ABSTRACT

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 tumours 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 oncolytic viruses may be failing to reflect their true potential in clinical trials. We argue that preventing non-CSC to CSC de-differentiation may be critical to achieving long-term treatment efficacy in future OV clinical trials.

INTRODUCTION

Glioblastoma is an incurable type of brain tumour 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 tumour, render therapeutic options for GBM limited [2]. Temozolomide (an alkylating agent) is given as first-line treatment with radiotherapy, resulting in a 14.6 month median survival compared to 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 tumour 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 anti-growth 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 tumour heterogeneity - characteristically seen in GBM [7,8]. Based on this view, any individual cancer cell can proliferate or cause tumour recurrence [9,10].

The CSC hypothesis argues an alternative; that a rare population of cancer-initiating cells with unlimited self-renewal, are responsible for tumour 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 tumour. Whilst CSCs can produce secondary tumours, 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 tumour penetration, the heterogenous nature of cells within the tumour, 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 quiescent, rendering less susceptibility to anti-proliferative treatment such as temozolomide [15]. This CSC hypothesis provides one explanation for the treatment resistant nature of certain tumours observed clinically. Current first-line treatment which indiscriminately kill the bulk of the tumour 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 tumours.

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 haematopoietic stem cells) was shown to generate acute myeloid leukaemia (AML) in the host immune-deficient mice [19]. Similar experiments followed for breast cancer, identifying a CD44+CD24- rare subpopulation capable of forming new tumours 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, characterised 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 tumour cultures and demonstrated that injecting as few as 100 cells into NOD-SCID mice was sufficient for tumorigenesis, whilst injecting up to 50,000 – 100,000 CD133- cells failed to form any tumour [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 vascular endothelial growth factor (VEGF) secretion.[31]. Importantly, GBM CSCs have been shown to have a greater drug resistance to conventional chemotherapy including doxorubicin, bischloroethylnitrosourea (BCNU) and temozolomide and this has been partly attributed to the down-regulation of autophagy proteins and the expression of multidrug resistance 1 (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% resepctively (compared to 12% and 6% respectively for all other non-virotherapy 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 oncolytic viruses 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 tumour 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 tumour cells, and inhibiting CSC plasticity in the tumour microenvironment that may be underpinning tumour

recurrence.**MAIN**

Mechanisms of action of oncolytic viruses

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 towards CSCs with tumour-selective conditional replication, possesses a highly appealing mechanism of therapy against aggressive treatment resistant solid tumours such as GBM. Though the exact mechanism of 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 tumour cells, will hijack the intracellular machinery to proliferate; inducing cytolysis in the process to release viral progeny for subsequent tumour cell infection.

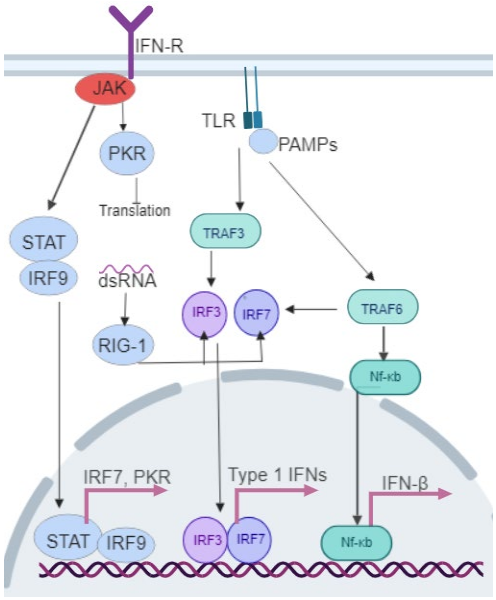

Figure 1. ***Disrupted intracellular viral defence mechanisms enable viral replication.*** *In infected cells, detection of viral elements by TLR and RIG-1 trigger signalling cascades through IRFs and IFN resulting in an inhibition of protein synthesis, and apotosis to limit spread of the virus. Cancer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

