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Virotherapy Against Malignant Glioma Stem Cells

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Abstract

Glioblastoma multiforme, the most common primary intracranial malignancy, is associated with very poor outcome despite advances in surgical techniques and chemo- and radiation therapy. Many novel treatment modalities are being investigated with varying amount of success. Evolution of cancer stem cell hypothesis provides a new venue for developmental therapeutics. In this review, we highlight the literature regarding the existence of glioma stem cells and their characteristics. We also discuss the potential for virotherapy, a novel therapeutic approach utilizing conditionally replicative viruses, to directly target this population of self-renewing cancer stem cells.

Keywords

Malignant glioma; glioblastoma multiforme; stem cells; virotherapy; conditionally replicative virus; adenovirus

1. Introduction

Gliomas, a group of malignancies derived from glial cells, represent the most common primary intracranial malignancy. According to CBTRUS 2007-2008 report, the incidence rate of gliomas is 16.5 cases per 100,000 persons per year in the United States [1]. Moreover, the incidence rate for malignant glioma is 7.3 per 100,000 persons per year. This translates to approximately 51,410 newly diagnosed cases of gliomas in the USA, of which more than 14,000 cases are expected to be malignant. Gliomas represent a spectrum of malignancies ranging from slow growing, non-infiltrative to rapidly multiplying and highly invasive. On the basis of their histological features, the World Health Organization (WHO) classifies astrocytomas into four grades: grade I (pilocytic astrocytoma), grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma) and grade IV (glioblastoma multiforme) [2]. Grade III and Grade IV gliomas are considered malignant and represent the focus of this review.

Malignant gliomas are histologically heterogeneous, expressing both differentiated and undifferentiated neural markers. Despite exponential increase in our knowledge about these malignancies at genetic and molecular levels, the outcome or survival in this group of patients has slowly improved over the past decade. From the time of diagnosis, the median survival is approximately fourteen months [3]. The pattern of treatment failure is most of the time associated with local recurrence. Historically glioma therapy consisted of gross total resection followed by local radiation. Subsequently, several studies involving addition of adjuvant systemic chemotherapy like nitrosourea [4], local chemotherapy in the form of Gliadel wafers

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Conflict of Interest

The authors have no conflict of interest.

[5,6] and finally oral alkylating agent like Temozolomide (TMZ) [7,8] showed small but significant increase in median survival. Thus, the current standard of care for patient with GBM includes gross total resection when possible followed by single-agent adjuvant therapy with TMZ and radiotherapy.

Even though different combinations of chemo- and radiation therapy have made a difference in terms of survival for GBM patients, the scrutiny of the pattern of failure has opened up many different avenues for targeting therapy. Some of the emerging concepts are individualized targeted therapy based on individual tumor's genetic signature in the form of immunotherapy, antibody targeting, virotherapy and gene therapy, and also targeting slow growing chemo and radio-resistant cancer stem cells.

2. Cancer Stem Cells

Solid tumors consist of heterogeneous mix of cells and structures, like neoplastic cells, stromal cells, inflammatory cells, and vascular structures. Recent literature in cancer biology suggests that just like in organogenesis, where a small group of slowly replicating cells possesses the unique property of producing differentiated cells and self-renewal, there exists a cellular hierarchy within the tumor. Not all the cells from the tumor can reproduce the original tumor with all the complexity and features of the original tumor when transplanted into an immunodeficient host. Hamburger et al. showed that only a small sub-group of tumor cells from different epithelial tumors had the capability of growing *in vitro* colony [9]. These observations led to the evolution of cancer stem cell (CSC) theory (Fig. 1), which implies that neoplastic clones are exclusively maintained by a very small fraction of transformed stem cells or progenitor cells with acquired stem cell like properties such as self renewal and differentiation [10].

CSC were first isolated and described in hematological malignancies. In early 1990's, Bonnet et al., reported prospective isolation of a small group of primitive hematopoietic stem cells in acute myeloid leukemia (AML) that were capable of initiating human AML in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice) [11]. Just like normal hematopoietic stem cells, these CSCs possessed differentiative and proliferative capacities and the potential for self-renewal [11]. This subset of cells was defined as CD34⁺CD38⁻; however, all the cells derived from these CD34⁺CD38⁻ cells were not CD34⁺CD38⁻ but a heterogeneous mix of differentiated and undifferentiated cell types.

Since this initial discovery, CSCs have been discovered in a growing number of solid malignancies. A number of cell surface markers have proved useful for the isolation of CSCs, including CD133, CD44, CD24, epithelial cell adhesion molecule (EpCAM), THY1, ATP binding cassette B5 (ABCB5) and Hoechst 33342 excluding side population cells [12]. In breast cancer, Al-Hajj et al., reported presence of CD44⁺CD24^{-/low}Lineage⁻ cells with stem cell like properties [13]. In head and neck squamous cell carcinoma (HNSCC), Prince et al., showed that of all the tumor cells, only a very small population of CD44⁺ and nuclear BMI1 expressing cells with primitive morphology were tumorigenic on serial passes [14]. Similar subpopulation of tumorigenic and self replicating CSCs were described by Fang et al. in metastatic melanoma [15] and Bapat et al. in ovarian cancer [16]. The hallmark feature of all these different subpopulations of CSCs are the ability to form non-adherent spheres in culture, tumorigenic phenotype on serial passage, and capacity to produce original tumor *in vivo* when transplanted in immunodeficient mice in very small numbers.

CD133 antigen, a pentaspan membrane glycoprotein and a marker of normal hematopoietic stem cells, has been subsequently identified in many solid tumors like colon [17], pancreas [18], lung [19], osteosarcoma [20], and brain tumors [21]. In all these studies, CD133⁺ cells represent a small subpopulation of cells with capabilities to recapitulate the original tumor.

When they are serially transplanted in SCID mice, they have the ability to give rise to both primitive undifferentiated CD133+ cells and more differentiated non-tumorigenic CD133- cells. In human pancreatic cancer, Hermann et al., demonstrated that CD133+ cells also have the potential to migrate and these migrating CD133(+) CXCR4(+) cancer stem cells were the main players in distant metastasis [18].

Thus, CSC can be defined as a small subpopulation of cancer cells with striking similarities to normal stem cells: self-renewal, multipotency, relative quiescence. Along with these properties, they also share other features that are similar to normal stem cells like activation of DNA repair mechanisms and expression on drug transporter. The same properties that render normal stem cells immunity from cytotoxic insults also protect these CSC from cytotoxic therapy. Thus, specifically targeting these cells may provide a much needed edge over cancer progression and recurrence.

