Curr. Treat. Options in Oncol. (2018) 19: 20
DOI 10.1007/s11864-018-0533-1

Hot Topic

Eradicating Cancer Stem Cells: Concepts, Issues, and Challenges

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Published online: 20 March 2018

Keywords Cancer stem cells · Chemoresistance · EpCAM · Wnt · Hh and Notch signaling

Opinion statement:

The cells of malignant cancers result in the evolution of cells with stem-like characteristics, commonly known as cancer stem cells (CSCs). Progress of anticancer therapies is severely hampered because of disease relapse mostly in a more aggressive form due to CSCs. These CSCs are more or less like embryonic or tissue stem cells, known for their capacity of self-renewal, exactly recapitulate of the original tumor. Deregulation of key stem cell pathways like Wnt, Hedgehog (Hh), and Notch is attributed towards the rise of CSCs. Recent breakthroughs offer better insights into CSC signaling. Scientists have developed several combinatorial therapies like targeting one/multiple of these CSC pathways. The article summarized various markers used to identify CSCs and discuss major signaling pathways in them. The futuristic probabilities to use CSC therapeutics in clinical development have been discussed. Our views have been highlighted on the future directions for targeting advances in the clinical development.

Introduction

Cancer is a complex disease with several deregulated pathways leading to disease progression. Worldwide, oncologists are working to understand the mechanisms of primary tumor initiation and propagation that can assist in elimination of cancer cells without altering normal mitotic cells. Another major obstacle to

improving overall cancer survival in current therapy is tumor relapse and metastasis that may be due to the existence of cancer stem cells (CSCs). Thus, there is a necessity to develop various therapeutic agents to target CSCs for prevention of relapse. Several agents targeting CSC signaling pathway (Wnt, Notch, and Hh pathways, etc.), either as single agents or in combination with standard chemotherapy, entered clinical trials as novel treatment strategies to control stem cell replication, survival, and differentiation. But, the genotype, phenotypes, and CSC of tumor microenvironment decide the success of these combination therapies in clinical trials.

The CSCs originated from small populations of phenotypically diverse cancer cells (tumor) with less proliferative potential and may result in disruption of normal stem cell self-renewal potential [1]. The CSCs exhibit some characteristic stem-like features of self-renewal and proliferation potential that lead to tumor progression. CSCs display radio and chemoresistance, thus are generally responsible for disease relapse.

Embryonic stem cells (ES cells) are derived from the blastocyst, an early-stage preimplantation embryo. ES are pluripotent in a manner to differentiate into three primary germ layers, i.e., ectoderm, endoderm, and mesoderm [2, 3]. Mesenchymal stem cells (MSCs) are multipotent stromal cells that differentiate into a variety of cell types like myocytes (muscle cells), adipocytes (fat cells), chondrocytes (cartilage cells), and osteoblasts (bone cells) [4–6]. The MSCs do not differentiate into hematopoietic cells, while the mesenchymal cells (embryonic connective tissue derived from the mesoderm) can differentiate into hematopoietic and connective tissue. MSCs were shown to localize in breast carcinomas as the tumor-associated stroma. The MSCs of breast cancer cell secrete CCL5/RANTES in response to signals released by cancer cells that act on the cancer cells in a paracrine fashion to enhance their motility, invasion, and metastasis that stimulate invasive behavior. So, the enhanced metastatic ability dependent on CCL5 signaling (through the chemokine receptor CCR5) that represents the reversible behavior changes in the phenotype of cancer cells metastatic spread [7].

Similar to these stem cells, induced pluripotent stem cells are generated in the laboratory directly from adult cells. The specific genes encoding transcription factors such as Oct3/4, Sox2, c-Myc, Klf4 [8] and OCT4, SOX2, NANOG, and LIN28 [9] could convert adult cells into pluripotent stem cells. These cells represent a single cell source to replace lost/damaged or diseased cells due to unlimited replicative as well as differentiative potential and thus are "The Choice" for regenerative medicine.

Signaling pathways in cancer stem cells

The origin and plasticity of cancer CSCs in malignancies is a controversial topic during the past decade. CSCs typically involve activation of signal transduction pathways, i.e., the Notch, Hedgehog (Hh), and Wnt pathways, for development and tissue homeostasis [10]. Wnt, Notch, Hh, and transforming growth factor β/bone morphogenetic protein (BMP) signaling network regulates the self-renewal of normal stem cells by maintaining the tissue homeostasis. These networks are generally disrupted in CSCs.

The Notch signaling plays an influential role in stem cell fate, differentiation, and cell cycle progression. Notch signaling can be either inhibitory or inductive. This pathway is over-activated in cancer and thus helps the CSCs in their maintenance [11]. For example, the Notch signaling specifically represses the neuronal pathway in the progenitor cells for gliogenesis during brain development, thereby serving as a gatekeeper for cell fate during its fate development. It directly influences for regulation of the proliferation and differentiation of intestinal stem and progenitor cells. It mainly targets the crypt base columnar (CBC) cell that helps to maintain the stem cell activity. The function of Notch inhibition is to induce rapid CBC cell loss, with reduced proliferation and efficiency of organoid initiation as well as apoptotic cell death [12]. In mammals, there are four transmembrane proteins act as Notch receptors (Notch1, Notch2, Notch3, and Notch4) and five delta-like proteins serve as ligands (DLL1, DLL3, DLL4) and Jagged proteins (JAG1 and JAG2) (Fig. 1). The Notch receptors contain an extracellular domain with EGF-like repeats for ligand binding and an intracellular domain (ICD). The activation of Notch signaling occurs by direct cell-to-cell contact. The ligand binds to the extracellular domain of the Notch receptor causes two proteolytic cleavages by γ-secretase and ADAM protease (a metalloprotease and disintegrin) results release of an active intracellular domain (NICD). The NICD translocate to the nucleus and binds to the CSL (CBF1/suppressor of hairless/LAG1)/RBPJ transcription factor. It activates transcription of specific target genes. The transcription repressed in the absence of the Notch signal as CSL/RBPJ bound by co-repressors. The gene expression activated in the presence of the Notch signal as the co-repressors displaced by NICD with mastermind-like proteins (MAML1, MAML2, or MAML3) [13, 14]. Notch signaling is highly active in neural stem cells, as it functions to prevent NSCs from entering the neuron lineage whereby suppression of neuron development promotes gliogenesis.

