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Latest Therapeutic Approaches Based on Cancer Stem Cells

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1. Introduction

A tumor is a caricature of normal tissue and appears undifferentiated because of the preponderance of undifferentiated proliferating stem cells in relationship to the number of cells that have differentiated and become benign. The hypothesis that cancer arises from de-differentiation suggests that cancer is a multigenic complex disease that broadly represents uncontrolled proliferation, blockage in cellular differentiation and metastasis [1]. Recently, cancer stem cells (CSCs) theory hypothesizes the presence of a hierarchically organized, relatively rare population of cells that possess self-renewal capacity and pluripotency, and can drive tumor initiation and maintenance, and the hypothesis has been generally accepted by most basic and clinical scientists.

In recent years the putative CSCs have become the focus of intense research, with the understanding the biological characteristics of the CSCs that have now been identified in several tumor types might prove useful in new therapeutic targets [2]. It is known that tumor ablation, hormonal therapy, radiotherapy, antiangiogenic, and chemotherapy, individually or in combination with, 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 cancer is often associated with intrinsic or acquired resistance to routine treatment, and the prognosis is poor. The CSC model has favored the conceptual development of targeted therapies towards molecular pathways relevant in CSC maintenance and progression, independent of their ability to induce cell death of non-CSC [3]. However, how to achieve CSC eradication is emergency to overcome the major barrier. It is thus evident that latest therapeutic approaches based on CSCs have suggested that developing novel strategies by focusing biological characteristics on the CSCs may allow for the discovery of effective methods to eradicate seeds of malignant tumor cells by specifically targeted therapy. In this article, we describe here latest therapeutic approaches based on CSCs over the past years, of which includes targeted therapy directed toward CSCs by

surface specific markers, CSC survival niche, blocking signal pathways, manipulation of miRNA and siRNA, screening directly drug-resistant CSCs in three dimensional cell culture and dendritic cell vaccination.

2. Therapeutic strategies to target CSCs

2.1 Targeted therapy directed toward CSCs

CSCs have been clearly identified in leukemias and some solid tumors. The major barrier to therapy is the CSCs with constitutive multidrug resistance (MDR). The approaches can be developed to eliminate the CSCs without excessive toxicity to normal stem cells if successful therapy awaits the discernment of biological and immunologic differences between the tumor and normal stem cells. Increasing evidence suggests that the treatment approaches may be acquired successfully by the targeted therapy directed toward CSCs.

2.1.1 Oncolytic viruses-based CSC therapies

Oncolytic viruses have undergone many developments and through multiple generations offer an effective way to specifically target and eradicate CSCs without damaging normal tissue and stem cells. Therefore, use of oncolytic adenoviruses or oncolytic reovirus presents an attractive antitumor approach for eradication of CSCs. For instances, 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 Ad5pk7-Delta 24. 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 Ad5pk7- Delta24 could kill $CD44^{+}CD24^{-/low}$ breast CSCs as well as committed breast cancer cells, making them promising agents against CSCs [4,5]. More recently, to test the efficacy of oncolytic reovirus to target and kill breast CSCs that were identified based on $CD44^{+}$ $CD24^{-/low}$cell surface expression and overexpression of aldehyde dehydrogenase *in vivo*, Marcato and his colleagues established palpable tumors in the mammary fat pads of immunodeficient NOD/SCID mice using a core biopsy sample of a primary infiltrating ductal carcinoma obtained from a breast cancer patient at the time of her primary surgery. The result showed that the oncolytic reovirus that induces inhibition of human breast cancer primary tumor samples xenografted in immunodeficient NOD/SCID mice also effectively targets and kills CSCs in these tumors. The experiments indicate that oncolytic reovirus has the potential to induce tumor regression in breast cancer patients. More important, the CSC population was equally reduced and was as susceptible to reovirus treatment as the non-CSC population [6].

2.1.2 Telomerase-based CSC therapies

Telomerase is the enzyme that synthesizes telomeric DNA, the terminal DNA at chromosome ends which, together with telomere-binding proteins, confers stability to hromosomes [7, 8]. Telomerase is expressed in almost all cancer cells and is required for long-term maintenance of telomeres and replicative immortality but is inactive in most normal somatic cells. The telomerase properties suggests that tumors are less likely to develop resistance to telomerase-based therapies than they are to other cancer targets that are members of a family of genes such as growth factor receptors or signal transduction enzymes. Putative CSCs are telomerase-positive and require telomerase to proliferate,

