HHS Public Access

Author manuscript

Pharmacol Ther. Author manuscript; available in PMC 2017 April 01.

Published in final edited form as:

Pharmacol Ther. 2016 April ; 160: 145–158. doi:10.1016/j.pharmthera.2016.02.008.

Cancer Stem Cells and Chemoresistance: The Smartest Survives the Raid

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Abstract

Chemoresistant metastatic relapse of minimal residual disease plays a significant role for poor prognosis of cancer. Growing evidence supports a critical role of cancer stem cell (CSC) behind the mechanisms for this deadly disease. This review briefly introduces the basics of the conventional chemotherapies, updates the CSC theories, highlights the molecular and cellular mechanisms by which CSC smartly designs and utilizes multiple lines of self-defense to avoid being killed by chemotherapy, and concisely summarizes recent progress in studies on CSC-targeted therapies in the end, with the hope to help guide future research towards developing more effective therapeutic strategies to eradicate tumor cells in the patients.

Keywords

Cancer stem cell; chemotherapy; chemoresistance; targeted therapy

1. Introduction

1.1. Chemotherapy and chemoresistance

Chemotherapy is one of the current mainstream anticancer therapies. It uses chemicals to kill rapidly dividing cancer cells. These chemicals or chemotherapeutic agents typically induce apoptosis by damaging DNA and/or inhibiting mitotic division (1–3). Despite being an important therapeutic option for most cancer patients and the many agents and strategies available, chemotherapy frequently misses slowly dividing and non-dividing cancer cells. It also leaves behind fast-dividing cancer cells that are less sensitive particularly when reduced doses are given to avoid side effects on rapidly dividing normal cells. The insensitivity of these cancer cells causes resistance to chemotherapy or *chemoresistance that* is a major cause of treatment failure with treatment strategies involving chemotherapy (4). Some of the tumor cells are already resistant to the “achievable” doses of anticancer drugs. This type of resistance present in therapy-naïve patients is named *intrinsic resistance*. Other tumor cells are initially sensitive but become resistant to the drug during the course of treatment. This

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Conflict of interest statement

The author declares that there are no conflicts of interest.

therapy-induced resistance is termed as *acquired resistance*. Although not pre-existing, *adaptive resistance* can occur immediately through a rapid rewiring of cancer cell signaling once treatment begins. Regardless of the resistance mechanisms, the chemoresistant cancer cells are the major cause of tumor recurrence or relapse and have garnered the most attention clinically (1, 2, 5).

1.2. Tumor recurrence or relapse

Tumor recurrence or relapse was initially tied to a small number of therapy-resistant leukemic cells that remained in the patient during treatment or post-therapeutic remission without overt symptoms or signs of disease. This phenomenon was termed as *minimal residual disease (MRD)*. It is the major cause of leukemic relapse. This terminology was then extended to non-leukemic cancer types particularly carcinomas and is divided into *local residual disease* and *metastatic residual disease* (or micrometastasis) depending upon the location of the residual disease or the recurrent tumor in relation to the primary tumor location. Importantly, minimal residual diseases are highly enriched for cancer stem cells (6).

1.3. Cancer stem cells

The cancer stem cell (CSC) concept was first reported more than a century ago on observing the histological similarities between tumors and embryonic tissues. A tumor contains heterogeneous populations of cells. CSCs are a very small subset of relatively quiescent cells in the tumor that are endowed with the ability to self-renew and differentiate into non-stem daughter cells that make the bulk of tumor, which is the central property of normal stem cells as well (7–10). The concept may explain not only why clonogenic capacity of most malignancies is very low but also why complete initial responses to treatment rarely leads to cures. The initial responses mirror the therapeutic effect on the cells that form the tumor bulk while leaving behind CSCs that are responsible for relapse, the real threat to tumor cell eradication (11, 12).

Growing evidence supports the notion that cancer is a disease driven by CSCs responsible for tumor initiation, growth, metastasis, therapy resistance, relapse and poor prognosis. The isolation of CSCs was first described in 1994 from leukemia and then from solid tumors of many cancer types including breast, lung, prostate, colon, brain, head and neck, skin, and pancreatic cancers (13). Despite extensive studies, there has been a long-lasting debate on the existence of CSCs and their roles in patients. For example, in contrast to the CSC model, a stochastic model describes that all tumor cells in the late stage of melanomas are equipotent and stochastically self-renew or differentiate, resulting in tumor heterogeneity with presumably different CSC types endowed with distinct therapy-resistant phenotypes (12, 14). As a result, more conservative terms instead of CSC have been used such as tumor-initiating cells, tumor-propagating cells, tumor-progenitor cells and tumor stem-like cells.

Direct *in vivo* evidence of CSC existence in mice came in three back-to-back research articles published in 2012 (15–17). The first group developed a genetically engineered mouse model of glioma. In that model, a nestin-DTK IRES-GFP transgene, alongside the HSV thymidine kinase gene was made that specifically labels quiescent adult neural stem

cells. This transgene was also expressed in a subset of spontaneous glioma tumor cells. After the tumor growth was transiently arrested using temozolomid followed by pulse-chase of the tumor growth, the tumor was able to re-grow only from the transgene-expressing cells. If these cells in the tumor were ablated with ganciclovir, the tumor growth and re-growth were inhibited. Thus the study concluded that a subset of quiescent endogenous tumor cells serve as CSCs responsible for sustaining long-term growth of glioma (15). The second group genetically labeled individual tumor cells *in vivo* and traced them over the progression of papilloma and squamous cell carcinoma. The study demonstrated that only a very small fraction of the labeled cells with CSC characteristics persist long term to grow and form a significant part of the tumor (16). The third group genetically labeled and traced primary intestinal adenoma cells that express the crypt stem cell marker Lgr5. The study revealed that these $Lgr5^+$ cells represent only 5–10% of the tumor cells and could self-renew as well as differentiate into all other adenoma cell types, indicating they are the CSCs (17).

Clinical evidence for CSCs came first from patient studies of MRDs. Post-therapeutic residual tumor cells of breast cancer are found enriched for CSCs (18). The presence of acute myeloid leukemic (AML) CSCs in the minimal residual diseases correlates with poor progression-free survival (19). The myelodysplastic syndrome (MDS) stem cells persist in the patients even with complete clinical and cytogenetic remissions (20). In a latest study the same group reported more direct evidence for CSC presence in patients (21). By establishing molecularly and functionally distinct and hierarchically organized stem and progenitor cell compartments of MDS and back tracking somatic genetic lesions, the group demonstrated that the rare $Lin^-CD34^+CD38^-CD90^+$ CD45RA MDS cell population functions as MDS-propagating cells in the patients. Elimination of this population is essential toward eradication of the entire MDS clone. This discovery strongly supports the CSC theory and could encourages development of CSC-targeted therapies and CSC-based relapse-predicting strategies.

