

Glioblastoma cancer stem cells: Basis for a functional hypothesis

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ABSTRACT

GBM Cancer stem cells (CSCs) are responsible for growth, recurrence and resistance to chemo- and radio-therapy. They are supposed to originate from the transformation of Neural stem cells (NSC) of the Sub-ventricular zone (SVZ) or Sub-granular zone (SGZ) of hippocampus. Alternatively, they can be the expression of a functional status of competence or dedifferentiated cells of the tumor re-acquiring stemness properties. The origin of gliomas has been put in relation with the primitive neuroepithelial cells of the SVZ or NSC or progenitors, as showed by the development of experimental tumors in rats by transplacental ethylnitrosourea administration. The demonstration of CSCs in GBM is based on Neurosphere (NS) and Adherent cell (AC) development in culture. NS share the same genetic alterations with primary tumors and express stemness antigens, whereas AC show differentiation antigens. NS are generated by the most malignant areas of GBM. CSCs are considered at the top of a hierarchy of tumor cells of which the most immature are Nestin⁺/CD133⁻ cells or established on the basis of EGFR amplification or delta-EGFR. NS in serum conditions differentiate and give origin to AC, the real nature of which is still a matter of discussion. Cells in culture could be simply *in vitro* entities depending on culture methodology. CSCs in GBM could be tumor cells at the end of a dedifferentiation process re-acquiring stemness properties, in an opposite way to what is realized in normal cytogenesis, where stemness is lost progressively with cell differentiation. This interpretation could fit with the origin of the two GBM types, primary and secondary. In primary GBM the tumor originates directly from stem

cells or progenitors from SVZ with an accelerated transformation, whereas secondary GBM originates by transformation from astrocytomas arisen through a slow transformation from migrating stem cells and progenitors.

Keywords: Glioblastoma; Stemness; Origin

1. INTRODUCTION

In the last decade a tremendous amount of contributions has been dedicated to the problem of Cancer stem cells (CSCs). In Glioblastoma (GBM) they have been repeatedly demonstrated, interpreted and discussed in relation with the methodology of culture and the starting conceptual viewpoint of the authors. Depending on their attitude as specialists of the field, pathologists/neuro-pathologists, neurobiologists or researchers simply using *in vitro* culture CSCs for resolving neuro-oncological problems, different opinions have been put forward going from CSCs as a special cell type to CSCs as a functional state or a sheer product of the culture. We wanted in this study to discuss the nature of CSCs on the basis of our experience of neuropathologists/neuro-oncologists based on the daily practice with CSCs in the context of diagnosis, genesis and prognosis of gliomas.

2. NERVOUS CYTOGENESIS AND CANCER STEM CELLS

As in other malignancies, in gliomas, Cancer stem cells (CSCs) represent a subset of rare tumor cells capable of self-renewal, tumorigenicity, differentiation and tumor regeneration [1-3] and at the top of a hierarchy of tumor cells [4]. They are supposed to originate from the transformation of Neural stem cells (NSCs), but also from restricted progenitors or more differentiated cells, capable of restoring self-renewal [5-7]. Their source has been identified in the germinal matrices, Sub-ventricular

zone (SVZ) or Sub-granular zone (SGZ) of the hippocampus. At variance, they may represent a functional status [8] or dedifferentiated tumor cells which re-acquire stem cell-like properties [2,9,10]. As a matter of fact, gliomas have been produced from dedifferentiated astrocytes [11,12]. Even though different from the Tumor initiating cells (TICs), identified in the early stages of the tumor, with less mutations and not yet showing the full characteristics of CSCs [13], the two terms are often used interchangeably [14] also for Glioblastoma multiforme (GBM) [15,16].

CSCs are generally considered as the real target of tumor therapies and from the neuropathologic view point, they are considered in the context of brain function and pathology and under the influence of microenvironments [17].

In the Central nervous system (CNS), NSCs and progenitors occur from the embryo to the adult. Primitive neuroepithelial cells reside in the germinative matrix and give origin to basal progenitors and radial glia, which are found in the telencephalon and generate neurons. In the adult, NSCs are found in the SVZ of the hemispheres, in niches with specific microenvironments [18], composed of A, B and C cells (neuroblasts, quiescent NSCs and transit-amplifying cells, respectively), surrounded by ependymal cells. They are in contact with vessels and send an apical process toward the ventricle, for the stemness maintenance [19]. One of the function of the niche is exactly that to preserve stemness of NSCs [20]. In the mouse, ependymal cells may exist expressing CD133/CD24 and capable of generating neurons, astrocytes and oligodendrocytes [21].

