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Cancer Stem Cells (CSCs) and Mechanisms of Their Regulation: Implications for Cancer Therapy

Bin Bao[1], **Aamir Ahmad**[1], **Asfar S. Azmi**[1], **Shadan Ali**[2], and **Fazlul H. Sarkar**[1,2,*]

[1]Department of Pathology, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan

[2]Department of Oncology, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan

Abstract

The identification of small subpopulations of cancer stem cells (CSCs) from blood mononuclear cells in human acute myeloid leukemia (AML) in 1997 was the landmark observation for recognizing the potential role of CSCs in tumor aggressiveness. Two critical properties contribute to the functional role of CSCs in the establishment and recurrence of cancerous tumors: their self-renewal capacity and their potential to differentiate into unlimited heterogeneous populations of cancer cells. These findings suggest that CSCs may represent novel therapeutic targets for the treatment and/or prevention of tumor progression as they appear to be involved in cell migration, invasion, metastasis, and treatment resistance, all of which lead to poor clinical outcomes. The identification of CSC-specific markers, the isolation and characterization of CSCs from malignant tissues, and targeting strategies for the destruction of CSCs provides a novel opportunity for cancer research. Described in this overview is the potential implication of several common CSC markers in the identification of CSC subpopulation restricted to common malignant diseases e.g., leukemia, breast, prostate, pancreatic and lung cancers. The role of microRNAs (miRNAs) in the regulation of CSC function is also discussed, as are several methods commonly used in CSC research. The potential role of the anti-diabetic drug metformin that has been shown to have effects on CSCs, and known function as an anti-tumor agent, provides an example of this new class of chemotherapeutics.

Keywords

CSCs; Cell surface markers; miRNAs; metformin

1. Introduction

While cancer stem cells (CSCs) were recognized several decades ago, it is only in the the last 15 years that they have been identified and characterized in hematological malignancies, such as leukemia (Bonnet and Dick, 1997) and other tumors. This led to an increased interest in the potential role of CSCs in tumor aggressiveness, treatment resistance, and tumor recurrence (relapse) and metastasis (Rasheed and Matsui, 2012;Sarkar *et al.*, 2009;Yu *et al.*, 2012b). Like normal pluripotent stem cells, CSCs are long-lived, and display quiescent potentials in a dormant state, and are responsible for angiogenic induction, apoptotic resistance, self-renewal and differentiation. These characteristics are orchestrated by a rare sub-population of tumor cells within the total tumor cells present in a tumor mass, namely CSC cells that express stem cell marker genes, including Oct4, Sox2, Nanog, c-kit,

[*]**Corresponding Author:** Fazlul H. Sarkar, Departments of Pathology and Oncology, Karmanos Cancer Institute, Wayne State University School of Medicine, 740 HWCRC, 4100 John R Street, Detroit, MI 48201. Phone: 313-576-8327; Fax: 313-576-8389; fsarkar@med.wayne.edu.

ABCG2, and ALDH (Charafe-Jauffret *et al.*, 2009;Croker *et al.*, 2009;Prud'homme, 2012;Yu *et al.*, 2012a). These characteristics suggest that CSCs themselves contribute to tumor development and progression. While the pathogenic effects of CSCs remains to be elucidated, it is widely believed that intrinsic and extrinsic alterations in the stem cell tumor microenvironment, together with mutations and epigenetic regulations, are mainly responsible for the development of CSCs that are involved in tumor initiation and progression (Figure 1) (Bao *et al.*, 2012a).

It is known that CSCs constitute only a small percentage (0.05-1%) of tumor cells within a tumor mass containing heterogeneous population of tumor cells within the tumor microenvironment (Li *et al.*, 2012;Yu *et al.*, 2012a;Yu *et al.*, 2012b). These CSCs have the capacity for self-renewal, giving rise to uncontrolled amplification of differentiated cell populations with altered molecular and cellular phenotypes. These eventually lead to the heterogeneous primary and metastatic tumor cells within a tumor mass that may be resistant to therapeutics and contribute to tumor recurrence (Li *et al.*, 2012;Yu *et al.*, 2012a). It is interesting to note that CSCs are important in the prognosis of many malignant diseases which has been demonstrated by the finding that they are present in the majority of malignant tumor tissues, and appear to be resistant to chemo-radiation therapy as compared to their differentiated progenies (Creighton *et al.*, 2010;Lee *et al.*, 2008). This may explain why tumor regression alone does not correlate with overall survival rate in cancer patients (Creighton *et al.*, 2010). Rather, it appears that tumor recurrence/relapse occurs because of the presence and sustenance of CSCs within the tumor microenvironment, even after conventional cancer therapy, which suggests that CSCs plays a critical role in treatment resistance, tumor metastasis and recurrence. The identification of CSC-specific markers, the isolation and characterization of CSCs from malignant tissues, and the development of strategies for targeted eradication of CSCs represent an important opportunity in cancer research.

Described in the present report are the implications of several common CSC markers and their relevance to common malignant diseases such as leukemia, breast, prostate, pancreatic and lung cancers. The importance of microRNAs (miRNAs) in the regulation of CSC characteristic is also considered, as are the methods commonly employed in CSC research. As an example of the possible success of this approach, the anti-tumor activity of metformin, an anti-diabetic agent and potential CSC regulator, is discussed.

2. CSCs and tumor aggressiveness

Cancer stem cells were first identified and characterized in the bone marrow of AML patients in 1997. Subsequent clinical and laboratory studies provided additional evidence supporting the role of CSCs in drug resistance and cancer metastasis, thereby contributing to the poor outcomes experienced by patients with pancreatic, prostate, liver, breast, and brain tumors (Bauerschmitz *et al.*, 2008;Lee *et al.*, 2008;Matsui *et al.*, 2008). For example, $CD133^+$ pancreatic CSC cells co-express the CXC chemokine receptor CXCR4 at the invading margins of human ductal pancreatic tumors (Lee *et al.*, 2008;Narducci *et al.*, 2006;Klein *et al.*, 2001). Both $CD133^+CXCR4^-$ and $CD133^+CXCR4^+$ CSC cells isolated from human pancreatic tumors are able to generate and reconstitute primary tumors in mouse xenograft models. However, only the $CD133^+CXCR4^+$ cells display a significant metastatic capacity in this animal model. As the inhibition of CXCR4 in these pancreatic CSCs prevents metastasis in this xenograft mouse model (Hermann *et al.*, 2007), it appears that $CD133^+CXCR4^+$ CSCs play a critical role in tumor metastasis although it is not the case for $CD133^+CXCR4^-$ cells. Moreover, the CSC-like glioma cells also contribute to resistance to radio-therapy via preferential activation of DNA damage response pathways, and by increasing DNA repair capacity (Bao *et al.*, 2006). Similarly, a subpopulation of

glioma cells with the phenotype of $CD133^+$, a CSC marker for various tumors, is enhanced after glioma radiation (Bao *et al.*, 2006). The $CD133^+$ glioma cell population surviving after treatment with ionizing radiation displays a significant increase in the proportions of CSCs relative to the large numbers of $CD133^-$ tumor cells, suggesting that they are responsible for the radiation-resistant phenotype in gliomas (Bao *et al.*, 2006).

It has been reported that mouse mammary tumor CSCs are responsible for cisplatin resistance (Shafee *et al.*, 2008) and that CSCs in human colorectal cancer tissue are responsible for resistance to chemotherapeutic agents (Dylla *et al.*, 2008). Moreover, human breast cancer cells containing aldehyde dehydrogenase $(ALDH)^+$ CSC cells have an increased metastatic capacity with distinct CSC molecular phenotypes, e.g., ALDH, Notch-2, and CXCR1 (Charafe-Jauffret *et al.*, 2009). This suggests that CSCs are also involved in the regulation of breast cancer metastasis.

The involvement of CSCs in drug resistance has been demonstrated in pancreatic, colon, breast, and brain tumors. Moreover, CSC-containing tumors display greater tumorigenic and metastatic potential *in vitro* and *in vivo* than non-CSC cancer cells. It has been documented that human pancreatic cancer tissues contain a small subpopulation of $CD133^+$ CSC phenotypic cells that are exclusively tumorigenic and highly resistant to standard chemotherapy (Hermann *et al.*, 2007). Elimination of these CSC populations suppresses the metastatic phenotype of pancreatic tumors without modulating their tumorigenic potential (Hermann *et al.*, 2007). Similarly, CSCs in pancreatic tumor tissues are associated with drug resistance and metastatic potential with pancreatic tumor cells having a CD44 positive CSC phenotype, which correlates with tumor histological grade and poor clinical outcomes (Hong *et al.*, 2009). These findings suggest that the CSCs, promote tumor aggressiveness. Given these findings CSCs appear to be an excellent target for treating malignancies.

