Perspective

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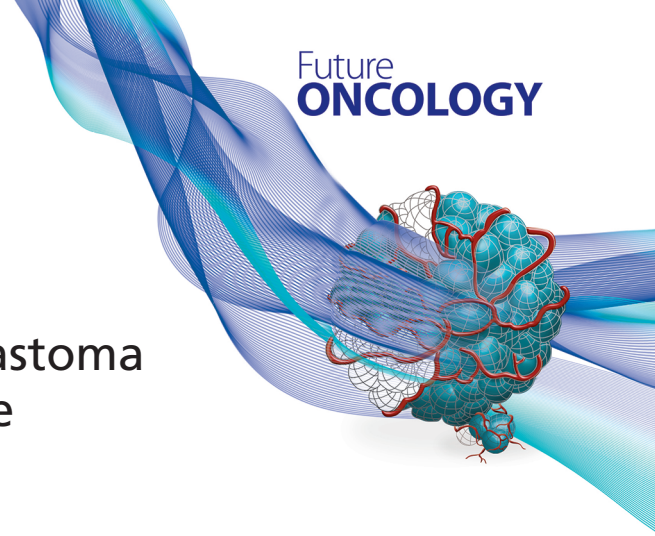

Cancer stem cell plasticity in glioblastoma multiforme: a perspective on future directions in oncolytic virotherapy

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The cancer stem cell (CSC) hypothesis suggests that a rare population of stem-like cells underpin tumorigenesis. Oncolytic viruses (OVs) demonstrate novel mechanisms of targeting the elusive CSCs with greater selectivity – promising therapeutic potential against solid tumors such as glioblastoma (GBM) that are resistant to conventional treatment. In general, OVs have failed to translate the efficacy from bench to bedside. The success of OVs rely on the hypothesis that eliminating CSCs is key to preventing recurrence. However, newly emerging evidence of CSC plasticity challenge this hypothesis by proposing that the CSC pool can be regenerated from non-CSCs post-treatment. We review this evidence surrounding the CSC hypothesis to propose an original perspective on why several advanced OVs may be failing to reflect their true potential in clinical trials. We argue that preventing non-CSC to CSC dedifferentiation may be critical to achieving long-term treatment efficacy in future OV clinical trials.

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Glioblastoma is an incurable type of brain tumor with a high recurrence rate and resistance to conventional surgical, radiation and pharmacological treatment [1]. Several factors including the difficulty for drugs to cross the blood–brain barrier, limited repair mechanisms of the brain as an organ and the treatment resistant nature of the tumor, render therapeutic options for glioblastoma (GBM) limited [2]. Temozolomide (an alkylating agent) is given as first-line treatment with radiotherapy, resulting in a 14.6 month median survival compared with 12.1 months in patients treated with radiotherapy alone hence the need for non-conventional treatment options to be explored [3]. The therapeutic potential of oncolytic viruses (OVs) has sparked interest in the last 20 years as they enable the selective destruction of tumor cells, and in particular, the cancer stem cells (CSCs) which are believed to be central to tumorigenesis.

Hanahan and Weinberg proposed in their seminal paper, eight physiological hallmarks of cancer; self-sufficient growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, metastasis, abnormal metabolic pathways and immune evasion [4]. Under the stochastic model, any cell can undergo transformation, acquiring these hallmarks through genetic mutations and epigenetic changes, resulting in uncontrolled proliferative potential [5,6]. This drives the accumulation of mutations resulting in tumor heterogeneity – characteristically seen in GBM [7,8]. Based on this view, any individual cancer cell can proliferate or cause tumor recurrence [9,10].

The CSC hypothesis argues an alternative; that a rare population of cancer-initiating cells with unlimited self-renewal, are responsible for tumor growth and recurrence [11,12]. CSCs reside at the top of this hierarchy, differentiating unidirectionally to produce a heterogenous progeny that comprises the bulk of the tumor. While CSCs can produce secondary tumors, the progeny cannot, though they carry the same genetic abnormalities as the hierarchical cell [13]. Despite advances, conventional therapy is faced with limitations posed by poor access or tumor penetration, the heterogenous nature of cells within the tumor and the challenges associated with targeting the elusive CSC subpopulation. CSCs express drug efflux pumps, ATP binding cassettes and upregulate DNA repair pathways that contribute to mechanisms of immune evasion [14–17]. Some maintain slow cell cycles or remain qui-

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escent, rendering less susceptibility to antiproliferative treatment such as temozolomide [15]. This CSC hypothesis provides one explanation for the treatment resistant nature of certain tumors observed clinically. Current first-line treatment which indiscriminately kill the bulk of the tumor but fail to eradicate this rare CSC population, is likely to permit recurrence to occur in the long term due to the enrichment of CSCs [18]. It is for this reason that we are interested in novel therapeutic approaches that may provide new avenues of approach in targeting CSCs in solid tumors.

Evidence for the CSC hypothesis, arose from a pioneering study by Dick and colleagues, where a transplanted population of human leukemic cells expressing $CD34^{+}CD38^{-}$ surface markers (found normally on hematopoietic stem cells) was shown to generate acute myeloid leukemia (AML) in the host immune-deficient mice [19]. Similar experiments followed for breast cancer, identifying a CD44+CD24− rare subpopulation capable of forming new tumors in NOD/SCID mice [20]. Subsequent experiments conducted across a range of solid cancers [21–24] including GBM [25,26] commonly identified the presence of stem-like cells in each cancer type, characterized by its capacity for self-renewal and aberrant differentiation, for which as few as 100 cells were required in a xenograft to initiate tumorigenesis [27].