It remains unclear, however, whether CSCs originate from normal stem cells or non-stem cells and what are the driving forces for the transformation process. Regardless, it seems that the physiological balance between the proto-oncogenes and tumor suppressor genes needs to be disrupted and in some cases the epithelial-mesenchymal transition (EMT) process must be involved for CSC formation (22, 23). Hypothesized origins and mechanisms of CSC formation are illustrated in Figure 1.

2. Mechanisms of CSC chemoresistance - the multiple lines of defense

Despite the intensive studies in the past, the mechanisms by which tumors become chemoresistant and the clinical causes of chemoresistance remain only partially understood (24, 25). Recent studies have suggested CSC as a main player for chemoresistance against a variety of drugs including cisplatin and/or paclitaxel (26–32), temozolomide (15, 30), etoposide (29), and doxorubicin or methotrexate (31, 33) in many types of cancer including gliomas and glioblastoma (15), breast (26, 27), colorectal (34–38), AML (39, 40), hepatic (33), lung (41), bone (42), pancreatic (30, 32), ovarian (28) and prostate (31) cancers. Thus, the cellular and molecular mechanisms underlying the CSC's chemoresistance have drawn accelerating attention for cancer research (37, 43–49). Although still poorly understood,

CSCs use a series of self-defense lines against attacks by the chemotherapeutic drugs. These defense lines are outlined in Figure 2 and discussed in details below.

2.1. Avoid cellular exposure to the drugs – the 1st line

2.1.1. CSC niches—Normal adult stem cells (ASCs) are usually located at places that are less exposed to potential external attacks. For example, the skin stem cells are housed in the bulge at the side of hair follicle away from the papilla and matrix that are connected with the capillary (50), the mammary stem cells hide under the luminal epithelium away from the acinar lumen (51), the intestinal stem cells are located at the very bottom of the crypt farthest away from the intestinal lumen (52), and the hematopoietic stem cells are shielded in the bone marrow and guarded by the hard bone (53).

CSCs are similarly protected physically albeit in slightly different ways. CSCs take the maximal advantage of the specific tumor microenvironments they are localized in. These particular compartments are called CSC niches. Like normal stem cell niches, CSC niches are thought to maintain a balance between self-renewal and differentiation and therapy resistance of CSCs through either extracellular matrix protein-cell or cell cell communication (54). Although the niche concept is generally accepted (55), the exact types of cells and proteins that constitute these niches remain largely elusive. Still under investigation, if not in debate (56) are the two CSC niche types, the *hypoxic niche* and the *perivascular niche*.

The *hypoxic niche* (12) has been demonstrated for hematopoietic stem cells (HSC) (57, 58), melanoma (59), leukemia (60, 61), brain (62–65), prostate (66, 67), lung (68), pancreatic (69) and breast (70) cancers. Hypoxia is a hallmark of cancer and an important factor for disease progression, therapeutic resistance and poor prognosis. Hypoxia regulates many aspects of cancer including cell survival, proliferation, differentiation, angiogenesis, metabolism, invasion and metastasis. The primary mediator of hypoxia is the hypoxia-inducible factor (HIF) subunits HIF1α and HIF2α, the regulators of tissue oxygen homeostasis (71). Hypoxia plays an important role for stem cell function via HIF (72). Deletion of *hif1α* leads to inhibition of HSC self-renewal (73) and the clonogenecity of leukemic CSCs (60, 61). In brain, pancreatic and breast cancers, hypoxia has been shown to promote the CSC expansion (62–65, 69, 70). Oxygen plays a crucial role in generating ROSs that mediate the anticancer effects of therapies such as radiotherapy. The low oxygen tensions in the hypoxic area of the tumor thus contain low levels of ROSs, reducing the risk for the cells located in the hypoxic area of being killed (71). Indeed, normal adult epithelial, glial and neuronal stem cells contain lower levels of ROSs than the differentiated progeny (74–77). Upon receiving irradiation, the MCF-7 breast CSCs in mammospheres showed a lower level of ROSs and DNA damage than cells in monolayer culture, suggesting high levels of free radical scavengers in the CSCs (56)

The hypoxic niche concept came at a bit surprising timing in that prior to it many studies had pointed to an essential role of endothelial cells and the *perivascular niche* for CSCs as was demonstrated for normal stem cells. Tumor vasculatures are required for delivery of nutrients and oxygen, recruitment of stromal cells, endocrine trafficking of growth factors and cytokines and disposal of metabolic wastes (78). In glioblastoma and squamous cell

carcinoma, CSCs are localized in close contact with endothelial cells (79–81). Anti-vascular endothelial growth factor (VEGF) receptor (VEGFR2) treatment causes CSC depletion resulting in inhibition of tumor growth or regression of tumor (12). These studies suggest that perivascular niches involving VEGF signaling are crucial for maintaining CSCs or tumorigenic potential. It should be noted, however, that anti-angiogenic therapies have not been as successful as expected in the many years of clinical trials. One of the reasons for the failure could be that the therapies enhance the hypoxic conditions good for maintaining the hypoxic niches of CSCs. As such, the vascular normalization in tumor has emerged as an important therapeutic strategy (82).

At the first thought, the two concepts for CSC niches seem paradoxical. However, they may coexist in distinct compartments of the same tumor and represent distinct requirements of CSCs. Indeed, studies have shown that HSCs reside in a vascular niche (83, 84) and in a hypoxic niche as well (57, 58). ER^+ and ER^- breast CSCs seem to have distinct preference to hypoxic and perivascular niches, respectively (57, 58, 83, 84). Subsets of CSCs in head and neck squamous cell carcinoma (HNSCC) differ intrinsically in their patterns of oxygen metabolism (68). These studies indicate that both of the niche types are required for CSCs and they may not be independent of each other. Rather, they may be interrelated and HIF-1, the critical regulator for both hypoxia and angiogenesis, may serve as the bridge or switchman between the two niche types. Whether or not the CSCs localized in the hypoxic niches are even the source of the CSCs localized in the perivascular niche (that houses CSCs ready to differentiate yet easier to kill) is worth further investigation. Nevertheless, HIF-1 may be an important molecular target for anti-CSC therapy (85).

