach developed for targeting CSCs is microRNA (miR) dependent. Transfection of miR34a and ectopic expression of miR206 reduces the stemness properties of breast cancer cells *in vitro* and *in vivo*

[146,147]. Mesenchymal Stem Cells (MSCs) are widely used to deliver specific miRs such as miR9 antisense oligonucleotide to control the expression of drug-resistant protein, p-glycoprotein in glioblastoma cells [148]. Along with these, novel drug delivery systems and unique secretory molecules such as exosomes have been identified to eradicate CSC subgroup from the heterogeneous tumors. The role of exosomes as information carriers and their existence in tumor microenvironment for maintaining the dynamic equilibrium state between non-CSCs and CSCs suggest that they might be an effective strategy for cancer therapeutics. For instance, molecular inhibitors such as GW4869, heparin are used to target molecules involved in exosomes regulating non-CSCs dedifferentiation pathway [149]. MSC derived exosomes are used to deliver synthetic miR122 to elevate the sensitivity of liver tumors to chemotherapeutic drug, sorafenib [150].

In addition to the above stated therapeutic approaches, numerous genomics, proteomics, bioinformatics tools and databases to integrate the genes were employed to understand the molecular mechanisms responsible for the origin of CSCs, tumor progression and metastasis in numerous cancers including lung [151] and in recurrent glioblastoma [152]. A summary of therapeutic strategies targeting CSCs are shown in Figure 3.

Figure 3: Strategies for targeting CSCs based on repressing surface marker expression, inhibiting the signaling pathways, promoting cell death pathways including apoptosis, inhibiting telomerase activity and drug efflux pumps, interfering the tumor microenvironment and epigenetic mechanisms, targeting self-renewal ability and cell differentiation process.

Future Directions

With the evolving techniques to identify and target CSCs in various tumors, it is quite logical to predict that future treatment strategies would include a combined therapy that addresses both CSCs and non-CSCs through in-vivo tracking, localization and targeting them at a microscopic level.

Summary

Compelling evidence demonstrates that the CSC population is accountable for the origin and development of cellular heterogeneity in tumors ensuing therapeutic resistance and cancer relapse. The effective strategy to improve cancer patient survival is through complete eradication of both CSCs and non-CSC populations. Comprehensive understanding of cellular heterogeneity in tumors, cellular plasticity and reprogramming mechanisms responsible for generating CSCs from different cell types offer new insights to develop effective methodologies to exterminate already existing CSCs and avert the generation of new diverse CSC subtypes. The therapeutic strategies that would be useful for targeting CSCs are based on surface marker, involving signaling cascades, epigenetic alterations or mechanisms that modulate CSC reprogramming and differentiation, molecules involved in immune evasion, tumor microenvironment, and tumor niche. Identification of unique dissimilarities owned by normal and CSCs can help in designing novel agents for targeting CSCs alone. Thus, targeting CSCs, the under bunker enemies, which are well protected in their niche and are resistant to chemo and radio-therapy would be crucial for cancer regression and for improving patient survival.

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