In GBM, Hemmati *et al.* isolated a subpopulation of cells able to form neurospheres in culture and found to express markers characteristic of neural stem cells – CD133, BMI1, Sox2 and musahi-1 [28]. Singh *et al.* first proposed CD133 (prominin-1) as a marker and isolated $CD133^{+}$ cells from human brain tumor cultures and demonstrated that injecting as few as 100 cells into NOD-SCID mice was sufficient for tumorigenesis, while injecting up to 50,000–100,000 CD133 – cells failed to form any tumor [29]. Other studies have highlighted other characteristics of GBM CSCs (also commonly referred to as glioma stem cells, GSCs or glioma CSCs) such as CD15 expression [30] and VEGF secretion [31]. Importantly, GBM CSCs have been shown to have a greater drug resistance to conventional chemotherapy including doxorubicin, bischloroethylnitrosourea and temozolomide and this has been partly attributed to the downregulation of autophagy proteins and the expression of MDR1 [32]. Most notably, it was shown that the relative proportion of $CD133^{+}$ cells increased GBM populations post-radiotherapy, providing evidence of treatment resistance, self-renewal and their role in tumorigenesis and recurrence, therefore lending support to the CSC model [33].

There are 19 active clinical trials across 9 different species of OVs against GBM – including several promising results in early clinical trials for Toca511+Toca FC, DNX2401 and PVS-RIPO that have been fast-tracked for approval [34–38] However, across the overall field of virotherapy in the last two decades, few have successfully translated efficacy from bench to bedside. A pooled analysis of recent OV trials for recurrent GBM demonstrated the 24-month and 36-month survival rates to be a modest 15 and 9%, respectively (compared with 12 and 6%, respectively, for all other nonvirotherapy trials) [39] OVs are yet to demonstrate efficacy and Phase II/III trials, hence the need for the field to continuously reshape future strategies.

We are therefore interested in whether OVs could target CSCs in GBM more effectively than conventional radiochemotherapy, based on recent evidence suggesting that the clearance of this rare subpopulation may prevent recurrence and tumorigenesis. This review will discuss recent key advances in oncolytic adenovirus, herpes simplex virus (HSV) and Zika virus (ZIKV) in particular, in order to first, illustrate the history of OV development that led to the current rationale for designing OVs to target CSCs in GBM specifically. The OVs discussed in this review are chosen because they highlight novel properties, or mechanisms of action that are likely to become of increasing interest. We then discuss the recently emerging evidence of CSC plasticity that may pose a challenge to this CSC hypothesis that many new OVs in development rely on being true for therapeutic success.

With a growing understanding of CSC plasticity, we propose our view that the focus of future research will turn to novel therapeutic agents that can inhibit signals in the tumor microenvironment that maintain the self renewal of the CSC subpopultation in GBM, which current conventional treatments fails to target. Finally, we provide a perspective on what a future curative virotherapy may look like. We propose that it will likely require a combination of targeting CSCs, non-CSC tumor cells and inhibiting CSC plasticity in the tumor microenvironment that may be underpinning tumor recurrence.

Mechanisms of action of OVs

If CSCs reside at the top of the cancer cell hierarchy and are the only cells that independently enable tumorigenesis, an OV that is tropic toward CSCs with tumor-selective conditional replication, possesses a highly appealing mechanism of therapy against aggressive treatment resistant solid tumors such as GBM. Though the exact mechanism of

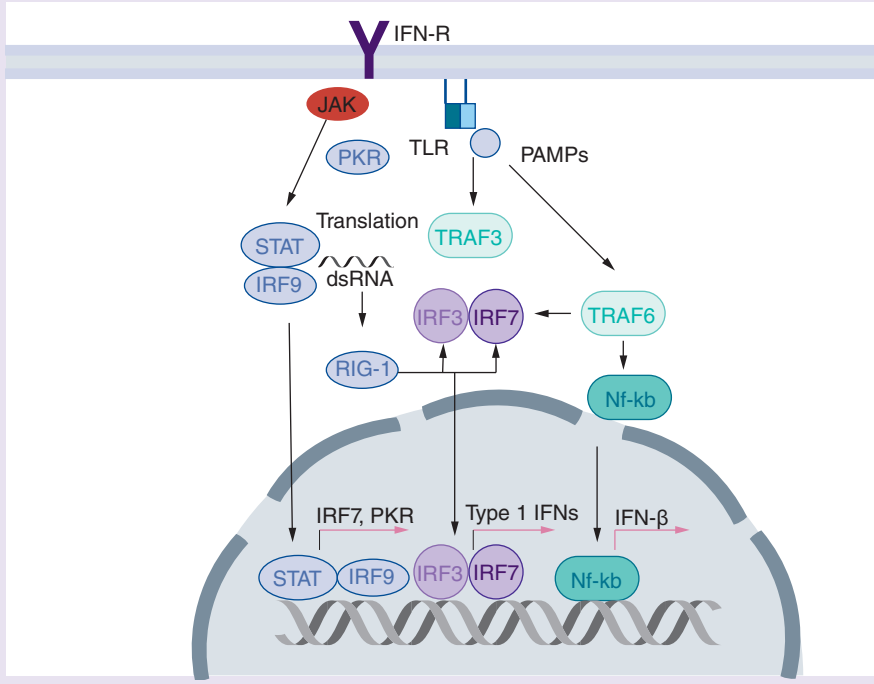

Figure 1. Disrupted intracellular viral defense mechanisms enable viral replication. In infected cells, detection of viral elements by TLR and RIG-1 trigger signaling cascades through IRFs and IFN resulting in an inhibition of protein synthesis, and apotosis to limit spread of the virus. Cancer cells downregulate RIG-1, IRF3 and IRF7, creating greater susceptibility for viral replication which can provide an advantage for oncolytic viruses.

