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Review

Frontiers in targeting glioma stem cells

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ABSTRACT

Patients with glioblastoma multiforme (GBM – WHO grade IV) seldom recover. This is due to the infiltrative nature of these tumours and the presence of cellular populations with ability to escape therapies and drive tumour recurrence and progression. In some cases, these resistant cells exhibit stem properties [glioma stem cells (GSC)]. This article aims at discussing relevant issues on GSC resistance to current therapies and outlines possible and promising avenues in regard to novel therapeutic strategies, such as pharmacological, immunological and viral interventions.

1. Introduction

Gliomas are highly lethal cancers containing self-renewing stem cells [glioma stem cells (GSC)] that contribute to therapeutic resistance, angiogenesis and tumour dispersal and can propagate tumours in secondary transplant assays. Whether GSC derive from normal neural stem cells (NSC), or from tumour cells that have reacquired stem cell-like properties still is an open question.[1] Molecular pathways that are typical of normal stem cells (e.g. the RAS/RAF/ERK pathway) are hyperactive and pathogenic in gliomagenesis and data in animal models indicate that in some cases, glioma tumours originate in the subventricular zone (SVZ), a stem cell niche in the adult brain. Further, GSC from different tumours may possess variable features with respect to resistance to therapies, gene expression profile and differentiation capacity that to some extent reflect the characteristics of different neural progenitor subtypes. So far yet, the possibility that GSC originate in a number of cases from de-differentiation of tumoural cells cannot be ruled out.[2,3] Although expression of different markers (CD133, Nestin, Sox-2 and Musashi-1) has been used for identification of GSC, the identity of GSC still remains elusive.[4] CD133 is the most studied and employed marker at present. CD133 (also referred to as prominin-1) is a transmembrane glycoprotein which has been identified as a cancer stem cell marker in several solid tumour types, including those of the brain. CD133 is often expressed on the surface of human GSC and CD133(+) cells may represent >85% of certain human and animal glioma cell lines.[5,6] Several studies have reported that expression of the CD133 stem cell antigen was prognostic of decreased overall survival in glioma patients (reviewed in Ref. 7). However, glioma tumours with a predominance of CD133(–) cells may be frequently found as well. For instance, Beier and coworkers[8] reported that 11 out of 15 primary glioblastomas contained significant cell populations positive for CD133 displaying

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self-renewal, tumourigenic and differentiation capacities. The remaining four tumours contained CD133(–) cells that fulfilled as well stem cell criteria (e.g. pluripotency and tumourigenicity in nude mice *in vivo*). Likewise, two distinct gene expression profiles were detected in nine glioma cell lines established under neural stem cell conditions.[9] Four cell lines were characterised by the expression of neurodevelopmental genes, a multipotent differentiation profile, expression of CD133 and formation of highly invasive tumours *in vivo*. The other five cell lines were characterised by expression of extracellular matrix-related genes, had a more restricted differentiation capacity, contained fewer CD133(+) cells and displayed reduced tumourigenicity *in vivo*. Prestegarden and colleagues[10] have recently observed, studying either primary glioblastoma multiforme (GBM) tumours and established glioma cell lines, that the ability to form tumours may be a general trait of glioma cells not exclusively linked to subpopulations with stem properties. To verify if CD133-expressing cells are essential for tumourigenesis, Nishide and coworkers[11] generated a glioma-initiating mouse cell line whose CD133(+) cells could be eliminated conditionally by a Cre-inducible diphtheria toxin fragment A (*DTA*) gene on the *CD133* locus. After induction of the *DTA* gene, the cell line depleted of CD133(+) cells maintained the capacity to form neurospheres *in vitro* and drove tumour development *in vivo*. Hence, at least in this mouse model, gliomagenesis was independent of CD133-expression. This conclusion was supported by the existence of two types of GSC within different regions of the same human GBM.[12] Albeit both types of GSC expressed CD133, they displayed diverse tumourigenic potential and distinct genetic anomalies, thus representing distinct cell targets independently of CD133 expression. Conversely, both CD133(+) and CD133(–) cells isolated from the rat C6 glioma cell line exhibited cancer stem-like cell features such as self-renewal, multilineage differentiation potentials *in vitro* and tumourigenic capacity *in vivo*, confirming that CD133 may fail to identify GSC.[13] In conclusion, CD133(+) GSC likely drive only an as yet unquantified percentage of human GBM, the remnant deriving from CD133(–) GSC with distinct phenotypical features.[4] A number of additional markers have been investigated to hallmark GSC including the aforementioned Nestin, Sox-2 and Musashi-1 as well as CD117, CD71, CD45, CD15 and A2B5,[14–16] but at the moment, CD133 remains prominent.

