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Review

Cancer stem cells – old concepts, new insights

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Cancer has long been viewed as an exclusively genetic disorder. The model of carcinogenesis, postulated by Nowell and Vogelstein, describes the formation of a tumor by the sequential accumulation of mutations in oncogenes and tumor suppressor genes. In this model, tumors are thought to consist of a heterogeneous population of cells that continue to acquire new mutations, resulting in a highly dynamic process, with clones that out compete others due to increased proliferative or survival capacity. However, novel insights in cancer stem cell research suggest another layer of complexity in the process of malignant transformation and preservation. It has been reported that only a small fraction of the cancer cells in a malignancy have the capacity to propagate the tumor upon transplantation into immuno-compromised mice. Those cells are termed 'cancer stem cells' (CSC) and can be selected based on the expression of cell surface markers associated with immature cell types. In this review, we will critically discuss these novel insights in CSC-related research. Where possible we integrate these results within the genetic model of cancer and illustrate that the CSC model can be considered an extension of the classic genetic model rather than a contradictory theory. Finally, we discuss some of the most controversial issues in this field.
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The idea that a cancer is constituted of a heterogeneous population of cells differing in morphology, marker expression, proliferation capacity and tumorigenicity has been around for over a century.[1–3] It is widely accepted that this heterogeneity can be explained by genetic and micro-environmental differences, together with the differentiation grade of individual cells.[4] However, the hypothesis that this occurs due to the fact that a tumor is hierarchically organized, with its own stem cell compartment called the cancer stem cells (CSC), is to this point highly controversial.[5,6] Although the cancer stem cell model is an old idea, serious attempts to gain insight in its nature only began in the 1970's.[7] The development of technical tools such as immunofluorescent flow cytometry revived research into the CSC theory, starting with malignancies of the hematopoietic system and later solid tumors.

'Cancer Stem Cells'

In the cancer stem cell model malignancies are viewed as abnormal organs with a stem cell compartment that drives the growth. (Figure 1) Cancer stem cells have been defined in analogy to normal stem cells, as cells that have the capacity to (1) self-renew, meaning undergo divisions that allow the generation of more CSCs and (2) give rise to the variety of differentiated cells found in the malignancy.[8] To date, the practical translation of this definition, and the gold standard for showing 'stemness' of cancer cells, is the ability to generate a phenocopy of the original malignancy in immuno-compromised mice. This experiment demonstrates the ability of specific cells to generate the variety of differentiated cells present within the original tumor. In addition, the xenotransplanted tumor must be serially transplanted into new recipient mice, which is believed to address the issue of self-renewal in this subset of cancer cells.

The term 'tumor-initiating cell' is also frequently used to describe cells with CSC capacities. Both terms can, and do, cause confusion about the cells they refer to.[8] CSC might imply that they are derived from normal stem cells that acquire the genetic hits necessary for malignant transformation. While this is likely to be a possibility in several malignancies, in other malignancies this seems to be more complex.[9] The term 'tumor-initiating cell' more closely reflects the experimental evidence that is available at present, but suggests that the tumor-initiating cell is the actual cell from which the tumor derived in the first place. This is likely not the case since, there is clear evidence that the CSC or tumor-initiating cell population can undergo changes as the disease progresses.[10,11]

Throughout this review we will use the term CSC and we will discuss their role in tumor growth. We will critically examine issues that relate to xenotransplantation, the plasticity of this cell population, their origin and the relation with the more classical genetic model.

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Abbreviations: CSC, cancer stem cell; CRC, colorectal cancer; CK-20, cytokeratin 20; HSC, hematopoietic stem cell; LIC, leukemia initiating cell; GMP, granulocyte–macrophage progenitor; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; EGF, endothelial growth factor; bFGF, basic fibroblast growth factor; TAC, transit amplifying cells; NOD/SCID, non-obese diabetic/severe combined immune-deficient; ESA, epithelial-specific antigen; ABC, ATP-binding cassette; SP, side population; MDS, myelodysplastic syndromes; CTC, circulating tumor cell; B-ALL, B-cell acute lymphoblastic leukemia
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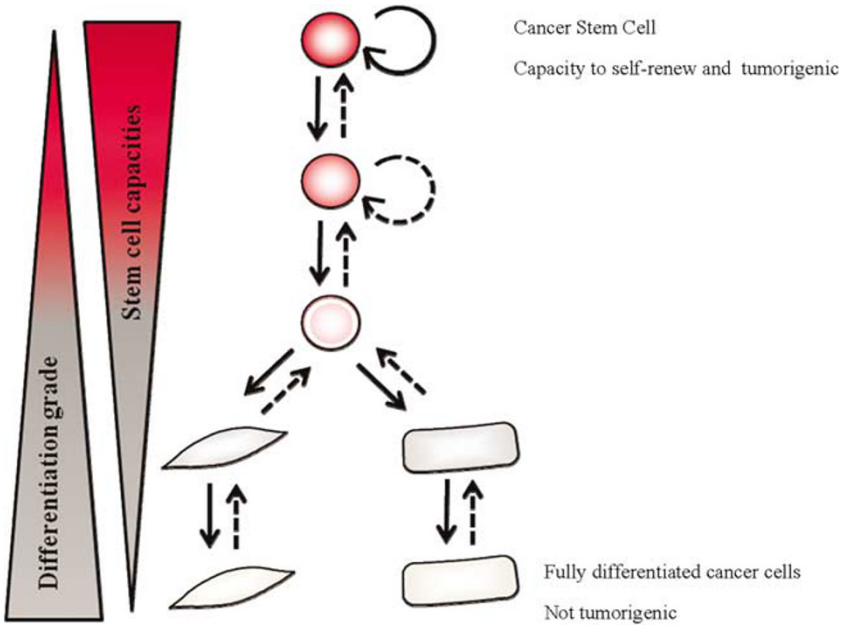


Figure 1 Hierarchical organization of a malignant clone. Depicted is the proposed organization of a malignant clone as predicted by the CSC model. The CSC on the top of the hierarchy (red) has the ability to self-renew, meaning generating more CSCs, and to spin off more differentiated cells (gray). It is to date not clear if the more differentiated cells can revert back and regain a more stem cell phenotype

'Cancer Stem Cells *versus* Genetics'

It is well established that cancer is in essence a genetic disease. The sequential accumulation of mutations in oncogenes and tumor suppressor genes, leading to a malignant clone, is a highly accepted and widely used paradigm in oncology research.[1,12] If the CSC theory is correct, then the result of this accumulation of genetic hits is at least one cell with CSC features that can give rise to more CSCs and to more differentiated progeny. At what stage in the process of malignant transformation this CSC arises, is highly disputed.

