Cancer stem cell niche: the place to be

Borovski, T.

Publication date
2012

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One Renegade Cancer Stem Cell?

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Cell Cycle 8:6, 803-808; 15 March 2009

Abstract

The CSC compartment represents the subpopulation of tumor cells with clonogenic potential and the ability to initiate new tumors. Besides self renewal, one of their main features is their ability to differentiate into the variety of cells within the tumor. The question remains whether this potential resides within the single CSC or whether many different CSCs are necessary to generate a heterogeneous population of tumor cells. There is an increasing amount of evidence showing that a single CSC indeed has the potential to reconstitute the complete tumor phenotype. This is likely to be a general phenomenon and it has been demonstrated in many tumors so far. Here we show that single GBM CSCs have multilineage potential, although not exclusively. Furthermore, our results show that CSCs originating from same tumor are not necessarily uniform in respect to their differentiation potential.

There are two opposing models to explain the initiation and development of tumors. One is the stochastic model which proposes that in principle all cells in the malignancy have the potential to drive tumor growth. In contrast, the hierarchical model suggests that only a small fraction of cells has tumor-initiating properties and can generate new tumors. The latter hypothesis is consistent with the cancer stem cell (CSC) theory. In this model, CSCs are defined as cells that have the property of self-renewal and are able to generate all more differentiated cell types in the tumor.1-3 In addition, they are able to initiate tumors that mirror the original malignancy after serial transplantation in a xenograft model.2 This means that, in analogy to normal stem cells, new CSCs with the same capacity to proliferate and differentiate as the parental cell can be generated after cell divisions. Simultaneously, progeny with the ability to differentiate is generated. It is believed that CSCs, like normal stem cells, accomplish this by asymmetric division, where one daughter cell retains a stem cell phenotype and one differentiates into the various cell types found in the tumor.4 It has been shown that CSCs are more resistant to genotoxic treatments when compared to more differentiated cell types within the tumor and therefore, it has been suggested that CSCs are responsible for tumor regrowth after treatment.5-8 Together, these features make CSCs a potential target for the development of novel therapies.

Convincing evidence that supports the CSC hypothesis was obtained first in acute myeloid leukemia.9 Since then the principle has been extended to solid malignancies as well, and CSCs have been described in brain, prostate, breast, colon, lung, pancreas and ovarian cancers.1 Markers that are normally associated with immature cells and normal stem cells have been successfully used to identify and isolate those CSCs. However, to date the 'gold standard' to confirm stem-cell properties of tumor cells is their ability to give rise to a new tumor that recapitulates the phenotype of the original one in immunocompromized mice and, in addition, can be serially transplanted.2 This indicates that newly generated tumors contain a population of functional stem cells and confirms their self-renewal properties.

Normal stem cells are required for tissue maintenance and repair. They can be found in nearly every major organ. In particular, they are required in tissues that undergo continuous and rapid cellular turnover, such as skin and gut epithelial cells or cellular components of blood. Therefore, there is a constant demand for stem cells to generate new mature cells through differentiation. Simultaneously, the stem cell pool needs to be kept constant and be replenished if necessary.10 There are several examples that illustrate the potential that resides within a single stem cell. One is the colon crypt, the main structural unit of the colon. Crypts are invaginations of the colon epithelial layer with the stem cells situated at the bottom wherefrom they simultaneously migrate upwards and start differentiating. There are three different lineages of colon epithelial cells and it is thought that the same stem cell can generate all these distinct intestinal cell types.11,12 This is

exemplified by the finding that crypts consist of clonal populations of cells. Firm evidence that this is indeed the case was obtained from a study of a *X0/XY* mosaic patient suffering from familiar adenomatous polyposis.13 Analysis of the patients' intestine revealed that the crypts were formed of *X0* or *XY* cells exclusively. Furthermore, it has been shown that certain mutations which are moderately common in colon stem cells were either present or completely absent per crypt.14-16 The existence of crypts in which all the lineages carry the same mutations indicates that the crypt most likely arose from the same mutated stem cell that had the potential to differentiate into all colon cell types. In addition, the newly identified stem cell marker Lgr5 was recently used to study the behavior and differentiation of intestinal stem cells.17 Cells which are Lgr5 positive could be marked irreversibly by induction of β-Gal expression. β-Gal positive cells were located at the bottom of the crypts and thought to mark stem cells. As expected, initially only a few cells at the base of the crypt were Lgr5 positive. At later time points β-Gal positive cells emanated from the crypts and were present along the sides of the villi. Importantly, all differentiated cell types of the intestine were identified among the daughter cells of the β-Gal positive cells. This demonstrates that Lgr-5 expressing cells are indeed stem cells that give rise to all diverse cell types of the intestinal epithelium.