Some cells of GBM show markers of neural progenitors/stem cells, so that its CSCs are considered as deriving from multipotent NSCs and responsible for growth, recurrence and resistance to therapies, as in other malignancies. To the first evidence that NSCs of the SVZ are involved in gliomagenesis [22,23], many studies pointed out the glioma origin from aberrant NSCs [2,24-28] and this was confirmed by experiments on animal models [29].

The origin of gliomas from the primitive neuroepithelial cells was demonstrated years ago by the experimental production of brain tumors in the rat with nitrosourea derivatives [30]. Ethylnitrosourea (ENU) was administered to the mother rat at the 17th day of gestation and Methylnitrosourea (MNU) to the adult rat [31]. In the first case tumors developed in the offspring migrating from the germinative matrices, proliferating and differentiating. The tumors appeared in the future hemispheres after some cell generations [22,23]. The latency period between the first hit, at the 17th day of gestation, and the first tumor development, was roughly two months and it corresponded in man to the period from i.u. life to the

fourth or fifth decade of e.u. life when astrocytomas appear. Tumors by MNU developed from the SVZ or from the SGZ of the hippocampus.

3. THE NATURE OF CSCS AND THE NICHES

GBMs are heterogeneous tumors with undifferentiated and differentiated glia cells and a genetic resemblance with NSCs, beside genetic alterations of the tumor transformation. The demonstration of CSCs in GBM is given by the formation of Neurospheres (NS) in culture media containing growth factors, sharing genetic properties with the primary tumor and antigenic properties with NSCs [32,33]. Adherent cells (AC) develop in serum-containing media which do not show the genetic properties of the tumor and express differentiation antigens [33-35].

The expression by CSCs of the surface glycoprotein CD133, is discussed as representative of stemness and conditioning tumorigenesis [28,36]. The differentiation between + and - cells, based on cell sorting, which is not exempt from impurity problems, is a problem [16]. However, it could be of marginal interest in comparison with other aspects, for example, the Phosphatase and tensin homolog (PTEN) status which correlates with NS growth. CD133⁺ and CD133⁻ cells form distinct self-renewing populations, hierarchically organized with CD133⁺/Nestin⁺ as the most immature cells. Cells expressing a range of markers could contribute to the aggressive growth of individual tumors [16]. A CSC hierarchy has been recognized also on the basis of amplified Epidermal growth factor receptor (EGFR) or delta-EGFR [37].

In the SVZ of the mouse, CD133⁺/CD24⁺ ependymal cells [21] form a second NSC population composed of and there is a high degree of plasticity in the of exchange between ependymal cells and astrocytes [38]. However, the stage-specific embryonic antigen-1 (SSEA1/CD15) [39] is not expressed in ependymal cells, but it can be positive in ependymoma-derived NS and ependymomas [38]. Among the many regulatory factors of CSCs and NSCs, B lymphoma Mo-MLV insertion region 1 homolog (BMI1) regulates p16 and p19 [40] and is largely expressed in gliomas [41] where it supports invasiveness.

Niches represent a crucial point in the relationship between GBM and its CSCs. To the SVZ [42] and SGZ of hippocampus [43] as niches for NSCs, the white matter could be added as well [44]. Stem cells and quiescent cells are regulated through a balance between proliferation and anti-proliferation signals [45] from which genetic events free cells for tumorigenesis [46]. There is a co-regulation between NSCs and vessels [19,20] and the same happens between microvasculature, cell proliferation and aggressiveness in GBM tumoral niches where stem cells through Vascular endothelial growth factor

(VEGF) support angiogenesis which in turn maintains stem cell survival [47,48], and hypoxia is crucial for both phenomena [49,50]. Proliferating tumor cells are supported by the activation of Hypoxia-inducible factor 1 (HIF-1) which regulates VEGF and platelet-derived growth factor B (PDGF-B) [51,52]. Subsets of Vascular endothelial (VE)-cadherin-positive (CD144⁺) cells showing CSCs features are capable to start *de novo* vascularisation by differentiating into endothelial cells [53,54].

