Targeting cancer stem cells in the clinic: Current status and perspectives.

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Abstract

Resistance to chemotherapy and cancer relapse are major clinical challenges attributed to a sub population of cancer stem cells (CSCs). The concept of CSCs has been the subject of intense research by the oncology community since evidence for their existence was first published over twenty years ago. Emerging data indicates that they are also able to evade novel therapies such as targeted agents, immunotherapies and anti-angiogenics. The inability to appropriately identify and isolate CSCs is a major hindrance to the field and novel technologies are now being utilized. Agents that target CSC-associated cell surface receptors and signaling pathways have generated promising pre-clinical results and are now entering clinical trial. Here we discuss and evaluate current therapeutic strategies to target CSCs.

Keywords: cancer stem cells, clinical trial, tumor initiating cells, cancer relapse, targeted therapy, personalized medicine

Contents

Abbreviations

Acute myeloid leukemia (AML)

Acute promyelocytic leukemia (APL)

ADAM (a disintegrin and metalloproteinase) enzymes

Aldehyde dehydrogenase (ALDH)

ATP – binding cassette (ABC)

Beckwith-Wiedemann syndrome (BWS)

Bone morphogenetic proteins (BMP)

Cancer stem cells (CSC)

Cancer-associated fibroblasts (CAF)

Chronic myelogenous leukemia (CML)

Cyclooxygenase -2 (COX-2)

Dendritic cells (DC)

DNA polymerase ŋ (Pol ŋ)

Doublecortin-like kinase 1 (Dclk1)

Epidermal growth factor receptor (EGFR)

Epithelial to mesenchymal transition (EMT)

FK506 binding like protein (FKBPL)

Focal adhesion kinase (FAK)

Glutathione (GSH)

Hedgehog (Hh)

Herpes Simplex Virus tyrosine kinase (Hsv-tk)

Inflammatory breast cancer (IBC)

Janus kinase/signal transducers and activators of transcription (JAK/STAT)

Mesenchymal stem cell (MSC)

mTOR (mammalian target of rapamycin)

Nanog (nanog homeobox),

Natural killer (NK)

Non-steroidal anti- inflammatory drug (NSAID)

Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)

Nucleotide excision repair (NER)

Octamer binding transcription factor 4 (Oct4)

P–glycoprotein (P-gp)

Reactive oxygen species (ROS)

Receptor tyrosine kinases (RTK)

Side population (SP)

Sex determining region Y – related high mobility group box 2 (Sox2)

Stem cell (SC)

Stromal cell – derived factor 1(SDF-1)

Temozolomide (TMZ),

Transforming growth factor-β (TGF-β)

Tumor associated macrophages (TAM)

Vasculogenic mimicry (VM)

γ – secretase inhibitor (GSI)

1. Timeline of the Cancer Stem Cell Theory

In the late eighties genetic changes in the cells of the colonic mucosa during colon cancer development, were noted to correlate with histopathological changes (Fearon & Vogelstein, 1990; Vogelstein et al., 1988). These observations supported the speculated theory at the time that malignant cells evolve through natural selection; a process highly reminiscent of Darwin's theory of evolution (Cahill, Kinzler, Vogelstein, & Lengauer, 1999; Cairns, 1975). The basic premise of this theory is that numerous random cellular mutations create a genetically variable heterogeneous cell population. Conventional cancer treatments then favor the growth of cells (clones) that possess alleles containing advantageous traits. These clones then proliferate more effectively, and over time, another mutation occurs to confer an even greater survival advantage. This process continues and researchers concluded that tumorigenesis and disease resistance are a consequence of a succession of clonal selection and expansion (Fig. 1A) (Greaves & Maley, 2012; Weinberg, 2013).

Later Dick and his colleagues, in landmark studies, used fluorescent activated cell sorting (FACS) analysis to separate subpopulations of acute myeloid leukemia (AML) cells (Bonnet & Dick, 1997). They demonstrated that $CD34^{+}$ $CD38^{-}$ cells represented less than 1% of the tumor mass but as few as 5000 cells in the subpopulation could form tumors in host mice, whereas 500,000 cells from the complete population where unable to form a tumor (Bonnet & Dick, 1997). This was the first solid evidence to cast serious doubt on the clonal expansion theory, which considered all malignant cells to have similar tumor initiating capacity. Dick's data suggested that a subpopulation of cells, known as tumor initiating cells or cancer stem cells (CSCs) were responsible for this observation. Whilst transplantation assays are successfully used for assessing the tumor initiating potential of a cell phenotype, they do not provide information on the fate of implanted cells. A commonly held theory is that CSCs are a small dormant subpopulation with the ability to persist for extended periods of time (Patel & Chen, 2012). Indeed, recent *in vivo* lineage tracing experiments, which permanently mark individual cells with a reporter gene, confirmed that the majority of tumor cells undergo terminal differentiation and only a small number of cells survive long term. Furthermore, there is rapidly

mounting evidence that asymmetric division of CSCs gives rise to committed progenitors which eventually undergo terminal differentiation (Blaas et al., 2016; Blanpain & Simons, 2013; Driessens, Beck, Caauwe, Simons, & Blanpain, 2012; Nassar & Blanpain, 2016; Schepers et al., 2012). Thus confirming the existence of a small number of viable tumor cells that linger during disease remission and which retain the ability to differentiate.

The current consensus now suggests that tumors display a similar hierarchical organization to normal tissue, with a small numbers of self-renewing stem cells at the top of the cellular pyramid (Fig. 1B) (Weinberg, 2013). The stem cell model explains the complex process of tumorigenesis and the CSC subpopulation plays a critical role in tumor initiation, propagation, relapse and distant metastasis (Banerjee & Kaye, 2013; Clevers, 2011; Lapidot et al., 1994; Reya, Morrison, Clarke, & Weissman, 2001). However, this is likely to be an over simplification of the process and integration of the clonal expansion theory and the CSC theory is probably more representative of the complex heterogeneity observed within tumors. A mixed clonal expansion/stem cell model is proposed, in which CSCs acquire genetic changes that are favorable for survival, resulting in clonal expansion of the original tumor initiating cell (Fig. 1C) (Kreso & Dick, 2014).

DNA barcoding is a powerful technique that has been utilized to track CSCs by exposing them to a library of viral vectors and integrating a unique identifying vector DNA sequence ("barcode") at a single copy level in individual cells (Bystrykh, Verovskaya, Zwart, Broekhuis, & de Haan, 2012). This technology has allowed the tracking, growth and differentiation of multiple sub clones within tumor xenografts and has recently confirmed that distant clones predominate at different stages of tumor progression (L. V. Nguyen, Makarem, et al., 2014; L. V. Nguyen, Cox, et al., 2014). These findings provide evidence for clonal evolution of the tumor initiating subpopulation. Interestingly, DNA bar coding has also challenged the premise that carcinogenesis and tumor heterogeneity is a slow event resulting from acquisition of multiple driver mutations. In fact heterogeneity occurred very rapidly after the initial transformation event, thus refuting the clonal evaluation theory of cancer (L. V. Nguyen et al., 2015).

2. Characteristics of Cancer Stem Cells

Similar to normal stem cells, CSCs are undifferentiated and harbor the capacity for unlimited self–renewal (Weinberg, 2013). CSCs are often described as rare, but it is likely their abundance varies within individual patients, tumor type, grade and treatment status (Tang, 2012). They have a quiescent, slowly cycling phenotype which may partly explain their role in therapy resistance and reoccurrence (L. Li & Clevers, 2010; Moore, Lyle, Moore, & Lyle, 2011). The CSC population is not a static entity and has the ability to interconvert between differentiated and stem-like states resulting in phenotypic plasticity (Cabrera, Hollingsworth, & Hurt, 2015). Currently, it is not clear how CSCs originate, although current thinking suggests that they are derived from either oncogenic mutations in normal stem cells or dedifferentiation of cells to a CSC phenotype (Reya et al., 2001; A Singh & Settleman, 2010; Visvader, 2011).

3. Isolation and Identification of Cancer Stem Cells

In order to develop therapeutically efficacious strategies to eradicate a tumor, it is essential that robust methodologies exist to identify CSCs from the bulk population of tumor cells. A number of different methods have been developed based on the biological characteristics of CSCs or on cell surface phenotype.

3.1 Cell surface phenotype

The majority of CSCs are validated using FACS sorting, through identification of cell surface markers, followed by validation using the *in vivo* limiting dilution transplantation assay, first pioneered by Dick *et al* (Bonnet & Dick, 1997). Al-Hajj *et al* provided the first evidence of CSCs within a solid breast tumor using the $CD44^{+}CD24^{-}$ phenotype (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003). This experimental design has been replicated in a wide variety of tumor types, and a large number of subpopulations with enhanced tumor initiating capacity have been

identified and validated (Table 1). Critics note that the cells experience stress during the cell sorting and transplantation process and the recipient murine microenvironment will differ drastically from the original niche. In addition, the timing, implantation site, developmental stage, host strain and sex of the mouse will influence the murine microenvironment and subsequent tumor initiation ability (LaBarge, 2010; Plaks, Kong, & Werb, 2015).

3.2 *Functional assays*

Assays based on the functional biological properties of CSCs provide highly valuable strategies for isolating CSCs, particularly in the absence of robust cell surface markers. Nevertheless, it is important to keep in mind that the selected cells in each assay may not be exclusively CSCs and the cells may only represent one of the putative CSC populations.