An important aspect of the CSC model is the implication that in a malignancy with a defined set of genetic alterations, cells with a different malignant potential are present. In a tumor both differentiated cells that have lost the capacity to propagate a tumor, and cells that retain a clonogenic capacity, exist. This implies that cells showing the same genotypic alterations can show a completely different potential to initiate a tumor in mice. We will present evidence that this proposed difference in malignant potential is not as surprising as it may initially seem.

It is believed that CSCs give rise to more differentiated progeny that have lost the ability for self-renewal and the capacity to initiate the formation of a tumor. (Figure 1) This would imply that remnant regulatory mechanisms remain present in cancer cells that guide the differentiation process in analogy to normal cell differentiation. Indeed, there are examples of malignant cells that are transformed in non-malignant cells by non-genetical means.[13–16]

Epigenetics. From studies addressing the question of whether malignant cells can give rise to benign offspring, it is clear that mutations are not the only factors that predict the malignant potential of cells.[13] It was already described in 1971 that malignant squamous cell carcinoma cells could give rise to more differentiated, non-malignant offspring.[14] In another study, performed in the 1970s, it was shown that subcutaneous injection of embryonal carcinoma cells gave rise to teratocarcinomas, while the same cells injected into a blastocyst gave rise to a normal chimeric mouse.[15] This concept was expanded in an elegant study by Hochedlinger *et al*.[16] They reported that transfer of a nucleus from a melanoma cell into an oocyte (to generate embryonic stem cells) generated chimeric mice with a normal phenotype, despite the fact that a clear increase in cancer incidence was found.[16] This work suggests that the epigenetic profile of a cell, in this case probably induced by the environment, and the proteins present in the cytoplasm of the oocyte at the moment of nuclear transfer, can compensate for mutations to a large extent. This difference in epigenetic profile could also explain the variety in tumorigenic potential in CSCs and differentiated cells in a malignancy. Indeed there is some evidence that epigenetic differences between CSCs and more differentiated cells exist, as there is for example, a hypermethylation described of TGFβ-R2 in the mammary carcinoma non-CSCs.[17]

Clonal selection. The proposed hierarchical organization of a malignancy could be easily integrated in the classical clonal selection theory of Nowell.[2] (Figure 2) This theory views a malignancy as a clonally-derived cell population, which acquires new potentially advantageous mutations that give rise to new more rapidly proliferating clones. This leads to a process referred to as 'tumor Darwinism', which selects for the cell type most suitable for unlimited proliferation in the given environment. When one integrates the CSC theory in

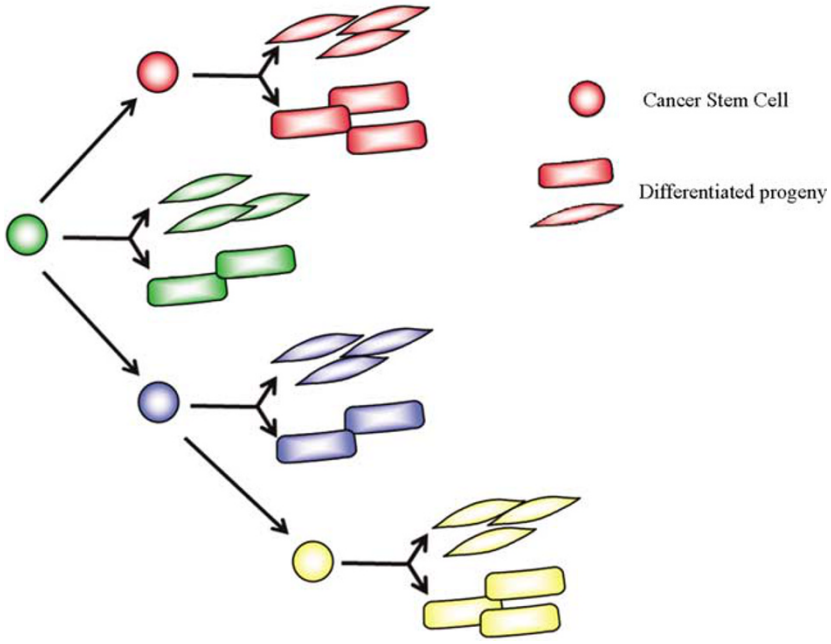


Figure 2 Clonal selection of hierarchical organized clones. Cancer stem cells with tumor initiating and tumor growth driving capacity give rise to more differentiated non-tumorigenic offspring. In this model selection pressure is predicted to act on the CSC level. CSCs acquire additional genetic alterations (here depicted by different colors) that can be beneficial for the clone 'blue' and 'yellow' or dreadful 'red'

this model, the selection pressure is predicted to act at the level of the CSC compartment, implying that certain new traits in CSCs result in an increase in expansion of the CSCs due to self-renewal by symmetrical divisions. This does not mean, however, that certain features present only in the more differentiated cells in the tumor could not be the subject of selection, especially if this increases the expansion rate of the CSCs from which they are derived. For example the more differentiated cells may provide the CSC from which they are derived and which they surround a possible advantage over other clones. In this respect one could think of growth factor production, promoting angiogenesis or the production of immunosuppressive cytokines. Although this suggests that purely genetic models of tumor selection could go hand in hand with the CSC hypothesis, several crucial issues remain. This will be discussed below.

