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New Opportunities and Challenges to Defeat Cancer Stem Cells

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Abstract

Cancer stem cells (CSCs) are a sub-population of cancer cells capable of self-renewal, proliferation, differentiation, plastic adaptation and immune regulation, thereby mediating tumorigenesis, metastasis and therapy resistance. CSCs are associated with cancer progression and clinical outcomes of cancer patients. Therefore successfully targeting CSCs is required to eradicate and cure cancer. Functional regulators of stem cell (stemness) signaling pathways in human cancers have brought new opportunities to target CSCs and reframe cancer-targeting strategies in clinical settings. However, challenges remain due to a lack of complete understanding of CSC plasticity/heterogeneity and limited efficacy of individual stemness inhibitors in cancer treatment. In this article, we review the CSC signaling pathways and the current state of CSC-targeting therapeutics in combinatory treatment in clinical trials.

Keywords

cancer stem cells; immune evasion; targeted therapy

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I Cancer stem cells as the underlying root of cancer

Cancer remains one of the most fatal diseases worldwide with approximately 14 million new cases and 8.2 million cancer related deaths every year [1]. Despite advances in treatment, cancer relapse, distant metastases and drug resistance continue to kill patients. Work over the past twenty years has identified a subset of cancer cells with tumorigenic and stem cell properties called cancer stem cells (CSCs) as the underlying root for tumor initiation, metastasis, relapse and drug resistance in both liquid and solid tumors [2-13].

The defining property of these CSCs is self-renewal contributed by both genetic mutations and abnormal epigenetic alternations [14]. The cell of origin of CSCs vary across different cancer types [15]. It has been hypothesized that CSCs originate from somatic stem cells, partially-differentiated progenitors, or differentiated cells that acquire stemness through various mechanisms [16-18]. CSCs may derive from transformation of non-cancerous stem cell populations within a tissue that are already capable of self-renewal and differentiation, such as hematopoietic stem cells, but may also arise from progenitor populations that have begun differentiation and are not considered capable of permanent self-renewal, such as common myeloid progenitors [19].

In this review we list major methods and markers used to identify CSCs, discuss key pathways that support CSC functions, and summarize current approaches utilizing these pathway targets to combat CSCs and cancers in the clinic. We also address challenges of CSC research and clinical applications. Finally, we highlight future directions and explore important novel avenues of research and how they apply to CSCs, including biomarker evaluation, immunotherapies and combination therapies, and big data-driven bioinformatics.

II CSCs are identified as essential therapeutic targets

The first evidence of mouse CSCs tracks back to 1937 when single transplanted mouse leukemic cells regenerated leukemia in recipient mice [20]. Human CSCs were first identified in the 1990s when Dick's group transplanted human acute myeloid leukemia (AML) into severe combined immunodeficiency disease (SCID) mice or NOD/SCID mice [21, 22], and found that only a fraction of the total leukemic cell population ($CD34^{++}CD38^{-}$) could regenerate AML in recipient mice due to their capacity for self-renewal, proliferation and differentiation into other cancer cells. Since this discovery, the gold standard assay to identify the tumorigenic properties of human CSCs has been their ability to generate human tumors in immune-deficient mice. Such use of *in vivo* CSC functional assays has helped create the alternative interchangeably-used terms in the literature, such as "tumor-initiating cell" and "tumorigenic cell" to describe putative CSCs. In Box I, we list common methods to identify CSCs, including cell sorting-based transplantation [4, 21-29], lineage tracing [15, 30-34], barcode tracing [35], and other functional assays [36] for representative CSC markers in solid tumors, hematologic malignancies, and metastases. It is anticipated that pooled CRISPR/Cas-mediated gene editing and modulation would also be used in identifying tumorigenic drivers and regulator markers of CSCs in various cancers.

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Notably, there has been a debate whether CSC models exist in melanoma. Studies by the Morrison group examined a list of markers, but failed to identify any that enrich CSCs from human melanoma or differentiate the tumorigenic capacity of individual melanoma cells [37]. In contrast, the Weissman group was able to successfully identify a specific CSC surface marker, CD271 in human melanoma [38]. The debating results illustrate some of the limitations of using biased markers to identify CSCs. Recent findings from Pascual et al. identified the fatty acid receptor CD36 as a target and marker of metastasis-initiating cells ($CD44^{bright}$) in melanoma and breast cancer since they rely on dietary lipids to promote metastasis [39]. In addition, heterogeneous CSC markers might be associated with distinct breast cancer subtypes, such as $CD44^{+}/CD24^{-}$ phenotype with basal-like or mesenchymal-like cancers [40, 41].

As the defining feature of CSCs is self-renewal, CSCs are often compared with the counterpart of non-malignant stem cells. Much like traditional stem cells, CSCs have the capacity to: 1) self-renew, 2) proliferate by asymmetric or symmetric divisions, and 3) differentiate into several cell types of the specific tissue or tumor. CSCs are often linked to altered proliferation, pluripotency (giving rise to phenotypically diverse differentiated tumor cell populations), persistence (with the ability to maintain a reservoir within the tumor), and resistance to traditional chemotherapy agents as well as radiation [42]. Distinct features have been observed in various CSCs versus other cancer cells, such as epithelial-mesenchymal transition [43], increased resistance to oxidative stress [44], induced reprogramming [45-48], enhanced metabolic alterations [49, 50] and improved DNA damage repair [51, 52]. More recently, the capacity and mechanisms of CSC regulators such as β-catenin and Myc in immune evasion and suppression have been investigated [53, 54], highlighting the necessity and potential of CSC-targeting effects in increasing the efficacy of immunotherapies.

The extent of similarity between CSCs and normal stem cells could cause toxicity as therapeutics are developed to target CSC stemness. The number of normal stem cells is usually regulated through strict balance of proliferation, differentiation, and apoptosis [42] while CSCs are abnormally regulated due to their genetic and epigenetic alterations [55]. One potential solution could be to target stemness linked to the genetic and abnormal epigenetic alterations (such as fusion genes PTPRK-RSPO3) specific to CSCs or cancer cells, allowing for a much more targeted therapy while sparing normal cells [56].