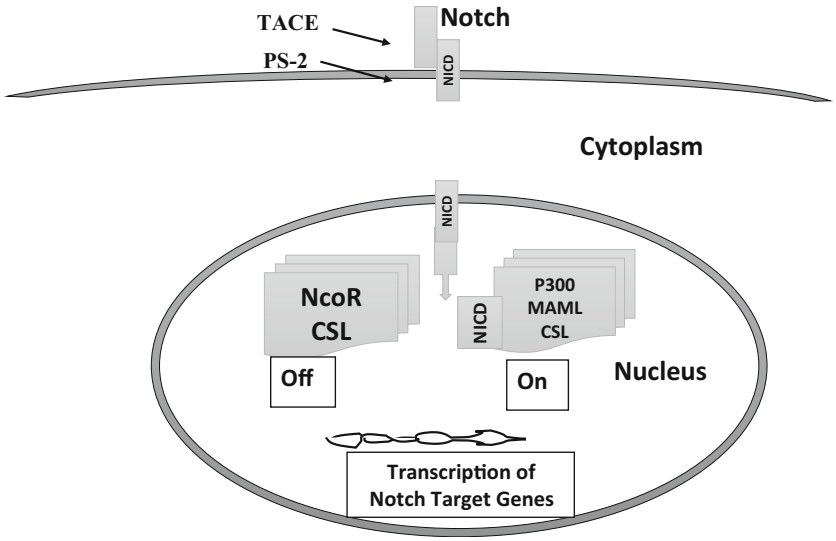

Fig. 1. Notch signaling pathway. Notch is cleaved via proteolytic cleavages (by TACE and γ-secretase. The Notch extracellular truncation (by TACE) then cleaved by the γ-secretase complex, releasing the active fragment, Notch intracellular domain (NICD) that binds to CSL in the nucleus. The CSL-NICD complex with p300 leads to the activation of Notch target genes. But, the transcription of Notch target genes is maintained in an inactive state through a repressor complex mediated by the CBF1, suppressor of hairless and lag-1 (CSL).

WNT signaling is transduced by Frizzled family receptors and LRP5/LRP6, a co-receptor to the β-catenin (Fig. 2). The positive regulators of canonical WNT pathway are casein kinase Iε (CKIε), and FRAT, whereas the negative regulators are APC, AXIN1, AXIN2, CKIα, NKD1, NKD2, βTRCP1, βTRCP2, ANKRD6, Nemo-like kinase (NLK), and peroxisome proliferator-activated receptor γ (PPARγ). Nuclear complex (comprising of T cell factor/lymphoid enhancer factor, β-catenin, BCL9/BCL9L, and PYGO) activates transcription of canonical WNT target genes (Glucagon (GCG), FGF20, DKK1, WISP1, MYC, CCND1). The non-canonical

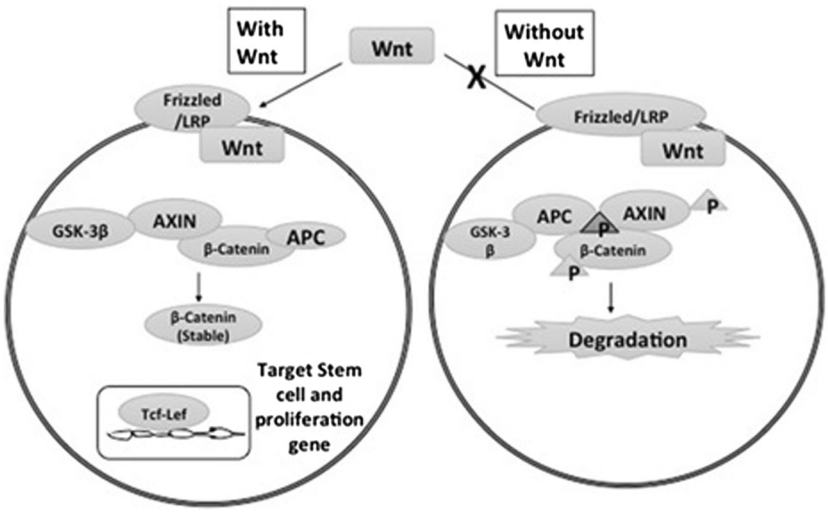

Fig. 2. WNT signaling pathway. The canonical signaling pathway activated when Wnt ligands bind to the Frizzled/LRP co-receptor complex. Axin results in the inactivation of the adenomatous polyposis coli (APC) destruction complex and β-catenin is stabilized and moves to the nucleus and binds to the T cell factor (TCF) and results in the expression of various target genes.

WNT signals also transduced via Frizzled family receptors. The loss-of-function mutation of negative regulators of the canonical WNT pathway and Epigenetic silencing has reported in a variety of human cancer.

Hh signaling pathways help in embryonic growth development as well as postembryonic regulation of stem cell number in epithelia of the skin and intestine [15]. The Hedgehog pathway is thought to play a role in regulating proliferation and survival of the neural stem cells [16, 17]. Patched receptors are located in the cilium that blocks smoothened entry in the absence of Hh ligand. The three secreted Hh ligands (SHh, IHh, DHh) binds to its receptor patched at the cell surface and allow it to move out of the primary cilium to release smoothened from its repressed state (Fig. 3). Smoothened then activates the expression of three glioma associated oncogene homolog (Gli) zinc-finger transcription factors (exist in repressor forms that prevent transcription of target genes) that are processed to activator forms and translocated to the nucleus to induce the transcription of Hh target genes. Depending on post-translational and post-transcriptional modifications, Gli1 and Gli3 acts as a transcriptional activator and repressor, respectively, while Gli2 can either repress or activate the gene expression. Robotnikinin and 5E1 antibodies prevent the interaction of Hh ligand with patch. GANT (Gli antagonists) blocks Gli transcription factors binding to DNA. Similarly, cyclopamine (Smo antagonists) and HPI 2,3,4 block the transportation of components in the signaling cascade. The aberrant activation of this pathway may contribute to tumorigenesis in many human cancers [11].

CSCs markers

The anti CSC therapy involves firstly the identification of various CSCs specific cell surface markers so as to correctly identify and target the stem cell population of a particular cancer. Here, we are briefly discussing various known cancer stem cell markers.

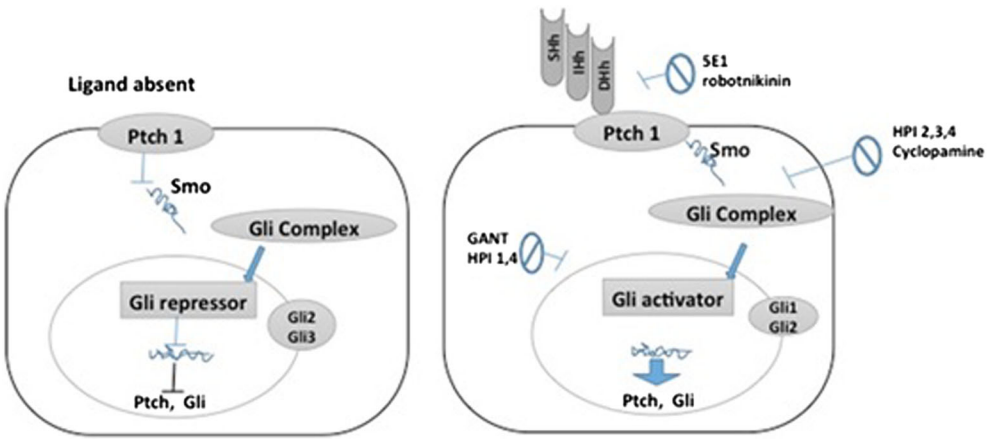

Fig. 3. A schematic of Hh pathway signal transduction. **a** Ptch blocks Smo entry in the absence of Hh ligand and Gli transcription factors remain in its repressor forms to prevent transcription of target genes. **b** Ptch binds to three homologs of Hh (SHh, IHh, DHh) at the cell surface that depressed Smo to activate Gli transcription factors. Gli translocated to the nucleus responsible for the transcription of Hh target genes. Cyclopamine, HPI 2,3,4, GANT, 5E1 antibodies, and robotnikinin block pathway activation by preventing signaling via interaction with different components of the pathways.