oncolysis varies across OVs, three major killing mechanisms are commonly shared. Firstly, almost all OVs elicit direct cytolysis by extensive replication. Typically, an attenuated virus infecting tumor cells, will hijack the intracellular machinery to proliferate; inducing cytolysis in the process to release viral progeny for subsequent tumor cell infection.

Secondly, OVs can be engineered to express viral proteins that either trigger pro-apoptotic pathways or are directly cytotoxic, such as the E3 adenovirus death protein [40]. This however often induces cytolysis prior to fully exploiting cellular resources to amplify viral progeny [41].

Lastly, as transformed cells often downregulate MHC for immune evasion, several genetically engineered OVs in trials are 'armed' with transgenes coding immunostimulatory molecules such as IL-2, IL-12 or GM-CSF capable of stimulating an anti-tumor immune response [42–44].

All of the mechanisms described are made possible by transformed cells, having broken innate antiviral and apoptotic pathways, which allows OVs to effectively proliferate within its host cell (Figure 1) [45,46]. The advantage of OVs compared with chemotherapy, is that the subsequent spread of the agent is spatially restricted to the target region due to tumor-selective replication and therefore reduce the likelihood of off-target effects [47]. Furthermore, a single low dose injection into a tumor site can sufficiently achieve therapeutic effects through viral amplification, overcoming the blood-brain barrier which poses a major limitation for pharmacological treatment options. As a caveat, all OVs are susceptible to neutralization by humoral and cell-mediated immune responses [48]. Therefore, immune cell recruitment is a double-edged sword as it presents a risk of complete viral clearance prior to infecting all the cancer cell targets within the tissue. Striking a balance between the anti-viral response and anti-tumor response is critical to treatment success.

Advances in engineered adenoviruses against GBM CSCs

A variety of viral species have been investigated for oncolytic potential in the context of GBM treatment. Notably, ONYX-015 is an adenovirus serotype 5 with *E1B* gene deletion, engineered to selectively replicate in and kill p53 mutant cells [49,50]. E1B proteins bind to p53 to inactivate the apoptotic responses and trigger S-phase entry [49].

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Deletion of these genes in a mutant adenovirus renders replication incompetency in normal cells, but capable in cells with mutant p53 genes, therefore conferring tumor selective conditional replication [49,51]. In contradiction to its initial proposed mechanism of action, later studies reported the loss of p53 alone to be insufficient for enabling ONYX-015 replication [52]. Tumor selectivity was conferred by YBX-1, expressed in tumor cells, substituting for the mRNA export function of the deleted *E1B* gene [53]. ONYX-015 has not progressed beyond Phase I clinical trials for GBM, having demonstrated only moderate efficacy at both preclinical and clinical stages [54,55]. ONYX-015 was one of the earliest forerunners for the field, and though it was granted license for the treatment of head and neck cancer, its failure in its application to GBM highlighted the extent of the GBM-specific challenges to overcome.

The CSC model would suggest that the therapeutic efficacy of an OV is a direct reflection of its ability to target the CSC population that is solely responsible for tumorigenesis and recurrence. This rationale has steered research toward developing OVs with greater tropism or selectivity for CSCs [56]. Jiang *et al.* were first to examine the efficacy of any OV against GBM CSCs specifically [57]. Immunoblotting experiments in isolated human GBM CSCs revealed high expression of adenoviral receptors and abnormal Rb pathways, thereby making adenovirus an appealing candidate for treating GBM. The authors demonstrated the efficacy of Delta-24-RGD (DNX-2401), a conditionally replication competent adenovirus with *E1A* deletion, against GBM CSCs xenografted in mice, to find effective autophagic oncolysis of infected cells, indicated by Atg5 and LC3-II protein accumulation [57]. In recent Phase I trials, Delta-24-RGD, demonstrated significant responses in patients with recurrent malignant glioma, with 20% of patients surviving >3 years post treatment, making it one of the most promising OVs in currently active clinical trials [37]. Two Phase I trials assessing the combination of Delta-24-RGD with temozolomide and with IFN-γ are also concurrently active.