2. Treatment resistance of GSC

Some recent results are in contrast with the idea that GSC are specifically resistant to therapies.[17,18] For instance, CD133(+) GSC have been defined as radioresistant as compared to CD133(–) cells. However, when compared to established glioma cell lines, six GSC cultures were more sensitive to ionising radiation (IR) than the established glioma cell lines.[19] Consistent with increased radiosensitivity, the double strand break (DSB) repair capacity as determined by neutral comet assay and resolution of gamma-H2AX and Rad51 foci was significantly reduced in GSC compared with the cell lines. Although G2 checkpoint activation was intact, in contrast to the established cell lines DNA synthesis was not inhibited in GSC after IR, indicating the absence of the intra-S-phase checkpoint. A minimal conclusion from this work is that the mechanisms through which CD133(+) GSC respond to IR are significantly different from those of the traditionally established glioma cell lines.[19] As a second instance, telomerase reverse transcriptase (TERT) activation has been proposed as a resistance mechanism in stem cells.[20] However, CD133(+) cells isolated from the glioma GOS-3 cell line showed a transcription downregulation of TERT (approximately 100-fold decrease) compared with CD133(–) cells.[21] A pronounced upregulation of CD133 and downregulation of telomerase expressions were produced as a consequence of decreasing serum supplement levels in GOS-3 cells indicating that telomerase is downregulated in GSC compared to non-stem cells. Despite those out-of-the-chorus-voices, growing evidence indicates that GSC may be at least one resistant cell population that causes relapse in gliomas (reviewed in Refs. 7,22). Merely to quote one recent example, the number of survived CD133(+) cells isolated from 20 primary GBM tumours and exposed to the chemotherapeutic agent VM-26 was much more than the number of CD133(–) cells.[23] Multiple mechanisms of resistance may be involved (Table 1). For instance, it has been recently shown that the induction of autophagy contributes to GSC radioresistance.[24] Treatment of glioma cells with IR induces autophagy to a wider extent in CD133(+) cells as compared to CD133(–) cells and the CD133(+) cells express more elevated levels of autophagy-related proteins. The autophagy inhibitor bafilomycin A1 and silencing of the autophagy factor ATG5 sensitize the CD133(+) cells to IR and significantly decrease survival of the irradiated cells and their ability to form neurospheres.[24] COX-2-dependent mechanisms of inflammation may also contribute to the resistant phenotype of GSC. Evidence for enhanced COX-2 expression in CD133(+) GSC and direct cell-based evidence of nuclear factor (NF)-kappa B-mediated COX-2 regulation by membrane-type-1 matrix metalloproteinase (MT1-MMP) have been reported suggesting that selective COX-2 inhibitors may be worth exploring to target GSC.[25,26] Further, GSC are completely resistant to tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) whereas non-stem cells reveal a moderate sensitivity to TRAIL.[27] GSC exhibit only low levels of caspase (CASP)-8 mRNA and protein, known to be indispensable for TRAIL-induced apoptosis and the CASP8 promoter is hypermethylated in GSC but not in non-stem cells.[27] The ATP-binding cassette sub-family G member 2 (ABCG2) plays a key role in glioma cells resistance to different chemotherapeutic drugs.[28] The ABC transporter function is increased in side population glioma cells and its expression level is positively associated with the increasing pathological grade of glioma.[28–31] For instance, CD133(+) GSC isolated from

Table 1 – Resistance mechanisms in GSC.