3. Identification of CSC markers in common malignant diseases

The identification and characterization of CSCs in malignant diseases provides insights as to ways in which to selectively inhibit or eradicate CSCs as a treatment for tumor aggressive phenotypes. Several common stem cell markers, including CSC-specific markers such as CD34, CD44, CD123, CD133, Oct4, Sox2, Nanog, c-kit, ABCG2, and ALDH have been identified in the CSC populations isolated from a wide variety of malignant diseases.

3.1. Leukemia

Leukemia is one of the most commonly diagnosed malignant diseases in children and adults (Siegel *et al.*, 2012). This includes acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and multiple myeloma (MM). Within each of these groups there is significant patient-to-patient heterogeneity in leukemic blast cell morphology. For instance, AML is classified into seven French-American-English subtypes, according to the maturation stage of the acute leukemias, the preferential expression of multi-lineage cell markers, and morphology among individual patients (Bennett *et al.*, 1985;Warner *et al.*, 2004). Targeting these different leukemic blasts, especially leukemia CSCs, while avoiding normal hematopoietic stem cells, provides an opportunity for the treatment or eradication of these conditions.

A small subpopulation of leukemic cells namely CSCs displaying the $CD34^+CD38^-$ cell surface phenotype were first identified in bone marrow samples of AML patients (Bhatia *et al.*, 1997;Bonnet and Dick, 1997) using flow cytometry-based sorting. The injection of 5,000 leukemic CSCs developed human leukemia in immune compromised mice (Bhatia *et al.*, 1997;Bonnet and Dick, 1997). These small subpopulations of leukemia CSC cells also

display a greater capacity for stem cell self-renewal as compared to normal adult bone marrow cells.

This seminal discovery led to the identification and characterization of CSCs in other cancers. Thus, several stem cell markers like CD34, CD38, HLA-DR, and CD71, which are shared with normal hematopoietic stem cells, have been identified in leukemic cells (Dick, 2005;Warner *et al.*, 2004). Some cell surface markers, such as CD90 (Thy-1), are differentially expressed between normal hematopoietic stem cells and leukemia CSCs, with CD90 being under-expressed in leukemic CSCs (Johnsen *et al.*, 2009;Warner *et al.*, 2004). This suggests that CD90 may be a useful marker for distinguishing leukemic CSCs from normal hematopoietic stem cell sub-populations (Blair *et al.*, 1997;Warner *et al.*, 2004). Loss of expression of stem cell factor c-kit, also known as CD117, is a consistent feature of leukemic CSCs ($CD34^+c\text{-}kit^-$) isolated from AML, but not of normal bone marrow hematopoietic stem cells ($CD34^+c\text{-}kit^+$) (Blair and Sutherland, 2000). It has also been reported that CD123 (IL-3Rα) is a unique marker of leukemic CSCs (Jordan *et al.*, 2000) and that it is essential in enhancing cell survival pathways in leukemia (Testa *et al.*, 2004). The expression of CD123 is significantly increased in the $CD34^+CD38^-$ CSC population isolated from leukemia patients (Budel *et al.*, 1989;Testa *et al.*, 2004). However, expression of CD123 is undetectable in $CD34^+CD38^-$ CSC cells isolated from the bone marrow of normal subjects (Jordan *et al.*, 2000). Moreover, activation of NF-κB increases the number of quiescent leukemia CSC populations, without affecting normal hematopoietic stem cell subpopulations (Warner *et al.*, 2004). These findings suggest that there are cell markers unique for leukemia CSCs that are not found in normal hematopoietic stem cells.

Monoclonal antibodies against CD44, CD47 and CD123 are effective in eliminating leukemic CSCs in AML. This finding is consistent with the inhibition of the IL-3-mediated signaling pathway between leukemic stem cells and supporting cells in NOD/SCID mice (Jin *et al.*, 2006;Jin *et al.*, 2009;Majeti *et al.*, 2009). Targeting these leukemic CSC related markers or proteins could provide an effective strategy for the eradication of CSC populations in leukemia. The identification and characterization of leukemic CSC-specific markers that are not shared with normal hematopoietic stem cells makes it possible to identify and develop novel therapeutic agents for the management of human leukemias.

3.2. Breast cancer

Breast cancer is the second most deadly malignancy for females, with one in eight women expected to develop the condition in their lifetime (Siegel *et al.*, 2012). Breast cancer affects 121 per 100,000 people, with a greater incidence among African Americans than other ethnic groups. Thanks to early detection and more effective treatment options the survival rate for breast cancer increased from 84% to 90% between 1987 and 2007 (Siegel *et al.*, 2012). Nonetheless, breast cancer remains a major cause of cancer-related death because of treatment resistance and metastatic disease, especially for those diagnosed at advanced stages of the disease.

Breast CSCs are a small subpopulation (0.1-1%) of breast cancer cells in primary tumors. A rare subset of breast CSC has a high capacity for self-renewal and is able to initiate tumorigenesis when transplanted into NOD/SCID mice (Al-Hajj *et al.*, 2003;Klonisch *et al.*, 2008). Several common CSC markers, including CD44, CD133, ALDH, c-kit, ESA and ABCG2, have been identified in primary breast cancer specimens (Prud'homme, 2012).

A critical role for $CD44^+$ in the development of this condition is indicated by the finding that injection of less than 100 breast CSCs with the phenotype of $CD44^+/CD24^-$ can result in 85% tumor formation in xenograft models, while injection of more than 10,000 non-CSC breast adenocarcinoma cells fails to do so (Al-Hajj *et al.*, 2003;Patrawala *et al.*, 2005).

Moreover, $CD133^+$ breast CSCs have characteristics similar to $CD44^+/CD24^-$ CSCs while $CD133^-$ breast adenocarcinoma cells do not generate tumors in mouse tumor xenograft models (Klonisch *et al.*, 2008) As there is no overlap in cell surface proteins between $CD133^+$ and $CD44^+/CD24^-$ CSCs (Al-Hajj *et al.*, 2003;Patrawala *et al.*, 2005), there is no universal CSC marker for each type of breast cancer. However, there is some overlap of CSCs among patients between $ALDH^+$ and $CD44^+/CD24^-$ cell subpopulations (Yu *et al.*, 2012a). Data indicate that breast cancer CSCs with a $CD44^+CD24^-ALDH^+$ phenotype have greater tumorigenic potential than CSCs with the $CD44^+CD24^-$ or $ALDH^+$ phenotype (Ginestier *et al.*, 2007). As ALDH is not expressed with CD44 and CD133 in ovarian tumors, it appears that CSCs with the phenotype of $CD44^+$, $CD24^-$, $CD133^+$, $ALDH^+$ have the most pronounced tumorigenic potential in breast cancer, making these CSC subpopulations are attractive targets for the treatment of breast cancer.

Expression of mucin 1 (MUC-1), a mediator of the growth of undifferentiated human embryonic stem cells (Hikita *et al.*, 2008) that is overexpressed in human estrogen positive and negative breast carcinomas, is associated with breast tumor cells and its side population cells, one type of so called CSC cells identified by Hoechst 33342 dye (Kufe, 2012;Engelmann *et al.*, 2008). This suggests that it is a potential CSC marker for breast cancer.

3.3. Prostate cancer

Prostate cancer is the most commonly diagnosed malignant disease in males, being the second leading cause of cancer-related death for men in the United States (Siegel *et al.*, 2012). As most prostate cancer patients are treatable, the survival rate has increased significantly over the years, although fatalities still occur, especially with aggressive phenotypes that are resistant to chemotherapy. Considerable effort has been expended to identify and characterize prostate CSC populations so as to target them in treating this condition. Subpopulations of CSCs (<0.1%) have been identified in primary prostate cancers that preferentially express the cell surface markers, CD44, CD133, stem cell antigen 1 (Sca-1), collagen receptor $\alpha 2\beta 1^{hi}$, CK5/14, CK8/18, CD49f, and ABCG2 (Klonisch *et al.*, 2008). However, different CSC subpopulations in prostate cancer are dependent on the enrichment of CSCs in different cellular compartments (Kasper, 2009). For example, the expression of CD44 in prostate CSCs is present in most prostate cancer basal cells, whereas CD133, $\alpha 2\beta 1^{hi}$, CK5/14, CK8/18 and ABCG2 are expressed in less than 1% of basal cells (Klonisch *et al.*, 2008). Nevertheless, there is some overlap of CSCs between the $\alpha_2\beta_1{}^{hi}$, $CD44^+$, and $ABCG2^+$ cell populations (Patrawala *et al.*, 2007). Subpopulations of CSCs in prostate cancer have high proliferative capacity, increased clonogenic potential, and a greater capacity for tumorigenesis and metastasis in xenograft models *in vitro* and *in vivo*. Injection of 1,000 prostate cancer cells with the $CD44^+CD24^-$ CSC phenotype consistently develops tumors in NOD/SCID mice, whereas the injection of non-CSC cancer cells does not (Hurt *et al.*, 2008). It has been reported that Sca-1, a cell surface protein, is a potential CSC marker that is unique to prostate cancer. It is found primarily in the proximal regions of the organ (Zhou *et al.*, 2007) in the same site as the CSC niche, a very small or limited area of tumor tissues where CSCs resides closely in a similar, permissive environment to retain the high capacity of self-renewal and multi-lineage differentiation potential as well as protect them from diverse genotoxic insults (Tsujimura *et al.*, 2002). The $Sca\text{-}1^+$ prostate epithelial cells that display an increased activation of Akt signaling by transfection of constitutively active AKT can initiate prostate cancer in xenograft models (Xin *et al.*, 2005). In addition, PTEN, a known tumor suppressor that targets primarily the PI3K/Akt/mTOR signaling, and c-kit are proposed to be potential markers of CSCs in prostate cancer (Klonisch *et al.*, 2008). The expression of these proteins is down-regulated in prostate cancer cells, especially prostate CSCs.

