SECTION I: CHARACTERIZATION OF CANCER STEM CELLS

1 Purification and characterization of cancer stem cells

Elaine M. Hurt and William L. Farrar

National Cancer Institute at Frederick, National Institutes of Health

The processes underlying the etiology of cancer have been the fodder for several theories for a century (for a discussion of the earliest theories, see the subsequent discussion and previous studies).[1,2] Central to all these theories is the cell of origin for the transformation from a normal to a cancerous cell. The prevailing hypothesis, until recent years, was that any cell that had acquired multiple genetic hits could give rise to a tumor.[3] The cancer stem cell hypothesis posits that only a small subset of cells, termed *tumor-initiating cells* or *cancer stem cells* (CSCs), is capable of giving rise to and maintaining tumors. Therefore all CSCs must display several characteristics: they must be the only cells that are capable of giving rise to a tumor (*tumorigenic*), they must be able to maintain the population of tumorigenic cells (*self-renewal*), and they must be able to give rise to the heterogeneous cells composing the entire tumor (*pluripotency*). When a CSC is transplanted into an immunocompromised mouse, self-renewal and pluripotency are vital for

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the formation of a tumor that recapitulates the original (reviewed by Wang and Dick[4]).

HISTORY OF CANCER STEM CELLS (CSCs)

Tumors are masses containing heterogeneous populations of cells with different biological characteristics.[5] Although there has been a marked increase in the number of publications regarding CSCs in the past 14 years, the notion that cancer cells have properties reminiscent of stem cells is not a new theory. This idea was first postulated by Rudolph Virchow and Julius Cohnheim in the nineteenth century.[1,6] Virchow's *embryonal rest hypothesis* noted the similarities between fetal tissue and cancer cells with respect to their ability to proliferate and differentiate.[2] Later, Cohnheim and Durante extended this by hypothesizing that there exist embryonal remnants in mature organs, and Beard hypothesized that cancer arises either from activated germ cells or from dislodged placental tissue. Within all these hypotheses, the basis for cancer was a cell that maintained the ability to differentiate, renew, and proliferate in a manner similar to cells of the developing embryo.

The first demonstration that tumors comprise cells with differential tumor-forming ability was in 1961. Southam and Brunschwig harvested recurrent cancer cells from patients and then autotransplanted the cells into different sites. To establish a new tumor, at least one million cells needed to be injected, and this only worked approximately 50% of the time.[7] Later studies showed similar results for colony initiation in vitro.[8,9] This suggested that not all the cells could initiate a tumor and that there existed a hierarchy of tumor-initiating cells.

The first demonstration of the hierarchy of cancer cells was done in leukemia by Lapidot and colleagues.[10] They demonstrated that $CD34^+CD38^-$ cells isolated from acute myeloid leukemia patients developed a tumor when injected into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, but injection of even larger numbers of the more differentiated cells, $CD34^+CD38^+$, did not initiate tumor formation. Moreover, the tumors formed by injection of the $CD34^+CD38^-$ cells were similar in morphology to the original disease present in the patient. Following leukemia, the first identification of CSCs in solid tumors was demonstrated in breast cancer by al-Hajj and colleagues in 2003.[11] Since then, CSCs have been identified in many solid tumors, including brain, prostate, pancreatic, liver, colon, head and neck, lung, and skin tumors.

IDENTIFICATION OF CSCs

Three methods are commonly employed for the isolation of CSCs. These methods include (1) the isolation by flow cytometric sorting of a side population (SP) based on Hoechst dye efflux, (2) sorting on the basis of cell surface marker expression, and (3) sphere culture. These methods lead to varying degrees of enrichment of CSCs, and each has its advantages and limitations.

Side populations

It was the observation of Goodell and colleagues that there was a small population of cells in bone marrow aspirates that did not accumulate Hoechst 33342 dye.[13] They further showed that this SP contained cells capable of repopulating the bone marrow. Using flow cytometry, the SP has been isolated from a variety of tumors, including leukemia,[14,15] ovarian cancer,[16] hepatocellular carcinoma,[17] brain cancer,[18–20] lung cancer,[21] thyroid cancer,[22] nasopharyngeal carcinoma,[23] prostate cancer,[24] breast cancer,[24] and other cancers. The SPs of all these tumor types have been shown to enrich cells with stemlike characteristics such as increased tumorigenicity,[14,16,17,19,21,23,24] expression of stemlike genes,[17,21–24] and self-renewal.[17,19,21,22,24]

