Controversies in Isolation and Characterization of Cancer Stem Cells

15

Ravi Gor and Satish Ramalingam

Abstract

Cancer is an uncontrolled growth of a cell in any part of the body. It has been more than a century for identification of cure for cancer/tumor, and still, we are unable to understand and treat the cancer completely. Current therapeutic techniques such as radiation, chemotherapy, surgery, etc. are failing to eradicate the cancer cells from its root and lead to its relapse in the short or long term. This is because of the small subpopulation of the cells within the tumor that are known as cancer stem cells (CSCs). These cells play an important role in supplying differentiated cells for the growth and development of the tumor. Along with this, they also maintain their population intact for the future requirement of the cells for tumor growth and its metastasis. In spite of several studies proving the presence of CSCs in various types of tumors, there is always a question about its existence and the way we characterize the CSCs based on the histotype-specific markers. There is a dire need for the compilation of research in this area to understand whether the cells, which are being confirmed as CSCs are really CSCs or not? In this chapter, we provide the various isolation and characterization techniques along with the latest CSC identification markers for different types of cancer, in addition we highlight the arguments and the limitations regarding the isolation and characterization of CSCs in this chapter.

Keywords

Cancer stem cells · Histotype-specific marker · Metastasis

R. Gor · S. Ramalingam (✉)
Department of Genetic Engineering, School of Bio-Engineering, SRM Institute of Science and Technology, Kanchipuram, Tamil Nadu, India

S. Pathak, A. Banerjee (eds.), *Cancer Stem Cells: New Horizons in Cancer Therapies*, https://doi.org/10.1007/978-981-15-5120-8_15

15.1 Cancer Stem Cell: Brief History and Current Status

It all started with one question, "How many cells are required for the induction of tumor?". Back in the year 1937, J. Furth and his group reported the transmission of mice leukemia with the transfer of a single transformed cell. The leukemia cell suspension was obtained by isolating the leukemic tumor and filtering it to remove larger particulates and this results in the single cell suspension. The cell suspension is diluted and a single cell is injected in the mice and checked for its tumor forming ability. Out of 97 mice, five developed carcinoma. Finally, they concluded that leukemia can be transmitted with a single transformed cell in an adult individual [1]. Later after half a decade, a publication by John E. Dick's and colleagues in 1994 and 1997 demonstrated that only a few rare cells (undifferentiated) of mouse acute myelogenous leukemia (AML) are capable of initiating this leukemia in other mice on transplantation. They concluded that these rare groups of cells ($CD34^+$ $CD38^-$) can produce a different lineage of cells and also maintain the undifferentiated form of themselves for a longer duration of time [2, 3].

Currently, we call them cancer stem cells, which are mostly like the hematopoietic stem cells present in the human body, which give rise to the blood cells, immune cells, etc. Cancer stem cells are called by many different names like "cancer-initiating cells," "cancer stem-like cells," and "tumor-initiating cells," but they all mean the same and possess key characteristics of stem cells. These are the population of cells found in the tumor which possess the features such as multipotentiality, self-renewal, clonogenicity, and treatment resistant which are the key features of a stem cell. Although these cells are found to be capable of recreating the original tumor independently, we are unable to uncover the origin or mechanism by which the tumor has got Cancer Stem cells. There are only possible theories suggesting that the CSCs came into existence due to mutation(s) in the tissue stem cell, or the transformed cancer cell has gained stem cell property by mutation(s) and became a Cancer Stem Cell. With these characteristics of CSCs, we are unable to eradicate the CSCs by conventional cancer therapy (Fig. 15.1) which will only wipe out the non-tumorigenic cells and CSCs will remain even after the treatment. Current methods of treatment includes chemotherapy, surgery, radiation, immunotherapy, etc. even if one CSC is left after the treatment, then it can regrow the tumor in the same place resulting in tumor recurrence. If the CSCs are killed along with the non-tumorigenic cells, it can result in the total eradication of the tumor. As a result of cancer stem cell targeting therapy the cancer can be eradicated from its root and there won't be any relapse of cancer.

The real challenge is how to select the putative cancer stem cells from the heterogeneous population of the cells in the tumor. Since they only comprise less than 1% of the total tumor cells, it's like finding a single nucleotide polymorphisms (SNP) in the whole genomic DNA sequence. We need to have some identification markers to target them. Around 40 cancer stem cell surface markers are being published and still counting for various types of cancer [4]. Including these cell surface markers, there are other stemness genes like BMI, β-catenin, OCT3/OCT4, SMO, SOX2, NANOG, NOTCH, etc. They play an important role in maintaining