Stem cells in the adult can be attracted from gliomas [55,56], likely by inflammatory mediators secreted in the damaged CNS from tumor cells, for example, C-X-C chemokine receptor type 4 (CXCR4) [57,58]. Migrating transformed stem cells can be attracted by hypoxia in a neo-niche with its specialized microenvironment [59] or they may contribute to the tumor mass.

Resistance to radio- and chemo-therapy is a feature of CSCs and it is one of the factors responsible for the failure of local control of GBM. Resistance to radio-therapy could be ascribed to the activation of the DNA damage response machinery that increases survival [46]. Inhibition of Checkpoint 1/Checkpoint 2 kinases (Chk1/Chk2) and Poly (ADP-ribose) polymerase 1 (PARP1) make the cells more vulnerable to radiation [60,61]. After irradiation, CD133⁺ cells accumulate in the irradiated areas [62] through the role played by BMI1 [63].

4. OUR EXPERIENCE ON CSCS AND ON THEIR IDENTIFICATION

In our collection of 21 GBM cell lines, NS developed in growth factor containing media and AC in serum condition in 10 and in 13 cases respectively. NS showed the same genetic alterations and stemness antigens, such as CD133, Musashi.1, Nestin, SOX2 and REST [33] as primary tumors whereas AC expressed differentiation antigens, such as GFAP, Galacto-cerebroside (GalC) and β -III Tubulin [33] (**Figure 1**, **Tables 1** and **2**). With the addition of serum to the medium, NS differentiated showing the same antigens as AC. In one case both cell types developed and in another case NS could be obtained from AC by the addition of factors. Our results corresponded, more or less, to previous ones [34,35]: In growth factor containing media NS grew and behaved like NSCs, with clonogenicity and tumorigenicity and with a gene expression profile similar to that of primary tumors; in serum containing media adherent cells developed with no resemblance either to NSCs or primary tumors. They were clearly differentiated.

Microarray studies showed a new categorization of GBM with three subtypes: A Proneural type (PN) with genes of normal brain and neurogenesis and a better prognosis; A Proliferation type (Prolif) with genes of cell proliferation and of poor prognosis and a mesenchymal

Table 1. Stemness and differentiation antigens in glioblastomas and cell lines.

Antigens	Primary tumors	Neurospheres	Adherent cells
CD133	+	++	-
Musashi.1	+	+	-
Nestin	++	++	-
SOX2	++	++	-
GFAP	+/-	-	+
GalC	-	-	+
β -III Tubulin	-	-	+

Table 2. Molecular genetics in glioblastomas and cell lines.

	EGFR amplification	PTEN mutations	TP53 mutations
Primary tumors	+	+	±
Neurospheres	+	+	±
Adherent cells	-	±	-

type (Mes) with genes of angiogenesis activation and poor prognosis [64]. In culture, cell clusters were identified corresponding to the NS and AC showing neurodevelopmental genes and extra-cellular matrix related genes and high and low tumorigenicity, respectively. GBM would be composed by the two phenotypes [34]. Serum cells, even if regaining tumorigenicity in later passages, underwent significant genomic alterations, genetically and biologically different from primary tumors.

NS appear to be the true CSCs and the relevant stem cell property goes lost when they are put in serum conditions; it is restored with the addition of growth factors, but it is very unlikely that they could have preserved the stem cell properties [35]. There are intermediate behaviours between NS and AC [34]. One wonders, therefore, whether AC are endowed with a partial stemness and what they really are. Monolayer systems of culture, *i.e.* plating NSCs on ornithine, laminin or fibronectin and making them grow flat and adherent with all the features of NS, give different views on CSCs summarized by the sentence “going round or going flat” [65,66]. It would be impossible to compare each other the results of both procedures also taking into account that it is not excluded that NSCs could be nothing else than “physiological players” or *in vitro* entities [67], in line with the repeatedly emphasized concept that culture methodology can influence the expression of CSCs [66,68]. Also in our culture conditions, a hierarchy of CSCs can exist, based not only on molecular features, but also on growth rate, clonogenicity and tumorigenicity, *i.e.* concerning stemness and aggressiveness or differentiation.

