# Chapter 16
# Targeting Cancer Stem Cells and the Tumor Microenvironment

**Alice Turdo, Matilde Todaro, and Giorgio Stassi**

**Abstract** Compelling evidence indicates that the survival and behavior of cancer stem cells (CSCs) are positively regulated by specific stimuli received from the tumor microenvironment, which dictates the maintenance of stemness, invasiveness, and protection against drug-induced apoptotic signals. CSCs are per se endowed with multiple treatment resistance capabilities, thus the eradication of CSC pools offers a precious strategy in achieving a long-term cancer remission. Numerous therapies, aimed at eradicating CSCs, have been elaborated such as: (i) selective targeting of CSCs, (ii) modulating their stemness and (iii) influencing the microenvironment. In this context, markers commonly exploited to isolate and identify CSCs are optimal targets for monoclonal antibody-based drugs. Furthermore, the molecules that inhibit detoxifying enzymes and drug-efflux pumps, are able to selectively suppress CSCs. Auspicious outcomes have also been reported either by targeting pathways selectively operating in CSCs (e.g. Hedgehog, Wnt, Notch and FAK) or by using specific CSC cytotoxic agents. Other compounds are able to attenuate the unique stemness properties of CSCs by forcing cell differentiation, and this being the case in ATRA, HDACi, BMPs and Cyclopamine, among others. Targeting the interplay between paracrine signals arising in the tumor stroma and the nearby cancerous cells via the inhibition of VEGF, HIF, CD44v and CXCR4, is increasingly recognized as a significant factor in cancer treatment response and holds alluring prospects for a successful elimination of CSCs. In the present chapter, we discuss the latest findings in the optimization and tailoring of novel strategies that target both CSCs and tumor bulk for the eradication of malignancies.

**Keywords** Cancer stem cells • Tumor microenvironment • Cancer therapy • Stemness modulator drugs • Targeted therapy

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## 1 Introduction

The concept that tumors are hierarchically organized and harbor cells with distinct tumor-initiating capabilities and self-renewal potential, referred to as cancer stem cells (CSCs), has long been observed in a variety of hematopoietic malignancies and solid tumors and is now well-recognized by the scientific community (Valent et al. 2012). By virtue of their innate plasticity, it is worth considering that CSCs fuel and succeed in tumor growth, treatment resistance, distant metastasis formation and patient relapse. Mechanistically, CSCs share several biological properties with normal adult stem cells that endow them with a survival advantage upon chemotherapeutic intervention. These include dormancy (quiescence), active DNA repair machinery, an enhanced reactive oxygen species (ROS) defence capability, a higher expression of multiple drug resistance (MDR) membrane transporters and anti-apoptotic proteins (Maugeri-Sacca et al. 2011; Zhou et al. 2014).

Thus, attractive emerging strategies have been developed to selectively target CSCs by using agents directed at CSC-surface markers, drug-detoxifying enzymes, drug efflux pumps or key signaling pathways sustaining the stemness properties of CSCs. Otherwise, stemness modulator drugs force CSCs to differentiate terminally, resulting in the loss of self-renewal potential and the gaining of susceptibility to cytotoxic therapies. To eventually overcome cancer resistance and relapse, a simultaneous delivery of stem cells targeting drugs or stemness modulator compounds, has been tested in combination with standard anticancer drugs to successfully eliminate CSCs, tumor bulk cells and spontaneously dedifferentiated non-CSCs (Chen et al. 2012; Chaffer et al. 2011). Of note, stem cell targeting drugs eradicate CSCs but at concentrations less toxic to non-CSCs. Conversely, stemness inhibiting drugs aim at reducing the stemness of CSCs and uniquely, at high doses, they may eliminate CSCs and non-CSCs with similar potency. Finally, paracrine signals between cancer cells and stromal cells are required to trigger an epithelial-to-mesenchymal transition (EMT) program. Besides the acquisition of a mesenchymal and invasive state, EMT seems to confer stem-like properties to neoplastic epithelial cells (Morel et al. 2008), and subsequently additional autocrine signals, arising from cancerous cells themselves, appear to maintain this mesenchymal state (Scheel et al. 2011). Therefore, specific molecular therapies that target CSC peculiarities and prominent tumor microenvironment signals may be powerful determinants in tumor shrinkage and successful elimination of CSCs (Fig. 16.1).

## 2 Selective Cancer Stem Cells Targeting Drugs

Proof of evidence that CSCs are endowed with self-renewal and differentiation capabilities is represented by the ability to engraft tumors when serially transplanted in immunocompromised mice. Further support, recently emerging from in vivo genetic cell fate tracking experiments, confirmed the capability of CSCs to seed a

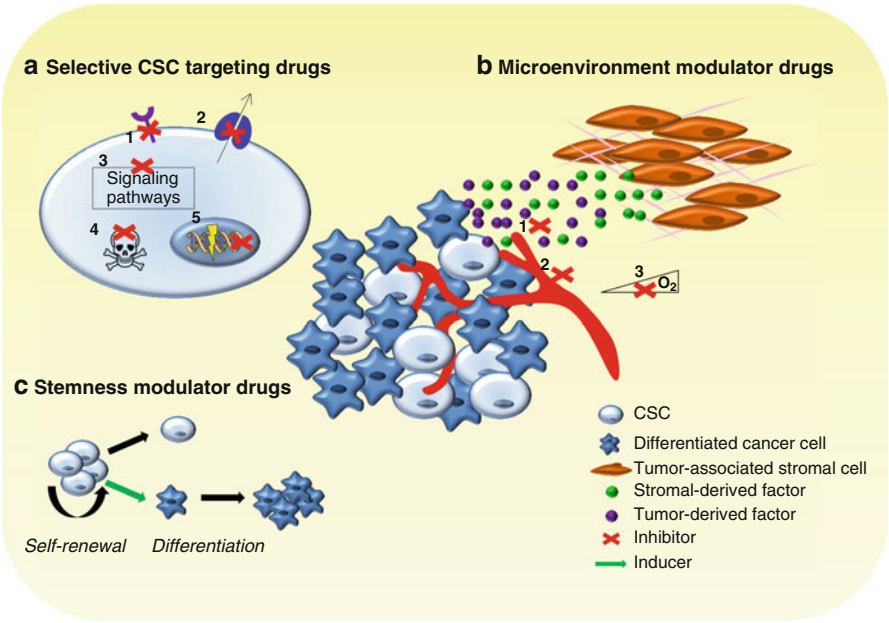


**Fig. 16.1** *Targeting cancer stem cells and the tumor microenvironment*. (**a**) Therapeutic approaches to selectively target CSCs use mAbs directed to CSC-surface markers (1), agents blocking drug efflux pumps (2), inhibitors of signaling pathways that take part in controlling the fate of CSCs (3), CSC-specific cytotoxic compounds (4) and inhibitors of the DNA repair machinery (5). (**b**) Microenvironment modulator drugs can impair the effect of stromal- and cancer-derived factors (1), inhibit angiogenesis (2) and counteract the pro-oxidant environment generated by tumor hypoxia (3). (**c**) Stemness modulator compounds force the differentiation of CSCs and in combination with standard chemotherapy contribute to the successful elimination of CSCs and tumor bulk. CSC: Cancer stem cell, mAB: monoclonal antibody

tumor and recapitulate its heterogeneity (Zhu et al. 2014; Schepers et al. 2012). The criteria used to identify CSCs in solid tumors and hematopoietic disorders include certain in vitro properties among which (i) CSCs can be distinguished and isolated with specific cell-surface marker profiles or intracellular molecules, (ii) CSCs are endowed with increased resistance to chemotherapeutic compound (CSCs are detectable for their high levels of detoxify enzymes and MDR) and (iii) the activation of CSCs-dependent pathways, which could offer a functional marker for their identification (Pattabiraman and Weinberg 2014).

## *2.1 CSC Surface Markers As a Therapeutic Target*

Thus, the ability to use CSCs' peculiar surface markers has been suggested as a promising therapeutic approach. One must bear in mind that some limitations do exist such as, the existence of inter- intra- tumor heterogeneity and splicing variants,

the different methodologies used for CSCs detection and the presence of some common markers shared by normal adult stem cells. For instance, CD44 is a transmembrane glycoprotein and the receptor for hyaluronic acid (HA) and osteopontin (OPN), among others. It is expressed in CSCs from distinct solid tumor types and H90, an anti-CD44 monoclonal antibody (mAb), was the first antibody that showed CSC targeting properties. In vivo administration of H90 interfered with acute myeloid leukemia (AML) stem cells' homing capability in the microenvironmental niche and maintained their stem cell status (Jin et al. 2006). Similarly, in a xenograft model initiated by triple negative breast cancer cells, the anti-CD44 mAb P245 inhibited tumor growth and recurrence if injected during the apparent tumor remission period achieved after treatment with doxorubicin and cyclophosphamide (Marangoni et al. 2009).