Mechanism	Refs.
Autophagy	24
COX-2-dependent inflammation	25,26
Down-regulated CASP-8	27
ABC transporter function	28–34
Notch signalling	36–38
DNA-damage checkpoint response	6,7,42

the U87 GBM line present positive immunohistochemical staining and elevated multidrug resistance (*MDR*)1 gene expression in comparison to the levels in the unsorted cell population and display stronger drug-resistance to conventional anti-cancer drugs, such as doxorubicin, etoposide, carboplatin and 1,3-bis(2-chloroethyl)-1- nitrosourea (BCNU).[32] In multidrug resistant side population cells, Akt, but not its downstream target mTOR, regulates ABCG2 activity and loss of phosphatase and tensin homologue (PTEN) increases the side population. This Akt-induced ABCG2 activation results from its transport to the plasma membrane. Temozolomide (TMZ), the standard treatment of gliomas, although not an ABCG2 substrate, increases the side population in GSC, especially in cells missing PTEN (Fig. 1A and B).[29–31,33] The activation of Tie2 receptor results in increased expression of ABC transporters in human glioma cell lines and may be important in modifying the evolution of gliomas during conventional chemotherapy regimens.[34] This role of Tie2 receptor in chemoresistance of gliomas has been determined by silencing *Tie2* using specific small interfering (si) RNA, with subsequent abrogation of chemoresistance.[34] Notch signalling may have a role in regulating radioresistance of GSC. The Notch signalling pathway promotes self-renewal and represses differentiation of a variety of adult stem cells including NSC[35] and aberrant Notch activity can be found in glioma.[36] Activation of Notch through expression of Notch intracellular domain (NICD) 1 promotes growth and neurosphere formation of the SHG-44 glioma cell line[37] and Notch inhibition by gamma-secretase inhibitors (GSI) induces apoptosis and differentiation in CD133(+) stem-like cells isolated from medulloblastoma cell lines thus limiting their tumourigenicity.[38] GSC may resist therapies for their relative