$CD133^+$ cells

CD133 was first reported to identify CSCs in brain tumors [18]. It is critical in regulating cell adhesion, proliferation, growth, survival, motility, migration, angiogenesis, and differentiation [19]. CD133 cell subpopulation from human brain tumors, are capable of self-renewal and exact recapitulation of the original tumor when transplanted into immune-deficient mouse brain.

CD133 antigen (prominin-1) is a member of pentaspan transmembrane glycoproteins (5-transmembrane, 5-TM), is encoded by the PROM1 gene in humans [20]. It is expressed by many types of normal stem cells like neural and hematopoietic stem cells and helps in stem cell migration and asymmetric division [21]. It has been widely identified in various human solid cancers like brain [22], liver [23], skin [24], prostate [25], ovary [26]. CD133 may serve as a marker to enrich tumor-propagating cells from $CD133^+$ but not $CD133^-$ glioblastomas to initiate tumorigenesis. The expression of the CD133 is also tumor dependent because $CD133^-$ compared with $CD133^+$ cancer cells were found deep within the structures of the glioblastomas [27, 28]. CD133 expression also detected in a subpopulation of prostate cancer cells co-expressed with CXCR4 [25]. CXCR4 is the receptor of CXCL12 (stromal cell derived factor-1), expressed in leukocytes, hematopoietic stem cells, and metastasizing cancer cells, and play a major role in cell trafficking.

EpCAM

EpCAM (epithelial cell adhesion molecule) is a transmembrane glycoprotein that causes $Ca2^+$-independent homotypic cell–cell adhesion in epithelia and involved in cell signaling. It is mainly expressed as cell surface molecule in most of the epithelial tumors, and identified as an early biomarker for HCC [29]. Also, the EpCAM has been firstly identified in premalignant hepatic tissue. It downregulated through epithelial-to-mesenchymal transition (EMT), and this transition might affect stem cell-like properties of tumor cells [30, 31].

EpCAM cleavage is catalyzed by ADAM17 (also known as TACE: tumor necrosis factor-α-converting enzyme) and a γ-secretase presenilin-2 that results in the release of a soluble extracellular domain (EpEX) of EpCAM into the area surrounding the cell, which may act as a homophilic ligand for non-cleaved EpCAM. The cleaved intracellular domain (EpICD) released into the cytoplasm of the cell thereby initiates signaling by association with proteins FHL2, β-catenin, and Lef inside the nucleus. This nuclear complex via binding to DNA further promotes the transcription of various genes like c-myc, e-fabp, thus promoting tumor growth [32, 33] (Fig. 4). EpCAM has also been involved in transcription of the Wnt/β-catenin signaling pathway [34]. Human colon cancer has been reported to investigate using EpCAM as marker for CSCs [35].

Aldehyde dehydrogenase

The ALDH gene superfamily comprises 19 isozymes, known for the physiological and detoxification mechanism involved in stem cell self-protection. The high aldehyde dehydrogenase 1 (ALDH1) activity has been reported in the isolated cancer cells that display CSC characteristics in vitro [36]. Also, the positive correlation has been demonstrated between the higher expression of ALDH1 with stage and grade of lung tumors of the 303 clinical

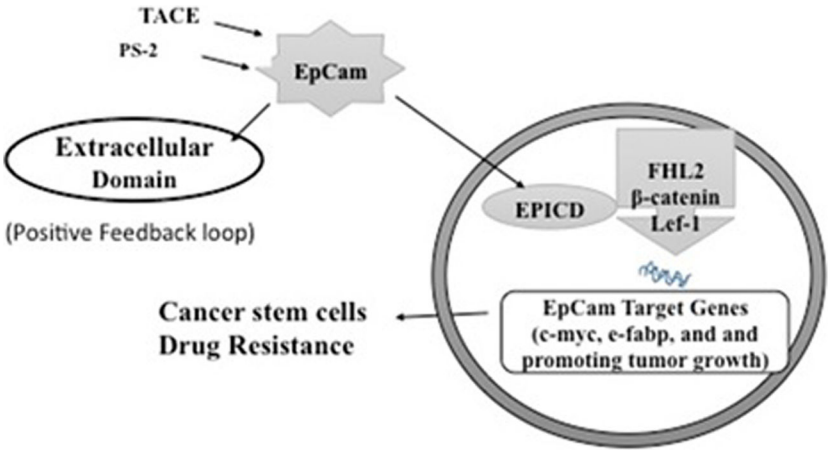

Fig. 4. EpCam in Oncogenicity. EpCam cleavaged by TACE (Tumor, Necrosis factor Alpha Converting Enzyme) and PS-2 (presenilin-2, γ-secretase complex), the intracellular domain (EpICD) translocates into the nucleus and associated with FHL2, β-catenin and Lef-1. Further, the EpICD contacts DNA at Lef-1 consensus sites.

specimens from three independent cohorts of lung cancer patients by Immunohistochemical analysis in the same study. In another study, the stem-like ALDHhiCD44^{+}CD24^{-} and ALDHhiCD44^{+}CD133^{+} cells are found to be important mediators of breast cancer metastasis [37].

CXCR4 receptor

The CXCR4 (fusin or CD184) is chemokine receptor expressed on the immune cell as well as the cells of central nervous systems with potent chemotactic activity for lymphocytes. The CD133^{+} cell population expresses the chemokine receptor CXCR4 in pancreatic cancer. In concert with its chemokine ligand SDF-1, the CXCR4 play an important role in both migration of normal stem cell and as well as metastasis of cancer cells. The metastatic ability of pancreatic cancer cells was significantly reduced upon elimination of CXCR4^{+} pancreatic CSC from highly metastatic pancreatic cancer cell lines, although the cells could still efficiently form primary tumors [38].