therefore, telomerase is an important drug target for CSCs. Imetelstat (GRN163L), a specificinhibitor of the reverse transcriptase activity of telomerase, in combination with effective tumor de-bulking agents, might help meet a major unmet need: that is notoriously resistant to standard chemotherapy and radiation [9]. The study indicated that the telomerase activities in established radioresistant (R) esophageal carcinoma cell lines (Seg-1R Seg-1R, Seg-1R side population (SP), and TE-2R) were significantly higher than in Seg-1, Seg-1R non-SP, and TE-2 cells, respectively. Using the telomerase-specific oncolytic adenoviral vector carrying apoptotic tumor necrosis factor-related apoptosis-inducing ligand and E1A gene (Ad/TRAIL-E1) may preferentially target CSCs because Seg-1R and TE-2R CSCs were more sensitive to Ad/ TRAIL-E1 than parental cells. Increased coxsackie-adenovirus receptor and elevated transgene expressions were found in the Seg-1R and TE-2R CSCs. Ad/TRAIL-E1 caused marked tumor growth suppression and longer survival in Seg-1R-bearing mice with no obvious toxicity [10]. In breast and pancreatic cancer cell lines, telomerase activity in the bulk tumor cells and CSC subpopulations were inhibited when these cells were treated with GRN163L *in vitro*. Additionally, *in vitro* treatment with GRN163L, but not control oligonucleotides, the breast and pancreatic cell lines also reduced the proliferation and self-renewal potential and resulted in cell death after less than 4 weeks of treatment. *In vitro* treatment of PANC1 cells showed reduced tumor engraftment in nude mice, concomitant with a reduction in the CSC levels. Significantly, the differences between telomerase activity expression levels or telomere length of CSCs and bulk tumor cells in these cell lines did not correlate with the increased sensitivity of CSCs to GRN163L, suggesting a mechanism of action independent of telomere shortening for the effects of GRN163L on the CSC subpopulations [11]. However, in human multiple myeloma CSCs derived from cell lines and primary clinical specimens, telomerase inhibition targets clonogenic myeloma CSCs through telomere length-dependent and independent mechanisms. This is because two weeks of exposure to GRN163L resulted in a significant reduction in telomere length and the inhibition of clonogenic myeloma growth both *in vitro* and *in vivo* ^{125}I-TSH binding inhibitor immunoglobuli.

No matter what mechanisms, through telomere length-dependent and independent mechanisms or mechanism of action independent of telomere shortening, these results suggest that GRN163L-mediated depletion of CSCs may offer an alternative way by which telomerase inhibition may be exploited for CSCs therapy [12].

2.2 Targeted therapy toward CSC by surface specific markers

CSC markers are expressed in the different cancers with the different patterns seen for the different histological types and degrees of differentiation. These markers provide the means to effectively target and eradicate CSCs.

2.2.1 ABCB5 and ABCG2 markers

ABCB5 molecule is one of ATP-binding cassette (ABC) transporters that have been identified in a variety of mammalian cells and represents a novel molecular marker for a distinct subset of chemoresistant, stem cell phenotype-expressing tumor cells among melanoma bulk populations, whereas, none of the benign nevi of non-melanoma patients demonstrated expression of ABCB5. One study indicated that 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 or ABCB5 blockade significantly reversed resistance of G3361 melanoma cells to doxorubicin [13, 14].
ABCG2, also called BCRP1 (breast cancer resistance protein 1) and another ABC transporters, often overexpress in therapeutics-resistant CSCs. More recent study showed that using ABCG2- molecule-expressing side population cells may identify cancer stem-like cells in a human ovarian A2780 cell line. The up-regulation of ABCG2 in cancer stem-like cells is closely associated with resistance to anticancer drugs vincristine, and after the ABCG2$^+$ cancer stem-like cells were incubated with the anti-ABCG2 monoclonal antibody, the cell survival rate was remarkably decreased compared with the ABCG2$^+$ cancer stem-like cells without monoclonal antibody incubation. The data suggest that the selective anti-ABCG2 monoclonal antibody can effectively targets and inhibits ovarian cancer stem-like cell growth in the A2780 cell line [15].

2.2.2 CD133 markers

CD133 molecule has been proposed as a surface marker of CSCs in a variety of solid primary tumors such as medulloblastomas, glioblastomas and subsequent CSCs in the cancers of epithelial tissues [16]. For noninvasive imaging of CSCs as well as CSC-specific therapies, Tsurumi et al used the CD133-specific monoclonal antibody AC133.1 for quantitative fluorescence-based optical imaging of mouse xenograft models based on isogenic pairs of CD133 positive and negative cell lines. They selected lentivirally transduced CD133-overexpressing U251 glioblastoma cells and HCT116 colon carcinoma cells that uniformly express CD133 at levels comparable to primary glioblastoma stem cells. The results indicated that the visualization and quantification of CD133 in overexpressing U251 xenografts was set up. The binding of i.v.- injected AC133.1 antibodies to CD133$^+$CSCs isolated from xenografts made efficient targeting and elimination of CSCs. This data showed that CD133 antibody-based CSC targeting is feasible [17]. In addition, a murine anti-human CD133 antibody (AC133) conjugated to a potent cytotoxic drug, monomethyl auristatin F, effectively inhibited the growth of Hep3B hepatocellular and KATO III gastric cancer cells by induced cell apoptosis in the cancer cells *in vitro*. Simultaneously, anti- CD133-drug conjugate treatment also resulted in significant delay of Hep3B tumour growth in SCID mice [15]. Certainly, it is pivotal to how select and develop the specific antibodies to CSCs as well as the targets they bind to and the drugs used in combination with them.