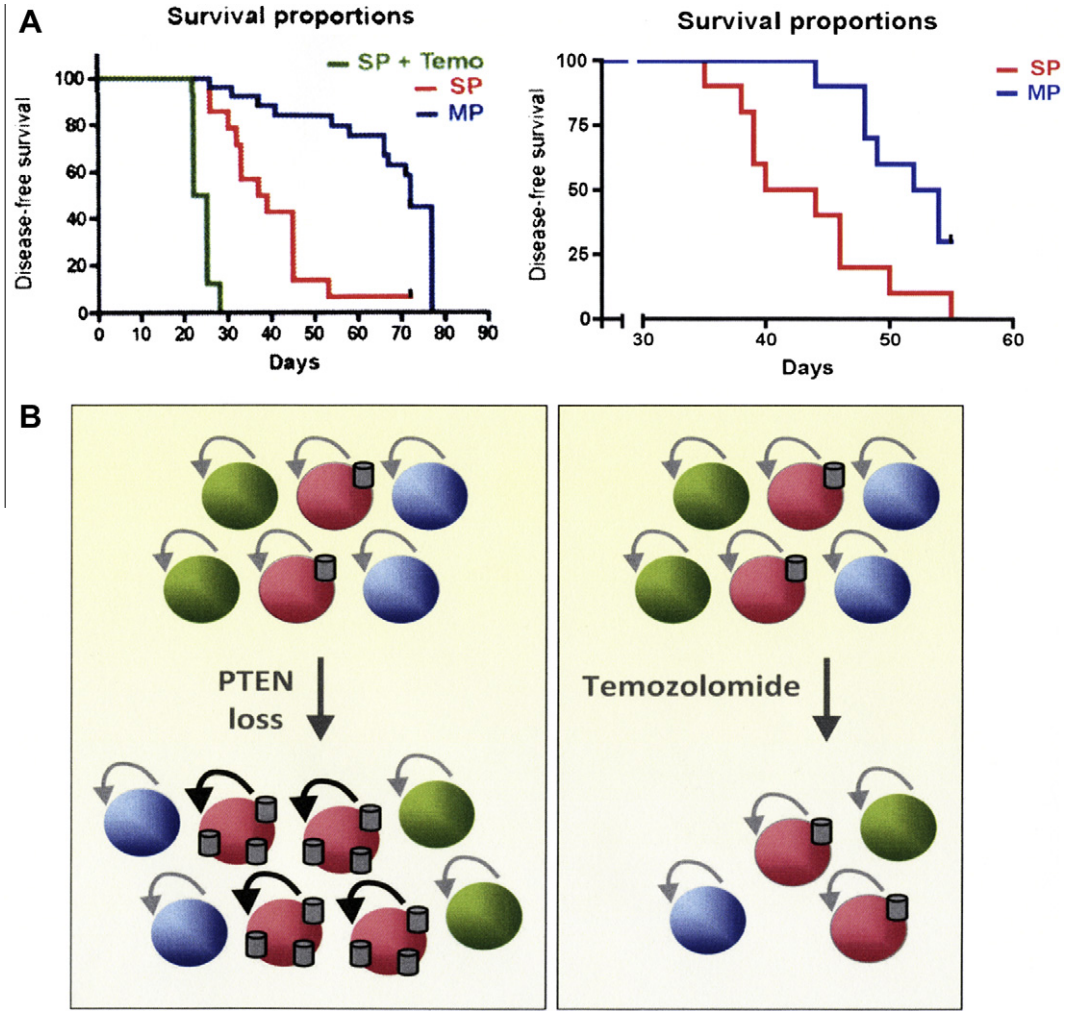


Fig. 1 – Side population phenotype of GSC. (A) Kaplan–Meier survival curve shows that side population cells (SP-red) isolated from mouse (left) and human (right) neurospheres with PTEN loss possess a higher tumourigenic potential than the main population cells (MP-blue); temozolomide treatment (green) increases the tumourigenicity of side population cells fraction (from Ref. 29, with permission). (B) Models to describe how loss of PTEN and treatment with temozolomide may lead to chemoresistance in glioma. (Left) Loss of PTEN leads to increased ABCG2 activity (represented by grey barrels) in a subset of cells, resulting in increased self-renewal and net chemoresistance. (Right) The alkylating agent temozolomide most effectively targets non-side population cells, leading to relative expansion of a chemoresistant (ABCG2-positive) side population fraction (from Ref. 31, with permission). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

