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Cancer stem cells: emerging actors in both basic and clinical cancer research

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Abstract: Cancer stem cells (CSCs) are a small subset of cancer cells within a tumor that are responsible for tumorigenesis and contribute to drug resistance. The CSC displays an anchorage-independent survival, active DNA-repair capacity, and relative quiescence and is capable of self-renewing and maintaining tumor growth and heterogeneity. At the molecular level, there are several signaling pathways (e.g., Wnt/β-catenin, Notch, and Hedgehog) to control CSC properties and alteration of these pathways has been recognized as an essential step for CSC transformation. Emerging evidence suggests that CSCs are clinically relevant. These cells are resistant to conventional chemotherapy and radiation treatment. Therefore, CSCs are thought to be the most important targets for anticancer therapy. In this review, we describe the characteristics of CSCs and how to isolate them based on some of their properties, as well as their importance in oncology.

Key words: Cancer stem cell, epithelial–mesenchymal transition, niche, drug resistance, oncology

1. Introduction

Over the past 50 years, many disease that cause death have dramatically decreased. Nevertheless, cancer deaths continue (Leaf, 2013). The International Agency for Research on Cancer, the specialized cancer agency of the World Health Organization, released the latest data on cancer incidence, mortality, and prevalence worldwide (Ferlay et al., 2013). According to GLOBOCAN 2012, an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared with 12.7 million and 7.6 million in 2008, respectively. The most commonly diagnosed cancers worldwide were those of the lung (13% of the total), breast (11.9%), and colorectum (9.7%). The most common causes of cancer death were cancers of the lung (19.4% of the total), liver (9.1%), and stomach (8.8%). GLOBOCAN 2012 predicted a substantive increase to 19.3 million new cancer cases per year by 2025 due to growth and aging of the global population (Ferlay et al., 2013). Despite these statistics, the ability to specifically target pathways altered in cancer raises the hope of developing therapies with high specificity and low toxicity. Therefore, it is important to target the 'right cells' (Wicha et al., 2006).

A large body of evidence is accumulating to indicate that most, if not all, malignancies can be viewed as abnormal organs with a stem cell compartment that drives the growth. Furthermore, besides tumor initiation, these tumor-initiating cells, also referred to as cancer stem cells (CSCs), are thought to be responsible for metastasis, recurrence, and drug resistance (Chen K et al., 2013). The CSC hypothesis has fundamental implications for understanding the biology of carcinogenesis as well as for developing new strategies for cancer prevention and eradication of malignancies.

2. Characteristics of CSCs

CSCs are cancer cells that possess characteristics associated with normal stem cells. At the molecular level, CSCs and normal stem cells share some common features, including the capacity for self-renewal (Reya et al., 2001), the ability to differentiate, active telomerase expression, activation of antiapoptotic pathways, increased membrane transporter activity, and the ability to migrate and metastasize (Wicha et al., 2006). In addition to these properties, they display an anchorage-independent survival, active DNA-repair capacity, and relative quiescence (slow cell cycling) (Dean et al., 2005; Wicha et al., 2006).

2.1. Concept of CSCs

Where CSCs come from is an intensely researched question. Some researchers suggest that CSCs may originate from mutated normal stem cells upon aberrant alteration of the self-renewal pathways (Reya et al., 2001). An alternative hypothesis is that CSCs originate from differentiated cells that have acquired stem-like features following

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multiple mutations. These features include the ability to self-renew and generate progenitors through asymmetric divisions to produce more committed progenitor cells or differentiated cells (Clarke et al., 2006). In addition, CSCs may be derived from progenitor cells that acquire a gain of function mutation to reactivate self-renewal pathways (Figure 1a) (Chaffer et al. 2011; Nguyen et al., 2012). CSCs may arise from normal stem cells, progenitor cells, or more differentiated cells through multiple mutations of genes as a result of their genomic instability (Li et al., 2009) or oncogene-induced plasticity (Rapp et al., 2008).