Hematological Malignancies

The hematological system is one of the most intensely studied and best understood human systems. The hierarchical organization of this system has been appreciated for over four decades.[18] The rare long term self-renewing stem cells or 'hematopoietic stem cells' (HSCs) at the top of the pyramid were identified by Weissman and coworkers in 1988.[19] This was achieved by sorting of a specific population of mouse bone marrow cells and subsequent transplantation into lethally-irradiated mice.[19] The HSCs give rise to more committed progenitors, the oligolineage precursors that subsequently produce all the different cellular blood components such as erythrocytes, B-cells, T-cells and macrophages. In the 1970s Fialkow *et al*.[20] extrapolated the then present knowledge of the hematopoietic system and initiated experiments that addressed the hierarchical organization of leukemia's. They showed that a variety of leukemias including acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) contain a diversity of cells that vary in differentiation lineages they follow, but were monoclonal in origin.[21]

In 1994, Dick *et al*.[22] were able to show that only the $CD34^+CD38^-$ cells in a human AML are able to transmit the disease into a NOD/SCID mouse.[23] This transplantation resulted in a phenocopy of the original leukemia, complete with a variety of more differentiated cells. Moreover, subsequent propagation of the leukemia in mice was possible. This demonstrates that the leukemia equivalent of the HSC is a $CD34^+CD38^-$ cell, also called the leukemia-initiating cell (LIC). It seems, however, that not all AML types follow this simple principle. For example, in a specific AML subtype characterized by its PML/RARA translocation, $CD34^+CD38^-$ purification did not result in engraftment.[22,24] Maybe the CSC in this type of AML expresses different markers which could imply a different cell of origin. After the identification of the CSC population in certain types of AML, other hematopoietic malignancies followed, including CML[25] and myelodysplastic syndromes (MDS).[26] Much of the present insights and concepts in the CSC theory are derived from studies in leukemias. Although tempting, care should be exercised when generalizing concepts derived from specific human malignancies or models.

Solid Malignancies

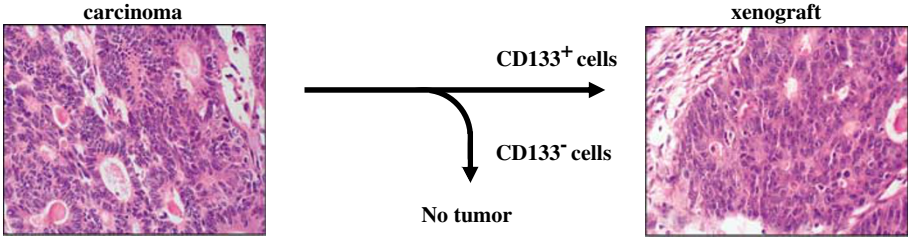
In similarity with the hematopoietic system, other organ systems also contain a stem cell compartment. For instance, the rapid turnover of the epithelial lining in the intestinal tract is tightly regulated by stem cells located at the bottom of the

crypts. These stem cells give rise to transit amplifying cells (TAC), which in turn provide the variety of differentiated cells found in the intestinal epithelium, including enterocytes, goblet cells and Paneth cells.[27] This analogy in hierarchical organization therefore induced speculation as to the existence of hierarchy in non-hematopoietic malignancies. Populations of cancer cells that match the described criteria for CSCs, that is, self-renewal and the ability to give rise to a variety of differentiated cells, have since been identified in a diversity of human malignant tumors. The first solid tumor type from which a CSC population was isolated using cell surface markers was breast cancer. In 2003 Al-Hajj *et al*.[28] showed that one hundred $CD44^+CD24^{low/-}ESA^+$ cells from breast carcinomas were able to grow a differentiated mammary carcinoma in SCID mice. This is in contrast to the $CD44^-$ and $CD24^+$ tumor cells that were not able to grow a tumor even when 10^5 cells were injected. Moreover, sequential transfer of the disease was possible in this setting. Currently, populations with CSC characteristics have been identified in brain tumors, including medulloblastoma and glioblastoma, head and neck squamous cell-, colon-, prostate-, lung-, pancreas-, ovarian- and hepatic carcinoma.[29–38] The CSCs in solid tumors are directly selected after dissociation based on the expression of cell surface proteins frequently associated with a primitive, non-mature cell type (Figure 3). These stem cell markers are often associated with stem cells or progenitor cells in the specific tissue from which the cancer arose, like CD133 in medulloblastomas.[29,39] However, in other instances the marker protein used to enrich the CSC population was not associated with the stem cells in the normal tissue. For example, CD133 is used to identify a colon CSC-containing population, but this is not known to be a stem cell marker in the normal colon.[31,40,41]

In addition to the direct isolation of populations of CSCs, there are attempts to culture the CSCs under specialized conditions – initially developed for the culturing of neuronal stem cells.[42] To initiate a CSC culture, tumor tissue is enzymetically digested and the cells are cultured in medium rich in EGF and bFGF. The cells are kept in low adhesion flasks, resulting in spheroid cultures. So far, reports of successful culture of populations of cells with CSC characteristics have been obtained for a diversity of brain tumors, colon carcinoma, breast cancer, lung cancer and melanoma.[38,41,43–46]

The above described CSC cultures are highly enriched for cells expressing the same markers as used for direct sorting of the CSC population in the particular malignancy. Although in the case of breast cancer mammary sphere cultures, there is some discrepancy in epithelial-specific antigen (ESA) expression, which is present on directly sorted breast CSCs but not on cultured mammary CSCs.[28,45]

a



b

Cancer type	Markers used for identification and/or purification of CSC population
Brain tumors (including: Glioblastoma and medulloblastoma	$CD133^{+(29)}$
Coloncarcinoma	$CD133^{+(31,41)}$,$CD44^+/Lin^-/ESA^{+(40)}$
Mamma carcinoma	$CD24^{-/Low}/CD44^{+(28)}$
Lung cancer	$CD133^{+(38)}$
Pancreascarcinoma	$CD44^+/CD24^+/ESA^{(33)}$ $CD133^{+(34)}$
Hepatocellular cancer	$CD133^{+(36,37)}$
Ovariumcarcinoma	Side population$^{(35)}$
Head and neck squamous cell carcinoma	$CD44^{+(30)}$
Prostate carcinoma	$CD44^+/\alpha2\beta1^{high}/CD133^{+(32)}$
Hematological malignancies	$CD34^+/CD38^{-(23)}$