GV5 is a recombinant human mAb that recognizes the extracellular domain of CD44's alternative splicing variant, termed CD44R1 (v8-v10). In athymic mice GV5 inhibited tumor formation, after the subcutaneous transplantation of larynx and cervix cancer cells, due to the induction of antibody-dependent cellular cytotoxicity (ADCC) and internalization of CD44R1 (Masuko et al. 2012). H4C4 is an anti-CD44 mouse mAb that decreased pancreatic CSC capabilities of in vitro tumor sphere formation and in vivo tumor growth. It also impaired metastasis formation and recurrence after radiotherapy via Nanog and STAT3 signaling pathway inhibition (Li et al. 2014). Finally, due to its promising preclinical results, RO5429083, which is a humanized mAb directed against an extracellular epitope of human CD44, has been evaluated in a phase I clinical study on CD44-expressing metastatic and/or locally advanced solid tumors. Another phase I clinical study is still ongoing involving patients with AML (http://www.cancer.gov/clinicaltrials).

MT110 is a bispecific bifunctional T-cell-engaging (BiTE) antibody that concomitantly binds to the epithelial cell adhesion molecule (EpCAM), a common CSC marker, and to the T-cell receptor complex CD3 which, leads to the activation of cytotoxic T-cells against EpCAM-expressing cells and causes cell death via redirected lysis. MT110 reduced the capacity of colon and pancreatic CSCs, co-cultured with peripheral blood mononuclear cells (PBMCs) as source of T-cells, to form spheres in vitro and to generate tumors in vivo (Herrmann et al. 2010; Cioffi et al. 2012). MT110, is in early stages of clinical trials for patients with locally advanced, recurrent or metastatic solid tumors, known to widely express EpCAM (http://www.cancer.gov/clinicaltrials).

Catumaxomab is a bispecific trifunctional antibody (Triomabs) binding to EpCAM and the CD3 complex in T-cells. In addition, it binds macrophages, natural killer (NK) and dendritic cells via its Fc fragment thus, synergizing the anti-tumor effects exerted by T-cells. When Catumaxomab is administered to patients with advanced solid cancers and suffering from malignant ascites, it activated peritoneal T-cells, stimulated the release of proinflammatory Th1 cytokines, decreased the peritoneal level of VEGF and eliminated $CD133^{+}/EpCAM^{+}$ CSCs (Jager et al. 2012). Catumaxomab has been approved in Europe for clinical use in the treatment of malignant ascites and the results, from a prospective randomized phase II/III

clinical trial, have been reported by Heiss et al. (2010). The ubiquitous expressed transmembrane antigen CD47 can trigger inhibition of phagocytosis (the so-called 'don't eat me' signal) on SIRPα-expressing phagocytic cells. CD47 blocking via the mouse mAb B6H12.2 favors the phagocytosis of human AML stem cells through mouse and human macrophages. Interestingly, B6H12.2 spares normal hematopoietic stem cells because they express low levels of CD47 (Majeti et al. 2009). 7G3 is a mouse mAb and recognizes the human interleukin-3 (IL-3) receptor α chain (CD123), which is overexpressed on AML blasts and $CD34^+$ AML stem cells. 7G3 inhibits the engraftment and homing of AML stem cells in immunocompromised mice through ADCC (Jin et al. 2009).

CSL362, a humanized anti-CD123 mAb with an increased affinity for human CD16, induces massive NK-mediated ADCC in both AML blasts and $CD34^+CD38^-CD123^+$ AML stem cells (Busfield et al. 2014). CSL362 is currently in the beginning stages of clinical trials for patients with AML (http://www.cancer.gov/clinicaltrials). A more detailed list of CSC specific markers and their use as putative therapeutic targets has been reviewed recently (Medema 2013; Naujokat 2014).

## *2.2 Targeting ABC Transporters in CSCs*

ATP-binding cassette (ABC) transporters have been used to identify CSCs because they are overexpressed on the membrane of both normal and cancer stem cells. ABC transporters enable the efflux of drugs and are responsible for MDR. Thus, CSCs are able to expel the Hoechst 33342 dye by adopting such machinery and thus creating a 'side population' (SP) which, can be isolated by fluorescence-activated cell sorting (FACS). ABCB1 (P-glycoprotein), ABCG2 and ABCC1 are the most extensively studied ABC transporters in stem cell biology. In order to avoid drug resistance, much effort has been devoted to the design of ABC transporter inhibitors which, selectively eliminate CSCs but spare normal stem cells. However, several ABCB1 inhibitors, such as verapamil, tariquidar, and quinidine, have shown little efficacy in clinical settings. The elimination of CSCs has not been successful perhaps due to: clinical studies that were not designed correctly, the choice of an incorrect ABC transporter as a target and other combinations of CSC targeting drugs would have been preferable (Dean et al. 2005). Some ABCG2 inhibitors showed high toxicity both in vitro and in vivo. Novel compounds are in preclinical studies such as the ABCG2 inhibitor YHO-13351 which, sensitized the human cervical carcinoma cell line to irinotecan and reduced the CSC population (Shishido et al. 2013). Xia et al. developed an image-based high-content screening system and identified 12 potent high drug efflux cancer cell inhibitors from 1280 screened compounds. These inhibitors sensitized lung cancer cells to chemotherapeutic drugs and possibly affected in vivo tumorigenic capabilities of the CSC compartment (Xia et al. 2010).

### 2.3 Molecules That Inhibit Pathways by Sustaining CSCs

CSCs are dependent on activated signaling pathways different from those sustaining the bulk population. Therefore, targeting the stemness determinants could effectively conduct to the most durable remission and prevent resistance to chemotherapy and radiotherapy. Being an important player in self-renewal and maintenance of CSCs (Chakrabarti et al. 2014), the Wnt signaling pathway has been targeted by both small-molecule and biologic inhibitors. The first class of compounds includes ICG-001 which, acts as an antagonist of CREB-binding protein (CBP)/β-catenin (Emami et al. 2004) and showed to selectively eliminate drug resistant leukemic stem cells (Takahashi-Yanaga and Kahn 2010). Moreover, the small LGK974 (Liu et al. 2013) and IWP2 (Chen et al. 2009) molecules target the porcupine enzyme which, is responsible for palmitoylation of Wnt ligands, a required step in activating their secretion. A LGK974-based phase I clinical trial on patients with solid tumors is still ongoing (http://www.cancer.gov/clinicaltrials). The second class of compounds includes, the humanized mAb OMP-18R5 that binds to the extracellular domain of multiple Frizzled (FZD) receptors and blocks the Wnt3A-induced downstream pathway. In preclinical settings, it reduces tumorigenic capabilities of human breast, pancreatic, colon and lung cancer cells, compared to standard chemotherapy (Gurney et al. 2012), and is currently in its early stages of clinical trial for patients with solid tumors (http://www.cancer.gov/clinicaltrials). The activation of the Hedgehog (Hh) pathway is mandatory for the maintenance of CSC properties in various human cancers. The molecules antagonist of smoothened (SMO), a G protein-coupled transmembrane serpentine receptor that usually acts as a signal transducer of the proximal Hh pathway, such as GDC-0449, inhibit cell growth and induce apoptosis of pancreatic CSCs (Singh et al. 2011). Interestingly, the antineoplastic compound mithramycin, showed properties that target $Sox2^+$ medulloblastoma stem cells and bear the aberrant Sonic hedgehog (Shh) pathway activation. Specific to this context, although $Sox2^+$ cancer cells were driven by Shh signaling, they were not affected by either the Shh-targeted therapy with GDC-0449 or antimitotic chemotherapy. This suggests the existence of heterogeneity even within the Shh medulloblastoma subgroup and that a combination of bulk targeting drugs and CSCs targeted therapy could lead to a more notable control of the disease (Vanner et al. 2014). GDC-0449 is in phase II of the clinical trial regarding the treatment of basal cell carcinoma (http://www.cancer.gov/clinicaltrials).