The process of carcinogenesis requires a series of mutations resulting in the acquisition of growth factor independence, resistance to growth-inhibitory signals, limitless replicative potential, tissue invasion, and metastasis (Hanahan and Weinberg, 2011). Related theories suggest that there are currently 2 accepted models as to how tumor heterogeneity arises. According to the CSC model, the heterogeneity and hierarchy among all of the cells within a tumor result from asymmetric division of CSCs. Therefore, based on this model, tumors are highly hierarchical with a unique self-renewing population of cells at the top of the hierarchy (Figure 1b). They are relatively less differentiated and quiescent cells that reside in a local microenvironment or 'niche' that controls their behavior. This population both self-renews and produces daughter progenitor cells that give rise to terminally differentiated cells. However, the clonal evolution model postulates that every cancer cell within a tumor has the same potential to act as a CSC (Hamburger et al., 1977; Pardal et al., 2003). Their variable activities are partially determined by some stochastically varying intrinsic factors that result from genetic/epigenetic changes during cancer development. These cells gain the ability to form tumors after accumulating mutations and then create clones of themselves with infinite lifespans. Thus, tumor cells with a growth advantage or the fittest subclones are selected and expanded upon selective pressure, similar to Darwinian evolution (Nowell, 1976).

2.2. Identification and isolation of CSCs

The discovery of a universal marker for CSCs has not yet been made. General methods for the identification and isolation of CSCs in malignancies include xenotransplantation assays, which are the gold-standard for identification of CSCs; sorting based upon cell surface markers; efflux of Hoechst 33342 or Rhodamine dyes; the enzymatic activity of aldehyde dehydrogenase (ALDH); and colony- and sphere-forming assays requiring specific culture conditions.

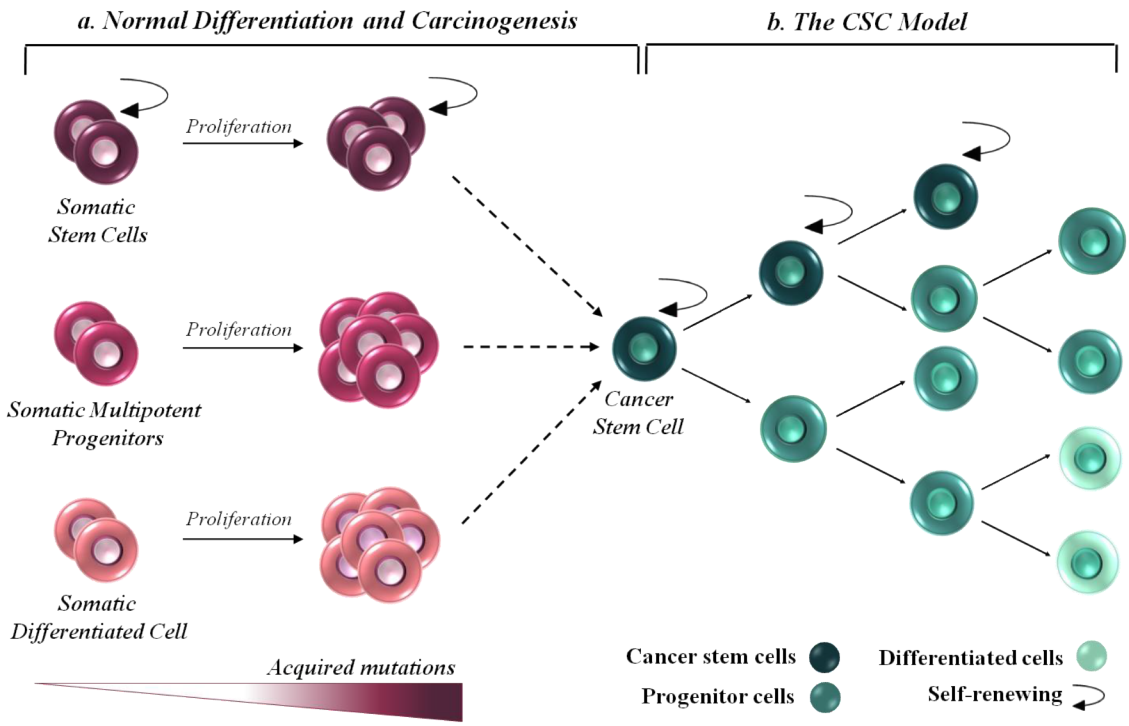

Figure 1. Hypothesis for the origin of cancer stem cells (**a**) and the role of cancer stem cells in tumorigenesis (**b**). CSCs arise from stem cells, progenitor cells, or differentiated cells present in adult tissue. CSCs divide asymmetrically and generate CSCs and more differentiated tumor cells, forming a hierarchical lineage system.