III Signaling pathways of CSC therapeutic targets

Over the last few decades the signaling pathways that regulate CSCs have been identified [57], facilitating the development of novel CSC targeted strategies. The best characterized CSC pathways include, but are not limited to, Sonic Hedgehog (Hh)/Patched (Ptch)/Smoothened (Smo), Notch/Delta-like ligand (DLL), CXC chemokine receptor1-2/CXCL8/FAK, and Wnt, which may also affect downstream effectors including the transcription factor activators and transcription factors such as β-catenin, STAT3, and NANOG. Figure 1 gives examples of some of the CSC signaling pathways that are currently being pursued as potential clinical targets to treat a number of cancer types. While advances in drug discovery have led to the development of several molecules targeting CSC regulatory players, several concerns have been raised in terms of their safety, efficacy and clinical impact. First, CSCs

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share expression of many genes and signaling pathways involved in regulating differentiation and self-renewal with normal stem cells. Therefore, the use of these drugs raise concerns about toxicity and off target effects. Secondly, the redundancy in the regulatory pathways creates challenging hurdles that can lead to limited efficacy. To tackle such obstacles clinical trials are currently utilizing high-throughput screening of combinatory approaches to target CSCs (such as NCT02654964). Thirdly, it is not clear whether targeting CSCs alone is sufficient to treat cancers. As cellular plasticity may exist to switch the lineage fate of CSCs [58, 59] or to de-differentiate cancer cells, it would be beneficial to target both CSCs and differentiated cancer cells using complementary, combinational therapies. Therapeutic interventions that target these cancer stemness pathways and show clear evidence of activity will help define the clinical impact of each putative signaling pathway in CSC maintenance and pool expansion.

Examples of CSC-targeted therapies that are in various stages of clinical development are presented in Table 1. Early phase clinical trials have shown promise for agents that target CSC pathways and preliminary results of single-arm studies have been reported as shown in Table 2. In general, CSC-targeted therapies were associated with manageable toxicities and the most common adverse reactions included Grade 1-3 fatigue, hematological events, and gastrointestinal events (Table 2). In addition to these early-phase studies, a number of CSC-targeted therapies are undergoing evaluation in randomized clinical trials (Table 3). Many of these studies are ongoing with no results published to date. Notably, these identified signaling pathways with developed therapeutics might not represent the complete picture of CSC regulation. Among the targeted CSC signaling pathways, Sonic Hedgehog pathway inhibitors have shown promising cancer-treating efficacy [60-62]. While many of the other approaches are combining with standard-of-care chemotherapy to treat cancer patients, the clinical efficacy of these individual pathway inhibitors is yet to be demonstrated in randomized clinical trials. Additional innovative strategies are sought to target CSCs in which plasticity and heterogeneity might be the major hurdles to defeat. Immunotherapies that overcome CSC-mediated immune suppression and evasion with diverse CSC recognition and targeting strategies could be one of the promising directions.

IIIa. The Sonic Hedgehog signaling pathway

The Sonic Hedgehog (Hh) pathway is crucial to development and patterning during embryogenesis and essential for the maintenance of CSCs [63]. Activation of the Hh pathway occurs when Hh protein ligand binds to and inhibits a transmembrane protein called Patched (Ptch), leading to activation of the protein receptor Smoothened (Smo) which regulates target genes involved in metastasis, proliferation, survival, and pathway auto-regulation. Numerous human malignancies are associated with Hh deregulation and have a profound effect on the outcome of treatment [64]. The Hh pathway may be important for the maintenance of CSCs in various tumor types, including glioma, small cell lung cancer, non-small cell lung cancer, colorectal, pancreatic, and gastric cancers [65-68]. As such, inhibiting any step of the Hh signaling pathway may reduce CSCs and overcome treatment resistance.

The most extensive studies on the pathway regarding CSCs have been done in basal cell carcinoma (BCC) and medulloblastoma [69]. Constitutive activation of Hh pathway via

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Ptch1 deletion in various hair follicle stem cell populations have demonstrated BCC-like neoplasms, suggesting the emergence of CSCs in BCC results from constitutive Hh signaling [70]. In medulloblastomas, 30% of all sporadic cases are a result of Hh pathway mutations, where dormant, therapy resistant $SOX2^+$ CSCs have been shown to be the key drivers of this population of medulloblastomas [71]. Two SMO inhibitors (LDE225/Sonidegib and GDC-0449/Vismodegib) have received FDA approval for treating basal cell carcinoma [72].

While there are many small drug inhibitors targeting Smo, vismodegib is currently being used in the clinic to effectively treat BCC [61]. Early results from vismodegib clinical trials suggest that it is effective at treating BCC in patients whose tumors display an activated Hh pathway, as assayed by the presence of GLI1 or PTCH2 [73]. Vismodegib is also being used in Phase 2 clinical trials to treat patients with Hh pathway mutations resulting in medulloblastoma [62].

IIIb. Notch signaling pathway

The Notch signaling pathway is one of the most extensively studied signaling pathways in CSC biology. [74]. The Notch pathway is activated via ligand-receptor interactions of 4 receptors (Notch-1-4) and 5 Notch ligands (Delta-like-ligand [DLL]-1, -3, -4 and Jagged-1, -2), leading to gamma-secretase-mediated cleavage of Notch, nuclear translocation of Notch Intracellular Domain (NICD), and NICD-transactivated target gene expression. In different cancers, the CSC-regulated Notch signaling can elicit either tumorigenic phenotypes (cervical, lung, colon, head and neck, prostate, brain/nerve, breast, and pancreatic cancer) or tumor-suppressive phenotypes (hepatocellular carcinoma, skin, and small cell lung cancer) [75-77]. Still, it is unclear how this dual role of Notch impacts clinical targeting of CSCs.

Elevated levels of Notch are often expressed in medulloblastoma and breast CSCs, making them ideal targets for Notch inhibition [78-80]. Studies show gamma-secretase inhibitor, GSI-18, prevents Notch receptor from becoming cleaved and translocated into the nucleus for transcriptional activation. GSI-18 can deplete CSC populations from medulloblastoma cell lines [79]. A different gamma-secretase inhibitor MK-0752 can also reduce CSCs and improve the activity of docetaxel in breast cancer cell lines [81]. Early staged clinical trials assessing the efficacy of MK-0752 in combination with other drugs, such as MTOR inhibitors or chemotherapies, demonstrate that while gamma secretase inhibitors can limit CSC populations in preclinical studies, it is still challenging to completely eradicate CSC subgroups in clinical settings of head and neck squamous cell carcinoma (HNSCC) and pancreatic ductal adenocarcinoma (PDAC) [82, 83]. Complete response in these studies was only observed in 1 out of 15 patients with HNSCC (Table 3) [82]. Partial response was observed in 1 out of the 15 patients with HNSCC and 11 out of 18 patients with PDAC [82, 83]. While partial response was promising for the PDAC patients, time to subsequent disease progression was only 38 weeks, suggesting the need for more effective therapies.