CD44

CD44, class I transmembrane glycoprotein acts as cell surface receptor for hyaluronic acid, involved in cell adhesion, promoting migration and metastasis of cancer cells in its standard or variant form [39]. In numerous studies, the role of CD44 in the initiation, metastasis, and promoting tumorigenesis has been reported [40–43]. CD44 leads to tumor progression via interaction with osteopontin [44, 45] and help in haematogenous spread through interaction with P- or L-selectins [46]. Also, it is responsible for enhancing tumor initiations via a complex signaling cascade initiated by neighboring receptors like tyrosine kinase [47]. The CD44 is widely used as a surface marker to isolate CSCs from the breast, prostate, pancreas, ovarian, and colorectal cancers [48, 49]. In prostate cancer, the CD44^{+}α2β^{+}D133^{+} population could differentiate to

mixed phenotype [50]. The high expression of $CD44^+$ cell has been reported in several prostate cell lines and xenograft tumors with increased proliferation in vitro and tumor initiation and metastasis in-vivo [51].

Bmi1

Bmi1 (B cell-specific Moloney murine leukemia virus integration site 1), a critical component of the polycomb repressive complex 1 (PRC1) is also used as biomarker of CSCs [52]. BMI1 also play a major role in cell immortalization and senescence and regulates the proliferation activity of normal, progenitor and stem cells. It is involved in the pathogenesis of multiple cancers and is frequently upregulated in varieties of cancer. BMI1 has been evidenced to have a positive correlation with clinical stage, grade, and poor prognosis by numerous researchers. Bmi1 has been reported to overexpress in human PanINs, pancreatic cancers, and cancer cell lines. The increased proliferation, larger in vivo tumors, in vitro invasion, more metastases, and gemcitabine resistance has been observed due to the overexpression of Bmi1 in MiaPaCa2 cells, while silencing of Bmi1 gave opposite results in Panc-1 cells. The Bmi1 increases CSC function and tumorigenicity in pancreatic adenocarcinoma [53]. Further, the transcription of the downstream INK4a/ARF gene is negatively regulated, and expression of P16 (ink4a)/P14(ARF) get inhibited in laryngeal CSCs, that help to maintain the higher ability of differentiation and proliferation. The knockdown of Bmi-1 expression in $CD133^+$ cells resulted in inhibition of colony formation, cell growth, cell invasion in vitro, and tumorigenesis vivo, via upregulation of p16(INK4A), and p14(ARF) [54].

CD24

CD24 is a heat stable cell surface protein anchored by glycosyl-phosphotidyl-inositol. It is widely expressed in numerous cancers like bladder, ovarian, renal, breast, prostate, and other human cancers [55] and is useful surface marker to isolate CSCs from solid tumors. It is also an alternate ligand for P-selectin, an adhesion receptor on platelets and endothelial cells [56], through which their interaction facilitates the passage of tumor cells in the bloodstream during metastasis. This metastatic association is more evidence for CD27 as a marker of CSCs'. The CD24 express in various cancers in combination with other markers such as CD44, CD29, and CD31 in different types of cancer. $CD44^+/CD24^+$ are designated as CSCs in pancreatic cancer [57]. Similarly, another study reported the high expression of $CD44^+/CD24^+$ cells in MDA-MB-468 cell line [58]. The NDRG2 and CD24 also regulate adhesion, migration, and invasion in hepatocellular carcinoma as NDRG2 (N-myc downstream-regulated gene 2) cause antitumor activity by regulating CD24 [55]. However, the lack of CD24 expression restricts its use as a potential target to control invasion and metastasis in breast and prostate cancer.

CD90 (Thy1)

CD90 (Thy1), a 25-37 KDa glycosylphosphatidyl inositol-anchored protein (GPI-AP) is expressed mainly in leukocytes and involved in cell-cell and cell-

matrix interactions [59]. It is generally expressed by cultured $CD133^+$ glioblastoma CSCs [22], bone marrow-derived MSCs [60], murine breast CSCs [61], and hepatic stem/progenitor cells (HSPCs) [62]. The $CD90^+$ cells exhibited tumorigenic capacity in human liver cancer cell lines. In the same study, it was reported that targeting CD44 prevented the formation of local and metastatic tumor nodules by the $CD90^+$ cells [63].

CD105

CD105 (Endoglin) is an accessory protein of the transforming growth factor-beta receptor system and is expressed on vascular endothelia [64]. This mesenchymal stem cell marker releases microvesicles that initiate angiogenesis to promote the formation of a premetastatic niche thereby acting as tumor-initiating cells in human renal cell carcinoma [65]. In a similar study, the $CD105^+$ cells and clones derived from renal carcinomas are reported as tumor-initiating cells with stem characteristics [66].

CD117

CD117 (mast/stem cell growth factor receptor (SCFR)), also known as proto-oncogene c-Kit or tyrosine-protein kinase Kit, is a type III receptor tyrosine kinase involved in signal transduction of several cell types. It is a better marker for gastrointestinal (GI) stromal tumors as compared to CD34. The expression of CD117 has been reported high in oral cancer stem-like cells. Also, the differentiation abilities, migration, invasion, and malignancy capabilities of $CD117^+$ cells are enhanced in vitro as well as in-vivo [67]. The CD117 and CD133 expressions appear to be limited in identifying MSCs as compared to CD44 in identifying oral CSCs [68].

SP

Side population (SP) has a characteristic to efflux the nucleic acid dye Hoechst 33,342 (binds to the AT-rich regions of the minor groove of DNA) through the expression of ATP-binding cassette protein (ABC) transporters [69]. This dye exclusion feature has a potential application to identify a unique population of cells with stem-like characteristics of tumor-initiating capacity to express stem-like genes, and resistant to chemotherapeutic drugs [70]. SPs are reported in various stem cells in cancerous and normal human tissues and cell lines with application to isolate and identify the CSCs. However, the expression lacks in some cells as in GI cancer cells [71]. Human hepatocellular carcinoma cell lines reported to have SP as a marker of CSCs like properties with metastatic potential also evidences to be the therapeutic resistance to HCC [72]. The SP cells reported to induce tumor formation in the mouse model as compared to non-SP population [73].

The expression of the ABCG2 transporter has been reported to be upregulated in SP cells from MCF-7 breast cancer [74], thyroid C6 glioma cell line [75], and GI cancer cell lines [76] in comparison to non-SP cells. Similarly, the expression of genes of WNT/beta-catenin signaling pathway was reported to be higher in SP cells of colon carcinoma cell lines compared [76]. The increased expression of C-kit/CD117 and decreased expression of AC133/CD71 and

CD56 has been reported in SP cells from neuroblastoma as compared to non-SP cells [77].

Strategies and concepts for therapeutic interventions

The most important aspect is to understand the interaction between the signaling and relevant therapeutic pathways to design safe and efficient therapy. Various concepts have been introduced to target against these signaling molecules as discussed below.