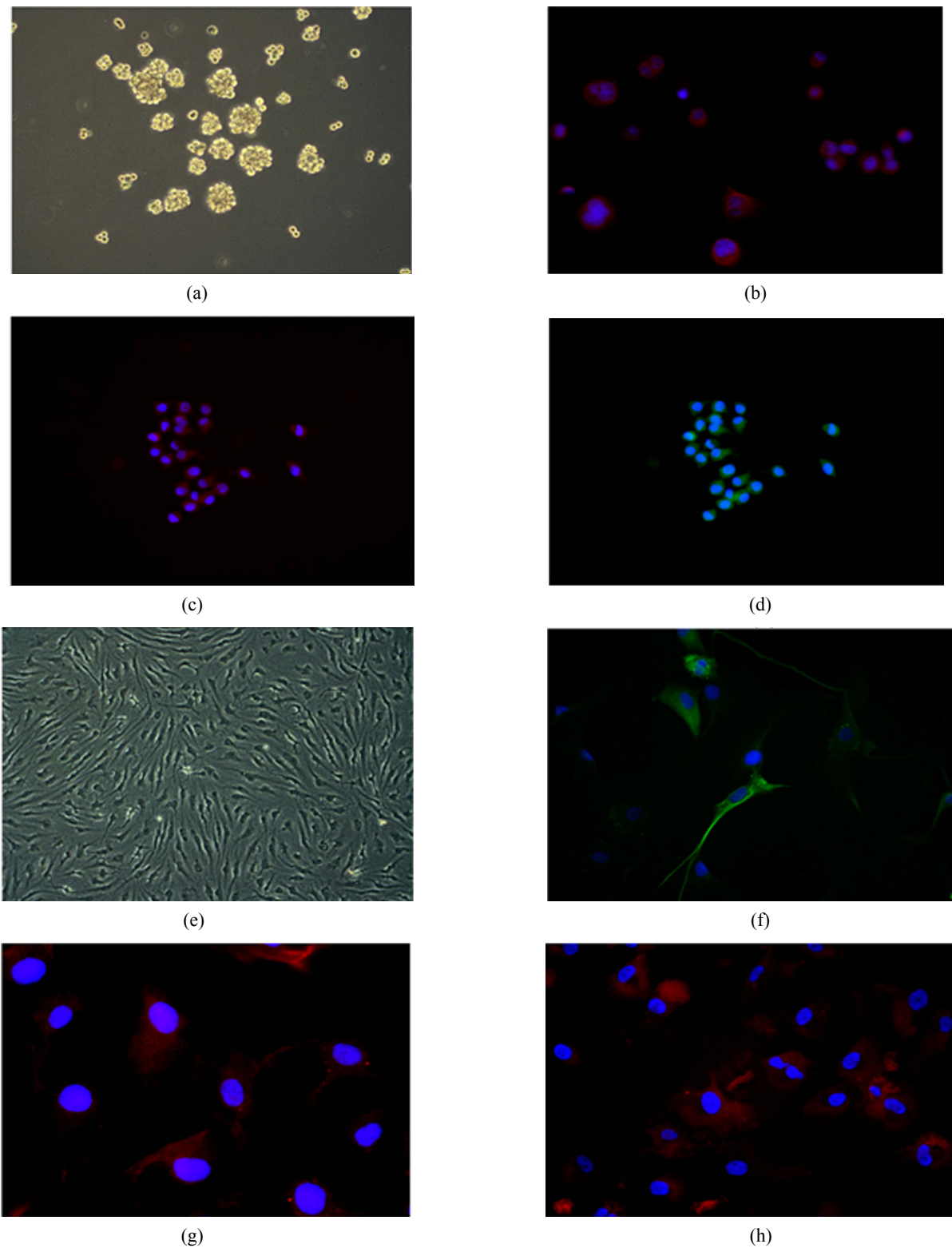


Figure 1. (a) Neurospheres in DMEM/F-12 medium with growth factors (10× magnification); (b) Nestin expression in cytoplasm. Nuclei are counterstained with DAPI (40× magnification); (c) Id. CD133 (20× magnification); (d) Id. Musashi.1 (20× magnification); (e) Adherent cells in DMEM with serum (10× magnification); (f) GFAP expression in cytoplasm. Nuclei are counterstained with DAPI (20× magnification); (g) Id. GalC (20× magnification); (h) Id. β -III Tubulin (20× magnification). Observations were made on a Zeiss Axioskop fluorescence microscope equipped with an AxioCam5MR5c and coupled to an Imaging system (AxioVision Release 4.5, Zeiss).