3. Glioma Stem Cells

Glioma cells with stem-like properties were first isolated by Ignatova et al., using a neurosphere assay (culture of clonally derived non-adherent neurospheres each of which arise from a single cell) [22]. Building on the same concept, Singh et al., [21] cultured primary brain tumor cells (dissociated into individual cells) in serum free medium with stem cell growth factors bFGF and EGF and showed that 0.3% to 25.1% of all cells in different tumor (higher frequency in more aggressive tumors) samples were capable of continuing to proliferate and expanding in tumor culture over time. These neurosphere forming cells, glioma stem cells, stained positive for undifferentiated neural stem cell marker nestin and CD133, a hematopoietic stem cell marker also present on normal human neural stem cell [23,24]. However, they lacked markers for differentiated neurons (beta tubulin III), astrocytes (GFAP) or oligodendrocytes. In contrast, CD133- cells adhered to the culture plates and did not proliferate or form spheres. In highly aggressive GBMs, the fraction of CD133+ cells ranged from 19-29% [25] but was lower in low grade glioma. All dissociated primary tumor spheres were capable of forming secondary tumor spheres on replating at serial dilution with multiple passages, demonstrating their self-renewing capabilities. At the same time, they maintained their original morphology *in vitro*. The frequency of sphere formation depended on the tumor's pathological subtype, with more aggressive tumors exhibiting increased self-renewal capacity compared to less aggressive tumors. When exposed to differentiating conditions, these cells lost expression of CD133 and nestin and instead expressed differentiated markers for the cell of origin. Cellular and genetic analysis of CSC showed that they were genetically transformed with enhanced self-renewal properties and possessed abnormal karyotype. This abnormal karyotype was not just limited to CD133+ cells, but this cytogenetic alteration was present in both CD133+ and CD133- cells suggesting that all the cancer cells were clonally derived. As few as 100 of these CD133+ cells, when serially transplanted in the brain of NOD-SCID (non-obese diabetic, severe combined immunodeficient) mouse, were able to produce exact phenocopy of patient's original tumor with all the histopathological features and cell surface markers of the original tumor. However, injection of 50,000 to 100,000 CD133- cells failed to produce any tumor in NOD-SCID mouse [25]. Immunohistological staining of the GBM xenograft revealed that only 19-22% of all cells were CD133+, and the majority of them were CD133-. Xenograft also stained positive for differentiated cell markers like GFAP; however, the cells staining for GFAP and CD133 were two distinct groups. These experiments clearly demonstrate the existence of a small population of CD133+ CSC that are undifferentiated and genetically altered. However, they possess characteristics of normal stem cells like relative quiescence, self-renewal, proliferation, and multipotency with the ability to recapitulate the original tumor with all its original heterogeneous characteristics.

The question of whether brain tumors arise from dedifferentiation of a normal brain cell or from the genetic transformation of normal neural stem cell or progenitor cell is yet to be answered. However, the existence of this functional hierarchy in malignant glioma provides some insight into the pattern of treatment failure in this group. Conventional therapies treat the entire tumor as a homogenous entity and are targeted towards rapidly multiplying bulk of tumor cells. However, they have no specificity towards CSC and most likely fail to target these slowly dividing mutated cells.

To establish a link between chemoresistance and CSC, Kang et al., exposed GBM cells to a lethal dose of carmustine (BCNU) and noticed that a small population of GBM cancer cells survived and proliferated. These were CD133+ and had the capacity for multipotency. These BCNU-resistant cells were able to give rise to the primary tumor when transplanted into severe combined immunodeficient (SCID) mouse brain [26]. Liu et al., reported that when compared with newly diagnosed GBM, recurrent GBM in the same patient has significantly higher level of CD133+ cells [27]. CD133+ cells expressed high level of anti-apoptotic genes and chemotherapy resistance genes like BCRP1, MDR1, MRP1 and MGMT [27-29], and these cells were significantly resistant to many chemotherapeutic agents including temozolomide, carboplatin, paclitaxel (Taxol) and etoposide (VP16) compared to autologous CD133- cells. In the same tumor, expression of multi drug resistance-associated proteins 1 and 3 was found to be markedly elevated when compared to non-CSC [30]. Eramo et al., treated glioma stem cells with a wide variety of chemotherapeutic agents for 48h and observed marked resistance of these cells to all compounds as compared to Jurkat cells. When these cells were allowed to recover after 24h of cytotoxic therapy, they showed remarkable recovery and proliferation compared to chemosensitive stem cells [31].

CSCs are not only chemoresistant, but they also show marked resistance towards radiation therapy, and tumors surviving radiotherapy are enriched in CSC. Bao et al., found that irradiation of *in vivo* glioma xenograft led to 3-5 fold increase in CD133+ cell population as compared to untreated xenografts [32]. Also, ionizing radiation (IR) treatment of short term culture from human glioma xenografts led to 4 fold increase in CD133+, and CD133+ cell population increased from 2-3% to 6-10% after radiation in freshly isolated GBM specimens. These findings suggest that IR leads to enrichment of CD133+ cells in the tumor and subsequent formation of more aggressive tumors with decreased latency on serial transplantation. Thus, with better understanding of cancer and CSC biology, it is becoming more evident that CSCs are a crucial player in glioma genesis and recurrence. It is probably the main factor leading to treatment failure. Novel therapies targeting this small group of cells instead of the bulk of the tumor might provide better success in treating this deadly disease.

4. Oncolytic Virotherapy

Viruses have shown promising results in two modern fields of emerging anti-tumor therapy: gene therapy and virotherapy. In gene therapy, viral vectors are used to transfer a therapeutic gene for mutation compensation, immunopotentialization, or prodrug activation in neoplastic cells. In virotherapy, oncolysis is achieved by intra-neoplastic cell virus replication mediated cell lysis. For effective oncolytic activity, a vector must be selective for the tumor target, should have minimal brain and systemic toxicities, and also have the capacity to penetrate and diffuse throughout the brain to reach all neoplastic foci residing beyond the resection border of the tumor. In addition, the viral vector has to remain active despite evoking an immune response. Anti-tumor effect of oncolytic virus is hypothesized to be a fine play between anti-tumor immune response and anti-viral immune response, as evident by the presence of both mononuclear and inflammatory infiltrate detected at the virus injection site [33,34].

Virotherapy vectors vary across the spectrum of viruses. NDV, a single-stranded RNA virus whose natural host is poultry, has a long history of being used as an oncolytic agent [35,36]. Both mesogenic (moderately pathogenic, capable of producing viable progeny and infect adjacent cells) and lentogenic (attenuated non-pathogenic, produces defective progeny and can't spread between tissue) forms of this virus have been evaluated as a potential candidate for oncolytic virotherapy. In a phase I/II clinical trial, Freeman et al., first reported favorable tolerability of systemic administration of a lentogenic NDV virus strain, NDV-HUJ, in GBM patients [37]. They administered NDV-HUJ virus systemically through peripheral or central line over 15 min. Analysis of blood, saliva, urine, tumor tissue and tumor cyst fluid from 5 of the 14 patients showed presence of infectious NDV particles, indicating the non-specificity of this virus for cancer cells.