Sphere forming assays were first used over 20 years ago in the neural stem cell field and are a widely accepted method to determine the self-renewal potential of CSCs (C. Lee, Yu, Wang, & Chang, 2015; Reynolds & Weiss, 1992). The assay involves plating single cells in suspension on an ultra-low attachment surface in serum free medium with the addition of growth factors (C. Lee et al., 2015). The undifferentiated cells survive to generate spheroids whilst all other cell types undergo anoikis (F. L. Shaw et al., 2012); a form of programmed cell death that occurs in anchorage-dependent cells. Mammospheres derived from breast cancer cells have an enriched $CD44^+$ $CD24^-$ phenotype and display greater tumorigenicity *in vivo* (Ponti et al., 2005). Likewise, tumorspheres derived from primary ovarian tumors and ascites fluid have an upregulation of stem cell-associated genes and high aldehyde dehydrogenase (ALDH) activity resulting in tumor generation, metastasis and chemotherapy resistance *in vivo* (Liao et al., 2014). The sphere forming assay has therefore emerged as a reliable screening method for the development of anti-CSC agents and has been utilized as an outcome measure in clinical trials (C. Lee et al., 2015). However, an important limitation of the sphere forming assay is its inability to detect quiescent CSCs, which reside in the G0 phase (Pastrana, Silva-Vargas, & Doetsch, 2011).

One way to overcome this drawback is to exploit a property known as label retention. This can be used to conduct live cell monitoring and to isolate CSCs for downstream functional assays (Kusumbe & Bapat, 2009; Szotek et al., 2008). Application of a dye such as, PKH26/PKH67 or BrdU marks cells that divide the least, while the dye gets diluted in frequently dividing cells (Guddati, 2012). The downfall of this method is that terminally differentiated cells can also be labelled with equal

efficiency (Guddati, 2012).

Another property exploited to identify stem cells is their ability to actively expulse substances (e.g. Hoechst 33342) from their cytoplasm via cell membrane transporter proteins, such as ATP – binding cassette (ABC) transporter proteins; ABCB1 (MDR1/P-glycoprotein), ABCC1 and ABCG2 (Donnenberg, Meyer, & Donnenberg, 2009). Overexpression of ABCB1/MDR-1 and ABCG2/BCRP has been shown in tissues of various malignancies and is associated with poor prognosis (Lingeng Lu et al., 2007; Martin et al., 2009) The degree of substance efflux correlates with the maturation state of the cell with the most primitive cells displaying the highest efflux capacity and cells displaying these characteristics have been termed the side population (SP) (Richard, Nair, Santhosh Kumar, & Pillai, 2013). In ovarian cancer, SP cells have the capacity for self-renewal and the production of non-SP offspring, significant chemo-resistance and enhanced tumorigenic properties (L. Hu, McArthur, & Jaffe, 2010; Lingeng Lu et al., 2007; Szotek et al., 2006). This constitutes a highly valuable technique, though a technically difficult strategy, for identification of CSC especially in the absence of tissue specific cell surface markers.

There are a number of pluripotency-associated transcription factors such as, Nanog (nanog homeobox), Oct4 (octamer binding transcription factor 4) and Sox2 (sex determining region Y – related high mobility group box 2) which are activated in both embryonic and CSCs. (A. Liu, Yu, & Liu, 2013). Emerging novel technologies are being developed to enable live cell mRNA quantification with the aim of identifying and FACs sorting viable CSCs based on expression levels of these transcription factors. Cells expressing Nanog have been successfully isolated from human tissues using the NanoFlare (SmartFlare) system (McClellan et al., 2015). The Smartflare RNA detection probe contains a gold nanoparticle conjugated with an oligonucleotide and a fluorophore and in the presence of the target RNA binding, the capture strand will leave the proximity of the gold and fluoresce (McClellan et al., 2015). A similar strategy, utilizing a GFP reporter driven by a Nanog promoter, has also been developed to enrich and track ovarian CSCs after treatment with cisplatin (Wiechert et al., 2016).

Finally, *in vitro* CSC differentiation can be monitored using a simple clonogenic-based assay. When cells are plated at low density and allowed to form colonies, a close correlation exists between the cell clone morphology and the stem cell hierarchy of the cancer cells. The spectrum of morphologies ranges from compact round colonies to loose packed cells; termed holoclones, meroclones and paraclones (Locke, Heywood, Fawell, & Mackenzie, 2005). Holoclones are considered to be enriched in CSCs in a multitude of tumor types and express high levels of stemness-related markers (Harper, Piper, Common, Fortune, & Mackenzie, 2007; H. Li, Chen, Calhoun-Davis, Claypool, & Tang, 2008; Z. Zhou et al., 2009).

The most common way to determine the frequency of self-renewing cells within a tumor is the gold standard limiting dilution cell transplantation assay, in which tumor cells are transplanted into recipient animals at increasing numbers; the proportion of animals that develop tumors is used to calculate the number of self-renewing cells within the original tumor sample (Bonnefoix, Bonnefoix, Verdiel, & Sotto, 1996; Dick, Bhatia, Gan, Kapp, & Wang, 1997). This model is commonly used in the pre-clinical evaluation of anti-CSC agents, however tumor engraftment may take months and is therefore a costly and labor intensive approach.

4. Signaling Pathways regulating stemness

A number of developmental pathways first identified in the regulation of normal stem cells, are also critical for the maintenance and self-renewal of CSCs. Notch signaling was the first pathway to be described and is a highly conserved developmental pathway which regulates cell fate during embryogenesis (Artavanis-Tsakonas, Rand, & Lake, 1999). It is an important short range communication system in cells and its activation in CSCs maintains the undifferentiated state (Borah, Raveendran, Rochani, Maekawa, & Kumar, 2015). Notch signaling between two neighboring cells is dependent upon a two-step proteolytic cleavage mediated by the ADAM (a disintergrin and metalloproteinase) enzymes and γ – secretase (Gu et al., 2012). In gliomas, Notch activation is associated with radio-resistance in CSCs and knockdown of Notch1 and 2, resulted in AKT inhibition

and enhanced radio-sensitivity in the CSC subpopulation, but not in the bulk of the glioma cells (J. Wang et al., 2010). Overexpression of Notch3 has been frequently found in primary ovarian cancer samples and correlates with poor survival and platinum resistance (McAuliffe et al., 2012; Joon T Park et al., 2006; Joon Tae Park et al., 2010). In addition, Notch1 and 2 down regulation is accompanied by an up regulation of Notch4 in tamoxifen resistant breast cancer cells (Lombardo et al., 2014). Furthermore, treatment with endocrine therapies decreased proliferation of the bulk breast cancer cells yet increased stemness through JAG1-NOTCH4 activation (Simões et al., 2015).

Wnt signaling is also associated with determining cell fate during development. Canonical Wnt signaling is mediated through translocation of β-catenin to the nucleus, which then directly interacts with Tcf3 to repress pluripotency transcription factors (Holland, Klaus, Garratt, & Birchmeier, 2013; Yi et al., 2011). The contribution of Wnt signaling in malignancy is elegantly demonstrated in colon cancer where aberrant Wnt/β-catenin signaling in the colonic epithelia drives tumorigenesis (Sparks, Morin, Vogelstein, & Kinzler, 1998). Moreover, colon CSCs contain high levels of β-catenin leading to drug resistance and metastasis (Vermeulen et al., 2010a). Linage tracing experiments have indicated Lgr5, a Wnt target gene, as a marker for stem cells in intestinal crypts (Barker et al., 2007). Lgr5 forms a complex with R-spondin and neutralizes two transmembrane E3 ligases, Rnf43 and Znrf3, resulting in the removal of Wnt from the cell surface thus initiating a negative feedback loop (de Lau, Peng, Gros, & Clevers, 2014). Indeed, lgr5 is expressed by colon CSCs and tumor regression occurs after genetic ablation of the $Lgr5^+$ population, followed by a tumor regrowth driven by differentiated cancer cells reverting to $Lgr5^+$ CSCs (Shimokawa et al., 2017). In gastric cancer cells, *Helicobacter pylori* activates Wnt/ β – catenin signaling which, in turn, up regulates Nanog and Oct4 to promote CSC-like properties (Yong et al., 2016).

The Hedgehog (Hh) signaling pathway orchestrates communication between cells during organogenesis and directs cell proliferation, cell fate, epithelial to mesenchymal transition (EMT) and motility (Cochrane, Szczepny, Watkins, & Cain, 2015). Indeed, Hh signaling is a direct transcriptional target of the pluripotency factor, Nanog, and it is believed to maintain the stemness

state in multiple cancers types (Cochrane et al., 2015; Po et al., 2010). Hh signaling components, such as Ptch1, Gli1 and Gli2, are highly expressed in normal mammary stem cells and down regulated upon differentiation (S. Liu et al., 2006).

The serine/threonine kinase, mTOR (mammalian target of rapamycin), is at the central crossroads of many intracellular signaling pathways and it exhibits control over an abundance of cellular processes including cell cycle progression, proliferation, autophagy and angiogenesis (Ciuffreda, Di Sanza, Incani, & Milella, 2010).The mTOR pathway and its upstream regulators, the PI3K/PTEN/AKT cascade, are altered in the majority of human cancers (Xia & Xu, 2015). PTEN loss is associated with prostate cancer development and indeed prostate specific deletion of PTEN in mice leads to development of cancer within nine weeks (Shunyou Wang et al., 2003). PTEN negatively regulates the basal cell population and loss of PTEN leads to basal cell proliferation with a subsequent expansion of the prostate CSC subpopulation (S. Wang et al., 2006). In glioma cells AKT, but not its downstream target mTOR, induces ABCG2 activation and loss of PTEN increases the side population (Bleau et al., 2009).