2.3 Targeted therapy toward CSC survival niche

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. The hypothesis that CSCs may exist in microenvironmental niches is also important in understanding the potential dynamic state of CSCs [19, 20]. 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.

2.3.1 Vascular niche

Oncogenic transformation and aberrant cellular differentiation are regarded as key processes leading to CSCs. Intracellular events involved in these changes profoundly impact the extracellular and systemic constituents of cancer progression, including the angiogenesis, vasculogenesis, activation of the coagulation system and formation of CSC-related and premetastatic niches. It is known that CSCs initiate tumor neovascularization and promote invasion by the secretion of vascular endothelial growth factor (VEGF) and recruit bone marrow-derived cells (BMDC), which play a critical role during the progression of cancer in tumor-bearing mice and cancer patients, to what is now referred to as the pre-metastatic niche. Although the mechanisms of CSC neovascularization is not clear an interfering with BMDC induction by blocking secretion of VEGF virtually eliminates metastasis in these cancers. For example, Bevacizumab, a monoclonal antibody against VEGF, and poly-ADP- ribose polymerase inhibitors, is currently in clinical trials for its antiangiogenic properties that have shown promising preclinical and clinical activities against metastatic colorectal cancer, particularly in combination with chemotherapy [21, 22]. Herbert et al treated the 402 patients with previously untreated metastatic colorectal cancer with irinotecan, bolus fluorouracil, and leucovorin (IFL) plus bevacizumab (5 mg per kilogram of body weight every two weeks), and the 411 patients by IFL plus placebo, respectively. The overall survival, the response rate, the duration of the response, safety were evaluated. The results showed that the median duration of survival was 20.3 months in the group given IFL plus bevacizumab, as compared with 15.6 months in the patients treated with IFL plus placebo, corresponding to a hazard ratio for death of 0.66 ($P<0.001$). The median duration of the response was 10.4 months in the patients treated with IFL plus bevacizumab, as compared with 7.1 months in the patients treated with IFL plus placebo ($P=0.001$).The data showed that the addition of bevacizumab to fluorouracil-based combination chemotherapy results in meaningful improvement in survival among patients with metastatic colorectal cancer[21].

Investigation of the role of CSCs in tumor vascularization and the interaction between CSCs and their vascular niches provides new insight into our understanding for tumorigenesis. A growing body of literature shows that CSCs preferentially produce higher levels of angiogenic factors, for instance, VEGF and interleukin 8 (IL-8, CXCL8). In an established breast carcinoma cell line MCF-7, an identified subpopulation of sphere-forming cells with CSC properties expressed higher levels of VEGF mRNA and detected higher amounts of VEGF protein in culture medium, which suggested CSCs might possess stronger proangiogenic capability than more differentiated tumor cells [23]. Evidence from the studies has indicated that CSCs might generate or transdifferentiate into endothelial cells (ECs) for neovascularization. 20%-78% of the ECs identified by CD105 expression exhibited amplification of the oncogene MYCN in the origin of the nephroblastoma, all of which strongly implicates the possible cancer cell origin of ECs in MYCN amplification [24] nephroblastoma. These studies suggested that CSCs might initiate and promote neovascularization at the early stage of tumor tumorigenesis and progression. The fact that vascular niches support self-renewal, proliferation and differentiation of CSCs suggests the combination of targeting CSCs and their vascular niche will provide more effective therapy for tumor treatment [25].

Tissue factor (TF) is a unique cell-associated receptor for coagulation factor VIIa, initiator of blood coagulation, and mediator of cellular signalling, all of which influence vascular homeostasis. TF pathway may play an important role in formation of the vascular niche for

tumor initiating CSCs, through its procoagulant and signal effects. It was noted recently that in human glioma cells, a transforming mutant of the epidermal growth factor receptor (EGFRvIII) triggers not only the expression of TF, but also of its ligand (factor VII) and protease activated receptors (PAR-1 and PAR-2). The tumor cells expressing EGFRvIII become hypersensitive to contact with blood borne proteases (VIIa, thrombin), which upregulate their production of angiogenic factors (VEGF and IL-8), and result in formation of the growth promoting microenvironment (niche). If TF was overexpression the change could accompany the features of cellular aggressiveness such as markers of CSCs (CD133), epithelial-to-mesenchymal transition (EMT) and expression of the angiogenic and prometastatic phenotype. If TF was blocked with blocking antibodies the change could inhibit tumor growth, angiogenesis, and especially tumor initiation upon injection of threshold numbers of tumourigenic cells. These observations suggest that both cancer cells and their adjacent host stroma contribute TF activity to the tumor microenvironment. Therefore, the TF pathway may play an important role in formation of the vascular niche for CSCs, through its procoagulant and signaling effects. Therapeutic blockade of these mechanisms could hamper CSC processes [26-28].