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-tumour 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 to chemotherapy, is that the subsequent spread of the agent is spatially restricted to the target region due to tumour-selective replication and therefore reduce the likelihood of off-target effects [47]. Furthermore, a single low dose injection into a tumour 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 neutralisation 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-tumour 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]. Deletion of these genes in a mutant adenovirus renders replication incompetency in normal cells, but capable in cells with mutant p53 genes, therefore conferring tumour 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]. Tumour selectivity was conferred by Y Box binding factor-1, expressed in tumour 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 towards 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-gamma 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-beta into the tumour microenvironment [58]. In a breakthrough study in 2017, with the aim of inducing a potent anti-tumour immune response, Freedman *et al.* successfully demonstrated that a modified adenovirus (EnAdenotucirev) could secrete a bispecific single chain antibody from infected tumour cells into the tumour 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 tumour 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 /tumour cell. EpCAM is expressed in 1x10^6 copies per cell whilst 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 tumour microenvironment and is therefore concentrated at the target site to maximise kinetics, whilst minimising potential off-target toxicity [61]. The use of BiTEs was previously limited by difficulty in delivery to deep tumour regions of interest, however oncolytic adenoviruses provide a novel mechanism of overcoming a major limitation in delivering therapeutic agents to brain tumour tissue. Gedeon *et al.* developed a bispecific antibody targeting EGFR variant III (EGFRvIII), a receptor variant found in some GBM tumours, 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-tumour immune response that is localised and concentrated at the tumour site [63].

Engaging a T cell response within the tumour microenvironment may be crucial in eliminating both CSCs and non-CSCs in GBM. CSCs are responsible for creating an immunosuppressive environment by shedding TGF-beta that inhibits T cell proliferation and promotes macrophage polarisation 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 tumour antigens post-therapy [65,66]. An initial tumour 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 pre-clinical 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 Herpes Simplex Virus (HSV) against CSCs

HSVs have been proposed as having greater oncolytic efficacy against GBM compared to adenovirus, along with a larger capacity for inserting transgenes to its genome [69,70]. This has enabled a range of genetically modified oHSVs to enter trials. The most advanced oHSV is G47-Delta (ICP6-, ICP34.5- and alpha47-), a 3rd generation OV engineered by Todo et al. by deleting the alpha47 gene from the 2nd generation oHSV, G207 [71]. In pre-clinical 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 2nd 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 tumour site [74]. Similarly, Zhu *et al.* demonstrated an advanced recombinant HSV with ICP34.5 and ICP6 deletion, and an endostatin-angiostatin fusion gene (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 tumour selectivity, whilst the expression of anti-angiogenic factors aims to augment efficacy by targeting the vascular niche in solid tumour [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 signalling in the tumour 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 signalling, poorly reflects the true *in vivo* microenvironment of a solid tumour.

Zika virus naturally possess a selectivity for GBM CSCs

Zika virus (ZIKV) is a naturally occurring ssRNA virus that preferentially replicates in neural progenitor cells (NPCs), which characteristically impairs neural development in the infected foetus, 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]. Musashi-1 (MS1) is a neural RNA binding protein highly expressed in neural progenitor cells 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 non-homologous end joining (NHEJ) 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 tumour 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 tumour 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 glial fibrillary acidic protein (GFAP).

By analysing 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 tumour 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.