The Notch signaling pathway is a well-recognized positive regulator of CSCs fate (Pannuti et al. 2010; Espinoza et al. 2013). The best way to target Notch activation, is to inhibit the proteolytic cleavage of the Notch intracellular domain (NICD) via the γ-secretase complex. γ-secretase inhibitors (GSIs) reduce self-renewal and tumorigenicity of GSCs and breast CSCs (Fan et al. 2010; Kondratyev et al. 2012). A phase I/II clinical trial that foresees the use of GSIs MK-0762 followed by docetaxel, whose purpose is killing breast cancer stem cells in advanced or metastatic breast cancer, has recently been completed (Schott et al. 2013). Antibodies targeting the Notch ligand Delta-like 4 (Dll4) such as the humanized mAb

OMP-21M18, have been developed and efficiently reduced CSC frequency in solid tumors (Hoey et al. 2009; Fischer et al. 2011). A comprehensive analysis of all ongoing and completed Notch clinical trials has recently been published (Andersson and Lendahl 2014). FAK activity seems to be critical for survival, migration and resistance to chemotherapy of CSCs (Sulzmaier et al. 2014; Schober and Fuchs 2011). Kang et al. demonstrated that the FAK inhibitor VS-6063 (which inhibits FAK autophosphorylation) overcomes resistance to paclitaxel in ovarian cancer by decreasing the AKT-dependent YB-1 phopshorylation which, in turn down-regulates the CD44 expression (Kang et al. 2013). Others showed that the up-regulation of CD44 favors breast cancer cell self-renewal, tumorspheres formation and induces paclitaxel resistance (To et al. 2010). Furthermore, CD44 up-regulates Nanog, responsible for increased ABCB1 expression and ovarian cancer cells acquired resistance to paclitaxel (Bourguignon et al. 2008). VS-6063 is currently in phase II of its clinical trial for *K-RAS* mutant non small cell lung cancer (NSCLC) patients. Similarly, other FAK inhibitors such as VS-4718 and PF-00562271, are in phase I of clinical evaluation (http://www.cancer.gov/clinicaltrials). Finally, the BMI-1 inhibitor PTC-209, has recently been proposed as an interesting small molecule affecting self-renewal of colorectal cancer cells with no systemic toxicity in preclinical settings (Kreso et al. 2014).

## *2.4 Agents That Selectively Eradicate CSCs*

A high-throughput screen for agents that selectively kill CSCs has been performed by Gupta et al. Among a library of 16,000 compounds tested, salinomycin induced breast CSC-specific toxicity. Breast cancer cells were initially forced to undergo an EMT by means of an E-cadherin knockdown. Pre-treatment with salinomycin inhibited tumorsphere formation in vitro and reduced tumor seeding ability in vivo by >100-fold, compared to paclitaxel. Salinomycin treatment also decreased tumor mass and metastasis and increased epithelial differentiation of breast CSCs in an immunocompromised mouse model (Gupta et al. 2009). Successively, similar results have been reached in some type of cancers, including leukemia, colorectal cancer, lung cancer, GIST and osteosarcoma. Some findings also suggested that, a combination of salinomycin and conventional cytotoxic drugs could be a much more efficient strategy than the use of a single agent to improve therapeutic outcomes (Bardsley et al. 2010; Koo et al. 2013). Moreover, being that salinomycin seems to be toxic to normal stem cells at concentrations also effective in CSCs (Boehmerle and Endres 2011) it will render its clinical use as a single agent difficult. Salinomycin acts as a $K^+$ ionophore in biological membrane that promotes mitochondrial and cytoplasmic $K^+$ efflux however, the exact mechanisms underlying its toxicity against CSCs still remains unclear. It has been shown that salinomycin is a powerful inhibitor of the multidrug resistance protein 1 (MDR-1) (P-glycoprotein/ABCB1) (Riccioni et al. 2010). It inhibits the phosphorylation of the Wnt co-receptor LRP6, induces apoptosis in chronic lymphocytic leukemia (Lu

et al. 2011) and is an antagonist of the mTORC1 signaling pathway in breast and prostate cancer cells (Lu and Li 2014). On the other hand, it encourages ROS production and inhibits oxidative phosphorylation in mitochondria (Ketola et al. 2012), resulting in the possible elimination of CSCs, which rely on this metabolic process. In addition, recent studies have unveiled that salinomycin induces cell growth inhibition and apoptosis in multi drug resistant ovarian cancer cell lines, by ablating the activity of the signal transducer and activator of transcription 3 (Stat3) and thus, diminishing the expression of Stat3 target genes, such as *cyclin D1, S-phase kinase-associated protein 2 (SKP2)* and *SURVIVIN* (Koo et al. 2013). This is not surprising if we consider the most recent evidence which highlights the major role that Stat3 plays in reducing the effectiveness of drugs treatment. Specifically, the inhibition of MEK in 'oncogene-addicted' cancer cells, (driven by activated EGFR, HER2, ALK, MET and KRAS pathways) triggers the feedback activation of Stat3 through IL-6R and FGFR, leading to treatment resistance (Lee et al. 2014). In line with these results, Kim et al. showed that the constitutive activation of the IL-6/Stat3/NF κB pathway in $p53^{-}PTEN^{-}$ non-transformed MCF10A, was dependent on the proteolytic degradation of SOCS3 and generated highly metastatic and EMT-like CSCs. Thus, proteasoma inhibition restored SOCS3 protein levels and the selective IL-6R antagonist, tocilizumab, repressed the CSC compartments, hampered tumor growth and dissemination in vivo (Kim et al. 2014).

## *2.5 PARPi Affects CSC Survival*

Recent breakthroughs displayed that inhibition of poly-ADP-ribose polymerase (PARP) could be a promising selective CSC-targeted therapy. Mechanistically, PARP is an abundant nuclear protein that mediates the repair of single strand breaks (SSBs) through base excision repair. The inhibition of PARP leads to the accumulation of SSBs that during replication are converted into double-strand breaks (DSBs), usually repaired by the homologous recombination (HR) pathway, mediated by BRCA1 or BRCA2 whereas in neoplastic cells with defective HR, the DSBs cannot be repaired and lead to cell death. It was shown that AZD2281, a PARP inhibitor (PARPi), preferentially targets glioblastoma stem cells (GSCs) and reduced their survival, expansion and tumor initiation capabilities, as well as having sensitized them to radiation therapy (Venere et al. 2014). Moreover, a PARPi, GPI 15427, was able to counteract GSC's resistance to temozolomide (Tentori et al. 2014). These examples opened a new road for the use of PARPi, even in the absence of mutations of *BRCA1/2*. This changed the classical idea of 'synthetic lethality' which exists between PARP and BRCA1/2 signaling pathways. Indeed, patients affected by triple negative breast cancer (non carriers of *BRCA1/2* mutations) have shown increased therapy response and survival following PARP inhibition (BSI-201) in combination with DNA-damaging chemotherapy. The latter of which may eventually obstruct the cellular DNA repair machinery and cause cell death (O'Shaughnessy et al. 2011). Moreover, deletions or mutations in other genes involved in key

genotoxic stress pathways such as *PTEN*, may sensitize them to PARPi administration (Mendes-Pereira et al. 2009). PARPi are currently under clinical evaluation in solid tumors as single agent or in combination with chemotherapy and detailed information about ongoing clinical trials has been published elsewhere (Curtin and Szabo 2013) (http://www.cancer.gov/clinicaltrials).

## 3 Stemness Modulator Drugs

Notwithstanding that CSCs embody a small portion of the tumor bulk, they are responsible for the heterogeneous cell population that constitutes the tumor mass and their intrinsic resistance to chemotherapy and radiotherapy shown by aggressive tumors. Indeed, CSCs possess both self-renewing capabilities, by means of generating two identical CSCs daughter cells through symmetrical division, and the ability to differentiate through asymmetrical division, yielding the multitude of cancerous cells that account for overwhelming tumor growth (Kreso and Dick 2014). As previously discussed, a prominent mechanism of therapeutic resistance includes an altered kinetic cell cycle in quiescent CSCs. They are spared by chemotherapy-induced cytotoxicity because they are not actively cycling cells but are capable of activating DNA repair mechanisms. Thus, forcing terminal differentiation of CSCs could be an extremely powerful weapon in preventing resistance and relapse. Ideally, a clinically effective response could be achieved by the simultaneous administration of anti-CSC therapy and conventional chemotherapy, in order to eliminate cytotoxic drug-susceptible non-CSCs and prevent their dedifferentiation in CSCs (Chaffer et al. 2011). Given that the development of clinical endpoints in this field may prove challenging, an emergent amount of stemness modulator drugs is already in clinical use and others are in preclinical or early stages of clinical evaluation. Some examples are listed below.