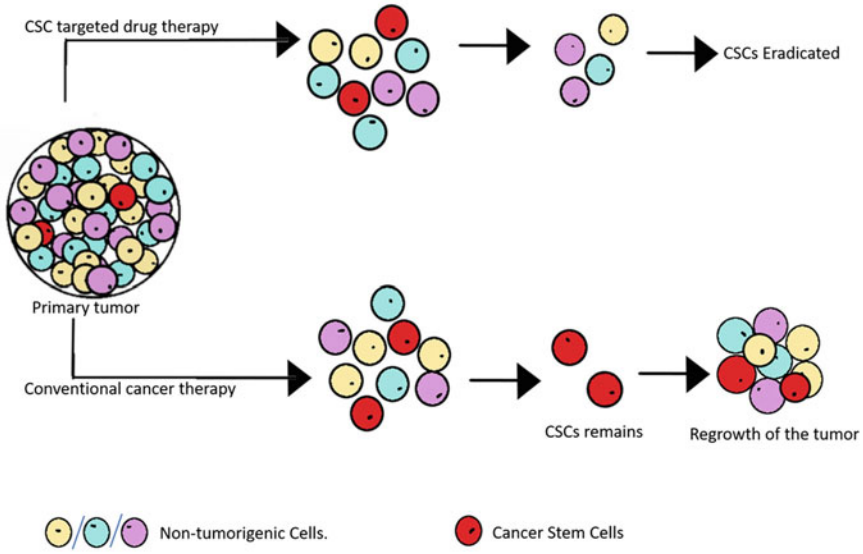

Fig. 15.1 Cancer stem cell hypothesis

the character of the cancer stem cell; evaluation of the expression of these genes by real-time PCR analysis can be used to characterize the isolated CSCs and to understand the molecular mechanism required to maintain the stemness. In the case of breast cancer, SOX2 levels are used as a prognostic marker for early detection of cancer recurrence [5]. In renal cell carcinoma, OCT4 and NANOG can be used as markers for prediction of poor prognosis of the disease [6]. This information regarding different cancer types can further be used for targeting them and fully eradicating them.

15.2 Isolation and Characterization Techniques

There are two major ways by which cancer stem cells can be identified, one with the help of cell surface marker dependent and another is independent of cell surface marker identification. The cell surface marker-dependent techniques involve fluorescence activated cell sorting (FACS) as a critical step in sorting the cells based on their surface markers. On the other hand, FACS is also used for detecting the intracellular marker such as ALDH1 to isolate cancer stem cells in different tumor types. Other identification techniques include phenotypic assay, cytotoxic drug effluxing assay, side population assay, sphere formation assay, somatic stem cell property, pulse-chase approach, etc. Almost all the isolation techniques have their pros and cons in isolating and characterizing the alleged cancer stem cell population, therefore combination of these techniques needs to be utilized for efficient isolation of CSCs.

15.2.1 Isolation Based on Cell Surface Markers

As the cells in the body have an identification mark showing self-cells, immune cells, etc. with the help of a cluster of differentiation protein collectively called CD, similarly, the CSCs can also be identified based on the CD proteins or other surface markers like EpCAM/ESA, etc. This can be achieved by advanced high-throughput machine called FACS; it can sort cells based on their surface marker using fluorescent labelled antibodies. For CSC characterization, different markers specific for mesenchymal and hematopoietic stem cells can be used, such as CD133, CD44, CD90, etc. Different combinations of these markers tell us about the presence of a very small population of the putative CSCs. A population of cells can be defined as a cancer stem cell if it can develop tumor after implantation to the immunodeficient host [7]. Also, it must continuously do that for multiple subculturing. To validate the ability of the isolated cells to imitate the tumor in vivo, the isolated cells are xenotransplanted to immunodeficient host (mice usually). If it gives rise to the tumor and further transplantation reproduces the result, then it can be concluded that the isolated population of cells are cancer stem cells.

The isolation protocol can be divided into three major steps, isolating the tumor sample from the patient, making a single cell suspension, and lastly cell labelling and flow cytometry analysis. The solid tumor is isolated from the patient with proper concern and must begin processing as soon as possible to maximize viability. To prepare a single cell suspension sample can be processed by a mechanical or enzymatical (overnight) method to make a suspension of cells from solid tissue. The goal is to break all the cell-cell connections or junctions so that we get a suspension with individual cells floating. To achieve better results, combination of chemical and mechanical dissociation is performed to provide maximum yield and viability of the cells schematic of the method is depicted in the figure (Fig. 15.2).

Further, the suspension is filtered through the cell strainer, and homogeneous cell suspension will be used in the process. Multiple cell wash is done and labelled with the fluorescent labelled antibody specific to the surface marker under study. Cells are sorted and transplanted to the immunodeficient mice to check its ability to form a tumor. FACS can also be used for isolation of the CSCs from the cell lines from the cell banks/working laboratory cell lines [45, 46]. $CD44^+$ gastric cancer stem cell that has been isolated by FACS shows stemness properties like differentiation and self-renewal. In gastric cancer, $CD44^+$ cells show more resistance for chemotherapy and also radiation-induced cell death. Also, knockdown of CD44 showed reduced tumor production in SCID mice and shows reduced spheroid colony formation [28]. When working with a large number of cells, magnetic bead-assisted sorting will be quicker than flow cytometry. Commonly used methods with magnetic beads are Dynabeads, magnetic-activated cell sorting (MACS), etc. According to the sample acquired and the number of marker needs to be accessed to separate the CSCs, FACS or magnetic bead-assisted sorting is carried out [45].