Reovirus selectively replicates in glioma cells, where stimulation of RAS pathway by PDGFR [38] or EGFR [39] inhibits RNA-activated protein kinase activation, and thus viral protein can be synthesized leading to tumor regression in intracranial human glioma xenografts in mice [40]. In a phase I trial, Forsyth et al., demonstrated that administration of live, replication competent and genetically unmodified reovirus, which uses activated ras signaling to enhance proteolytic virus disassembly [40,41], directly into the tumors of patients with malignant gliomas is safe and well tolerated with no evidence of clinical encephalitis [42].

In humans, wild type HSV causes necrotizing hemorrhagic encephalitis. However, genetically engineered HSV vectors have been tested for treatment of malignant glioma. G207, a genetically engineered HSV -1, has deletion of γ 34.5 gene at both loci and a disabling insertion of *lacZ* in *U_L 39*, the gene encoding for the large subunit of viral ribonucleotide reductase. As a result of these mutations, the virus can only replicate in rapidly dividing cells but not in quiescent cells, and mutants have been shown to be virtually lacking in virulence even after direct inoculation of high titers into the CNS [43,44]. Moreover, G207 retains thymidine kinase activity so it is susceptible to anti-viral therapy with agents like acyclovir. In a phase I trial, Markert et al., demonstrated that it is safe to administer G207 directly to the enhancing portion of glioma [45]. In phase 1N, they demonstrated that it is also safe to directly inoculate into the brain surrounding the tumor [46]. Virus was found to preferentially target cancer cells and spare normal nervous tissue, and single fraction of radiation increased the oncolytic properties of G207. The same mutation that rendered mutant HSV-1 more selective for replication in human gliomas also attenuated the viral progeny, thus decreasing its treatment power [43,47, 48]. To overcome this limitation, Kambara et al., generated a mutant in which one copy of the γ ₁ 34.5 gene is reinserted into the UL39-deleted, γ ₁ 34.5-deleted viral genome, under control of a tumor-specific promoter called nestin. In cultured and primary human glioma cell lines, replication, cellular propagation, and cytotoxicity of rQNestin34.5 was significantly enhanced compared with control virus; however, it remained quantitatively similar to that of control virus in normal human. In glioma cell lines infected with rQNestin34.5, the level of phosphoeukaryotic initiation factor 2A was lower than that of cells infected by control rHsvQ1, confirming selective ICP34.5 expression in glioma cells. *In vivo* study of rQNestin34.5 showed significantly more potent inhibition of tumor growth compared with control virus [49].

Conditionally replicative adenoviruses, or CRAds, are naturally selected or genetically engineered adenovirus that preferentially replicate in neoplastic cells and by lysis of the malignant cells, releases many progeny which in turn infect neighboring neoplastic cells [50-53]. The tropism of CRAd can be improved by mainly modifying the events in viral replication pathway.

- First, deletion of viral genomic regions that are not required for replication in cancer cell with specific pathway alterations [54-59].
- Second, facilitation of viral transduction in malignant cells [54,60-62].

- Third, transcriptional targeting of viral genes or transgenes using tumor specific promoters [49,63-66].

Deletion of viral genome responsible for bypassing cancer cell's antiviral proteins helps viral vectors to be able to replicate within cancer cells with disrupted antiviral mechanisms. One such genetically altered virus is ONYX-015, which replicates more efficiently in cells with defective p53 pathway due to deletion of viral genomic region coding for E1B 55K. In a phase I trial of ONYX-015, Chiocca et al., showed that injection of 10^{10} plaque forming units of ONYX-015 virus into brain tissue adjacent to a freshly excised glioma is well tolerated in human [67]. Another such vector is Ad5-Delta24 which carries a 24-bp deletion in the E1A region responsible for binding Rb protein and can replicate in and lyse cancer cells with great efficiency. Lysis of most human glioma cells was observed within 10 - 14 days after infection with Ad5-Delta24 at 10 PFU/cell. *In vivo*, a single dose of the Ad5-Delta24 virus induced a 66.3% inhibition, and multiple injections showed an 83.8% inhibition of tumor growth in nude mice. However, normal fibroblasts or cancer cells with restored Rb activity were resistant to the Delta24 adenovirus [56].

Facilitating viral transduction, the process of virion entry into the host cell leads to enhanced tropism of CRAd for glioma. Entry of adenoviral particle into the cell is mediated by interactions between specific cell receptors and viral proteins. Malignant gliomas express a variety of receptors like PDGFR, EGFR, or $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, which can be targeted for viral transduction. Adenovirus serotype 5, one of the most popular adenoviral vectors, requires expression of CAR, an adhesion protein, to achieve transduction. CAR is widely expressed in most tissues but is inconsistently expressed in gliomas [54,61,68-71]. This limits the possibility of developing efficient anti-cancer therapy based on the use of CRAd with wild-type knob domain. In order to address this limitation, Suzuki et al., designed a new vector, Ad5-Delta24RGD, which was obtained by incorporation of Arg-Gly-Asp (RGD) motif, known to interact with αv integrin (abundant expression in glioma), in the HI loop of fiber knob of Delta24 adenovirus [72]. Lamfers et al., showed that Ad5-Delta24RGD has a stronger oncolytic effect than the non-RGD-expressing variant in a broad panel of primary glioma cells, in particular on those with low CAR expression. In subcutaneous glioblastoma xenografts in nude mice expressing low levels of CAR, intratumoral injections of Ad5-Delta24RGD resulted in complete tumor regression in 9 of 10 mice and long-term survival in all treated mice. Low-dose irradiation before Ad5-Delta24RGD infection decreased viability of glioma cells more effectively than Ad5-Delta24RGD alone, such that the same therapeutic effect was achieved when a 10-fold lower viral dose was applied. *In vivo* treatment of GBM xenograft bearing nude mice with low dose radiation and Ad5-Delta24RGD vector showed tumor regression and long term survival in every mouse [54].