3.4. Pancreatic cancer

Pancreatic ductal adenocarcinoma is currently the fourth leading cause of cancer-related deaths in the United States (Siegel *et al.*, 2012). It is also one of the most lethal malignancies, with a 5-year survival rate of less than 5% (Edwards *et al.*, 2005). It is estimated that each year in the United States, 44,000 patients are diagnosed with pancreatic cancer (greater than 90% as pancreatic ductal adenocarcinoma), with 37,000 dying from this malignancy. Due to the absence of specific symptoms, the lack of early sensitive detection tests, and its rapid and insidious growth, pancreatic cancer is typically diagnosed at an advanced and incurable stage. Thus, the overall survival of patients is approximately 5–6 months, even with conventional therapy for locally advanced and metastatic disease.

Pancreatic CSC populations account for less than 1% of all pancreatic cancer cells. They have the capacity for self-renewal and uncontrolled potential of differentiated progeny. Pancreatic CSC populations express the cell surface markers $CD44^+$, $CD24^+$ and epithelial-specific antigen $(ESA)^+$ (Lee *et al.*, 2008;Klonisch *et al.*, 2008). When transplanted into NOD/SCID mice, a $CD44^+CD24^+ESA^+$ CSC subpopulation isolated from human primary pancreatic cancers readily formed tumors, while cancer cells lacking these cell surface markers were poorly tumorigenic (Lee *et al.*, 2008;Klonisch *et al.*, 2008). The CSC marker-positive cells display a 100-fold increased capacity for the development of tumors and exhibit tumor morphology similar to primary pancreatic cancer. Moreover, these CSCs maintain their cell surface marker phenotype after repeated passages as xenografts in immunocompromised mice (Li *et al.*, 2007;Lee *et al.*, 2008). Unlike normal pancreatic epithelial cells and non-CSC-like cancer cells, these pancreatic cancer CSC phenotypic cells also display a strong transcriptional up-regulation of sonic hedgehog (SHH) and the polycomb group (PCG) gene family member Bmi-1. All of these are known mediators for maintaining CSC characteristics (Li *et al.*, 2007;Lee *et al.*, 2008). Pancreatic cancers contain 1–3% of $CD133^+$ cancer cells, some of which show high expression of CXCR4, a pro-invasive marker. These $CD133^+CXCR4^+$ cells, but not $CD133^+CXCR4^-$ cells, have significant metastatic capacity. The selective inhibition by AMD3100 of CXCR4 signaling in $CXCR4^+$ CSC cells blocks tumor tissue invasion (Hermann *et al.*, 2007), suggesting a potential role of CXCR4 in pancreatic tumor metastasis. Accordingly, it remains possible that there is more than one type of CSC sub-population in pancreatic cancer tissues, which would be consistent with the known heterogeneity of most human tumors (Klonisch *et al.*, 2008).

Putative human pancreatic CSC-like cells derived from MiaPaCa-2 sphere-forming cells obtained from the mouse xenograft tumors showed increased expression of CD44, EpCAM, and enhancer of zeste homolog 2 (EZH2), which is an epigenetic mediator involved in the regulation of CSC characteristics (Bao *et al.*, 2012b). Expression of EZH2 increases in various tumors, including pancreatic cancer (Bao *et al.*, 2012b;Toll *et al.*, 2010). Injection of as few as 5,000 of these CSC-like sphere cells into SCID mice produces tumors within 2–3 weeks, whereas it is necessary to inject 10^7 non-CSC parental cancer cells to achieve comparable tumor incidence. As these pancreatic CSC-like cells display an increased capacity for migration and invasion, aggressiveness, and self-renewal capacity (Bao *et al.*, 2012b), it is possible that EZH2 can be used as a CSC marker and might be a therapeutic target in the treatment of pancreatic cancer.

3.5. Lung cancer

In the United States, 226,000 people are diagnosed with lung cancer each year, with 160,000 individuals dying annually from this malignancy (Siegel *et al.*, 2012). Although there has been significant progress over the past decade in the diagnosis and the treatment of this condition, prognosis remains poor due to treatment resistance, rapid tumor growth, and

metastatic capacity. A small subpopulation of lung CSC cells appears to be responsible for the aggressive phenotypes of lung cancer. This group expresses typical stem cell markers, such as CD133, CD44, ALDH, Oct4, and Nanog (Eramo *et al.*, 2010;Wu *et al.*, 2012). The remarkable heterogeneity among lung cancers in terms of cell origin, biology, etiology, and molecular/genetic pathogenesis influences treatment strategies and prognosis. This makes it important to be able to distinguish non-small cell lung cancer (NSCLC), which accounts for 40% of lung tumors, and small cell lung cancer (SCLC), which accounts for 20% of lung tumors, on the basis of CSC characteristics. Although there are controversies regarding lung cancer CSC markers, CD133, CD44, ALDH, Oct4, Nanog, and ABCG2 have been associated with NSCLC, whereas only CD133 and ALDH have been found in association with SCLC (Kitamura *et al.*, 2009;Nurwidya *et al.*, 2012;Wu *et al.*, 2012). High levels of the tumor metastatic marker urokinase plasminogen activator receptor (u-PAR), podocalyxin-like protein 1 (PODXL-1), a marker of embryonic and hematopoietic stem cells, and B cell-specific Mo-MULV integration site 1 (Bmil-1), a member of polycomb group protein family, are considered potential markers for CSCs in SCLC ((Kitamura *et al.*, 2009). Lung CSCs with the $CD44^{+}CD133^{+}$ phenotype display an increased capacity for self-renewal and show unlimited differentiated progeny of heterogeneous populations of NSCLC cancer cells in comparison with cancer cells lacking these markers (Eramo *et al.*, 2010). Expression of ABCG2, a marker for drug resistance, epithelial-specific antigen (ESA), stem cell factor c-kit, and CXCR4 is increased in lung CSC subpopulations of NSCLC (Sung *et al.*, 2008;Ho *et al.*, 2007;Wu *et al.*, 2012). The atypical protein kinase C (PKC) iota, an oncogene for NSCLC, is required for bronchioalveolar stem cell growth *in vitro* and *in vivo* and for lung tumor formation in the PCK iota-deficient K-ras mouse model (Regala *et al.*, 2009). Expression of Rac-1, a small GTP binding protein of the Rho family, is increased in CSC populations associated with NSCLC (Akunuru *et al.*, 2011). Because Rac-1 is a signal transducer for several oncogenic pathways involved in cell survival, proliferation, migration and invasion in tumors, it appears to be essential for the K-ras mediated tumor growth of lung tumors (Kissil *et al.*, 2007). In addition, RAC-1 is a downstream target of PKC iota, with PCK iota/Rac-1 signaling required for the activation of the K-ras signaling pathway in NSCLC tumors (Fields and Regala, 2007). Therefore, Rac-1 and/or PKC iota are potential CSC markers for NSCLC. Inasmuch as CD133, CD44, and ALDH are common markers for both normal and cancer stem cells, there are no selective markers for lung CSC populations. This makes it necessary to identify and use multiple markers for characterizing the lung CSC subpopulations (Wu *et al.*, 2012).