In effort to limit Notch signaling abnormalities, an array of antibodies have been designed to target distinct components of the pathway. The first therapeutic drug to selectively target the Notch pathway and enter clinical trials was the anti-DLL4 antibody, demcizumab. Demcizumab has been expected to block tumor angiogenesis, diminish CSCs, and alleviate

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immune suppression. A phase 1b clinical trial, combining demcizumab with chemotherapies, carboplatin and permetrexed, was well tolerated by patients [84, 85]. The combination is now being evaluated in phase 2 clinical trials (NCT02289898) which however has not appeared to meet its expected endpoint based on the OncoMed updates in 2017 [86]. MEDI0639, also an anti-DLL4 antibody, was evaluated in phase 1 clinical trials for solid tumors, but achieved minimal disease control [87]. DLL3 has also been shown to be a promising target to limit CSCs. A recent phase 1 clinical trial using the anti-DLL3 drug rovalpituzumab tesirine (SC16LD6.5) was designed to treat small-cell lung cancer patients who frequently express high levels of DLL3 [88]. This trial confirmed 18% of patients achieved the objective response, including 38% of patients with high DLL3 expression levels [88]. This study demonstrates that despite patients expressing high levels of DLL3, combinatorial approaches may be more effective to combat pathway redundancies.

The anti-Notch1 antibody, brontictuzumab has been evaluated in a phase 1 clinical trial as a single agent in patients with advanced solid tumors or blood cancers [89, 90]. This early study shows brontictuzumab is generally well tolerated and demonstrates moderate anti-tumor activity in patients. Finally, tarextumab is an anti-Notch2/3 antibody that can trigger tumor cell differentiation, limit angiogenesis, and delay tumor recurrence after cytotoxic therapies [91]. After being well tolerated by patients in phase 1b clinical trials, tarextumab has recently entered phase 2 clinical trials in combination with either etoposide and platinum or nab-paclitaxel and gemcitabine to combat untreated extensive small-cell lung cancer and metastatic pancreatic cancer, respectively (Tables 2 and 3) [92, 93]. Encouraging anti-tumor activity of tarextumab was observed for both phase 1b studies, however, final safety, immunogenicity, and correlation to clinical response are not yet determined.

IIIc. FAK signaling pathway

Focal adhesion kinase (FAK) is a cytoplasmic non-receptor tyrosine kinase that plays a significant role in tumor cell survival by orchestrating cellular signaling through integrins and growth factor receptors and functions in multiple steps of tumorigenesis [94]. This includes regulation of cell motility, invasion, cell survival, and transcription that promotes epithelial-mesenchymal transition. FAK also contributes to breast CSC proliferation and cell survival in a kinase-independent manner [95-97].

In vitro, FAK inhibitors exhibit enhanced activity in combination with cytotoxic drugs or agents targeting angiogenesis, such as sunitinib [94]. Preclinical data supports using FAK inhibition to treat tumors with upregulated CSC pathways [94]. A recently completed phase 2 randomized clinical trial assessed the FAK inhibitor defactinib as a maintenance therapy in patients with malignant pleural mesothelioma (NCT01870609) [98]. Patients who had 4 or more cycles of chemotherapy were recruited with the purpose of defactinib preventing tumors from reappearing by CSC initiation [98]. However, defactinib lacks efficacy as a single agent to control malignant mesothelioma [98]. A future promising direction might be utilizing FAK inhibitors with immune checkpoint inhibitors to target immunosuppressive pancreatic cancer microenvironment [99].

The binding of IL-8 to CXCR1 can also promote CSCs by triggering FAK phosphorylation. This activated cascade can then phosphorylate AKT and activate the WNT pathway for self-

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renewal. FAK signaling has been proposed to protect CSCs from a FASL/FAS-mediated bystander effect by limiting downstream signaling of FAS during chemotherapy in models of breast cancer *in vitro* and *in vivo*, suggesting a combined chemotherapy and CXCR1 inhibitor regimen could provide a strategy to effectively target breast CSC compartments [100]. Currently, an ongoing phase 2 clinical trial is testing this concept using paclitaxel in combination with the CXCR1 inhibitor reparixin to treat breast cancer patients after a phase 1b clinical study showed safety and tolerability, as well as promising responses in triple negative breast cancer patients [101].

IIId. Wnt signaling pathway

The canonical Wnt signaling pathway is another developmental cascade involved in multiple biological processes including regulation of gene transcription for embryogenesis, development, cell proliferation, survival, renewal, and differentiation [102-104]. Lineage tracing assays in intestinal adenomas demonstrated that a small fraction of tumor cells positive for *Lgr5*, a Wnt target gene associated with somatic stem cells in the colon, and these tumor cells were largely responsible for the abnormal growths [31]. Other lineage tracing studies of Wnt target *Axin2* and *Lgr5* have also been utilized to identify cells with self-renewing capacity in several tissue types [105-107]. Still, the reach of Wnt signaling seems limitless, playing a role in numerous cancers. Deletion of β-catenin has limited tumorigenesis in mixed lineage leukemia and facilitated tumor regression by depleting CSCs in epidermal tumors [33, 108]. Thus, targeting CSC Wnt/β-catenin signaling or further downstream transcription factors in this pathway are attractive strategies for CSC-targeted cancer therapy.

Due to the cascading nature of the pathway, Wnt inhibitors have emerged to target various components of the pathway. For example, vantictumab (OMP-18R5) blocks Wnt signaling by binding multiple FZD receptors (FZD1,2,5,7, and 8) and has been found to decrease CSC numbers and proliferation in patient-derived xenograft (PDX) models of breast, pancreatic, and lung cancers [109]. Currently, vantictumab is being administered in phase 1b clinical trials to breast, pancreatic, and lung cancer patients in combination with standard chemotherapy regimens (clinicaltrials.gov).