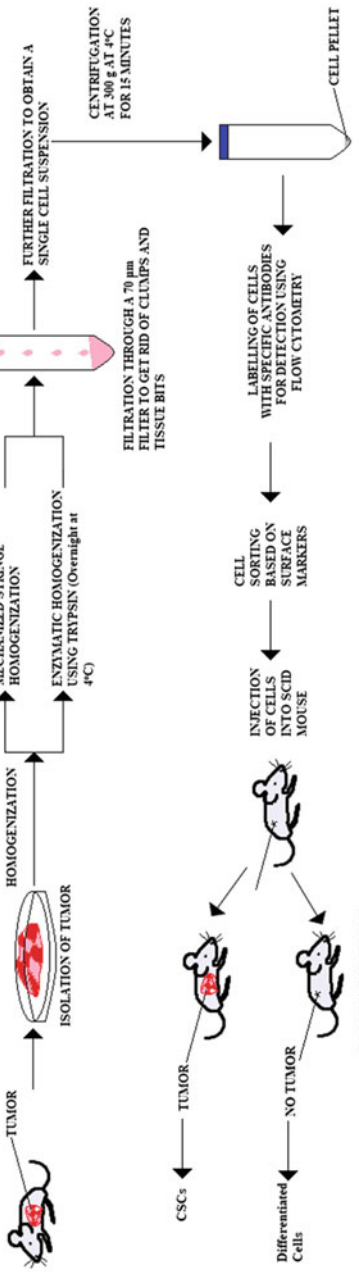

Fig. 15.2 General overview of cancer stem cell isolation by FACS

15.2.2 Isolation Independent of Cell Surface Marker

15.2.2.1 Side Population (SP) Assay

Stem cells have a high capacity to outflow antimitotic drugs. These cells come under a subset of stem cells and called "side population." Side population assay checks for the ability of the CSCs to remove the drug out from the system rapidly as their characteristic for chemoresistance. Normally the differentiated cells will take up the chemical in and process it or be targeted by the same. CSCs can achieve drug resistance with the high expression of ABC transporter protein family members. It is an ATP-dependent transporter or also called a drug effluxing pump and is used to translocate molecules across membranes [47–49]. It has been analyzed in glioblastoma [50], colon carcinoma [51], breast cancer [52], and other types of cancer [53, 54] that ABC proteins provide high chemoresistance to the normal stem cells as well as CSCs with comparison to the differentiated cells [55–57]. Hoechst 33342 dye is used in this assay; it is a nucleic acid stain and emits blue fluorescence when bound to dsDNA. In the heterogeneous population, differentiated tumor cells will keep the dye, whereas the CSCs stream out the dye with high efflux capacity. In the case of neuroblastoma cells, SP was capable of sustaining expansion in vitro and also shows the asymmetrical division, generating SP and non-SP (differentiated) cells. High level of ABCG2 and ABCA3 expression was found to help in better survival by expelling cytotoxic drugs [58]. The advantage of SP assay is there is no requirement of the cell-specific marker to isolate the CSCs. Since the population of CSCs are less in number which makes it difficult to even isolate them with the help of FACS (<2%) [59], the longer incubation with the dye increases apoptosis in a glioma cell line [60].

15.2.2.2 Sphere Formation Assay

Sphere formation assay can enrich CSCs from the solid tumor without utilizing the cell sorting and surface marker. The solid tumors are grown in the non-adherent condition. In this assay, mitogenic growth factors are provided, and the media is devoid of the serum to provide a non-adherent environment. Mitogenic growth factors including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), etc. are supplied depending on the specific cell line. As a result of providing a non-adherent environment, the primitive cells will form a sphere by clustering together, and the differentiated cells die because of no communication to the neighboring cells. Cell sphere was first found in the culture of adult mouse striatum (part of the basal ganglia of the brain) forming sphere in the absence of adhesion factors or supplementary substrate. With all the provided mitogenic growth factor, only the primitive cells survived and the differentiated cells died [61]. With this finding even only cancer stem cells can be grown in the non-adherent environment and be isolated from the heterogeneous population of cells. After a decade it has been shown that a CD133+ cell from human brain tumor grew as a neurosphere in a non-adherent environment [62]. In case of the C6 glioma cell line, only the SP cells survived in the serum-free, growth factor supplemented media and the non-adherent environment by forming a tumorsphere. It has also reported that the C6SP cells can

generate the SP and non-SP cells in the culture, and they are responsible for the malignancy [63].

15.2.2.3 Pulse-Chase Approach

One characteristic of a CSC is slow proliferation, i.e., as compared to the other cells in the surrounding, they will be in the quiescent stage and only divide when needed to produce the differentiated cells. Slowly dividing cells will retain DNA analog for a long time because of no cell division taking place, and this will be termed as a label-retaining cell or stem cell. The more the label retained in the cell, chances of it to be CSCs are more. Firstly, the cells are labelled with BrdU (^{3}H-thymidine or 5-′-bromodeoxyuridine), and then it will be examined regularly to check for the cells with label retention. As the dividing cell's label will be diluted due to the DNA replication during cell division, the selected cells with high label retention are tested with other assays to confirm the CSCs [64–66]. In the case of prostate cancer (PCa) cells with the help of BrdU, pulse-chase assays reveal that $CD44^+$ cells colocalize with intermediate label-retaining cells. Further, it was concluded that these cells are more proliferative, tumorigenic, metastatic, and clonogenic than the $CD44^-$ PCa cells [67].

15.2.2.4 Aldehyde Dehydrogenase (ALDH) Activity

ALDH1 is used as an internal marker for the identification of CSCs in many cancers like breast, colon, etc. ALDH is an enzyme which catalyzes the pyridine nucleotide-dependent oxidation of aldehydes to acids. For example, in case of retinoid signalling ALDH1 catalyzes the conversion of retinol to retinoic acid (RA) in the cytoplasm and finally the RA activates genes which will help in the regulation of stem cell and cancer stem cells. ALDH is substrate nonspecific; by this property it protects the organism from potentially harmful xenobiotics and makes stem cells resistant to the aldehyde-specific xenobiotics [68]. Commercially available fluorescent ALDEFLUOR assay kit can be used to identify the ALDH activity with the help of the ALDH substrate and an ALDH inhibitor (diethylaminobenzaldehyde) as a negative control. By ALDEFLUOR assay, isolation of CSC from breast cell line is done [69] also from acute myeloid leukemia, and multiple myeloma CSCs are isolated with this method [70, 71]. Further, isolated cells, i.e., ALDELFUOR positive and negative cells, are collected, and tumor xenograft studies, expression of stemness genes, etc. can be studied to validate the isolated cancer stem cells. It is found that in renal cell carcinoma (RCC), the number of $ALDH1^+$ cells is doubled in the metastatic ACHN cell line than compared to the primary KRY/Y cell line. Also, the $ALDH^+$ cells show higher sphere-forming ability than that of $ALDH^-$ cells [72].