4. The role of miRNAs in the regulation of CSC characteristics

MicroRNAs (miRNAs), non-protein-coding RNAs 18–24 nucleotides in length, are post-transcriptional regulators of mRNAs that binds to specific sites in the 3’ untranslated region (3’-UTR) of their target mRNAs. This attachment results in either mRNA degradation or inhibition of protein synthesis (Liu and Tang, 2011), making miRNAs useful tools in characterizing the role of select proteins in cell function. Moreover, miRNAs have an important role in tumorigenesis, with alterations in their expression in cancers associated with clinical outcome, therapy resistance, and tumor recurrence/relapse (Fabbri *et al.*, 2007;Liu and Tang, 2011). It is also known that miRNAs regulate the CSC characteristics by affecting signaling pathways and CSC signature genes. Particular miRNAs, which are differentially expressed in CSCs or CSC-like cells in various tumors, are potential CSC markers, as discussed below.

4.1. Let-7/miR-200

The members of the Let-7 and miR-200 family participate in the development and progression of tumors by targeting multiple cell signaling pathways involved in cell survival. Moreover, it has been demonstrated that the expression levels of let-7 and miR-200

are related to clinical outcomes in cancer patients (Olson *et al.*, 2009;Peter, 2009;Wendlandt *et al.*, 2012). As the expression of both of these miRNA family members is either lost or significantly reduced in leukemia, breast, prostate, pancreatic, and lung cancers, it is possible that they may be tumor suppressors. Indeed, let-7 family members are negative regulators of the epithelial-to-mesenchymal transition (EMT), a developmental event associated with treatment resistance, metastasis, and tumor recurrence. These effects are similar to those observed with CSCs that are, in part, mediated through regulation of PTEN and CSC gene signature marker Lin28B in pancreatic and prostate cancer cells (Kong *et al.*, 2010;Chang *et al.*, 2011;McCarty, 2012;Li *et al.*, 2009;Peter, 2009). The miR-200 family members also inhibit the EMT phenotype by directly targeting the EMT regulators, ZEB1 and ZEB2 (Peter, 2009;Kent *et al.*, 2009;Li *et al.*, 2009), and, like CSC, miR-200 decreases the expression of Bmil-1, Suz12, and Notch-1, known regulators of CSC and EMT phenotypes and function, in various cancer cells (Bao *et al.*, 2011;Iliopoulos *et al.*, 2010;Leal and Lleonart, 2012). The let-7 family members also inhibit the expression of EZH2, a major epigenetic component of the polycomb repressive complex 2 (PRC2) that inhibits the expression of developmental genes in embryonic and adult stem cells (Kong *et al.*, 2012b). Thus, EZH2 may be a regulator in maintaining the characteristics of CSCs (Chang and Hung, 2012). Down-regulation of let-7 and miR-200 family members occurs in CSCs of various tumors, including breast cancer (Golestaneh *et al.*, 2012;Yu *et al.*, 2007;Shimono *et al.*, 2009), suggesting that the let-7 and miR-200 families play critical roles in the regulation of CSCs via regulation of multiple signaling pathways implicated in survival and the acquisition of EMT. For this reason, these miRNAs could serve as CSC markers and therapeutic targets.

4.2. miR-21

It appears that miR-21 acts as an oncogenic molecule by targeting multiple survival signaling pathways (Pang *et al.*, 2010). Increased expression of miR-21 in pancreatic, prostate, lung, and breast cancer tumors, as well as in leukemia, is associated with poor clinical outcomes (Dillhoff *et al.*, 2008;Moriyama *et al.*, 2009) and the suppression of PTEN expression (Ali *et al.*, 2010;Bao *et al.*, 2012b). In addition, miR-21displays anti-apoptotic activity and enhances the proliferative, invasive and angiogenic potentials in a wide variety of tumor cells (Moriyama *et al.*, 2009;Zhang *et al.*, 2007). Expression of miR-21 is increased significantly in CSC populations as compared to non-CSC cancer cells while forced overexpression of miR-21 by its mimic cells enhances survival of bone marrow mesenchymal stem cells (Golestaneh *et al.*, 2012;Han *et al.*, 2012). Conversely, functional loss of miR-21 increased apoptosis of mesenchymal stem cells (Nie *et al.*, 2011).

4.3. miR-34a

It has been reported that miR-34a is under-expressed in a variety of tumors, including those associated with pancreatic, prostate, breast and lung cancers, and with leukemia (Kent *et al.*, 2009;Kong *et al.*, 2012a). Decreased levels of miR-34a are also thought to be associated with poor clinical outcomes (Kent *et al.*, 2009;Kong *et al.*, 2012a). Mounting evidence suggests that miR-34a may act as a tumor suppressor by inhibiting cell survival, proliferation, invasion, and metastasis, which are mediated, in part, through activation of p53 and inactivation of Cyclin D1, E2F1/2, and CDK6 in tumor cells (Aranha *et al.*, 2011;Guo *et al.*, 2011;Lodygin *et al.*, 2008;Sun *et al.*, 2008;Wang *et al.*, 2011). It has been shown that miR-34a inhibits the expression of CSC signature genes such as CD44, CD133, and Notch-1, which is consistent with its ability to attenuate the self-renewal capacity of many tumor cells (Kong *et al.*, 2012a;Liu and Tang, 2011;Nalls *et al.*, 2011). The expression of miR-34a has been found to be significantly decreased in CD133+ glioma CSC-like cells (Sun *et al.*, 2012). This suggests the loss of miR-34a acts as a tumor suppressor in the regulation of the CSC function, and points to the possibility of employing miR-34a as a CSC

marker and therapeutic target. A strategy to up-regulate miR-34a in tumors would be a novel approach for the treatment of cancer.

5. Common experimental methods used in CSC research

Summarized on Table 1 are several common methods or techniques that have been used to better understand the biology of CSCs.

6. The anti-diabetic drug metformin as a potential anti-tumor agent targeting CSC subpopulations

Metformin, a biguanide, oral hypoglycemic agent, is the most commonly used drug for the treatment of type 2 diabetes mellitus (DM). Metformin reduces blood glucose levels by the down-regulation of hepatic gluconeogenesis and up-regulation of glucose uptake in peripheral tissues, such as skeletal muscle and fat (Shaw *et al.*, 2005). It also enhances the insulin sensitivity of tissues, thereby reducing insulin levels overall.

Epidemiological and clinical studies indicate a reduced incidence of breast and pancreatic cancers in DM patients taking metformin, and that its consumption improves the clinical outcome of cancer patients (Bowker *et al.*, 2006;Heikkinen *et al.*, 2007;Landman *et al.*, 2010;Libby *et al.*, 2009;Monami *et al.*, 2009). While these findings suggest that metformin has anti-tumor effects, the mechanism of this action remains undefined. One possibility is that the metformin-induced increase in insulin sensitivity inhibit cancer cell growth by activation of AMP kinase (AMPK) which, in turn, inhibits the PI3K/Akt/mTOR signaling pathway via phosphorylation of mTOR (Dowling *et al.*, 2007;Zakikhani *et al.*, 2006) and a rapid inhibition of cellular protein synthesis and growth (Cazzaniga *et al.*, 2009;Goodwin *et al.*, 2009;Martin-Castillo *et al.*, 2010). Moreover, metformin can directly inhibit tumor cell growth and proliferation by regulating the cyclin D1-mediated cell cycle, p53 expression, and phosphorylation in breast and pancreatic cancers (Ben, I *et al.*, 2010;Feng *et al.*, 2007). Metformin can also decrease the production of inflammatory cytokines, including TNF-α, IL-6, and VEGF by inactivation of NF-κB and HIF-1α (Ersoy *et al.*, 2008;Lund *et al.*, 2008;Huang *et al.*, 2009). It is also possible that the anti-tumor activity of metformin *in vitro* and *in vivo* may be associated with inhibition of the insulin/IGF-1 pathway through AMPK activation (Kisfalvi *et al.*, 2009;Rozengurt *et al.*, 2010) by inactivation of breast $CD44^+$/$CD24^-$ CSC cells and the EMT phenotype (Hirsch *et al.*, 2009;Vazquez-Martin *et al.*, 2010) or by inhibiting cell growth, clonogenic potential, migration/invasion, and CSC self-renewal capacity in gemcitabine-resistant pancreatic cancer cells (Bao *et al.*, 2012c). It has also been found that metformin inhibits the expression of the CSC surface markers CD44 and EpCAM, expression of CSC genes such as EZH2, Notch-1, Nanog, and Oct4, and the miRNA expression of let-7 and miR-200 family in the CSC-like sphere cells of gemcitabine-resistant cells (Bao *et al.*, 2012c). These findings indicate that the anti-tumor effects of metformin may involve the targeting of CSC subpopulations, providing additional proof in support of the importance of CSC cells in cancer.