Another Wnt inhibitor also in Phase 1b clinical trials is Ipafricept which binds and sequesters Wnt directly to block the cascade [110] (clinicaltrials.gov). Lipid modification is necessary for all Wnt proteins, therefore targeting the enzyme, Porcupine (Porcn), which facilitates lipid modifications of Wnt as it binds Fzd or as it is secreted from a ligand expressing cell, works as an alternative form to sequester Wnt from activating the pathway. Porcn inhibitor LGK974 is currently being used in combination with EGFR and B-raf inhibitors to treat colorectal cancer patients with aberrant Wnt signaling in Phase 1b clinical trials [111]. Additionally, dual targeting of the Wnt pathway and microtubule function shows considerable promise as an effective therapy against cancer pathologies [112].

IIIe. Signal transducer and activator of transcription 3 (STAT3) signaling

STAT3 is one of the seven STAT family members that have been identified and primarily activated by Janus kinases (JAK)-mediated phosphorylation of a specific tyrosine residue

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[113]. Once activated, dimerized STATs translocate to the nucleus and initiate transcription of the target genes. STAT3 is excessively active in many cancers and plays a major role in tumor growth and metastasis [114], notably converting non-CSCs to CSCs by upregulating the expression of the reprograming factor OCT 3/4, and subsequent expression of several stem cell genes and suppressed cell commitment genes [114, 115]. Targeting STAT3 activation inhibits tumor growth and metastasis both in-vitro and in-vivo without affecting normal cells, therefore suggesting that STAT3 could be a valid molecular target for cancer therapy [114]. A phase 3 study recently completed the evaluation of the STAT3 inhibitor napabucasin (BBI608) plus best supportive care treatment in advanced colorectal carcinoma (CO.23 study) [116]. Although this monotherapy study was stopped early due to minimal differences between the napabucasin and placebo treatment groups, patients who showed expression of pSTAT3 in their tissue samples had improved survival, suggesting napabucasin may be useful for selective patients with targeted biomarker expression [116]. Currently, another phase 3 study is assessing combinational napabucasin and paclitaxel treatments in patients with advanced gastric or gastroesophageal junction cancer who progressed after initial chemotherapy treatment (BRIGHTER study) [117].

IIIf. NANOG signaling

NANOG is a homeodomain-containing transcription factor that is part of the key set of transcription factors involved in the maintenance of pluripotency and self-renewal in undifferentiated embryonic stem cells [118]. In CSCs of various cancers, NANOG expression levels are higher including breast, cervix, oral, kidney, prostate, lung, gastric, brain, pancreas, and ovarian cancer [47, 119-125]. Increased expression of NANOG is shown as an indicator of a poor prognosis for ovarian, colorectal, and breast cancer patients [118]. In oral squamous cell and lung adenocarcinoma, higher expression of NANOG was associated with advanced cancer stage and shorter patient survival rate. NANOG expression has also been shown to be higher in CSCs than in other cancer cells in several types of cancers [118]. $CD133^{+}$ cancer cells express significantly higher levels of NANOG in comparison to $CD133^{-}$ cancer cells. Whereas, in prostate cancer cell lines, NANOG induction results in upregulation of CD133 and ALDH1. Functional studies have shown that NANOG is not only a marker for CSCs, but also promotes CSC characteristics across multiple cancers. Overexpression of NANOG in cancer cells has been linked to increased proliferation rate in vitro and tumor growth in vivo [118].

Amacasertib (BBI503) which targets multiple kinases in the NANOG pathway is currently being evaluated in clinical trials (NCT02232633, NCT02232620, NCT02232646, and NCT02432690). Since colorectal cancer patients often express high levels of NANOG which is associated with poor prognosis, a phase 1 clinical trial used amacasertib to treat patients (NCT01781455). This study demonstrated a disease control rate of 56% in patients with high NANOG expression compared to 13% in patients that were NANOG negative [126]. These results highlight the potential benefits of CSC biomarkers and how they can help personalize patient care.

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IV. CSCs and resistance to therapy

The percentage of CSCs in a tumor is determined mainly by: 1) the characteristics of the CSC population that initiated tumorigenesis, 2) the “niche” or microenvironment, and 3) the frequency with which new or additional CSCs are created and non-CSCs are removed. Tumor type or stage of progression may determine the percentage of CSCs, where larger numbers of CSCs may indicate: 1) worse clinical outcomes, 2) higher rate of proliferation, 3) greater genetic instability, and 4) lack of differentiation. In addition, CSCs gain selective advantage in distinct microenvironment conditions, such as hypoxic environments or in the presence of chemotherapy [127]. CSCs have numerous properties that allow them to persist through chemotherapy, radiation and other treatments [2, 24, 28, 53, 58, 59, 128]. First, CSCs have plastic cell cycle kinetics, allowing a sub-population of CSCs to remain quiescent even as others are proliferating. Thus, certain CSCs are able to evade the numerous therapies that target rapidly proliferating cells [129]. Additionally, CSCs have more efficient DNA repair mechanisms than other cancer cells [24, 130, 131], and exposure to DNA damage (through ionizing radiation, for example) triggers recruitment of cell cycle inhibitors, allowing more time for the DNA damage to be repaired [51, 52]. Furthermore, CSCs express transporter proteins that can pump chemotherapy agents out of the cells [132]. CSCs express high levels of metabolic regulators, such as ALDH and oxidant scavengers, which are able to metabolize chemotherapeutic agents or reactive oxygen species (ROS) [2] to reduce damage from ROS [2]. Finally, stemness regulators such as SOX2 and EZH2 work in the absence of p53 and RB1 to confer lineage plasticity, allowing CSCs to survive anti-tumor therapies [58, 59]. Thus, the intrinsic heterogeneity, plasticity and adaptive resistance of CSCs to stress contribute to the protection of CSCs, allow them to foster the tumor mass and establish secondary tumors, and provide resistance to anti-tumor therapies [127, 133]. Whether the current CSC identification and targeting strategies are sufficient and effective enough to overcome a wide array of mechanisms CSCs possess that enable them to resist anti-tumor therapies is an open question. More importantly, it is uncertain whether these properties would also enable CSCs to resist the newly-developed and to-be-developed anti-CSC therapeutics.