2.1.2 CSC-specific stroma—In addition to physically protecting the niches, other components of tumor microenvironment including extracellular matrix (ECM), cancer-associated fibroblasts, immune cells and inflammatory cells (86, 87) also play a role in protecting CSCs against both chemotherapy and other therapies (88). They provide CSCs with resistant signaling stimuli through surface receptors to activate other lines of defense for CSCs. For example, tumor myofibroblasts secrete growth factors such as hepatocyte growth factor (HGF) and periostin to activate Wnt signaling required for tumor stemness in colorectal or breast cancer (89, 90). Stromal cells secrete high levels of HGF which makes co-cultured human cancer cells of many types acquire resistance to various anticancer drugs particularly RAF inhibitors (91). Other growth factors or cytokines including interleukin 6 (IL-6), fibroblast growth factor (FGF) and neuregulin 1 are reported to help form the so-called ‘chemoresistant niche’ of CSCs by activating various survival signaling pathways (92, 93). In addition, the ECM receptors integrins are highly associated with CSC survival and drug resistance (94, 95), suggesting critical roles for the ECM. Lastly, the stem cell surface marker CD44, initially identified as the lymphocyte homing receptor, is commonly expressed in embryonic and adult stem cells as well as CSCs (96). It binds its ligand hyaluronan to regulate many aspects of stem cell function including self-renewal, ECM retention in the niche, proliferation, differentiation and homing or metastasis. CD44 signaling also helps maintain the genomic integrity of stem cells by protecting and repairing DNA from oxidative damage. In addition, CD44 is implicated in regeneration of CSCs responsible for chemoresistance and relapse.

2.2. Avoid molecular exposure to the drugs- the 2nd line

2.2.1. ATP-binding cassette transporters (ABCT) and drug efflux—What if the CSCs have failed to avoid a contact with the drugs? At the organismic level, a toxic chemical accidently taken in is detoxified and filtered out primarily by the liver. The first thing a CSC does once the drug has entered the cytoplasm, however, is to activate the 2nd line of defense, i.e., the drug efflux mechanisms to pump the drug out of the cell. This mechanism has been taken advantage of for isolating CSCs as the side-population (SP) cells by flow cytometry that do not accumulate the drug-mimicking dye. The transmembrane proteins of the ABCT family are the main players of drug efflux (97). Among 49 family members three have been extensively studied and correlated with drug resistance. They are the multidrug resistance protein 1 (MDR1 or ABCB1), multidrug resistance-associated protein 1 (MRP1 or ABCC1) and breast cancer resistance protein (BCRP or ABCG2) (97). They can eliminate various types of chemotherapeutic drugs such as taxanes, topoisomerase inhibitors and antimetabolites. MDR1 are normally expressed on the surface of epithelial cells of intestine and ductules of pancreas, bile and kidney and overexpressed in many tumor types associated with intrinsic resistance (98, 99). Its expression can also be induced by chemotherapy which is associated with acquired resistance (100). MDR1-associated resistance has been seen in kidney, colon, liver, prostate, lung and breast cancers as well as leukemia (101–103). MRP1 has been used as a marker of prediction for chemoresistance (104). BCRP is associated with drug resistance in breast cancer and leukemia (105, 106). Interestingly, MDR1 and BCRP can also perform efflux of small molecule drugs (107). Clinical trials of MDR1 inhibitors such as zosuquidar and tariquidar, however, have been disappointing in breast (108) and squamous cell carcinoma (108, 109) even when used in combination with docetaxel (109). This suggests that a high degree of functional redundancy among the ABCT family members is a critical factor for this particular defense line of CSCs. Indeed, CSCs of several cancer types show high expression of multiple ABCTs (110, 111). The NCI60 panel studies indicated a role in drug resistance for majority of the family members (112). As such, finding the ‘Achilles heel’ of the redundancy is key for ABCT-targeted anti-CSC therapy.

2.3. Avoid conditions needed for the drugs to act- the 3rd line

2.3.1. Intracellular drug inactivation—What if the drug efflux has failed and the drug has invaded the cytoplasm of CSCs? Interestingly, the poison filtering organs like the liver lack ABCTs. However, liver cells are highly drug-resistant because the cells contain enzymes such as the cytochrome-c p450 that can effectively detoxify the drugs in the cytoplasm. On the other hand, some therapeutic drugs are given in a pro-form that needs to be activated after administration. Tumor cells that lack the drug inactivating enzymes or possess the drug activating enzymes are sensitive to the drugs. Conversely, high levels of drug inactivating enzymes or low expression of the drug activating enzymes would make the cells resistant to the drugs as in the case of CSCs. Below are a few examples of such enzymes. The *thiol glutathione* inactivates platinum drugs (113). *Thymidine phosphorylase* converts capecitabine into 5-fluorouracil (5-FU) (114). The antimetabolite drugs such as 5-FU and methotrexate need to be converted further into their most active forms to be therapeutically effective, which does not occur when the target cells lack the enzyme

activities (115, 116). For example, in CSCs expression of the thymidine phosphorylase can be inhibited via epigenetically silencing mechanisms such as gene promoter methylation catalyzed by the *DNA methyltransferases (DNMTs)*, resulting in capecitabine resistance (117). Using inhibitors of DNMTs can thus sensitize the CSCs to capecitabine. Epigenetic silencing can also activate drugs. For example, *UDP glucuronosyltransferase 1 (UGT1A1)* is an enzyme that inactivate the topoisomerase I inhibitor irinotecan which is associated with the drug resistance. If UGT1A1 gene promoter is methylated in the cells, irinotecan activity is maintained that sensitizes the cells (118, 119). The *aldehyde dehydrogenase (ALDH)* is a major marker of CSCs in many cancer types (120) associated with poor prognosis (121–123). ALDH catalyzes the oxidation of aldophosphamide to carboxyphosphamide, the primary mechanism of cyclophosphamide detoxification (124) that occurs in normal stem cells to protect them. This same mechanism, however, is hijacked by CSCs to counteract cyclophosphamide and various relevant chemotherapeutic drugs including temozolomide (125), 4-hydroperoxycyclophosphamide (126), irinotecan (38), paclitaxel, epirubicin and doxorubicin (127, 128).