The transforming growth factor-β (TGF-β) superfamily of proteins are potent, context dependent tumor suppressor or tumor promoters. The family comprises more than 30 members including TGF-β isoforms, actins, NODAL, bone morphogenetic proteins (BMP) and growth and differentiation factors (Wakefield & Hill, 2013). TGF-β, as a pleiotropic cytokine, promotes the differentiation of stem cells into neurocytes, smooth muscle cells, dendritic cells, but on the other hand, it inhibits the differentiation of stem cells into myotubes, adipocytes and endothelial cells (M.-K. Wang et al., 2012). TGF-β-related pathways with regards to CSCs remain largely undefined; however TGF-β deficient transgenic mice may provide some insights. TGF-β deficient mice closely resemble Beckwith-Wiedemann syndrome (BWS), including the hallmark characteristic of adrenal cytomegaly (Rao et al., 2017). BWS is a human stem cell overgrowth disorder and is associated with an 800-fold increased risk of childhood neoplasms (Weksberg, Shuman, & Beckwith, 2010). Interestingly, there are cases of multiple tumor types developing within the same organ simultaneously, such as

mesenchymal hamartoma, capillary hemangioma hepatoblastoma and cholangiocarcinoma occurring in the organ of one patient (Hadzic & Finegold, 2011; Keller, El Demellawy, Quaglia, Finegold, & Kapur, 2015). This suggests that dysfunctional processes are occurring as stem cells differentiate into mature adult cell types in the absence of TGF-β. Indeed, epigenetic silencing of β2-spectrin (encoded by STBN1), a SMAD adapter for TGF- β signaling is associated with BWS (Yao et al., 2010). Chen *at al*, showed double heterozygous *Sptbn1*$^{+/-}$ *Smad3*$^{+/-}$ mice have defective TFG-β signaling and develop tumors phenotypically similar to BWS. Whole transcriptome RNA sequencing of mouse embryonic fibroblasts from the mice revealed an increase in stemness genes (including CD133, NANOG, OCT4, SOX2), and knockout of any element of the TFG-β mediated β2SP/SMAD3/CTCF complex resulted in an increase in the ALDH^{+} cell populations (J. Chen et al., 2016).

Bone morphogentic proteins (BMPs) are a family of evolutionarily conserved secreted growth factors that are processed by Noggin and Chordin before binding to receptors on the plasma membrane of cells (Ashok Singh & Morris, 2010). BMPs have both pro- and anti-tumor activity and control a range of oncogenic cell processes including proliferation, angiogenesis and differentiation (Marshall, Reynolds, & Laywell, 2007). In glioblastoma, a BMP target gene Distal-less homeobox 2 (DLX2) promotes apoptosis and differentiation of CSCs and this could be targeted using the anti-epileptic compound valproate (Raja et al., 2017). In the intestinal epithelium, BMP signaling maintains Lgr5 stem cells homeostasis and prevents premalignant hyper proliferation via SMAD signaling (Qi et al., 2017). On the other hand, BMP-2 promoted cell migration and induced EMT in breast cancer cells (H. Jin et al., 2012). Furthermore, BMP-2 induced ubiquitin mediated degradation of the cancer suppressor retinoblastoma (Rb) via PI3K/AKT signaling and crosstalk between CD44 and Rb upregulated CD44 expression (N. Ning et al., 2012).

The epidermal growth factor receptor (EGFR) is a transmembrane protein that belongs to the ErbB family of receptor tyrosine kinases (RTK) (Normanno et al., 2006). The EGFR is overexpressed in inflammatory breast cancer (IBC); an aggressive subtype with high metastatic potential (Masuda et al., 2012). Recently, it has been reported that EGFR regulates IBC stem cells though cyclooxygenase

- 2 (COX -2), a key inflammatory mediator which in turn activates Nodal signaling (X. Wang et al., 2017). Indeed lapatinib, a dual tyrosine kinase inhibitor of the EGFR receptor and the HER2 receptor, has been shown to prolong progression free survival in IBC patients (Kaufman et al., 2009). On the other hand, mutations in EGFR also occur in lung cancer, however, targeting these mutations with inhibitors in clinical trials has resulted in adverse outcomes (Kelly et al., 2008). This is supported by *in vitro* data that confirms that treatment of EGFR-mutated lung cancer cell lines with erlotinib, a EGFR kinase inhibitor, enriches for CSCs though EGFR- dependent activation of Notch3 (Arasada, Amann, Rahman, Huppert, & Carbone, 2014).

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is the principle signaling mechanism for a wide variety of cytokines and growth factors (Rawlings, Rosler, & Harrison, 2004). This pathway has been associated with the regulation of normal stem cell fate and studies have indicated its upregulation in CSCs from a range of cancers (Birnie et al., 2008; Stine & Matunis, 2013; J. Zhou et al., 2007). Chemotherapy and radiation resistance have been linked to STAT3 activation (Groner & Von Manstein, 2017). Furthermore, IL-6 secretion from mesenchymal stem cells resulted in resistance to chemotherapy in osteosarcoma cells (Di et al., 2014). IL-6 regulates Oct4 gene expression through a IL-6-JAK1-STAT3 signal transduction pathway and has a role in the conversion of non-stem cells into cancer stem cell like cells (Kim et al., 2013).

The nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) family of transcription factors consists of five members, p65, RelB, c-Rel, p105/p50, and p100/p52 and it operates through two major pathways; canonical and non-canonical (Hayden & Ghosh, 2008). The canonical pathway occurs downstream of inflammatory stimuli, such as lipopolysaccharide (LPS) and results in a loss of IκBα which enhances NF-κB nuclear accumulation and promotes transcription of target genes (Ghosh & Hayden, 2012). The non-canonical pathway is activated in development and leads to stabilization of NF-κB inducing kinase (NIK) which results in an RelB-52 dimer that locates to the nucleus to regulate transcription (Ghosh & Hayden, 2012). The NFκB pathway has been studied extensively in cancer biology and its activation is associated with a number of the hallmarks of cancer, such as

proliferation, anti-apoptosis and angiogenesis (Baldwin, 2012; Bassères & Baldwin, 2006; C. Y. Wang, Mayo, Korneluk, Goeddel, & Baldwin, 1998). In ovarian cancer, TLR 2 - NFκB signaling regulates the stem cell related gene, CD44 and Nanog expression (Chefetz et al., 2013). In addition, depending upon the tumor site, CSCs are reported to have higher levels of p65, acetylated p65 or increased nuclear localization of p65 (Garner et al., 2013; Rajasekhar, Studer, Gerald, Socci, & Scher, 2011). Given the extensive interest in NFκB signaling in the cancer field, targeting this pathway induces apoptosis and reduces tumor growth, however, many studies do not investigate the effect specifically on the CSC subpopulation (Xue et al., 2011). A compound which blocks IκBα degradation selectively induces apoptosis in acute myelogenous leukemia stem cells as opposed to normal hematopoietic stem cells (Guzman et al., 2005).

Interestingly, in *in vivo* models of HER-2 breast cancer, both canonical and non- canonical NFκB pathways contribute to stemness (Pratt et al., 2009). HER-2 targeted therapies have been a milestone in cancer research and there is evidence that trastuzumab, a HER-2 monoclonal antibody, targets HER-2 positive CSCs as well as the HER-2 positive bulk population (Magnifico et al., 2009). Targeting of CSCs may explain the clinical efficacy of trastuzumab in HER-2 negative cancers (Ithimakin et al., 2013). There is currently a large Phase III clinical trial evaluating whether women with low HER2 expression can benefit from the combination adjuvant treatment with chemotherapy plus trastuzumab (NCT01275677) and the results from this study will inform us of the clinical utility of targeting $HER2^+$ CSCs independent of tumor subtype.

Focal adhesion kinase (FAK), encoded by PTK2, is a cytoplasmic protein tyrosine kinase that is overexpressed and activated in a range of primary and advanced cancers and it correlates with poor survival (Sulzmaier, Jean, & Schlaepfer, 2014). FAK controls a number of functions in tumor cells including cell movement, invasion, survival, gene expression, and CSC self-renewal (Sulzmaier et al., 2014). NFκB and p53 activate and repress the PTK2 promoter, respectively (Cance & Golubovskaya, 2008; Corsi, Rouer, Girault, & Enslen, 2006). In addition the FAK promoter contains four Nanog binding sites and Nanog binds to the FAK promoter and upregulates its activity (Ho et al., 2012).FAK

directly phosphorylates Nanog and siRNA knockdown of Nanog reversed FAK overexpression (Ho et al., 2012). In the MMTV-PYMT mouse model of breast cancer metastasis, conditional embryonic FAK deletion suppressed CSC generation (M. Luo et al., 2009). In addition, expression of the CSC marker CD133, activated SRC-FAK signaling pathway and promotes cell migration (C. Liu et al., 2016).

5. THE TUMOR MICROENVIRONMENT AND CANCER STEM CELL NICHE

Cancer cells recruit a complex assemblage of cells such as immune cells, vascular and lymphatic cells, cancer-associated fibroblasts (CAFs), pericytes and adipocytes to create the tumor microenvironment. Communication between the multiple cell types is mediated through a dynamic network of cytokines, chemokines, growth factors and matrix remodeling enzymes (Balkwill, Capasso, & Hagemann, 2012; Hanahan & Weinberg, 2011).