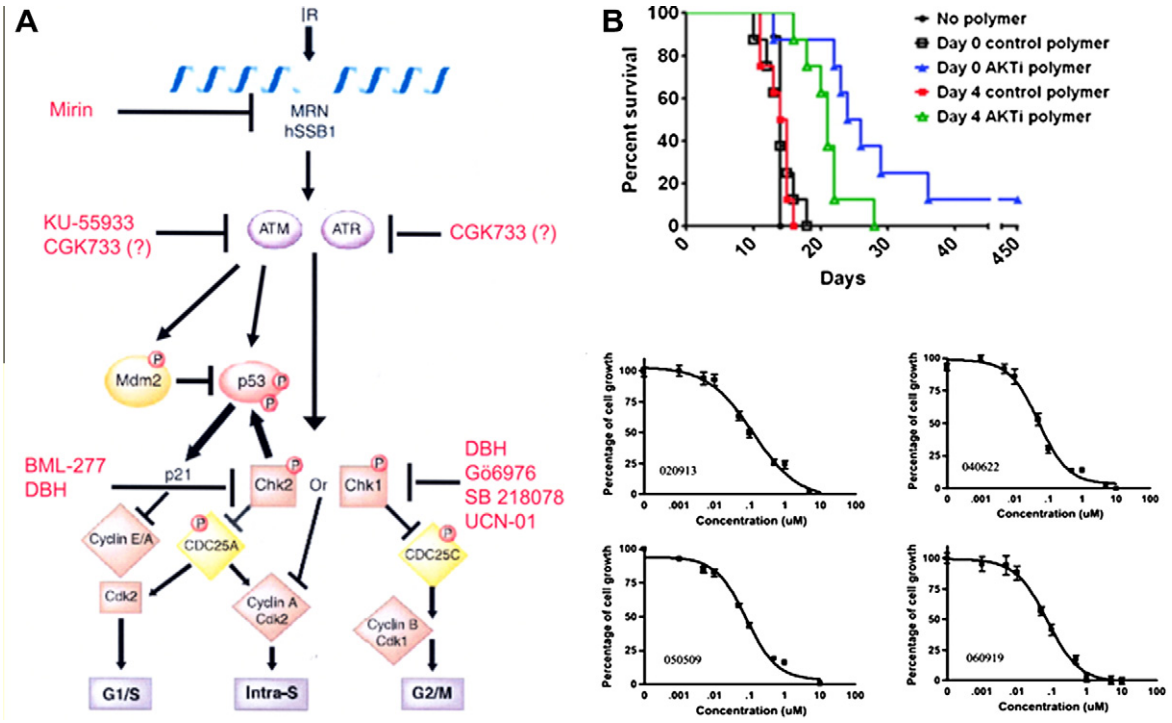


Fig. 2 – Pharmacological targeting of glioma stem cells. (A) Cell cycle checkpoint pathways, possible targets in GSC. Once DNA damage is identified with the aid of MRN and hSSB1 sensors, the checkpoint transducers ATM and ATR undergo conformational change and/or localisation, resulting in their activation. ATM and ATR activate a series of downstream molecules, including the checkpoint kinases 1 (Chk1 – right) and 2 (Chk2 – left). These kinases are responsible for inhibitory phosphorylation of cell division cycle (CDC) 25, a phosphatase required for activation of cyclin-dependent kinases (Cdk) resulting in cell cycle arrest. Mirin (6-(4-hydroxyphenyl)-2-thioxo-2,3-dihydro-4(1H)-pyrimidinone) is a specific inhibitor of MRN[90]; KU-55933 is a specific inhibitor of ATM[61]; CGK733 has been described as a specific inhibitor of ATM and/or ATR[91] but the article has been subsequently retracted[92]; BML-277,[65] debromohymenialdisine (DBH),[42] Gö6976,[64] SB218078[67] and UCN-01[64] are specific inhibitors of Chk1 and/or Chk2 kinases (modified from Ref. 41, with permission). (B) Top. Kaplan–Meier survival curves for control F344 rats (black dots), animals that received simultaneous tumour implantation (day 0) of polymers either empty (black squares) or containing the AKT inhibitor A-443654 (blue), and animals treated 4 d after tumour implantation (day 4) with either empty (red) or A-443654 containing polymers (green). Animals treated with polymers containing A-443654 on day 0 or 4 had significantly extended survival compared with control animals (from Ref. 18, with permission). Bottom. The AKT inhibitor A-443654 inhibits GSC lines. Cell proliferation assays of four GSC lines treated with increasing concentrations of A-443654 for 48 h (from Ref. 18, with permission). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