There is an increasing number of examples that demonstrate the generation of a complete organ from a single stem cell. Among others, these kinds of studies were performed in the mammary gland. The mammary gland is comprised of two different groups of epithelial cells, luminal and myoepithelial cells that both branch out and form ducts and lobulo-alveolar units. It has been speculated that these two groups of cells originate from the same stem cell. Convincing proof of this idea has recently been obtained by Shackleton et al.18 A stem-cell enriched population was isolated from a mouse mammary gland based on cell-surface markers (Lin- CD29 hi CD24+) and a single cell was injected into cleared mammary fat pads of recipient female mice. This resulted in outgrowths of the ductal-alveolar network. Furthermore, histological analysis revealed that these mammary structures formed functional mammary glands that contained both types of epithelial cells. Similar experiments have been performed with prostate stem cells. A single adult prostate stem cell (Lin-, Sca-1+, CD133+, CD44+, CD117+) could generate functional prostate tissue after transplantation in vivo.19 Consistently, a single muscle stem cell transplanted into the muscle of mice is capable of self-renewal and production of more progenitors that contribute to the muscle fibres.20 To demonstrate the properties contained within a single hematopoietic stem cell (HSC), specific markers were used for purification and characterization of HSCs (CD34 lo/-, c-Kit+, Sca-1+, Lin-).21 One of those cells has been injected into lethally irradiated mice and this lead to the reconstitution of the lymphohematopoietic system in 21% of the mice. This illustrates the plasticity imposed on stem cells by their microenvironment and that indeed one single somatic stem cell is capable to regenerate a whole organ in vivo.

Malignancies can be seen as an abnormal growth of the organ from which they originate. It has long been hypothesized that cancers contain a population of stem cells which are at the heart of cancer growth, in analogy to the way normal tissues are generated from somatic stem cells.22 Recent advances have yielded evidence that this is indeed the case, at least for some cancers. CSCs are supposed to have similar properties as normal stem cells, with respect to self-renewal and multilineage differentiation potential.2 However, as a result of acquired mutations CSCs may have acquired unregulated self-renewal that results in the generation of cancers. The same principles that have been employed to demonstrate the identity of normal stem cells have been used to identify CSCs.

One type of tumor that contains a population of cells which per definition are CSCs is the teratoma. Teratomas are the result of abnormal development of totipotent embryonic stem cells. The main hallmark of teratomas is the diversity of the cell types found in the tumor tissue which can resemble normal derivatives of all three germ layers and sometimes reaches a complete structure of certain organs such as eyeballs, hair or teeth. First attempts to address the question of whether this potential resides within a single embryonic carcinoma cell were undertaken a few decades ago. In 1964 it has been shown that one single teratoma cell deposited intraperitoneally into mice can give rise to tumors in 11% of the cases.23 These tumors were true teratocarcinomas as they were comprised of a variety of differentiated tissues from each of the three germinal layers. Furthermore, it has been shown that teratoma cells, on one hand, can give rise to teratocarcinomas in mice when injected subcutaneously. On the other hand, the same cells injected into the cavity of the blastocyst gave rise to tumor-free mosaic offspring.24 Therefore, in addition to demonstrating the differentiation potential of teratoma cells, these two experiments indicate that most likely the combination of both, mutations and epigenetic changes, regulated by the tumor microenvironment, determines the tumorigenic potential of a cell.25