5. CSCS AND THE TUMOR PHENOTYPE

In our cell line series a correlation was found between NS generation in culture and the phenotype of primary tumors. This was characterized by the highest degree of malignancy with high cell density, high small vessel density, the highest proliferation index and the occurrence of necroses and of perivascular cuffings of tumor cells, expressing much more Nestin than GFAP, and other stemness antigens [33]. It could correspond to the intra-tumoral niches with hypoxic regions where CSCs are promoted or maintained [69-71]. The perivascular location of CSCs is in line with the close relationship among endothelial cells, Nestin and CD133⁺ cells, with the enhancement of their self-renewal by endothelial cells and the support of CSCs to the vasculature development [46]. This inter-relation might be read in a dynamic perspective, *i.e.* in a functional way, as if stemness may be transient and reversible and niche-dependent [72].

In general, quantitative differences in stemness properties, clonogenicity, tumorigenicity exist between NS and AC and within each category, corresponding to a kind of hierarchy of CSCs [16,37]. Stemness could be distributed in a spectrum covering all the tumor cells with a *crescendo* from quiescent highly differentiated cells, where it is nil, to those in which it reaches the highest degree of expression. As a matter of fact, either NS or AC grow in culture with different rates [2,33] and the capacity to generate NS in culture conditions is not uniform for the different areas of GBM [24,33,73]. Areas increasingly different from the most malignant phenotype seem to progressively generate less NS or AC until to zero. Immature cells expressing Nestin, likely to be CSCs, have been found lining central necroses inside proliferating areas, where HIF-1 is highly expressed [73]; these sites may roughly correspond to those of the tumor where usually the highly malignant phenotype occurs. Practically, all the cells of a tumor are hierarchically distributed with respect to the capacity to produce NS in culture conditions or AC in serum condition. Obviously this does not resolve the question whether stem cells, at the top of the hierarchy represent a cell type or a functional status [66].

6. SIGNIFICANCE OF THE STEM CELL STATUS AND THE ORIGIN OF GBM

Of course, there are genetic determinants of stem cell identity and in the first row there is BMI1 which is believed to represent an oncogenic addition which distinguishes CSCs from NSCs [74,75]. Other pathways are Notch, Hedgehog, Bone morphogenetic protein (BMP), SRY (sex determining region Y)-box 2 (SOX2), Signal transducer and activator of transcription 3 (STAT3) [76-78] and c-MYC [79]. The regulatory factors can be

activated or deactivated in the different contexts. In the conception that CSCs are the product of tumor cell dedifferentiation which follows mutation accumulation in the course of malignant transformation [2,8,9], the re-acquisition of stemness properties by the cells could be linked to the activation of the before mentioned pathways. At this point, the two GBM subtypes must be discussed as for their different origin.

Secondary GBM (sGBM) develops from a previous astrocytoma, whereas primary GBM (pGBM) is a *de novo* tumor. They differ as for the genetic configuration, age, and growth speed [80], but not for location and phenotype; at the most they can differ for the spreading modalities [9]. It is not known how *de novo* tumors arise, whereas it is believed that secondary ones originate through anaplasia, *i.e.* through dedifferentiation of tumor cells which follows mutation accumulation [81]. Generally, it is known that GBMs originate either from NSCs or from astrocytes [11] and this could correspond to the distinction between pGBMs and sGBMs. Obviously, it is likely that the two GBM subtypes must originate *ab initio* from the same CSCs. The development of GBM in the emisphere, far away from the SVZ, could be in contrast with its origin from NSCs of the same region, but this can be got over if we refer to the concept of asymmetric division and of migration of progenitors [82]. A path has been traced from mitotically active precursors to the developed tumors [12], which recognizes in transiently dividing progenitors and in somatic stem cells the elements where mutations accumulate; they express also EGFR, present in normal progenitors of SVZ [83]. These cells are the possible source of pGBMs, whereas for sGBMs it is mandatory to refer to a previous astrocytoma.