Figure 3 Direct isolation of cancer stem cell-containing populations in various malignancies. (**a**) Colorectal cancer can be serially transplanted by isolation of the $CD133^+$ cell fraction. $CD133^-$ cells do not have the ability to initiate a subcutaneous xenograft in mice. The typical morphology of the colorectal cancer and marker expression are preserved. (**b**) Table describing the direct isolation of cancer stem cell containing populations in various malignancies

It is described that cells cultured under stem cell conditions are able to obtain a more differentiated morphology and expression of differentiation markers, when cells are transferred to differentiating culture conditions. For glioblastoma cells it is shown that the differentiated cells lose the CD133 stem cell marker and gain GFAP and Tuji expression, glial and neuronal cell markers respectively.[44,47] In the case of colon CSC cultures, differentiation of CSC in matrigel results in crypt-like structures that lose CD133 expression and start to express CK20 and villin, proteins normally expressed in differentiated colon cells[41,48] (Figure 3a). In addition, the differentiated cells start producing mucin, which indicates functional differentiation into a goblet-like cell type.[48]

Once these cultured CSC have undergone differentiation these cells no longer retain tumorigenic potential upon injection into mice[41] or show a completely different morphology to the original tumor, with less differentiation and invasive growth.[44] Whether this *in vitro* differentiation program is reversible, and whether such cells can regain their original tumorigenicity remains to be established, as does the relationship between *in vitro* and *in vivo* differentiated CSCs.

Caveats

As highlighted by others, the practice used to identify CSCs in a given malignancy causes some concern with regards to interpretation of the results so far.[5,6,8] The fact that testing for CSC capabilities of cell populations involves xenotransplantation and, in the case of solid malignancies, complete tissue disruption during dissociation, could mean that we simply select for cells better equipped for survival in mice, or for cells that are more independent of their microenvironment. In addition, the non-orthotopic injection of cancer cells, for example subcutaneous for colorectal cancer, provides the cells with a completely different environment. This could also influence the outgrowth potential of certain cancer cell subsets.

Finally, immuno-compromised mice are not completely devoid of an immune system. The remnant immune response observed in for example NOD/SCID mice could be one mode of selective pressure – to explain the observation that only a subset of tumor cells is able to give rise to a phenocopy of the original human malignancy.

In most studies performed to date human material was transplanted into mice. This possibly introduces a so called xenotransplantation bias. A bias possibly caused by mouse chemokines having different affinities for human receptors and altered downstream signaling features. In apparent agreement with this potential criticism is the observation that transplantation of leukemic cells derived from a variety of transgenic mouse strains into other non-immuno-compromised, syngeneic mice showed that an invariably high percentage of cells was able to propagate the disease.[49] The authors conclude from this that the relative low frequency of LIC (e.g. one in every 10^6 cells in human AML) is due to the fact that only a very limited number of leukemia cells are capable of rapid adaption to a foreign, mouse milieu. Whether this observation is due to the nature of these particular mouse models or is a general phenomenon when tumors are transplanted into a syngeneic background needs to be further elucidated. In a comment to the above mentioned study by Kennedy *et al*.[50] emphasis was paid to experimental data showing a comparable LIC frequency in xenografts *versus* syngeneic approaches for a particular form of B-cell acute lymphoblastic leukemia. In addition, Kennedy *et al*.[50] stress that the definition of a CSC does not refer to any of the relative amount of CSCs present in a malignancy. It is highly possible that certain less differentiated, homogeneous malignancies contain high relative numbers of cells fulfilling the criteria for CSCs.

In solid malignancies, examples of mouse–mouse transfers exist, where it is indeed only a subset of malignant cells expressing markers associated with immature cell types that are able to initiate a new malignancy. For example in a recent study using the MMTV-Wnt-1 mouse model for breast cancer it was observed that only the $Thy1^+CD24^+$ population, that makes up about 1–4% of total tumor cells, is able to propagate the disease in a syngeneic background.[51] This illustrates that the differences in tumor propagating capacities between subsets of malignant cells cannot be explained only by human-to-mouse transplantation biases.

Lastly, the ability of certain cells to grow a complete new tumor in a mouse does not necessarily reflect their growth promoting capacities and features within a malignancy.

CSC entity. In the studies performed so far populations of cells, often as little as 10^2, enriched for CSC markers are injected. However, this raises doubt as to whether the 'entity' with CSC features is a single cell or whether the diversity of injected cells is necessary to grow the tumor.[28,29,31] The last point is significant since, due to technical limitations, these injected populations invariably show a marked (0.2–5%) contamination with cells showing a non-CSC phenotype.[28,29,31] This allows for alternative interpretations of the data, that is, the cells bearing the CSC markers only facilitate the outgrowth of the non-CSCs. There are even some indications that the non-CSCs in mammary cancer, though clonally related to their CSCs, sometimes show a different genotype to the CSCs, suggesting two parallel operating clones.[17]

Seminal studies on HSCs and mammary epithelial stem cells have shown that one single stem cell can give rise to a complete hematopoietic system and a functional mammary gland respectively.[52,53] In analogy with these observations, we have addressed this question by single-cell cloning of colon CSCs and transplanting their progeny subcutaneously into a mouse. The single CSC-derived tumors that arose were similar to the original parental human tumor in morphology and marker expression and were able to give rise to new CSC cultures. We found evidence for differentiated progeny (goblet cell-like, enterocyte-like and neuroendocrine-like) within these tumors (submitted data). Using single cell propagation, similar results have been obtained for teratocarcinomas[54] and breast cancer cells,[55] although the latter study was performed using a rat mammary carcinoma cell line that has been in culture for almost 30 years. Therefore, this data shows that, in principle, a single CSC contains all the information necessary to grow a phenotypically identical tumor. Because by definition, single-cell propagation of a malignancy results in a monoclonal cancer, the heterogeneity in a tumor with respect to the

presence of genetically different CSC clones cannot be investigated in this manner.