Another receptor that holds promise for viral retargeting is epidermal growth factor receptor (EGFR). EGFR has negligible expression in normal mitotically quiescent neural tissue but is highly expressed in high grade gliomas, and this expression is associated with EGFR gene amplification [39,73,74]. Upon binding its ligand, EGFR is internalized and leads to activation of PI3K [75], which is required for adenovirus entry into host cells [76]. To circumvent the lack of fiber receptors and to target adenoviral gene transfer specifically to tumor cells, Miller et al., used a bispecific antibody conjugate to ablate adenoviral binding to fiber receptors and retarget binding to the epidermal growth factor receptor (EGFR). EGFR-targeted adenoviral gene transfer was EGFR specific, independent of fiber-fiber receptor interactions and significantly enhanced adenoviral gene delivery to 7 of 12 established glioma cell lines and to 6 of 8 cultured primary gliomas [61]. In an alternative approach Van Beusechem et al., designed an adenoviral vector lacking CAR and αv integrin binding capacities, together with bispecific single-chain antibodies targeted toward human EGFR. These vectors efficiently and selectively targeted both alternative receptors on the surface of cancer cells and primary human tumor

specimens. With primary glioma cell cultures, EGFR targeting augmented the median gene transfer efficiency of doubly ablated adenoviral vectors 123-fold. EGFR-targeted doubly ablated adenoviral vectors had a 5- to 38-fold-improved tumor-to-normal brain targeting index compared to native vectors. They transduced organotypic glioma spheroids with efficiencies similar to those of native adenoviral vectors, while exhibiting greater-than-10-fold-reduced background levels on normal brain explants from the same patients [60].

Neoplastic phenotype is a result of a series of modifications in gene expression, with activation of specific promoter elements that enhance tumor growth and invasion. This phenomenon of cancer cells can be exploited to develop viral vector where tumor specific promoter can be incorporated into viral genome to drive the expression of viral genes and transgenes. These tumor-specific promoter driven vectors limit their respective gene's expression and oncolysis to specific neoplastic cells bearing the specific promoter. Gliomas express many different promoters, such as tissue specific promoters like GFAP [77] and myelin basic protein (MBP) [64] and tumor specific promoters like nestin [54], human telomerase reverse transcriptase (hTERT) [78], E2F1 [66], CXCR4 [79,80], midkine [81], and survivin [82].

Telomerase composed of functional or template RNA component (hTR) and telomerase catalytic protein subunit (hTERT) is over-expressed in majority of gliomas with no expression in normal brain. Activation of telomerase is tightly regulated at the transcriptional level of hTERT. Komata et al constructed an expression vector consisting of the constitutively active caspase-6 (rev-caspase-6) under the hTERT promoter (hTERT/rev-caspase-6) and investigated its antitumor effect in malignant glioma cells. The results showed that the hTERT/rev-caspase-6 construct induced apoptosis in hTERT-positive malignant glioma cells, but not in hTERT-negative astrocytes, fibroblasts, and alternative lengthening of telomeres cells. In addition, the growth of subcutaneous tumors in nude mice was significantly suppressed by the treatment with hTERT/rev-caspase-6 construct [78].

Malignant gliomas have disrupted pRB function, either because of RB-1 gene mutations or as a result of mutations affecting upstream regulators of pRB such as cyclin D1 or p16/INK4a/MTS1, and tumor suppression effect of pRB has been linked to its ability to repress E2F-responsive promoters such as the E2F-1 promoter. Adenoviral vectors containing transgenes driven by the E2F-1 promoter showed tumor-selective gene expression *in vivo*, leading to eradication of established gliomas with significantly less normal tissue toxicity as seen with standard adenoviral vectors [66]. ICOVIR-5 adenovirus, which encompasses an early 1A adenoviral (E1A) deletion in the retinoblastoma (Rb) protein-binding region, substitution of the E1A promoter for E2F-responsive elements, and an RGD-4C peptide motif inserted into the adenoviral fiber to enhance adenoviral tropism, showed a dramatic addition of ICOVIR-5 to the E2F1 oncogene *in vitro* and *in vivo* due to the occupancy of the ectopic adenoviral E2F1-responsive elements by the endogenous E2F1 protein resulting in high level of E1A expression in cancer cells and potent anti-glioma effect [83]. Combination of ICOVIR-5 with RAD001 or TMZ showed a potent anti-glioma effect *in vitro* and *in vivo*, resulting in a dramatic extension of the median [84].

Chemokine C-X-C motif receptor 4 (CXCR4), the inhibitor and apoptosis protein (IAP) survivin, and the growth factor midkine are tumor specific promoters that play an important role in tumor growth and survival and are up-regulated in the majority of all gliomas [79-82]. Ulasov et al., compared feasibility of transcriptional targeting of glioma cells by comparing the activity of survivin, midkine and CXCR4. In this study, the authors designed replication deficient adenoviral vectors that incorporate these promoters upstream of luciferase reporter gene. Infection of glioma cell lines, primary brain tumor and normal brain by these vectors showed that all three promoters are over-expressed in various glioma cell lines and can drive luciferase expression in these cell lines with less than 2% expression in normal brain tissue.

However, of the three promoters, only survivin showed 10,000-fold increased expression in tumor cells compared to normal tissue. Based on these results, the authors tested the cytopathic effect of a novel oncolytic vector CRAd-Survivin-pk7, a chimeric vector containing a pk7 fiber modification and a survivin promoter driving E1A replication, in tumor cells. The results demonstrate that CRAd-S-pk7 vector effectively replicates in many glioma cell lines like U-87MG, U-251MG, A172, Kings-1, No. 10 and U-118MG and leads to tumor oncolysis in these cell lines with minimal viral replication and toxicity in normal human brain. Intratumoral injection of CRAd-S-pk7 in U-87MG glioma xenograft inhibited tumor growth by more than 300% with 67% of the mice surviving long term (>120 days). Analysis of xenograft tumor tissue indicated increased adenoviral infectivity, decreased mitotic activity, and enhanced tumor apoptosis [85]. These results suggest that a combination of transductional approach (pk7 modification) and a transcriptional approach (incorporation of the survivin promoter) results in both enhanced replication and enhanced cell lysis; thus revealing a promising potential vector for clinical use in glioma gene therapy.

5. Targeting Glioma Stem Cells with Virotherapy

Among all the emerging therapeutic options for GBM, virotherapy has shown significant promise in terms of targeting glioma cells (Fig. 2). However, one of the major limitations of oncolytic viral vectors is poor replication and poor transduction of neighboring tumor cells following intracranial injection. Part of this problem is due to the need for attenuation of viral backbone, which has shown to decrease viral propagation and spread. Attenuation of E1B in ONYX-015 and deletion of γ 34.5 (RL1) in G207 are associated with decreased viral replication [86]. The clinical studies of these viruses have failed to show efficacy partly due to the fact that the virus does not replicate with robust efficiency [67,87]. One of the ways to circumvent around this problem is to design vectors that are not just targeted towards tumor cells but specifically target the small population of chemo- and radiation resistant CSCs. This can be achieved by use of tumor specific promoters or modifications of viral capsids to enhance viral transduction. Fueyo et al., used Delta-24-RGD, an oncolytic adenovirus with enhanced tropism to glioma cells and selective replication in cells with abnormal p16INK4/Rb pathway, to target CSCs and showed that brain tumor stem cells are susceptible to adenovirus mediated cell death via autophagy. Fueyo et al., isolated p16INK4/Rb negative and CAR and/or Arg-Gly-Asp (RGD)-recognizing integrins $\alpha\beta$ 3 and $\alpha\beta$ 5 positive neurosphere forming cells from four surgical glioma specimens of GBM. These stem cells were then treated with Delta-24-RGD adenovirus [56]. Delta-24-RGD treatment led to a drastic reduction in cell viability as compared to the non-treated group. Electron microscopic analysis of the Delta-24-RGD treated cells showed presence of cytoplasmic autophagic vacuoles and remarkable induction of endogenous Atg5, a key molecule in the conversion of LC3-I to -II and essential for autophagosome formation and autophagic cell death, 48 and 72 hrs post treatment. Treatment of xenografts derived from brain tumor stem cells with Delta-24-RGD led to statistically significant improvement in survival of glioma bearing mice. Immunohistochemical staining of the treated tumor revealed high expression of E1A and hexon implicating efficient adenoviral infection and replication *in vivo* and immunofluorescence analysis identified high expression of Atg5. This proves that glioma stem cells are susceptible to adenovirus mediated cell death via autophagy both *in vivo* and *in vitro*.

