[Frontiers in Bioscience E3, 701-710, January 1, 2011]

CD133 negative cancer stem cells in glioblastoma

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1. ABSTRACT

Glioblastomas (GBM) are paradigmatic for the investigation of cancer stem cells (CSC) in solid tumors. Recently, the discovery of CD133$^-$ CSC in addition to CD133$^+$ CSC has substantially added to our understanding of the complexity of GBM CSC. This review gives an overview on our current knowledge on CD133$^-$ cells in GBM and describes five different hypothesizes on the nature of CD133$^-$ cells in GBM. In addition, we summarize the current knowledge on tumorigenic CD133$^-$ CSC, list available markers, describe the current controversies on the origin of CD133$^-$ CSC, and discuss how the heterogeneity of CSC may correspond to the molecular heterogeneity of GBM.

2. INTRODUCTION

Solid and hematological tumors show marked intratumoral heterogeneity. Only a low but variable proportion of cells shows clonogenic growth *in vitro* or is tumorigenic *in vivo* (1). A popular explanation for the heterogeneity was provided by the stochastic model suggesting that all tumor cells have in principle the same ability to maintain tumor growth or to cause tumor relapses (2). Park, Hamburger, and Salomon were the first to postulate a hierarchical organization (3, 4) within a tumor with tumor stem cells (also referred to as cancer stem cells (CSC), or tumor initiating cells) giving rise to more differentiated and less tumorigenic cells. Still, reliable markers for the further in depth characterization of CSC

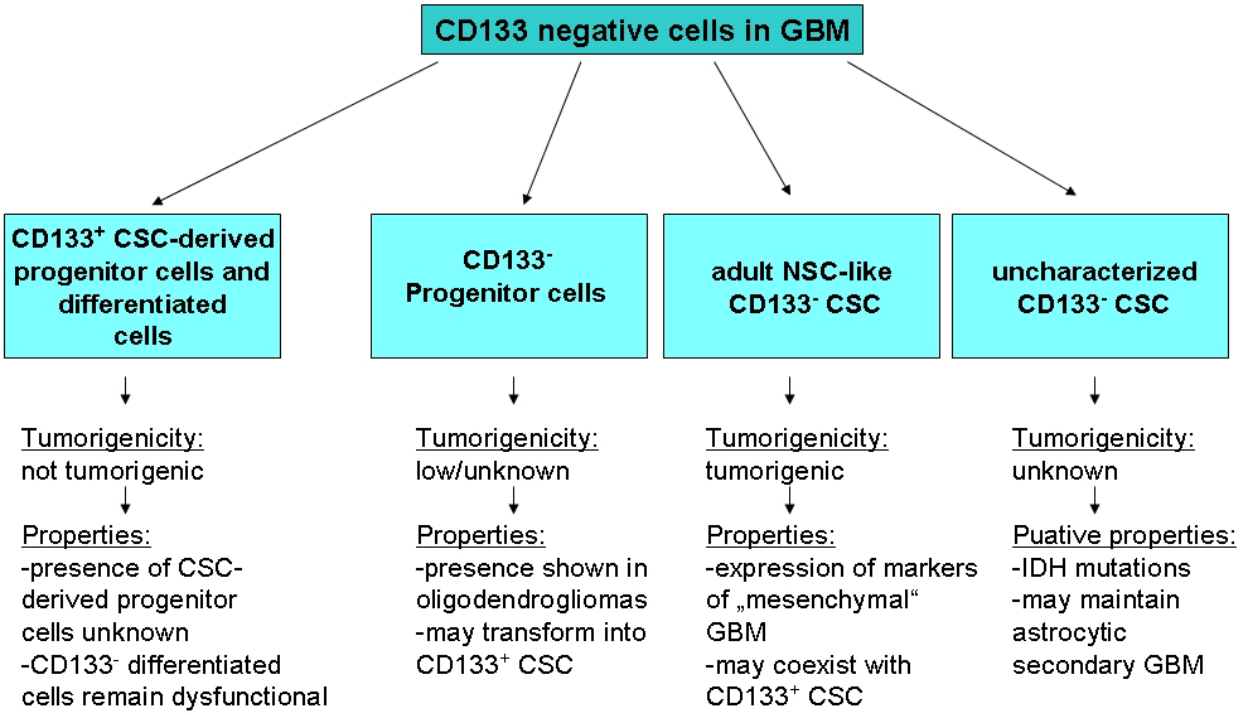

Figure 1. Overview over different hypotheses on CD133 negative cells in GBM.

were lacking at that time and impaired the detailed investigation of this concept in the 80ties. With our increasing knowledge on somatic stem cells in various tissues, stem cell specific markers were discovered that also characterized stem cell-like tumor cells in derived malignancies (5-7).