Colon cancer is one of the most studied and best understood malignancies, yet it remains the second leading cause of cancer-associated death.26 Recently, the hypothesis that CSCs drive tumorigenesis in colorectal cancer and that a single CSC has the potential to recapitulate the original tumor phenotype gained support by experiments conducted in our laboratory.27 We utilized a method to generate single-cell derived CSC cultures by single-cell sorting GFP transduced spheroid colon CSC cultures. Different numbers of CD133+ cells were deposited into varying numbers of CD133- cells from a GFP- subculture. In all the cases only GFP+ spheres were obtained, indicating that only CD133+ cells have clonogenic potential in vitro. Moreover, subcutaneous injection of single-cell derived GFP+ CSCs into NOD-SCID mice lead to the growth of adenocarcinomas. The morphology of these tumors resembled that of the original malignancy and importantly, all epithelial structures within the tumor were derived from GFP+ cells. Further,

all tumors showed heterogeneous cellular morphology, protein expression and differentiation along different intestinal cell lineages. Cells that expressed markers associated with enterocyte, goblet and neuroendocrine cells were present. These single-cell derived xenografts retained a CD133+ compartment that displayed CSC properties. Moreover, we were able to confirm these findings by direct ex vivo sorting of single colon CSCs from human colon carcinomas. Odoux et al. used a similar approach to investigate the properties of single-cell derived colon CSC.28 They found, in agreement with our results, that a single cell has the potential to generate the full spectrum of differentiated cells found in the original tumor. In addition, they performed a karyotype analysis to assess the presence of chromosomal instability. While the same aberrations observed in the parental cells were also seen in the clonally derived tumor cells, surprisingly, some clones also had aberrations unique to each culture. These results open the question whether differences seen among clones, derived from a common progenitor, were present in the original isolate.

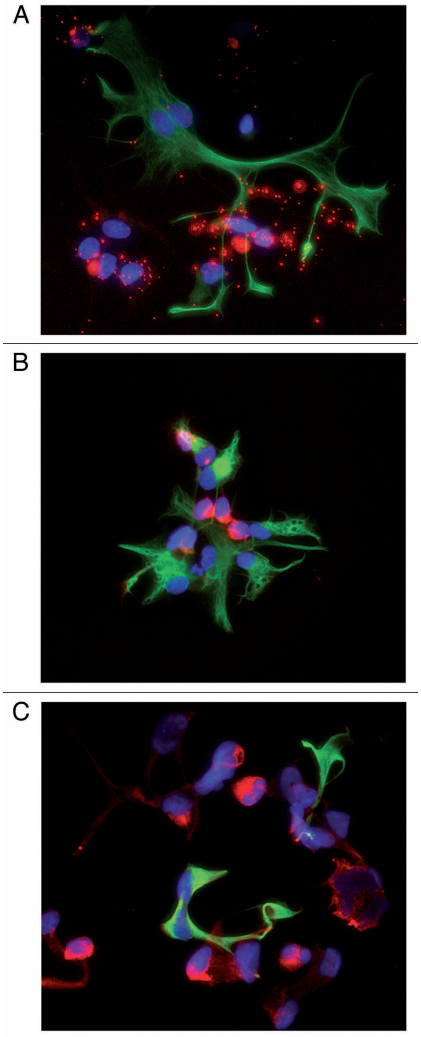
The presence of a small, genetically identical population of CSCs in all tumors has been challenged recently also for a mouse model of lymphoma.29 Kelly et al. demonstrated that as many as one in ten tumor cells were able to propagate lymphomas, fulfilling the postulates of cancer stem cells. Additionally, it has been demonstrated that as many as one in four melanoma cells is able to propagate tumor growth in a highly immunodeficient model.30 While a syngeneic system was used in the first publication, the authors of the second paper demonstrated that the tumorigenicity in xenograft models depended on the grade of immunodeficiency of the host. Interestingly, in the latter model the xenografts obtained from one patient specimen were heterogeneous in nature. This raises the question whether some tumors can contain several genetically or epigenetically distinct populations of cells with cancer stem-cell like properties. In order to address this question, we choose to analyze the CSC populations in glioblastoma multiforme.

Gliomas are the most common type of primary brain tumors, amongst which glioblastoma multiforme (GBM) is the most frequent and aggressive one.31 Mainly because of their ability to infiltrate into the healthy surrounding tissue, so far all the attempts to develop effective treatments against this disease failed. Despite progress in the research of GBMs, patients still have a median survival of less than a year.32 Thus, a better understanding of initiation and formation of GBM is necessary for development of new, more effective therapeutic approaches.