2.2.1. Cell surface markers

Many groups have isolated CSCs from primary tumors and cell lines by flow cytometry, fluorescence-activated cell sorting, or magnetic cell separation according to specific cell surface markers, most notably CD133, CD44, CD24, CD34, and CD38. Experimentally, CSCs are currently identified by using cell-surface markers and their ability to reestablish a new tumor with identical heterogeneity by transplanting them into new immunodeficient hosts in limiting dilutions (Bonnet and Dick, 1997). The first evidence supporting the hierarchical tumor model and a role for CSCs in tumor formation came from studies on acute myeloid leukemia (AML) (Lapidot et al., 1994; Bonnet and Dick, 1997). NOD/SCID mice receiving cancer cells from AML patients developed hematopoietic malignancy only when given cells expressing related stem cell markers, which in this case was $CD34^{+}CD38^{-}$. The first report for identification of CSCs in solid tumors came from Al-Hajj et al. (2003). They identified $ESA^{+}CD44^{+}CD24^{-/low}$ lineage cells as breast CSCs. There are many other studies that have shown the existence of these cells within different solid tumors, including those of the brain (Singh et al., 2003, 2004), prostate (Collins et al., 2005), colorectum (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), head and neck (Prince et al., 2007), lung (Eramo et al., 2008), ovary (Curley et al., 2009), and skin (Fang et al., 2005). Here we summarize the identified CSC markers varying by tumor types (Table 1). Although great progress has been made in understanding CSC surface molecules, it should be realized that these markers are not perfect for defining the tumor-initiating cell because some cells that do not belong to the CSC compartment may also express these markers. Furthermore, it has also been demonstrated that cell surface markers could be dynamically and reversibly expressed by tumorigenic cells (Quintana et al., 2010). Therefore, it is clearly insufficient to define a cancer stem cell based solely on surface markers.

2.2.2. Dye exclusion assays

The side population (SP) discrimination assays are based on the differential potential of cells to exclude the fluorescent DNA-binding dye Hoechst 33342 via the ATP-binding cassette (ABC) family of transporter proteins expressed within the cell membrane. Shi et al. (2012) showed that purified SP cells from lung cancer cell lines exhibited more enhanced tumorigenicity than corresponding non-SP cells and expressed SMO at higher levels, a critical mediator of

Table 1. CSC markers and associated tumor types.

Cancer type	Cell surface markers															References
	CD44	CD24	CD90	ALDH	CD133	BMI-1	ALDH1	ABCG2	CD29	CD166	Lgr5	CD13	CD171	CD15	α_6-integrin	
Breast	+	-	+	+	+											Lobba et al., 2012; Medema, 2013; Liu et al., 2014
Colorectal	+				+				+	+	+					Todaro et al., 2010; Wang et al., 2012
Glioma			+		+								+	+	+	Brescia et al., 2012, 2013; Medema, 2013
Head and neck	+	+					+									Chen et al., 2009; Han et al., 2014
Liver	+		+		+							+				Haraguchi et al., 2010; Medema, 2013
Lung	+	+	+	+	+			+								Salama et al., 2012; Yan et al., 2013
Pancreatic	+	+			+		+	+								Pascal et al., 2007; Immervoll et al., 2008; Medema, 2013
Prostate	+				+	+	+									Richardson et al., 2004; Patrawala et al., 2006; Medema, 2013

Hedgehog (Hh) signaling. Patrawala et al. (2005) identified SP cells in glioma and breast cancer cell lines that, compared to non-SP cells, were enriched with tumorigenic stem-like cancer cells. These findings support the isolation of SPs via Hoechst staining as an identification method for CSCs. Although the use of Hoechst 33342 dye enables isolation of Hoechst-negative CSC SPs, the possible toxicity of the dye may cause side effects during cell sorting (Siemann et al., 1986; Erba et al., 1988). Furthermore, studies in other cancers suggest that SPs are neither necessary nor sufficient for conferring a CSC phenotype (Broadley et al., 2007; Burkert et al., 2008; Mitsutake et al., 2011).

2.2.3. ALDH activity

CSCs can upregulate the expression of detoxification enzymes as an alternative mechanism for drug resistance besides the drug efflux transporters. ALDHs belong to the oxidoreductase family, which oxidizes a wide range of endogenous and exogenous aldehydes to their corresponding carboxylic acid. Increasing evidence has suggested that ALDH activity can be used either alone or in combination with cell surface markers to identify CSCs in hematologic malignancies (Cheung et al., 2007) and solid tumors, including those of breast (Ginestier et al., 2007), colon (Huang et al., 2009), bladder (Su et al., 2010), lung (Sullivan et al., 2010), and skin (Luo et al., 2012). It has also been used as a prognostic indicator of metastases and poor survival (Charafe-Jauffret et al., 2010). However, ALDH does not appear to be a CSC marker in all tumor types (Yu et al., 2011).