2.3.2 Tumor-associated ECM

An increasing body of evidence has shown that the host microenvironment undergoes extensive change during the evolution and progression of CSCs. This involves the generation of cancer-associated fibroblasts (CAFs), which lead to enhanced angiogenesis, increased tumor growth and invasion by release of growth factors and cytokines that activate the adjacent extracellular matrix (ECM) and induce the selection and expansion of CSCs. The tumor- associated ECM may be modified and lead to altered signaling in tumor cells. This activated ECM also conferred chemoresistance mediated by β_1-integrins that adhere to fibronectin, leading to the activation of β_1-integrin [29,30]. Thus, anti-ECM agents may have a place in overcoming resistance to chemotherapy. The expression of 'tumor specific' ECM proteins has been exploited to target delivery of bioactive molecules to tumors: these ECM components are highly abundant in tumors and are often more stable than antigens located on the cell surface of tumor cells. Radiolabelled antibodies specific to fibronectin and tenascin-C domain C (TNC) domains A1 and D have been used successfully in the clinic to treat glioma and lymphoma [31]. However, the ECM in solid tumors affects the effectiveness of therapeutics through blocking of intratumoral diffusion and/or physical masking of target receptors on malignant cells. Based on the situation, the researchers used monoclonal antibodies towards tumor-associated ECM isoforms such as TNC to target ECM. Using antibody phage technology, a human monoclonal antibody to the C domain of TNC has been generated, and this scFv protein shows a highly selective uptake in gliomas, making it a promising tool for the future [32]. In immunohistochemical studies of tumor sections from breast cancer patients and xenografts, Beyer's group observed colocalization of ECM proteins and Her2/neu, a tumor-associated antigen that is the target for the widely used monoclonal antibody trastuzumab (Herceptin). They tested whether intratumoral expression of the peptide hormone relaxin (Rlx) would result in ECM degradation and the improvement of trastuzumab therapy by a hematopoietic stem cell (HSC)-based approach to deliver the Rlx gene to the tumor. HSC-mediated intratumoral Rlx expression resulted in a decrease of ECM proteins and enabled control of tumor growth, in mouse models with syngeneic breast cancer tumors. If trastuzumab therapy was combined with Rlx expression

a significant delay of tumor growth emerged in a model with Her2/neu-positive BT474-M1 tumors as well as more treatment- refractory tumors derived from HCC1954 cells [33].
These studies above-mentioned suggested that the CAFs in tumor-associated ECM plays a critical role in the regulation of tumor behavior—that of modulating the CSC phenotype. The researchers try to find the methods to specifically kill CAFs. For example, Loeffler et al constructed an oral DNA vaccine targeting fibroblast activation protein, which is specifically overexpressed by fibroblasts in the tumor stroma. Through $CD8^{+}$T cell-mediated killing of CAFs, the vaccine successfully suppressed primary tumor cell growth and metastasis of multidrug-resistant murine colon and breast carcinoma. Furthermore, tumor tissue of fibroblast activation protein -vaccinated mice revealed markedly decreased collagen type I expression and up to 70% greater uptake of chemotherapeutic drugs. This strategy opens a new venue for the combination of immuno-and chemotherapies [30]. Another study indicated that the co-culture of colon cancer cells with myofibroblasts or myofibroblast conditioned medium resulted in enhanced nuclear β-catenin, increased Wnt activity and enhanced clonogenic activity, with enhanced tumorigenicity when coinjected into mice. These studies suggest that the stem cell phenotype is plastic and is dependent on the tumor-associated ECM, and that targeting the CSC-tumor- associated ECM interface may be the most effective approach to overcome stem cell resistance to current therapies[34].Elucidating the nature of the interactions between the tumor and the multiple facets of the microenvironment will allow us to harness this relationship for clinical benefit.

2.4 Targeted therapy toward CSCs by blocking signal pathways

In order to effectively target CSCs, a detailed understanding of the signal pathways regulating the growth and self-renewal of CSCs is needed. It is now clear that most CSCs depend on more than one signal pathway for their growth, survival, invasion and metastasis. Moreover, multiple cell signal pathways may control a given step in tumorigenesis. Thus agent or drug that inhibits multiple pathways or their combination is needed for CSC treatment [35, 36].