quiescence and metabolic inertia as compared to non-stem cells.[39] In response to DNA damage, eukaryotic cells activate cell cycle checkpoints – complex kinase signalling networks that prevent further progression through the cell cycle.[40] The initial event of the DNA-damage checkpoint response is sensing of DNA damage by the Mre11-Rad50-Nbs1 (MRN) complex and human single strand DNA binding protein (hSSB) 1 with subsequent activation of the ataxia telangiectasia mutated (ATM) and Rad3-related (ATR) kinases (Fig. 2A).[41] Once activated, ATM and ATR phosphorylate downstream effector proteins to initiate cell cycle checkpoints at the G1/S, intra-S and G2/M boundaries. Checkpoint proteins 1 and 2 (Chk1 and Chk2) are key downstream checkpoint substrates of ATM and ATR. In turn, the concerted action of Chk2 kinase and p53 through p21 and cell division cycle (CDC) 25A alter the activity of cyclin E/A – cyclin-dependent kinase (Cdk) 2 protein complexes at the G1-S interface and the intra-S-phase checkpoint. Further, after DNA damage, Chk1 phosphorylates CDC25C to inhibit its activity towards cyclin B-Cdk1 and the cell arrests in G2/M. We and others[6,42] have recently observed that the activation of the DNA-damage checkpoint response may be constitutive in GSC. In particular, the population doubling time is significantly increased in GSC compared to non-stem cells and enhanced activation of Chk1 and Chk2 kinases can be found in untreated CD133(+) compared to CD133(–) cells. Neither DNA base excision nor single strand break (SSB) repair nor resolution of pH2AX nuclear foci are increased in CD133(+) compared to CD133(–) cells.[6,7] GSC thus display elongated cell cycle and enhanced basal activation of checkpoint proteins that might contribute to their radioresistance, giving them time to repair DNA damage before arrival of the replication fork.[6,7] Overcoming GSC resistance will likely increase neurotoxicity due to damage on normal stem and progenitor cells.[43,44] A recent analysis of the literature has shown that radiotherapy and chemotherapy of brain tumours may pay the price of adverse effects on attention, executive functions,