It is generally thought that the SP is the result of the dye being extruded out of the cell by an ATP-binding cassette (ABC) transporter.[25] Indeed, bone marrow cells isolated from *abcg2*$^{-/-}$ mice lack an SP,[26] strong evidence that the bone marrow SP is a result of the efflux of Hoechst dye mainly by ABCG2. Moreover, the SP of neuroblastomas had increased expression of ABCG2 and ABCA3,[18] and the SP isolated from the breast cancer cell line, MCF7, has been studied extensively and has increased expression of ABCG2.[24,27] However, expression of ABCG2 alone may not identify the CSCs in all tumor types. In prostate cancer, the SP enriched tumor-initiating cells and ABCG2 expression, but purified ABCG2^{+} cells did not show increased tumorigenesis compared to ABCG2^{-} cells.[24] Thus the authors concluded that the SP is enriched for CSCs and that the SP contains the ABCG2^{+} cells, but it is still a heterogeneous population, and ABCG2^{+} cells are not the tumor-initiating cells. The expression of multidrug resistance 1 (MDR1), another drug transporter, is also not correlated with the SP in acute myeloid leukemia.[15] Therefore the lack of Hoechst staining in the SP may not rely entirely on efflux by drug transporters.

There is some evidence that suggests that the presence of the SP may be a result of inefficient dye uptake as a reflection of the presence of largely quiescent cells, another characteristic of stem cells. In prostate cancer, Bhatt and colleagues demonstrated that the CSC population was composed of the $G_{(0)}$ cells contained within the SP, whereas the $G_{(1)}$ cycling cells in the SP were the more differentiated transit-amplifying cells.[28] Likewise, Ho and colleagues showed that lung cancer SP cells were also largely quiescent.[21] This might, in part, explain the results of Patrawala and colleagues, which showed that the SP displayed increased tumorigenicity, but the ABCG2^{+} population did not.[24] It may be that the CSCs, which are generally quiescent, are unstained by the Hoechst dye rather than actively transporting the dye out via a transporter.

Although the SP has been shown in many tissue types to enrich for CSCs,[29] it is generally agreed that it does not represent a homogeneous population of CSCs. Furthermore, in some cases, such as in nontransformed renal cells[30] and skin cells,[31,32] the SP does not appear to enrich cells with stem cell characteristics. Further limitations of this method of isolation have to do with the procedure itself, in which the parameters of Hoechst 33342 concentration, staining time, and staining temperature are critical. An excellent protocol can be

found online (http://www.bcm.edu/labs/goodell/protocols.html). However, dye concentrations and staining time can vary with different cell preparations, and Hoechst staining needs to be carefully controlled every time it is performed.[29] Moreover, there have been reports that the dye can have deleterious effects on cells. For example, in the rat C6 glioma cell line, Shen and colleagues demonstrated that incubation with Hoechst 33342 for prolonged periods of time leads to increased apoptosis.[20] This problem raises the possibility that differences seen in tumorigenicity between SP and non-SP cells may be due to a toxic effect of the Hoechst dye, specifically in the non-SP population.

Cell surface markers

Cell surface markers have been used as a means of identification and isolation. Most of the markers utilized to date are based on knowledge of tissue development or are derived from hematopoietic or embryonic stem cells. The two most commonly used surface markers used to identify CSCs are CD133 and CD44.

Prominin-1 (CD133) was originally identified on rat neuroepithelial stem cells[33] in 1997. Later that year, a monoclonal antibody (AC133) was made to $CD34^+$ stem cells isolated from fetal liver, bone marrow, and cord blood,[34] and subsequent cloning identified it as the human homolog of prominin-1. Prominin-1 is a transmembrane glycoprotein with five membrane-spanning domains and two large N-glycosylated extracellular loops that is localized to plasma membrane protrusions and microdomains (reviewed by Bauer et al.[35]). The function of prominin-1 is not entirely known, however. A single nucleotide deletion of *PROMININ-1* is responsible for an inherited form of retinal degeneration.[36] Despite the unknown cellular function of prominin-1, it has been found to be a marker for many of the CSCs identified to date, including those from gliomas,[37,38] colon,[39,40] lung,[41] liver,[42] and prostate[43] (Table 1–1). Although prominin-1 marks a tumor-initiating population in many solid tumors, it does not appear to have a significant role in maintaining properties of CSCs. In colorectal tumor cells isolated from patients, the knockdown of prominin-1 did not result in any significant decrease in the tumorigenic capacity of these cells.[44] However, the knockdown of CD44 inhibited tumor formation in these same cells.[44]