15.2.2.5 Tumorigenicity Assay Also Known as Reestablishing Heterogeneity

Along with the slowly dividing, self-renewal property, a cancer stem cell must be able to generate a heterogeneous population again at the new site of growth; this property is called tumorigenicity. All the cells which are isolated as prospective cancer stem cells are further analyzed to check its ability to imitate the same cell

heterogeneity when injected into immunocompromised mice. This was first demonstratd by J. Furth et al., to know how many cells are required for it to generate leukemia in immunodeficient mice. As per their research a solid tumor is isolated, and after processing tumor to a single cell suspension and limiting dilution, the specific amount of the cells was counted and injected subcutaneously into NOD-SCID mice [1]. This research has made one thing clear that there is a small population of cells responsible for the tumor regeneration, and the easier way to know them well is by tumor xenograft model. It's the best alternative to the marker-dependent CSC isolation. Limiting dilution assay is a labor-intensive process and time demanding, but the results are highly acceptable. As the smaller number of cells are being introduced to check tumorgenicity, the identity of the same can be known very well.

15.3 Controversies in Cancer Stem Cells

Discovery of the CSCs is a great finding that has provided a reason behind resistance to cancer therapy or cancer survival even after an enormous effort or ways to treat cancer, and tumor recurrence. This in turn also enabled us to identify new approach for cancer therapy, that is, to target the CSCs to totally eradicate the roots of the cancer. However the CSC hypothesis remains controversial; it is because of the divergence in defining cancer stem cells, reliance only on cell surface markers, and lack of standard functional assays [73]. CSCs are known by many different terminologies like cancer stem cells, tumor-initiating cells, or cancer stemlike cells. The CSCs and tumor-initiating cells cannot be considered as a same population of cells. This is because the tumor-initiating cells means that the isolated cell can generate tumor after implantation, whereas CSCs can repopulate the tumor as well as the original heterogeneity. Assays must be done to check the tumorigenicity as well as to demonstrate the cellular heterogeneity.

15.3.1 Relying on the Cell Surface Markers

As depicted in Table 15.1, there are multiple CSC markers for a single type of cancer based on different research group findings. CD133 marker is expressed in multiple tumor-like glioblastoma [42], ovarian cancer [20], prostate cancer [8], etc. One research group found that CD133 is not found to be a CSC marker for non-small cell lung cancer [74]. Whereas the other group found that CD133 shows the ability of a tumor-initiating cell by resistance to cisplatin in case of NSCLC [75]. Apart from this, the combination of markers shows tumorigenic properties rather than individual markers alone [75]. All these finding suggest that we do not have a universal marker for individual cancer type, and we must find the distinctive small population of cell lines on the top hierarchy. As different research groups utilizes various combination of cell surface markers to isolate CSCs. Since these subpopulations of CSCs in the heterogeneous tumor are very less, as reported in the case of pancreatic cancer, only

Table 15.1 List of cancer stem cell markers of various cancer types

Sr. no	Cancer type	Markers	References
01	Prostate cancer	$CD44^+/\alpha_2\beta_1^{hi}/CD133^+$	[8]
02	Colon cancer	$EpCAM^{hig}/ CD44^+/ CD166^+$	[9, 10]
		$CD26^+$	[11]
		$DCLK1^+$	[12, 13]
03	Pancreatic cancer	$CD44^+/CD24^+/ESA^+$	[14]
04	Breast cancer	$CD44^+/CD24^-/$ $lowLineage^-$	[15]
		$CD44^+/CD24^{-/low}/$ $ALDH^{high}$	[16]
		$Thy^+/CD24^+$	[17]
05	Lung cancer	$CD133^+$	[18]
06	Non-small cell lung cancer	$CD24^+/CD38^-$	[19]
07	Ovarian cancer	$CD133^+$	[20]
		$ALDH^+$	[21]
		$CD44^+/CD177^+$	[22]
08	Liver cancer	$CD133^+$	[23–25]
		$EpCAM^+/AFP$	[26]
		$CD13^+$	[27]
09	Gastric cancer	$CD44^+$	[28]
		$CD133^+/CD44^+/ CD24^+$	[29]
10	Melanoma cancer	$CD271^+$	[30]
		$CXCR6^+$	[31]
11	Acute myeloid leukemia (AML)	$CD34^+/CD38^-$	[2, 3]
12	Chronic myeloid leukemia (CML)	$CD34^+/CD38^-$	[32]
13	Acute lymphocytic leukemia (ALL)	$BCR/ABL^-/ALL^-$	[33]
14	Chronic lymphocytic leukemia (CLL)	$CD19^+/CD5^+$	[34]
15	Head and neck squamous cell carcinoma (HNSCC)	$ALDH^+/CD133^+/CD44^+$	[35]
16	Cervical cancer	ABCG2-positive	[36]
		$OCT3/4/BCRP/CD133^+$	[37]
		CD49f	[38]
		ALDH1	[39]
17	Renal cell carcinoma	$ALDH1/CD44^+/CD24^{-/}$	[40]
		$CD133^+/CXCR4^-$	[41]
18	Glioblastoma	$CD133^+/SSEA1^+$	[42]
19	Esophageal carcinoma	$\alpha6^{bri}/CD71^{dim}$	[43]
20	Bladder cancer	$EMA^-/CD44v6^+$	[44]

0.2–0.8% of cells show increased tumorigenic potential compared with non-tumorigenic cancer cells [14]. Also in the case of glioblastoma (GBM), $CD133^+$ is a putative CSC marker, but recently it has been challenged by other

groups. In the culture of $CD133^+$ CSCs, the $CD133^-$ population of cells are unable to form a tumor. But individually isolated $CD133^-$ shows tumorigenic potential which was not reported earlier [76]. The $CD133^+$ CSC culture only maintains a small set of primary glioblastomas. This means that $CD133^+$ cells are an early differentiated cell from the parental CSC, and we need to trace the first line of cells which give rise to the different progenies making a heterogeneous population of tumor. From this, we can assume that there might be another tumor-initiating cell that exists in the heterogeneous population of tumor cells which recapitulates the original tumor and maintains the heterogeneity. As we have advanced in the CSC isolation techniques from serial dilution to FACS, MACS, and transplantation to NOD/SCID mice, maybe soon we might be able to isolate and characterize CSCs with a universal marker for each cancer type.