In the adult brain, self renewing neural stem cells (NSC) have been described in the early 90ties (8) that were characterized by stem cell markers like nestin and CD133 (9). In 2002, Ignatova and coworkers were the first to propagate stem-cell like tumor cells isolated from human GBM that were further characterized by Galli *et al.* (10, 11). Singh *et al.* discovered that only a subgroup of tumor cells expressed the stem cell marker CD133 (12) and that only 100 $CD133^+$ cells were tumorigenic when injected into the brain of immunodeficient mice (13). Even more important, more than 100.000 $CD133^-$ did not induce tumors but integrated into the healthy brain parenchyma. The authors concluded that CD133 is a marker for CSC in brain tumors and their study became the starting point for the investigation of CSC in GBM during the last 6 years (12, 13).

Although the initial experiments by Singh *et al.* have been reproduced (14-17), their results remain controversial: Several authors failed to detect CD133 expression on tumor cells with stem cell properties (14, 16, 18-20), or found relevant tumor formation by both, $CD133^-$ and $CD133^+$ cells within one tumor (21-23). As a consequence, CD133 is the most intensively investigated marker for GBM so far. This review therefore aims to summarize the controversial data on $CD133^-$ GBM cells with and without stem cell properties and to describe soundly documented findings on $CD133^-$ CSC in addition to emerging concepts on the role of $CD133^-$ cells (Figure 1). To date, five different hypothesizes on $CD133^-$ tumor cells have been raised in the literature. The available evidence for these subtypes varies with substantial experimental data for some subtypes and rather speculative reports for others. In addition, it remains unknown if there is an overlap between postulated subtypes. However, an overview over the concepts that are currently discussed is required to give a full and structured overview on this evolving field in neurooncology. The following postulated $CD133^-$ cell types in GBM are described in the first part of the review:

1. Differentiated, non-tumorigenic $CD133^-$ cells derived from $CD133^+$ GBM CSC

2. $CD133^-$ progenitor cell-like tumor cells with limited proliferative potential

3. Uncharacterized $CD133^-$ CSC that can not be propagated in medium optimized for NSC

4. $CD133^-$ CSC coexisting next to $CD133^+$ CSC

5. Adult NSC-like $CD133^-$ CSC

The second part of the review focuses on adult NSC-like $CD133^-$ CSC, summarizes different CSC markers, discusses the relationship between $CD133^-$ CSC and molecular defined GBM subtypes, and gives an

outlook on putative therapeutical implications of the heterogeneity of CSC.

3. HYPOTHESES ON CD133 NEGATIVE CANCER CELLS IN GBM

3.1. Differentiated, non-tumorigenic CD133$^-$ cells derived from CD133$^+$ GBM CSC

In 2004, Singh *et al.* published that CD133$^-$ cells within brain tumors were virtually devoid of tumorigenic cells. Conversely, CD133$^+$ were highly tumorigenic and 100 cells injected into the brains of nude mice sufficed to induce tumors that closely resembled the initial tumors. These experiments suggesting that differentiated CD133$^-$ cells derive from CD133$^+$ CSC in GBM, have been reproduced in the meantime (14-17, 24). However, reports on tumorigenic CD133$^-$ CSC (14, 16, 18-20) and the presence of tumorigenic CD133$^-$ and CD133$^+$ CSC within one tumor (21-23, 25) have substantially challenged this concept. At the moment there is no consensus in the literature if GBM exclusively maintained by CD133$^+$ CSC actually exists. This implies that the lack of tumorigenicity of the entire CD133$^-$ fraction, as proposed by Singh *et al.*, remains vague (13). Although the impact of technical and methodological differences between the reports remains unknown (21, 26, 27), it appears likely that the molecular heterogeneity of GBM contributes to the conflicting experimental reports (28, 29). In line with this idea, analysis of a larger series of 22 GBM samples suggests that only CSC lines derived from a subset of GBM samples are actually exclusively maintained by CD133$^+$ CSC (16). The data published by Ogden *et al.* support the idea of intertumoral heterogeneity (20): in this report, in only 1 out of 6 GBM the CD133$^-$ (A2B5$^{+/-}$) fraction of cells was not tumorigenic. In the remainder (n=4 out of 6), CD133$^-$ /A2B5$^+$ were able to form GBM-like lesions *in vivo*.