7. Perspective and conclusion

A considerable body of evidence supports the hypothesis that a very small population of CSCs is associated with an aggressive tumor phenotype characterized by increased cell survival, migration, invasion, metastatic capacity, treatment resistance, and tumor recurrence, all of which ultimately contribute to poor prognosis. Although there have been efforts to characterize CSCs, their pathogenesis and molecular interactions in the tumor microenvironment are not well defined. The identification of CSC-specific markers, and the isolation and characterization of CSCs in malignant tissues will provide insights that will be

of value in designing strategies for the development of chemotherapeutics that is expected to reduce tumor aggressiveness by targeting CSCs. Drug-induced modification of CSC-associated markers will modulate the phenotype and consequent function of these cells. For example, miRNAs such as, let-7, miR-200, miR-21 and miR-34a are possible targets as they play key roles in CSC regulation via multiple signaling pathways that regulate cell growth and survival. Because these miRNAs can be differentially expressed in the CSCs or CSC-like cells of various tumors, they may also be useful as CSC markers. The validity of this approach is suggested by the finding that metformin, an anti-diabetic drug, displays anti-tumor effects that may be due to the targeted elimination of CSCs. Additional clinical and preclinical work is required to demonstrate conclusively the therapeutic benefit of metformin, and CSC-targeting drugs in general, for the management of particular cancers.

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Reference List

Akunuru S, Palumbo J, Zhai QJ, Zheng Y. Rac1 targeting suppresses human non-small cell lung adenocarcinoma cancer stem cell activity. PLoS One. 2011; 6(2):e16951. [PubMed: 21347385]

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A. 2003; 100(7):3983–3988. [PubMed: 12629218]

Ali S, Ahmad A, Banerjee S, Padhye S, Dominiak K, Schaffert JM, Wang Z, Philip PA, Sarkar FH. Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of miR-200 and miR-21 expression by curcumin or its analogue CDF. Cancer Res. 2010; 70(9):3606–3617. [PubMed: 20388782]

Aranha MM, Santos DM, Sola S, Steer CJ, Rodrigues CM. miR-34a regulates mouse neural stem cell differentiation. PLoS One. 2011; 6(8):e21396. [PubMed: 21857907]

Bao B, Ahmad A, Li Y, Azmi AS, Ali S, Banerjee S, Kong D, Sarkar FH. Targeting CSCs within the tumor microenvironment for cancer therapy: a potential role of mesenchymal stem cells. Expert Opin Ther Targets. 2012a

Bao B, Ali S, Banerjee S, Wang Z, Logna F, Azmi AS, Kong D, Ahmad A, Li Y, Padhye S, Sarkar FH. Curcumin Analogue CDF Inhibits Pancreatic Tumor Growth by Switching on Suppressor microRNAs and Attenuating EZH2 Expression. Cancer Res. 2012b; 72(1):335–345. [PubMed: 22108826]

Bao B, Wang Z, Ali S, Ahmad A, Azmi AS, Sarkar SH, Banerjee S, Kong D, Li Y, Thakur S, Sarkar FH. Metformin Inhibits Cell Proliferation, Migration and Invasion by Attenuating CSC Function Mediated by Deregulating miRNAs in Pancreatic Cancer Cells. Cancer Prev Res (Phila). 2012c; 5:355–364. [PubMed: 22086681]

Bao B, Wang Z, Ali S, Kong D, Li Y, Ahmad A, Banerjee S, Azmi AS, Miele L, Sarkar FH. Notch-1 induces epithelial-mesenchymal transition consistent with cancer stem cell phenotype in pancreatic cancer cells. Cancer Lett. 2011; 307(1):26–36. [PubMed: 21463919]

Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature. 2006; 444(7120):756–760. [PubMed: 17051156]

Bauerschmitz GJ, Ranki T, Kangasniemi L, Ribacka C, Eriksson M, Porten M, Herrmann I, Ristimaki A, Virkkunen P, Tarkkanen M, Hakkarainen T, Kanerva A, Rein D, Pesonen S, Hemminki A. Tissue-specific promoters active in CD44+CD24-/low breast cancer cells. Cancer Res. 2008; 68(14):5533–5539. [PubMed: 18632604]

Ben SI, Le Marchand-Brustel Y, Tanti JF, Bost F. Metformin in cancer therapy: a new perspective for an old antidiabetic drug? Mol Cancer Ther. 2010; 9(5):1092–1099. [PubMed: 20442309]

Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C. Criteria for the diagnosis of acute leukemia of megakaryocyte lineage (M7). A report of the French-American-British Cooperative Group. Ann Intern Med. 1985; 103(3):460–462. [PubMed: 2411180]

Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci U S A. 1997; 94(10):5320–5325. [PubMed: 9144235]

Blair A, Hogge DE, Ailles LE, Lansdorp PM, Sutherland HJ. Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo. Blood. 1997; 89(9):3104–3112. [PubMed: 9129012]

Blair A, Sutherland HJ. Primitive acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo lack surface expression of c-kit (CD117). Exp Hematol. 2000; 28(6):660–671. [PubMed: 10880752]

Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997; 3(7):730–737. [PubMed: 9212098]

Bowker SL, Majumdar SR, Veugelers P, Johnson JA. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. Diabetes Care. 2006; 29(2):254–258. [PubMed: 16443869]

Budel LM, Touw IP, Delwel R, Clark SC, Lowenberg B. Interleukin-3 and granulocyte-monocyte colony-stimulating factor receptors on human acute myelocytic leukemia cells and relationship to the proliferative response. Blood. 1989; 74(2):565–571. [PubMed: 2546627]

Cazzaniga M, Bonanni B, Guerrieri-Gonzaga A, Decensi A. Is it time to test metformin in breast cancer clinical trials? Cancer Epidemiol Biomarkers Prev. 2009; 18(3):701–705. [PubMed: 19240238]

Chang CJ, Hsu CC, Chang CH, Tsai LL, Chang YC, Lu SW, Yu CH, Huang HS, Wang JJ, Tsai CH, Chou MY, Yu CC, Hu FW. Let-7d functions as novel regulator of epithelial-mesenchymal transition and chemoresistant property in oral cancer. Oncol Rep. 2011; 26(4):1003–1010. [PubMed: 21725603]

Chang CJ, Hung MC. The role of EZH2 in tumour progression. Br J Cancer. 2012; 106(2):243–247. [PubMed: 22187039]

Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, Hur MH, Diebel ME, Monville F, Dutcher J, Brown M, Viens P, Xerri L, Bertucci F, Stassi G, Dontu G, Birnbaum D, Wicha MS. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. Cancer Res. 2009; 69(4):1302–1313. [PubMed: 19190339]

Creighton CJ, Chang JC, Rosen JM. Epithelial-mesenchymal transition (EMT) in tumor-initiating cells and its clinical implications in breast cancer. J Mammary Gland Biol Neoplasia. 2010; 15(2):253–260. [PubMed: 20354771]

Croker AK, Goodale D, Chu J, Postenka C, Hedley BD, Hess DA, Allan AL. High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. J Cell Mol Med. 2009; 13(8B):2236–2252. [PubMed: 18681906]

Dick JE. Acute myeloid leukemia stem cells. Ann N Y Acad Sci. 2005; 1044:1–5. [PubMed: 15958691]

Dillhoff M, Liu J, Frankel W, Croce C, Bloomston M. MicroRNA-21 is overexpressed in pancreatic cancer and a potential predictor of survival. J Gastrointest Surg. 2008; 12(12):2171–2176. [PubMed: 18642050]

Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. Cancer Res. 2007; 67(22):10804–10812. [PubMed: 18006825]

Dylla SJ, Beviglia L, Park IK, Charter C, Raval J, Ngan L, Pickell K, Aguilar J, Lazetic S, Smith-Berdan S, Clarke MF, Hoey T, Lewicki J, Gurney AL. Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. PLoS One. 2008; 3(6):e2428. [PubMed: 18560594]

Edwards BK, Brown ML, Wingo PA, Howe HL, Ward E, Ries LA, Schrag D, Jamison PM, Jemal A, Wu XC, Friedman C, Harlan L, Warren J, Anderson RN, Pickle LW. Annual report to the nation on the status of cancer, 1975-2002, featuring population-based trends in cancer treatment. J Natl Cancer Inst. 2005; 97(19):1407–1427. [PubMed: 16204691]

Engelmann K, Shen H, Finn OJ. MCF7 side population cells with characteristics of cancer stem/progenitor cells express the tumor antigen MUC1. Cancer Res. 2008; 68(7):2419–2426. [PubMed: 18381450]

Eramo A, Haas TL, De MR. Lung cancer stem cells: tools and targets to fight lung cancer. Oncogene. 2010; 29(33):4625–4635. [PubMed: 20531299]