Immune evasion is another key hallmark of cancer [4]. GBM cancer cells have been shown to evade immune detection by downregulating MHC and secreting immunosuppressive cytokines including IL-6, IL-10 and TGF-β into the tumor microenvironment [58]. In a breakthrough study in 2017, with the aim of inducing a potent antitumor immune response, Freedman *et al.* successfully demonstrated that a modified adenovirus (EnAdenotucirev) could secrete a bispecific single chain antibody from infected tumor cells into the tumor microenvironment, pioneering a new approach that uses OVs as an effective vector for targeted delivery of immunostimulatory agents [59]. This 'bispecific T-cell engager' (biTE) is engineered to bind EpCAM, a marker expressed on the target tumor cell, to cause EpCAM cross-linking with CD3 on T cells to activate $CD4^+$ and $CD8^+$ cytotoxic T cells [59]. EnAd is able to stimulate a T cell mediated immune response in addition to direct oncolysis of the tumor cell. EpCAM is expressed in 1×10^6 copies per cell while MHC is expressed <100,000 per cell, thus enabling higher probability of T-cell engagement [59,60]. BiTE expression is also spatially limited to the tumor microenvironment and is therefore concentrated at the target site to maximize kinetics, while minimizing potential off-target toxicity [61]. The use of BiTEs was previously limited by difficulty in delivery to deep tumor regions of interest, however oncolytic adenoviruses provide a novel mechanism of overcoming a major limitation in delivering therapeutic agents to brain tumor tissue. Gedeon *et al.* developed a bispecific antibody targeting EGFR variant III (EGFRvIII), a receptor variant found in some GBM tumors, that is expressed exclusively in cancer and presents minimal risk of cross reactivity [62]. Encoding anti-EGFR biTE into an OV vector, has produced an OV in the pipeline that is the first of its kind against GBM, with a promising new mechanism of eliciting a specific anti-tumor immune response that is localized and concentrated at the tumor site [63].

Engaging a T-cell response within the tumor microenvironment may be crucial in eliminating both CSCs and non-CSCs in GBM. CSCs are responsible for creating an immunosuppressive environment by shedding TGF-β that inhibits T-cell proliferation and promotes macrophage polarization into M2 [64]. Adjuvant immunotherapy may therefore be key to preventing recurrence. There is some evidence to suggest that the therapeutic benefit of chemo-radiotherapy may largely depend on the immune responses generated from liberated tumor antigens post-therapy [65,66]. An initial tumor reduction to minimal residual disease may reflect an immunologically sustained equilibrium maintained by the presence of a high number of $CD4^+$ and $CD8^+$ T cells [65–67]. The specificity, low molecular weight, and preclinical efficacy, provide promise of a new class of therapeutics that can elicit a T cell mediated immune response in an otherwise, highly immunosuppressive microenvironment [68].

Efficacy of HSV against CSCs

HSVs have been proposed as having greater oncolytic efficacy against GBM compared with adenovirus, along with a larger capacity for inserting transgenes to its genome [69,70]. This has enabled a range of genetically modified oncolytic HSVs (oHSVs) to enter trials. The most advanced oHSV is G47-Delta *(ICP6-, ICP34.5-* and *alpha47-*),

a third generation OV engineered by Todo *et al.* by deleting the *alpha47* gene from the second generation oHSV, G207 [71]. In preclinical studies conducted in human GBM CSC models, Wakimoto *et al.* found that G47-Delta was able to kill GBM CSCs and effectively eliminate the ability of any viable cells to form secondary neurospheres, hence limiting the self-renewal property of CSCs [72]. G47-Delta has demonstrated promising results in 2019 Phase II trials in Japan with a 92% 1-year survival rate in patients [73]. M032, currently in Phase I trials, is a second generation oHSV with a *ICP34.5* deletion, expressing IL-12 as a means of eliciting an immune response and an anti-angiogenic effect at the tumor site [74]. Similarly, Zhu *et al.* demonstrated an advanced recombinant HSV with *ICP34.5* and *ICP6* deletion, and a *VAE* insertion, was able to effectively kill the majority of glioma CSCs *in vitro* and destroy the vascular niche by disrupting the function of microvascular endothelial cells [75]. Deletions of the neurovirulence genes, *ICP34.5* and *ICP6*, prevent viral replication in normal cells and confers tumor selectivity, while the expression of anti-angiogenic factors aims to augment efficacy by targeting the vascular niche in solid tumor [76]. The authors also note that a few CSCs escaped therapy, but were found to have lost their self-renewal ability. These findings support earlier studies by the Rabkin group that showed that G47-Delta killed GBM CSCs and eliminated the neurosphere-forming ability of viable cells [72]. Though the paper concluded this to be significant evidence of inhibition of CSC activity, the limitations of their experiment being *in vitro*, render it unconvincing. Surviving CSCs were resuspended in serum-free medium and observed for 14 days to examine if further neurospheres could be generated. Critically, the experiments fail to account for the major role of signaling in the tumor microenvironment from niche cells that influence the differentiation of CSCs. The secondary neurosphere-formation assays conducted in small suspensions of only 1–10 dissociated cells, in the absence of niche signaling, poorly reflects the true *in vivo* microenvironment of a solid tumor.