Studies using CSC-targeted therapies as monotherapy have shown promise in early phase studies, but so far, have not shown adequate activity in randomized trials. One solution would be combining anti-CSC approaches with other therapies to not only eliminate the bulk of dividing and differentiated components of the tumor responsible for tumor-associated symptoms, but also to remove the minority CSC population that is resistant to therapies, fuels tumor growth, and contributes to disease relapse and metastases [134]. Indeed many of the randomized trials have combined the listed anti-CSC therapeutics with standard-of-care chemotherapies (Table 3). Another solution might be that the CSC hypothesis requires adjustments about how CSCs are diagnosed and treated in tumor. Possible adjustments could include evaluations of CSC biomarkers based on their levels of pluripotency (dedifferentiation), plasticity and heterogeneity instead of certain pathway markers, development of efficacious approaches to selectively target CSCs without affecting normal SCs, and blocking the mechanisms of CSC resistance to anti-tumor treatment using innovative targeting techniques, such as immunotherapy.

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V. Immunotherapy and CSCs

Recent studies demonstrated the immune suppressing roles of cancer stemness regulators such as β-catenin in the Wnt signaling pathway [53]. cMyc as one of the pluripotency transcription factors upregulates both innate immune inhibitor CD47 and adaptive immune checkpoint molecule PD-L1 in human tumor cells [54]. This suggests that CSCs might not only passively evade immune attacks, but actively suppress immune responses. Durable cancer-controlling effects of immune checkpoint blockade antibodies have brought a new era of cancer immunotherapy, leading to more successful eradication of CSCs and other cancer cells in the clinic. Targeting CSCs using immunotherapy can take the form of either generating an immune response directed at CSC-specific antigens, blocking the function of those CSC-specific proteins or by inhibiting components of the immune checkpoint system. Inhibition of immune checkpoints could thereby raise the immune system's sensitivity to tumor/CSC antigens and trigger a response by the immune system against cells that would normally be unaffected. We will summarize below a few CSC-specific antigen therapies tested against CD133, and CD47 as well as immune checkpoint blockade therapy using anti-PD-1 and anti-CTLA-4 antibodies.

Recently, Huang et al.[135] produced a bi-specific antibody against CD3 and CD133, binding cytokine-induced killer (CIK) $CD3^+$ cells to $CD133^+$ CSCs from pancreas and hepatic cancers. This antibody should bring CSCs into contact with CIK cells, targeting the $CD133^+$ cells for death. In nude mice, CIK cells bound to this antibody effected the destruction of $CD133^+$ tumor cells more than unbound CIK cells or cells bound to only a CD3-specific antibody, suggesting that the antibody was successfully recruiting the immune system to attack CSCs.

CD47 has been identified as a surface marker for all cancer cells, but has been of particular clinical interest as a marker of leukemic stem cells [136] and other CSCs [137-142]. CD47 functions as a potent anti-phagocytic agent, allowing cells to evade the innate immune system. When leukemia CSCs express lower levels of CD47, they are preferentially phagocytosed by macrophages, meaning that $CD47^+$ leukemia cells predominate [143]. Blockade of CD47 could therefore be a potent treatment targeting leukemia CSCs, and has been shown to work as such by Liu et al. [144] in PDX models of leukemia.

Targeting the immune checkpoint molecules of PD-1 and CTLA-4 with blocking antibodies has proven to be a productive treatment for some patients, particularly among those with lung cancer and melanoma of high mutation rates. Immune checkpoint blockade inhibits the interaction of PD-1 and CTLA-4 with their ligands, allowing cytotoxic T cells to attack cancer cells despite the presence of inhibitory ligands on cancer cells. Although CSCs may express many immunogenic antigens, the expression of PD-1 ligand PD-L1 and CTLA-4 ligands CD80 and CD86 prevent the cytotoxic activity of the immune system from attacking them [145]. The durable response that some patients have to immune checkpoint blockade therapy may be attributable to its effect on CSC populations, although this hypothesis has yet to be proven. However, current immune checkpoint blockade to inhibit PD-1 and CTLA4 pathways is only suitable for a small set of cancer types and subtypes with relatively high

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mutation loads. To effectively target CSCs in the majority of cancers with low mutation frequencies, new immune-regulating approaches are to be discovered and explored.

VI Future trends and strategies of targeting CSCs –bioinformatics and big data

Identifying targets for novel drugs in CSCs is complicated by both their relative rarity in a tumor sample and the difficult task of accurately characterizing them. New developments in high-throughput sequencing, including single-cell sequencing, will help provide a better definition of CSCs and may identify new targets for treatment. By sequencing individual tumor cells and looking for populations with expression patterns associated with stemness, relatively complete genomic and epigenomic profiles of CSCs can be identified without labor-intensive and condition-compromised tumor initiation assays.

Tirosh et al.[146] performed single-cell RNA-seq on whole primary lesions from six oligodendroglioma patients, hoping to identify intratumoral heterogeneity and potentially characterize CSCs from within those lesions. Among all cancer cells, they identified three common expression patterns – one with genes associated with oligodendrocytic markers, one with genes associated with astrocytic markers, and a third that had more intermediate expression patterns for oligodendocytic genes and astrocytic genes, but also with genes important for neuronal development and proliferation, suggesting a stem-cell like population. Expression patterns from within this sub-population may be useful for the identification of novel drug targets for oligodendroglioma CSCs. Another method is barcode-tagging and tracing in vivo used to identify $CD109^{+}$ metastatic CSCs in lung cancer [35].

We anticipate that the understanding and targeting of CSCs will be inevitably tailored and accelerated by big interactive data-driven artificial intelligence and deep learning. Esteva *et al.* recently utilized deep learning algorithms to develop convolutional neural networks to identify skin cancer with dermatologist-level capacity [147]. With the recent boom of multi–omic studies, machine-learning and bioinformatic approaches, big data mining-assisted decision making has emerged as a new tool to study cancer and would benefit ongoing and future CSC-targeting trials. However, there continues to be a translational disconnect between the research bench and the clinic. Despite the numerous ongoing clinical trials, only 4-5% of cancer patients are enrolled. Major hurdles include a lack of data sharing, overwhelming disorganization, insufficient data access, and unbridged collaboration between specialized data-mining experts and cancer biologists. Much of the data gathered is not being used at a maximum potential. For this reason, moving towards a more uniform system of data collection and dissemination may mobilize and integrate the entire cancer community's effort to identify and target CSCs, find cure, and stop cancer.