Taken together, it is unknown if a strict hierarchical organization with CD133$^+$ CSC and differentiated, non-tumorigenic CD133$^-$ cells actually exits in GBM. However, the discrepancy of several high quality reports points towards interindividual differences between the GBM samples used suggesting that a subgroup of GBM may be maintained exclusively by CD133$^+$ CSC and thereby comprises a completely non-tumorigenic CD133$^-$ cell compartment.

3.2. CD133$^-$ progenitor cell-like tumor cells with limited proliferative potential

While a subgroup of CD133$^-$ GBM does not grow at all *in vitro*, transient growth of tumor cells that fail to further proliferate infinitely has also been reported in a small fraction of GBM samples that were all characterized by oligodendroglial differentiation (30). In this study, the reduced proliferative capacity was associated with low CD133 expression (less than 3% of all cells) and a reduced differentiation potential of the CD133$^-$ tumors cells as compared to "normal" GBM derived CD133$^+$ CSC. The combination of reduced differentiation potential and limited proliferative capacity suggested that CD133$^-$ progenitor cell-like cells but not CSC actually maintain these tumors. Although all tumors were classified histologically as high-grade oligodendrogliomas (WHO °III) or GBM with oligodendroglial differentiation, the prognosis of the patients varied substantially. Patients with CD133$^-$ progenitor cell-like tumor cells survived significantly longer as compared to tumors maintained by CD133$^+$ CSC which further advocates that the less malignant tumors may be maintained by CD133$^-$ progenitor cell-like cells rather than by CSC. A second study supported this hypothesis: Rebetz *et al.* reported that low-grade gliomas were characterized by the expression of markers typical for glial progenitor cells (PDGFR-alpha, A2B5, O4, and CD44) but not of neural markers (synaptophysin and NSE). This phenotype inversely correlated with the expression of CD133. Tumors with CD133 expression were characterized by a broader differentiation pattern mainly towards cells with a neuron-like phenotype. Again, the progenitor cell-like phenotype was associated with a better prognosis (31). Histologically, some tumors maintained by progenitor-like cells have been classified as GBM and may therefore account for a part of the CD133$^-$ GBM. The lack of CD133 expression in tumors derived from progenitor-like cells was also described in a mouse model of medulloblastoma (32). In these tumors that were induced by activation of shh signaling in cerebellar progenitor cells, tumors were maintained by SSEA-1/CD15 positive cells but not by CD133$^+$ cells.

Taken together, experimental evidence suggests that a small fraction of GBM (mainly with oligodendroglial differentiation) may be maintained by CD133$^-$ progenitor cell-like tumor cells with limited proliferative potential. Importantly, the similarities of progenitor-like tumor propagating cells and NSC derived progenitor cells does not prove that the malignant cells develop from their non-malignant counterparts. At the moment it remains unknown if this "subtype" represents a own entity, if the reported data corresponds to the low tumorigenic type III cells reported by Chen *et al.* (25), or the less tumorigenic CSC reported by Gunther *et al.* (33).