Survivin is a tumor specific promoter [88,89], and an adenoviral vector using survivin promoter and binding to heparan sulfate proteoglycans (CRAd-Survivin-pk7) has shown to greatly enhance antitumor efficacy in experimental glioma model [85]. In a recent study, Nandi et al., showed that survivin is a radiation inducible promoter and when exposed to 2Gy of radiation, increases transgene expression by 10 folds in glioma cell lines. Radiation therapy has also shown to increase CD133+ cells in glioma cell cultures implicating their increased proliferative capacity following radiation. Upon infection of CD133+ cells with CRAd-S-pk7 from glioma

cell lines and primary tumor samples, the authors found significantly higher toxicity in cells and tumor tissue infected by the vector compared to wild type virus. Addition of 2Gy of radiation was shown to further enhance this virolytic effect of CRAd-S-pk7 with increased viral replication in virus infected radiated cells. *In vivo*, tumors formed from CD133+ enriched cells treated with CRAd-S-pk7 showed most significant tumor growth inhibition compared to untreated group. Of note, the combination of CRAd-S-pk7 treatment with radiation therapy led to a 100-fold increase in viral replication compared to CRAd-S-pk7 treatment alone [90]. This great synergistic effect seen in combination treatment of CRAd-S-pk7 with radiation is also evident when it is combined with chemotherapeutic agent Temozolomide. TMZ is known to induce glioma cell death via autophagy [91]. Ulasov et al., have recently shown that combination of adenoviral virotherapy and temozolomide chemotherapy eradicates malignant glioma via autophagic and apoptotic cell death *in vivo* [92]. This combination therapy revealed an increased expression of pro-apoptotic proteins p53 and BAX, and a decreased expression of anti-apoptotic protein BCL-2 implicating apoptosis as one of the cytotoxic mechanisms of combination therapy. At the same time treatment with TMZ followed by CRAd-S-pk7 led to a significant increase of acidic vesicular organelles (AVO), characteristic feature of autophagy, and microtubule-associated protein 1 light-chain 3 (LC3), a protein playing crucial role in autophagosomes formation, in comparison to TMZ or CRAd-S-pk7 treatment alone, suggesting enhanced induction of autophagy in combination therapy. *In vivo* evaluation in mice with U87MG intracranial glioma xenografts with combination therapy revealed that the effect of combination therapy is additive, and 90% of all mice survived long term. Immunohistochemical analysis of the tumor treated with combination therapy showed an increase in the expression of both LC3 and cleaved Caspase-3 over time, proving that both autophagy and apoptosis are responsible for cell death *in vivo*.

Neuronal cell adhesion molecule, L1CAM, regulates brain development and is expressed in gliomas. L1CAM (+) and CD133 (+) cells are found cosegregated in gliomas, and levels of L1CAM have been seen to be higher in CD133 (+) glioma cells than normal neural progenitors. Using a lentiviral-mediated short hairpin RNA (shRNA) interference to target L1CAM in CD133(+) glioma cells., Bao et al., showed potent disruption of neurosphere formation, induction of apoptosis, and inhibition of growth specifically in glioma stem cells [93]. L1CAM knockdown decreased expression of the basic helix-loop-helix transcription factor Olig2 and up-regulated the p21 (WAF1/CIP1) tumor suppressor in CD133 (+) glioma cells. Targeting L1CAM by shRNA in glioma cells before injection into immunocompromised mice or directly in established tumors suppressed tumor growth and increased the survival of tumor-bearing animals [93]. L1CAM represents another glioma stem cell specific target that can be potentially targeted using viral vectors.

6. Conclusions

Transcriptional control of viral gene expression using tissue specific promoter has shown promising results in targeting just malignant cells and sparing normal tissue. This approach can be fine tuned by identification of stem cell specific promoters and thus designing vectors that drive transgene expression using these stem cell specific promoters. Cancer stem cells show marked similarity with normal stem cells, which represent the biggest challenge in the way of developing vectors driven by stem cell specific promoters. The same promoter that will drive viral gene expression in CSC, if present in normal stem cell, will also drive same viral gene expression in normal stem cells leading to normal stem cell lysis. Thus, it is critical to identify promoters that are not only specific for stem cell but restricted to CSC.

One of the hallmark features of glioma stem cells is the expression of a cellular surface glycoprotein containing five transmembrane regions and two glycosylated extracellular loops called CD133. Designing a viral vector that will specifically bind to CD133 will provide

increased specificity in targeting these cells for virolytic therapy. However, one of the main limitations of this technique is the fact that CD133 is expressed not only in glioma stem cells but in other parts of the body. A recent study showed that human AC133 gene has at least 9 distinctive 5'-untranslated region (UTR) exons, resulting in the formation of at least 7 alternatively spliced 5'-UTR isoforms of AC133 mRNA, which are expressed in a tissue-dependent manner. Transcription of these AC133 isoforms is controlled by five alternative promoters, P1-P5 depending on their location relative to different exons, In vitro methylation of P1 and P2 completely suppresses their activity, suggesting that methylation plays a role in their regulation [94]. Finding the brain specific isoform of AC133 and directing vectors to the specific isoform will also increase the specificity of these vectors.

Optimal strategy for targeting glioma stem cells with viral vector will involve transduction of viral vectors in only CD133+ cells and transcription of viral gene driven by glioma specific promoter. Finally, the synergistic effect between virotherapy and chemo- and radiation therapy needs to be further evaluated to achieve optimal outcome for patients.