VII. Concluding Remarks

Cancer stem cells (CSCs) are a sub-population of cancer cells capable of self-renewal, proliferation, differentiation, plastic adaptation and immune regulation, thereby mediating tumorigenesis, therapy resistance and metastasis. Successful cancer treatment is complex

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and involves direct anticancer activity of eliminating both CSCs and other tumor cells, changing immune responses, and altering the tumor microenvironment. Therapies directed only toward differentiated cancer cells risk relapse and proliferation of more aggressive tumor cells due to not eliminating the pool of CSCs. In the recent trials, anti-CSC therapies have been benefited in combination with other approaches targeting bulk tumor cells, such as radiation and chemotherapies. However, lack of efficacy has been the hurdle for many individual pathway-specific anti-CSC therapeutics, thus demanding better identification and targeting strategies to specifically, effectively, and rapidly eliminate CSCs and tumor burden, as well as limit toxic side effects on normal SCs and organs.

Moving forward, the CSC model has profound implications for the development of new treatments for cancer. Many current therapies are based on the ability to shrink or de-bulk tumors as assessed by Response Evaluation Criteria in Solid Tumors (RECIST). Unfortunately, tumor shrinkage does not correlate well with patient survival for many tumor types. Therefore, using tumor shrinkage as an indicator of activity for an agent that targets CSCs in either preclinical models or phase 1/2 clinical trials may have to be re-examined. In addition, development of adjuvant therapies may have to be completely reanalyzed based on the CSC model (see outstanding questions). A recent study demonstrated that targeting Lgr5+ CSCs are more critical for metastasis than the primary colon cancer [148]. It is likely that targeting CSCs is more crucial to remove secondary tumors than for shrinking primary tumors.

Many of the listed CSC-targeting therapeutics have not yet shown promising efficacy as single agents to treat cancer in randomized clinical trials. Not only is there a need to develop the innovative CSC-targeted therapies, much work still needs to be done to evaluate combinations of CSC-targeted therapies with other therapies, such as immunotherapy that provide the most benefit in a particular tumor type. As many identifiers of CSCs are surface markers, use of antibodies to invoke an immune response may be represented as another tactic but requires targeting heterogeneous CSC populations in combination with the blockade of immune checkpoints, including PD-1 and CTLA4. Finally, the particular choice of CSC-targeted therapy may depend on better assays and strategies to detect and target CSCs. Thus, validated biomarker assays for CSC defining and specific features for self-renewal, plasticity and motility will need to be developed and these assays will help to define patient populations who will benefit most from a particular CSC-targeted therapy.

While the heterogeneity, evolution, plasticity and motility of CSCs need to be better recognized in both cancer initiation and progression, we need to keep in mind that understanding and targeting CSCs must be the entire cancer community's team effort. Open and shared resources with integrated and interactive big data on CSCs will empower us to take advantage of super computation-based artificial intelligence, thereby hopefully outsmarting intelligent CSCs and other cancer cells to defeat cancer.

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Box I

Identification methods and markers of CSCs

CSC Identification Methods

1. Fluorescence-activated cell sorting (FACS) and serial transplantations *in vivo* [4, 21-29]. This is the first method used to identify human CSCs xenografting sorted cells into immune-deficient mice.
2. Lineage tracing of CSCs with putative promoter-driven reporters *in vivo* [15, 30-34]. It examines the CSCs in intact tumor microenvironment without dissociation and sorting-mediated disruption. It is suitable for syngeneic mouse tumor models and can study immune –CSC interactions.
3. Barcode-tagging and tracing via RNA-sequencing to identify metastatic CSCs [35]. It enables simultaneous tracing of all tagged cancer cells for systematic, large scale, high throughput analyses. Chuang et al [35] used this approach and identified CD109 and the Jak-Stat pathway as critical drivers of lung cancer metastasis.
4. Other complementary assays *in vitro*, such as spheres and reporter systems [36] which normally require validation analyses *in vivo*.

Representative CSC Markers

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Tumor Type	CSC markers
Solid tumors	
Brain [149, 150]	CD133, CD49f, CD90, CD44
Breast [4]	CD44, CD24 (negative/low), EpCAM, ALDH
Colon [25, 27, 151]	CD133, CD44, CD44v6, CD166, EpCAM, CD24, Lgr5
Gastric [152-159]	ALDH1, CD24/CD44, CD54/CD44, EpCAM/CD44, CD71-negative, CD90, CD133
Lung [26, 160]	Sca 1, CD34, CCA, CD133, ABCG2,
Melanoma [38, 161]	CD271, CD20
Pancreatic [134]	CD133, CD44, EpCAM, CD24
Prostate [162]	CD133, CD44, CD24 (negative)
Skin [163]	SOX2
Hematologic maligencies	
Acute myeloid Leukemia [22]	CD34, CD38
Leukemia [164]	CD34, CD38-negative, CD71-negative, CD90-negative, CD117-negative, CD123
Metastases	
Pancreas [165]	CD133, CXCR4
Breast [10, 39]	CD44, CD36
Melanoma [39]	CD44, CD36
Lung [35]	CD109
Colon [49, 148]	CD110, LGR5

ABCG2, ATP-binding cassette subfamily G member 2; ALDH, aldehyde dehydrogenase; CCA, Clara cell-specific marker; CSC, cancer stem cell; CXCR4, C-X-C chemokine receptor type 4; EpCAM, epithelial cell adhesion molecule; LGR5, Leucine-rich repeat-containing G-protein coupled receptor 5.