2.2.4. Anchorage-independent cell culture

CSCs can be enriched as a subpopulation of cells propagating as nonadherent spheres in serum-free medium supplemented with several growth factors, resulting in the survival of a small portion of cells forming floating spheres with enriched stem cell properties. In these culture conditions, nonstem cancer cells undergo anoikis, a programmed cell death associated with loss of adhesion to substrate, thus selecting for the CSC-like subpopulation (Dontu et al., 2003). This sphere technology is now largely used in cancer research to isolate cancer-initiating cells. The growth of these spherical colonies is considered to be indicative of the cells' self-renewal ability. Cariati et al. (2008) identified a subpopulation of cells within the breast cancer cell line MCF-7 that is capable of growth in anchorage-independent conditions as spherical organoids. These cells also display resistance to proapoptotic agents, greater tumorigenicity than their parental line, and overexpression of the adhesion molecule α6-integrin (Cariati et al., 2008). They are capable of in vivo tumor formation at limiting cell dilutions and express high levels of stem cell markers, such as Oct4 (Ponti et al., 2005).

These in vitro assays have many limitations. For example, the cells are under selection pressure exerted by the culture conditions, leading to selection of only the cell populations that are able to survive and proliferate under such specific conditions (Han et al., 2013). In addition, in vitro assays measure ex vivo proliferation instead of true self-renewal and they cannot show the tumor-formation ability of CSCs. Therefore, in vitro assays must be confirmed by in vivo assays (Han et al., 2013).

2.3. Signaling pathways in CSCs

There are several signaling pathways (Notch, Wnt/β-Catenin, and Sonic Hedgehog) and molecules (Oct-4, bone morphogenic protein) that regulate self-renewal ability of cancer stem cells and maintain stem cell proliferation (Pazarbaşı et al., 2011; Routray and Mohanty, 2014). The core stem cell signaling pathways Hh, Wnt, and Notch are deregulated in most cancers and act as survival pathways for CSCs (Ponti et al., 2006; Song et al., 2007).

The Hh signaling pathway regulates stem cell maintenance, tissue polarity, cell proliferation, and differentiation (Jena et al., 2012). It has been suggested that the deregulated Hh pathway plays a role in the development of several other types of cancer, including lung, prostate, breast, and pancreas (Gupta et al., 2010). The Hh signaling pathway also plays a key role in the epithelial–mesenchymal transition (EMT) process and maintenance of CSCs (Li et al., 2012). Recently, it was shown that Hh genes are highly expressed in both mammospheres that include normal stem/progenitor cells and $CD44^{(+)}/CD24^{(-/low)}$ CSCs (Liu et al., 2006). The Notch signaling pathway is responsible for the cell fate determination through regulation of apoptosis, cell proliferation, and differentiation (Wang et al., 2009). The Notch signaling pathway regulates the formation of CSCs and the acquisition of EMT properties, which are associated with drug resistance (Wang et al., 2009, 2010). The other signaling pathway, Wnt, is deregulated in cancers and this deregulation promotes the carcinogenic process (Reya and Clevers, 2005). The Wnt pathway also contributes to the malignant transformation of stem/progenitor cells (Reya and Clevers, 2005). In addition, it has been showed that the Wnt pathway contributes to radioresistance in glioblastoma multiforme and head and neck CSCs (Chang et al., 2008; Kim et al., 2012).

2.4. CSC niche and hypoxia

CSCs require a special microenvironment called the CSC niche that regulates their self-renewal ability and keeps their undifferentiated state under control (Yi et al., 2013). The tumor microenvironment gives rise to tumor stroma and is composed of CSCs, carcinoma-associated fibroblasts, adipocytes, endothelial cells and immune cells, secreted growth factors, and networks of cytokines (Albini and Sporn, 2007; Ye et al., 2014). Stromal cells within the niche may secrete some factors that regulate CSC self-renewal properties (Medema, 2013).