Stem cell-based concepts for chemo- and radioresistance

The reason of current anticancer therapies failure may be due to the relative ineffectiveness of radiation and drug therapy on CSCs, which makes up for a loss of the tumor cells to these conventional therapies. The signaling inhibitors have therapeutic regimens that could be managed to reverse or prevent chemo- or radioresistance. Targeting CSCs with marker such CD133 and EpCAM/ESA might be a strategy to improve the cancer therapy by enabling specific interventions [78]. The CD44 could be a probable therapeutic target in solid tumors while leukemic stem cell reduction in number is also reported in acute myeloid leukemia by targeting CD44 function [79]. The activation of BMI1 and a polycomb group family member results in chemoresistance of CSCs although its mechanism of action is not entirely understood [80]. The 50% inhibition growth of the $CD44^{+}CD117^{+}$ cells has been reported in the 2D cell culture while inhibiting the cell growth by 34.4, 40.8, 34.8, and 21.9% in 3D culture assays by anticancer drugs 5FU, cisplatin, docetaxel, and carboplatin respectively. So, the $CD44^{+}CD117^{+}$ cells of human epithelial ovarian cancer acquired the properties of more chemoresistance in the 3D culture as compared to 2D [81]. The investigation on the tumorigenic process in the central nervous system is crucial to develop therapies targeted to the brain tumor stem cell (BTSC). The identification and purification of human brain tumor CSCs of different phenotypes has been reported by Singh et al. 2004 [18]. The clinical samples of MB patients reported a highest self-renewal capacity of the BTSC as compared to low-grade gliomas. Further, the $CD133^{+}$ cells (expressed on isolated cell fraction of BTSC) could differentiate into tumor cells resembled the tumor from the patient phenotypically. Similarly, the injection of 100 $CD133^{+}$ cells (brain tumor fraction) capable of initiating tumor in NOD-SCID mouse brains (non-obese diabetic, severe combined immunodeficient) that further serially transplanted to make phenocopy of the original tumor from the patient. On the other side, the injection of 105 $CD133^{-}$ cells engrafted did not cause a tumor [18]. Thus, it could be a useful tool for investigation of human brain tumor pathogenesis, and could provide some cellular target for more efficient cancer therapies in future.

Epithelial-to-mesenchymal transition (EMT), a morphogenetic process in which the repression of E-cadherin expression leads to a loss of cell adhesion of epithelial tumor cells that attains a mesenchymal-like phenotype [30]. EMT causes cancer progression in the bladder at the initial stages

that further becomes a reason for drug resistance in the later stage and finally metastasis. Thus EMT along with Sonic Hedgehog (Shh) signaling pathway, may boost bladder cancer progression [82]. The DNA damage checkpoint activation and an increase in DNA repair capacity promote radioresistance in glioma stem cells. During radiation therapy, the CD133-expressing tumor cells (from human glioma xenografts and primary patient glioblastoma specimens) are more efficient to activate the DNA damage checkpoint and repair radiation-induced DNA damage as compared to CD133-negative tumor cells [83]. Several marker proteins described above have similar implications in chemoresistance of specific malignancies for potential clinical targets.

Notch/wnt-targeting with chemotherapy combination to target CSCs

The chemotherapy in combination with targeting CSCs signaling pathways could be novel vision to develop the new strategies for cancer treatment. A traditional Chinese medicine named Cantharidin is an active constituent of mylabris, which induces β-catenin phosphorylation resulting in its repression. The cantharidin and its derivant, norcantharidin repress β-catenin pathway in pancreatic cancer cells [84]. The Notch-targeting and chemotherapy combination could be efficient approach to target CSCs and the tumor cells. McAuliffe et al., 2012 [85•] used specifically cisplatin/GSI combination to eradicate both CSCs and the bulk of tumor cells that give a synergistic cytotoxic effect in Notch-dependent tumor cells by improving the DNA damage response, G_2M cell cycle arrest and apoptosis [85•]. Similarly, the improved survival benefit for GBM has been demonstrated when NOTCH/γ-secretase inhibitor (GSI) RO4929097 combined with temozolomide and radiotherapy (standard of care treatment) resulted in reduced tumor growth and prolonged survival compared to dual combination in Glioblastoma multiforme [86••]. The glioma stem cell marker SOX2, CD133, and Nestin were reduced via combined treatments as discussed. Similarly, the NOTCH1 inhibitor DAPT (GSI-IX) reduces CSCs frequency either alone or in combination with cisplatin, docetaxel, and 5-fluorouracil (chemotherapeutic agents) [87••]. Another Wnt/beta-catenin signal inhibitors, HC-1 and 5-fluorouracil (chemotherapy) can suppress the tumor in oral squamous cell carcinoma cells [88••]. These strategies encourage the additional translational and clinical studies to improve cancer remedy further.

Challenges in therapeutic strategies

Though significantly improved anticancer therapeutics are in clinical use, yet the tumor recurrence is frequent. This could be due to insufficient understanding of mechanistic pathways in chemoresistance and tumor progression. CSCs are assumed to maintain tissue homeostasis and tumor growth similar to normal stem cells. Currently, there are several attempts to address the issues of CSCs in cancer therapeutics as discussed here.

Targeting Wnt signaling to eradicate CSCs

As discussed earlier, the Wnt signaling pathways regulate morphology, cell proliferation, motility, and fate during embryonic development and are

conserved throughout evolution. Wnt/beta-catenin is reported to be essential to maintain intestinal stem cells and intestinal homeostasis [89] that could be futuristic therapeutic approach to target aberrant Wnt signaling to treat colorectal cancer. Total five IWR (Inhibitors of Wnt Response) compounds act as inhibitors of Wnt response (IWR compounds, 1–5) and four inhibitors of Wnt production (IWP compounds, 6–9) act against the Wnt/β-catenin pathway that appear to only affect β-catenin levels has been observed in zebrafish by Chen et al. 2009. The IWR compounds decreased number of the bromodeoxyuridine (BrdU)-labeled cells at the base of the intestinal folds in IWR-1-treated fish, indicates a loss of function of stem/progenitor cell. They also reported that the prolonged treatment (longer than 5 days) of IWR compounds results in gross histological changes in the architecture of GI tissue as exhibited by lethargy and decreased appetite. But, this transient Wnt/β-catenin response inhibition did not alter the ability of stem cells to self-renew permanently as zebrafish resume regenerative processes after 7 days of IWR compound removal [90]. So, the anticancer therapeutic goal could be achieved by attaining a short-term repression of pathological Wnt response without acquiring a permanent damage to normal stem cell function. Also, the β-catenin pathway inhibitor, FH535, represses pancreatic CSC stemness in vitro as well as angiogenesis [91].

A small synthetic compound β-catenin inhibitor, CWP232228 (U.S. Patent 8,101,751 B2) inhibit the growth of breast CSCs and tumor cells by antagonizes β-catenin binding to TCF in the nucleus that further suppresses the formation of tumor and metastasis without any toxicity in vitro as well as in-vivo [92]. Recently, Kim et al. (2016) demonstrated for the first time that CWP232228 also target liver CSCs by a molecular mechanism involving Wnt/β-catenin signaling and thereby suppresses liver cancer. This could be a significant clinical approach to treat liver cancer resistant to chemotherapy as liver CSCs are responsible for tumor relapse [93••]. Further, a novel Wnt signaling inhibitor, Z86 (isopropyl 9-ethyl-1-(naphthalen-1-yl)-9H-pyrido[3,4-b]indole-3-carboxylate) has been reported, which inhibits the GSK3β (Ser9) phosphorylation that leads to phosphorylation and degradation of β-catenin. They observed that the Z86 inhibited the growth of colorectal cancer cells selectively and caused obvious G1 arrest via Wnt signaling [94•].