CD44 is a glycoprotein that is the receptor for hyaluronan (HA), a major component of the extracellular matrix (reviewed by Misra et al.[45]). As a result of binding HA, CD44 activates many receptor tyrosine kinases, including EGFR and ERBB2, in many cancer types.[46] This leads to increased proliferation and survival via activation of the MAPK and PI3K/AKT pathways, respectively.[47] CD44 also plays an important role in invasion of a variety of tumor cells, including breast,[48] prostate,[49] and mesotheliomas,[50] and in lymphocyte homing to the bone marrow[51] and has been positively correlated with the number of circulating prostate cancer cells in the bloodstream.[52] CD44, either alone or in combination with other surface markers, has been used to isolate cells with stem cell properties from multiple tumor types, including breast,[12,53] prostate,[54,55] colon,[56] pancreas,[57] and head and neck squamous cell carcinomas[58] (Table 1–1).

Table 1–1. Cell surface phenotypes of cancer stem cells

Tumor type	Phenotype	Fraction (%)	Reference
Breast	$CD44^+CD24^-$	11–35	(12)
Brain	$CD133^+$	5–30	(37, 38)
Prostate	$CD44^+CD133^+\alpha2\beta1^{hi}$ or $CD44^+CD24^-$	0.1–3	(43) (55)
Pancreatic	$CD44^+CD24^-ESA^+$	0.2–0.8	(57)
Hepatocellular	$CD133^+$	1–3	(42)
Colon	$CD133^+$ or $ESA^{hi}CD44^+$	1.8–24.5	(39, 40, 56)
Head and neck	$CD44^+$	<10	(58)
Lung	$CD133^+$	0.3–22	(41)
AML	$CD34^+CD38^+$	0.2–1	(10, 60)
Multiple myeloma	$CD138^+$	2–5	(59)
Melanoma	$CD20^+$	~20	(65)

Other markers used for the identification of CSCs tend to be more specific to the organ, and the choice is generally gleaned from knowledge of how that tissue develops. For instance, CD138 is a marker for terminally differentiated B cells (plasma), and multiple myeloma (a plasma cell malignancy) CSCs are $CD138^-$ cells.[59] Likewise, CSCs from acute myelogenous leukemia are $CD34^+CD38^-$ cells,[10,60] the same markers used to identify normal early hematopoietic progenitor cells.

Although using surface markers allows for the definition of a precise population, as opposed to both SPs and spheres, there are several limitations to this method of isolation as well. The number of CSCs usually identified by this method is almost always low (Table 1–1), requiring a large number of cells to be sorted. This is especially problematic when isolating cells from tumor samples that are often small in size. Also, isolation of CSCs from tissue requires the cells to be enzymatically dissociated, usually with collagenase and other proteolytic enzymes, which can damage some of the surface antigens expressed.[61] Probably the greatest drawback of using surface markers for the identification of CSCs is the choice of the markers themselves. As outlined earlier, the markers often come from what is known about the development of the tissue and from markers of stem cells from systems in which a hierarchy of differentiation has been clearly established such as CD133 from the hematopoietic system.

Culture of nonadherent spheres

In addition to both SPs and cell surface markers, CSCs have been isolated by their ability to form spheres in culture. The ability of CSCs to form spheres in culture was first demonstrated in the central nervous system. In 1992, Reynolds and Weiss demonstrated that cells isolated from the striatum of adult mouse brain could be clonally expanded by culturing spheres and that these cells could generate both astrocytes and neurons.[62] In humans, $CD133^+$ cells isolated from human fetal brain were shown to form spheres in vitro.[37] Further studies have

demonstrated that brain tumors also contain $CD133^+$ cells that are capable of giving rise to neurospheres.[37] Subsequently, the ability of purified CSCs to form spheres in culture has been demonstrated for breast,[12,53] prostate,[54,55] colon,[40,63] pancreatic,[64] and melanoma CSCs.[65]

Because it has been demonstrated that purified CSCs can give rise to spheres in culture, some researchers have used sphere cultures to enrich CSCs. For example, it was shown that cultures of breast cancer under low-adherent sphere-forming conditions enriched the $CD44^+CD24^-$ population, the surface marker phenotype associated with breast CSCs, by 40% to 98% and that the spheres were more tumorigenic in immunocompromised mice.[66] Likewise, researchers have enriched for CSCs from brain,[37,38,67,68] colon,[40] pancreas,[64] bone sarcomas,[69] and melanomas[65] by using sphere culture conditions. In all cases, these spheres are enriched in the surface markers reported by others to represent the tumor-initiating population in the respective tissue, except for the spheres generated from bone sarcomas, in which surface marker expression remains to be determined.