What if the drug inactivation mechanisms fail in the cytoplasm? Theoretically, the CSC would do something to protect important cytoplasmic components from being damaged and to prevent the drug from further entering the nucleus and subsequently damaging the genomic DNA. Chemotherapy has been shown to activate autophagy. *Autophagy* is a cellular protective mechanism that promotes cell survival by sacrificing some relatively less important cytoplasmic components in response to catastrophic stresses derived from metabolic, therapeutic or mechanical pressure. Several reports have shown that blocking autophagy pathways using an inhibitor of autophagy such as chloroquine and hydroxychloroquine can sensitize cancer cells to chemotherapies (129, 130). Although extensive studies are needed in order to understand the details regarding role of autophagy for chemoresistance, it would be interesting to test if there are any ABCTs expressed on the membrane of autophagosomes. Do these ABCTs cleanse the drug that has already entered the cytoplasm by pumping the drug from the cytoplasm into autophagosomes? Then does the cell somehow send the drug-loaded autophagosomes out of the cell? Recent studies have shown that in addition to the plasma membrane, nuclear membrane also expresses some of the ABCTs (131–134). Do these ABCTs, like the cell surface ABCTs, pump the drug that has entered the nucleus out of it so that the nuclear DNA can be protected? This would be another interesting possibility to look into.

2.3.2. Quiescence and dormancy—What if the drug has already entered the cytoplasm and even nucleus and is ready to attack the vital intracellular machineries? What does the cell do in this phase to avoid the state susceptible to the potential attack? One of the ‘safe’ states is non-dividing, i.e., quiescence or dormancy. As described above, chemotherapy (as well as radiotherapy) targets fast proliferating cells. It is generally believed that both normal ASCs and CSCs stay in the quiescent phase of the cell cycle most of the times (135–137). Therefore, unlike the rapidly dividing cells, the relatively quiescent CSCs can avoid the attacks from most of the chemotherapeutic drugs. A recent study tracing glioma stem cells in a transgenic mouse model (15) has demonstrated that the slow-cycling tumor cells survived the alkylating drug temozolomide, which eventually led to recurrence. The tumor bearing

mice survived for a much longer time if the slow-cycling cells were removed from the tumor. In melanoma, expression of the H3K4 demethylase JAR-ID1B is restricted to a small subset of slow-cycling cells with a doubling time of > 4 weeks. Knockdown of JARID1B from these cells leads to their failure to expand, resulting in reduction in tumor growth and metastases (138). A very small subset of slowly cycling cells specifically retaining the lipophilic labeling dye DiI or the carboxyfluorescein diacetate succinimidyl ester was identified in pancreatic, colon and breast tumors to be responsible for the invasive potential, chemoresistance and relapse (139, 140). Several studies have shown that induction of dormant leukemic CSCs to enter the cell cycle using granulocyte colony-stimulating factor (G-CSF), promyelocytic leukaemia protein (PML) inhibitors or histone deacetylase inhibitors can prime the sensitization of the otherwise slow-cycling CSCs to the subsequent chemotherapies (39, 141, 142).

2.4. Fix damages caused by the drugs - the 4th line

2.4.1. DNA-damage response and repair—The vast majority of chemotherapeutic drugs kill cells by inducing DNA damage (143). Fast dividing cells are sensitive to chemotherapy because they give the drug more chances to interact with and damage their nuclear DNA. DNA damage is inevitable as long as the cell has exposed its DNA to the drug even if to a much slower rate as in the case of slow-cycling CSCs. In normal cells there exist a variety of DNA damage response (DDR) and DNA repair mechanisms that constantly monitor and repair DNA damage to maintain the genomic stability and protect the cell from DNA damage-mediated death. Unfortunately, these mechanisms are inherited by tumor cells and even enhanced in CSCs (144). Unless un-repairable DNA damage occurs, DNA repair is another main reason for chemoresistance of tumor cells particularly CSCs (143, 145).

DNA repair mechanisms are largely categorized into two classes, 1) those that fix single-strand damages including *dealkylation* (that removes alkyl group from DNA to restore normal DNA structure), *base excision repair (BER* that removes the wrong base to fix minor, non-structure-distorting damage to DNA), *nucleotide excision repair (NER* that replaces a fragment of the strand containing a DNA adduct to fix larger, helix-distorting damage), *mismatch repair (MMR* that corrects miscopied DNA sequences overlooked by DNA polymerases that are responsible for microsatellite instability of the genome) and *error-prone repair (EPR* that fixes the damaged strand based on a guess of identity of the nucleotide damaged using enzymes lacking proofreading capability), and 2) those that fix double-strand breaks including *homology-directed repair (HDR*, a sister chromatid based re-synthesis of DNA to fill the gap formed after the double strand break is excised) and *non-homologous end joining (NHEJ* that re-synthesizes DNA using the resection-generated single strand DNA on the end of the DNA as template to fill the gap formed after the double strand break is excised when sister chromatid is not available particularly on the G1 phase of the cell cycle).

It is an obvious anticancer strategy to combine DNA-damaging agents with DNA repair inhibitors. Inhibiting poly (ADP-ribose) polymerase 1 (PARP1) prevents BER, leading to the double-strand breaks. However, if the cancer cells have a functional double strand break repair mechanism such as the expression of breast cancer associated genes (BRCAs),

inhibiting PARP1 alone would not cause un-repairable, death-inducing DNA damage. Cancer cells that do not express functional BRCAs (146) are particularly sensitive to PARP1 inhibitors, suggesting that targeting redundant DNA repair mechanisms simultaneously for induction of so-called *synthetic lethality* is important to sensitize cancer cells to DNA damaging drugs (147). Notably, aberrant high expression of the excision repair cross-complementing 1 (ERCC1) protein has been associated with chemoresistance to cisplatin in cancer of the lung, stomach and ovary (148, 149) but not testis where there is only marginal expression of ERCC1 (150). MMR deficiency, such as hypermethylation of MLH1 or MLH2, mediates the microsatellite instability that has been linked to resistance of numerous cancer types to a variety of chemotherapeutic agents including the alkylating agents procarbazine, temozolomide, the busulfan, cisplatin and carboplatin, the antimetabolite 6-thioguanine, and the topoisomerase II inhibitors etoposide and doxorubicin (151).