15.3.2 Model for Tumor Heterogeneous Population

As we call tumor a heterogeneous population of cells, this plays a key role in the development and growth of the tumor. Currently, there are two models representing the heterogeneous population in the tumor, CSC model and the stochastic model. As depicted in Fig. 15.1, the CSC model suggests that the growth and progression of many cancers are driven by a small uncommon subpopulation of cells called CSCs. They mimic normal tissue development by working as stem cells in the normal tissues. Whereas the stochastic model predicts that the reaction of a cancer cell is random and influenced by the environment in which it is, i.e., intrinsic and/or extrinsic factors [77]. Interleukin 6 (IL6) can induce transformation of non-stem cancer cells (NSCCs) to form CSCs. CSCs are shown to have more amount of IL6 as compared to the NSCCs and therefore it is hypothesized that the non stem cancer cells having increased IL6 expression can instruct the NSCC to dedifferentiate to CSCs. This has been reported in breast and prostate cancer cell lines and also from the human breast tumors [78]. In the case of colon cancer, $CD133^+$ cells are potential cancer stem cell population from SW620 human colon cancer cells [79]. A recent study has demonstrated that with in situ immunofluorescence the division types of CSC from the non-stem cancer cell (NSCC) are observed. Results show that even non-stem cancer cells can differentiate into CSCs due to extrinsic factors like radiation in their study [80]. The CSCs which we are identifying may not be the universal CSCs for a particular cancer type, because of the plasticity of cell and their niche. Although both the models are based on theoretical and experimental studies and support the cancer therapy targeting CSCs along with the heterogeneous populations, it would be better if a combination of these two models is created which will provide clarity regarding the cell responsible for repopulating and maintaining the heterogenicity of the parent tumor.

15.3.3 The Problem in the NOD/SCID Mice System and FACS-Mediated Isolation of CSCs

The presence of CSCs in the heterogeneous population of cells in the tumor is very rare. Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice are used as an in vivo model system to validate the tumorigenic ability of CSCs. Studies have indicated that only a rare 0.1–0.0001% of the heterogeneous population of cells in the human cancer cells can initiate a tumor, in diverse cancer types. Not all the NOD/SCID mice are equally immunocompromised, which can lead to variation in the result from one research group to another research group. There are no defined criteria for the compromised immunity of NOD/SCID mice, how much of their immunity is compromised is not mentioned while injecting the cells for tumor formation. In the case of acute myeloid leukemia (AML), it has been shown by Quintana et al. using higher immunocompromised NOD/SCID mice as a xenotransplantation system there is increase in the percentage of the tumorgenic cells in the tumor population. Their study focuses on injecting the isolated cells from tumor into two different mice one with higher immunocompromised and against the regular NOD/SCID mice and comparing the percentage of tumor-initiating cells in both the experiment. Results from that study suggested that the percentage of tumor-initiating cells increased in number by 25–27% by limiting dilution and single-cell transplantation in NOD/SCID mice [81–83]. In AML, it has been shown that $CD34^{+}/CD38^{-}$ cells have an ability to repopulate the tumor. Fluorescent-activated cell sorting uses the fluorescently conjugated antibodies for the cell surface marker, and based on the expression of the antigen, different fractions were collected and transplanted into NOD/SCID mice. In this study, $CD34^{+}/CD38^{-}$ cells were isolated from AML, the antibodies itself affected the survival of transplanted cells which is Fc-mediated, and this was overcome by treating the mice with immunosuppressive antibodies. Further when the inhibitory effect is prevented, most of the cells were found to be leukemia-initiating cells. This is another example to show the increase in the leukemia-initiating cells [84]. This finding was carried out on the same AML on which the CSC hypothesis is established, resulting in having multiple tumor-initiating cell phenotypes. So, it can be concluded that if the test system is not evenly immunocompromised from one laboratory to another, the data generated can be error-prone. Also, the antibodies we use to isolate the single cells from the population of cells must also be studied for its effects on the sorted cells and their ability to produce a tumor. It questions all the studies based on the CSCs in a different solid tumor, whether the cells isolated are really CSC or not, and further validation of the isolated cells needs to be done.

15.4 Conclusion and Future Perspectives

Multiple evidences and researches prove that only a small distinctive population of cells are capable of generating tumors and original heterogenicity in many different cancer types. Many methods are being developed to isolate and characterize the