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References

1. CBTRUS. Statistical Report: Primary Brain Tumors in the United States, 2000–2004. 2008.
2. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol* 2007;114:97–109. [PubMed: 17618441]
3. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005;352:987–996. [PubMed: 15758009]
4. Stewart LA. Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. *Lancet* 2002;359:1011–1018. [PubMed: 11937180]
5. Westphal M, Hilt DC, Bortey E, Delavault P, Olivares R, Warnke PC, et al. A phase 3 trial of local chemotherapy with biodegradable carmustine (BCNU) wafers (Gliadel wafers) in patients with primary malignant glioma. *Neuro Oncol* 2003;5:79–88. [PubMed: 12672279]
6. Westphal M, Ram Z, Riddle V, Hilt D, Bortey E. Gliadel wafer in initial surgery for malignant glioma: long-term follow-up of a multicenter controlled trial. *Acta Neurochir (Wien)* 2006;148:269–275. discussion 275. [PubMed: 16482400]
7. Yung WK, Albright RE, Olson J, Fredericks R, Fink K, Prados MD, et al. A phase II study of temozolomide vs. procarbazine in patients with glioblastoma multiforme at first relapse. *Br J Cancer* 2000;83:588–593. [PubMed: 10944597]
8. Brada M, Hoang-Xuan K, Rampling R, Dietrich PY, Dirix LY, Macdonald D, et al. Multicenter phase II trial of temozolomide in patients with glioblastoma multiforme at first relapse. *Ann Oncol* 2001;12:259–266. [PubMed: 11300335]
9. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science* 1977;197:461–463. [PubMed: 560061]
10. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001;414:105–111. [PubMed: 11689955]
11. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997;3:730–737. [PubMed: 9212098]
12. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 2008;8:755–768. [PubMed: 18784658]

13. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* 2003;100:3983–3988. [PubMed: 12629218]
14. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci U S A* 2007;104:973–978. [PubMed: 17210912]
15. Fang D, Nguyen TK, Leishear K, Finko R, Kulp AN, Hotz S, et al. A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res* 2005;65:9328–9337. [PubMed: 16230395]
16. Bapat SA, Mali AM, Koppikar CB, Kurrey NK. Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. *Cancer Res* 2005;65:3025–3029. [PubMed: 15833827]
17. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007;445:111–115. [PubMed: 17122771]
18. Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 2007;1:313–323. [PubMed: 18371365]
19. Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, et al. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ* 2008;15:504–514. [PubMed: 18049477]
20. Tirino V, Desiderio V, d'Aquino R, De Francesco F, Pirozzi G, Galderisi U, et al. Detection and characterization of CD133+ cancer stem cells in human solid tumours. *PLoS ONE* 2008;3:e3469. [PubMed: 18941626]
21. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003;63:5821–5828. [PubMed: 14522905]
22. Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia* 2002;39:193–206. [PubMed: 12203386]
23. Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, et al. Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci U S A* 2000;97:14720–14725. [PubMed: 11121071]
24. Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, et al. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood* 1997;90:5013–5021. [PubMed: 9389721]
25. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. *Nature* 2004;432:396–401. [PubMed: 15549107]
26. Kang MK, Kang SK. Tumorigenesis of chemotherapeutic drug-resistant cancer stem-like cells in brain glioma. *Stem Cells Dev* 2007;16:837–847. [PubMed: 17999604]
27. Liu G, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir IR, et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer* 2006;5:67. [PubMed: 17140455]
28. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med* 2005;352:997–1003. [PubMed: 15758010]
29. Bi CL, Fang JS, Chen FH, Wang YJ, Wu J. Chemoresistance of CD133(+) tumor stem cells from human brain glioma. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 2007;32:568–573. [PubMed: 17767043]
30. Salmaggi A, Boiardi A, Gelati M, Russo A, Calatuzzolo C, Ciusani E, et al. Glioblastoma-derived tumorspheres identify a population of tumor stem-like cells with angiogenic potential and enhanced multidrug resistance phenotype. *Glia* 2006;54:850–860. [PubMed: 16981197]
31. Eramo A, Ricci-Vitiani L, Zeuner A, Pallini R, Lotti F, Sette G, et al. Chemotherapy resistance of glioblastoma stem cells. *Cell Death Differ* 2006;13:1238–1241. [PubMed: 16456578]
32. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 2006;444:756–760. [PubMed: 17051156]

33. Parker JN, Gillespie GY, Love CE, Randall S, Whitley RJ, Markert JM. Engineered herpes simplex virus expressing IL-12 in the treatment of experimental murine brain tumors. *Proc Natl Acad Sci U S A* 2000;97:2208–2213. [PubMed: 10681459]
34. Fulci G, Breymann L, Gianni D, Kurozumi K, Rhee SS, Yu J, et al. Cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses. *Proc Natl Acad Sci U S A* 2006;103:12873–12878. [PubMed: 16908838]
35. Bar-Eli N, Giloh H, Schlesinger M, Zakay-Rones Z. Preferential cytotoxic effect of Newcastle disease virus on lymphoma cells. *J Cancer Res Clin Oncol* 1996;122:409–415. [PubMed: 8690751]
36. Cassel WA, Garrett RE. Newcastle Disease Virus As An Antineoplastic Agent. *Cancer* 1965;18:863–868. [PubMed: 14308233]
37. Freeman AI, Zakay-Rones Z, Gomori JM, Linetsky E, Rasooly L, Greenbaum E, et al. Phase I/II trial of intravenous NDV-HUJ oncolytic virus in recurrent glioblastoma multiforme. *Mol Ther* 2006;13:221–228. [PubMed: 16257582]
38. Guha A, Dashner K, Black PM, Wagner JA, Stiles CD. Expression of PDGF and PDGF receptors in human astrocytoma operation specimens supports the existence of an autocrine loop. *Int J Cancer* 1995;60:168–173. [PubMed: 7829210]
39. Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, et al. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature* 1985;313:144–147. [PubMed: 2981413]
40. Coffey MC, Strong JE, Forsyth PA, Lee PW. Reovirus therapy of tumors with activated Ras pathway. *Science* 1998;282:1332–1334. [PubMed: 9812900]
41. Alain T, Kim TS, Lun X, Liacini A, Schiff LA, Senger DL, et al. Proteolytic disassembly is a critical determinant for reovirus oncolysis. *Mol Ther* 2007;15:1512–1521. [PubMed: 17519890]
42. Forsyth P, Roldan G, George D, Wallace C, Palmer CA, Morris D, et al. A phase I trial of intratumoral administration of reovirus in patients with histologically confirmed recurrent malignant gliomas. *Mol Ther* 2008;16:627–632. [PubMed: 18253152]
43. Markovitz NS, Baunoch D, Roizman B. The range and distribution of murine central nervous system cells infected with the gamma(1)34.5- mutant of herpes simplex virus 1. *J Virol* 1997;71:5560–5569. [PubMed: 9188630]
44. Mineta T, Rabkin SD, Yazaki T, Hunter WD, Martuza RL. Attenuated multmutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nat Med* 1995;1:938–943. [PubMed: 7585221]
45. Markert JM, Medlock MD, Rabkin SD, Gillespie GY, Todo T, Hunter WD, et al. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. *Gene Ther* 2000;7:867–874. [PubMed: 10845725]
46. Markert JM, Liechty PG, Wang W, Gaston S, Braz E, Karrasch M, et al. Phase Ib Trial of Mutant Herpes Simplex Virus G207 Inoculated Pre-and Post-tumor Resection for Recurrent GBM. *Mol Ther.* 2008
47. Chung RY, Saeki Y, Chiocca EA. B-myb promoter retargeting of herpes simplex virus gamma34.5 gene-mediated virulence toward tumor and cycling cells. *J Virol* 1999;73:7556–7564. [PubMed: 10438845]
48. Glorioso JC, Fink DJ. Herpes vector-mediated gene transfer in treatment of diseases of the nervous system. *Annu Rev Microbiol* 2004;58:253–271. [PubMed: 15487938]
49. Kambara H, Okano H, Chiocca EA, Saeki Y. An oncolytic HSV-1 mutant expressing ICP34.5 under control of a nestin promoter increases survival of animals even when symptomatic from a brain tumor. *Cancer Res* 2005;65:2832–2839. [PubMed: 15805284]
50. Heise C, Hermiston T, Johnson L, Brooks G, Sampson-Johannes A, Williams A, et al. An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nat Med* 2000;6:1134–1139. [PubMed: 11017145]
51. Alemany R, Balague C, Curiel DT. Replicative adenoviruses for cancer therapy. *Nat Biotechnol* 2000;18:723–727. [PubMed: 10888838]
52. Jiang H, Conrad C, Fuego J, Gomez-Manzano C, Liu TJ. Oncolytic adenoviruses for malignant glioma therapy. *Front Biosci* 2003;8:d577–588. [PubMed: 12700119]
53. Lin E, Nemunaitis J. Oncolytic viral therapies. *Cancer Gene Ther* 2004;11:643–664. [PubMed: 15286681]