The two GBM types differ for the expression of mutated Isocitrate dehydrogenase 1-2 (IDH1-2), occurring in sGBM, anaplastic and diffuse astrocytomas and oligodendrogliomas and not in pGBM [84,85]. The mutual exclusion of IDH mutations with EGFR amplification and its association with 1p-19q co-deletion, TP53 mutations and younger age [86-90] are relevant to the timing of IDH mutations, which must be placed between precursors and progenitors. All this means that sGBM originate from tumor cells which have already reached the site of tumor development and the stage of precursors, whereas pGBM originate from cells which transform by mutation accumulation during migration and before reaching the stage of progenitors; they reach the site of development already possessing the genetic equipment of malignancy and keeping or re-acquiring again, at the same time, the features of stem cells. This is something similar to the concept of "maturation arrest" [91] of cells that accumulate mutations and transform before reaching the full maturity or differentiation.

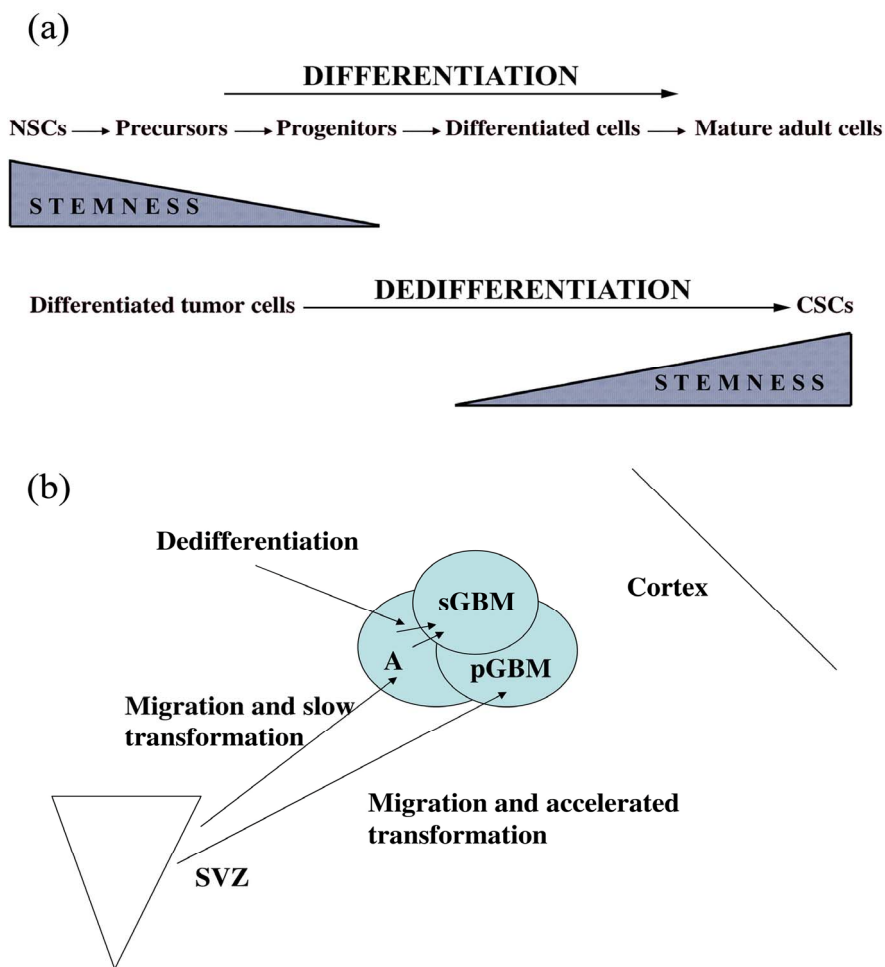


Figure 2. (a) Stemness in differentiation and dedifferentiation; (b) Origin of pGBM and sGBM.

The stemness properties would be acquired in sGBM by dedifferentiation and in pGBM by their preservation (**Figure 2(a)**). The process would be substantially the same, but more rapid and accelerated in pGBM, as already pointed out [17] (**Figure 2(b)**). Stemness would be a condition which is progressively lost during the normal nervous cytogenesis and progressively acquired during dedifferentiation in tumors. In sGBM to obtain NS in culture conditions would be more difficult, because few cells with dedifferentiation reach the stage of stemness, whereas in pGBM it is easier, because most cells already possess it. Stemness would be in this way more a transient status than a fixed feature of a given cell type. The microenvironmental influences are very important in modifying the stemness status, even conceiving GBM in a neo-darwinistic interpretation [17,92].

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