### *3.1 ATRA Induces Differentiation of CSCs*

Among these, all-trans-retinoic acid (ATRA), a derivate of vitamin A, has already been demonstrated to be a potent differentiation-inducing drug and a successful treatment strategy, in combination with arsenic trioxide, for AML patients carrying the PML-RARα fusion protein (Zhou et al. 2005). Campos et al. (2010) reported that ATRA induced differentiation and radio- and chemo-sensitization of stem-like glioma cells. Given that, ALDH is a common marker of breast CSCs and a detoxifying enzyme responsible for the oxidation of intracellular aldehydes as well as of retinol to retinoic acid; it was shown that DEAB-mediated ALDH inhibition increased the CSC compartment by abrogating CSC differentiation. Conversely, ATRA treatment induced differentiation of breast CSCs and decreased the stem population (Ginestier et al. 2009). Similarly, Hammerle et al. (2013) suggested that

the neuroblastoma stem cells' response to 13-cis-retinoic acid (RA), could be enhanced by the proteasome inhibitor MG132. Interestingly, a combination of CSC genomics with connectivity map, analyzed a database of 6100 gene expression profiles of four breast cancer cell lines, treated with different concentrations of approximately 1000 FDA approved drugs. This revealed that ATRA is negatively associated with CSC-enriched gene expression signature. ATRA induced apoptosis, hampered mammosphere formation and forced differentiation of fulvestrant-resistant cells. Intriguingly, in the same study, a MEK inhibitor, selumetinib, sensitized the *K-RAS* mutant breast cancer cell line, which was enriched with CSCs, to the ATRA treatment (Bhat-Nakshatri et al. 2013).

### 3.2 SAHA Modulates Differentiation and Apoptosis of CSCs

Suberoylanilide hydroxamic acid (SAHA), also called vorinostat, a potent inhibitor of the histone deacetylase (HDAC) family, caused differentiation and apoptosis of several tumor type cells. In an in vivo prostate cancer tumor model, SAHA hampered tumor growth with low systemic toxicity (Butler et al. 2000). Additionally, HDAC inhibitors can be therapeutically exploited to specifically target slow cycling cells. For instance, SAHA, coupled with imatinib mesylate, successfully fostered apoptosis in quiescent chronic myelogenous leukemia stem cells and offered a novel strategy to overcome chemoresistance and the difficulties in targeting dormant cells (Zhang et al. 2010).

### 3.3 BMPs: An Actor of Balance Between Differentiation and Stemness

It is the general understanding that the bone morphogenic protein family (BMPs) is required to inhibit the stem cell state and mesenchymal traits in a variety of normal and cancerous epithelial tissues (Scheel et al. 2011; Cordenonsi et al. 2011) and promote differentiation of adult and pluripotent stem cells (Varga and Wrana 2005). Mechanistically, BMPs are members of the transforming growth factor-β (TGF-β) superfamily and bind to a combination of type I receptors (anaplastic lymphoma kinase 2 (Alk2), Alk3 (or BMPR1A), and Alk6 (or BMPR1B)) and type II receptors (BMPR2). They activate either the canonical BMP signaling pathway, through phosphorylation of smads receptors, or the PI3K/AKT-mediated non canonical BMP signaling pathway. Specifically, a BMP7 variant (BMP7v) abrogated in vitro proliferation of glioblastoma stem cells (GSCs) as well as the expression of stem associated markers and endothelial cord formation. In a glioblastoma orthotopic mouse model, BMP7v impaired tumor growth, invasion and angiogenesis (Tate et al. 2012). Likewise, our group demonstrated that BMP4 enhanced colorectal CSCs' differentiation and apoptosis and it their sensitized them to 5-fluorouracil

and oxaliplatin treatment. However, the *SMAD4*-defective tumors carrying either mutations in *PI3K* or loss of *PTEN* are refractory to the treatment mentioned above thus, confirming the BMP4-mediated activation of both canonical and non canonical pathways (Lombardo et al. 2011). On the contrary, molecules such as Coco, an antagonist of TGF-β ligands, reverse the effect of BMP thereby, enhancing the self-renewal of metastasis-initiating cells (Gao et al. 2012).

## 3.4 Resveratrol Affects CSC Self-Renewal

A number of epidemiological studies have proposed that resveratrol, a polyphenolic compound with which, many plant species are enriched with, exerts several biochemical activities associated with tumorigenesis such as, inhibition of inflammation, cell proliferation and angiogenesis as well as, sensitizing tumor cells to chemotherapy (Harikumar et al. 2010). Even though the influence of resveratrol on CSCs is still under evaluation, recent evidence showed that *KRAS*$^{G12D}$ mice, which spontaneously develop aggressive pancreatic cancer, treated with resveratrol developed smaller tumors (dimension and weight). Moreover, patient-derived pancreatic cancer and mice-derived *KRAS*$^{G12D}$ CSCs, lost their self-renewal capability in presence of resveratrol, possibly by the inhibition of Nanog, Sox-2, c-Myc and Oct4. In the same study, patient-derived CSCs underwent resveratrol-evoked apoptosis by activating caspase 3/7 and inhibiting XIAP and Bcl-2. Migration and invasion were suppressed following the inhibition of EMT related markers such as ZEB-1, SLUG and SNAIL (Shankar et al. 2011). Similarly, in Glioblastoma multiforme (GBM), resveratrol induced apoptosis and differentiation of stem-like cells and sensitized them to radiotherapy in vitro and in vivo, via disruption of STAT3 signaling (Yang et al. 2012). Thereafter, Sato et al. mechanistically explained the inhibitory effect observed after resveratrol treatment on self-renewal and the tumorigenicity of CSCs. Indeed, resveratrol promoted the phosphorylation and activation of p53, which in turn may directly favor Nanog degradation via proteasome machinery (Sato et al. 2013).

## 3.5 Cyclopamine Limits the Self-Renewal of CSCs

An additional plant-derived compound, the steroidal alkaloid cyclopamine, is a potent cancer preventing compound that directly binds to the heptahelical bundle of SMO (Chen et al. 2002). As already discussed in the present chapter, Hh signaling is essential for the maintenance of stem-like traits in multiple myeloma, leukemia and gastric cancer, among others (Peacock et al. 2007; Dierks et al. 2008; Song et al. 2011). Hh pathway inhibition through cyclopamine inhibited tumorsphere formation in vitro and the establishment of orthotopic glioblastoma tumors (Clement et al. 2007). The newly synthesized cyclopamine-derived inhibitor of the Hh pathway,

IPI-926, ameliorated cyclopamine characteristics such as oral bioavailability, higher metabolic stability, and a better pharmacokinetic profile (Tremblay et al. 2009). Cyclopamine and IPI-926 limited self-renewal potential of B-cell acute lymphocytic leukemia (B-ALL) cells (Lin et al. 2010). Interestingly, delivery of conventional chemotherapy, such as gemcitabine, to the tumor site, may be potentiated by the simultaneously administration of IPI-926. Indeed, in vivo inhibition of the Hh pathway increased intratumoral drug absorption in a gemcitabine-resistant pancreatic ductal adenocarcinoma model thus, making IPI-926 an important therapeutic strategy for the management of pancreatic cancer chemoresistance (Olive et al. 2009). IPI-926 is undergoing early step clinical trials for solid malignancy in combination with standard chemotherapy (Jimeno et al. 2013) (http://www.cancer.gov/clinicaltrials).

### *3.6 Curcumin Promotes CSC Differentiation*

Curcumin (diferuloylmethane) derives from the Indian spice plant turmeric. Extensive preclinical studies showed its therapeutic potential in a variety of human diseases, including cancer. Due to its pleiotropic activities, curcumin is able to modulate a variety of normal or aberrant biological processes, hence it has been selected as a promising anti-cancer drug in several clinical trials (Gupta et al. 2013). Moreover, studies have shown that curcumin displayed capability of eliminating colon CSCs either alone or in combination with standard chemotherapy, such as FOLFOX (5-fluorouracil and oxaliplatin) and dasatinib (Nautiyal et al. 2011; Yu et al. 2009). Furthermore, Curcumin promotes GSCs terminal differentiation, which culminated in autophagy. Whereas, in an intracranial glioblastoma xenograft model, it repressed their self-renewal capability and tumorigenicity (Zhuang et al. 2012). Intriguingly, breast CSCs, derived from the MCF7 cell line, displayed inhibition of tumorsphere formation and the Wnt signaling pathway (Kakarala et al. 2010).