Rapidly growing evidence supports a role of DNA repair for CSC chemoresistance. It has been shown that DNA damage checkpoint and repair proteins such as the ATM, Chk1/2, p53, BRCAs and XRCC5 are aberrantly overexpressed or overactivated in CSCs but not in non-CSCs (152), and is further activated by DNA-damaging therapy such as radiation rendering delayed cell division and prolonged DNA repair time leading to resistance (153–155). Indeed, inhibition of the DNA repair pathways can sensitize CSCs of various cancer types to chemotherapy or radiotherapy. Inhibition of Chk1 or ATM sensitizes the $CD24^+$/$CD44^+$/ESA^+ pancreatic or breast CSCs to gemcitabine (156) or radiation (157) treatment. Knockdown of the DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT) or inhibition of the G_2 checkpoint kinase Wee1 sensitizes gliomas to temozolomide as well as radiotherapy (158–160). Inhibition of DNA-PKcs sensitizes glioblastoma CSCs to radiation-induced death (161). BMI1, the polycomb group E3-ubiquitin ligase, plays a critical role in assembling DNA repair machinery. Recent studies have shown that BMI1 expression is higher and enhanced by irradiation in CSCs (162–165). Ablation of bmi1 in CSCs promotes double-strand breaks and subsequent radiosensitivity whereas overexpressing BMI1 increases radioresistance (163, 166). These data support DNA repair pathway targeted therapies to overcome CSC resistance.

2.5. Lift up the pro-survival and anti-apoptotic power - the 5th line

2.5.1. Over-activation of pro-survival signaling—If left unrepaired, DNA damage will eventually lead to programmed cell death or apoptosis. Apoptosis is a physiological program controlled by a tightly regulated balance between pro-survival, anti-apoptotic mechanisms and pro-apoptotic mechanisms that eliminate sick, aged or unwanted cells. However, in cancer cells, this balance is frequently and aberrantly tilted to prevent apoptosis even when DNA repair fails. The same is more evident in CSCs. Similar to normal stem cells CSCs rely on specific signaling pathways for maintaining essential proliferation, survival and the balance between self-renewal and differentiation. In response to therapies, CSCs either over-activate pro-survival and anti-apoptotic signaling or down-regulate pro-apoptotic signaling as another mechanism of resistance to therapies.

Several pro-survival signaling pathways have been indicated to play a role in CSCs including the pathways of Notch, Hedgehog (Hh), Wnt, epidermal growth factor receptors

(EGFR), bone morphogenetic proteins (BMP), insulin growth factor (IGF), extracellular signal regulated kinases (ERKs), phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB or AKT), Janus kinase/signal transducers and activators of transcription (JAK/STAT), and nuclear factor (NF)-κB. Inhibition of these pathways has led to abrogation of CSCs and tumorigenicity (167–173). *Notch* pathway regulates cell interaction, proliferation, differentiation and apoptosis. Notch signaling can be blocked by impeding its interaction with ligand, inhibiting γ-secretase or interfering with its co-activator MAML1 (167). Inhibition of Notch signaling has been shown to cause a reduction in Akt activity and Mcl-1 expression in glioblastoma CSCs (174) and enhance CSC apoptosis in medulloblastoma (175). Clinical trials of the Notch inhibitors are in progress for treating glioblastoma, leukemia and breast cancer (167). *Hh* pathway plays a role in maintaining tissue polarity and stemness. Overactivation of Hh pathway was found in CSCs of breast cancer and chronic myeloid leukemia (CML) (167). Inhibition of Hh pathway using cyclopamine reduces glioblastoma stemness (176, 177). Clinical trials of the Hh inhibitors are underway for cancers of the breasts, prostate, lungs, and brain (167). *Wnt* signaling regulates cell fate determination and tissue self-renewal. This pathway is highly associated with CSC activity (167). Its overactivation has been shown in 5-FU-resistant colorectal CSCs and silencing its downstream main effector TCF4 sensitizes the tumor to radiotherapy (178). *EGFR* plays a critical role in cancer resistance to various chemotherapies (179–182). EGFR-targeted therapies sensitize various tumor types to agents such as 5-FU, irinotecan, paclitaxel and TRAIL (180–182). Glioblastoma CSCs are resistant to irradiation with aberrantly high *Akt* activity and double strand break DNA repair capacity. Inhibiting Akt in the CSCs by blocking EGF (168, 183) or Wnt (184) signaling suppresses the cell proliferation and enhances the irradiation-induced DNA damages. Inhibition of Akt has achieved similar sensitizing effect in breast CSCs (185). Forced expression of *BMP* receptor 1B leads to differentiation and reduced tumorigenecity of patient glioblastoma CSCs (169). Knockdown of *IGF-R* sensitizes the resistant non-small cell lung cancer (NSCLC) CSCs to radiation (172). Inhibition of *STAT3* induces apoptosis in the CSCs in HNSCC (170) and NSCLC (171) tumors resulting in reduced stemness and tumorigenecity. Inhibition of *NF*-κB hinders the stemness of CSCs in pancreatic cancer (173) and glioblastoma (186). It should be noted that these stem cell signaling pathways often act together and for this reason, simultaneous inhibition of more than one of them is likely needed for effective CSC targeting.

2.5.2. Overactivation of anti-apoptotic and overinhibition of pro-apoptotic signaling—Chemoresistance of cancer cells are typically linked to aberrantly overexpressed or overactivated anti-apoptotic proteins including the BCL-2 family proteins, inhibitors of apoptosis (IAPs) and the caspase 8 inhibitor FLIP (187) and aberrant reduction in expression or activity of the pro-apoptotic proteins such as BIM. These proteins are localized at mitochondria where they play counteracting roles against each other (188–190). Drugs mimicking the IAP antagonist second mitochondria-derived activator of caspases (SMAC) or causing IAP protein degradation have been shown to sensitize various tumor types to chemotherapy and their clinical trials are underway (191). Germline deletion of bim gene has been reported in an Asian population of cancer patients with CML and EGFR-mutated lung cancer intrinsically resistant to tyrosine kinase inhibitor (TKI) therapies (192). Treatment with TKIs induces BIM expression and apoptosis in these cancer cells with

inhibition of Akt and Erk (193–195). For this reason, BIM expression has recently been used to predict clinical responsiveness to inhibitors of EGFR, HER2 and PI3K (196). A recent study has shown that treatment with pro-apoptotic BH3 peptide can sensitize chemoresistant cells of several cancer types to chemotherapy (197). This indicates that BCL-2 family proteins play a critical role for cancer cell survival and chemoresistance. It is shown that the glioblastoma CSCs ($CD133^+$) isolated from primary cultures express higher levels of BCL-2, BCL_{XL}, XIAP and FLIP than the non-CSCs ($CD133^-$) (29), and the CSCs are resistant to TRAIL-induced cell death (198). Inhibiting Bcl-2 sensitizes these CSCs to TRAIL death effect both in culture and in tumor-bearing mice (199). Silencing c-FLIP sensitizes CSCs to cisplatin or TRAIL therapy (200, 201). Inhibition of Bcl-2, survivin and XIAP sensitizes prostate CSCs to apoptosis (202, 203). These results hold promise for tilting down anti-apoptotic activity to effectively sensitize cancer to chemotherapy (204).