2.4.1 Targeting Hh signal pathway

The Hh signal pathway plays a critical role in development and is usurped by transformed cells for tumor initiation, progression, and metastasis. The hedgehog proteins are secreted signal proteins that were initially identified in *Drosophila* as segment polarity genes. Hh can function both as a morphogen and a mitogen. As a morphogen, Hh induces cell differentiation in a concentration-dependent manner. As a mitogen, it drives the proliferation of precursor cells and mediates the interaction between the epithelial and mesenchymal compartments [37, 38]. Hh signal pathway activation is noted in the neighboring stromal cells but not in the epithelial cells consistent with a paracrine signal mechanism [39], and only the stromal compartment is competent to activate Hh signaling [40]. This observation is consistent with Hh signaling in CSCs that do not express markers of epithelial differentiation. In order to determine whether increased Hh activity is a property of all pancreatic tumor cells or is selective for the CSCs, Li et al. demonstrated that Hh transcripts were 4-fold upregulated in the bulk pancreatic xenograft cells and 43-fold upregulated in the $CD44^{+}CD24^{+}ESA^{+}$ pancreatic CSCs as compared with normal pancreatic epithelial cells using quantitative real time-PCR [41]. Aberrant activation of the Hh pathway is common in basal cell carcinoma, medulloblastoma, a tumor of cerebellar granule neuron

progenitor cells, and rhabdomyosarcoma, a muscle tumor and breast tumors. The modulation of Hh pathway activity in these cell types results in decreased tumorigenic potential and depletion of the CSC compartment [42].

As the Hh pathway represents an attractive target for drug development, it has shown promise in Phase I clinical trials of advanced basal cell carcinoma and medulloblastoma with GDC-0449, an Hh pathway inhibitor [43, 44]. In addition, a role for Hh signaling in CSCs was demonstrated by specific deletion of smoothened in BCR-ABL positive chronic myeloid leukemia stem cells, which prevented tumor-initiation, and treatment with cyclopamine increased survival of mice transplanted with BCR-ABL leukemia cells [45-47]. Aldehyde dehydrogenase has been identified as one of putative markers of pancreatic CSCs. The initial study suggests that inhibition of the Hh pathway results in depletion of the pancreatic CSC compartment and is a putative mechanism for metastasis. Mueller et al further extended these findings by demonstrating that dual inhibition of the Hh pathway and the mTOR pathway in combination with gemcitabine is required to completely eliminates both CD133$^+$ and CD24$^+$CD44$^+$EpCAM$^+$ pancreatic CSC populations *in vitro*. In contrast, inhibition of Hh pathway alone in combination with gemcitabine abolished the CD133$^+$CXCR4$^+$ migratory CSC population. Similarly, in a mouse model of orthotopic pancreatic cancer, treatment with cyclopamine, rapamycin (mTOR inhibitor) and gemcitabine was required to fully inhibit growth at the primary site and resulted in a significant overall survival benefit. However, treatment with cyclopamine and gemcitabine resulted in complete inhibition of metastatic activity. Thus, they concluded that dual pathway inhibition is required for complete abrogation of tumorigenic potential [38, 48].

2.4.2 Targeting Notch-1 signaling pathway

Notch-1 and its ligands (Delta and Jagged) control an ancestral pathway of cell division and organ formation which is conserved in humans [20]. Notch-1 controls cell cycle progression from G0 to G1. The Notch-1 pathway is important as it precedes activation by oncogenes. Notch-1 activates *c-myc* and *hypoxia* genes, which in turn activate expression of embryonic stem cell (ESC) genes. Notch activation starts from a signal from Delta and Jagged on the neighboring cell. The signal 'pulls and breaks' the Notch heterodimer. The Notch-1 extracellular domain, N1ECD, is endocytosed together with Delta/Jagged in neighboring cells. The intracellular domain of Notch-1 protein (N1ICD) binds its suppressor, Numb, and pushes it for degradation. Signals from Notch-1 co-activator family, Mastermind-like-1 members, enhance Notch-1 signaling [49,50]. Currently, the Notch pathway is one of the most intensively studied putative therapeutic targets in CSCs, and several investigational Notch inhibitors are being developed. However, successful targeting of Notch signaling in CSCs will require a thorough understanding of Notch regulation and the context-dependent interactions between Notch and other therapeutically relevant pathways. Understanding these interactions will increase our ability to design rational combination regimens that are more likely to prove safe and effective. Additionally, to determine which patients are most likely to benefit from treatment with Notch-targeting therapeutics, reliable biomarkers to measure pathway activity in CSCs from specific tumors will have to be identified and validated [51,52].

2.4.3 Targeting Wnt/β-catenin signaling pathway

Wnt/β-Catenin is an essential component of both intercellular junctions and the canonical Wnt signaling pathway and the aberrant activation of Wnt signaling is involved in tumor

development and progression. Therefore, the Wnt signaling pathway is clearly important for the self-renewal and maintenance of stem cells, as seen by the effectiveness of inhibiting Wnt/β-catenin signaling in blocking human U251 glioma cells [53, 54]. The study showed 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. The acetaminophen is an anti-inflammatory, antipyretic and analgesic drug that is already in clinical use for drug and induces differentiation of CSCs, such as a human breast cancer cell line (MDA-MB-231 cells that contains cancer stem cell-like cells). In 2011 Takehara et al demonstrated that the increased susceptibility of MDA-MB-231 cells to acetaminophen seems to involve suppression of expression of multidrug efflux pumps, which suggests that this induction of differentiation is mediated by inhibition of a Wnt/β-catenin canonical signal pathway. Treatment of MDA-MB-231 cells with acetaminophen *in vitro* resulted in the loss of their tumorigenic ability in nude mice. Furthermore, administration of acetaminophen inhibited the growth of tumor xenografts of MDA-MB-231 cells in both the presence and absence of simultaneous administration of doxorubicine, a typical anti-tumor drug for breast cancer. The result indicates that acetaminophen may be beneficial for breast cancer chemotherapy by inducing the differentiation of CSCs [55]. It is thus evident 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 [56].