There are some chemoprevention agents as nonsteroidal anti-inflammatory drugs and PPARγ agonists eg. ZTM000990 and PKF118-310 that could inhibit the canonical WNT signaling pathway [95]. The small molecules as well as Anti-WNT1/WNT2 monoclonal antibodies are proven to be effective antitumor approaches in vitro [96]. Further, the drug resistance could be overcome by the blended effect of serpentine receptor smoothened (SMO) antagonists with imatinib mesylate and cyclopamine that could overcome the imatinib resistance of CSCs [97]. Moreover, the treatment of advanced basal cell carcinoma (BCC) could be possible by administration of a small molecule anti-smoothened (SMO) agent vismodegib (GDC0449; Roche, FDA approved since 2012) that is regulated by the sHh signaling pathway. Porcupine and Wntless help to regulate the secretion on Wnt proteins. LGK974 agent targets Porcupine to inhibit Wnt ligand secretion from the endoplasmic reticulum [98]. Recently, a clinically approved drug, axitinib, is reported to block Wnt/β-catenin signaling in

cancer cells, zebrafish, and Apc(min/+) mice, thereby could have therapeutic benefits to cancer patients with aberrant nuclear β-catenin activation [99•]. Another Wnt pathway inhibitor small molecule, XAV939, stimulates degradation of β-catenin by axin stabilization through inhibition of the poly-ADP-ribosylating enzymes tankyrase 1, and tankyrase 2 [100•].

Targeting Hh signaling to eradicate CSCs

Hh pathway represents a potentially therapeutic target for CSC elimination as associated with the stem cell maintenance of a variety of cancers including multiple myelomas and colorectal cancer [101]. The experiments involving novel Hh signaling pathway inhibitors suggest the need to optimize the disease-specific factors in human cancers. This pathway is inappropriately activated in MB cancers. The first inhibitor cyclopamine (teratogen) was an alkaloid isolated from the corn lily. Several phase 1 clinical trial drugs, like Genentech's GDC-0449 showed positive results in patients with advanced BCC. Metastatic MB treated with GDC-0449 results in rapid regression of the tumor and reduction of symptoms [102]. GDC-0449 inhibits Hh signaling by targeting the serpentine receptor smoothened (SMO), and thus shows anti-tumor activity. The SMO has an amino acid substitution at its conserved aspartic acid residue with no effect on Hh signaling, but suppress this pathway by disrupting its ability to bind SMO [103]. Similar mutation altered the same amino acid in a GDC-0449-resistant mouse model of MB. It concludes that mutations in a serpentine receptor with features of a G protein-coupled receptor could evaluate for drug resistance in human cancer. Some inhibitors named GSIs (γ-secretase inhibitors): MK0752 (Merck), R04929097 (Roche), PF-03084014 (Pfizer) LY450139 (Eli Lilly), BMS-unknown (BMS) have already undergone a clinical-phase trial. Other Hh inhibitors, such as vismodegib, BMS-833923, saridegib (IPI-926), sonidegib/erismodegib (LDE225), PF-04449913, LY2940680, LEQ 506, and TAK-441 (specifically targeting Smoothened) are reported to be effective as monotherapy in BCC and MB patients [104]. The Hh signaling mechanisms could be considered a new therapeutic strategy that holds a considerable scope for anticancer therapy.

Targeting Notch pathway signaling to eradicate CSCs

Notch signaling (evolutionary conserved) is self-fate differentiation pathway that is highly relevant to cancer cell signaling including CSCs. The complex signaling pathway is involved in various functional activities. It is activated by ligands present on neighboring cells, inducing proteolytic cleavage, translocation and further activation of downsteam signaling [105]. Nowadays, there are certain notch inhibitors that prevent the proteolytic cleavage and the final release. The notch signaling can also be blocked by monoclonal antibodies. Anti-DLL4 mabs, enoticumab, results in disorganization of angiogenesis. Notch overexpression cause also causes resistance to sunitinib [98]. The Notch3 overexpression leads to CSC expansion and increase platinum chemoresistance in tumor cells, but tumor sensitivity increases to platinum by a Notch pathway inhibitor named as γ-secretase inhibitor (GSI) that can reduce the CSCs, further demonstrating that the Notch3 siRNA knockdown also increases the response to platinum

therapy. They also reported that the cisplatin/GSI combination is an efficient treatment to eradicate both CSCs and the bulk of tumor cells and this combination have a synergistic cytotoxic effect in Notch-dependent tumor cells via enhancing the DNA damage response, G_2M cell cycle arrest, and apoptosis [85•]. The selective γ-secretase inhibitor (PF-03084014) alone and with gemcitabine combination results tumor regression in three of four subcutaneously implanted xenograft models of pancreatic cancer xenografts. The PF-03084014 acts by inhibiting nuclear Notch 1 intracellular domain cleavage and Hes-1, Hey-1 (Notch targets) activation. The PF-03084014 and GEM combination could be effective to suppress tumor cell proliferation, induction of apoptosis, curb CSCs, inhibition of tumor growth, delayed tumor recurrence, and prevention of metastatic spread in pancreatic ductal adenocarcinoma patients clinically by inhibiting notch pathway [106]. Thus, the Notch pathway is immerging to be used as therapeutic targets to eradicate CSCs.

Targeting miRNAs to eradicate CSCs

miRNAs are small non-coding RNAs that regulate the gene expression by binding to its target mRNA or via translational modulation. Differential expression profiles of various miRNAs in CSCs make them potential biomarkers for therapeutic resistance and their biological specificity in targeting the various properties of CSCs. Novel anticancer therapies are based on the manipulation of oncogenic or tumor suppressor miRNAs by reducing or increasing their expression levels respectively [107]. Some CSCs specific miRNAs in specific cancer types may regulate certain CSC biological phenotypes (Table 1). There are certain oncogenic miRNAs such as LIN-28B, miR-9, miR-181, and miR-215 that are responsible for tumor initiation or therapy resistance caused by CSCs [133].