The role of autophagy in cancer is paradoxical as it functions both to suppress tumor progression and promote cell survival in response to catastrophic stresses (205). CSCs take advantage of the pro-survival side of this double-edged sword feature of autophagy. Many anticancer therapies including chemo-, radio- and targeted therapies have been shown to activate autophagy and subsequent therapy resistance in cancer cells, and inhibiting autophagy sensitizes tumor to chemotherapies resulting in tumor regression (129, 130). In addition, resistance to drug-induced differentiation may also play a role in protecting CSCs from chemotherapeutic death effect.

2.6. Regenerate self - the 6th line

2.6.1. Epithelial-mesenchymal transition (EMT)—EMT is physiologically designed for the epithelial cells to change so that the cells can migrate away during embryonic developmental processes such as gastrulation. During EMT, epithelial cells undergo morphological changes into fibroblast-like mesenchymal phenotype. Typically, the epithelial cells change from a polarized monolayer where they are connected side-by-side and shoulder-by-shoulder via various types of cell-cell junctions into isolated or scattered cells that lose cell-cell interactions and become more motile and invasive. At the molecular level, an expression switch from epithelial marker genes such as E-cadherin and β-catenin to mesenchymal marker genes such as vimentin and fibronectin is turned on by various types of EMT-inducing transcription factors (EIFs) such as Snail, Twist, Zebs and krüppel-like factor 8 (KLF8). These EIFs have recently been found to work closely with microRNAs particularly the miR200 family and miR146a to control EMT, CSC induction and therapy resistance (206–216) (A recent review has more details (217)). In addition to embryonic development, wound healing also requires EMT on the wound edges so that the cells can migrate efficiently towards the center of the wound to eventually heal the wound. Invasive tumor cells hijack this wound healing mechanism to dissociate from the primary tumor mass, migrate away and invade through the stromal matrix including basement membrane that constrains the tumor mass and envelops the vascular vessels to eventually enter the circulation and reach distant metastatic sites (218).

Importantly, EMT has recently been linked to both CSCs and therapy resistance of cancer. Cells undergone EMT gain CSC-like features and CSCs exhibit a mesenchymal phenotype

(22, 219, 220). EMT induced by Twist (22), Snail (221) and KLF8 (214) is all accompanied by the induction of CSC properties. Mouse mammary stem cells exhibit a mesenchymal phenotype (22, 222) and transforming growth factor beta (TGF-β) induces both EMT and cell renewal capabilities in human mammary epithelial cells (220). Association of EMT with the CSC properties was also found in metastatic breast (223) and colorectal (224) cancer cells. Many other EMT-regulating signaling pathways such as the Wnt, Notch and Hedgehog pathways were also found to be critical for CSCs (222, 225, 226). Lastly, CSCs express high levels of EMT markers (12). Drug resistance is an intrinsic mechanism of normal stem cells that protects them like seeds for embryonic development or tissue regeneration and repair. Like normal stem cells, CSCs have been found to associate with EMT as well as acquired therapy resistance in various types of cancer (12). Mesenchymal (but not epithelial) phenotype and/or gene expression signature has been linked to therapy resistance in many cancer types including cancers of the lungs (227, 228), head and neck (229, 230), pancreas (44, 231, 232), colon (233), breast (214, 234–238) and ovaries (231, 239) against targeted therapies using erlotinib, gefitinib, lapatinib or tamoxifen as well as chemotherapies using cetuximab, taxol, paclitaxel, vincristine and oxaliplatin, gemcitabine or oxaliplatin. Various studies have provided evidence supporting a role of Notch signaling for cancer chemoresistance (214, 240–243).

2.6.2. CSC plasticity and dynamics—For more than a century, “terminally differentiated cell” has been the terminology used to describe cells that have completed cellular differentiation pathway and believed to no longer be capable of proliferating. The vast majority of this cell category happens to be a component of the epithelia that cover the skin surface or line the lumen of the tubular or ductal organs and are the tumor origin of more than 80% of all cancer types. This terminology, however, has no longer been correct since the induced pluripotent stem cells (iPSCs) were created via dedifferentiation of the “terminally differentiated” cells by re-activation of a few reprogramming transcription factors (RPFs) (244–247) as illustrated above in Figure 1. Similarly to RPFs, EIFs specifically grant the stem capability back to “terminally differentiated” normal or non-tumorigenic epithelial cells. This EMT-associated regeneration of stem or stem-like cells may be critical for refreshing adult epithelial stem cell pool in response to their loss due to any cause. For example, depletion of hair follicle stem cells is followed by repopulation of the stem cells from differentiated skin cells (12). However, the same mechanism could contribute to the initiation of tumorigenic CSCs as is seen in the cells overexpressing the EIFs Twist, Snail or KLF8 (22, 214, 221). This same mechanism could also be utilized in cancer to regenerate CSCs from non-stem cancer cells in the tumor in response to loss of CSCs due to anticancer therapies (248–250). Thus, regeneration of CSCs by EMT is a likely mechanism for chemoresistance of cancer and relapse just as normal ASCs repopulate and redistribute when encountering massive damage by radiation. Indeed, rapid repopulation of CSCs is believed to occur in human tumors during radiotherapy (251), and redistribution of CSCs to the quiescent phase of the cell cycle makes the cells more resistant to radiotherapy (137, 252). The regeneration process could be responsible for the heterogeneous phenotypes of CSCs within a same tumor as recently discovered in melanoma (138). This mechanism of CSC plasticity associated with EMT provides a new challenge for the concept of anti-CSC monotherapy that has been thought to be capable of eradicating the source of tumor.