CSCs are believed to reside in anatomically distinct regions of the tumor microenvironment; termed the 'niche' (Fuchs & Horsley, 2011; Medema & Vermeulen, 2011). There is evidence that circulating CSCs occupy native stem cell niches, such as the hematopoietic stem cell niche in the bone marrow or around blood vessels in the perivascular niche, where they benefit from signals that enhance stemness and deter differentiation (Oskarsson, Batlle, & Massagué, 2014; Shiozawa et al., 2011). A characteristic of the tumor microenvironment is chronic inflammation, however, the CSC niche is believed to suppress natural killer (NK) cells and $CD8^+$ T cells in order to evade immune surveillance (Ghebeh, 2013). In glioma, CSCs secrete a host of cytokines to propagate $FOXP3^+$ regulatory T cells and modulate the immune response to Th2, a humoral response antagonistic to cytotoxic Th1 response (Wei et al., 2010). Tumor associated macrophages (TAM) also suppress the adaptive immune response by releasing anti-inflammatory factors, such as IL-10, to attenuate the Th1 response and hinder the cytotoxicity of T lymphocytes and NK cells (Raggi, Mousa, Correnti, Sica, & Invernizzi, 2016). The presence of M2-like TAMs is correlated with poor prognosis and an increased number of CSCs, attributed to their ability to induce EMT and chemoresistance via tumor necrosis

TGF-β (Q.-M. Fan et al., 2014; Y. Hu et al., 2016). In glioblastoma multiformes, CSCs secrete periostin in order to recruit TAMs through integrin $\alpha\beta_3$ and blocking this signaling with a RGD peptide prevented their recruitment (W. Zhou et al., 2015).

Mesenchymal stem cells (MSCs) in the bone marrow support and maintain gastric CSCs via up regulation of WNT and TGF-β signaling (Nishimura, Semba, Aoyagi, Sasaki, & Yokozaki, 2012). Furthermore, they are recruited from the bone marrow and engraft at the tumor site where they trans-differentiate into fibroblasts, perivascular cells and macrophages. In breast cancer, MSCs promote CSC self-renewal through cytokine loops involving IL-6 (S. Liu et al., 2011). IL-1 secretion from tumor cells induces prostaglandin E2 secretion by MSCs which in turn acts in a paracrine fashion to induce formation of CSCs though β-catenin signaling (H.-J. Li, Reinhardt, Herschman, & Weinberg, 2012).

The MYC oncogene is a transcription factor that is over expressed in many cancers (Tansey, 2014). Recently, it has been shown that MYC regulates the expression of two immune checkpoint proteins; the innate checkpoint protein CD47 and the adaptive checkpoint inhibitor PD-L1 (Casey et al., 2016). Upregulation of CD47 is a method by which lung CSCs evade phagocytosis and treatment with anti-CD47 antibodies enabled macrophage phagocytosis leading to improved survival in xenograft models (L. Liu et al., 2017). Targeting the PD-1/PD-L1 axis has shown great promise in a variety of malignancies. In advanced Merkel-cell carcinoma, a malignancy with an average survival of 3 months, treatment with pembrolozumab (an PD-1 antibody) resulted in 67% of patients having progression free survival (PFS) at six months (Nghiem et al., 2016). Indeed, pembrolozumab has been approved for adults and children with advanced solid tumors with either high levels of microsatellite instability or mismatch repair deficiency – a first for the FDA to give approval without stating tumor type (FDA, 2017). There is some evidence that expression of PD-L1 is associated with $CD44^+$ cells in primary human head and neck squamous cell carcinoma (Y. Lee & Sunwoo, 2014). These results suggest that preferential PD-L1 expression on CSCs may be a mechanism for evading the immune response and it provides a rationale for targeting the PD-1 axis to allow co-targeting of the CSC

subpopulation. However, in advanced melanoma patients, approximately one quarter who responded to PD-1 blockage had disease progression at a median of 21 months (Ribas et al., 2016). Recent studies indicate that the JAK, MAPK and interferon signaling pathways are contributing to cells evading anti PD-1 therapy and this approach may need to be combined with other therapies in relapsing patients (Ayers et al., 2017; Hugo et al., 2016; Zaretsky et al., 2016).

Factors produced by endothelial cells and CSCs have the ability to transform normal fibroblasts into cancer associated fibroblasts (CAFs) (Kalluri & Zeisberg, 2006). CAFs generally reside at the tumor margins, but some infiltrate the tumor, and secrete a variety of growth factors to enhance stemness and angiogenesis (Balkwill et al., 2012; Madar, Goldstein, & Rotter, 2013; Plaks et al., 2015). In colon cancer, CAFs secrete hepatocyte growth factor (HGF) to regulate Wnt activity and they are a major source of IL-6 in the microenvironment (Huynh et al., 2016; Vermeulen et al., 2010b). In the breast cancer setting, CAFs and adipocytes secrete leptin and stromal cell – derived factor 1 (SDF-1) to enhance stemness and self-renewal associated pathways (Giordano et al., 2016). In breast cancer, knockout of TIMP genes from fibroblasts induces CAFs that secrete exosomes enriched in ADAM10, leading to increased expression of ALDH though Notch receptor activation and cell motility though GTPas RhoA (Shimoda et al., 2014).

6. ANGIOGENESIS AND CANCER STEM CELLS

Pre-clinical animal studies of anti-angiogenic agents often demonstrated substantial tumor growth delay and regression, however, this has not necessarily translated to improvement in patient outcomes (Jayson, Kerbel, Ellis, & Harris, 2016). There is emerging evidence in glioblastoma, a highly angiogenic malignancy, that the $CD133^+$ stem cell like sub-fraction has the capability to differentiate along the endothelial lineage, in addition, to traditional tumor lineages (R. Wang et al., 2010). Endothelial cells secrete IL-8 leading to the up regulation of the IL-8 receptors, CXCR1/CXCR2, on CSCs (Infanger et al., 2013). Interestingly, treatment with a vascular endothelial growth factor (VEGF) inhibitor abrogated the maturation of tumor endothelial progenitors into endothelium, but not

the differentiation of the CSC fraction into endothelial progenitor cells (R. Wang et al., 2010). Furthermore, in glioblastoma, CSCs are recruited towards endothelial cells through the SDF1/CXCR4 axis and TGF-β leading to differentiation of CSCs into pericytes and subsequent remodeling of the perivascular niche (Cheng et al., 2013; Sharma & Shiras, 2015). In a mouse glioma model, osteopontin expression in the perivascular niche promoted the CSC phenotype via the γ-secretase-regulated intracellular domain of CD44 (Pietras et al., 2014). In bevacizumab – resistant glioblastoma, hypoxia induced activation of STAT3 leading to expression of the cell adhesion molecule 1 (ICAM-1). ICAM knockdown in glioma stem cells resulted in decreased tumor size and inhibited macrophage invasion into the tumor site *in vivo* (Piao et al., 2017).

Nitric oxide released from endothelial cells promotes stemness via activation of the Notch pathway and, in turn, CSCs secrete VEGF thus suggesting a complementary relationship between stemness and angiogenesis (S. Bao et al., 2006; Charles et al., 2010; Eyler et al., 2011; Lv, Wang, Song, Pang, & Li, 2016). The capacity of cancer cells to promote microcirculation via the formation of a *de novo* vessel like network, without endothelial cells, is termed vasculogenic mimicry (VM) (Y.-L. Fan, Zheng, Tang, & Liang, 2013; Seftor et al., 2012). VM tumor cells show significant expression of both endothelial and tumor phenotypes and thus may represent incomplete differentiation of CSC into endothelial lineages (Ricci-Vitiani et al., 2010). This is considered to be an important resistance mechanism for anti-VEGF therapies. Worryingly, although treatment with bevacizumab (anti – VEGF therapy) inhibits primary tumor growth, studies suggest it accelerates metastasis by promoting VM formation and upregulation of CSCs through HIF-1α (Conley et al., 2012; Soda, Myskiw, Rommel, & Verma, 2013; Xu et al., 2012)

7. CANCER STEM CELLS AND RESISTANCE TO THERAPY

It is well established that CSCs are resistant to ionizing radiation and conventional chemotherapy treatments and there is emerging evidence that CSCs can evade novel immunogenic therapies (Chang

et al., 2016; Radvanyi, 2013; Yoshida & Saya, 2016; J. Zhao, 2016). Furthermore, they are thought to be a central cause of tumor heterogeneity, a key feature of therapy resistance (Yoshida & Saya, 2016). Tumor plasticity is a huge challenge when developing targeted anti-CSC agents, especially when specifically targeting cell surface phenotype markers.

7.1 Slow cell cycle

The majority of chemotherapy agents only exhibit their actions under proliferative conditions. CSCs reside in the G0 phase of the cell cycle and are thus inherently resistant to drugs dependent on the cell cycle (Yoshida & Saya, 2016). Repeated radiotherapy of glioma CSCs upregulates the IGF (insulin like growth factor) type 1 receptor and subsequently increases IGF1 secretion. When cells are in a resting state, this chronic activation of IGF1 inhibits PI3-AKT signaling and activates Fox03a resulting in a slow cycle and enhanced self-renewal (Osuka et al., 2013). Using a genetically engineered model of glioma, Parada *et al* elegantly demonstrated the importance of quiescent CSCs to tumor relapse post chemotherapy. They introduced a transgene expressing GFP and the Herpes Simplex Virus tyrosine kinase (Hsv-tk) suicide gene under a Nestin promoter that labelled both quiescent normal neural stem cells that reside in the sub-ventricular zone and a subset of glioma tumor cells with CSC properties. After administration with temozolomide (TMZ), tumor regrowth originated from the quiescent $GFP^{+}Nestin^{+}$ transgene subpopulation, which re-entered the cell cycle. Lineage ablation of the $GFP^{+}Nestin^{+}$ CSCs with chronic ganciclovir administration significantly arrested tumor growth and combined treatment with TMZ prevented tumor development (J. Chen et al., 2012).