Although culturing for spheres is an easier method of enrichment in comparison to sorting for SPs or surface markers, it is not without limitations. Perhaps the biggest drawback is that the spheres still represent a heterogeneous population, with only a portion of the cells capable of self-renewal.[66,70] Furthermore, immunohistochemical staining of spheres generated from prostate cell lines show that the spheres are heterogeneous for markers of CSCs.[54] Furthermore, differences in the enrichment of CSCs in spheres due to differences in sphere size, passage, culture medium, and technique can be demonstrated in neurosphere cultures.[71]

PROPERTIES AND CHARACTERIZATION OF CSCs

Despite the isolation methodology, establishing that a subpopulation of cells is indeed a CSC population relies on validation of several of the biological characteristics of CSCs, including tumorigenicity, self-renewal, and the ability to histologically recapitulate the tumor of origin. Indeed, a 2006 American Association for Cancer Research workshop concluded that "cancer stem cells can thus, only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor."[72]

Tumorigenicity

At the heart of the definition of CSCs is their ability to induce tumor formation. Most experiments demonstrating tumorigenicity utilize one of two immunodeficient mouse models: the nude mouse or the Non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse. The nude mouse, a result of a mutation in the *FOXN* gene, is athymic, resulting in the hairless phenotype that gives it its name and in a lack of mature T cells.[73] The NOD/SCID mouse model[74]

Table 1–2. Numbers of CSCs required for tumor formation and sites of injection

Tumor type	Site of injection	Numbers of cells tested	Lowest number required	Reference
Brain	Brain	100–100,000	100	(38)
Prostate	Subcutaneous	100–10,000	100[a]	(54, 55)
	Prostate	100–1,000,000	1,000	(79)
Pancreatic	Subcutaneous	100–10,000	100	(57)
Hepatocellular	Intrahepatic	50,000–300,000	50,000	(42)
	Subcutaneous	1,000–1,000,000	1,000	(42)
Colon	Renal capsule	100–250,000	100	(39)
	Subcutaneous	3,000–100,000	3,000	(40)
Head and neck	Subcutaneous	2,000–650,000	5,000	(58)
Lung	Subcutaneous	10,000–500,000	10,000	(41)

[a] Ten $CD44^+$ cells from a single cell line, LAPC-9, were able to induce a tumor in one-fourth of mice tested. This was the only cell line in which 10 cells were tested.

is the result of a cross of the SCID mouse model, which lacks both T and B lymphocytes,[75] and the NOD mouse model, which lacks natural killer cells and antigen-presenting cells. The result is a mouse model that has functional defects in both innate and adaptive immunity. Both models result in a mouse that does not reject xenografts.

Historically, researchers needed to inject millions of cells to establish a tumor. This was first demonstrated in 1961, when researchers harvested recurrent cancer cells from patients and then autotransplanted the cells. Tumors formed only when patients received injections of one million cells.[7] The requirement for millions of cells to establish a tumor led investigators to hypothesize that there are only a limited number of cells that are able to initiate and maintain the tumor. Theoretically, implantation of a single CSC should be capable of generating the entire tumor in a mouse model. Therefore one important test of a prospective CSC population is the ability to form tumors at low cell densities. Most tests of CSC-induced tumorigenesis have used anywhere between 100 and 1,000 cells as the lowest number of cells injected (Table 1–2). In four cancers – brain,[38] prostate,[54,55] colon,[39] and pancreatic[57] – as few as 100 cells were able to give rise to tumor formation in a significant number of the animals tested. Patrawala and colleagues were able to show that 10 $CD44^+$ cells isolated from the prostate cancer cell line LAPC-9 were able to give rise to a tumor in one-fourth of mice.[54]

Self-renewal

The CSC must have the ability to sustain itself and continue to give rise to cells with equal abilities of tumorigenicity and recapitulation of the original tumor. This ability of the CSC to give rise to another CSC is termed *self-renewal*. Self-renewal maintains a reservoir of CSCs when the CSCs undergoes either asymmetrical or symmetrical division (reviewed by Huntly and Gilliland[6]). Asymmetric division forms one more differentiated cell and one CSC. Symmetrical division

results in the CSC forming either two differentiated cells or two CSCs. This behavior is critical because it allows the CSC to expand its numbers.

Self-renewal has been experimentally demonstrated in two major ways: (1) by serial transplantation of tumors and (2) by showing the ability of CSCs to initiate spheres or soft agar colonies over multiple generations. Although serial transplantation of a tumor is the most rigorous proof of the ability of the CSC to self-renew, it is also lengthy and more expensive than culture techniques. Culture methods rely on the assumption that the ability to form a sphere or a colony in soft agar is a surrogate for tumor formation. As long as the spheres or colonies have been shown to be more tumorigenic than nonspheres or the total cell population in a mouse model, this is a fair assumption.