Ersoy C, Kiyici S, Budak F, Oral B, Guclu M, Duran C, Selimoglu H, Erturk E, Tuncel E, Imamoglu S. The effect of metformin treatment on VEGF and PAI-1 levels in obese type 2 diabetic patients. Diabetes Res Clin Pract. 2008; 81(1):56–60. [PubMed: 18358555]

Fabbri M, Ivan M, Cimmino A, Negrini M, Calin GA. Regulatory mechanisms of microRNAs involvement in cancer. Expert Opin Biol Ther. 2007; 7(7):1009–1019. [PubMed: 17665990]

Feng Z, Hu W, de SE, Teresky AK, Jin S, Lowe S, Levine AJ. The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. Cancer Res. 2007; 67(7):3043–3053. [PubMed: 17409411]

Fields AP, Regala RP. Protein kinase C iota: human oncogene, prognostic marker and therapeutic target. Pharmacol Res. 2007; 55(6):487–497. [PubMed: 17570678]

Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell. 2007; 1(5):555–567. [PubMed: 18371393]

Golestaneh AF, Atashi A, Langroudi L, Shafiee A, Ghaemi N, Soleimani M. miRNAs expressed differently in cancer stem cells and cancer cells of human gastric cancer cell line MKN-45. Cell Biochem Funct. 2012

Goodwin PJ, Ligibel JA, Stambolic V. Metformin in breast cancer: time for action. J Clin Oncol. 2009; 27(20):3271–3273. [PubMed: 19487373]

Guo Y, Li S, Qu J, Wang S, Dang Y, Fan J, Yu S, Zhang J. MiR-34a inhibits lymphatic metastasis potential of mouse hepatoma cells. Mol Cell Biochem. 2011; 354(1-2):275–282. [PubMed: 21553024]

Han M, Wang Y, Liu M, Bi X, Bao J, Zeng N, Zhu Z, Mo Z, Wu C, Chen X. MiR-21 regulates epithelial-mesenchymal transition phenotype and hypoxia-inducible factor-1alpha expression in third-sphere forming breast cancer stem cell-like cells. Cancer Sci. 2012; 103:1058–1064. [PubMed: 22435731]

Heikkinen S, Auwerx J, Argmann CA. PPARgamma in human and mouse physiology. Biochim Biophys Acta. 2007; 1771(8):999–1013. [PubMed: 17475546]

Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschen C. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell. 2007; 1(3):313–323. [PubMed: 18371365]

Hikita ST, Kosik KS, Clegg DO, Bamdad C. MUC1* mediates the growth of human pluripotent stem cells. PLoS One. 2008; 3(10):e3312. [PubMed: 18833326]

Hirsch HA, Iliopoulos D, Tsichlis PN, Struhl K. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. Cancer Res. 2009; 69(19):7507–7511. [PubMed: 19752085]

Ho MM, Ng AV, Lam S, Hung JY. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. Cancer Res. 2007; 67(10):4827–4833. [PubMed: 17510412]

Hong SP, Wen J, Bang S, Park S, Song SY. CD44-positive cells are responsible for gemcitabine resistance in pancreatic cancer cells. Int J Cancer. 2009; 125(10):2323–2331. [PubMed: 19598259]

Huang NL, Chiang SH, Hsueh CH, Liang YJ, Chen YJ, Lai LP. Metformin inhibits TNF-alpha-induced IkappaB kinase phosphorylation, IkappaB-alpha degradation and IL-6 production in endothelial cells through PI3K-dependent AMPK phosphorylation. Int J Cardiol. 2009; 134(2):169–175. [PubMed: 18597869]

Hurt EM, Kawasaki BT, Klarmann GJ, Thomas SB, Farrar WL. CD44+ CD24(-) prostate cells are early cancer progenitor/stem cells that provide a model for patients with poor prognosis. Br J Cancer. 2008; 98(4):756–765. [PubMed: 18268494]

Iliopoulos D, Lindahl-Allen M, Polytarchou C, Hirsch HA, Tsichlis PN, Struhl K. Loss of miR-200 inhibition of Suz12 leads to polycomb-mediated repression required for the formation and maintenance of cancer stem cells. Mol Cell. 2010; 39(5):761–772. [PubMed: 20832727]

Jin L, Hope KJ, Zhai Q, Smadja-Joffe F, Dick JE. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. Nat Med. 2006; 12(10):1167–1174. [PubMed: 16998484]

Jin L, Lee EM, Ramshaw HS, Busfield SJ, Peoppl AG, Wilkinson L, Guthridge MA, Thomas D, Barry EF, Boyd A, Gearing DP, Vairo G, Lopez AF, Dick JE, Lock RB. Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells. Cell Stem Cell. 2009; 5(1):31–42. [PubMed: 19570512]

Johnsen HE, Kjeldsen MK, Urup T, Fogd K, Pilgaard L, Boegsted M, Nyegaard M, Christiansen I, Bukh A, Dybkaer K. Cancer stem cells and the cellular hierarchy in haematological malignancies. Eur J Cancer. 2009; 45(Suppl 1):194–201. [PubMed: 19775618]

Jordan CT, Upchurch D, Szilvassy SJ, Guzman ML, Howard DS, Pettigrew AL, Meyerrose T, Rossi R, Grimes B, Rizzieri DA, Luger SM, Phillips GL. The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. Leukemia. 2000; 14(10):1777–1784. [PubMed: 11021753]

Kasper S. Identification, characterization, and biological relevance of prostate cancer stem cells from clinical specimens. Urol Oncol. 2009; 27(3):301–303. [PubMed: 19414117]

Kent OA, Mullendore M, Wentzel EA, Lopez-Romero P, Tan AC, Alvarez H, West K, Ochs MF, Hidalgo M, Arking DE, Maitra A, Mendell JT. A resource for analysis of microRNA expression and function in pancreatic ductal adenocarcinoma cells. Cancer Biol Ther. 2009; 8(21):2013–2024. [PubMed: 20037478]

Kisfalvi K, Eibl G, Sinnett-Smith J, Rozengurt E. Metformin disrupts crosstalk between G protein-coupled receptor and insulin receptor signaling systems and inhibits pancreatic cancer growth. Cancer Res. 2009; 69(16):6539–6545. [PubMed: 19679549]

Kissil JL, Walmsley MJ, Hanlon L, Haigis KM, Bender Kim CF, Sweet-Cordero A, Eckman MS, Tuveson DA, Capobianco AJ, Tybulewicz VL, Jacks T. Requirement for Rac1 in a K-ras induced lung cancer in the mouse. Cancer Res. 2007; 67(17):8089–8094. [PubMed: 17804720]

Kitamura H, Okudela K, Yazawa T, Sato H, Shimoyamada H. Cancer stem cell: implications in cancer biology and therapy with special reference to lung cancer. Lung Cancer. 2009; 66(3):275–281. [PubMed: 19716622]

Klein RS, Rubin JB, Gibson HD, DeHaan EN, Alvarez-Hernandez X, Segal RA, Luster AD. SDF-1 alpha induces chemotaxis and enhances Sonic hedgehog-induced proliferation of cerebellar granule cells. Development. 2001; 128(11):1971–1981. [PubMed: 11493520]

Klonisch T, Wiechec E, Hombach-Klonisch S, Ande SR, Wesselborg S, Schulze-Osthoff K, Los M. Cancer stem cell markers in common cancers - therapeutic implications. Trends Mol Med. 2008; 14(10):450–460. [PubMed: 18775674]

Kong D, Banerjee S, Ahmad A, Li Y, Wang Z, Sethi S, Sarkar FH. Epithelial to mesenchymal transition is mechanistically linked with stem cell signatures in prostate cancer cells. PLoS One. 2010; 5(8):e12445. [PubMed: 20805998]

Kong D, Heath E, Chen W, Cher M, Powell I, Heilbrun L, Li Y, Ali S, Sethi S, Hassan O, Hwang C, Gupta N, Chitale D, Sakr WA, Menon M, Sarkar FH. Epigenetic silencing of miR-34a in human prostate cancer cells and tumor tissue specimens can be reversed by BR-DIM treatment. Am J Transl Res. 2012a; 4(1):14–23. [PubMed: 22347519]

Kong D, Heath E, Chen W, Cher ML, Powell I, Heilbrun L, Li Y, Ali S, Sethi S, Hassan O, Hwang C, Gupta N, Chitale D, Sakr WA, Menon M, Sarkar FH. Loss of Let-7 Up-Regulates EZH2 in Prostate Cancer Consistent with the Acquisition of Cancer Stem Cell Signatures That Are Attenuated by BR-DIM. PLoS One. 2012b; 7(3):e33729. [PubMed: 22442719]

Kong D, Li Y, Wang Z, Banerjee S, Ahmad A, Kim HR, Sarkar FH. miR-200 regulates PDGF-D-mediated epithelial-mesenchymal transition, adhesion, and invasion of prostate cancer cells. Stem Cells. 2009; 27(8):1712–1721. [PubMed: 19544444]

Kufe DW. MUC1-C oncoprotein as a target in breast cancer: activation of signaling pathways and therapeutic approaches. Oncogene. 2012 (in press).