memory and graphomotor speed, especially in long-term survivors.[45] Hence, preventive or therapeutic interventions for treatment-induced cognitive dysfunction should also be developed, a task that might be as demanding as tumour control.

3. Targeting glioma stem cells

3.1. *Pharmacological targeting*

Knowledge of potential molecular targets along disregulated pathways ensued in a number of proposals for targeted therapies using small-molecule inhibitors (Table 2).[46] Therapeutic targeting of stem cell populations *via* small molecule-cerebrospinal fluid (CSF)-directed therapy has proved successful in the treatment of medulloblastoma, another brain tumour thought to be derived from stem cells and might be a worth exploring possibility for limiting high grade glioma recurrence as well.[47] As aforementioned, activation of the Akt pathway is often observed in GBM.[18] Activation occurs following a number of genomic alterations including genomic amplification of epidermal growth factor (*EGF*) receptor, *PTEN* deletion or phosphatidylinositol 3-kinase (*PI3K*) mutations. Using a human glioma cell line with an activated Akt pathway secondary to a PI3K mutation, Gallia and coworkers[18] have characterised a number of PI3K/Akt small-molecule inhibitors. One of them, A-443654, showed the greatest inhibitory capacity and was selected for further *in vitro* studies on a panel of GBM cell lines. All cell lines were sensitive to A-443654 by an apoptotic mechanism of cell death. A-443654 was further tested *in vivo* in a rat orthotopic GBM tumour system (Fig. 2B, top). Implantation of polymers containing A-443654 caused significantly extended survival of animals as compared to control animals when A-443654-containing polymers were implanted simultaneously to the tumour (day 0) or 4 d later (day 4). A-443654 inhibited growth of GSC and traditional non-stem GBM cell cultures to a similar extent (Fig. 2B, bottom). Hence, the local application of an Akt small-molecule inhibitor may be effective against intracranial orthotopic GBM with no observed resistance of GSC. Albeit the cellular effects of transforming growth factor (TGF)-beta may be modulated and even reversed by a multitude of factors and cross-signalling pathways,[48] a predominant role of TGF-beta and leukaemia inhibitory factor (LIF) in inducing the self-renewal capacity and prevent the differentiation of GSC has been recently described.[49,50] Self-renewal of GSC, but not of normal human neuroprogenitors, may be induced by TGF-beta through the induction of LIF and the subsequent activation of the JAK-STAT pathway. TGF-beta and LIF promote GSC-driven tumourigenesis *in vivo* and in some patients elevated expression of LIF correlating with high expression of TGF-beta and neuroprogenitor cell markers has been observed. Hence, GSC tumourigenicity may in part be dependent on TGF-beta and LIF expression[49,50] and compounds designed to inhibit LIF or the JAK-STAT pathway (e.g. P6) could improve therapies against glioma by targeting GSC. The effect of TGF-beta was blocked by a TGF-beta receptor I (TβRI) inhibitor as well.[49] TGF-beta- driven mechanisms of GSC fuelling have been described by Ikushima and colleagues as well.[51] The stem cell marker Sox2 is induced by TGF-beta *via* Sox4 activation. Inhibitors of TGF-beta cause GSC differentiation and loss of tumourigenicity and this effect is attenuated by concomitant expression of Sox2 or Sox4. Overall survival of animals bearing GSC-driven orthotopic glioma tumours is increased after treatment of GSC with TGF beta inhibitors thus confirming that the TGF-beta – Sox4–Sox 2 pathway is a possible therapeutic target.[51] Bone morphogenetic proteins (BMP) by interacting with their cognate receptors (BMPR) trigger the Smad signalling cascade that ultimately stimulates differentiation of human glioma cells.[52] In particular BMP4 elicits a reduction in proliferation and increases expression of differentiated neural markers, without affecting cell viability. The size of the CD133(+) side population and the growth kinetics of glioblastoma cells are concomitantly reduced, indicating that BMP4 triggers a reduction in the *in vitro* cancer stem cell pool. Experiments conducted in mice bearing orthotopic glioma tumours showed that *in vivo* delivery of BMP4 effectively blocked the tumour growth and extended survival of animals. Hence, the BMP signalling system, which regulates differentiation of normal neural precursors, may intervene in differentiation of GSC as well, thus identifying a possible tool to limit cell proliferation and prevent tumour progression and recurrence.[52,53] Interferon (IFN)-beta has been further proposed as a potential therapeutic agent for inducing the terminal differentiation of GSC.[54] IFN-beta reduces the proliferation and self-renewal of GSC by inducing their differentiation through STAT3 signalling and, under those conditions, cytotoxicity of TMZ is increased.[54] The EGF receptor (EGFR) and/or its mutational variants such as the EGFR class III variant (EGFRvIII) often are over-expressed in GBM.[55] Enhanced EGFR signalling stimulates proliferation, angiogenesis and tissue invasion by glioma cells and inhibits their apoptosis. A number of inhibitors of EGFR kinase have been produced during past years.