CSCs prefer to reside in a hypoxic microenvironment to maintain their homeostasis, rather than normal stem

cells that prefer a glycolytic microenvironment (Pani et al., 2010). Hypoxia contributes to self-renewal in both stem and nonstem cell populations in glioblastoma. Hypoxia leads to an increase in the expression of Oct-4, Nanog, and c-Myc, which results in neurosphere formation, which is a stem cell property (Heddleston et al., 2009). HIF1α and HIF2α are hypoxia inducible factors (HIFs), which play an essential role in cancer hypoxia and are shown to be associated with poor prognosis (Li and Rich, 2010). HIF2α is highly expressed in glioma and neuroblastoma CSCs, and loss of this transcription factor leads to a decrease in proliferation and self-renewal properties of CSCs (Li and Rich, 2010). HIF2α regulates cancer stem cell function through activation of Oct-4 (Covello et al., 2006). In addition, Oliveira-Costa et al. (2011) determined a correlation between the hypoxia markers (HIF1α and CAIX) and $CD44^{(+)}CD24^{(-/low)}$ phenotype in breast invasive ductal carcinomas. Das et al. (2008) identified a highly aggressive SP that localizes and migrates to hypoxic regions in solid tumors in several tumor cell lines, including neuroblastoma, rhabdomyosarcoma, and small-cell lung carcinoma. This study is support for the hypothesis that a CSC niche is characterized by a hypoxic environment. Therefore, targeting hypoxic CSC niches may be a promising strategy for the elimination of CSCs.

2.5. CSCs and EMT

EMT is a biological process enabling epithelial cells to acquire a migrant mesenchymal phenotype and is currently viewed as one of the essential steps during metastasis (Gupta and Massagué, 2006; Routray and Mohanty, 2014). In this process, epithelial cells lose cell-cell adhesion and cell polarity and decrease the expression of epithelial cells markers while upregulating mesenchymal markers such as vimentin, N-cadherin, and fibronectin. Among these, E-cadherin is considered a hallmark of EMT. Loss of E-cadherin expression in epithelial cells results in increased motility (Di Croce et al., 2003) while forced expression of this protein in highly invasive epithelial tumor cell lines is sufficient for reversal of the epithelial phenotype (Vleminckx et al., 1991).

It has been shown that EMT promotes a CSC-like phenotype in differentiated cancer cells (Mani et al., 2008). It has also been demonstrated that the transient induction of EMT in mammary epithelial cells results in an increase in mammosphere-forming ability. The mesenchymal phenotype marker Zeb1 can facilitate the acquisition of stem cell-like properties (Peter, 2010). Santisteban et al. (2009) observed that the induction of EMT by an immune response against an epithelial breast cancer led to the outgrowth of tumor in vivo. Moreover, the resulting mesenchymal tumor cells had a $CD44^{+}CD24^{-/low}$ phenotype with the ability to reestablish an epithelial tumor and increased drug resistance, which is consistent with breast CSCs. Liu et al. (2012) demonstrated that stem cell phenotype, survival, and metastasis of murine breast cancer cells were inhibited when TGF-β signaling, a major inducer of EMT, was blocked. Thus, the future appears to hold potential for eradication of cancer by the understanding of the biology of CSCs and the EMT process.

3. Importance of CSCs in oncology

Drug resistance, which may be inherited or acquired, is a major issue in oncology. It is blamed for the poor prognosis and then death of patients. CSCs are thought to be responsible for drug resistance because these cells have been shown to be relatively more resistant against classical anticancer drugs in vitro than their parental cell lines. In our studies in which parental MCF-7 cell line and MCF-7-derived CSCs were used, relatively higher doses (about 2-fold) of a palladium-based novel compound were required to kill the same percentage of CSCs (unpublished data). This suggests that CSCs are intrinsically more resistant to anticancer compounds. However, in recent years, several compounds were found to be effective against cancer stem cells (Table 2). In this context, we observed that the same palladium-based compound had a considerable antigrowth effect on most prostate cancer cell lines and primary cultures. Importantly, it also successfully inhibited the viability of prostate cancer stem cells ($\alpha2\beta1integrin^{hi}/CD133^{+}$) (Ulukaya et al., 2013).

In terms of the poor prognosis-causing effect of CSCs, a hematological study of 1047 AML patients was published (Gentles et al., 2010). This study clearly showed that patients who had higher activity in leukemic stem cell genes had poorer prognosis than those who had lower activity. This is highly likely to apply to solid tumors. For example, a panel of 66 markers of stemness were analyzed in 62 colon cancer patients (Giampieri et al., 2013). A significantly different median relapse-free survival was observed between 2 groups (22.18 vs. 42.85 months, $P = 0.0296$). This analysis showed that expression levels of colon cancer stem cell genes might be relevant in determining an increased risk of relapse in resected colorectal cancer patients. Aldehyde dehydrogenase 1A1 (ALDH1A1) has been characterized as a CSC marker in different types of tumors. In a study with clear cell renal carcinoma (Wang K et al., 2013), the Kaplan–Meier survival analysis demonstrated that ALDH1A1 overexpression was significantly associated with shorter recurrence-free survival and overall survival ($P = 0.003$ and $P = 0.008$, respectively). CD133 is one of the major stem cell markers. It was found in osteosarcoma patients that CD133 expression was positively correlated with lung metastasis ($P = 0.002$) and shorter overall survival time using the Kaplan–Meier method as compared by log-rank test ($P = 0.000$) (He et al., 2012). This research area has also led to clinical trials to test usefulness in humans, as shown in Table 3.