It has already been demonstrated that GBM CSCs are more resistant to therapy than the bulk of the tumor cells.7,32 In addition, it has been reported that, based on the molecular signature of high grade gliomas, these tumors can be classified into three distinct subgroups where the most

aggressive forms are enriched for genes normally expressed in neural stem cells.33 In addition, upon recurrence tumors have a tendency to shift towards this more aggressive, less differentiated phenotype.

The presence of different cell types in gliomas, along with the shift of tumor phenotype during progression, poses a challenge for an overly simplified view of CSC. How is the presence of neuronal, as well as glial, markers in the same tumor explained? We decided to assess the differentiation potential of GBM CSCs cultures in order to address the question whether one single population of CSCs is indeed responsible for the tumor phenotype, or several competing clonescan co-exist in one tumor to produce the diversity of phenotypes observed.



Glioma cancer stem cells are usually isolated and propagated from patient specimens under stem-cell conditions. This means non-adherent growth as spheres in serum-free medium supplemented with bFGF and EGF. Under these conditions the GBM CSCs retain the ability to induce phenocopies of the original human malignancy upon transplantation into immunocompromized mice.34 In addition, such GBM CSC cultures can differentiate among different cell lineages in vitro. Some examples of differentiated CSC cultures from our laboratory are shown in Figure 1. GBM073 cultures become positive for the early oligodendrocyte marker O4 and the astrocyte marker GFAP (Fig. 1A). In contrast, the GBM lines 408 (Fig. 1B) and 081 (Fig. 1C) show expression of the neural marker β3-Tubulin and GFAP. The CSC hypothesis postulates that a single, multipotent population of cells in those cultures should be capable to give rise to those differentiation patterns. If this would be universally true, single-cell cloning of GBM CSC cultures should yield clones that mirror the differentiation potential of the parental culture.

Figure 1. Marker expression of differentiated GBM CSCs cultures. (A) After 10 days of growth factor withdrawal, and addition of serum, the cells adhered and differentiated. Immunofluorescence staining was performed on differentiated GBM CSCs isolated from different tumor specimens. GBM073 were co-stained for the early oligodendrocyte marker O4 (red) and the astrocyte marker GFAP (green); (B) GBM408 and (C) GBM081 were co-stained for the neural marker β3-Tubulin (red) and GFAP (green); nuclei were stained with DAPI (blue).

We thus generated single-cell derived CSCs from one of our cultures by plating them at clonal density (Fig. 2). Upon differentiation, the parental line GBM006 showed mainly β3-Tubulin and GFAP expression with few cells being negative for both markers (Fig. 2A). However, the differentiation patterns of the single-cell derived spheroids showed an unexpected variety. Although the majority of the clones were positive for both β3-Tubulin and GFAP upon