In prostate CSCs, miR21 modulates the proliferation in CSCs [134]. Lower expression of miR-34a in prostate CSCs leads to activation of androgen receptor and CD44. It is also an important regulator in glioblastoma CSCs by causing cell cycle arrest, apoptosis, and xenograft tumor regression [135]. In breast CSCs, it directly targets CD44 to restrict CSCs. It has been reported that p53 directly targets miR34b/miR34c and this miRNA was downregulated in p53 deficient ovarian carcinoma cells [136]. In hepatocellular stem cells (HpSCs), the highly expressed miR-181 promote its differentiation and directly targets CDX2, GATA6, and NLK [137•]. The miR-181 is highly expressed in $EpCAM^+AFP^+$ hepatic CSCs and its inhibition leads to reduced CSC renewal and tumor initiation (130). In hematopoietic stem cells (HSCs), miR-128 and miR-181 prevents differentiation whereas the expression of miR-16, miR-103, and miR-107 inhibits proliferation [138•]. Higher expression of miR-200c inhibits breast CSC-mediated colony formation and suppressed tumorigenesis in mice. It has also been found that the let-7 miRNAs suppress oncogenes like RAS and HMGA2 and inhibit CSC growth [139]. The miRNAs are also involved in regulating signaling pathways involved in CSCs. In CSCs, the Wnt signaling is activated by the APC mutation that causes β-catenin nuclear accumulation and activation. It has been reported that miR-320 can inhibit β-catenin expression by targeting the 3′UTR of β-catenin mRNA [133]. Importantly, miRNAs can regulate the

Table 1. microRNAs involved in regulation of cancer stem cells

Sr.	miRNAs	Transcriptional targets	Relevance to cancer progression and its biological functions	References
1.	miR-1246	CCNG2	Pancreatic cancer: induces chemoresistance and CSC-like properties	[108]
2.	miR-495	E-cadherin and REDD1	Breast cancer: promotes oncogenesis and hypoxia resistance	[109]
3.	miR-371-373 cluster	Wnt/β-catenin, DKK1 Myc	Promotes cell growth and invasive activity of cancer cell	[110, 111]
4.	miR-216a/217	PTEN and SMAD7	Liver cancer: regulates the properties of CSCs	[112]
5.	miR-210	PTEN and SMAD7	Hepatocellular carcinoma: increased proliferation, migration and metastatic ability	[113]
6.	miR-210	BASP1, Wnt/β-catenin	Pancreatic cancer: increased cell migration and invasion	[114]
7.	miR-130b	P53-induced nuclear protein 1	Lung cancer: increased migratory potential and neoplastic properties	[115]
8.	miR-29a	P53-induced nuclear protein 1	Acute myeloid leukemia: regulates hematopoietic stem cells	[116]
9.	miR-21	Nanog, Oct4 and EZH2	Acute myeloid leukemia: regulates hematopoietic stem cells	[113]
10.	miR-18	DLL4, inhibitor of Notch signaling	Glioma: promotes tumorigenic potential of GSCs	[117]
11.	Let-7	Lin-28	Colon adenocarcinomas: promotes cell migration, invasion and transforms immortalized colonic epithelial cells Pancreatic cancer: increased pluripotency	[117, 118]
12.	miR-487b	SUZ12, BMI1, WNT5A, MYC, and KRAS	Lung cancer: increased proliferation and invasion	[119]
13.	miR-451	SMAD 3 and 4	GBM: controls GBM stem cells differentiation	[120]
14.	miR-326	Hh smoothened signal transducer	Chronic myeloid leukemia: increased cell proliferation and decreased apoptosis	[121]
15.	miR-204	Sox4 and Ephrin receptor EphB2	Glioma: involved in GSCs self-renewal and invasion	[122]
16.	miR-200 family	VEGFR1, VEGFR2 and EMT-related transcription factors	Pancreatic cancer: regulates CSCs properties	[117]

Table 1. (Continued)

Sr.	miRNAs	Transcriptional targets	Relevance to cancer progression and its biological functions	References
17.	miR-200a	ZEB1, ZEB2, SNAIL & SLUG, N-cadherin, ZEB1, vimentin	Pancreatic cancer: increased cell migration and invasion	[123]
18.	miR-181	ATM	Breast cancer: regulates the properties of CSCs	[124]
19.	miR-150	MYb	Acute myeloid leukemia: blocking of myeloid differentiation	[125]
20.	miR-143/145 cluster	KRAS2 and its downstream effector RREB1	Pancreatic cancer: regulates CSCs survival	[126]
21.	miR-145	Oct4, Sox2, Nanog, Klf4 as well as Kras and Rreb1	Pancreatic cancer: increased pluripotency	[117]
22.	miR-128	Histone methylation [H3K27me(3)], Akt phosphorylation, p21(CIP1) Bmi-1	Glioma: increased self-renewal and proliferation	[127]
23.	miR-107	Nanog, Oct3/4, and Sox2	Head and neck squamous cell carcinoma: Increased CSC proliferation	[128]
24.	miR-100/let-7a-2/miR-125b-1cluster	Myc	Liver cancer: regulates the properties of CSCs	[111]
25.	miR-34 family	Notch and Bcl-2	Pancreatic cancer: involved in self-renewal of CSCs	[110, 129]
26.	miR-29b-1	CD133, N-Myc, CCND2, E2F1 and E2F2, Bcl-2, IAP-2, Oct3/4, Sox2 and Nanog	Osteosarcoma: increased proliferation, self-renewal and chemoresistance	[130]
27.	miR-27a	14-3-3θ, Bax and Bad	Acute leukemia: regulate apoptosis	[131]
28.	miR-23b	Cell cycle arrest	Glioma: inhibits proliferation	[132]

signal transductions between these pathways and form a CSC signaling network with the pathway molecules, thus controlling CSC self-renewal and differentiation. Therefore, the CSCs modulation by miRNAs may help in the cancer therapeutics.

Inhibitors of histone deacetylase and DNA methyltransferase to eradicate CSCs

Aberrant DNA methylation is an important hallmark of cancer. Histone acetyltransferases (HAT) catalyze the acetyl group transfer from acetyl-co-A to the e-amino site of lysine that results in chromatin decondensation, whereas the histone deacetylases (HDAC) act on lysine residues that suppress gene