ZIKV naturally possess a selectivity for GBM CSCs

ZIKV is a naturally occurring ssRNA virus that preferentially replicates in neural progenitor cells (NPCs), which characteristically impairs neural development in the infected fetus, manifesting clinically as microcephaly [77,78]. NPCs are similar to CSCs in their capacity for self-renewal, tumorigenesis and differentiation, which raised interest in using ZIKV as a therapeutic to target GBM CSCs [79,80]. MS1 is a neural RNA binding protein highly expressed in NPCs and is essential for neurodevelopment [81]. Musashi RNA binding proteins have also been implicated as drivers for glioblastoma and has been highlighted as a potential therapeutic target [82]. In one study, depletion of MS1 produced a decreased expression of a DNA-PK subunit resulting in less nonhomologous end joining based repair, and therefore has a direct impact on the susceptibility of GBM to radiotherapy [82]. MS1 has been shown to be an excellent marker for neural stem cells in healthy brain tissue, and also correlated well with the stage of malignancy and proliferative activity of tumor cells in human glioma [83]. It has been shown that effective replication of ZIKV is conditional on the presence of MS1, which directly interacts with the ZIKV genome [81]. A modified live attenuated ZIKV (ZIKV-LAV) with a 10 nucleotide deletion has been tested in a mouse model of human glioblastoma, demonstrating significant reduction of tumor growth and prolonged animal survival, with evidence of selective elimination of GBM CSCs [79]. ZIKV was shown to have specific tropism for $SOX2^+$ glial stem cells (infecting 60–70%) but not differentiated glioma cells (infecting only up to 20%) marked by GFAP. By analyzing the transcription profiles of ZIKV-infected CSCs, the group found that ZIKV infection activated TNF pathways and the upregulation of CXCL10, a cytokine shown to inhibit tumor angiogenesis [84] and recruit $CXCR3^+$ T cells [85], suggesting that ZIKV-LAV may stimulate a GBM CSC-targeted immune response. However this study had conducted its experiments in immune-deficient mice lacking T cells, therefore, the full extent to which the oncolytic activity is potentiated by T cell mediated immune mechanisms is yet to be shown [79].

According to the current widely accepted understanding of the CSC model, an OV capable of killing the majority of CSCs and eliminating self-renewal and differentiation ability in the CSCs that escape, would be a means of curing GBM, if it were combined with a therapy that eliminate the non-CSC population. Chen *et al.* showed that ZIKV-LAV, with unparalleled tropism for glial stem cells/NPCs, would be a strong candidate OV to achieving effective clearance of the GBM CSC subpopulation. However, ZIKV is unlikely to be stable with significant genetic modification that is possible in larger viruses such as HSV. A double hit therapy comprised of treatment with recombinant 'armed' HSV followed by ZIKV-LAV may elicit synergistic effects through each viral agent targeting a CSC subpopulation that is not targeted by the other.

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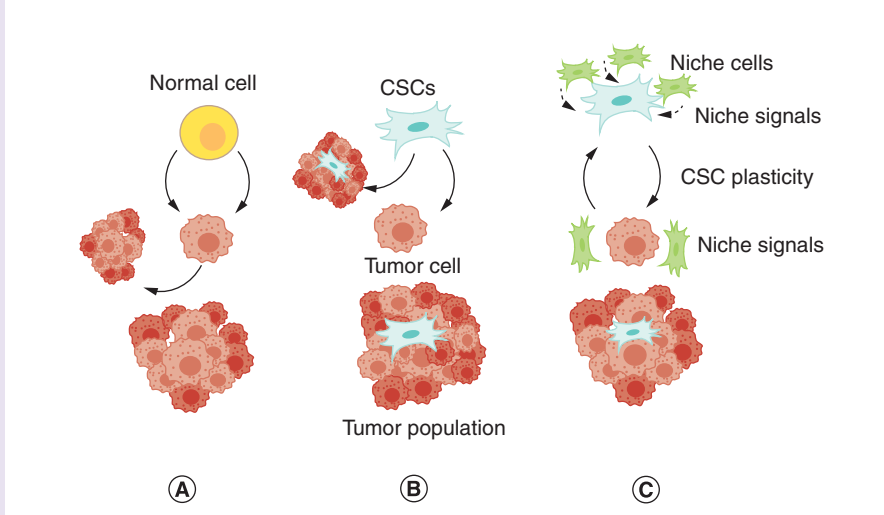

Figure 2. Concepts of cancer cell hierarchy. Orange – normal cell, Red – tumor cell, Blue – Cancer Stem Cell (CSC), Green – Niche cells in the tumor microenvironment. Niche signals include PI3K, Notch and Wnt pathways which influence the control of tumor cells entering quiescence, differentiation and epithelial-mesenchymal transition. **(A)** Stochastic model – all cells are capable of acquiring mutations to undergo transformation and possess equal tumorigenic potential. **(B)** CSC hypothesis model – a rare subpopulation of stem-like cells are tumorigenic and differentiate to give rise to a heterogenous population of tumor cells. **(C)** Updated CSC model to include CSC plasticity. Depletion of the CSC population results in the tumor microenvironment signaling to promote the dedifferentiation of cells from non-CSC to CSC states.

CSC plasticity & the role of the microenvironment

Recently emerging studies suggest that even an ideal OV that fully eliminates the CSC population in a tumor, may not adequately prevent tumor recurrence in the long-term. This leads us onto the concept of CSC plasticity – a challenge to the current CSC hypothesis, suggesting that the dedifferentiation of non-CSCs into CSCs after CSC depletion, provide a potential mechanism of enabling tumor recurrence post-treatment [86,87].