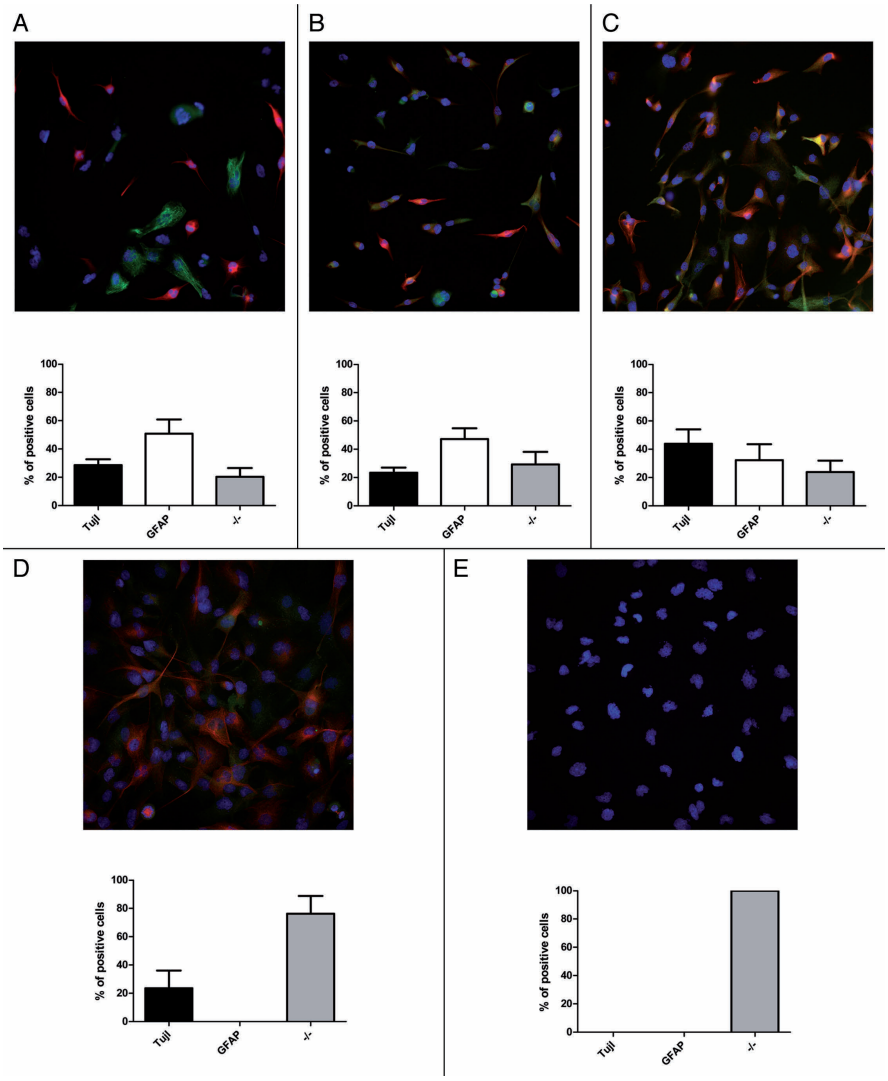


Figure 2. Marker expression of differentiated GBM006 parental cultures and single-cell derived clones (A) Immunofluorescence staining on parental GBM006 culture upon differentiation and quantification of marker expressing cells, as well as cells that do not express either of the markers: β3-Tubulin (red)/GFAP (green); Immunofluorescence staining of single-cell derived clones after differentiation for β3Tubulin (red)/GFAP (green) and quantification of differentiated cells per lineages. (B) clone 1; (C) clone 7; (D) clone 6; (E) clone 9.

differentiation, the ratio of the positive cells varied greatly. While in some cases the differentiation patterns of clones resembled the parental one (Fig. 2B), in others marker expression was shifted towards one of the two lineages (Fig. 2C). In addition, a few clones showed uni-lineage differentiation (Fig. 2D) and one clone did not express either of the two markers (Fig. 2E).

What do those results imply in the context of the CSC hypothesis?

If a single, genetically stable, CSC population is responsible for tumor growth then a change in the tumor-microenvironment could potentially be responsible for the observed change in tumor phenotype. However, in our experiment all clones were cultured and differentiated under the same conditions. Further, all clones we obtained appear to have a stable phenotype in our cultures. An alternative explanation for our observations would thus discard the strict requirement of a single population of CSCs and take into account a diversity of the CSC population with respect to differentiation programs. In that respect, our results would indicate that the original tumor contained several different clones and that CSCs isolated from the same tumor are not necessarily uniform in respect to their differentiation abilities. It is likely that all clones arose from one initial cell, but due to the diverse selective pressures in different areas of the tumor acquired different mutations.35 These mutations could influence their differentiation preferences, giving rise to several distinct daughter CSCs. Another explanation of the varied differentiation patterns of different clones could be different cells of origin. There are, for example, conflicting data regarding the cell of origin of GBM. It is proposed that gliomas arise from multipotent stem cells, partially differentiated unipotent progenitors or from mature glial cells that dedifferentiate.36 All three hypotheses are potential explanations for the diverging differentiation patterns of single-cell derived CSCs seen in our GBM clones and in other tumors. It remains to be determined if this diversity of CSCs is a common feature.37 In that respect, it's notable that the GBM CSCs mirror the diversity of the tumor phenotypes observed in vivo. Colon carcinomas, on the other hand, do not show this variety, which is consistent with the finding that even genetically different clones yield very similar in vivo differentiation patterns.28

What are the implications for the development of a CSCs-targeted therapy?

It has been shown in colon cancer already that inhibitors of the Notch pathway can drive colon cancers into differentiation.38 This kind of manipulation could be used for driving cells into differentiation or redirecting overall differentiation towards the cell type most sensitive to therapy. The presence of different CSC populations in the tumor would complicate this endeavor, as one can expect that the different CSC populations react differently to this treatment. Thus targeting one type of CSCs alone might not be enough for efficient tumor treatment.

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