### *3.7 Metformin in CSC Biology*

Metformin is a well-established oral anti-diabetic drug of the biguanide class. It is an agonist of the adenosine monophosphate-activated protein kinase (AMPK) and an inhibitor of PI3K, mTOR and IGF. It has gained attention for its in vitro and in vivo antitumor effects and is now being tested in several advanced clinical trials (Rattan et al. 2012) (http://www.cancer.gov/clinicaltrials). Metformin has also emerged as an important factor to counteract the retention of stemness and the activation of the EMT program of some cancer populations (Rattan et al. 2012). Metformin was able to inhibit the expression of Oct4 in the MCF7 cell line, mediated by 17-β-estradiol treatment, and to reduce the fraction of $CD44^{high}/CD24^{low}$

cells (Jung et al. 2011). In line with these results, Vazquez-Martin et al. observed that metformin deprived basal-like breast cancer cells of the stem compartment and suppressed an EMT program activation through the transcriptional repression of ZEB1, TWIST1, SNAI2 and TGF-β (Vazquez-Martin et al. 2010). Metformin depleted the CSC pool in both gemcitabine-sensitive and -resistant pancreatic cancer cells, by decreasing the expression of CSC-specific markers such as EpCAM, Notch, Nanog, and CD44, as well as reexpressing miRNAs, (e.g. let7a, let7b, miR-200b, and miR-200c) usually associated with cellular differentiation (Bao et al. 2012). The studies performed by Oliveras-Ferraros et al. attempted to anticipate the possible mechanisms of acquired resistance to metformin treatment. They observed that the potential of metastatic dissemination of breast stem-like cells seemed to be fueled by the chronic administration of metformin to the estrogen–dependent MCF7 cell line. Thus, the drug selected for the emergence of resistant cells, leads to a transcriptome reprogramming which, drives them towards a metastatic stem-like profile (Oliveras-Ferraros et al. 2014).

## 4 Microenvironment Modulator Drugs

### *4.1 Targeting the CSCs Vasculature Niche*

There is proof of evidence that tumor-associated stroma and the extracellular matrix, are an extremely powerful source of herotypic signals, responsible for the activation of an EMT program on cancer cells and possibly to nurture the CSCs within their niche. Among the stromal compartment, endothelial cells play a major role in supporting the self-renewal capability of CSCs and in building up all the vasculature architecture needed from these cells to provide nutrients and an easy route to metastatic dissemination. While the contribution of endothelial cells to tumor angiogenesis is self-evident, our understanding on CSC survival and drug resistance is still incomplete. Pioneer work from Calabrese et al., showed how the formation of a vascular niche is directly involved in the function of CSCs. Interestingly, glioblastoma stem cells (GSCs) can be induced to differentiate in either endothelial cells or pericytes, as a consequence of their undifferentiated state and their strict dependence on microvasculature stimuli (Calabrese et al. 2007). Tumor vasculature is classically composed of a network of tortuous, saccular and extremely permeable vessels, endothelial cells that are abnormally covered by pericytes and an irregular basal membrane. As a result, cancer cells can easily penetrate into the bloodstream and colonize distant metastatic sites, and a higher interstitial hydrostatic pressure, due to plasma leakage, may impair the delivery of chemotherapeutic drugs to the tumor site (Jain 2005). Vascular endothelial growth factor (VEGF) was identified as an endothelial compartment mitogen which has a prominent role in positively regulating physiological and pathological angiogenesis. The mammalian VEGF family consists of five heparin-binding homodimeric glycoprotein of 45 kDa referred to as, VEGFA (VEGF), VEGFB, VEGFC, VEGFD and Placental growth factor (PlGF).

The predominant VEGF molecules are represented by several spliced variants denoted as, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148,}$ $VEGF_{165}$, $VEGF_{183,}$ $VEGF_{189}$, and $VEGF_{206}$ (Tischer et al. 1991). They are commonly secreted by macrophages, neutrophils, fibroblast and several cancer cells but not by endothelial cells themselves. VEGF receptors consist of VEGFR1 (FLT1), VEGFR2 (FLK1) and VEGFR3 (FLT4). VEGFR1 is able to bind VEGF, VEGFB and PlGF. VEGFR2 is activated by VEGF, VEGFC and VEGFD. Lastly, VEGFR3 is primarily involved in lymphangiogenesis as a receptor for VEGFC and VEGFD. Although all VEGFRs are tyrosine kinase receptors, VEGFR2, in response to VEGF stimulation, has captured the most attention as the predominant effector in cancer initiation and progression. This is explained by the fact that VEGFR1 binds VEGF with a higher affinity than VEGFR2 but conversely exhibits weaker tyrosine kinase activity in response to its ligand (Ellis and Hicklin 2008). In this context, Park et al. also proposed that VEGFR1 could act as a 'decoy' receptor able to negatively regulate VEGF activity, by preventing its binding to VEGFR2 (Park et al. 1994).

The binding of VEGFs to their cognate receptors induces dimerization and autophosphorylation of the intrinsic receptor's tyrosine residues and consequently activates the dominant PI3K-AKT, MAPK and FAK pathways. It is now well established that VEGFs and VEGFRs are expressed in a variety of tumors (including colon, breast, lung, prostate, and ovarian cancer). VEGF signaling interferes in cancer biology and interestingly in CSC function, independently of angiogenesis and in autocrine fashion. Conversely, it is popular belief that tumors rely on the classical paracrine VEGF-mediated sprouting angiogenesis, the increased permeability and the influence from the immune cells and the tumor microenvironment's fibroblasts (Goel and Mercurio 2013). The realization that VEGF signaling is a crucial determinant in EMT-induced cancer stemness, is becoming an emerging theme. Indeed, VEGF-mediated angiogenesis by itself is not sufficient but required to increase tumor initiating capacity and dissemination of breast cancer cells undergoing EMT, also suggesting that additional factors from the microenvironment are required (Fantozzi et al. 2014). For instance, a fraction of $CD133^{+}$ GSCs showed a 10–20-fold increase of VEGF secretion and displayed strongly angiogenic and hemorrhagic tumors through the enhancement of resident endothelial cell function and recruitment to the tumor site of bone marrow-derived endothelial progenitors (Bao et al. 2006). In murine models, GSCs may be induced to differentiate into endothelial cells and to directly contribute to tumor vasculature architecture, as proven by the positivity of those cells to VEGFR2 (Ricci-Vitiani et al. 2010). These findings clearly establish that VEGF, secreted by tumoral cells, acts as a paracrine factor to sustain angiogenesis and as an autocrine factor to boost cancer stemness.

Folkman (1971) was the first scientist to introduce the pioneer idea that solid neoplasms were always sustained by new vessel growth and envisioned angiogenesis as a new target for cancer treatment. In 2004, for the first time the FDA approved an anti angiogenic compound, called Bevacizumab, for clinical use in combination with standard chemotherapy. It is a humanized monoclonal antibody specific to

VEGF that prevents the interaction of VEGF to its receptor. It became the standard means of treatment for metastatic HER2 negative breast cancer, metastatic colorectal cancer, glioblastoma, advanced or metastatic non-small-cell lung cancer, advanced renal-cell carcinoma and recently, for persistent, recurrent, or metastatic cervical cancer (Tewari et al. 2014). Later, Aflibercept was approved as a 'decoy' receptor for VEGFA, VEGFB and PlGF (Patel and Sun 2014). The inhibition of VEGFR kinase activity, is another valid approach to counteract tumor angiogenesis. Sunitinib targets multiple receptor tyrosine kinases including PlGFR and VEGFRs in unresectable, local, advanced or metastatic disease in well differentiated pancreatic neuroendocrine tumors, renal-cell carcinomas, and imatinib-resistant gastrointestinal tumors. Similarly, Sorafenib inhibits Raf kinases, VEGFRs and PlGFR in thyroid, liver and hepatocellular carcinoma (Santoni et al. 2014). Since 1971, lots of studies have been published in the field and seemed promising but little efficacy has been shown yet. Besides their remarkable activity in the inhibition of primary tumor growth, anti-angiogenic drugs failed in producing lasting responses and patients' illnesses eventually progress (Bergers and Hanahan 2008). This could be partially explained by the fact that alternative adaptive resistance mechanisms, used to overcome the drug-mediated anti angiogenic effect, can occur. This could be the case when there is: an activation of alternative angiogenic pathways, including Fibroblast growth factor 1 (FGF1) and FGF2, Ephrin A1 (EFNA1) and EFNA2 and Angiopoietin1 (ANGPT1), the recruitment of proangiogenic cells, and the increased coverage of pericytes to support vessel integrity. Interestingly, in an in vivo engineered model of KRAS-driven pancreatic ductal adenocarcinoma, resistant to anti-VEGF therapy, the MEK inhibitor substantially decreased the release of granulocyte–colony stimulating factor (G-CSF) by the tumor cell, which is usually responsible for the recruitment and mobilization of pro-tumorigenic and pro-metastagenic $CD11b^+$ $Gr1^+$ myeloid-derived suppressor cells. $CD11b^+$ $Gr1^+$ cells also helped the establishment of metastases by secreting matrix metalloproteinases (MMPs) as well as the Bv8 molecule, endowed with pro angiogenic features. This study revealed that a combination of MEK inhibitor and anti-VEGF therapy substantially decreased tumor burden and angiogenesis (Phan et al. 2013). Likewise, anti angiogenic therapy eradicated the brain tumor stem cell niche in an in vivo model of c6 rat glioma cell line and enhanced the effect of the conventional cytotoxic agent, cyclophosphamide (Folkins et al. 2007).