Of major importance to begin answering the questions that relate to transplantation biases will be the development of methods to isolate the CSCs in mouse models of solid cancer. This will give insight into the possible xenotransplantation bias in relation to the functional definition so far. For the study of hematological malignancies, mouse models are frequently used and the mouse CSCs in leukemias are much better characterized than the human equivalents. However, in solid malignancies there are currently no studies describing the isolation of a CSC population in a mouse model.

Plasticity. Widespread pluripotency and 'plasticity' are features that have been observed in normal adult stem cells. For example, bone marrow-derived stem cells that give rise to hepatocytes,[56] and neuronal stem cells have been shown to transdifferentiate into hematopoietic stem cells.[57] This raises questions about the very concept of a stem cell. Is a stem cell a concrete, cellular entity, or possibly a more functional entity (reviewed in Blau *et al*.[58] and Zipori[59])? The same question applies to CSCs. In case of CSCs the inherent features may reflect a transient state that is regulated by micro-environmental parameters, rather than by cell intrinsic properties. Throughout this review we will use the term 'plasticity' to describe the phenomenon that phenotypically differentiated cells can de-differentiate and acquire stem cell features such as self-renewal and the capacity to generate cells in a variety of differentiation lineages. This possible plasticity of the CSC compartment in a malignancy is a much debated issue within the CSC model. When de-differentiation of differentiated tumor cells into CSCs exists, this would imply that the CSC compartment is not stable over time. This issue will be addressed in more detail below.

With respect to the potential existence of plasticity of the CSC compartment careful examination of the CSC cultures is potentially useful. CSC cultures provide an enormous potential, but could also be flawed by cellular selection. There is evidence that culturing of dissociated tumor cells, under stem cell conditions, minimizes genetic alterations, which are normally found when primary cancer cells are cultured under classical conditions in an attempt to generate a cell line.[44] In addition, the tumors that arise after transplantation of the cells cultured under stem cell conditions are genotypically and phenotypically more closely related to the original malignancy, than the tumors that arise after injection of adherent cultures from the same malignancy. This indicates that CSC cultures are a major improvement in comparison with the regular method of cancer cell line generation. But, caution is warranted since the limited success rate of culture initiation may reflect selection for CSCs that contain special characteristics, such as niche-independent growth.

The question arises as to whether the CSC culturing method gives the CSCs a selective advantage over the non-CSC, or whether the culture environment may induce a 'stemness' program in the more differentiated cells. Both bFGF and EGF, which are added to the medium of CSC cultures, have been described to be able to induce this de-differentiation.[60,61] If de-differentiation occurs *in vitro*, this would imply that the differentiation program is in principle reversible. Extrapolating this to the situation in a tumor would mean that the CSC population in a malignancy is unstable over time. However, it is also reported that $CD133^-$ cells in medulloblastoma are not able to initiate a CSC spheroid culture, indicating that this plasticity is not a frequent event *in vitro*.[47] We observed the same for $CD133^-$ colon carcinoma cells. (Vermeulen *et al*. submitted) However, the situation in an established tumor could be completely different. The existence of a CSC niche that not only prevents differentiation, but possibly even induces de-differentiation, and the induction of a CSC phenotype of more differentiated tumor cells could support this idea.[13,62] If this is the case this would severely change the CSC model, since it implies that the hierarchical organization of a malignancy is not as rigid as proposed. (Figure 1) In addition, the therapeutic benefit of drugs that specifically target the CSCs would be considerably limited.

More extensive and intriguing insights into the role and importance of the CSC compartment in the development and preservation of a malignancy is expected from the development of technically challenging mouse models, in which the CSCs can be targeted specifically. A pre-requisite of these models must be the possibility to selectively impair the CSC function by either killing, inactivating or differentiating the CSC after a malignancy occurs. This will address the question of whether there is a need for CSCs in the expansion of a given malignancy together with the question of whether plasticity concerning the CSC population exists.

CSC markers. The membrane markers used to identify CSC populations, such as CD133, CD44 and CD24 as well as the functional characteristic of Hoechst 33342 exclusion, which indicates a side population (SP) of cells that express high levels of ATP-binding cassette transporter proteins, ask for a more careful examination. Many of the markers are associated with a stem cell phenotype in the tissue the malignancy occurred in.

The term 'marker' implies that those proteins are simply a read out for a particular stem cell phenotype, but possibly the proteins themselves fulfill an important role in the process of engraftment that is eventually used to determine the tumorigenic potential of this population.

For example, CD44, which is suggested to be a CSC marker in colon cancer, has also been described to enhance the engraftment of colorectal cancer cell lines in both a subcutaneous, and a liver injection model.[63] In analogy, the SP analysis that is performed to select cells with stem cell characteristics leads to 'loading' of the non-SP cells with Hoechst 33342 that is potentially toxic and hampering the viability and thus engraftment of those cells.[64] More insight as to the functions of the markers used for identification of CSC is required, together with studies addressing the contribution of the proteins used for selection on the engraftment potential of certain subpopulations of cells.