54. Lamfers ML, Grill J, Dirven CM, Van Beusechem VW, Georger B, Van Den Berg J, et al. Potential of the conditionally replicative adenovirus Ad5-Delta24RGD in the treatment of malignant gliomas and its enhanced effect with radiotherapy. *Cancer Res* 2002;62:5736–5742. [PubMed: 12384532]
55. Suzuki K, Fueyo J, Krasnykh V, Reynolds PN, Curiel DT, Alemany R. A conditionally replicative adenovirus with enhanced infectivity shows improved oncolytic potency. *Clin Cancer Res* 2001;7:120–126. [PubMed: 11205899]
56. Fueyo J, Gomez-Manzano C, Alemany R, Lee PS, McDonnell TJ, Mitlianga P, et al. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. *Oncogene* 2000;19:2–12. [PubMed: 10644974]
57. Bischoff JR, Kim DH, Williams A, Heise C, Horn S, Muna M, et al. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* 1996;274:373–376. [PubMed: 8832876]
58. Khuri FR, Nemunaitis J, Ganly I, Arseneau J, Tannock IF, Romel L, et al. a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat Med* 2000;6:879–885. [PubMed: 10932224]
59. Heise C, Sampson-Johannes A, Williams A, McCormick F, Von Hoff DD, Kim DH. ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. *Nat Med* 1997;3:639–645. [PubMed: 9176490]
60. van Beusechem VW, Grill J, Mastenbroek DC, Wickham TJ, Roelvink PW, Haisma HJ, et al. Efficient and selective gene transfer into primary human brain tumors by using single-chain antibody-targeted adenoviral vectors with native tropism abolished. *J Virol* 2002;76:2753–2762. [PubMed: 11861842]
61. Miller CR, Buchsbaum DJ, Reynolds PN, Douglas JT, Gillespie GY, Mayo MS, et al. Differential susceptibility of primary and established human glioma cells to adenovirus infection: targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. *Cancer Res* 1998;58:5738–5748. [PubMed: 9865732]
62. Fueyo J, Alemany R, Gomez-Manzano C, Fuller GN, Khan A, Conrad CA, et al. Lang, Preclinical characterization of the antiglioma activity of a tropism-enhanced adenovirus targeted to the retinoblastoma pathway. *J Natl Cancer Inst* 2003;95:652–660. [PubMed: 12734316]
63. Vandier D, Rixe O, Besnard F, Kim M, Rikiyama T, Goldsmith M, et al. Inhibition of glioma cells in vitro and in vivo using a recombinant adenoviral vector containing an astrocyte-specific promoter. *Cancer Gene Ther* 2000;7:1120–1126. [PubMed: 10975672]
64. Shinoura N, Saito K, Yoshida Y, Hashimoto M, Asai A, Kirino T, et al. Adenovirus-mediated transfer of bax with caspase-8 controlled by myelin basic protein promoter exerts an enhanced cytotoxic effect in gliomas. *Cancer Gene Ther* 2000;7:739–748. [PubMed: 10830721]
65. Kohno S, Nakagawa K, Hamada K, Harada H, Yamasaki K, Hashimoto K, et al. Midkine promoter-based conditionally replicative adenovirus for malignant glioma therapy. *Oncol Rep* 2004;12:73–78. [PubMed: 15201962]
66. Parr MJ, Manome Y, Tanaka T, Wen P, Kufe DW, Kaelin WG Jr, et al. Tumor-selective transgene expression in vivo mediated by an E2F-responsive adenoviral vector. *Nat Med* 1997;3:1145–1149. [PubMed: 9334729]
67. Chiocca EA, Abbed KM, Tatter S, Louis DN, Hochberg FH, Barker F, et al. A phase I open-label, dose-escalation, multi-institutional trial of injection with an E1B-Attenuated adenovirus, ONYX-015, into the peritumoral region of recurrent malignant gliomas, in the adjuvant setting. *Mol Ther* 2004;10:958–966. [PubMed: 15509513]
68. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, et al. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 1997;275:1320–1323. [PubMed: 9036860]
69. Tomko RP, Xu R, Philipson L. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc Natl Acad Sci U S A* 1997;94:3352–3356. [PubMed: 9096397]
70. Asaoka K, Tada M, Sawamura Y, Ikeda J, Abe H. Dependence of efficient adenoviral gene delivery in malignant glioma cells on the expression levels of the Coxsackievirus and adenovirus receptor. *J Neurosurg* 2000;92:1002–1008. [PubMed: 10839262]