There is growing evidence in support of a unifying model suggesting that some cancer cells are capable of transitioning between CSC and non-CSC states, in a phenomenon termed 'CSC plasticity' (Figure 2) [86,88]. Stimuli within the tumor microenvironment may induce dedifferentiation of tumor cells into acquiring stem-like characteristics and vice versa, in solid tumors [89]. Targeting Hedgehog-, Notch- and PI3K-activating signals in the perivascular niche may prevent GBM CSC self-renewal and migration [64]. The reciprocal signaling between differentiated tumor cells and CSCs within the niche, is yet to be fully understood. Differentiated GBM cells in niche express BDNF that binds to NTRK2-receptor on GBM CSCs which promote VGF expression [90]. This enables autocrine signaling to the CSC to maintain self-renewal and a paracrine signal to differentiated GBM cells. Disruption of niche signaling pathways may prevent the self-renewal of surviving CSCs post-therapy, along with preventing the dedifferentiation of surviving differentiated tumor cells that recognize the depletion of the CSC subpopulation. Targeting cell signaling pathways in the tumor microenvironment may be a likely new avenue of approach for adjuvant therapy. This may also explain the unknown mechanism through which several of the oHSVs previously described in this article, may be eliminating the self-renewal capability in surviving CSCs [72,75].

In a study, first conducted in a mouse model of xenografted colorectal cancer, the cancer organoids were modified to express diptheria toxin receptors under Lgr5. Lgr5+ CSC cells in the xenograft were selectively ablated by diptheria toxin treatment that resulted in the cessation of tumor growth [88]. After the treatment was removed, tumor growth re-emerged, coinciding also with the regeneration of Lgr5+ CSCs – leading to the speculation that non-CSCs were plastic; capable of dedifferentiation into CSCs to replace the lost subpopulation. Several studies have demonstrated that the tumor microenvironment of the primary tumor regulates this phenomenon where cancer cells are able to readily convert between nontumorigenic and tumorigenic states through a number

of extracellular signals and transcription factors [91,92]. Activation of the Ras-MAPK pathway in human mammary epithelial cells has been shown to induce epithelial–mesenchymal transition (EMT) and enable to acquisition of stem-like characteristics in a mammary tumor progression model [93]. This bidirectional plasticity of cancer cells is supported by evidence from Chaffer *et al.* demonstrating that plastic non-CSCs maintain the ZEB1 promoter in a bivalent chromatin configuration [94]. The expression of ZEB, SNAI and TWIST family of genes induce EMT, and by maintaining bivalency, these cells are capable of responding to signals in the tumor microenvironment such as TGF-β which has been shown to induce mesenchymal phenotype in GBM via activation of the ZEB1 pathway [95].

However, despite being a solid tumor, evidence describing the EMT-like process in GBM has been limited. In GBM, proneural–mesenchymal transition may underpin the molecular events that lead to enhanced invasive capability that is described as EMT in other solid tumors. Cancer cells acquire stem-like characteristics through EMT such as metastatic potential and resistance to conventional therapies [96]. *In vitro* studies of mesenchymal GBM cells induced by TGF-β demonstrate enhanced invasive potential and migration [95]. The TGF-β pathway is involved in maintaining the stemness of GBM CSCs [97]. Several studies have produced promising results in experiments applying TGF-β inhibitors to target CSCs in glioblastoma models [98,99]. Studies applying exogenous TGF-β to glioma cells found increased glioma cell motility through increased integrin expression along with upregulated MMP-2 and MMP-9 activity [100,101]. This mechanism enables GBM tumor cells to dedifferentiate to restore the CSC pool post-treatment, which may underpin an explanation for the observation by Bao *et al.* of that $CD133^+$ cell proportions increased in a GBM culture post radiotherapy [33].

Both *in vitro* and *in vivo* studies have documented the clonal heterogeneity in GBM CSCs and the ability of differentiated GBM CSCs to revert to GSCs in response to insults to the tumor microenvironment, such as exposure to temozolomide or radiation [102]. Interestingly, Maracto *et al.* found that treatment of a tumor xenograft population with a reovirus does not alter the CSC:non-CSC proportions within the tumor population unlike radiotherapy. This lends support to the idea that OVs may be employing an entirely different mechanism of killing CSCs without causing the molecular or physiological disruptions to the signaling pathways in the tumor microenvironment that would normally induce signaling for extensive re-enrichment of the CSC pool [103]. Future research could extend this study to address whether this is a phenomenon exclusive to reoviruses, and also whether this phenomenon is observed only in xenografted tumors.

With this in mind, a future virotherapy strategy should consider employing mechanisms to inhibit EMT; encoding genes that express inhibitors that target signaling pathways in the niche to prevent the re-generation of CSCs from the non-CSC pool [104]. Our perspective is that preventing the regeneration of CSCs from dedifferentiating tumor cells, may be equally as important as eliminating present CSCs when testing the efficacy of OVs in an *in vivo* trial.