Obviously, elimination and prevention of the regeneration of CSCs are equally important for CSC-targeted therapy.

2.7. Perish together - the last line of defense

The large overlap of the mechanisms of action in CSCs with those in normal stem cells or non-stem cells makes CSC targeting difficult because of likely toxic side effects on the normal cells. This makes CSC-specific targeted therapy particularly important to prevent normal cells and eventually the patient from being harmed alongside the CSCs.

3. An update on CSC-targeted therapy studies

The definition of targeted anticancer therapy is two-fold: 1) To disable the molecules in the tumor cells being targeted and thus the tumor cells. 2) To destroy the tumor cells being targeted by cancer cell-specific molecule-guided delivery of non-targeted drug, mostly chemotherapeutic drugs. Several scores of CSC-targeted small molecule compounds have been reported as outlined in Table 1. These compounds are designed or selected to directly inhibit CSC. Not surprisingly, most of these compounds target the survival/apoptosis and stem signaling pathways in CSCs. Others target the ABCTs and EMT. While this list of compounds is rapidly growing, CSC-specific molecule, such as CD44, directed delivery of non-targeted drugs has drawn increasing attention (253, 254).

4. Conclusion

Studies on CSCs have raised tremendous hope for designing tumor cell eliminating anticancer therapy. These same studies have also revealed significant challenges due to the multiple step defense strategy used by CSCs against chemotherapy, leading to the important clinical concerns such as chemoresistance, metastasis and relapse. Since other types of anticancer therapies, particularly radiotherapy, share much in therapeutic mechanisms of action, the mechanisms underlying CSCs chemoresistance might as well be applicable to other types of therapy such as radiotherapy, targeted therapy as well as anti-stromal therapy. The similarity in self-defense between CSCs and normal stem cells and even non-stem cells requires development of smart CSC-specific anticancer therapy. The CSC plasticity and regeneration underscore the importance of combination of CSC-specific therapy with other therapies including the mainstream conventional therapies such as chemotherapy and radiotherapy to prevent regeneration, repopulation and redistribution of CSCs from non-stem tumor cells. Lastly, patient's individual difference in CSC properties may require patient stratification as well for effective CSC-based anticancer strategies.

Acknowledgments

I am grateful to some of my lab members including Justin Gray, Debarati Mukherjee, Satadru K. Lahiri and Lin Yu for their critical discussions and technical assistance. This work was supported by the NIH-NCI grant (R01CA132977) to J.Z. I apologize to colleagues whose important contributions were not cited due to page limitations. I also wish to dedicate this work to the memory of my father who lost his battle against cancer during my preparation of this manuscript.

Abbreviations

5-FU	5-fluorouracil
ABCT	ATP-binding cassette transporter
ALDH	aldehyde dehydrogenase
AML	acute myeloid leukemia
ASC	adult stem cell
BCRP or ABCG	breast cancer resistance protein
BER	base excision repair
BMP	bone morphogenetic proteins
BRCA	breast cancer associated gene
CML	chronic myeloid leukemia
CSC	cancer stem cell
DDR	DNA damage response
DNMT	DNA methyltransferase
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ERCC1	excision repair cross-complementing 1
EMT	Epithelial-mesenchymal transition
EIF	EMT-inducing factor
EPR	error-prone repair
ERK	extracellular signal regulated kinase
FGF	fibroblast growth factor
G-CSF	granulocyte colony-stimulating factor
HDR	homology-directed repair
HGF	hepatocyte growth factor
Hh	Hedgehog
HIF	hypoxia-inducible factor
HSC	hematopoietic stem cells
IAP	inhibitor of apoptosis
iPSC	induced pluripotent stem cell
IGF	insulin growth factor
IL	interleukin

JAK	Janus kinase
KLF8	krüppel-like factor 8
MDS	myelodysplastic syndrome
MRD	minimal residual disease
ROSs	reactive oxygen species
MDR1 or ABCB1	multidrug resistance protein 1
MMR	mismatch repair
MRP1 or ABCC1	multidrug resistance-associated protein 1
NER	nucleotide excision repair
NF-κB	nuclear factor-kappa B
NHEJ	non-homologous end joining
HNSCC	head and neck squamous cell carcinoma
NSCLC	non-small cell lung cancer
PARP-1	poly (ADP-ribose) polymerase 1
PI3K	phosphatidylinositol-3-kinase
PKB or AKT	protein kinase B
PML	promyelocytic leukaemia
RPF	reprogramming factors
SMAC	second mitochondria-derived activator of caspase
SP cell	side-population cell
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor beta
TKI	tyrosine kinase inhibitor
TSG	tumor suppressor gene
UGT1A1	uridine diphosphate (UDP) glucuronosyltransferase 1
VEGF	vascular endothelial growth factor

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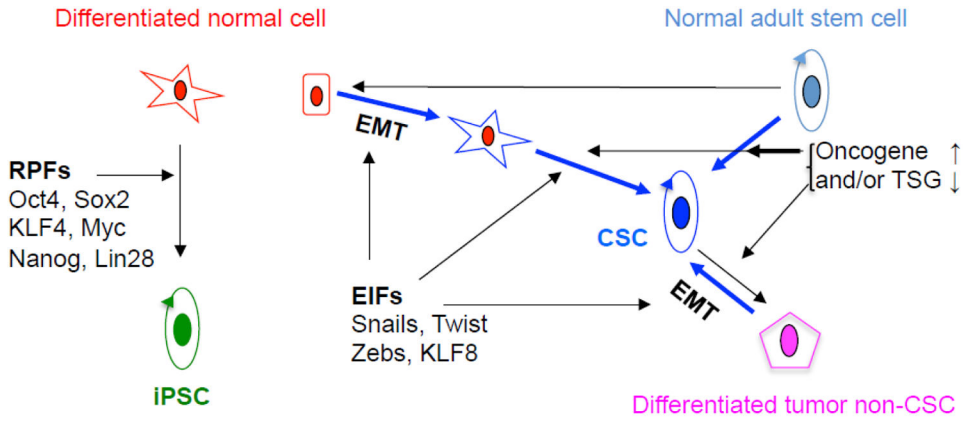

Figure 1.