71. Grill J, Van Beusechem VW, Van Der Valk P, Dirven CM, Leonhart A, Pherai DS, et al. Combined targeting of adenoviruses to integrins and epidermal growth factor receptors increases gene transfer into primary glioma cells and spheroids. *Clin Cancer Res* 2001;7:641–650. [PubMed: 11297260]
72. Suzuki K, Fueyo J, Krasnykh V, Reynolds PN, Curiel DT, Alemany R. A conditionally replicative adenovirus with enhanced infectivity shows improved oncolytic potency. *Clin Cancer Res* 2001;7:120–126. [PubMed: 11205899]
73. Wong AJ, Bigner SH, Bigner DD, Kinzler KW, Hamilton SR, Vogelstein B. Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification. *Proc Natl Acad Sci U S A* 1987;84:6899–6903. [PubMed: 3477813]
74. Ekstrand AJ, James CD, Cavenee WK, Seliger B, Pettersson RF, Collins VP. Genes for epidermal growth factor receptor, transforming growth factor alpha, and epidermal growth factor and their expression in human gliomas in vivo. *Cancer Res* 1991;51:2164–2172. [PubMed: 2009534]
75. Hu P, Margolis B, Skolnik EY, Lammers R, Ullrich A, Schlessinger J. Interaction of phosphatidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors. *Mol Cell Biol* 1992;12:981–990. [PubMed: 1372091]
76. Li E, Stupack D, Klemke R, Cheresch DA, Nemerow GR. Adenovirus endocytosis via alpha(v) integrins requires phosphoinositide-3-OH kinase. *J Virol* 1998;72:2055–2061. [PubMed: 9499060]
77. Wilcox ME, Yang W, Senger D, Rewcastle NB, Morris DG, Brasher PM, et al. Reovirus as an oncolytic agent against experimental human malignant gliomas. *J Natl Cancer Inst* 2001;93:903–912. [PubMed: 11416111]
78. Komata T, Kondo Y, Kanzawa T, Hirohata S, Koga S, Sumiyoshi H, et al. Treatment of malignant glioma cells with the transfer of constitutively active caspase-6 using the human telomerase catalytic subunit (human telomerase reverse transcriptase) gene promoter. *Cancer Res* 2001;61:5796–5802. [PubMed: 11479218]
79. Zhou Y, Larsen PH, Hao C, Yong VW. CXCR4 is a major chemokine receptor on glioma cells and mediates their survival. *J Biol Chem* 2002;277:49481–49487. [PubMed: 12388552]
80. Oh JW, Drabik K, Kutsch O, Choi C, Tousson A, Benveniste EN. CXC chemokine receptor 4 expression and function in human astrogloma cells. *J Immunol* 2001;166:2695–2704. [PubMed: 11160334]
81. Mishima K, Asai A, Kadomatsu K, Ino Y, Nomura K, Narita Y, et al. Increased expression of midkine during the progression of human astrocytomas. *Neurosci Lett* 1997;233:29–32. [PubMed: 9324232]
82. Yang L, Cao Z, Li F, Post DE, Van Meir EG, Zhong H, et al. Tumor-specific gene expression using the survivin promoter is further increased by hypoxia. *Gene Ther* 2004;11:1215–1223. [PubMed: 15141159]
83. Alonso MM, Cascallo M, Gomez-Manzano C, Jiang H, Bekele BN, Perez-Gimenez A, et al. ICOVIR-5 shows E2F1 addiction and potent antiglioma effect in vivo. *Cancer Res* 2007;67:8255–8263. [PubMed: 17804740]
84. Alonso MM, Gomez-Manzano C, Jiang H, Bekele NB, Piao Y, Yung WK, et al. Combination of the oncolytic adenovirus ICOVIR-5 with chemotherapy provides enhanced antiglioma effect in vivo. *Cancer Gene Ther* 2007;14:756–761. [PubMed: 17557108]
85. Ulasov IV, Zhu ZB, Tyler MA, Han Y, Rivera AA, Khramtsov A, et al. Survivin-driven and fiber-modified oncolytic adenovirus exhibits potent antitumor activity in established intracranial glioma. *Hum Gene Ther* 2007;18:589–602. [PubMed: 17630837]
86. Yazaki T, Manz HJ, Rabkin SD, Martuza RL. Treatment of human malignant meningiomas by G207, a replication-competent multimutated herpes simplex virus 1. *Cancer Res* 1995;55:4752–4756. [PubMed: 7585498]
87. Markert JM, Medlock MD, Rabkin SD, Gillespie GY, Todo T, Hunter WD, et al. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. *Gene Ther* 2000;7:867–874. [PubMed: 10845725]
88. Van Houdt WJ, Haviv YS, Lu B, Wang M, Rivera AA, Ulasov IV, et al. The human survivin promoter: a novel transcriptional targeting strategy for treatment of glioma. *J Neurosurg* 2006;104:583–592. [PubMed: 16619663]

89. Ulasov IV, Rivera AA, Sonabend AM, Rivera LB, Wang M, Zhu ZB, et al. Comparative evaluation of survivin, midkine and CXCR4 promoters for transcriptional targeting of glioma gene therapy. *Cancer Biol Ther* 2007;6:679–685. [PubMed: 17404502]
90. Nandi S, Ulasov IV, Tyler MA, Sugihara AQ, Molinero L, Han Y, et al. Low-dose radiation enhances survivin-mediated virotherapy against malignant glioma stem cells. *Cancer Res* 2008;68:5778–5784. [PubMed: 18632631]
91. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ* 2004;11:448–457. [PubMed: 14713959]
92. Ulasov IV, Sonabend AM, Nandi S, Khramtsov A, Han Y, Lesniak MS. Combination of adenoviral virotherapy and temozolomide chemotherapy eradicates malignant glioma through autophagic and apoptotic cell death in vivo. *Br J Cancer* 2009;100:1154–1164. [PubMed: 19277041]
93. Bao S, Wu Q, Li Z, Sathornsumetee S, Wang H, McLendon RE, Hjelmeland AB, Rich JN. Targeting cancer stem cells through L1CAM suppresses glioma growth. *Cancer Res* 2008;68:6043–6048. [PubMed: 18676824]
94. Shmelkov SV, Jun L, St Clair R, McGarrigle D, Derderian CA, Usenko JK, et al. Alternative promoters regulate transcription of the gene that encodes stem cell surface protein AC133. *Blood* 2004;103:2055–2061. [PubMed: 14630820]

Figure 1. Cancer Stem Cell Hypothesis

According to cancer stem cell hypothesis, tumor burden is maintained by a small group of self-renewing and differentiating cells, aka cancer stem cells (CSC). Conventional therapy targeting tumor bulk leads to tumor regression, however, presence of CSCs in the residual tumor leads to tumor recurrence. Goal of novel stem cell targeted therapy is to eradicate the population of CSC and absence of tumor sustaining CSCs will lead to tumor eradication overtime.

Figure 2. Stem cell targeted virotherapy

Viral vectors targeting cancer stem cells are genetically modified to recognize and multiply only in CSCs leading to destruction of CSCs and resulting in further infection of neighboring stem cells. Repetition of this cycle leads to eradication of the pool of multipotent CSCs which eventually leads to eradication of the tumor.