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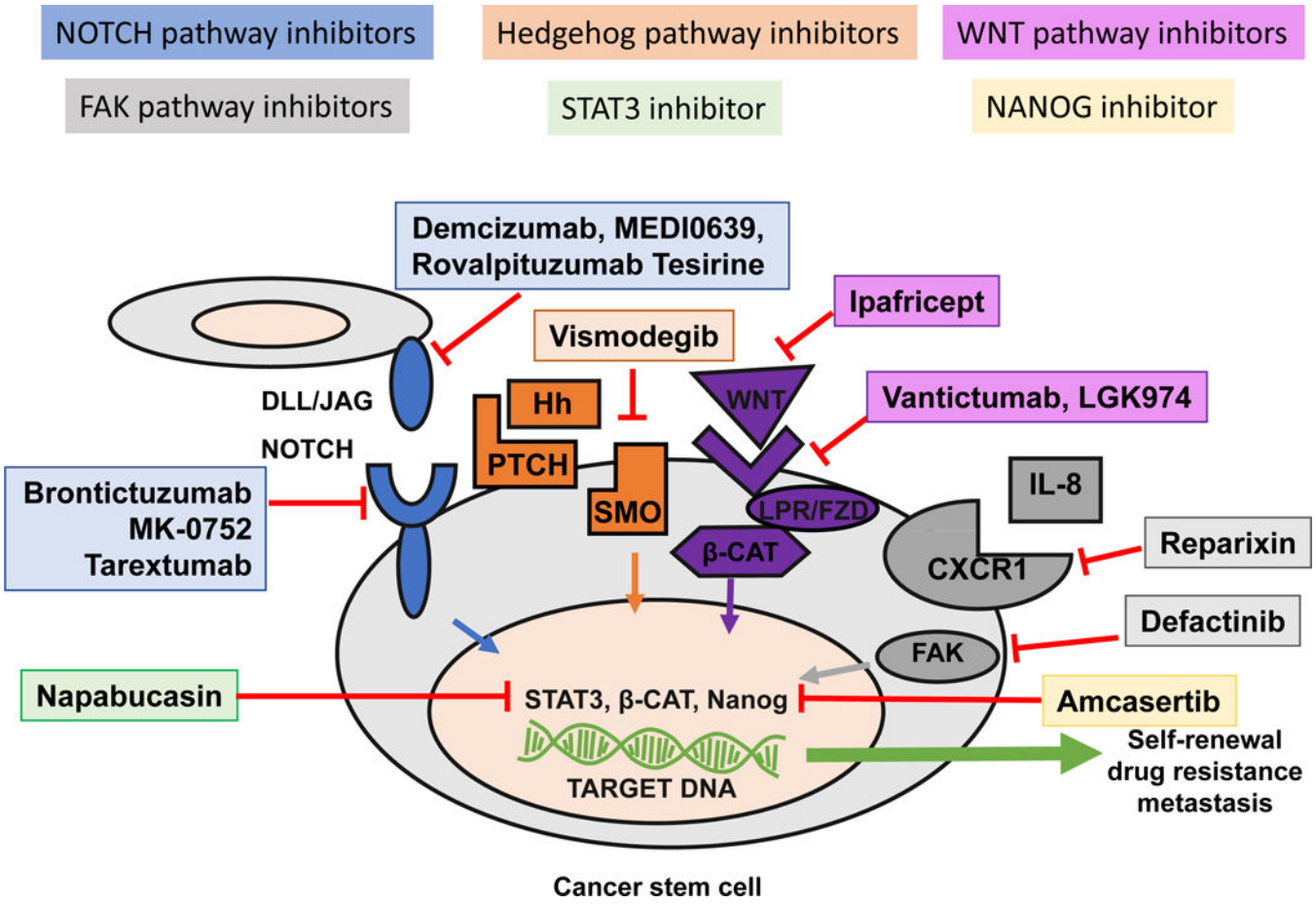

Figure 1. Targeting CSC pathways with newly developed drugs

The schematic displays targeting strategies to combat CSCs by blocking stemness signaling pathways. NOTCH receptors can be inhibited by brontictuzumab, MK-0752, and tarextumab while NOTCH ligands can be inhibited by demcizumab, MEDI0639, and rovalpituzumab tersirine. These agents all block NOTCH signaling, ultimately preventing intracellular Notch cleavage and translocation into the nucleus. The hedgehog pathway can be blocked using the smoothened receptor antagonist Vismodegib and Sonidegib, which prevent smoothened activation and downstream signaling. WNT ligands can be inhibited by ipafricept and WNT receptors can be inhibited by vantictumab and LGK974. Reparixin can block FAK signaling by inhibiting CXCR1 thereby blocking IL-8 binding which normally triggers FAK phosphorylation and downstream signaling. FAK can also be targeted directly by defactinib. Finally, napabucasin can preventSTAT3 transcriptional activation while amcasertib inhibits Nanog transcriptional activation. While these agents are designed to limit CSCs by inhibiting mechanisms of self-renewal, drug resistance, and metastasis, their efficacy in the randomized trials has yet to be demonstrated. Additional innovative strategies are sought to target CSC plasticity and heterogeneity, including immunotherapies that overcome CSC-mediated immune suppression and evasion with diverse CSC recognition and targeting, as well as big data and super computation-based bioinformatic intelligence.

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Table 1

Clinical drugs targeting CSC pathways.

Targeted CSC Therapy	Targeted CSC Pathway(s)	Mechanism of Action Notes
Brontictuzumab (OMP-52M51) http://www.oncomed.com/	Notch	An anti-Notch1 antibody
Demcizumab (OMP-21M18) http://www.oncomed.com/	Notch/DLL-4	A humanized IgG2 antibody that targets DLL-4
MEDI0639 https://www.medimmune.com/	Notch/DLL-4	An immunoglobulin G1 lambda (IgG1λ) monoclonal antibody that selectively binds to DLL-4
MK-0752 http://www.merck.com/	Notch/γ-secretase	A γ-secretase inhibitor
Rovalpituzumab tesirine (SC16LD6.5) https://www.abbvie.com/landing/stemcentrx.html	Notch/DLL-3	An anti-DLL3 antibody drug conjugate
Tarextumab (OMP-59R5) http://www.oncomed.com/	Notch	An anti-Notch-2/-3 antibody
Vismodegib https://www.gene.com/	Hedgehog/Smoothened	An antagonist of the Smoothened receptor, part of the Hh pathway; FDA-approved for treatment of basal cell carcinoma
Defactinib (VS-6063) http://www.verastem.com/	FAK	A focal adhesion kinase (FAK) inhibitor
Reparixin http://www.dompe.com/en/	CXCR1	An inhibitor of human CXCR1/R2 receptor activation
Ipafricept (OMP-54F28) http://www.oncomed.com/	Wnt	A fusion protein formed with a truncated Frizzled-8 receptor and the Fc fragment of human immunoglobulin IgG1 that acts as a decoy receptor for Wnt ligands
LGK974 [166]	Wnt/Porcupine	Porcupine inhibitor
Vantictumab (OMP-18R5) http://www.oncomed.com/	Wnt/Frizzled	A fully human IgG2 monoclonal antibody targeting the Frizzled receptors 1, 2, 5, 7 and 8
Napabucasin (BBI608) http://www.bostonbiomedical.com	STAT3/β-catenin/NANOG	A CSC inhibitor that targets STAT3
Amcasertib (BBI503) http://www.bostonbiomedical.com/	NANOG	A multi-kinase inhibitor of NANOG pathway

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Table 2

Early phase (1/1b/2), single-arm trials of CSC-targeted therapies.