Even upon anti-VEGF therapy, functional vessels tightly covered by pericytes have been observed. Indeed, endothelial cells can recruit perycites to protect themselves from anti angiogenic treatments and preserve their vascular structure. An attractive hypothesis suggested that $CXCR4^+$ GCSs were mobilized towards the tumor site through an SDF-1 gradient and, upon TGF-β release by endothelial cells, were forced to differentiate in pericytes and contributed to tumor vasculature and growth (Cheng et al. 2013). Moreover, Conley et al. showed that, hypoxic conditions limit the effectiveness of the antiangiogenic agents bevacizumab and sunitinib, by increasing breast CSC populations (Conley et al. 2012).

### *4.2 Therapeutic Implications of Neuropilins in CSCs Biology*

VEGF receptors can functionally interact with other receptors and foster CSC-driven tumor growth and progression. Within the same context, Neuropilins (NRPs) were described earlier as neuronal receptors for the semaphoring family and also involved in axon guidance. They act as transmembrane glycoproteins with a short cytoplasmic domain that lacks intrinsic catalytic activity and function as co receptors of VEGFR1 and VEGFR2. NRP1 is commonly expressed by endothelial cells and tumor cells (Soker et al. 1998). Upon autocrine VEGF stimulation, NRP1 promotes stemness and renewal of VEGFR2$^+$ squamous skin CSCs (Beck et al. 2011). Similarly, viability, self-renewal and tumorigenicity of CD133$^+$ GSCs rely on autocrine VEGF/VEGFR2/NRP1 signaling and are maintained by a continuous secretion of VEGF (Hamerlik et al. 2012). Cao et al. showed that VEGF and NRP1 induced a dedifferentiated phenotype in vitro and promoted tumor formation in vivo (Cao et al. 2012). α6β1 integrin is necessary for the tumorigenicity of some subpopulations of breast CSCs and GSCs (Goel et al. 2014; Lathia et al. 2010). In triple negative breast cancers, NRP2 resulted preferentially expressed in breast CSCs and associated with α6β1 integrin. Upon VEGF stimulation of the NRP2- α6β1 complex, the focal adhesion kinase (FAK) mediated the activation of MAPK signaling and the subsequent expression of GLI1, an effector of the non canonical Hedgehog pathway. GLI1 in turn, induced BMI1 and positively fed back to the NRP2 expression, thus contributing to tumor initiation (Goel et al. 2013). NRP2 is also associated with aggressive prostate cancer and its expression is forced by PTEN loss. Activation of the VEGF/NRP2 axis culminates in BMI1 expression, which represses the transcription of the insulin like growth factor 1 receptor (IGF1R), commonly responsible for tumor progression. Interestingly, single targeting of NRP2 led to compensatory IGF-1R activation (Goel et al. 2012). Therefore, these findings offer a perfect example of how an ideal combination of conventional chemotherapy, stemness modulator drugs (in this case anti-NRP specific antibodies), and anti IGFR antibodies could reduce tumor bulk, overcome treatment resistance and prevent relapse (Fig. 16.2).

For instance, multiple compensatory signals could be activated when a single anti-angiogenic treatment is administrated, regardless of possible collateral stimulation of pathways involved in invasiveness or tumor cell stemness. Given that Bevacizumab does not inhibit VEGF binding to NRPs, Pan et al. (2007) generated two anti-NRP1 monoclonal antibodies specific to the binding site of semaphorin and VEGF on NRP1. This caused a reduction in cell proliferation as well as vascular density in a NSCLC in vivo model, assuming that the inhibition of NRP1, impairs vascular remodeling and thus rendering vasculature more responsive to anti VEGF treatment. In contrast with these findings, Snuderl et al. recently showed that the exclusive targeting of the PlGF/NRP1 pathway with the previously used phase I clinical trials, TB403 and 5D11D4, respectively an anti-murine PlGF antibody and an anti-human/murine PlGF antibody, reduced primary tumor burden and progression of medulloblastoma. PlGF seemed to be secreted by the tumor stroma,

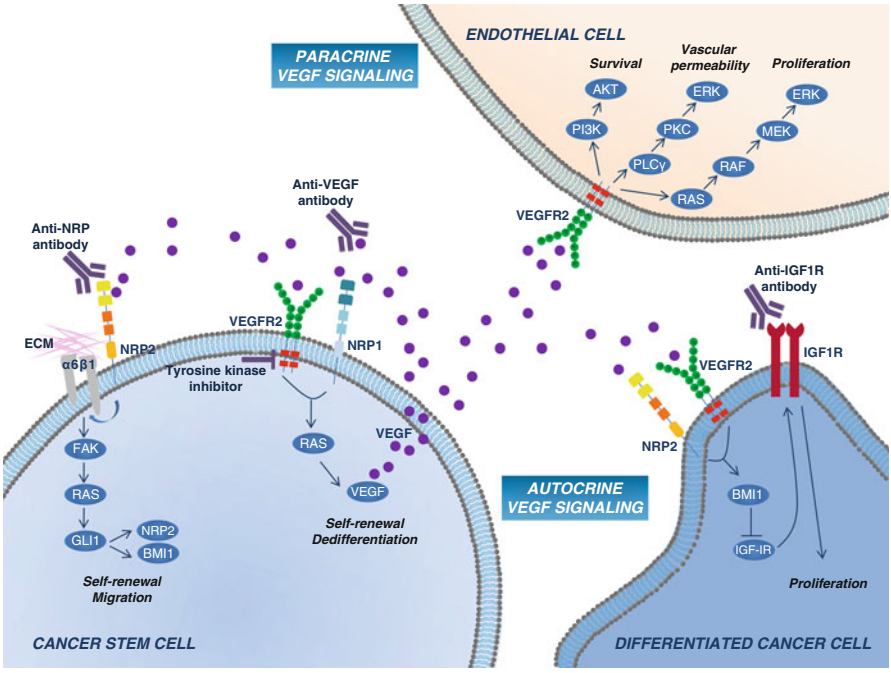


**Fig. 16.2** *Therapeutic strategies to inhibit VEGF signaling in tumor cells*. Besides regulating the common paracrine pathway on endothelial cells to sustain angiogenesis, VEGF signaling, when potentiated by NRPs, exerts its role in the autocrine stimulation of CSC self-renewal and migration. NRP2 can also interact with α6β1 integrin and trigger the integrin-mediated activation of FAK signaling cascade that culminates in the induction of BMI1 and NRP2. NRP1 interaction with VEGFR2 promotes the release of VEGF in the extracellular compartment, sustaining both the autocrine loop and the paracrine endothelial cell activation. Inhibition of VEGF signaling can be achieved mainly by mAb targeting VEGF and small molecules TKIs. mAbs directed against NRPs have been developed and proved to hamper self-renewal and tumorigenic capabilities of CSCs. However, inhibition of NRP2 can lead to compensatory IGF1R expression via BMI1 down-regulation, supporting the importance of multiple therapy administration aimed at targeting both NRPs and IGF1R. Vascular endothelial growth factor (VEGF), Neuropilin (NRP), cancer stem cell (CSC), focal adhesion kinase (FAK), monoclonal antibody (mAb), tyrosine kinase inhibitor (TKI), insulin-like growth factor 1 receptor (IGF1R), extracellular matrix (ECM)

following tumor-derived Shh stimulation. PlGF only interacts with NRP1 rather than with VEGFR1 on medulloblastoma cells, for the enhancement of tumor spread. Authors suggested that the use of anti-NRP1 and –PlGF, in concert with standard chemotherapy, could make an additional improvement in the clinical setting (Snuderl et al. 2013).

Another example of multiple compensatory signaling activation was shown by Lu et al.. Indeed, bevacizumab treatment fostered an invasive phenotype in an in vivo model of GBM. The inhibition of VEGF suppressed the recruitment of the protein tyrosine phosphatase 1 B (PTP1B) from the VEGFR2/MET complex,

consequently restoring hepatocyte growth factor (HGF)-mediated MET phosphorylation and tumor invasiveness. Authors suggested that in selected patients with GBM, tumor recurrence could be avoided by the combined use of anti VEGF and anti MET treatments (Lu et al. 2012).