2.5 Targeted therapy toward CSCs by manipulation of miRNA and siRNA therapy

2.5.1 Manipulation of miRNA therapy

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. 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. Although a relatively new field, there is already a clear and definitive role for miRNA that function as tumor suppressors were found to be markedly down regulated in malignant transformation and tumor progression. Thus, miRNAs have spurred studies to investigate whether miRNAs play an important role in the CSC phenotype.

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 CSCs. The data showed that the mRNA encoding BMI1 was specifically targeted by miR-200c, miR-200b, and miR-183. The three miRNA clusters had decreased expression in freshly isolated $CD44^{+}CD24^{-/low}$ breast CSCs compared to cells in the tumor bulk. 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 [57,58].

Prostate CSCs with enhanced clonogenic and tumor-initiating and metastatic capacities are enriched in the $CD44^{+}$ cell population. If miR-34a was overexpressed in bulk or purified

$CD44^+$ prostate cancer cells purified from xenograft and primary tumors the clonogenic expansion, tumor regeneration, and metastasis were inhibited. However, the expression of miR-34a antagomirs in $CD44^-$ prostate cancer cells facilitated tumor development and metastasis. Administratively delivered miR-34a inhibited prostate cancer metastasis and extended survival of tumor-bearing mice. The identified $CD44^+$ prostate cancer cells may be as a direct and functional target of miR-34a and the CD44 knockdown phenocopied miR-34a overexpression in inhibiting prostate cancer regeneration and metastasis. The study suggests that miR-34a is underexpressed in tumorigenic $CD44^+$ prostate cancer cells, and that it has potent antitumor and antimetastasis effects and is a novel therapeutic agent against prostate CSCs [59]

The symmetric division of CSCs is one mechanism enabling expansion in their numbers as tumor grow, while EMT is an increasingly recognized mechanism to generate further CSCs endowed with a more invasive and metastatic phenotype. Since the EMT of ovarian cancer cells located at the periphery of primary tumors is essential to this process, molecular interventions that can block EMT are of potential clinical significance [60]. It was found that the members of the miR200 family of microRNAs have been implicated in EMT in other cancers. In 2011 Chen et al [61] tested gene expression profiles of two ovarian cancer cell lines with different metastatic potentials by using quantitative reverse transcription polymerase chain reaction. The results showed that molecular profiling of two ovarian cancer cell lines with differing metastatic potentials identified significant differences in previously established epithelial and mesenchymal cell biomarkers, such as E-cadherin, ZEB1, ZEB2, miR-205 and miR-200 family microRNAs. They demonstrated that ectopic overexpression of miR-200 family microRNA (miR-429), in mesenchymal-like ovarian cancer cells resulted in reversal of the mesenchymal phenotype (mesenchymal- epithelial transition, MET). The data indicate that miR-429 may not only be a useful biomarker of EMT in ovarian cancer, but also of potential therapeutic value in abating ovarian cancer metastasis.

More recently, Strauss et al [62] developed a subpopulations of ovarian cancer cells that simultaneously express epithelial and mesenchymal markers, and the subpopulations are not homogenous, however, the subsets that can be distinguished based on a number of phenotypic features including the subcellular localization of E-cadherin, and the expression levels of Tie2, CD133, and CD44. A cellular subset (E/M-MP) (membrane E-cadherin^{low}/cytoplasmic E-cadherin^{high}/ CD133^{high}, CD44^{high}, Tie2^{low} is highly enriched for CSCs. The group demonstrated that E/M-MP cells are able to differentiate into different lineages under certain conditions, and have the capacity for self-renewal. Trans-differentiation of E/M-MP cells into mesenchymal or epithelial cells is associated with a loss of stem cell markers and tumorigenicity. Xenograft tumor growth *in vivo* is driven by E/M-MP cells, which give rise to epithelial ovarian cancer cells. In contrast, E/M-MP cells differentiate into mesenchymal cells *in vitro*, which involves pathways associated with the EMT. The study provides a better understanding of the phenotypic complexity of ovarian cancer and has implications for ovarian cancer or ovarian CSC therapy. In addition, miRNAs might play important roles in stemness maintenance of ovarian or colon CSCs, and targeting miRNA may provide a new strategy for CSC therapy by impairing resistance to chemotherapy [63-65].