CSC plasticity therefore highlights the inherent limitations of our current approach to clinical trials. The efficacy of any CSC-tropic OV, as a monotherapy in a given clinical trial, may not be a true reflection of its therapeutic potential if treatment is unable to prevent the dedifferentiation of non-CSCs (Figure 3). In theory, it is possible that a highly potent OV against CSCs, as part of an adjuvanted therapy or combination approach, may yield long-term recurrence-free outcomes through complete clearance of both CSC and non-CSC subpopulations and by inhibiting the dedifferentiation pathways responsible for regeneration. However, when administered as a stand-alone treatment, the dedifferentiation of untargeted non-CSCs may re-enrich the CSC pool post-treatment. Therefore, more trials in the future should be encouraged to assess the efficacy of CSC-tropic OVs co-administered with non-CSC targeting treatment. Supporting this argument, in one study, co-administration of oHSV (G47-Delta) with temozolomide has been shown to elicit higher rates of remission free survival in preclinical models of glial stem cell derived tumors, compared with oHSV alone, supporting the notion that GBM CSCs and non-CSCs ought to both be targeted simultaneously in order to provide the best chances of remission free survival [105]. The authors attributed this synergistic effect to the relocalization of tumor cell DNA repair proteins to the oHSV thus preventing repair of temozolomide-induced DNA damage. However, future studies could be designed using similar immunocytochemistry methods to elucidate whether the adjuvanted therapy (oHSV + temozolomide) elicits a difference in the rate of regeneration of the CSC subpopulation compared with the oHSV monotherapy arm. In the most promising clinical trials of OVs targeting GBM – several authors note a number of complete responders to virotherapy (some with progression free periods of 3–4 years), however, this is followed by the appearance of new enhancing lesions several months after complete response [37]. This phenomenon observed in clinical trials lends support to our hypothesis that GBM CSC regeneration underpins a delayed tumor recurrence that occurs after the elimination of the CSC subpopulation with a highly effective virotherapy.

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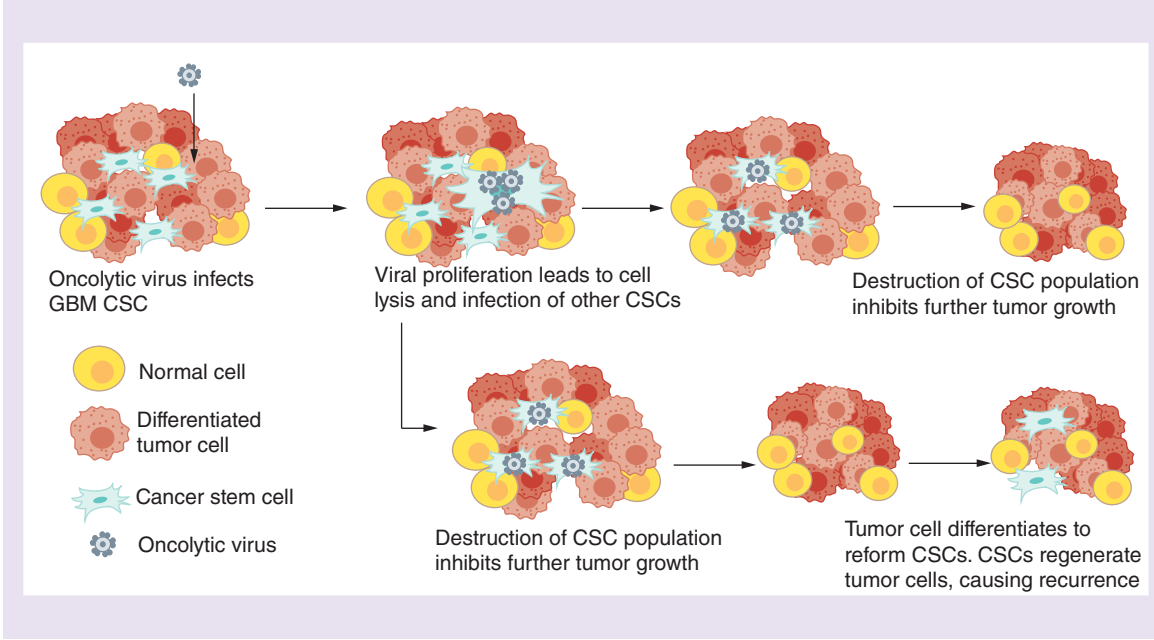

Figure 3. Cancer stem cell plasticity post-treatment with oncolytic virotherapy. The ideal virotherapy targeting glioblastoma cancer step cell (GBM CSCs) would eliminate this rare subpopulation of tumorigenic cells and prevent further tumor growth. New evidence suggests that GBM CSCs may be regenerated from the dedifferentiation of tumor cells after virotherapy and cause recurrence in the long-term. This is enabled by Wnt and Notch signaling pathways in the tumor microenvironment promoting the dedifferentiation of tumor cells into GBM CSCs.

Molecular mechanisms of CSC epigenetics

Resistance to chemotherapy has been attributed to a range of both intrinsic and acquired mechanisms including the ability of CSCs to remain quiescent [15,16]. CSCs are capable of upregulating developmental programs mediated by Notch signaling that enable them to enter a slow-cycling state that has been proposed as one possible mechanism underpinning their evasion of antiproliferative treatment such as temozolomide [106]. Quiescent cells in the intestinal and stomach epithelia have been shown to bidirectionally transition into stem cell states to replace fast cycling stem cells upon injury [107,108]. A similar mechanism may underpin the enrichment of CSCs in solid tumors. By targeting the epigenetic remodeling pathways that enable CSCs to enter slow-proliferative states, rendering them to greater susceptibility for temozolomide mediated killing. Studies by Takebe *et al.* have demonstrated the potential of targeting Notch pathways as a cotarget of conventional treatment, in order to target CSCs, while preventing potential escape mechanisms [109,110]. It has previously been shown that Wnt signaling pathways are preferentially activated in GBM cell cultures post-treatment with ionizing radiation [111]. This is co-observed with enhanced clonogenicity and CSC enrichment. Pharmacological inhibition of Wnt signaling significantly reduced the survival of the GBM cells. Applying this evidence, future OVs may be engineered to express WNT inhibitory factor-1 to promote cellular senescence and inhibit the signaling pathways that drive stemness in GBM [112].