### *4.3 Targeting Microenvironment Stimuli*

AMD3100 is an antagonist of CXCR4. This drug, in combination with G-CSF to improve hematopoietic stem cell mobilization to peripheral blood for autologous transplantation, was approved in 2008 by the FDA for clinical use as a treatment for non-Hodgkin's lymphoma and multiple myelomas (DiPersio et al. 2009b; DiPersio et al. 2009a).

Commonly used for leukemia in several clinical trials, AMD3100 prevents $CXCR4^+$ leukemia cell recruitment to the SDF-1-secreting bone marrow microenvironment, thus rendering cancerous cells more susceptible to cytotoxic drugs (Burger and Peled 2009). In agreement with this, invasive $CD133^+$ pancreatic CSCs expressed CXCR4 and predominantly metastasize in the liver, being attracted by a gradient of SDF1, which is secreted by the stroma compartment (Hermann et al. 2007).

Recently, CXCR4-SDF1 signaling has been identified as the driving force behind the establishment of bone metastasis in triple negative breast cancers. Particularly, CAF-rich stroma found in primary breast cancer secretes SDF-1 and IGF and selects tumor cell clones with high Src activity and thus, characterized by an activation of PI3K-AKT pathway. Src hyperactive clones were primed for bone metastasis because endowed with a greater chance of survival in the bone environment enriched with SDF-1 and IGF. Mechanistically, human mesenchymal stem cells were stimulated with a conditioned media from MDAMB231 cell line to constitutively secrete SDF-1 and IGF. Subsequently, authors cotransplanted breast cancer cell lines and stromal cells in an orthotopic mouse model. Following an in vivo treatment with CXCR4 inhibitor (AMD3100) and IGF1R inhibitor (BMS754807), the recovered cells were reimplanted and resulted in tumors, low in bone metastasis, compared to reimplanted cells from untreated tumors (Zhang et al. 2013).

Similarly, we recently showed that in colorectal cancer, the exposure to SDF1, HGF and OPN, increased the migratory capabilities of colorectal CSCs and induced the CD44v6 expression, an alternative splicing isoform of CD44, on transiently amplifying progenitors. Interestingly, in untreated colorectal CSCs, CD44v6 was already highly expressed whereas, it was lower in sphere-derived differentiated progeny and bulk primary cells. CD44v6 acts as a coreceptor of the tyrosine kinase receptor MET, and together with its ligand, the pleyotropic cytochine HGF, cooperates to promote survival and migration through the PI3K-AKT pathway. When blocking SDF-1-CXCR4 activity with AMD3100, it reduced the invasive potential and abrogated the CD44v6 expression induced by HGF and OPN. Similarly the PI3K inhibitor, BKM120, killed $CD44v6^+$ colorectal CSCs and impaired metastatic dissemination (Todaro et al. 2014). It is worth considering that targeting these

powerful effectors in the tumor microenvironment could have tremendous therapeutic implications. In this context, the use of compounds which, target both MET and HGF, are still under evaluation in several clinical trials (Peters and Adjei 2012) and only few of them were recently approved by the FDA. Although discovered as a MET tyrosine kinase inhibitor, Crizotinib was approved at the end of 2013 exclusively for the treatment of NSCLC as an ALK blocking compound (Malik et al. 2014). Similarly, Cabozantinib is a multi-kinase inhibitor against VEGFR1, 2 and 3, RET, MET, TIE-2 and KIT and is currently administered uniquely for progressive medullary thyroid cancer (Elisei et al. 2013). Clinical trials for prostate, brain, breast, and NSCLC are still undergoing (http://www.cancer.gov/clinicaltrials).

## *4.4 Hypoxia as a Therapeutic Target*

Evidence that CD44 variant isoforms (CD44v) could promote survival and multidrug resistance has been shown by Ishimoto et al. In gastrointestinal cancer cells, CD44v enhanced the synthesis of reduced glutathione (GSH), the predominant intracellular antioxidant factor, by physically interacting with and stabilizing the cystine transporter subunit (xCT) at the plasma membrane. xCT is the light chain subunit of the cysteine-glutamate exchange transporter, which exchanges intracellular glutamate for extracellular cysteine, required for GSH synthesis. GSH protects the cell against reactive oxygen species (ROS) damages and suppresses $p38^{MAPK}$ activation, leading to cancer cell proliferation and resistance to ROS-inducing agents, such as docetaxel and cisplatin. As a result of these findings, in vivo exposure to sulfasalazine, a selective xCT inhibitor, induced $p38^{MAPK}$ signaling, enhanced response to chemotherapy, and avoided CD44-dependent tumor growth. Therefore, authors suggested that either sulfasalazine or CD44v-target therapy could abrogate ROS defense capabilities of CSCs and in turn sensitize to conventional cancer treatments (Ishimoto et al. 2011).

Normal stem cells as well as CSCs, harbor low levels of ROS and possess an efficient defense mechanism against oxidative stress (Diehn et al. 2009). An increase in ROS levels can occur in response to either environmental extrinsic (e.g. CAFs, CAMs, and hypoxia) or intrinsic oxidative stress (e.g. ROS producing enzyme and Jun D down-regulation), along with iron chelators, nitric oxide (NO), and genetic alterations in PTEN, von Hippel-Lindau (VHL), succinate dehydrogenase (SDH), RAS-MAPK, and PI3K-AKT accounts for the hypoxia-inducible factor 1 α (HIF-1α) stabilization (Moeller et al. 2004; Li et al. 2007; Lu and Kang 2010). As well as under normoxia, HIF-1α exerts its role in shielding tumor cells from oxygen deprivation and thus aids in meeting the metabolic requirements of the expanding tumor mass. The HIF family of transcription factors has a prominent role in a finely tuned and well characterized oxygen-sensor mechanism. They comprise a heterodimer of an oxygen dependent α–subunit (either HIF-1α, HIF-2α or HIF-3α) together with a constitutively expressed β-subunit (HIF-1β). Under normoxic conditions and

in presence of iron, prolyl hydroxylases (PDH) modifies Pro402 and Pro564 of HIF-1α and promotes the interaction with VHL, leading to ubiquitination and proteasomal degradation. It prevents HIF-1α to dimerize with HIF-1β and to bind with the coactivator CBP/p300 to the hypoxia response element (HRE) in the promoters of hypoxia-target genes, regulating proliferation/apoptosis, glycolysis, angiogenesis, and invasion/metastasis (Harris 2002). A high HIF-1α level is observed in many human cancers and is associated with poor prognosis in brain, breast, ovary, cervix, colorectal, prostate, bladder, and oropharynx cancers (Semenza 2003; Talks et al. 2000). Particularly, HIF-1α has been reported to be hyperactivated in TNBCs and necessary for the maintenance of the $CD44^{high}CD24^{low}$ cell population. Chen et al. identified XBP1, a component of the unfolded protein response (UPR) pathway, as a major controller of HIF-1α transcriptional activity in TNBCs. It is required for tumor relapse in a murine model and directly enriches the $CD44^{high}CD24^{low}$ population in vitro. XBP1 can also be associated with poor prognosis, suggesting that combinatory therapy using stem cell targeting drugs, such as inhibitors of the UPR pathway and standard chemotherapy may improve cancer therapeutic intervention (Chen et al. 2014).

A tight relationship exists between hypoxia and tumor dissemination. Low oxygen levels in tumor microenvironment promote the overexpression of EMT master regulators such as SNAIL, TWIST, and ZEB1, while it attenuates E-cadherin expression. Matrix remodeling requires basal membrane degradation via HIF-1α-dependent production of MMP2 and cathepsin D (CTSD). The so-called "invasive–switch" is guided by hypoxia and sustained by MET and lysyl oxidase (LOX) expression. Hypoxia facilitates both intravasation and extravasation of tumor cells through the increased production of VEGFA. Meanwhile, CXCR4, OPN, and Angiopoietin-like 4 (ANGPTL4) increase the chance of homing and outgrowth to secondary organs (Catalano et al. 2013).

HIF-2α also contributes to the hypoxia-driven "angiogenic-switch" and is directly linked to stem cell biology as a regulator of *OCT4* (Covello et al. 2006) and *c-MYC* (Gordan et al. 2007). Given that it displays a restricted tissue-specific expression pattern compared to its homologs, little attention has been given to addressing its pro angiogenic and pro tumorigenic features (Gordan et al. 2007). One key study showed the preferential expression of HIF-2α on GSCs compared to the differentiated and normal counterpart and its association with poor survival in glioblastoma patients. Authors underlined that HIF-2α may support the CSCs niche by providing survival and metabolic advantages through the modulation of *OCT4*, *GLUT1*, and *SERPINB9* expression. This suggests that new therapeutic approaches should be aimed at targeting stem cell specific molecules involved in neoangiogenesis (Li et al. 2009)

On the contrary, besides being a member of the HIF system, HIF-3α's role in the tumor hypoxia-inducible adaptive response system, is not well characterized. Indeed, it lacks the transactivation domain and likely functions as a negative regulator of HIF-1α and HIF-2α due to sequestration of HIF-1β (Kaur et al. 2005).