3.3. Uncharacterized CD133$^-$ CSC that can not be propagated in medium optimized for NSC

Wang *et al.* reported a 96% "take rate" for the *in vivo* propagation of GBM samples (n=29) that remained close to 100% at subsequent passages suggesting that almost all GBM were maintained by CSC (34). Conversely, several laboratories reported that only approximately 40% of all GBM samples give rise to CSC lines that can be propagated using standard conditions *in vitro* (11, 16, 35, 36). The standard conditions include the culture of CSC in serum-free medium supplemented with growth factors that have been optimized to propagate NSC (8). Although protocols used in different laboratories varied substantially, all groups used at least EGF and bFGF (27) suggesting that a significant proportion of GBM are maintained by tumor cells requiring additional growth factors. If new culture conditions (e.g. the use of laminin coated culture dishes) will increase the proportion of CSC proliferating *in vitro*, remains uncertain given that the new protocols have not been tested in a larger set of samples (19, 37). In addition, the rate of *in vivo* "take rate" is variable and depend on the genetic background of the mice used as well as the

injection technique (38). Although technical problems may influence the rate of successful propagation of CSC, the better prognosis of patients without proliferating CSC *in vitro* (30, 35, 36) strongly suggest that a yet uncharacterized subgroup of CSC maintains at least some of the GBM. Because CD133 expression correlates with the likelihood to growth under standard medium conditions (36) most of these yet uncharacterized CSC will express no or low CD133 on the cellular surface. Nevertheless, the biological properties of these uncharacterized CSC, e.g. the expression of CD133, have yet to be determined.

3.4. CD133$^-$ CSC coexisting next to CD133$^+$ CSC

Several recent papers reported that CD133$^+$ CSC may coexist next to CD133$^-$ within GBM. Ogden *et al.* showed that CD133$^+$ and CD133$^-$ cells were tumorigenic if they co-express the glial progenitor marker A2B5 (20). A similar report by Joo *et al.* confirmed that both CD133$^+$ and CD133$^-$ cell derived from the same GBM were able to form tumors after injection in nude mice (22). A recent and very detailed study investigated CSC lines derived from primary GBM in detail. In this paper, Chen *et al.* found three types of stem cells: CD133$^-$ type I CSC that formed CD133$^+$ and CD133$^-$ CSC. CD133$^+$ type II CSC that give rise to CD133$^+$ and CD133$^-$ CSC. The less malignant CD133$^-$ CSC type III differentiated only in CD133$^-$ progeny. All three reports uniformely report that CD133$^+$ CSC may coexists next to CD133$^-$ CSC within the same GBM. It remains unknown if the coexistence of CD133$^+$ and CD133$^-$ is a hallmark of all GBM or present only in a subgroup. Ogden *et al.* found this phenotype in 4 out of 6 GBM samples investigated (20). However, the absolute number of GBM investigated in this report is to low to approximate a proportion.

Reports on a marked spatial heterogeneity added to the complexity of GBM CSC. CSC in the core of GBM showed higher CD133 expression and an increased chemoresistance by overexpression of MGMT while peripheral GBM expressed less CD133 (39). A second paper confirmed the difference of CD133 expression between peripheral and central tumor samples. Nevertheless, peripheral CSC also expressed CD133 though to a lower percentage (39-41). Importantly, all three reports uniformly described that CSC from different regions of GBM show distinct biological differences (39-41). This raises the intriguing question if the intratumoral variability described may correspond to the CD133$^+$ and CD133$^-$ CSC lines described in paragraph 3.1 and 3.5 (16, 29, 33) or if it rather reflect the adoption to the hypoxia (39, 42-44) present in the central parts of GBM. At the moment, no definitive experimental data has been published to finally answer this question.