CSC plasticity and the role of the microenvironment

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

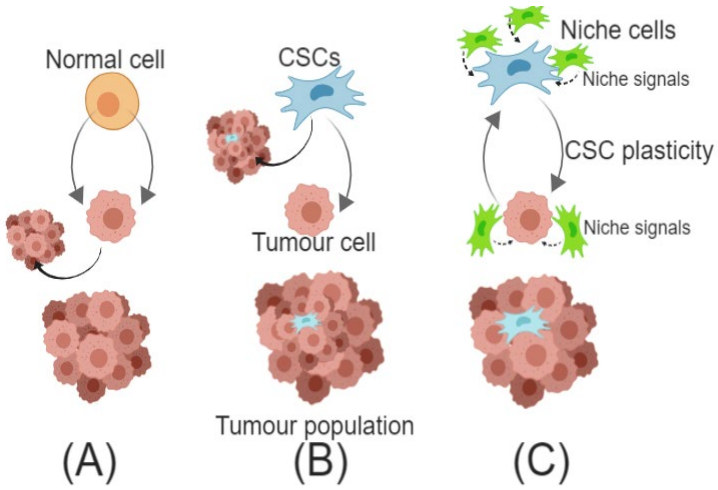

Figure 2. Concepts of cancer cell hierarchy. *Orange – normal cell, Red – tumour cell, Blue – Cancer Stem Cell (CSC), Green – Niche cells in the tumour microenvironment. Niche signals include PI3K, Notch and Wnt pathways which influence the control of tumour cells entering quiescence, differentiation and EMT.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 tumour cells. (C) Updated CSC model to include CSC plasticity. Depletion of the CSC population results in the tumour microenvironment signalling to promote the de-differentiation of cells from non-CSC to CSC states.*

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 tumour microenvironment may induce dedifferentiation of tumour cells into acquiring stem-like characteristics and vice versa, in solid tumours [89]. Targeting Hedgehog-, Notch- and PI3K- activating signals in the perivascular niche may prevent GBM CSC self-renewal and migration [64]. The reciprocal signalling between differentiated tumour 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 signalling to the CSC to maintain self-renewal and a paracrine signal to differentiated GBM cells. Disruption of niche signalling pathways may prevent the self-renewal of surviving CSCs post-therapy, along with preventing the dedifferentiation of surviving differentiated tumour cells that recognise the depletion of the CSC subpopulation. Targeting cell signalling pathways in the tumour 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 which resulted in the cessation of tumour growth [88]. After the treatment was removed, tumour growth re-emerged, coinciding also with the re-generation of Lgr5+ CSCs – leading to the speculation that non-CSCs were plastic; capable of de-differentiation into CSCs to replace the lost subpopulation. Several studies have demonstrated that the tumour microenvironment of the primary tumour regulates this phenomenon where cancer cells are able to readily convert between non-tumorigenic 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 tumour 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 tumour microenvironment such as TGF-beta which has been shown to induce mesenchymal phenotype in GBM via activation of the ZEB1 pathway [95].

However, despite being a solid tumour, evidence describing the EMT-like process in GBM has been limited. In GBM, proneural-mesenchymal transition (PMT) may underpin the molecular events that lead to enhanced invasive capability that is described as EMT in other solid tumours. 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-beta demonstrate enhanced invasive potential and migration [95]. The TGF-beta pathway is involved in maintaining the stemness of GBM CSCs [97]. Several studies have produced promising results in experiments applying TGF-beta inhibitors to target CSCs in glioblastoma models [98,99]. Studies applying exogenous TGF-beta 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 tumour cells to de-differentiate 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 tumour microenvironment, such as exposure to temozolomide or radiation [102]. Interestingly, Maracto *et al.* found that treatment of a tumour xenograft population with a reovirus does not alter the CSC:non-CSC proportions within the tumour 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 signalling pathways in the tumour microenvironment that would normally induce signalling 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 secondly, whether this phenomenon is merely an artefact observed only in xenografted do tumours.