7.2 Efflux of cytotoxic agents

ALDH is a common CSC marker and a member of the $NAD(P)^{+}$ dependent super family of enzymes. Their role is to catalyze the oxidation of aldehydes to carboxylic acids, which arises as a result of chemotherapy, radiation or oxidative stress (Marchitti, Brocker, Stagos, & Vasiliou, 2008; Vasiliou,

Thompson, Smith, Fujita, & Chen, 2013). ALDH expression can be downregulated by retinoic acid, a well-established treatment in acute promyelocytic leukemia (APL), and has shown promising chemo- and radio- sensitizing effects in an range of pre-clinical solid cancer models (Stacy, Jansson, & Richardson, 2013).

The ABC transporter family includes 49 proteins, including P–glycoprotein, and results in ATP dependent efflux of cytotoxic drugs from cells, thus providing a resistance mechanism to both conventional chemotherapy agents and more novel molecularly targeted therapies (Cojoc, Mäbert, Muders, & Dubrovska, 2015; Vasiliou, Vasiliou, & Nebert, 2009). Inhibitors of ABC transporters have the potential to chemo-sensitize CSCs, however, the ABC transport family play a role in normal physiological functions such as the blood brain barrier, GI tract and the blood testis barrier (Stacy et al., 2013). The first developed ABCG2 inhibitor, fumitremorgin C, had severe neurotoxic side effects and altered the pharmacokinetics of co-administered cytotoxics resulting in severe side effects which limited its use clinically (Stacy et al., 2013).

7.3 RESISTANCE TO REDOX STRESS

Reactive oxygen species (ROS) is a collective term for highly chemically reactive molecules derived from molecular oxygen that result in peroxidation of nucleic acids, lipids, amino acids and carbohydrates (Kobayashi & Suda, 2012). ROS are produced as a bi-product of cellular reactions, but are also triggered by chemotherapy and radiation. In general, ROS are elevated in malignant cells due to increased metabolism, however, the slow cycling of CSCs is thought to contribute to lower ROS levels; thus rendering CSCs intrinsically resistant to oxidative stress-based therapies (Ding et al., 2015). Variant isoforms of the major CSC marker, CD44, contribute to low ROS levels through up regulation of reduced glutathione (GSH), a primary intracellular antioxidant (Nagano, Okazaki, & Saya, 2013).

7.4 INCREASED DNA REPAIR RESPONSE

Chemotherapy agents, such as platinum based drugs and radiotherapy, exert their effect through the DNA damage pathway and CSCs display enhanced DNA repair capacity (Maugeri-Saccà, Bartucci, & De Maria, 2012). Furthermore, stem cell niches are located in hypoxic regions thus minimizing oxidative DNA damage (Cabarcas, Mathews, & Farrar, 2011). Glioblastoma CSCs display enhanced repair capability through up regulation of PARP1 and sensitization to radiation occurs by treating with PARP inhibitors (Venere et al., 2014). On the other hand, Yajing *et al* noted that breast CSCs are resistant to PARP inhibition though a RAD51 dependent mechanism (Yajing Liu et al., 2017). Furthermore, ovarian CSCs exhibit increased nucleotide excision repair (NER) efficiency through elevated expression of DNA polymerase ŋ (Pol ŋ) and thus rendering cells resistant to cisplatin; a mainstay treatment of ovarian cancer (Srivastava et al., 2015).

8. CURRENT APPROACHES TO TARGETING CANCER STEM CELLS

In light of the role of CSCs in clinical relapse and metastasis, it is essential that novel therapeutic strategies designed to specifically target CSCs are developed in order to effectively eradicate cancer (Fig. 2).

8.1 PHENOTYPIC MARKER BASED TARGETING OF CANCER STEM CELLS

The majority of stem cell markers are not good candidates for antibody treatment as many fail to distinguish normal stem cells from CSCs. However, in the case of colorectal cancer, lineage tracing experiments showed that doublecortin-like kinase 1 (Dclk1) is not a marker for normal stem cells in the intestine but instead represents CSCs that continuously produce tumor progeny in polyps (Chandrakesan et al., 2017; Nakanishi et al., 2013). Lineage ablation of $Dclk1^{+}$ CSCs resulted in regression of tumour polyps without damage to the intestine (Nakanishi et al., 2013).

8.1.1 CD44

CD44 is the most common CSC marker and plays a major role in enhancing stemness and communication with the microenvironment (Naor, Sionov, & Ish-Shalom, 1997). When designing targeted therapies, the presence of CD44 on normal cells and the resemblance of CD44 to other molecules, such as lyve 1, must be considered (Yan, Zuo, & Wei, 2015). Furthermore, the development of CD44-based therapies is complicated by alternative splicing and post translational modifications (Naor et al., 1997). Nevertheless, anti-CD44 antibodies have demonstrated promise in pre-clinical studies (L. Jin, Hope, Zhai, Smadja-Joffe, & Dick, 2006). They promote terminal differentiation of acute myeloid leukemia (AML) blasts, retard tumor growth in solid cancers and decrease metastasis formation (Charrad et al., 1999; Thapa & Wilson, 2016). However, resistance to CD44 therapy has been described in the AML setting through regulation of the bone marrow stromal cells via the PI3K/AKT-p27 axis (P. Chen et al., 2015).

In the clinic, an antibody against CD44v6 labelled with 186Re or conjugated with the microtubule targeting cytotoxic agent, mertansine, stabilized heavily pre-treated metastatic breast cancer patients, however, serious dose limiting toxicities occurred (Tijink et al., 2006). Furthermore, the immunogenic accumulation of the antibody in skin keratinocytes caused mild skin disorders in 75% of patients and a case of fatal toxic epidermal necrolysis stopped the trial prematurely (Rupp et al., 2007).

A second humanized anti-CD44 antibody specifically designed to inhibit CD44 – HA interactions has recently completed Phase 1 clinical trials and data suggests that CD44 isoform status is a potential novel biomarker for patient response (Birzele et al., 2015). A third, humanized CD44 antibody, R05429083, designed to target a glycosylated epitope, has been shown to have dual action by targeting the CSCs and expanding the NK cell population in a pre-clinical model of neck squamous cell carcinoma (Perez et al., 2014). It is currently in clinical trial in patients with CD44 expressing metastatic tumors or AML (NCT01358903 and NCT01641250).

A6, a peptide derived from human urokinase plasminogen activator, binds to CD44 and inhibits angiogenesis, migration and metastasis formation (Piotrowicz et al., 2011). It is well-tolerated and completed Phase II clinical trial in ovarian patients after first line chemotherapy with asymptomatic CA125 progression and in recurrent ovarian cancer patients. A6 therapy prolonged PFS in patients treated after first line chemotherapy but had no activity in patients with persistent or recurrent ovarian cancer (Ghamande et al., 2008; Gold et al., 2012). The researchers contributed its lack of clinical efficacy in recurrent disease to A6's ability to only delay formation of metastasis coupled with no anti-proliferation action (Gold et al., 2012).

Our own group has characterized a novel protein with anti-CSC activity called FK506 binding like protein (FKBPL). FKBPL was initially found to have potent anti-angiogenic activity via a CD44-dependent mechanism (Valentine et al., 2011; Yakkundi et al., 2013). The active domain of the protein was identified and a therapeutic 24 mer peptide based on this region was developed and termed AD-01 (Robson & James, 2012). ALM201, a truncated 23 mer peptide, has now entered a Phase I cancer clinical trial (Eudract number 2014-001175-31). FKBPL and its peptide derivatives also display potent anti-CSC activity both *in vitro* and *in vivo* using models of breast cancer via a CD44 dependent mechanism (McClements et al., 2013). Overexpression of FKBPL or treatment with its therapeutic peptides (AD-01/ALM201) induces differentiation of the CSC by reducing Sox, Oct, Nanog. We also showed that tumor *FKBPL* and *Nanog* inversely correlated with survival outcomes in patients with breast cancer (McClements et al., 2013). Furthermore, and in support of our own data, FKBPL was identified as one of the top hits, together with WNT (SFRP1), PI3K/AKT (FOXO3A), in a shRNA genetic screen to identify genes that upon knockdown enhance mammosphere formation in breast cancer cells, reinforcing FKBPL's endogenous role in CSC signaling (Smit et al., 2016).

8.1.1.2 CD133

The function of CD133 in normal tissues is unknown, however, is it thought to have a role in organizing cell membranes (Irollo & Pirozzi, 2013). It is a key CSC marker in many tissues and a CD133 antibody conjugated with a cytotoxic agent has resulted in promising *in vivo* results in

hepatocellular and gastric cancer (Smith et al., 2008). However, as the biological role of CD133 is still not clarified, this is a controversial targeting strategy. Furthermore, it has been suggested that an undefined glycosylated state of CD133 may regulate the CSC phenotype rather than the expression of CD133 itself, thus complicating the development of CD133 targeting therapies (Bidlingmaier, Zhu, & Liu, 2008). More recently bi-specific antibodies, which act by arming activated T cells to $CD133^+$ cells have been developed (L. Zhao et al., 2015). This approach is currently in clinical trials in a range of cancers; safety and efficacy data will be eagerly awaited (NCT02541370).

8.1.1.3 CD117 (c-kit)

CD117 or c-kit is a transmembrane receptor with tyrosine kinase activity. Its ligand, stem cell factor (SCF), has an important role in maintaining survival and differentiation of hematopoietic stem cells. $CD117^+$ cells in ovarian cancer display high tumorigenic potential and the ability to differentiate into $CD117^+$ and $CD117^-$ cells (L. Luo et al., 2011). Imatinib is a tyrosine kinase inhibitor that selectively inhibits c-kit, BCR/ABL and PDGF receptors and is approved for the treatment of chronic myelogenous leukemia (CML) and unresectable $CD117^+$ gastrointestinal stromal tumors (Druker, Talpaz, & Resta, 2001; Heinrich et al., 2006). It was used as a monotherapy in clinical trials in solid tumors, including breast and ovarian, but results were disappointing (Alberts et al., 2007; Cristofanilli et al., 2008). However, imatinib selectively targeted lung CSCs, thus enhancing the anti-tumor effect of cisplatin; indicating it may have efficacy in combination therapies (Levina et al., 2010).