3.5. Adult NSC-like CD133$^-$ CSC

Not all CD133$^-$ CSC fail to proliferate in NSC medium. Although CD133$^+$ CSC maintain tumor growth in most GBM, a substantial proportion of GBM are maintained by CD133$^-$ CSC that can be propagated *in vitro* using growth conditions that favor the growth of NSC (14, 16, 19, 22, 33). Although a detailed study is lacking, the available data suggests that approximately 30% of all primary GBM samples that can be propagated *in vitro* are maintained by CD133$^-$ CSC (13, 16). As reported by Beier *et al.*, these CSC show an adherent growth pattern and were characterized by a distinct molecular profile (16). Günther *et al.* further characterized growth pattern, marker expression, differentiation pattern, tumorigenicity, and transcriptional profile of CD133$^-$ CSC. Similar to the report by Beier *et al.*, the authors found an adherent or semi-adherent growth pattern *in vitro*. On a functional level, CD133$^-$ CSC lines were less tumorigenic and CD133$^-$ CSC lines showed a reduced spontaneous oligodendroglial differentiation. Still, cells expressing markers of all three neural lineages have been detected in all CSC lines. On the transcriptional level, both subtypes differed significantly and microarray data derived from CD133$^+$ and CD133$^-$ CSC lines formed two independent clusters. The main differences between both groups comprised ECM related molecules and genes associated with NOTCH signaling (33). Given the distinct growth pattern of CD133$^+$ and CD133$^-$ CSC lines, the study was controversial and it remained unclear if the two molecular phenotypes result in different growth patterns or vice versa. An extensive bioinformatic analysis of GBM CSC lines by Lottaz *et al.* suggested that the molecular phenotypes do not represent an *in vitro* artifact but actually reflect two distinct entities (29). The authors referred to the two clusters as CD133$^+$ "type I CSC lines" and CD133$^-$ "type II CSC lines". Notably, both, CD133 expression and growth pattern showed a highly significant correlation to the molecular profile but did not unequivocally indicate group assignment. Only the transcriptional profile allowed a reliable classification. As a consequence, a 24-genes signature that allows the classification of yet unclassified GBM CSC lines was established (45). Using the signature genes, 3 of 7 GBM CSC lines described by Pollard *et al.* (19) were classified as type II. Notably, only one of the three CSC lines actually lacked CD133$^+$ expression. In line with the report by Wang *et al.*, (18) who reported on a gain of CD133 expression in a xenotransplantation model. This suggests that a loss or gain of CD133 (and possibly other positional identity markers) expression of CSC *in vitro* or *in vivo* contributes to the inconsistency of CD133 expression and transcriptional profile. This variable CD133 expression substantially impairs the usability of CD133 to differentiate between type I and type II CSC. In addition, CD133 expression may also reflect cellular stress (43, 44), only the glycosylation but not the protein indicates stemness (46, 47), and different epitopes were referred to as CD133 (26). These problems further complicate the use of CD133 as marker for type I CSC. Nevertheless, CD133 expression remains the only established marker at the moment to differentiate between type I and type II CSC and the two types will subsequently be referred to as "CD133$^+$ type I CSC" and "CD133$^-$ type II CSC" in this manuscript. Additional markers like CD44 may be helpful too, but larger studies confirming the usability of these markers are lacking (45). New markers like SSEA-1/CD15 for CD133$^-$ type II CSC are currently under investigation, still their practicability and specificity to identify different subtypes of CSC is unknown (14).

3.6. Markers for CD133$^-$ CSC

New markers are required to further investigate CSC in CD133$^-$ GBM and CSC lines. Two important

markers have been established recently allowing to enrich CSC. A2B5 labels surface ganglioside epitope and is marker for glial progenitor cells in the SVZ and the white matter of the CNS. A2B5$^+$ cells in the healthy brain develop directed from NSC. They have lost the ability to differentiate in neuronal cells but have conserved the capacity to differentiate into mature astrocytic and oligodendrocytic cells (48). Two reports now show that A2B5 also labels CSC in CD133$^+$ and CD133$^-$ GBM and CSC lines suggesting that A2B5 represents a suitable marker for CSC in CD133$^-$ CSC lines (20, 49). Another recently described marker for CD133$^-$ CSC is SSEA-1/CD15/Lex. A cluster of surface proteins and glycolipids sharing a common pentasaccharide (Lewis X antigen - Lex) are recognized by specific antiantibodies. SSEA-1 labels cancer stem cells in murine models of medulloblastoma (32, 50). In GBM derived CD133$^-$ CSC lines, SSEA-1 surface expression allows an at least 100 fold enrichment of tumorigenic cells (14) making SSEA-1 a promising stem cell marker if CD133 expression is lacking, if CSC lines have lost their hierarchical organization, or if several CSC populations coexists within a GBM or CSC line. If additional markers like the side population (27, 51-53), IL6 receptors, or Podoplanin (54) may help to improve the detection of CSC is yet unknown.