Serial transplantation of CSCs

Serial transplantation involves isolating the prospective CSCs, initiating a tumor in a mouse model, and subsequently removing the tumor to reisolate the cells with the prospective CSC phenotype and retesting tumorigenicity with these isolated CSCs. In theory, the CSCs isolated from any generation of tumor should be able to give rise to a subsequent tumor. The first isolation of CSCs from a solid tumor was from breast cancer by al-Hajj and colleagues in 2003.[12] They found that as few as 100 $CD44^+CD24^-$ cells could form a tumor in immunocompromised NOD/SCID mice. Furthermore, $CD44^+CD24^-$ cells were able to give rise to tumors when serially transplanted into NOD/SCID mice through four passages. The vast majority of cells isolated within the primary and subsequent tumors were more differentiated, and these cells were unable to generate a tumor. This provided the most compelling demonstration that the $CD44^+CD24^-$ breast cancer cells were indeed CSCs. Since this initial demonstration of self-renewal by serial transplantation, CSCs isolated from several solid tissues have been demonstrated to have self-renewal capabilities by serial transplantation. These include CSCs isolated from brain (two generations),[38] prostate (two generations),[54] pancreas (two generations),[57] hepatocellular carcinoma (two generations),[42] colon carcinoma (three and four generations, respectively),[39,40] and head and neck carcinomas (three generations).[58]

In vitro renewal of CSCs

Demonstration of the ability to self-renew in a culture system provides a shorter, less expensive alternative to mouse models. This in vitro technique employs the same principal as the in vivo self-renewal assay. Nonadherent spheres or colonies in soft agar are formed, dissociated, and replanted to determine the ability of the cells to form new spheres or colonies. Several CSCs have been shown to have in vitro self-renewal capacities, as measured by their ability to form spheres or colonies through multiple generations. For example, Ricci-Vitiani and colleagues were able to demonstrate the ability of spheres derived from the colon to reform spheres up to 10 generations.[40] They also demonstrated that the spheres are able to induce tumor formation. Similar in vitro self-renewal assays have been performed with CSCs isolated from prostate cancer[76] and lung cancer.[41]

Establishment of tumor heterogeneity

Not only must a CSC be able to self-renew, but it must also be able to differentiate to recapitulate the heterogeneity seen in tumors (reviewed by al-Hajj and Clarke[77]). They are the putative population responsible for generation and maintenance of a heterogeneous population of cells. Again, two approaches have been taken: one is to examine the heterogeneity of CSC-derived tumors, and the other is to determine the ability of CSCs to differentiate in vitro.

The heterogeneity of a CSC-derived tumor has been demonstrated either by flow cytometry of surface markers or by immunohistochemistry. Al-Hajj and colleagues were the first to demonstrate the ability of a CSC to give rise to a heterogeneous tumor population.[12] They showed, by flow cytometry, that injection of mammary $CD44^{+}CD24^{-}$ cells led to tumors with a diverse surface phenotype reminiscent of the original tumor, with only a minority of cells retaining the $CD44^{+}CD24^{-}$ phenotype. Since then, all solid-tumor CSCs listed in Table 1–1 have been shown to differentiate into other cell types.

The ability of a CSC to differentiate in vitro into the other cell types present in a tumor has also been demonstrated. This has been demonstrated for brain,[78] prostate,[43,55] colon,[40] and lung cancers.[41] Differentiation in culture has been shown to occur in the presence of serum both with and without other factors known to induce differentiation in the specific tissue type being studied. The in vitro differentiated cells not only lose markers of CSCs, but also lose tumorigenic potential.[41,78]

CONCLUDING REMARKS

The field of CSC research is a rapidly moving field that is still in its infancy. The information that we glean from CSCs isolated from one type of cancer is not always applicable to another cancer, type. This has already been the case when choosing cell surface markers for the isolation of the CSCs. It is too early to take much of what has been hypothesized and determined for a single cancer and use it as dogma. Therefore, before further classifying a subpopulation of cells, no matter the method of isolation, investigators must first determine if the population has properties of CSCs, including self-renewal, increased tumorigenic potential, and the ability to recapitulate tumor heterogeneity.

ACKNOWLEDGMENTS

This publication has been funded in part with federal funds from the National Cancer Institute, National Institutes of Health (NIH), under contract N01-CO-12400. This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute.

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