CSCs from the mixed population of the cells. There are different types of cancer, and current research has found multiple combinations of the CSCs in a single type of cancer which is identified, verified, characterized, and published. But, can we rely on these data? Since multiple CSCs are identified for a single cancer type, how do we decide which CSC needs to be targeted for eradication of cancer from its root? It's been more than half a decade but still, there is no exact definition for the CSCs, and different researchers call it with different names like CSCs, tumor-initiating cells, etc. but there are no standard meaning or definition. CSC means the cell which can recapitulate the parental tumor and keeps original heterogeneity, whereas tumor-initiating cells are the cells which can form a tumor after transplanted into NOD/SCID mice. Recent publications raise questions regarding the existence of CSCs based on the experiment performed by John E. Dickand colleagues based on which the CSC theory is established. Research shows the standard assays to identify tumor-initiating cells fail to detect the exact population of cells which are responsible for tumor regeneration. A proper experimental system must be established to perform a solid functional analysis of CSCs isolated from the parental tumor. The in vivo xenograft assays must be refined for proper characterization of CSCs. Not only the isolation and identification of CSCs are important but also the understanding of the gene expression of the CSCs versus normal stem cancer cells is imperative. Unanticipated intrinsic or extrinsic factors also play an important role in the fate of the cells to be normal or cancer stem cells. Also, the stemness genes are the same that help the CSCs and normal stem cells to maintain their stem cell property. A better understanding of it helps in targeting the cells overexpressing those genes and not only relying on the cell surface markers. Further, we need to design a better model for tumor heterogenicity to make a clear understanding regarding the cell responsible for the heterogeneous population. We conclude that, although we have come a long way with the understanding of CSCs but with many assumptions has lead to the controversies like broad definition, limited assays and their standard of quality to determine the CSCs, relying mainly on the surface markers, etc. A standard definition and list of rigorous assays need to be made mandatory for the isolation and characterization of the CSCs and this needs to be followed by the research group aound the world to isolate, analyse and understand the CSCs. Analysing the cells by using surface markers, Side population analysis, intracellular markers, spheroid assays etc. alone will not provide a strong supporting data for a population of cells to be CSCs, because of the limitations in each of these methods, however combining these methods together could provide a strong supportive evidence. Therefore a guidlines listing a combinative approach to be made for the identification of CSCs is obsolutely essential. This will further reduce the possible controversies arising due to the limitations of different methods that are currently used by the research groups worlwide. The current strategies to understand CSCs is not bound to any rules and to get more clarity about these small subpopulation of cells a standard international guidelines must be made and followed. It's high time now to clear these black spots in the CSC theory and direct research in the aim of curing this dreaded disease and eliminating its existence from mankind for a better future.

Acknowledgments The authors would like to thank the SRM Institute of Science and Technology for the infrastructure and fellowship to Ravi Gor. We would also like to thank the funding support provided to us from Science and Engineering Research Board (EMR/2017/002874), and the Department of Biotechnology, (BT/PR26189/GET/119/226/2017).

References

1. Furth J, Kahn MC (1937) The transmission of leukemia of mice with a single cell. Am J Cancer. https://doi.org/10.1158/ajc.1937.276
2. Lapidot T et al (1994) A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. https://doi.org/10.1038/367645a0
3. Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. https://doi.org/10.1038/nm0797-730
4. Kim WT, Ryu CJ (2017) Cancer stem cell surface markers on normal stem cells. BMB Rep. https://doi.org/10.5483/BMBRep.2017.50.6.039
5. Finicelli M et al (2014) Expression of stemness genes in primary breast cancer tissues: the role of SOX2 as a prognostic marker for detection of early recurrence. Oncotarget. https://doi.org/10.18632/oncotarget.1936
6. Rasti A et al (2018) Co-expression of cancer stem cell markers OCT4 and NANOG Predicts poor prognosis in renal cell carcinomas. Sci Rep. https://doi.org/10.1038/s41598-018-30168-4
7. Clarke MF et al (2006) Cancer stem cells—perspectives on current status and future directions: AACR workshop on cancer stem cells. Cancer Res 66:9339–9344
8. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ (2005) Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 65:10946–10951
9. Dalerba P et al (2007) Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A. https://doi.org/10.1073/pnas.0703478104
10. Chu P et al (2009) Characterization of a subpopulation of colon cancer cells with stem cell-like properties. Int J Cancer. https://doi.org/10.1002/ijc.24061
11. Pang R et al (2010) A subpopulation of CD26 + cancer stem cells with metastatic capacity in human colorectal cancer. Cell Stem Cell. https://doi.org/10.1016/j.stem.2010.04.001
12. Kantara C et al (2014) Curcumin promotes autophagic survival of a subset of colon cancer stem cells, which are ablated by DCLK1-siRNA. Cancer Res. https://doi.org/10.1158/0008-5472.CAN-13-3536
13. Nakanishi Y et al (2013) Dclk1 distinguishes between tumor and normal stem cells in the intestine. Nat Genet. https://doi.org/10.1038/ng.24813
14. Li C et al (2007) Identification of pancreatic cancer stem cells. Cancer Res 67:1030–1037
15. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A. https://doi.org/10.1073/pnas.0530291100
16. Ricardo S et al (2011) Breast cancer stem cell markers CD44, CD24 and ALDH1: expression distribution within intrinsic molecular subtype. J Clin Pathol. https://doi.org/10.1136/jcp.2011.090456
17. Cho RW et al (2008) Isolation and molecular characterization of cancer stem cells in MMTV-Wnt-1 murine breast tumors. Stem Cells. https://doi.org/10.1634/stemcells.2007-0440
18. Tan Y, Chen B, Xu W, Zhao W, Wu J (2014) Clinicopathological significance of CD133 in lung cancer: a meta-analysis. Mol Clin Oncol. https://doi.org/10.3892/mco.2013.195
19. Karimi-Busheri F, Rasouli-Nia A, Zadorozhny V, Fakhrai H (2013) CD24+/CD38- as new prognostic marker for non-small cell lung cancer. Multidiscip Respir Med. https://doi.org/10.1186/2049-6958-8-65
20. Baba T et al (2009) Epigenetic regulation of CD133 and tumorigenicity of CD133+ ovarian cancer cells. Oncogene. https://doi.org/10.1038/onc.2008.374