2.5.2 Manipulation of siRNA therapy

RNA interference (RNAi) is triggered by short interfering RNAs (siRNAs) of between 19 and 21 nucleotides in length, which induces the targeted cleavage of mRNA with sequences

of homology to the siRNA. Because of its high degree of specificity and efficacy, the potential for RNAi-based therapeutics have been employed as transcriptional inhibitors of oncogene and growth factor signaling [66]. To this end, several groups are attempting to develop effective vehicle for delivering siRNA to *in vivo* growing tumors. Octamer 4 (Oct4), a member of the POU family of transcription factors, plays a key role in the maintenance of pluripotency and proliferation potential of embryonic stem cells. Continuous Oct4 expression in epithelial tissues is observed to lead to dysplastic disorders by inhibiting cellular differentiation in a manner similar to that in embryonic cells. Oct4 has also been reported to be an oncogenic fate determinant. High levels of Oct4 increase the malignant potential of embryonic stem cells-derived tumors whereas inactivation of Oct4 induces a regression of the malignant component [67]. The role of Oct4 in CSC-like cells (CSCLC) was recently evaluated by Hu et al [68]. They found that almost all murine Lewis lung carcinoma 3LL cells and human breast cancer MCF7 cells express Oct4 at high levels *in vitro*. This expression of Oct4 is effectively reduced by siRNA that finely causes cell apoptosis. The signal pathway Oct4/Tcl1/Akt1 is involved in this process. The repression of Oct4 reduces Tcl1 expression and further down-regulates the level of p-Ser.473-Akt1. Only approximately 5% of tumor cells were detected to express Oct4 in established 3LL and MCF7 tumor models, respectively, *in vivo.* siRNA against Oct4 successfully reduces the CSCLCs and remarkably inhibits tumor growth. Oct4 might maintain the survival of CSCLCs partly through Oct4/Tcl1/ Akt1 by inhibiting apoptosis. The results strongly suggestes that targeting Oct4 may have important clinical applications in cancer and CSC therapy.

Emerging data indicate that transglutaminase 2 (TG2) has closely relationship among the EMT, and CSCs in inflammation and cancer. TG2 is a structurally and functionally complex protein implicated in such diverse processes as tissue fibrosis, wound healing, apoptosis, neurodegenerative disorders, celiac disease, atherosclerosis and cancer. Depending on the cellular context, TG2 can either promote or inhibit cell death. Increased expression of TG2 in several types of cancer cells or CSCs have been associated with increased cell invasiveness, cell survival and decreased survival of patients with cancer. Down-regulation of TG2 by siRNA or its inhibition by small molecule inhibitors has been shown to significantly enhances the therapeutic efficacy of anticancer drugs and inhibit metastatic spread [69].

2.6 Targeted directly drug-resistant CSCs in 3D cell culture

Tissues and organs are three dimensional (3D). Cell behavior, which includes survival, motility, and differentiation, mainly depends on its 3D growth environment. Cells growing on flat two-dimensional (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 that display enhanced cell biological activities and promote normal cell polarity and differentiation. In addition, signal pathway and other cellular functions also differ in 3D compared with 2D systems [70, 71].

Cukierman and his colleagues revealed their approach that an *in vivo*-like ovarian stromal 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 [72]. Because the vast majority of studies to identify cancer-associated genes and therapeutic targets use adherent cells grown in 2D on a plastic substrate, the multicellular composition of these 3D tumor spheroids presents both challenges and opportunities for their imaging and characterization. Robertson et al

described approaches to image 3D breast CSC spheroid structures, allowing for characterization of specific traits of CSCs, including self-renewal as assessed by their clonogenic growth, their ability to retain a nucleoside analog label such as 5-ethynyl-2'-deoxyuridine, and their expression of specific surface markers. They explored the major challenges of imaging and analyzing the activities of breast CSCs when cultured as 3D tumor spheroids and provide insight into potential solutions that allow multicellular tumor spheroids to be imaged and analyzed to further characterize signatures and therapeutic targets of CSCs [73]. Botchkina et al reported that the selected CSC phenotype was isolated from three independent invasive colon cancer cell lines, HCT116, HT29 and DLD-1. In 3D culture, the colonospheres induced by purified $CD133^{high}/CD44^{high}$ expressing cells contained some minority cell populations with high levels of expression of Oct4, Sox2, Nanog and c-Myc, which are essential for stem cell pluripotency and self-renewal. Using the new-generation taxoid SB-T-1214 at concentration 100 nM^{-1} microM for 48 hr not only induced growth inhibition and cell apoptosis in these three types of colon cancer spheroids, but also mediated massive inhibition of the stem cell-related genes and significant down-regulation of the pluripotency gene expression. Importantly, viable cells that survived this treatment regimen were no longer able to induce secondary floating spheroids and exhibited significant morphological abnormalities [74]. Although emerging reports indicate that *in vitro* 3D culture system was also used for cardiac stem cells and ovarian cancer cell lines[75, 76], we know very little about cell-based assays used for screening target stem cell or CSC drugs in 3D cultures at present. 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 CSCs, and at the same time to minimize unwanted side effects of the drugs to host tissues [77].