8.2 IMMUNOLOGY APPROACHES TO TARGETING CSCS

The literature clearly demonstrates that CSCs have distant gene expression profiles and express different antigens (Pan et al., 2015). Therefore the ability of immunotherapies to target multiple antigens makes them a promising approach for heterogeneous CSC populations.

$CD8^+$ T cells undergo differentiation into cytotoxic T lymphocytes and memory $CD8^+$ T cells (Ahlers & Belyakov, 2010; Y. Huang, Shah, & Qiao, 2007). In 1999, Bonnet *et al* generated CSC – specific $CD8^+$ T cells from human acute myeloid leukemia and tumor regression occurred in *in vivo* models (Bonnet, Warren, Greenberg, Dick, & Riddell, 1999). Further studies have shown that CSC specific T cells can be generated *in vitro* for subsequent adoptive transfer for CSC-specific T cell mediated killing. Visus *et al* generated CSC specific $CD8^+$ T cells by using an antigenic peptide for ALDH1A1 and the adoptive transfer of $CD8^+$ T cells eliminated ALDH1A1 cells in squamous cell carcinoma of the head and neck xenografts (Visus et al., 2007, 2011). However, this strategy may lead to resistance as CSCs can evolve to escape T cell-mediated attack. CSC from colorectal patients show weak immunogenicity and high levels of IL-4, leading to neutralization of the cytotoxic T cell lymphocytes (Volonte et al., 2014). A further limitation is clonal evolution of the CSC subpopulation leading to antigen loss and new CSC antigen expression. One way to overcome this may be the use of antigen presenting cells, such as dendritic cells (DC), in a cancer vaccine approach.

Immature DCs in peripheral tissue efficiently capture antigens and activate mature, antigen loaded DCs to initiate the differentiation of antigen specific T cells into effector T cells (Trombetta & Mellman, 2005). The use of tumor lysates from CSCs would allow personalized targeting of multiple antigens simultaneously and therefore loss of tumor antigen as a means of resistance would be less likely. Ning *et al* isolated ALDH expressing cells as an antigen source to prime DCs and demonstrated that $ALDH^+$ primed DC vaccination was more effective at preventing metastasis and primary tumor growth, compared to $ALDH^-$ primed DC vaccination (Ning Ning et al., 2012). In addition, CSC based DC vaccination may have a valuable role in targeting micro metastasis. In a murine mouse model, treatment of the primary tumor with radiation increased the percentage of CSCs, whilst combination treatment with radiation and CSC-DC vaccine resulted in a significant decrease in CSCs in the primary tumors and a reduction in spontaneous lung disease (Lin Lu et al., 2015).

DCs have been successfully generated *ex vivo*, loaded with different forms of antigens, activated and injected into patients (Palucka & Banchereau, 2012). Indeed, clinical studies have shown that DC based vaccines are safe and can induce the expansion of circulating tumor antigen specific $CD4^+$ T cells and $CD8^+$ T cells (Palucka & Banchereau, 2012). Phase II and Phase III clinical trials are currently underway, utilizing a proteome based screen of patients peripheral blood, to develop personalized CSC specific DC vaccination in glioblastoma and brain metastasis from other primary sites (NCT01782287, NCT01782274, NCT01759810)

8.3 TARGETING CSC SIGNALING PATHWAYS

CSCs exploit self-renewal pathways that also have critical roles in embryonic development and differentiation and many new agents have entered clinical trial as summarized in Table 2.

Inhibitors of γ-secretase activity were the first Notch targeting agents in clinical development. In pre-clinical mouse models, they abrogated recurrence when used as a combination therapy and demonstrated anti-CSC activity in patient-derived samples (Grudzien et al., 2010; Pandya et al., 2011). They showed some promise in early clinical trials, however, they exhibited dose-limiting side effects due to goblet cell metaplasia (Messersmith et al., 2015; Milano et al., 2004). γ – secretase has at least 90 substrates and inhibition may lead to systemic toxicity or off target effects (Takebe et al., 2015). Currently, it is not clear which class of γ – secretase inhibitors (GSIs) exhibit greatest safety/efficacy and information from pre-clinical and clinical trials is needed to precisely target this protease.

The Notch ligand, DLL4, has been another popular targeting strategy in cancer. Treatment with anti-DLL4 monoclonal antibody results in disorganized angiogenesis due to its effect on endothelial cells and in clinical trial inhibition of DLL4 has demonstrated good safety and preliminary efficacy (Chiorean et al., 2015; Takebe et al., 2015). Moreover, anti-DLL4 therapy upregulates markers of differentiation (i.e. ATOH1) in colon cancer cells suggesting it promotes differentiation of the stem/progenitor cells (Fischer et al., 2011). Anti-DLL4 treatment has synergistic activity with

chemotherapy agents indicating that differentiation of resistant CSCs sensitizes cells to cytotoxic effects (Fischer et al., 2011). MEDI10639 is an anti-DLL4 monoclonal antibody and pre-clinical studies have shown a reduction in the CSC subpopulation. In a Phase 1 trial in small cell lung cancer, pre and post treatment biopsies were collected and there was a > 50% decrease in *ex vivo* sphere formation in three out of eight patients and expression levels of CSC genes were decreased in three out of seven patients (NCT01577745).

The most clinically advanced Hh targeting therapy is vismodegib, which is approved by the FDA and EMA for the treatment of metastatic basal cell carcinoma (Sekulic et al., 2012; Takebe et al., 2015). It is a direct competitive antagonist of SMO, a Hh ligand, and it has shown some efficacy in targeting glioblastoma CSCs in a Phase II trial in patients with relapsed disease undergoing debulking surgery (Fecher & Sharfman, 2015). Patients received vismodegib or control for one week prior to surgery and until disease progression. There was no difference in progression free survival (PRS) or overall survival (OS) in the glioblastoma patients, but there was a reduced capacity of $CD133^+$ cells to form *ex vivo* neurospheres (Sloan et al., 2014).

Wnt/β-catenin inhibitors include several approved drugs such as non-steroidal anti-inflammatories (NSAIDs), COX-2 inhibitors and glitazone anti-diabetic agents, and pre-clinical data suggest they have the ability to inhibit stemness (Moon et al., 2014; Takebe et al., 2015). An antibody targeting Frizzled receptors, OMP-18R5, has shown promising anti-CSC activity in several cancers, however, there are concerns regarding safety given the essential role of Wnt in homeostasis (Gurney et al., 2012; Kahn, 2014)

Gemcitabine treats the bulk of pancreatic cancer cells, but enriched the $CD133^+$ CSC population. However, combination treatment with gemcitabine, rapamycin (an mTOR inhibitor) and cyclopamine (a hedgehog inhibitor) suppressed CSCs and increased survival (Mueller et al., 2009). Everolimus, an mTOR antagonist, reduced the expression of AKT1 and p-AKT and targeted CSCs in HER2 expressing breast cancer and combination treatment with trastuzumab proved even more effective at targeting CSCs (Yan Liu et al., 2014; Zhu et al., 2012). Metformin is the most widely prescribed type

2 diabetes drug and treatment reduces the risk of cancer in diabetic patients (Evans, Donnelly, Emslie-Smith, Alessi, & Morris, 2005). It activates the LKB1/AMPK axis and thereby directly inhibits the mTORC1 complex (R. J. Shaw et al., 2005). Many studies have shown that treatment with metformin targets the CSC component by disruption of mTOR signaling (B. Bao et al., 2012; K.-M. Lee et al., 2014; Q. Liu et al., 2016; Snima, Pillai, Cherian, Nair, & Lakshmanan, 2014). A novel dual mTORC2/mTOR1 inhibitor targets acute myeloid leukemia precursors more effectively than rapamycin (Altman et al., 2011).

Targeting STAT3 was shown to reduce glioblastoma brain tumor stem cells in pre-clinical orthotropic models (Stechishin et al., 2013). WP1066, a STAT3 inhibitor, is currently in Phase 1 clinical trial from patients with recurrent malignant glioma and brain metastasis from melanoma (Clinical trials ID: NCT01904123).

Small molecule FAK inhibitors have been in clinical trial for a number of years and Phase 1 trials have demonstrated that they are well-tolerated with low adverse events. PF-00562271 displayed nonlinear kinetics and was discontinued whilst the later generation PF-04554878 had favorable pharmacokinetics and patients with a range of tumors exhibited stable disease at Phase 1 (Clinical trial ID: NCT00787033). There is evidence that FAK inhibitors preferentially target CSCs in *ex vivo* patient models and xenografts and FAK inhibition reduced phosphorylation of β-catenin (Kolev et al., 2017). Pre-clinical studies have shown increased susceptibility to FAK inhibition in cells with KRAS in association with INK4A/ARF deficiency (Konstantinidou et al., 2013). Indeed defactinib, an oral FAK inhibitor is currently in clinical trial in KRAS mutation non-small lung cancer (Clinical trial ID: NCT01778803). The study is completed and although the full study results are not published, data presented at IASLC (2015) suggest a response rate of 12 week PFS in 28% of heavily pre-treated patients (Keegan, 2015). Therefore, results indicate that FAK inhibitors could be a viable approach for the treatment of CSCs in the future.