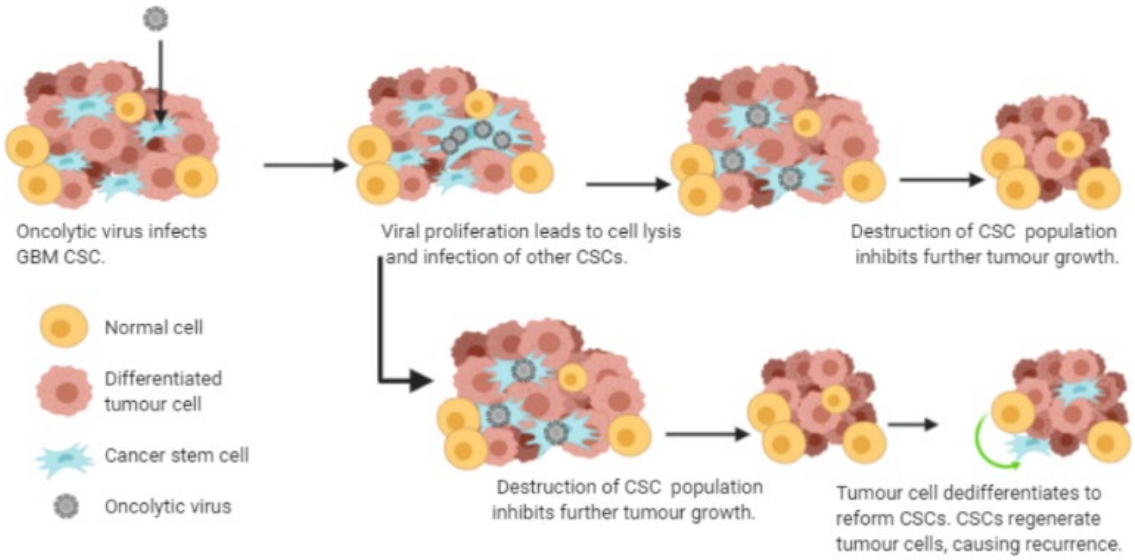

Figure 3. Cancer stem cell plasticity post-treatment with oncolytic virotherapy. *The ideal virotherapy targeting GBM CSCs would eliminate this rare subpopulation of tumorigenic cells and prevent further tumour growth. New evidence suggests that GBM CSCs may be regenerated from the dedifferentiation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

With this in mind, a future virotherapy strategy should consider employing mechanisms to inhibit EMT; encoding genes that express inhibitors that target signalling 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 tumour 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 de-differentiation of non-CSCs (Figure 3). In theory, it is possible that a highly potent oncolytic virus 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 sub-populations and by inhibiting the de-differentiation pathways responsible for regeneration. However, when administered as a stand-alone treatment, the de-differentiation 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 pre-clinical models of glial stem cell derived tumours, compared to 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 relocalisation of tumour 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 to 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 tumour recurrence that occurs after the elimination of the CSC subpopulation with a highly effective virotherapy.

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 signalling that enable them to enter a slow-cycling state that has been proposed as one possible mechanism underpinning their evasion of anti-proliferative 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 tumours. By targeting the epigenetic remodelling 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 co-target of conventional treatment, in order to target CSCs whilst preventing potential escape mechanisms [109,110]. It has previously been shown that Wnt signalling pathways are preferentially activated in GBM cell cultures post-treatment with ionising radiation [111]. This is co-observed with enhanced clonogenicity and CSC enrichment. Pharmacological inhibition of Wnt signalling 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 signalling 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 transcription 3 (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 tumour 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 signalling in the tumour 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 de-differentiation 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 de-differentiation 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 tumour 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 PERSPECTIVES

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 de-differentiating non-CSCs in the in vivo environment. Future studies ought to identify the signalling mechanisms in the tumour 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 signalling in the tumour 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 oncolytic virus (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 tumour 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 tumour 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 tumours such as GBM.

EXECUTIVE SUMMARY

- The focus in the field of research has been towards targeting CSCs. A number of engineered OVs including oHSVs and ZIKV have been shown to be highly effective at eliminating GBM CSCs.
- This field of research is beginning to recognise the potential difference in efficacy between OV monotherapy compared to OV co-administered with chemotherapy, checkpoint inhibitors or even with other OVs. Future studies are likely to shift its focus towards the latter approach in light of CSC plasticity
- Our understanding of the relationship between the tumour microenvironment and tumour cell plasticity is limited. Mapping out these signalling pathways will enable us to identify novel targets that may direct what genes are encoded into 'armed' OVs.
- Disruption of reciprocal signalling in the niche may prevent the dedifferentiation of tumour cells.
- Engaging macrophage and T cell responses at the tumour site has been a challenge due to the highly immunosuppressive nature of the GBM niche. OVs encoding BiTEs have demonstrated efficacy in immune stimulation in GBM models and may overcome these hurdles.

KEYWORDS

Cancer, glioblastoma, stem, oncolytic virus, virotherapy, plasticity

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