3.7. Relationship of molecular GBM subtypes to CD133$^-$ CSC

As proposed by Howard Fine and others (55), the work by Lottaz *et al.* also provided first experimental evidence that different cells of origin may give rise to different types of CSC (29). This bioinformatic study confirmed a previous report suggesting that GBM CSC divide into two groups on the transcriptional level (33). In extension of this earlier study, the authors also compared the transcription profile of CSC and 5 putative cell populations of origin. As suggested by previous reports (11, 56, 57), all CSC closely clustered with adult and fetal NSC. Surprisingly, CD133$^+$ type I CSC clustered together with fetal NSC and all CD133$^-$ type II CSC showed high similarity to adult NSC. This suggests that GBM CSC develop from two different cells of origin resembling either adult or fetal NSC respectively. Given that residual fetal NSC appear unlikely in patients in the sixth decade, the transformation of another cell type into an immature, fetal NSC-like cell type a possible first step in the genesis of CD133$^+$ type I CSC and derived GBM.

As discussed in paragraph 3.4, it remains unproven if CD133$^-$ CSC lines represent an entity of their own. The spatial heterogeneity of CSC within GBM provides a tempting alternative explanation for CD133$^+$ type I and CD133$^-$ type II CSC: Type I CSC originate from the core of GBM while type II CSC stem from the periphery of the tumor. However, the intertumoral heterogeneity of GBM may explain also explain the heterogeneity of CSC and vice versa. Therefore, a crucial question refers to the relationship of CD133$^+$ type I and CD133$^-$ type II CSC to the different molecular subtypes described in GBM. Several classifications of GBM based on their transcriptional profile have been proposed (28, 58, 59). Phillips *et al.* analyzed more than 300 different high-grade glioma samples (including high grade astrocytomas (WHO °III) and GBM) and described three distinct classes of GBM that resemble different stages of neurogenesis: "proneural", "mesenchymal", and "proliferative" glioma. "Proneural" gliomas had a substantially better prognosis as compared to "mesenchymal" and "proliferative" tumors (60) most likely because the patients were younger and because most WHO °III tumors were classified as "proneural" (61). Similar subtypes corresponding to "mesenchymal" and "proneural" GBM have been recently described based on the analysis of the transcriptional profile or proteomic approaches (61-64). In contrast, the "proliferative" subtype has not been reproduced in a recent study (61). In this study, Verhaak *et al.* extended the classification by integrating data from the human Genome atlas and transcriptional data: In addition to the "proneural" and the "mesenchymal" phenotype, the authors identified "classical" GBM and "neural" GBM.

"Classical GBM" had a three fold overexpression of EGFR or showed vIII EGFR mutations. In contrast "neural" GBM expressed markers of mature neurons like NEFL or GABRA1. Tumors characterized by "proneural" signature genes are younger but were resistant to therapy. The better prognosis described by Phillips *et al.* could not be reproduced after exclusion of WHO °III tumors and the isolated analysis of GBM (65). "Proneural" GBM were characterized by PDGFR-alpha and frequent IDH1 mutations. "Mesenchymal" tumors were diagnosed in older patients but tend to respond better to therapies (60, 61, 66). In addition, the analysis of transcriptional profiles of "mesenchymal" tumors showed mutations in the NF1 signaling pathway and co-mutations with PTEN both activating the AKT signaling pathway. The overexpression of markers like CD44, YKL40, MET, and MERTK indicate astroglial/mesenchymal differentiation reminiscent to the epithelial-mesenchymal transition seen in epithelial tumors outside the CNS (67). Carro *et al.* further analyzed the transcriptional profile of "mesenchymal" GBM and found that the transcription factors C/EBPbeta and STAT3 act as master regulator for the mesenchymal transformation (68).

Using a proteomic approach Brennan *et al.* identified three groups that closely resembled the results by Verhaak *et al.*: tumors that depended on the NF-1 cocluster, the EGF cocluster, and the PDGF cocluster. Similar to "mesenchymal" phenotype derived from the genomic and transcriptional analysis, the NF-1 cocluster was characterized by the expression of YKL-40 and IRS-1. The transcriptional analysis then confirmed that the NF-1 cocluster on the protein level corresponded to the "mesenchymal" phenotype identified in transcriptional and genomic studies. In addition, the authors could show that the PDGF cocluster corresponded to the previously described "proneural" phenotype identified in GBM (63).