transcription and compact chromatin [140, 141]. This effect of HDAC is associated with numerous signaling pathways that regulate and maintain the stem cell pluripotency [142]. The polycomb genes are frequently downregulated in CSCs by DNA or histone methylation [143]. The histone deacetylases are reported to be a key regulator of the cell cycle and active genes in the S phase of the cell cycle, regulated by E2F transcription factors, are silenced by retinoblastoma protein (Rb) that bind to the E2F activation domain. The active transcriptional repression by Rb results in chromatin structure modification [144]. The effect of acetylation of histone 3 on chromatin organization as a marker of chromatin compaction further explored by researchers in head and neck squamous cell carcinoma (HNSCC). The hypoacetylated HNSCC cells with microenvironmental cues (e.g., microvasculature endothelial cells) induce tumor acetylation as compared to control. Interestingly, the CSCs numbers are reduced and clonogenic sphere formation is inhibited due to chemical inhibition of histone deacetylases that induced EMT transition with BMI-1 accumulation and expression of the vimentin mesenchymal marker in HNSCC cells [145]. The DNA methylation at the 5-position of cytosines (5 mC) is frequently altered in cancers and is an important epigenetic modification for tissue differentiation [146]. The CpG hypermethylation is reported to suppress tumor-suppressive genes whereas the hypomethylation results in expression of oncogenes. So, the hyper- and hypomethylation contribute differently to carcinogenesis. Till date, two therapeutic strategies have been reported to inhibit the DNA hypomethylation named as DNA-hypomethylating or DNA-demethylating agents [147, 148]. Recently, it has been shown that IL-6/JAK2/STAT3 pathway upregulates DNMT1 and further the downregulation of p53 and p21 resulted from DNA hypermethylation enhances lung cancer initiation and CSC proliferation. Thereby, the proliferation of lung CSCs is controlled by DNMT1 inhibition and blockage of the IL-6/JAK2/STAT3 pathway [149]. Targeting DNA methyltransferases (DNMT) 1, DNMT3A and DNMT3B via DNA methylation inhibitors 5-Azacytidine (Aza) and 5-Aza-2′-deoxycytidine (Aza-dC) is also emerging as a successful epigenetic therapy to treat patients with DNA hypermethylation-induced cancer diseases such as hematologic neoplasms especially with myelodysplastic syndrome [150]. Further, the novel therapies targeting key molecules that maintain the CSCs are emerging such as Cyclooxygenase-2 (COX-2) that regulate DNA methylation. The stemness of CSCs was restrained by targeting of aberrant methylation by using COX-2 inhibitors [148]. The HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) reported to significantly inhibit mantle cell lymphoma growth either by G_1S or G_2M arrest or by inducing apoptosis without any toxic side effects [151]. EZH2 is the catalytic subunit of PRC2 (polycomb repressive complex 2) with histone methyltransferase activity and catalyzes di-methylation (H3K27me2) and trimethylation (H3K27me3) of H3K27. It functions as a transcriptional repressor in gene silencing and facilitates histone methyltransferase activity [152] and is overexpressed in many types of tumors. The PRC2 catalyzes trimethylation of lysine 27 on histone H3 (H3K27me3) via the histone methyltransferase, EZH2 [153]. Researchers are targeting EZH2 as a novel cancer treatment. The EZH2 inhibitors such as EPZ-6438 (E7438), has been reported to be effective against lymphoma in a Phase I study. Similarly, another EZH2 inhibitor, 3-deazaneplanocin A (DZNep), exhibits anticancer activity due to its methionine metabolism inhibition [154]. The Histone deacetylase inhibitors

suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA) resulted in cell cycle arrest, apoptosis and growth inhibition of SCCHN (squamous cell carcinoma of the head and neck) cell lines in a dose-dependent manner. The expression of CD44 and ABCG2 CSC markers, mRNA expression levels of stemness-related genes and the EMT phenotype of CSCs, was also decreased by HDACi treatment in same cell lines. The synergistic effect of HDACi in combination with chemotherapeutic agents named cisplatin and docetaxel also reported on SCCHN cell lines [142]. It is clear that the HDAC inhibitors modify the tumor plasticity by reducing the CSC population. HDACi creates a homogeneous population with biologically defined and predictable behavior of cancer cells. So, the HDAC inhibition may constitute a novel strategy to disrupt the CSC population in head and neck tumors.

Conclusion and future perspective

The EpCAM, Wnt, Hh, and Delta/Notch pathways associated with CSC self-renewal and proliferation that may be inhibited to potentiate the current research for better therapeutically target these cells. Various markers could be used as prognostic to evaluate the clinical as well as predictive outcome of various drugs response in cancer patients. Targeting the self-renewal pathways in CSCs using Wnt Ligand inhibitors, Hh ligand Inhibitors, GLI Antagonists, SMO Inhibitors, Anti-DLL4 Antibodies, Notch inhibitors, γ –Secretase Inhibitors, results in hopeful outcomes to give effective cancer remedy. miRNAs as well as histone modifications may be targeted as an add-on therapy to increase the therapeutic potential. The improvements of new strategies are presently delayed due to the absence of reliable markers for the identification of CSCs.

Acknowledgements

All the authors thank the Vice-Chancellor of CUPB for the support.

Funding Information

The work was supported by CUPB-RSM and UGC startup grants.

Compliance with Ethical Standards

Conflict of Interest
Gurpreet Kaur, Praveen Sharma, Nilambra Dogra, and Sandeep Singh declare they have no conflict of interest.

Human and Animal Rights and Informed Consent
This article does not contain any studies with human or animal subjects performed by any of the authors.

Abbreviation

ABC, ATP-binding cassette; *ALDH1*, aldehyde dehydrogenase 1; *APC*, adenomatous polyposis coli; *BCC*, basal cell carcinoma; *Bmi 1*, B cell-specific Moloney murine leukemia virus integration site 1; *BrdU*; bromodeoxyuridine; *BTSC*, brain tumor stem cell; *CBC*, Crypt base columnar; *CCL5*, Chemokine C-C motif ligand 5; *CCR5*, Chemokine C-C Motif Receptor 5; *CKIε*, Casein Kinase Iε; *CSCs*, Cancer stem cells; *CXCL12*, C-X-C motif chemokine ligand 12; *CXCR4*, Chemokine receptor type 4; *DKK1*, Dickkopf related protein 1; *DLL*, Delta-like ligands; *EMT*; Epithelial-to-mesenchymal transition, *EpCAM*, Epithelial cell adhesion molecule; *ES cells*, Embryonic stem cells; *ESA*; Epithelial surface antigen; *FGF20*, Fibroblast growth factor 20; *GBM*, Glioblastomas; *GCG*, Glucagon; *GI*, Gastrointestinal; *GPI-AP*, Glycosylphosphatidylinositol-anchored protein; *GSI*, Gamma secretase inhibitors; *Hh*, Hedgehog; *HpSCs*, Hepatocellular stem cells; *HSPCs*, Hepatic stem/progenitor cells; *ICD*, Intracellular domain; *IWR* compounds, Inhibitors of Wnt response compounds; *JAG1 and JAG2*, Jagged proteins; *MB*, Medullo-blastoma; *MSCs*, Mesenchymal stem cells; *NDRG2*, N myc downregulated gene 2; *NICD*, Notch intracellular domain; *NLK*, Nemo-like kinase; *NOD-SCID*, Non-obese diabetic- severe combined immunodeficient; *PPARγ*, Peroxisome proliferator-activated receptor γ; *SCFR*, Stem cell factor receptor; *SDF-1*, Stromal cell-derived factor; *SHh*, Secreted Hedgehog ligands; *Shh*, Sonic Hedgehog; *SMO*, Small molecule anti-smoothened; *SP*, Side population; *TACE*, Tumor, necrosis factor alpha-converting enzyme; *TCF*, T cell factor; *WISP1*, WNT1- inducible signaling pathway

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- •• Of major importance

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