Date initiated	Estimated Completion date	Treatment	Tumor Type	NCT#	Outcomes
03-01-10	10-2015	Vismodegib + nab-p + gem [167]	Pancreatic	NCT01088815	DCR: 42/49; grade ≥3 AE: neutropenia (37.5%), anemia (21.4%), neuropathy (16.1%), fatigue (9.4%)
04-01-10	10-2014	MK-0752 + gem [83]	Pancreatic	NCT01098344	PR: 11/18 AE: diarrhea, nausea, vomiting, fatigue, thrombocytopena, transaminitis
08-25-10	09-2016	Demcizumab + carbo/pem [84]	NSCLC	NCT01189968	Well tolerated from initial report; regimen in ongoing randomized phase 2 study (final report to be published) AE: fatigue, nausea, hypertension
02-09-11	08-2015	MK-0752 + ridaforolimus [82]	HNSCC	NCT01295632	CR: 1/15; PR: 1/15; SD: 1/15; AE: stomatitis, diarrhea, decreased appetite, hyperglycaemia, thrombocytopenia, asthenia and rash
12-01-11	01-2020	LGK974 + EGFR inhibitor + B-raf inhibitor PDR001	Solid tumors	NCT01351103	To be determined
04-05-12	03-2017	MEDI0639 [87]	Solid tumors	NCT01577745	PR: 1/19; SD: 7/19; AE: increased aspartate aminotransferase, increased brain natriuretic peptid, and fatigue
05-29-12	08-2016	Ipafricept [168]	Solid tumors	NCT01608867	
07-11-12	04-2016	Tarextumab + nab-p + gem [92]	Pancreatic	NCT01647828	No grade ≥ 3 AEs; SD: 6/25
01-17-13	12-2015	Napabucasin[169]	Solid tumors	NCT01775423	Well tolerated; 15 mg/kg dose selected for phase 2; AE: diarrhea, fatigue, anemia
01-24-13	12-2017	Amcasertib (BBI503) [126]	CRC	NCT01781455	DCR: 56% (in pts with high NANOG expression) AE: diarrhea, abdominal pain, fatigue, nausea
01-24-13	07-2016	Brontictuzumab (OMP-52M51) [89, 90]	Solid and hematologic tumors	NCT01778439	DCR (CR+PR+SD) in biomarker-positive for NANOG (BP+) pts:56%; DCR in biomarker-negative (BP-) pts:13% (P=.040); OS in BP+ pts: 38 weeks; OS in BP- pts: 16 weeks
05-16-13	04-2018	Tarextumab + etoposide/platinum [89, 90, 93]	SCLC	NCT01859741	Well tolerated; AE: diarrhea; final report is not yet published
07-11-13	01-2017	Rovalpituzumab tesirine (SC16LD6.5) [88]	SCLC	NCT01901653	Well tolerated; 15 mg/kg dose selected for phase 2; AE: diarrhea, fatigue, nausea, anemia;ORR: 84%
08-30-14	06-2018	Napabucasin + paclitaxel [170]	Pancreatic	NCT02231723	OR: 11/60; DCR: 46/65; AE: thrombocytopenia, pleural effusion, increased lipase

carbo=carboplatin; CRC=colorectal cancer; CR=complete response; OR=objective response; ORR= overall response rate; pts= patients; DCR=disease control rate; DLT=dose-limiting toxicity; AE= most common treatment-related adverse effects; MTD=maximum tolerated dose; GEJ=gastroesophageal junction; gem=gemcitabine; nab-p=nab-paclitaxel; pem=pemetrexed; NSCLC=non-small cell lung cancer; SCLC=small cell lung cancer, HNSCC=head and neck squamous cell carcinoma

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Table 3

Recently completed or ongoing randomized trials (phase 2/3) of CSC-targeted therapies.

Trial Name Treatment	Tumor Type	NCT#	Phase	Outcomes
ALPINE [171] Tarextumab + gem/nab-p vs gem/nab-p + placebo	Pancreatic	NCT01647828	2	Study completed; Addition of TRXT to Nab-P+Gem did not improve OS in 1st line mPC. A potential detrimental effect on PFS and ORR was seen in subjects with N3 < 25%ile (ASCO 2017 Abstract 279)
BRIGHTER [117] Napabucasin + paclitaxel vs paclitaxel + placebo	Gastric/GEJ	NCT02178956	3	Study is ongoing (Active, not recruiting)
CO.23 [172][116] Napabucasin + BSC vs placebo + BSC	CRC	NCT01830621	3	Study closed; No significant difference between treatment groups AE: diarrhea
COMMAND [98] Defactinib vs placebo	Pleural mesothelioma	NCT01870609	2	The study was terminated; interim analysis-DSMB stated good safety profile but lack of efficacy; results not yet published
DENALI Demcizumab + carbo/pem vs carbo/pem + placebo	NSCLC	NCT02259582	2	Study is ongoing, (Active, not recruiting)
fRida [101] Reparixin + paclitaxel vs paclitaxel + placebo	Breast	NCT02370238	2	Study is currently recruiting
PINNACLE Tarextumab + etop/platinum vs etop/platinum + placebo	SCLC	NCT01859741	2	Study is ongoing (Active, not recruiting)
YOSEMITE Demcizumab + gem/nab-p vs gem/nab-p + placebo	Pancreatic	NCT02289898	2	Study is ongoing (Active, not recruiting)

BSC=best supportive care; carbo=carboplatin; CRC=colorectal cancer; CR=complete response; DCR=disease control rate; AE= most severe adverse effect; etop=etoposide; GEJ=gastroesophageal junction; gem=gemcitabine; nab-p=nab-paclitaxel; NSCLC=non-small cell lung cancer; SCLC=small cell lung cancer