Origin of the Cancer Stem Cell

As appreciated from the definition of a CSC, this cell is not necessarily derived from a normal tissue stem cell. Although it is tempting to speculate that normal stem cells, which are present for long enough in the human body to acquire the

amount of mutations (estimated to be 4–6) necessary to give rise to a malignant clone, are the origin of CSC. However, for this kind of reasoning it is also important to take the amount of cells into account. Since HSC are estimated to make up only 0.005% of the bone marrow, there are 20 000 times more non-stem cells present.[65] This large difference in vast numbers could easily compensate for the fact that the lifespan of stem cells is longer. Alternatively, hypothetical mutations caused in differentiated cells, which prevent the cells entering apoptotic clearance could be very well possible. However, not necessary *per se*, there are indications, at least in hematological malignancies that the initial genetic hits resulting in leukemia occur in the normal stem cell compartment of an individual. In both AML and CML, the chromosomal aberrations that characterize the disease in the majority of cases, the AML1–ETO translocation and the BCR–ABL translocation respectively, are also found in 'normal' HSC and in functionally differentiated cells of these patients.[66,67] This indicates that one of the first genetic lesions occurs in normal HSCs. Alternatively, the first genetic lesions convert the more differentiated cells back into a cell that is able to function as a HSC.

Indeed, the cell of origin does not always have to be a stem cell, as shown in a study by Krivtsov *et al*.[68] The MLL-AF9 fusion protein was introduced into committed GMPs, which were then transferred into sublethally-irradiated mice, which resulted in the development of AML in those animals. Intriguingly, the leukemias that these recipient mice developed were transplantable into secondary recipients indicating that the transformed GMPs acquired the ability for self-renewal. Similar observations have arisen from a mouse model for MLL[69] and in human ALL.[9]

The situation in CML is even more complex when one takes a closer look at the course of the disease. CML usually starts with the so-called chronic phase-CML, in which the BCR-ABL translocation is present in the leukemic clone, but the cells undergo fairly normal differentiation in most lineages.[70] This initial stable phase is followed by the blast crisis. The blast crisis-CML is characterized by the emergence and accumulation of more undifferentiated blasts. This progression of the disease is also accompanied by an increase in the amount of granulocyte–macrophage progenitor-like (GMP-like) cells. It is reported that those GMP-like cells acquire the capacity to self-renew, and subsequently during the transformation of chronic phase-CML into blast crisis-CML, another 'class' of CSCs seems to emerge.[10,71]

In solid malignancies there is much less evidence for a stem cell origin of a tumor. Recently, it was reported that the epigenetic profile, as analyzed by promoter methylation, in colorectal cancer samples is similar to the profile found in embryonic stem cells.[72] For breast cancer, it is reported that the gene expression profile of the $CD44^{+}CD24^{-}$ CSCs is much more similar to the normal breast epithelial stem cells, to the more differentiated cells in the same tumor.[17] This, combined with the observation that the initial lesions observed in different mouse models of cancer concern the stem cell region of the tissue make it likely, but not *per se* true, that stem cells are the seeds of cancer.[73,74] For several solid malignancies, including intestinal malignancies and non-small cell lung cancer, it is described that the premalignant situation is characterized by an increase in the amount of stem cells.[75–77] This points to the idea that one of the first genetic hits results in an expansion of the stem cell pool harboring specific genetic lesions that subsequently increase the chance that one of these premalignant stem cells acquires more oncogenic hits that ultimately results in a malignant cell. However, there are other explanations possible. It could be that disruption of tissue integrity, by uncontrolled expansion of cells, results in an increase in stem cells in an attempt to repair the damage.

In addition to the studies in hematological malignancies, it is also reported for solid tumors that introduction of oncogenes in committed progenitors can give rise to malignant clones. For example when ras and c-myc are introduced into oligodendrocyte progenitors, tumors with a glioma multiforme phenotype arise upon *in vivo* transplantation.[78] This indicates that also for solid malignancies to occur there is no absolute prerequisite for genetic mutation of normal stem cells. It is important to note that the term cancer stem cell, therefore, does not necessarily refer to a stem cell origin.

Oncogene introduction. To discuss the origin of the CSCs it is important to re-examine the classical oncogenic transformation studies. It was shown that normal rodent cell can be transformed into malignant cells by the introduction of two specific oncogenes.[79,80] In the following 15 years researchers tried to achieve the same with normal human cells, but in a landmark study by Hahn *et al*.[81] it was described that the malignant transformation of human cells depends on the reactivation of telomerase activity. This strategy was then applied to transform normal human breast epithelia cells into tumorigenic cells. This was successful, as judged by their ability to grow a subcutaneous tumor upon transplantation into an immuno-compromised mouse.[82]

So, how should we view these results in relation to the CSC model? It is clear from these studies that a cell population with a high proliferation capacity can be created by oncogene introduction. However, the resulting tumors show an undifferentiated phenotype that possibly reflects a lack of epigenetic regulation, which may also be necessary for maintaining an hierarchy in a malignant clone.

In a recent extension of these studies, Weinberg and coworkers introduced a set of oncogenes into two different types of normal primary mammary epithelial cells.[83] The two cell types were isolated by two different culture methods. The cells that were transformed after a period of culturing with regular cell culture conditions (normal tissue culture plastic and serum supplemented medium) gave rise to less tumorigenic, non-metastatic and not well differentiated squamous cell carcinomas. In contrast, the cells that were transformed after being cultured in medium supplemented with high concentrations of EGF and insulin, gave rise to highly tumorigenic and metastatic adenocarcinomas. This is the first report of the generation of artificial CSCs. It remains elusive, however, if those observed differences are truly due to transformation of different cell types present in human breast tissue. An alternative explanation is that the culture methods introduce differences in cells that were in origin of the same type.[83]

Metastasis and Cancer Stem Cells

If the cancer stem cell theory is correct, that is, the only cells capable of initiating and driving tumor growth are CSCs, it is logical to assume that all metastases arise from CSCs. In the 1950s it was illustrated, by ethically controversial auto-transplantation experiments, that the development of a metastasis is a fairly inefficient process since only a small minority of all tumor cells is able to generate a distant recurrence.[84] Moreover, evidence is accumulating that numbers of circulating tumor cells in peripheral blood correlate with disease progression and prognosis.[85] However, the numbers of cells shed into the bloodstream, estimated to be as high as 10^6 cells per gram of cancer tissue daily,[86] is not reflected in the amount of distant metastasis found.[85] This again demonstrates the metastatic inefficiency. This is widely attributed to the prevailing idea that there is only a small number of cells present in a tumor that have acquired the necessary genetic alteration to make successful metastasis possible, that is, the capacity to migrate, evade the immune system, home and colonize a distant site.