21. Landen CN et al (2010) Targeting aldehyde dehydrogenase cancer stem cells in ovarian cancer. Mol Cancer Ther. https://doi.org/10.1158/1535-7163.MCT-10-0563
22. Zhang S et al (2008) Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res. https://doi.org/10.1158/0008-5472.CAN-08-0364
23. Ma S et al (2007) Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology. https://doi.org/10.1053/j.gastro.2007.04.025
24. Suetsugu A et al (2006) Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells. Biochem Biophys Res Commun. https://doi.org/10.1016/j.bbrc.2006.10.128
25. Yin S et al (2007) CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. Int J Cancer. https://doi.org/10.1002/ijc.22476
26. Yamashita T et al (2008) EpCAM and α-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. Cancer Res. https://doi.org/10.1158/0008-5472.CAN-07-6013
27. Haraguchi N et al (2010) CD13 is a therapeutic target in human liver cancer stem cells. J Clin Invest. https://doi.org/10.1172/JCI42550
28. Takaishi S et al (2009) Identification of gastric cancer stem cells using the cell surface marker CD44. Stem Cells 27:1006–1020
29. Chen T et al (2012) Identification and expansion of cancer stem cells in tumor tissues and peripheral blood derived from gastric adenocarcinoma patients. Cell Res. https://doi.org/10.1038/cr.2011.109
30. Civenni G et al (2011) Human CD271-positive melanoma stem cells associated with metastasis establish tumor heterogeneity and long-term growth. Cancer Res. https://doi.org/10.1158/0008-5472.CAN-10-3997
31. Taghizadeh R et al (2010) Cxcr6, a newly defined biomarker of tissue-specific stem cell asymmetric self-renewal, identifies more aggressive human melanoma cancer stem cells. PLoS One. https://doi.org/10.1371/journal.pone.0015183
32. Jørgensen HG, Holyoake TL (2007) Characterization of cancer stem cells in chronic myeloid leukaemia in biochemical society transactions. https://doi.org/10.1042/BST0351347
33. Bernt KM, Armstrong SA (2009) Leukemia stem cells and human acute lymphoblastic leukemia. Semin Hematol. https://doi.org/10.1053/j.seminhematol.2008.09.010
34. Gross E, Quillet-Mary A, Ysebaert L, Laurent G, Fournie JJ (2011) Cancer stem cells of differentiated B-cell malignancies: models and consequences. Cancers. https://doi.org/10.3390/cancers3021566
35. Krishnamurthy S, Nör JE (2012) Head and neck cancer stem cells. J Dent Res. https://doi.org/10.1177/0022034511423393
36. Villanueva-Toledo J, Ponciano-Gómez A, Ortiz-Sánchez E, Garrido E (2014) Side populations from cervical-cancer-derived cell lines have stem-cell-like properties. Mol Biol Rep. https://doi.org/10.1007/s11033-014-3047-3
37. Qi W et al (2014) Sorting and identification of side population cells in the human cervical cancer cell line HeLa. Cancer Cell Int. https://doi.org/10.1186/1475-2867-14-3
38. López J, Poitevin A, Mendoza-Martínez V, Pérez-Plasencia C, García-Carrancá A (2012) Cancer-initiating cells derived from established cervical cell lines exhibit stem-cell markers and increased radioresistance. BMC Cancer. https://doi.org/10.1186/1471-2407-12-48
39. Ginestier C et al (2007) ALDH1 Is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell. https://doi.org/10.1016/j.stem.2007.08.014
40. Debeb BG et al (2010) Characterizing cancer cells with cancer stem cell-like features in 293T human embryonic kidney cells. Mol Cancer. https://doi.org/10.1186/1476-4598-9-180
41. Varna M et al (2015) Stem cells increase in numbers in perinecrotic areas in human renal cancer. Clin Cancer Res. https://doi.org/10.1158/1078-0432.CCR-14-0666

42. Son MJ, Woolard K, Nam DH, Lee J, Fine HA (2009) SSEA-1 Is an enrichment marker for tumor-initiating cells in human glioblastoma. Cell Stem Cell. https://doi.org/10.1016/j.stem.2009.03.003
43. Croagh D, Phillips WA, Redvers R, Thomas RJS, Kaur P (2007) Identification of candidate murine esophageal stem cells using a combination of cell kinetic studies and cell surface markers. Stem Cells. https://doi.org/10.1634/stemcells.2006-0421
44. Yang YM, Chang JW (2008) Bladder cancer initiating cells (BCICs) are among EMA-CD44v6+ subset: novel methods for isolating undetermined cancer stem (initiating) cells. Cancer Investig. https://doi.org/10.1080/07357900801941845
45. Dobbin ZC, Landen CN (2013) Isolation and characterization of potential cancer stem cells from solid human tumors-potential applications. Curr Protoc Pharmacol. https://doi.org/10.1002/0471141755.ph1428s63
46. Aplin AC, Nicosia RF (2016) The aortic ring assay and its use for the study of tumor. Tumor Angiogenes Assays Methods Protoc 1464:63–72
47. Keysar SB, Jimeno A (2010) More than markers: biological significance of cancer stem cell-defining molecules. Mol Cancer Ther. https://doi.org/10.1158/1535-7163.MCT-10-0530
48. Ding XW, Wu JH, Jiang CP (2010) ABCG2: a potential marker of stem cells and novel target in stem cell and cancer therapy. Life Sciences. https://doi.org/10.1016/j.lfs.2010.02.012
49. Zhou S et al (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med. https://doi.org/10.1038/nm0901-1028
50. Li WQ et al (2010) Downregulation of ABCG2 expression in glioblastoma cancer stem cells with miRNA-328 may decrease their chemoresistance. Med Sci Monit
51. Paduch R, Jakubowicz-Gil J, Niedziela P (2010) Hepatocyte growth factor (HGF), heat shock proteins (HSPS) and multidrug resistance protein (MRP) expression in co-culture of colon tumor spheroids with normal cells after incubation with interleukin-1β (IL-1β and)/or camptothecin (CPT-11). Indian J Exp Biol
52. Chuthapisith S, Eremin J, El-Sheemey M, Eremin O (2010) Breast cancer chemoresistance: emerging importance of cancer stem cells. Surg Oncol. https://doi.org/10.1016/j.suronc.2009.01.004
53. Misawa A et al (2010) AP-1-dependent miR-21 expression contributes to chemoresistance in cancer stem cell-like SP cells. Oncol Res. https://doi.org/10.3727/096504010X12828372551759
54. Tang QL et al (2011) Enrichment of osteosarcoma stem cells by chemotherapy. Chin J Cancer. https://doi.org/10.5732/cjc.011.10127
55. Donnenberg VS, Donnenberg AD (2005) Multiple drug resistance in cancer revisited: the cancer stem cell hypothesis. J Clin Pharmacol. https://doi.org/10.1177/0091270005276905
56. Dean M, Fojo T, Bates S (2005) Tumour stem cells and drug resistance. Nat Rev Cancer. https://doi.org/10.1038/nrc1590
57. Eyler CE, Rich JN (2008) Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. J Clin Oncol. https://doi.org/10.1200/JCO.2007.15.1829
58. Hirschmann-Jax C et al (2004) A distinct 'side population' of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci U S A 101:14228–14233
59. Gilbert CA, Ross AH (2009) Cancer stem cells: cell culture, markers, and targets for new therapies. J Cell Biochem. https://doi.org/10.1002/jcb.22350
60. Shen G et al (2008) Identification of cancer stem-like cells in the C6 glioma cell line and the limitation of current identification methods. Vitr Cell Dev Biol Anim. https://doi.org/10.1007/s11626-008-9115-z
61. Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science. (80-). https://doi.org/10.1126/science.1553558
62. Singh SK et al (2003) Identification of a cancer stem cell in human brain tumors. Cancer Res 63:5821–5828
63. Kondo T, Setoguchi T, Taga T (2004) Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci 101(3):1–6