Taken together, although additional phenotypes of GBM have recently been described (a.o. "proliferative", EGF-cocluster, "neural" GBM) all recent studies confirmed the presence of a "proneural" and a "mesenchymal" phenotype. How do these subtypes relate to CD133$^-$ type II and CD133$^+$ type I CSC? Lottaz *et al.* analyzed 100 glioma

samples (described by (60)) and questioned if the 24 signature genes characterizing $CD133^+$ type I CSC and $CD133^-$ type II CSC^- lines *in vitro* may help to identify distinct molecular subtypes in GBM *in vivo*. The signature genes clearly identified two distinct groups within the samples. In addition, the CSC-derived gene signature reliably differentiated between "proneural" and "mesenchymal" GBM. Almost all GBM samples classified as "proneural" were also characterized by signature genes for $CD133^+$ type I CSC. Conversely, signature genes for $CD133^-$ type II CSC were overexpressed in "mesenchymal" GBM. The match between the CSC derived classification and the "proneural" and "mesenchymal" phenotype was almost perfect with a p-value of 10^{-10} (45). These data suggests that $CD133^-$ type II CSC may maintain "mesenchymal" GBM and that $CD133^+$ type I CSC may give rise to "proneural" GBM. However, transcriptional similarities between CSC lines and tumor samples do not prove an actual biological connection between the CSC phenotype *in vitro* and the transcriptional/proteomical phenotype *in vivo*. A detailed conformational study is required to prove this model.

3.8. Therapeutical implications of $CD133^-$ CSC

Tumors characterized by signature genes of $CD133^-$ type II CSC do not have a different prognosis as compared to $CD133^+$ type I CSC (own unpublished observation). The same applies for the classification into "proneural" and "mesenchymal" GBM (61) if low grade gliomas (WHO °II-°III) being mainly classified as "proneural" were excluded. Murat *et al.* identified a HOX cluster including CD133 that was associated with resistance of GBM towards standard therapy comprising radiotherapy and temozolomide (66). In line with these results, "proneural" GBM responded worse to therapy as compared to "mesenchymal" GBM. This points towards distinct differences between both GBM subtypes and their associated CSC subtypes and may harbor therapeutic implications. However, $CD133^+$ type I and $CD133^-$ type II CSC lines do not differ with respect to their susceptibility towards temozolomide *in vitro* (69). The transcriptional comparison of $CD133^+$ type I and $CD133^-$ type II CSC unveiled the differential expression of ECM molecules, transcripts associated with the WNT signaling pathways, and genes of the TGF-beta signaling (33, 45). This indicates that the differentiation between $CD133^+$ type I or $CD133^-$ type II CSC driven tumors will be relevant for the personalized therapy of patients treated with therapies targeting these three signaling pathways (70-73).

4. ACKNOWLEDGEMENT

This work was supported by the NGFNplus Brain Tumor Network. Subproject 7 No. 01GS0887.

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Abbreviations: CSC: cancer stem cell; NSC: neural stem cell; GBM: glioblastoma multiforme; TGF-beta: transforming growth factor beta; WNT: wingless; PDGFR: Platelet derived growth factor receptor; NSE: neural specific enolase; FGF: fibroblast growth factor; EGF: epithelial growth factor; IDH: isocitrate dehydrogenase; SSEA-1: stage specific embryonic antigen; NEFL: neurofilament light polypeptide; WHO: World health organisation; NF: neurofibromatosis, CD: cluster of differentiation; EGFR: epithelial growth factor receptor; MET: met protooncogene; GABRA1: Gaba receptor alpha 1; PTEN: phosphatase and tension homologue; MERTK: MER receptor tyrosine kinase; CNS: central nervous system; STAT3: signal transducer and activator of transcription 3; IRS: insulin receptor substrate; IL6: interleukin 6, shh: sonic hedgehog

Key Words: CD133, Glioblastoma, Mesenchymal, Cancer Stem Cell, Transcriptional Profile, Review

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