Landman GW, Kleefstra N, van Hateren KJ, Groenier KH, Gans RO, Bilo HJ. Metformin associated with lower cancer mortality in type 2 diabetes: ZODIAC-16. Diabetes Care. 2010; 33(2):322–326. [PubMed: 19918015]

Leal JA, Lleonart ME. MicroRNAs and cancer stem cells: Therapeutic approaches and future perspectives. Cancer Lett. 2012 (in press).

Lee CJ, Dosch J, Simeone DM. Pancreatic cancer stem cells. J Clin Oncol. 2008; 26(17):2806–2812. [PubMed: 18539958]

Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. Identification of pancreatic cancer stem cells. Cancer Res. 2007; 67(3):1030–1037. [PubMed: 17283135]

Li Y, Kong D, Ahmad A, Bao B, Sarkar FH. Pancreatic cancer stem cells: Emerging target for designing novel therapy. Cancer Lett. 2012

Li Y, VandenBoom TG, Kong D, Wang Z, Ali S, Philip PA, Sarkar FH. Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. Cancer Res. 2009; 69(16):6704–6712. [PubMed: 19654291]

Libby G, Donnelly LA, Donnan PT, Alessi DR, Morris AD, Evans JM. New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes. Diabetes Care. 2009; 32(9):1620–1625. [PubMed: 19564453]

Liu C, Tang DG. MicroRNA regulation of cancer stem cells. Cancer Res. 2011; 71(18):5950–5954. [PubMed: 21917736]

Lodygin D, Tarasov V, Epanchintsev A, Berking C, Knyazeva T, Korner H, Knyazev P, Diebold J, Hermeking H. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. Cell Cycle. 2008; 7(16):2591–2600. [PubMed: 18719384]

Lund SS, Tarnow L, Stehouwer CD, Schalkwijk CG, Teerlink T, Gram J, Winther K, Frandsen M, Smidt UM, Pedersen O, Parving HH, Vaag AA. Impact of metformin versus repaglinide on non-glycaemic cardiovascular risk markers related to inflammation and endothelial dysfunction in non-obese patients with type 2 diabetes. Eur J Endocrinol. 2008; 158(5):631–641. [PubMed: 18426821]

Majeti R, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs KD Jr, van RN, Weissman IL. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. Cell. 2009; 138(2):286–299. [PubMed: 19632179]

Martin-Castillo B, Vazquez-Martin A, Oliveras-Ferraros C, Menendez JA. Metformin and cancer: Doses, mechanisms and the dandelion and hormetic phenomena. Cell Cycle. 2010; 9(6):1057–1064. [PubMed: 20305377]

Matsui W, Wang Q, Barber JP, Brennan S, Smith BD, Borrello I, McNiece I, Lin L, Ambinder RF, Peacock C, Watkins DN, Huff CA, Jones RJ. Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. Cancer Res. 2008; 68(1):190–197. [PubMed: 18172311]

McCarty MF. Metformin may antagonize Lin28 and/or Lin28B activity, thereby boosting let-7 levels and antagonizing cancer progression. Med Hypotheses. 2012; 78(2):262–269. [PubMed: 22129484]

Monami M, Lamanna C, Balzi D, Marchionni N, Mannucci E. Sulphonylureas and cancer: a case-control study. Acta Diabetol. 2009; 46(4):279–284. [PubMed: 19082520]

Moriyama T, Ohuchida K, Mizumoto K, Yu J, Sato N, Nabae T, Takahata S, Toma H, Nagai E, Tanaka M. MicroRNA-21 modulates biological functions of pancreatic cancer cells including their proliferation, invasion, and chemoresistance. Mol Cancer Ther. 2009; 8(5):1067–1074. [PubMed: 19435867]

Nalls D, Tang SN, Rodova M, Srivastava RK, Shankar S. Targeting epigenetic regulation of miR-34a for treatment of pancreatic cancer by inhibition of pancreatic cancer stem cells. PLoS One. 2011; 6(8):e24099. [PubMed: 21909380]

Narducci MG, Scala E, Bresin A, Caprini E, Picchio MC, Remotti D, Ragone G, Nasorri F, Frontani M, Arcelli D, Volinia S, Lombardo GA, Baliva G, Napolitano M, Russo G. Skin homing of Sezary

cells involves SDF-1-CXCR4 signaling and down-regulation of CD26/dipeptidylpeptidase IV. Blood. 2006; 107(3):1108–1115. [PubMed: 16204308]

Nie Y, Han BM, Liu XB, Yang JJ, Wang F, Cong XF, Chen X. Identification of MicroRNAs involved in hypoxia- and serum deprivation-induced apoptosis in mesenchymal stem cells. Int J Biol Sci. 2011; 7(6):762–768. [PubMed: 21698002]

Nurwidya F, Murakami A, Takahashi F, Takahashi K. Lung cancer stem cells: Tumor biology and clinical implications. Asia Pac J Clin Oncol. 2012; 8:217–222. [PubMed: 22897822]

Olson P, Lu J, Zhang H, Shai A, Chun MG, Wang Y, Libutti SK, Nakakura EK, Golub TR, Hanahan D. MicroRNA dynamics in the stages of tumorigenesis correlate with hallmark capabilities of cancer. Genes Dev. 2009; 23(18):2152–2165. [PubMed: 19759263]

Pang Y, Young CY, Yuan H. MicroRNAs and prostate cancer. Acta Biochim Biophys Sin (Shanghai). 2010; 42(6):363–369. [PubMed: 20539944]

Patrawala L, Calhoun T, Schneider-Broussard R, Zhou J, Claypool K, Tang DG. Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and A. Cancer Res. 2005; 65(14):6207–6219. [PubMed: 16024622]

Patrawala L, Calhoun-Davis T, Schneider-Broussard R, Tang DG. Hierarchical organization of prostate cancer cells in xenograft tumors: the CD44+alpha2beta1+ cell population is enriched in tumor-initiating cells. Cancer Res. 2007; 67(14):6796–6805. [PubMed: 17638891]

Peter ME. Let-7 and miR-200 microRNAs: guardians against pluripotency and cancer progression. Cell Cycle. 2009; 8(6):843–852. [PubMed: 19221491]

Prud'homme GJ. Cancer Stem Cells and Novel Targets for Antitumor Strategies. Curr Pharm Des. 2012; 18:2838–2849. [PubMed: 22390767]

Rasheed ZA, Matsui W. Biological and clinical relevance of stem cells in pancreatic adenocarcinoma. J Gastroenterol Hepatol. 2012; 27(Suppl 2):15–18. [PubMed: 22320910]

Regala RP, Davis RK, Kunz A, Khoor A, Leitges M, Fields AP. Atypical protein kinase C{iota} is required for bronchioalveolar stem cell expansion and lung tumorigenesis. Cancer Res. 2009; 69(19):7603–7611. [PubMed: 19738040]

Rozengurt E, Sinnett-Smith J, Kisfalvi K. Crosstalk between insulin/insulin-like growth factor-1 receptors and G protein-coupled receptor signaling systems: a novel target for the antidiabetic drug metformin in pancreatic cancer. Clin Cancer Res. 2010; 16(9):2505–2511. [PubMed: 20388847]

Sarkar FH, Li Y, Wang Z, Kong D. Pancreatic cancer stem cells and EMT in drug resistance and metastasis. Minerva Chir. 2009; 64(5):489–500. [PubMed: 19859039]

Shafee N, Smith CR, Wei S, Kim Y, Mills GB, Hortobagyi GN, Stanbridge EJ, Lee EY. Cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors. Cancer Res. 2008; 68(9):3243–3250. [PubMed: 18451150]

Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, DePinho RA, Montminy M, Cantley LC. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science. 2005; 310(5754):1642–1646. [PubMed: 16308421]

Shimono Y, Zabala M, Cho RW, Lobo N, Dalerba P, Qian D, Diehn M, Liu H, Panula SP, Chiao E, Dirbas FM, Somlo G, Pera RA, Lao K, Clarke MF. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell. 2009; 138(3):592–603. [PubMed: 19665978]

Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012; 62(1):10–29. [PubMed: 22237781]

Sun F, Fu H, Liu Q, Tie Y, Zhu J, Xing R, Sun Z, Zheng X. Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest. FEBS Lett. 2008; 582(10):1564–1568. [PubMed: 18406353]