Cancer stem cell (CSCs) origins and mechanisms of formation. The three hypotheses are highlighted in bold blue arrows. 1. The epithelial to mesenchymal transition (EMT) induced by EMT-inducing factors (EIFs) turns normal epithelial cell (the red square-shaped) to fibroblast-like cell (the blue star-shaped). The latter subsequently becomes CSC particularly with mutations of oncogenes and/or tumor suppressor genes (TSGs). This process mirrors the formation of induced pluripotent cell (iPSC, green round-shaped) from normal fibroblast (red star-shaped) activated by the reprogramming factors (RPFs) shown on the left. 2. Normal adult stem cell (ASC, light-blue round shaped) is transformed into CSC by mutations of oncogene(s) and/or TSG(s). 3. EMT drives the tumor non-CSC (pink pentagon-shaped) back to be CSC.

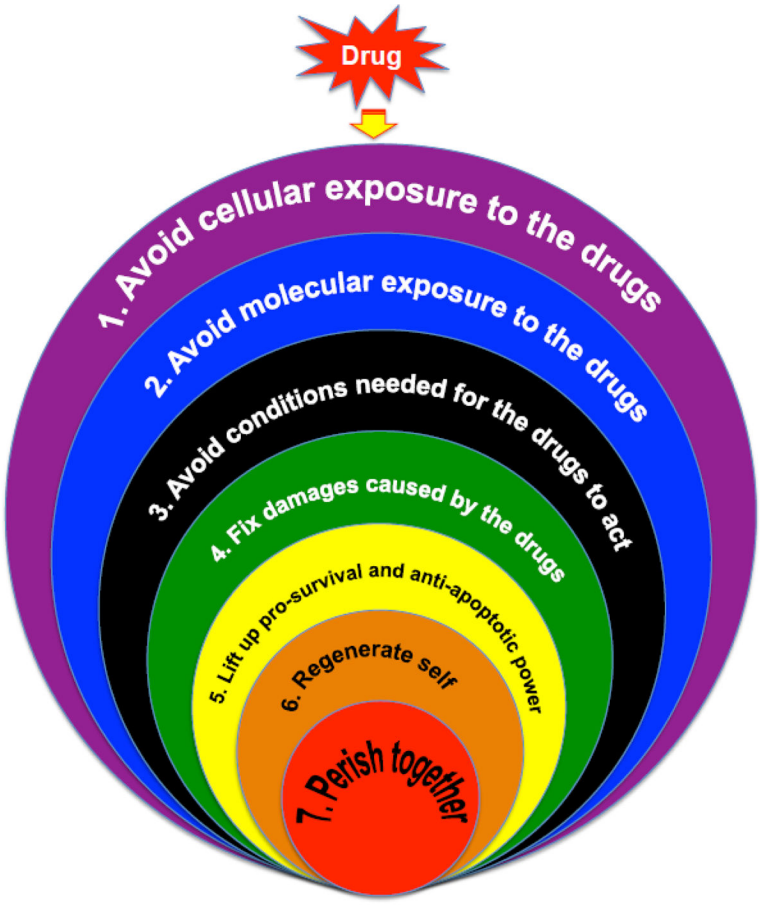

Figure 2.
The multiple lines of defense cancer stem cells use to resist chemotherapy.

Table 1

CSC-targeted small molecule monotherapy studies

Mechanisms	Agents	Cancer Types	References
Targeted			
Survival/apoptosis			
NF-κB	Eriocalyxin	Ovarian	(255)
NF-κB	MK-5108	Ovarian	(256)
NF-κB	Morusin	Cervical	(257)
NF-κB	Parthenolide	Multiple myeloma	(258)
NF-κB	Andrographolide	Multiple myeloma	(258)
STAT3	Galiellalactone	Prostate	(259)
STAT3	STX-0119	Glioblastoma	(260)
STAT3	Tanshinone IIA	Breast	(261)
STAT3	WP1193	Glioblastoma	(262)
Akt, mTOR	Rottlerin	Pancreatic	(263)
Akt, MAPK	Sorafenib	Glioblastoma	(264)
Akt, MAPK	Strigolactone	Breast	(265)
Akt	Harmine	Glioblastoma	(266)
Metabolism	Niclosamide	Ovarian	(267)
Metabolism	3-bromopyruvate	Pancreatic	(268)
Metabolism	2-deoxyglucose	Breast	(269)
MAPK	Icaritin	Breast	(270)
MAPK	Imatinib	Glioblastoma	(271)
Survivin	LLP-3	Glioma	(272)
Bcl-2	ABT-737	Glioma	(199)
FAK, ROCK1	Atorvastatin	Prostate	(273)
AMPK	Metformin	Pancreatic	(274)
Telomerase	MST312	Lung	(275)
Mcl-1	Omacetaxine	CML	(276)
Stem signaling			
Wnt	Phosphosulindac	Breast	(277)
Wnt	Salinomycin	Endometrial	(278–281)
Wnt	Acetaminophen	Breast	(282)
Wnt	Celecoxib	Colon	(283)
Wnt, IGF-1R	Adamantyl retinoid	Pancreatic	(284)
Wnt	Oximatrine	Breast	(285)
Wnt, hedgehog	Sulforaphane	Breast, pancreatic	(286–288)
Hedgehog	Cyclopamine	Glioblastoma	(176)
Hedgehog	Erismodegib	Prostate	(203)
Hedgehog	Vismodegib	Pancreatic	(289)

Mechanisms	Agents	Cancer Types	References
Hedgehog	GANT-61	Pancreatic	(290)
Notch	DAPT	Ovarian	(291)
Notch	MRK-003	Breast	(292)
Notch	Retinoic acid	Brain	(293, 294)
Notch	RO4929097	Melanoma	(295)
Stem factors	SB-T-1214 (taxoid)	Colon	(281)
c-Met	Cabozantinib	Pancreatic	(296)
ABCTs			
ABCG2	Curcumin	Colon	(297)
ABCG2	Imatinib, nilotinib	Hematopoietic	(298)
ABCG2	Mithramycin	Lung, esophageal	(299)
DNA repair			
ATM, ATR	CCT129202	Lung	(300)
PARP-1	NSC747854	Lung	(301)
CSC niches/stroma			
HIF1α	17-AAG	Lymphoma	(302)
Glycosaminoglycan	G2.2	colon	(303)
EMT			
EMT/Stem factors	Resveratrol	Pancreatic	(304)
EMT/NF-κB	Lupeol	Head and neck	(305)
EMT	Cystatin C	Breast	(306)
EMT	Dasatinib	Breast	(307)
Drug metabolism			
AhR	Tranilast	Breast	(308)