Table 2. Compounds or molecules that target CSCs.

Molecule	Mode of action	Reference
INFα, arsenic trioxide (As_2O_3)	Induction of quiescent cells to proliferate	Ito et al., 2008; Essers et al., 2009
All-trans retinoic acid (ATRA)	Inhibition of ALDH activity	Croker et al., 2012
Curcumin	Inhibition of cell migration, invasion, and colony formation in vitro and tumor growth and liver metastasis in vivo; inhibitor of EMT	Chen CC et al., 2013; Chen WC et al., 2013
Niclosamide	Inhibition of stemness signaling pathways (Wnt, Notch, and Hh)	Wang et al., 2013
Metformin	Inhibition of tumor growth	Hirsch et al., 2009
Piperine	Inhibition mammosphere formation and percent of $ALDH^+$ cells	Kakarala et al., 2010
Sulforaphane	Decreased $ALDH^+$ cell population and reduced size and number of primary mammospheres	Li et al., 2010
Cyclopamine	Inhibition of Hh signaling pathway	Bar et al., 2007
Salinomycin	Inhibition of tumorsphere formation and expression of Oct-4, Nanog, and Sox2	Wang, 2011
	Inhibition of mammary tumor growth in vivo and induction of increased epithelial differentiation of tumor cells, loss of expression of breast cancer stem cell genes identified from breast tissues isolated directly from patients	Gupta et al., 2009
Silibinin	Inhibition of self-renewal and sphere formation by suppressing the PP2Ac/AKT Ser473/mTOR pathway	Wang et al., 2012
Resveratrol	Inhibition of pluripotency maintaining factors and EMT	Shankar et al., 2011

Table 3. CSC clinical trials. This table was generated from the website clinicaltrials.gov.

Study number	Year	Title	Recruitment	Clinical trial number
#1	2006	Isolation and Characterization of Mammary Stem Cells	Completed	NCT00340392
#2	2008	Biopsy of Human Tumors for Cancer Stem Cell Characterization: A Feasibility Study	Completed	NCT00610415
#3	2010	Interrogation of Wnt, Notch and Hedgehog Activity in Primary Tumor Samples	Recruiting	NCT02006550
#4	2011	Impact of Pretreatment With Metformin on Colorectal Cancer Stem Cells (CCSC) and Related Pharmacodynamic Markers	Terminated	NCT01440127
#5	2011	Cancer Stem Cell Biomarkers as a Predictor of Response to Trastuzumab in Samples from Patients with Breast Cancer Previously Treated in the NSABP-B-31 Trial	Active, not recruiting	NCT01424865
#6	2011	Cancer Stem Cell Markers and Prognostic Markers in Circulating Tumor Cells	Recruiting	NCT01286883
#7	2012	Invasiveness and Chemoresistance of Cancer Stem Cells in Colon Cancer	Recruiting	NCT01577511
#8	2013	A Comprehensive Study to Isolate Tumor-Initiating Cells from Human Epithelial Malignancies	Recruiting	NCT01060319
#9	2013	Cancer Stem Cells in Multiple Myeloma	Recruiting	NCT01820546
#10	2014	The Immunotherapy of Nasopharyngeal Cancer Using Cancer Stem Cells Vaccine	Recruiting	NCT02115958
#11	2014	Cancer Stem Cells Vaccine Therapy in Treating Hepatocellular Cancer Patients	Recruiting	NCT02089919
#12	2014	Vaccine Therapy in Treating Lung Cancer Patients With Cancer Stem Cells	Recruiting	NCT02063893

4. Conclusion

CSCs are an emerging topic in the field of both basic and clinical cancer research. They can be isolated on the basis of some cell surface antigens and/or other properties (e.g., dye exclusion, ALDH activity), although better isolation procedures are still required. The close link between CSCs and EMT, circulating tumor cells that may be used in clinics for the prediction of response to treatment in the near future, is also an emerging new field. In addition, because they are thought to be resistant to chemotherapy, new strategies aiming at the elimination of CSCs may open new avenues for better management of cancer patients.

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