Sun L, Wu Z, Shao Y, Pu Y, Miu W, Yao J, Wu Y, Yang ZX. MicroRNA-34a Suppresses Cell Proliferation and Induces Apoptosis in U87 Glioma Stem Cells. Technol Cancer Res Treat. 2012; 11(5):483–490. [PubMed: 22568628]

Sung JM, Cho HJ, Yi H, Lee CH, Kim HS, Kim DK, Abd El-Aty AM, Kim JS, Landowski CP, Hediger MA, Shin HC. Characterization of a stem cell population in lung cancer A549 cells. Biochem Biophys Res Commun. 2008; 371(1):163–167. [PubMed: 18423378]

Testa U, Riccioni R, Diverio D, Rossini A, Lo CF, Peschle C. Interleukin-3 receptor in acute leukemia. Leukemia. 2004; 18(2):219–226. [PubMed: 14671644]

Toll AD, Dasgupta A, Potoczek M, Yeo CJ, Kleer CG, Brody JR, Witkiewicz AK. Implications of enhancer of zeste homologue 2 expression in pancreatic ductal adenocarcinoma. Hum Pathol. 2010; 41(9):1205–1209. [PubMed: 20573371]

Tsujimura A, Koikawa Y, Salm S, Takao T, Coetzee S, Moscatelli D, Shapiro E, Lepor H, Sun TT, Wilson EL. Proximal location of mouse prostate epithelial stem cells: a model of prostatic homeostasis. J Cell Biol. 2002; 157(7):1257–1265. [PubMed: 12082083]

Vazquez-Martin A, Oliveras-Ferraros C, Barco SD, Martin-Castillo B, Menendez JA. The anti-diabetic drug metformin suppresses self-renewal and proliferation of trastuzumab-resistant tumor-initiating breast cancer stem cells. Breast Cancer Res Treat. 2011; 126:355–364. [PubMed: 20458531]

Wang X, Meyers C, Guo M, Zheng ZM. Upregulation of p18Ink4c expression by oncogenic HPV E6 via p53-miR-34a pathway. Int J Cancer. 2011; 129(6):1362–1372. [PubMed: 21128241]

Warner JK, Wang JC, Hope KJ, Jin L, Dick JE. Concepts of human leukemic development. Oncogene. 2004; 23(43):7164–7177. [PubMed: 15378077]

Wendlandt EB, Graff JW, Gioannini TL, McCaffrey AP, Wilson ME. The role of MicroRNAs miR-200b and miR-200c in TLR4 signaling and NF-kappaB activation. Innate Immun. 2012

Wu X, Chen H, Wang X. Can lung cancer stem cells be targeted for therapies? Cancer Treat Rev. 2012; 38(6):580–588. [PubMed: 22436486]

Xin L, Lawson DA, Witte ON. The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis. Proc Natl Acad Sci U S A. 2005; 102(19): 6942–6947. [PubMed: 15860580]

Yu C, Yao Z, Jiang Y, Keller ET. Prostate cancer stem cell biology. Minerva Urol Nefrol. 2012a; 64(1):19–33. [PubMed: 22402315]

Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J, Song E. let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell. 2007; 131(6):1109–1123. [PubMed: 18083101]

Yu Y, Ramena G, Elble RC. The role of cancer stem cells in relapse of solid tumors. Front Biosci (Elite Ed). 2012b; 4:1528–1541. [PubMed: 22201973]

Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res. 2006; 66(21):10269–10273. [PubMed: 17062558]

Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. Dev Biol. 2007; 302(1):1–12. [PubMed: 16989803]

Zhou Z, Flesken-Nikitin A, Nikitin AY. Prostate cancer associated with p53 and Rb deficiency arises from the stem/progenitor cell-enriched proximal region of prostatic ducts. Cancer Res. 2007; 67(12):5683–5690. [PubMed: 17553900]

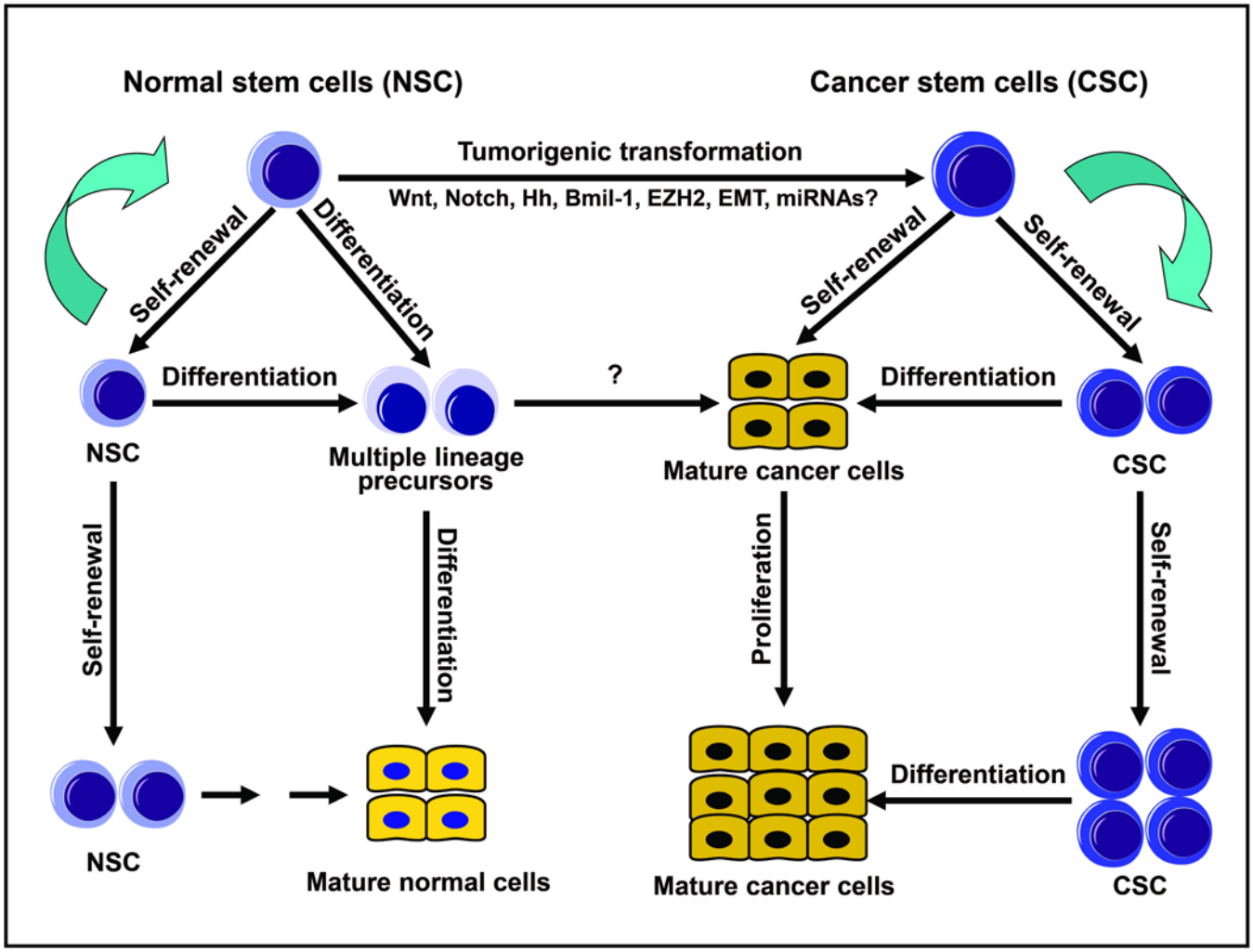

Figure 1.
The implication of stem cells in the development and progression of tumor. NSC: normal stem cells; CSC: cancer stem cells; Hh: hedgehog; Bmil-1: polycomb complex protein; EZH2: enhancer of zeste homolog 2; miRNAs: microRNAs.

Table 1

Most Common Experimental Methods/Assays in CSC Research

Methods/Assays	**Biological Significance in CSC Research**
1. Clonogenic assay (Ali *et al.*, 2010)	Evaluation of proliferative potential of CSCs
2. Sphere formation assay (Bao *et al.*, 2012b)	Evaluation of CSC self-renewal capacity
3. Immunostaining assay for FACS*, image microscopy, or flow cytometry (Kong *et al.*, 2009)	Isolation of pure CSC subpopulation and/or evaluation of the expression of CSC markers
4. Real-time RT-PCR assay (Bao *et al.*, 2012b)	Evaluation of the expression of CSC marker mRNAs and CSC-related miRNAs
5.Mouse xenograft tumor assay of CSC or CSC-like sphere cells (Bao *et al.*, 2012b)	Evaluation of CSC or CSC-like sphere cells-initializing tumor formation

* FACS (fluorescence activated cell sorting).