As previously discussed, preclinical data provide evidence that hypoxic tumor cells play a pivotal role in tumor progression and resistance to therapies. Moreover,

the pro metastatic effect elicited by angiogenesis-induced hypoxia can compromise clinical outcomes in patients. Thus, targeting intratumoral hypoxia can be considered the gold standard to be exploited in neoplastic malignancy. Nevertheless, it is clear that hypoxia is heterogeneously diffused within a given tumor cell population and is endowed with an even more differentiated extension among patient tumors. Based on this observation, an appropriate measuring of tumor hypoxia either by direct or indirect methods, will facilitate the selection of the patient's treatment as well as, the monitoring of their treatment-response (Wilson and Hay 2011). However, an interesting finding recently reported for the first time is that, a chemotherapeutic agent, in this case doxorubicin, can stabilize HIF-1α even in normoxic cells. Indeed, doxorubicin increased the expression of STAT1, with consequent stimulation of iNOS, intracellular synthesis of NO and HIF-1α accumulation (Cao et al. 2013).

In recent years, several drugs have been designed to selectively target chemo- and radio-resistant hypoxic cancer cells. According to the action mechanism, they could be tentatively categorized as (a) agents targeting HIF-1α DNA binding, (b) agents attenuating HIF-1α protein translation, (c) agents inducing HIF-1α protein degradation, (d) prodrugs inducing hypoxia-mediated cytotoxicity (e) HRE-driven expression of enzymes converting prodrugs and (f) agents targeting downstream HIF pathway effectors.

Specifically, HIF-1α function can be directly targeted via chetomin, a small molecule that precludes HIF-1α binding to the transcriptional coactivator p300/CBP (Kung et al. 2004). Similarly, the proteasome inhibitor bortezomib, which has been approved by the FDA for clinical use in multiple myeloma and mantle cell lymphoma patients refractory to at least one prior therapy, affects the C-terminal activation domain (CAD) of HIF-1α. It was shown that bortezomib enhanced the HIF-1α hydroxylation of Asn803 residue, by the dioxygenase factor-inhibiting hypoxia 1 (FIH-1), causing the inhibition of p300-HIF interaction (Kaluz et al. 2006). Intriguingly, anthracyclines, such as doxorubicin and daunorubicin, block HIF-1 binding to HRE sequence, providing new evidence in refining their use as antiangiogenic drugs (Lee et al. 2009).

HIF-1α expression can be modulated by the topoisomerase I inhibitor topotecan, one of the first hypoxia inhibitor ever tested on humans and currently approved for the treatment of small cell lung cancer and recurrent cervix carcinoma. Cardiac glycoside digoxin inhibited the translation of HIF-1α in an mTOR-independent manner. In preclinical settings, PX-478 appeared to inhibit HIF-1α mRNA expression and translation, and foster HIF-1α degradation by preventing its deubiquitination (Onnis et al. 2009). Contrasting data have been generated regarding the contribution of the mTOR pathway in the modulation of hypoxia. Several mTOR inhibitors, such as everolimus and temsirolimus, have been approved by the FDA for clinical use in renal cancer patients and displayed remarkable antiangiogenic activity and inhibition of HIF-1α (Del Bufalo et al. 2006). Hypoxia, especially in early stage tumors, may negatively regulate HIF-1α expression according to the intensity and duration of oxygen deprivation (Wouters and Koritzinsky 2008). Another indirect mechanism of HIF-1α inhibition includes the targeting of upstream

pathways (e.g. PI3K-AKT and RAS-MAPK) involved in HIF-1α protein translation (Poon et al. 2009). Interestingly, the tumor suppressor p53 mediates apoptosis under hypoxic conditions. However, cancer cells with dysregulated p53, escape programmed death and p53-mediated HIF-1α inhibition (Ravi et al. 2000). P53 may either interact with HIF-1α or mediate its degradation through HDM2 (Ravi et al. 2000) or compete with HIF-1α for p300 thus, blocking its transcriptional activity (Schmid et al. 2004). Agents targeting p53, aim at reactivating mutant p53. This is the case of RITA (reactivation of p53 and induction of cell apoptosis), which induces DNA damage in order to stimulate p53-evoked cell apoptosis and inhibits MDM2 to prevent p53 degradation. This mechanism seems to be hypoxia-independent (Yang et al. 2009).

HIF-1α degradation may be forced by the inhibition of chaperone HSP90. In normoxia and hypoxia, the HSP90 antagonists GA and 17-AAG mediate elimination of HIF-1α through E3 ubiquitin ligase and reduces angiogenesis in vivo (Isaacs et al. 2002). Trichostatin A is an inhibitor of HDAC and promotes proteasome-dependent HIF-1α degradation in osteosarcoma (Yang et al. 2006). Similarly, HDAC inhibitors FK228 and LAQ824 resulted in the abrogation of HIF-1α activity (Mie Lee et al. 2003; Qian et al. 2006). Of note, SAHA, the potent pan HDAC inhibitor, may act together with TRAIL, in breast cancer orthotopic models and down-regulate both VEGF and HIF-1α (Shankar et al. 2009).

One promising approach seeks to develop prodrugs that can be reduced by hypoxia in prodrug radicals, as intermediate products. In normoxia, they can be re-oxidized and converted back by oxygen while in hypoxic cells they can be either further reduced or fragmented so as to generate an active toxic drug. Examples of bioreactive prodrugs still in clinical development include RH-1, mitomycin C, AQ4N, PR-104, and SR4233. Some concerns have been reported regarding the prodrugs' penetration into poorly perfused tumors and their toxicity. The activation of aerobic reductase also in normal tissues or the additional generation of DNA reactive cytotoxins, make it hard to combine bioreductive prodrugs with standard chemotherapy (Wilson and Hay 2011).

Moreover, in tumoral cells prodrugs can be converted into cytotoxins by a hypoxia-regulated expression vector which, encodes the enzyme responsible for this reaction. Hypoxia targeted gene therapy has been tested in a preclinical setting and consists in the expression, in tumoral cells, of plasmid vector carrying genes driven by a promoter containing HRE and encoding: thymidine kinase (TK), cytosine deaminase (CD), uracil phosphoribosyltransferase (UPRT), and flavoprotein cytochrome c P450 reductase (CPR) (Patterson et al. 2002; Hsiao et al. 2014). A triple suicide gene therapy has proven to enhance cytotoxicity to ganciclovir and 5 fluorocytosine and sensitize colorectal cancer cells, both in vitro and in vivo, to radiotherapy by simultaneous expression of TK, CD and UPRT (Hsiao et al. 2014).

Finally, multiple agents also aim at targeting the downstream component of the HIF signaling pathway such as the LOX inhibitors, β-aminoproprionitrile (βAPN) or anti-LOX antibody, which binds the LOX active site and blocks its enzymatic function (Erler et al. 2009)

## 5 Challenges and Limitations of Targeting Cancer Stem Cells and Their Niche

Conventional anti-cancer drug development has been focused on the identification of cytotoxic chemotherapeutic agents that can target deregulated pathways and molecular markers in tumor cells. Despite all efforts, patients undergoing chemotherapy, after an apparent remission, often relapse and develop more aggressive diseases. This emphasizes that CSCs may be responsible for therapy failure due to the specific activated mechanisms which are peculiar to the undifferentiated status of these cells. In this context, novel compounds have been precisely designed to eliminate CSCs or affect their microenvironment and, administered in concert with conventional chemotherapy, can lead to tumor bulk shrinkage and ablate resistance and relapse. Of note, there is a need to refine such therapies to counteract their side effects. Indeed, such approaches could impair normal stem cell niches, have 'off target' effects on signals required for normal cells survival or, and as well as standard treatments, they should be administered at concentrations harmless to patients.

## 6 Conclusion and Future Perspectives

The reviewed data show only a partial portion of the existing therapies in the field. Anyhow, they seek to emphasize that despite of the efforts that have been made to develop powerful CSCs targeted therapy, multiple obstacles still need to be faced for the achievement of long lasting clinical benefits. The future use of appropriate tumor models and technologies reflecting the phenotypic, genetic and epigenetic tumor heterogeneity constantly evolving to counteract the hostile milieu, will possibly overcome the achieved disappointing results. Moreover, a multitude of new inhibitors are currently being investigated and will possibly conduct to some encouraging experimental evidence.

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