This genetic framework for metastasis is somewhat conflicting with new insights gained by microarray studies, which point towards the fact that gene expression profiles of the complete tumor can predict metastatic behavior of the malignancy.[87,88] Moreover, gene expression profiles of the primary malignancy are similar to that of the metastases that arise from it.[88–90] However, a small amount of cells that accumulated the genetic mishaps to facilitate metastasis is not very likely to dominate the gene expression profile of the primary tumor. This implicates that those observations cannot be readily explained by the model described above that a small clone with metastatic capacity is responsible for metastatic spread of a tumor. Instead, these findings rather suggest that metastasis is a feature of the dominant clone in the primary tumor and is possibly executed by the CSCs.

Note that the CSC model is in perfect accordance with the classical 'seed and soil' hypothesis of Paget.[91] A CSC seeds reach a fertile soil – the distant metastatic site where the local environment stimulates the outgrowth of the CSC with certain defined genetic alterations. The genotype of the CSC in turn drives the differentiation process in a specific manner corresponding to the phenotype of the primary malignancy. This results ultimately with a metastasis showing morphological resemblance to the primary tumor and, moreover, a similar gene expression profile.[92]

For breast cancer, several studies have addressed the relation between mammary CSC and metastasis.[93] It was reported that the percentage of $CD44^+CD24^-$ CSCs corresponds with the chance of developing metastasis, which could reflect a stochastic process of CSC dissemination from the primary tumor.[94] In addition, a gene expression profile derived from $CD44^+CD24^-$ cells generated a predictive profile for the development of a metastasis in breast cancer patients.[95] However, it is not clear if this profile gives insight as to the intrinsic CSC properties required to metastasize or whether it simply reflects the relative amount of CSCs in the samples tested. The idea that the CSC phenotype plays a role in the process of metastasing was also suggested from a study, which examined the bone marrow of breast cancer patients. It was reported that the percentage of cancer cells detected in bone marrow is highly enriched for the CSC phenotype.[93] In the primary tumor the amount of $CD44^+CD24^-$ cell is around 10%, while in the bone marrow it is as high as 72%. This shows that the cells bearing CSC markers are either better capable of disseminating from the tumor or better suited for survival outside of the primary malignancy. An alternative explanation could be that the microenvironment of the bone marrow promotes the expression of CSC-associated markers.

Implications of the CSC Model for Therapy

Despite the immense body of knowledge present concerning the development of malignancies, the genetic lesions present and the signal transduction pathways involved, the overall survival of cancer patients has only increased modestly during the past few decades.[96] The identification and characterization of CSCs might lead to the development of new therapies. If CSCs are the only cells in a malignancy with the ability to expand and promote tumor growth, and more importantly with the capacity to metastasize, then novel cancer therapies should target the CSC population. At present, there is some evidence that the CSCs are relatively resistant to chemotherapeutic and radiotherapeutic approaches. So, new treatment modalities to target these cells directly would be of immense benefit.

In hematological malignancies it appears that most CSCs are in a quiescent state, which means they are non-dividing and subsequently much less sensitive to classical antiproliferative chemotherapeutic regimens.[97,98] The high expression of drug transporters found on normal HSC and CSC also indicates that these cells are better capable of dealing with chemotoxic agents (Reviewed in Dean *et al*.[99]). Hence, current treatments are unlikely to effectively kill the CSC population.

In fact, in glioblastoma it was shown that after irradiation the fraction of $CD133^+$ cells, believed to be the CSCs, actually increased after treatment, possibly reflecting an intrinsic difference in radiosensitivity.[100] In this study, it was reported that the difference was due to differential activation of the DNA damage response.

For colon carcinomas it was shown by our group that CSCs show more chemoresistance than differentiated tumor cells from the same patient. In addition, we are able to sensitize the colon CSCs with anti-IL-4 combination treatment.[48] The combination of a chemotoxic agent and a treatment modality that increases the sensitivity of the CSCs could be an important new concept in cancer therapy.

An important point is that caution is warranted when interpreting the chemo sensitivity of CSCs and more differentiated cells in a tumor especially in *in vitro* settings. As described above, CSCs are often cultured as non-adherent free-floating spheroids. It is described that aggregates of cells, such as spheroids, display a phenomenon that is referred to as multicellular resistance.[101] This multicellular resistance is believed to be mediated, among other things, by an increase in extra cellular matrix production and drug permeability. This could explain the differences observed in chemo sensitivity between CSC cultures and adherent growing cells, regardless of any possible CSC phenotype.[102]

This proposed difference in therapy resistance could explain the observation in patients that even after effective therapy, that is, when the tumor seems to be eradicated completely, there can be a recurrence of the malignancy. This is referred to as 'minimal residual disease' and it has also been suggested to be caused by the fact that CSC have evaded the therapy, while the more differentiated cancer cells are targeted very effectively by the agent.[103,104] However, this concept does not explain another frequent observation in the clinic. The recurrence of the disease after an initial round of treatment often causes a significant decrease in sensitivity to the therapy; the bulk of the tumor cells also show this change in susceptibility. (Figure 4) For example, in CML patients treated with imatinib mesylate, resistance often occurs by a single mutation in the BCR-ABL fusion gene.[105] If the CSC theory holds, then the applied therapy has selected for specific, more resistant CSC clones that were present in the malignancy or are induced during therapy. Only resistant clones are then able to grow out. If this model is correct, clear selection occurs by conventional treatment at the level of the CSCs. This would imply that research aimed at identifying the mechanisms of therapy resistance between sensitive and insensitive clones is possibly more fruitful than identifying the difference in sensitivity between the CSCs and the more differentiated progeny. (Figure 4)