When targeting signaling pathways, the role of the microenvironment to cause resistance must be taken into account. IL-6 and IL-8 abrogated the pre-clinical efficacy of the Notch inhibitor R04929097 and, indeed, in Phase 1 clinical trials only patients presenting with low baseline levels of IL-6 and IL-8 derived clinical benefit (He et al., 2011). Reparixin is an allosteric inhibitor of CXCR1/2 and has demonstrated pre-clinical efficacy against breast CSCs and a Phase 1b clinical trial explored its effect on CSCs in metastatic breast cancer patients in combination with paclitaxel. A 30% response rate was reported with no serious adverse effects or pharmacokinetic interactions (Anne F. Schott et al., 2017). Vismodegib, a SMO inhibitor, is approved for clinical use in the treatment of metastatic basal cell carcinoma, however, the overall response rate is only 48% and an additional 20% of patients will develop acquired resistance (Atwood et al., 2015; Sekulic et al., 2012). The resistance is due to a compensatory up regulation of other signaling pathways, such as PI3K signaling, and structural alterations to the ligand binding pockets (Atwood et al., 2015; Buonamici et al., 2010). There have been many studies conducted investigating the regulation of CSCs, however our current understanding is limited and precise, tissue-specific mechanisms are yet to be elucidated. Complex signaling patterns are known to play a role in the normal maintenance of stem cells within embryogenesis and tissue homeostasis and therefore it is not surprising that cross talk exists when targeting a single signaling pathway. In the skin, systemic treatment with a Notch inhibitor leads to a compensatory up regulation of Hh signaling (Collu, Hidalgo-Sastre, & Brennan, 2014). This phenomenon is not isolated to cancer patients; Phase III clinical trials of Notch inhibitors to treat Alzheimer's disease demonstrated a fivefold increase in non-melanoma skin cancers in the treatment cohort mediated through Hh signaling (Doody et al., 2013). Another challenge in the field is developing effective pharmacodynamic biomarkers for novel agents targeting CSC signaling pathways. This is especially important for choosing dose ranges in clinical trials and for assessing *in vivo* efficacy. Molecular biomarkers for Notch inhibition include analysis of hair follicles for downstream inhibition of transcriptional markers however, this may not reflect Notch inhibition within tumor tissue (Krop et al., 2012).

9. Summary and Perspectives

There is compelling evidence that cancer is a disease with a stem cell hierarchy, similar to normal tissues. Cancer stem cells have the ability to evade current cancer therapies, potentially including novel immunotherapies, resulting in tumor relapse and metastatic disease. Given the difficulty in targeting metastatic disease, it is imperative that strategies targeting CSCs are devised and one hindrance to the field is the controversy regarding the identification and isolation of CSCs. Currently, this is primarily based on cell surface markers, however, the extent to which these populations are "true" CSCs remains to be answered. Novel technologies such as live cell RNA detection may advance CSC identification, but are unlikely to yield a universal CSC marker. It is clear that the cellular signaling pathways regulating the CSC phenotype do not operate in isolation and a combination of agents is likely to be the most robust targeting strategy. There are a significant number of clinical trials investigating the efficacy of anti-CSC agents and tumor volume is a common clinical end point. Given that the CSCs are a rare subpopulation, a reduction in tumor volume is unlikely and therefore progression free and survival endpoints are more appropriate and ultimately more for cancer clinical trials. Spheroid assays have been used as a surrogate marker for CSCs in clinical trials, although surgical removal and labor intensive preparation of the tissue is required. Targeting of CSCs in the clinic remains in its infancy, and asystems based, personalized medicine approach will ultimately enable the stratification of patients to the most appropriate anti- CSC agent. CSC-dendritic cell vaccination approaches are a promising, personalized approach at targeting CSC, however, they are not without their risks and their success will most likely depend upon a better understanding of the basic immunology. During the progression of the disease, clonal evolution of the CSC population is likely to occur, leading to the need for of multiple anti-CSC agents at different stages in a patient's cancer journey.

Conflict of Interest

The authors declare there is no conflict of interest

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Figure 1 The Evolution of the Cancer Stem Cell Theory

A. In the clonal evolution model the multiple cell populations are a result of a succession of genetic mutations. There is no cellular hierarchy and later successions are likely to proceed more rapidly because the cells have acquired mutations to proliferate faster. **B.** Cancer stem cells (CSC) divide asymmetrically and give rise to one stem cell and one more differentiated cell. A cell, which has exited the stem cell state, is termed a transit amplifying cell or progenitor cell. These cells are intermediates and divide symmetrically before eventually entering a fully differentiated post meiotic state. **C.** A mixed model of clonal evolution and CSC may underlie tumorigenesis and heterogeneity. The tumor will be initiated by a CSC (CSC1) and during the course of disease progression, other distant CSCs (CSC2 and CSC3) may arise due to clonal evolution of CSC1. The more aggressive CSC mutant will become dominant and drive tumor growth and resistance to therapies.

Figure 2 Strategies to target Cancer Stem Cells

Many strategies aimed at eradicating cancer stem cells have been developed and the main areas have been summarized. Targeting of cell surface markers (blue boxes), cell signaling pathways (green boxes), modulation of the immune system (red box) and inhibiting drug afflux pumps (purple box) are alluring methods to either eradicate cancer stem cells or sensitize them to chemotherapy.

Table 1: Cell surface phenotype to identify Cancer Stem Cells

Cancer	**Cell surface phenotype**	**Validation technique**	**Reference**
Breast	$CD44^+$ $CD24^-$ ESA^+	Xenotransplant of human primary tissue sorted by FACS	(Al-Hajj et al., 2003)
	$ALDH^+$	Xenotransplant of human primary tissue sorted by FACS	(Ginestier et al., 2007)
Prostate	Sca-1	Xenotransplant of murine prostate cells sorted by FAC	(Xin, Lawson, & Witte, 2005)
	$CD133^+$	Microarray analysis of $CD133^+$ and $CD133^-$ cell isolated from human primary prostate cells	(Shepherd et al., 2008)
	$CD44^+/ \alpha_2\beta_1{}^+$ /$CD133^+$	In vitro characterization of self-renewal, differentiation, invasion from primary tissue sorted by magnetic beads	(Collins, Berry, Hyde, Stower, & Maitland, 2005)
	$PSA^{-/low}/ALDH^+/CD44^+/\alpha_2\beta_1{}^+$	Xenotransplantation of DU145 cell line sorted by FACS	(Salvatori et al., 2012)
Ovarian	$CD44^+CD117^+$	Xenotransplant of human primary tissue sorted by FACS	(S. Zhang et al., 2008)
	$CD133^+ALDH^+$	Xenotransplant of human primary	(Kryczek et al.,

	$CD133^+CXCR4^+$	tissue sorted by FACS; Xenotransplant of human primary tissue and cell lines sorted by FACS Xenotransplantation of cell lines sorted by FACS	2012; Silva et al., 2011) (Cioffi et al., 2015)
Lung	$CD133^+$ $CD44^+$ $ALDH^+$	Xenotransplant of human primary tissue sorted by FACS Xenotransplant of human cell lines sorted by FACS Xenotransplant of human cell lines sorted by FACS	(Eramo et al., 2008) (Leung et al., 2010) (F. Jiang et al., 2009)
Glioblastoma	$CD133^+$ $CD15^+$	Gene expression analysis of human primary derived neurospheres; Chemo sensitivity of primary cell lines sorted by FACS Linage tracking and xenotransplantation of primary and xenograft derived tumor cells sorted by FACS	(Hemmati et al., 2003; G. Liu et al., 2006) (Son, Woolard, Nam, Lee, & Fine, 2009)

Colon	CD133	Magnetic cell sorting of primary colon tumor cells and xenotransplantation	(O'Brien, Pollett, Gallinger, & Dick, 2007; Ricci-Vitiani et al., 2007)
	$CD44^{+}$/$EpCAM^{high}$/$CD166^{+}$	Xenotransplant of human primary tissue sorted by FACS	(Dalerba et al., 2007)
Liver	$CD133^{+}$	Xenotransplantation of human cell line and primary tissue sorted by FACS	(Ma et al., 2007)
	$CD90^{+}$	Xenotransplantation of human cell line and primary tissue sorted by FACS	(Yang et al., 2008)
	$CD24^{+}$	Xenotransplantation of human cell line and primary tissue sorted by FACS	(T. K. W. Lee et al., 2011)
Pancreas	$CD44^{+}CD24^{+}ESA^{+}$	Xenotransplantation of primary tissue sorted by FACS	(C. Li, Lee, & Simeone, 2009)
	$CD44^{+}CD133^{+}ESA^{+}$	Xenotransplantation of human cell line sorted by FACS	(Bin Bao et al., 2014)

Head and Neck	$CD44^+$	Xenotransplantation of primary tissue sorted by FACS	(Prince et al., 2007)
	$CD44^+ALHD^+ Lin^+$	Xenotransplantation of primary tissue sorted by FACS	(Krishnamurthy et al., 2010)
	$CD133^+$	Cell line and patient derived culture of oraspheres	(Chiou et al., 2008)
Multiple myeloma	$CD138^-$	Xenotransplantation of cell line and primary tissue sorted by FACS	(Matsui et al., 2004)
Esophageal	$CD44^+$	In vivo tumorgenicity of primary derived cell lines	(J.-S. Zhao et al., 2011)
	$P75^{NTR}$ (CD271)	Xenotransplantion of cell lines by FACS sorting	(S.-D. Huang et al., 2009)
	$ICAM1^+$	Xenotransplantion of cell lines by FACS sorting	(Tsai et al., 2015)
Gastric	$CD44^+ EpCAM^+$	Xenotransplantation of primary tissue sorted by FACS	(Han et al., 2011)
	$CD44^+CD24^+$	Xenotransplantation of primary tissue and cell lines sorted by FACS	(C. Zhang, Li, He, Cai, & Yang, 2011)
	$CD44^+CD54^+$	Xenotransplantation of	(T. Chen et al.,