More recent trials have examined the response in OV therapy adjuvanted with agents such as checkpoint inhibitors and have yielded promising results attributed to the recruitment of T cell mediated responses [113]. The Rabkin group modified G47-Delta oHSV to express IL-12, and combined this with treatment with anti-PD1 and anti-CTLA4 in a triple combination therapy, which elicited curative results in two GBM models [113]. The group attributed this to the synergistic effects from macrophage, $CD4^+$ and $CD8^+$ T-cell recruitment. IN GBM, CSCs have been shown to activate STAT3 resulting in the suppression of T-cell activation and proliferation [114]. Therefore, OVs employing mechanisms that stimulate immunological recruitment may be able to overcome the immunosuppressive signals in the tumor microenvironment and enable effective clearance of CSCs. Yet to be assessed, is how much of this therapeutic efficacy demonstrated in the study by *Saha et al.* is owed to the inhibition of reciprocal signaling in the tumor microenvironment between CSCs and the niche cells as previously discussed. The successful recruitment of T cells and the expression of IL-12 by the oHSV are likely to be inhibiting the expression of TNF and IL-6 in the microenvironment that would be driving dedifferentiation and CSC plasticity.

Though there is compelling evidence in support of CSC plasticity being responsible for OV treatment failure, it is still a hypothesis. To date, we have yet to directly observe any evidence of EMT and dedifferentiation in GBM following treatment with OVs. Furthermore, there is not a clear distinction between GBM CSCs and non-CSCs, despite several markers including CD133 having been identified [33]. Therefore, further studies including single cell sequencing studies conducted in GBM cells post therapy, are required at this stage in order to find conclusive evidence of CSC plasticity underpinning tumor recurrence in GBM. CSC plasticity is unlikely to be the sole reason for OV treatment failure. However, a growing body of evidence suggests that this is an avenue that warrants further investigation.

Future perspective

OVs possess the potential to efficiently target and clear CSCs, with clever new approaches for treating GBM. Emerging evidence of CSC plasticity highlight mechanisms by which even an effective CSC-targeting OV will yield suboptimal efficacy in clinical trials due to the regeneration of the CSC pool from dedifferentiating non-CSCs in the *in vivo* environment. Future studies ought to identify the signaling mechanisms in the tumor microenvironment that underpin the epigenetic changes that ultimately result in the re-enrichment of the CSC pool.

Currently, 'armed' OVs in the pipeline such as Adv/HSV are engineered for characteristics such as enhanced tropism, conditional replication and immunostimulation, and more recently, the production of BiTEs. Encoding transgenes that directly inhibit both signaling in the tumor microenvironment and intracellular epigenetic pathways that induce EMT, may be more likely to yield long-term recurrence-free outcomes. We also propose that co-administering a CSC-tropic OV (e.g., ZIKA-LAV) with an effective non-CSC targeting treatment such as temozolomide, checkpoint inhibitors, radiotherapy or another OV, may yield unforeseen synergistic effects by preventing cells escaping treatment by moving in or out of the CSC pool. We discuss evidence suggesting that this may be a major enabler of tumor recurrence that past clinical trials testing single OVs administered alone, have failed to account for.

The challenges posed by CSC plasticity is most likely to be overcome in future trials through an adjuvant therapy approach rather than through the discovery of a highly efficacious single viral agent. It is our view that we already have an arsenal of potent OVs that have proven their efficacy in preclinical GBM models. The failure of a range of these OVs to translate efficacy from bench to bedside, calls for reconsideration of our strategy. We must aim to understand what underpins tumor cell regeneration – CSC plasticity may provide an explanation for the failures observed in clinical trials. Based on the evidence discussed, we are yet to elucidate the full synergistic potential of co-targeting CSCs and non-CSCs; the effects of which may be particularly prominent in aggressive, treatment-resistant solid tumors such as GBM.

Executive summary

Mechanisms of action of oncolytic viruses

- The focus in the field of research has been toward targeting cancer stem cells (CSCs). A number of engineered oncolytic viruses (OVs) including oncolytic herpes simplex viruses and ZIKV have been shown to be highly effective at eliminating glioblastoma (GBM) CSCs.

Advances in engineered adenoviruses against GBM CSCs

- This field of research is beginning to recognize the potential difference in efficacy between OV monotherapy compared with OV co-administered with chemotherapy, checkpoint inhibitors or even with other OVs. Future studies are likely to shift its focus toward the latter approach in light of CSC plasticity.

CSC plasticity & the role of the microenvironment

- Our understanding of the relationship between the tumor microenvironment and tumor cell plasticity is limited. Mapping out these signaling pathways will enable us to identify novel targets that may direct what genes are encoded into 'armed' OVs.
- Disruption of reciprocal signaling in the niche may prevent the dedifferentiation of tumor cells.

Future Perspective

- Engaging macrophage and T-cell responses at the tumor site has been a challenge due to the highly immunosuppressive nature of the GBM niche. OVs encoding bispecific T-cell engagers have demonstrated efficacy in immune stimulation in GBM models and may overcome these hurdles.

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