Nonetheless, the design of therapies that selectively target the putative CSC compartment could be of great benefit in future treatment modalities. To identify drugs that specifically target CSCs, assays should be developed that prevent, the very likely scenario, that those drugs do not target the normal tissue stem cells as well.[71,106] For this, it is essential to find ways to discriminate between functions of normal- and cancer stem cells. With respect to this, interesting observations have been reported in hematological malignancies. In a conditional PTEN deletion model it was found that, in contrast to CSCs, normal HSCs were depleted after PTEN deletion.[107–109] This indicates there are indeed mechanistic differences between the regulation of leukemia stem cells and normal HSCs that can possibly be exploited. Furthermore, it is reported that both IL3 receptor alpha and CD96 are exclusively expressed on AML stem cells but not on normal HSCs.[110,111] These could be useful targets for specific elimination of the CSC compartment in AML.[112,113]

Another option that is getting a lot of attention is the therapeutic angles that exist to target a possible CSC niche. At this point it remains speculative, although, whether CSC reside in a niche,[114,115] its existence could provide us with a 'drugable' target.[115] Promising studies show that the adhesion molecule CD44, implicated in HSC-niche interaction, is necessary for AML engraftment in NOD/SCID mice.[116] Moreover, CD44 is necessary for the preservation of a CSC compartment in AML.[117] This may indicate that CD44 blockage results in disruption of the CSCs from their niche. Preliminary studies in solid tumors also point towards the fact that CSCs reside in a niche. For gliomas it is postulated that vascular endothelial cells provide a niche for the glioma CSCs, similar to the situation with normal neuronal stem cells.[62,118] Disruption of this niche by anti-VEGF treatment resulted in depletion of the $CD133^+$ cells in a mouse xenotransplantation model.[62] Although the CSC niche is an intriguing new concept, more evidence is anxiously awaited and needed to advance this field.

Besides a paradigm shift in the target chosen for anti-tumor therapy, the CSC hypothesis has a more practical implication. If the rare fraction of CSC should be eliminated to obtain therapeutical success in the long term, then the way in which new drugs are tested should be re-evaluated. Nowadays, common practice is that the first clinical trials are performed on

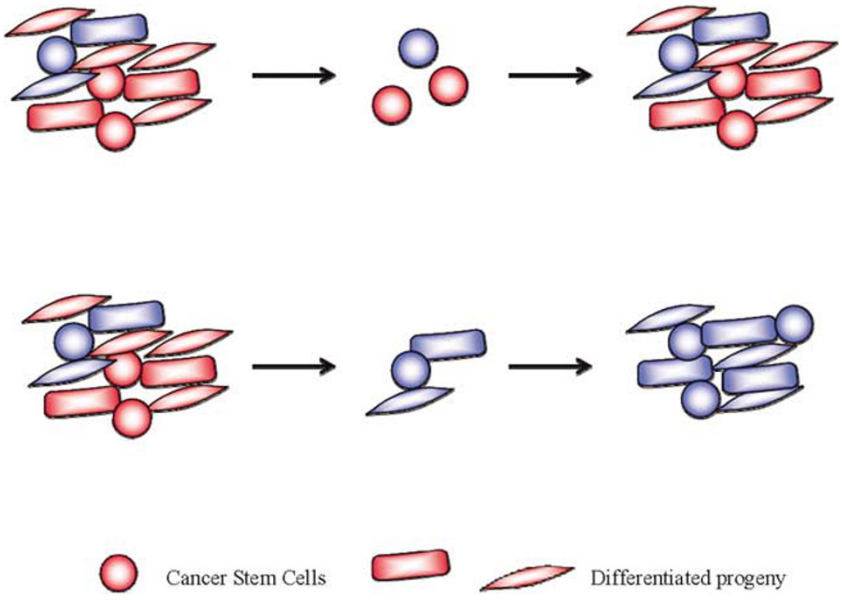


Figure 4 Mechanisms of therapy resistance. The substrate of minimal residual disease is proposed to consist of relatively therapy-resistant CSCs. (upper panel) After therapy the more differentiated cancer cells are deceased and the CSCs maintain present and grow back the tumor, including the variety of differentiated cancer cells. Alternatively, a subset of cancer cells has acquired an additional (epi)genetic hit, depicted here as blue color in the nucleus, resulting in higher therapy resistance. (lower panel) After therapy it will be those CSCs that re-grow the malignancy. This results in a tumor, including the differentiated cells present, that is relatively therapy resistant

late-stage patients and response is mainly scored as a reduction in tumor load. In this setting it is possible that promising new drugs that target the CSC compartment are overlooked, because their influence on tumor mass is small in the short term while their effect is evident on the CSCs.[96]

Synthesis

Cancer stem cell research has provided us with important new insights for future oncological research, but definitive proof of the CSC model is still lacking in most malignancies. Although current data is in perfect accordance with a hierarchical organization of malignancies, other explanations are feasible. With respect to this, research should be aimed at clarifying the role of the protein surface molecules that are used to identify the CSC population in transplantation experiments. In addition, to date basically all CSC research involves transplantation assays for characterization of CSCs. Therefore technically challenging mouse models are awaited that elucidate the role of the proposed CSC compartment in an established malignancy.

In this review, we have tried to integrate the CSC model into the dominant genetical view of cancer. This integrative model is possibly much more fruitful in guiding oncological research in the future.

Besides the conceptual insights, CSC related research has provided us with important technical advancements, for example in cell culturing techniques that will be very important in the near future. It has been clearly shown that the culture method applied to culture cells with an immature phenotype has large advantages over regular culture methods. The gene expression profile mimics the original human malignancy much closer and also the morphology is better conserved in mouse transplants. This indicates that this is potentially a superior model system to study the biology of a variety of malignancies, regardless of the validity of the CSC model.

With respect to therapies the CSC model can possibly explain some clinical observations, including minimal residual disease and treatment resistance. In addition, if the CSC model is true it would have great consequences and challenges for the development of new therapeutic agents.

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