64. Welm BE et al (2002) Sca-1 pos cells in the mouse mammary gland represent an enriched progenitor cell population. Devl biol 56:42–56
65. Cotsarelis G, Sun T, Lavker RM (1990) label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61:1329–1337
66. Kim SJ et al (2004) Methods in cell physiology isolation of nuclei from label-retaining cells and measurement of their turnover rates in rat colon. Am J Physio Cell Physiol 3104:1464–1473
67. Patrawala L et al (2006) Highly purified CD44 þ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells:1696–1708. https://doi.org/10.1038/sj.onc.1209327
68. Sládek NE (2003) Human aldehyde dehydrogenases: potential pathological, pharmacological, and toxicological impact. J Biochem Mol Toxicol. https://doi.org/10.1002/jbt.10057
69. Charafe-Jauffret E et al (2009) Breast cancer cell lines contain functional cancer stem sells with metastatic capacity and a distinct molecular signature. Cancer Res. https://doi.org/10.1158/0008-5472.CAN-08-2741
70. Cheung AMS et al (2007) Aldehyde dehydrogenase activity in leukemic blasts defines a subgroup of acute myeloid leukemia with adverse prognosis and superior NOD/SCID engrafting potential. Leukemia. https://doi.org/10.1038/sj.leu.2404721
71. Corti S et al (2006) Identification of a primitive brain-derived neural stem cell population based on aldehyde dehydrogenase activity. Stem Cells. https://doi.org/10.1634/stemcells.2005-0217
72. Khan MI et al (2015) Current approaches in identification and isolation of human renal cell carcinoma cancer stem cells. Stem Cell Res Therapy. https://doi.org/10.1186/s13287-015-0177-z
73. Lathia JD (2013) Cancer stem cells: moving past the controversy. CNS oncology. https://doi.org/10.2217/cns.13.42
74. Salnikov AV et al (2010) CD133 is indicative for a resistance phenotype but does not represent a prognostic marker for survival of non-small cell lung cancer patients. Int J Cancer. https://doi.org/10.1002/ijc.24822
75. Bertolini G et al (2009) Highly tumorigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. Proc Natl Acad Sci U S A. https://doi.org/10.1073/pnas.0905653106
76. Beier D et al (2007) CD133+ and CD133- glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res. https://doi.org/10.1158/0008-5472.CAN-06-4180
77. Shackleton M, Quintana E, Fearon ER, Morrison SJ (2009) Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell 138:822–829
78. Iliopoulos D, Hirsch HA, Wang G, Struhl K (2011) Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion. Proc Natl Acad Sci U S A. https://doi.org/10.1073/pnas.1018898108
79. Ricci-Vitiani L et al (2007) Identification and expansion of human colon-cancer-initiating cells. Nature. https://doi.org/10.1038/nature05384
80. Wang W et al (2014) Dynamics between cancer cell subpopulations reveals a model coordinating with both hierarchical and stochastic concepts. PLoS One. https://doi.org/10.1371/journal.pone.0084654
81. Wang JCY, Dick JE (2005) Cancer stem cells: lessons from leukemia. Trends Cell Biol. https://doi.org/10.1016/j.tcb.2005.07.004
82. Blair A, Hogge DE, Ailles LE, Lansdorp PM, Sutherland HJ (1997) Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo. Blood. https://doi.org/10.1182/blood.v89.9.3104
83. Quintana E et al (2008) Efficient tumour formation by single human melanoma cells. Nature. https://doi.org/10.1038/nature07567
84. Taussig DC et al (2008) Anti-CD38 antibody – mediated clearance of human repopulating cells masks the heterogeneity of leukemia-initiating cells. Blood. https://doi.org/10.1182/blood-2007-10-118331