2.7 Targeted therapy toward CSCs by dendritic cell vaccination

There is growing evidence that were based on the use of tumor-homogenate pulsed dendritic cells (DC) for patients with recurrent gliomas (GBM) and provided encouraging results. Xu's study showed that CSCs express high levels of tumor-associated antigens as well as major histocompatibility complex molecules and they explored the suitability of CSCs as sources of antigens for DC vaccination again human GBM. This DC vaccination 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. In using a 9L CSC brain tumor model, vaccination with DCs loaded with 9L CSCs, but not daughter cells or conventionally cultured 9L cells, induced cytotoxic T lymphocytes against CSCs, and prolonged survival in animals bearing 9L CSC tumors [78]. Murine brain tumor GL261 GBM cell line may mimic the growth of human GBM-CSC because the characterization i*n vivo* and *in vitro* demonstrates that GL261-NS (neurospheres) satisfy criteria used to identify CSCs and are more immunogenic than GL261-AC (Adherent Cells). DC from the bone marrow of syngeneic mice were then used for immunotherapy of GL261-NS and GL261-AC tumors. The results showed that DC loaded with GL261-NS (DC-NS) lysates protected mice against tumors from both GL261-NS (cured 80%) and GL261-AC(cured 60%), respectively, whereas DC-AC cured only 50% of GL261-AC tumors. The study also indicated that GL261-NS expressed higher levels of MHC and costimulatory molecules (CD80 and CD86) than GL261-AC and that DC-NS splenocytes had higher lytic activity than DC-AC splenocytes on both GL261-NS and GL261-AC. Immunohistochemistry showed that DC-NS vaccination was associated with robust tumor infiltration by $CD8^{+}$ and

CD4+ T lymphocytes. The results indicate that only DC vaccination against neurospheres can restrain the growth of a highly infiltrating and aggressive model of GBM and may have implications for the design of novel, more effective immunotherapy trials for malignant GBM and possibly other malignancies. These data suggest that DC targeting of CSC provides a higher level of protection against GL261 GBM, a finding with potential implications for the design of clinical trials based on DC vaccination [79, 80]. These findings suggest that understanding how immunization with CSCs as sources of antigens for DC vaccination generates superior antitumor immunity may accelerate development of CSC-specific immuno-therapies and CSC vaccines [77, 81].

3. Conclusions

We are still in the early days of clinically validating CSCs as a cancer target. We do not fully understand the role of CSC biology and cancer pathophysiology. Undoubtedly, proper characterization and refinement of the tools used for the identification, isolation, and propagation of CSCs may lead to a better understanding of how these cells initiate and sustain tumor growth.

Currently, novel therapeutic approaches to eliminate CSCs are imperative because CSCs may escape standard therapies and cause disease recurrences and/or metastasis after apparently complete remissions. Developing effective cancer treatment by focusing therapy on the relatively more malignant and quiescent cells could be a direct result of the application of CSC hypothesis to tumor growth. In order to achieve this goal it is important to determine which cancers possess a CSC traits and which do not, and to address technical issues related to tumorigenesis assays [3, 82]. It is known that CSC targeting is essential, but recently it has been speculated that non-CSCs in a tumor need to be targeted as well. These non-CSCs could form CSCs and might even sustain the tumor even after CSCs have been destroyed [83]. Furthermore, innate immunity including natural killer cells and gammadelta T cells and adaptive immunity (cytotoxic T lymphocyte-based cellular immunity and antibody-based humoral immunity) can recognize CSCs *in vitro* efficiently, of which CSC-specific monoclonal antibody therapies are also efficient *in vivo*[84]. Taken together, targeted therapies on the CSC compartment could provide cancer curability through targeted therapy directed toward CSCs, and along with the conventional treatments, gene therapy and nanotechnology as well as new technologies could provide sophisticated multifunctional agents to target CSCs, all of which has begun to revolutionize approaches for simultaneous drug design, targeting, imaging, and therapy of CSCs [2, 77,85].

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Over the last thirty years, the foremost inspiration for research on metastasis, cancer recurrence, and increased resistance to chemo- and radiotherapy has been the notion of cancer stem cells.The twenty-eight chapters assembled in Cancer Stem Cells - The Cutting Edge summarize the work of cancer researchers and oncologists at leading universities and hospitals around the world on every aspect of cancer stem cells, from theory and models to specific applications (glioma), from laboratory research on signal pathways to clinical trials of bio-therapies using a host of devices, from solutions to laboratory problems to speculation on cancersâ€™ stem cellsâ€™ evolution. Cancer stem cells may or may not be a subset of slowly dividing cancer cells that both disseminate cancers and defy oncotoxic drugs and radiation directed at rapidly dividing bulk cancer cells, but research on cancer stem cells has paid dividends for cancer prevention, detection, targeted treatment, and improved prognosis.

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