	$CD90^+$	tumorspheres derived from cell isolated from patient blood Xenotransplantation of human primary cells sorted by FACS	2012) (J. Jiang et al., 2012)
Acute myeloid leukemia	$CD34^+CD38^-$	Xenotransplantation of human tissue sorted by FACS	(Bonnet & Dick, 1997)
Melanoma	$CD271^+$ $ALDH^+$	Xenotransplantation of human tissue sorted by FACS Xenotransplantation of human tissue sorted by FACS	(Boiko et al., 2010; Civenni et al., 2011) (Y. Luo et al., 2012)

Table 2 Clinical trial targeting cancer stem cells via signaling pathways (clinicaltrial.gov accessed 22nd Nov 2017)

Trial Identifier	Intervention	Primary outcome	Condition	Target	Trial Status
NCT02753127	BBI-608 (napabucasin) in Combination With 5-Fluorouracil, Leucovorin, Irinotecan (FOLFIRI)	Overall survival [Time frame 36 months]	Metastatic colorectal cancer	STAT3 inhibitor	Phase 3 Enrolling (estimated completion 2020)
NCT01553851	GSK1120212 in Surgically Resectable Oral Cavity Squamous Cell Cancer	Changes in CD44 expression and Intracellular Phospho-ERK1/2 Staining	Oral Cavity Squamous Cell Cancer	MEK1/2 inhibitor	Phase 2 complete (June 2015) Study results significant reduction in Ras/MEK/ERK pathway activation and in clinical and metabolic tumor responses in OCSCC patients (Uppaluri et al.,

					2017)
NCT01190345	Pre-operative bevacizumab in combination with conventional chemotherapy in breast cancer receiving neo-adjuvant treatment	Anti-cancer stem cell ($ALDH^+$) activity [Time Frame:4 months]	Breast cancer	Bevacizumab (anti VEGF antibody)	Phase 2 (status unknown)
NCT01579812	Metformin administered in combination with chemotherapy	Recurrence-Free Survival [Time Frame:5 years]	Ovarian, Fallopian Tube, and Primary Peritoneal Cancer	Type 2 anti-diabetic drug	Phase 2 (Estimated end date: Feb 2018)
NCT01624090	Mithramycin IV in cycles	Objective response rate [Time Frame: Every 8 weeks until disease progression	Lung Cancer, Esophageal Cancer, Mesothelioma, Gastrointestinal	Mithramycin: RNA synthesis inhibitor	Phase 2 (Estimated completion Aug 2020)

		or unacceptable toxicity]	Neoplasms, Breast Cancer		
NCT01861054	Reparixin on CSCs in the primary tumor and the tumoral microenvironment in an early breast cancer population	Markers of Cancer Stem Cells (CSCs) in the primary tumor and the microenvironment [Time Frame: Day 21]	Breast Cancer	Inhibitor of CXCL8 receptor CXCR1 and CXCR2 activation	Phase 2 (Study terminated; Enrollment target not reached)
NCT01195415	Vismodegib and Gemcitabine Hydrochloride in Treating Patients With Advanced Pancreatic Cancer	Median Percent at Baseline and 3 Weeks in CD44+/ CD24+/ ESA+ Cells From Needle Biopsy Calculated Using FACS	Recurrent Pancreatic Carcinoma Stage IV Pancreatic Cancer	Hedgehog inhibitor	Phase 2 complete Study results published – no significant decrease in $CD44^{+}CD24^{-}$ population
NCT00645333	MK-0752 Followed by Docetaxel in Advanced	Dose Limiting Toxicity (DLT)	Metastatic Breast Cancer	γ-secretase inhibitor	Phase 1

	or Metastatic Breast Cancer	[Time Frame: first 21 days]			Phase 2 Completed Study results published – manageable toxicity and evidence of decrease CSC in patients undergoing serial biopsies(A. F. Schott et al., 2013)
NCT01088815	GDC-0449 in combination with chemotherapy (gemcitabine and nab-Paclitaxel).	Progression free survival with the combination of GDC-0449 with Gemcitabine and nab-paclitaxel. [Time Frame: 2 years]	Metastatic Pancreatic Cancer	Hedgehog inhibitor	Phase 2 Status unknown

NCT02370238	Paclitaxel in Combination With Reparixin or Placebo for Metastatic Triple-Negative Breast Cancer (FRIDA)	Progression Free Survival (PFS) [Time Frame:18 months]	Metastatic Triple-Negative Breast Cancer (FRIDA)	An inhibitor of CXCL8 receptor CXCR1 and CXCR2 activation	Phase 2 (estimated end date February 2018)
NCT02279719	BBI608 in Combination With Sorafenib, or BBI503 in Combination With Sorafenib in Adult Patients With Hepatocellular Carcinoma	Phase 1:Phase 2 Dose (RP2D) by assessing dose-limiting toxicities (DLTs [Time Frame:6 weeks] Assessment of the preliminary anti-tumor activity by performing tumor assessments every 8	Advanced hepatocellular carcinoma who have not received systemic chemotherapy	STAT3 inhibitor BBI-608 BBI503 – Nanog inhibitor	Phase 1 Phase 2 (estimated end date December 2017)

		weeks (Phase 2 portion) [Time Frame:6 months]			
NCT01951690	VS-6063 in Patients With KRAS Mutant Non-Small Cell Lung Cancer	PFS at 12 weeks (PFS12) within each cohort. [Time Frame: From baseline through 12 weeks of treatment]	KRAS mutant non-small cell lung cancer (NSCLC)	FAK inhibitor	Phase 2 (Complete)
NCT00949325	Temsirolimus (Torisel) plus liposomal doxorubicin (Doxil)	Incidence of dose limiting toxicities [Time Frame:2 months]	Advanced Soft Tissue and Bone Sarcomas	mTOR inhibitor	Phase 1/Phase 2 (complete)
NCT02315534	BBI608 in Combination With Temozolomide	Determination of the Recommended Phase 2 Dose (RP2D) by assessing dose-limiting	recurrent or progressive glioblastoma	STAT3 inhibitor	Phase 1 Phase 2 (estimated completion date Dec 2017)

		toxicities (DLTs) (Phase 1 portion) [Time Frame:4 weeks] Progression Free Survival (PFS)-6 (Phase 2 portion) [Time Frame:6 months]			
NCT01255800	Clinical activity of the combination of ipilimumab (IPI) -926 in combination with cetuximab	Dose-limiting toxicities	Recurrent Head and Neck Cancer	Hedgehog inhibitor	Phase 1 (complete)
NCT03030287	OMP-305B83, when given in combination with paclitaxel	Dose limiting toxicities (DLT)	Platinum Resistant Ovarian, Primary Peritoneal or Fallopian Tube Cancer	Anti DLL4/VEGF bispecific antibody	Phase 1 (estimated completion June 2019)

NCT02722954	demcizumab, when given in combination with pembrolizumab	Dose limiting toxicities (DLT)	Locally Advanced or Metastatic Solid Tumors	Anti DLL4 antibody	Phase1b (Estimated completion Dec 2018)
NCT03035253	OMP-305B83, when given in combination with FOLFIRI chemotherapy regimen	Dose limiting toxicities (DLT)	Metastatic Colorectal Cancer	Anti DLL4 antibody	Phase 1b (estimated completion Jan 2019)
NCT01189929	Gemcitabine and Demcizumab (OMP-21M18) With or Without nab-paclitaxel	Maximum tolerated dose of demcizumab	Locally Advanced or Metastatic Pancreatic Cancer	Anti DLL4 antibody	Phase 1b (complete)
NCT01192763	RO4929097 before surgery	Notch activity (expression of Hes-1) [Time Frame: Up to day 3 (of course 1)]	Adenocarcinoma of the Pancreas (All stages)	γ-secretase inhibitor	Phase 1 (terminated)

		Frequency and severity of adverse events			
NCT01189942	OMP-21M18, when given in combination with FOLFIRI chemotherapy regimen	Maximum tolerated dose of OMP-21M18 plus FOLFIRI	Advanced Colorectal Cancer	Anti DLL4 inhibitor	Phase 1 complete
NCT01189968	Demcizumab (OMP-21M18), when given in combination with carboplatin and pemetrexed	Maximum tolerated dose of demcizumab (OMP-21M18) plus carboplatin and pemetrexed	Non Small Cell Lung Cancer	Anti DLL4 inhibitor	Phase 1 (complete)
NCT03113643	SL-401 in Combination With Azacitidine	Maximum Tolerated Dose	Acute Myeloid Leukemia Myelodysplastic Syndrome	Anti – IL3 agent	Phase 1 (estimated end date May 2023)

NCT01943292	Dose Escalation Study of VS-6063	Safety and Tolerability	Non Hematologic Cancers	FAK inhibitor	Phase 1 (complete)
NCT01849744	Dose Escalation Study of VS-4718	Safety and Tolerability	Non Hematologic Cancers Metastatic Cancer	FAK inhibitor	Phase 1 (terminated)
NCT01991938	Dose Escalation Study of VS-5584	Safety and tolerability	Non Hematologic Cancers Metastatic Cancer Lymphoma	PI3K/mTOR kinase inhibitor	Phase 1(terminated)
NCT01281163	lapatinib ditosylate and Akt inhibitor MK2206	Safety and tolerability	metastatic breast cancer	AKT inhibitor	Phase 1 (terminated)