Table 2 – Potential targets for future GSC therapy.

Target	Refs.
Pharmacological	
Akt pathway	18
TGF-beta pathway	49–51
BMP pathway	52,53
STAT3 pathway	54
EGFR pathway	56–58
Notch pathway	59,60
DNA damage response	6,7,42,88,89
Immunological	
IL6 signalling	70
GSC specific antigens	71
SOX6 antigen	72
NK cells-mediated killing	73
EGFR	74
VEGF	75–77
Viral	
Infection with oHSV vectors	81
Infection with lentiviral pseudotyped vectors expressing HSV-1-tk	83
Infection with lentiviral vectors expressing DCX	84
Expression of the *Prkg2* gene encoding cGKII	86
Lentivirus-mediated silencing of *SirT1*	87

The effects of two of them, erlotinib and gefitinib, on the proliferative activity of GSC have been investigated by Griffero and colleagues.[56] Seven GSC lines (GBM 1–7) were characterised for their tumourigenic properties, expression of tumour stem cell markers (CD133, nestin) and differentiation capacity. The GSC cultures were exposed to increasing concentrations of erlotinib and gefitinib and their survival evaluated after 1–4 d. Most GSC lines were sensitive to both inhibitors, although GBM2 was completely insensitive and GBM7 was sensitive to the highest concentration only. EGFR was inhibited by erlotinib and gefitinib in all GSC, independently of the cytotoxic effect. Akt phosphorylation was completely insensitive to both drugs in GBM2 and sensitive to the highest concentration only of erlotinib in GBM7, thus correlating with the antiproliferative and apoptotic effects of the inhibitors. Low expression of PTEN homologue was further observed in GBM2, also correlating with the complete insensitivity of this tumour to the drugs. Hence EGFR inhibitors may be useful to limit proliferation of GBM cells *in vitro*, but only under conditions of PTEN expression and Akt inhibition. Transfer of these results to the bedside is unfortunately a far cry. A recent review of published clinical studies failed to observe effects of EGFR inhibitors on glioma patients significant enough to warrant a change in clinical practice. In a few studies, tissue analyses of PTEN and Akt were reported, with inconsistent results on efficacy correlation.[57] Likewise, results from a recent clinical trial showed that cetuximab, a chimeric monoclonal antibody that inhibits EGFR did not demonstrate in fact any significant benefit in patients with recurrent glioma.[58] Inhibition of Notch pathway might be considered to augment the efficacy of radiotherapy against gliomas.[59,60] GSI reduce Notch signalling and enhance the cytotoxic effect of IR specifically against GSC and this effect is mediated by diminished Akt activity. The DNA-damage checkpoint response was not affected. Similar sensitising effects and reduced capacity of GSC for xenograft tumour formation were further obtained after knockdown of Notch1 or Notch2.[59,60] The DNA damage response pathway may represent one possible target for GSC-specific therapy (Fig. 2A). 2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (KU-55933) is an ATP-competitive inhibitor that specifically targets ATM as compared to other phosphatidylinositol 3′-kinase-like kinases.[61] Cellular inhibition of ATM by KU-55933 resulted in ablation of IR-dependent phosphorylation of a range of ATM targets, including p53, gammaH2AX and Nijmegen breakage syndrome (NBS) 1. Exposure to KU-55933 significantly sensitised HeLa cells to the cytotoxic effects of IR and to the DNA DSB-inducing chemotherapeutic agents etoposide, doxorubicin and camptothecin. Inhibition of ATM may be a promising approach to sensitise GSC to radiation. One further GSC sensitising drug might be Gö6976 which showed ability to abrogate S and G2 arrest and enhance the cytotoxicity of the topoisomerase I inhibitor SN38 in p53-defective cells.[62] Cell viability studies demonstrated that Gö6976 was relatively nontoxic and in a comparative analysis of the specificities of 65 commonly used small molecule kinase inhibitors for inhibition of a panel of 80 purified protein kinases, Gö6976 exhibited specificity against Chk1.[63] At sub-micro molar concentrations, the specificity of Gö6976 against Chk1 was over 40-fold that of Chk2, 100-fold that of mitogen activated protein kinase-activated protein kinase (MAPKAP-K) 2 and 30-fold that of mitogen activated protein kinase kinase (MKK) 1 and MKK2.[63] These properties make Gö6976 a promising candidate for preclinical and clinical studies aimed at targeting Chk1.[62,64] A number of additional inhibitors of checkpoint proteins are available or under development (Fig. 2A).[42,65–67] Their use may be worth exploring to reverse the cell cycle block of GSC, push them into proliferation and sensitise them to radiotherapeutic and chemotherapeutic agents.[7]

3.2. *Immunological targeting*

The immuno-suppressive role of GSC has been demonstrated in a number of recent studies. Di Tomaso and colleagues[68] described lower immunogenicity and higher suppressive activity of GSC as compared to non-stem glioma cell lines. Consistently, Wei and colleagues[69] have observed that GSC markedly inhibited T-cell proliferation and activation, induced regulatory T cells and triggered T-cell apoptosis. Hence, GSC may contribute to tumour evasion from immunosurveillance thus making approaches that enhance the immunological response to GBM worth investigating (Table 2). For instance, GSC growth and survival may be spurred by interleukin (IL) 6 signalling and targeting IL6 may be a research possibility.[70] The IL6 receptors interleukin 6 receptor alpha (IL6Ralpha) and glycoprotein 130 (gp130) are preferentially expressed in GSC. Increased survival of mice bearing GSC-driven glioma tumours has been observed after targeting IL6Ralpha or IL6 expression and decreased tumour growth and proliferative capacity of GSC is evident after treatment with IL6 antibody (Fig. 3A). In patients, elevated IL6 ligand and receptor expression are associated with reduced survival indicating the potential clinical utility of treatments targeting IL6 signalling.[70] Likewise differentiated tumour cells, GSC are targeted and killed by cytolytic T lymphocytes (CTL) through a perforin-mediated mechanism. Using a panel of early-passage human GSC, Brown and colleagues[71] have demonstrated that GSC derived from high-grade gliomas may be recognised and eliminated by CD8(+) CTL (Fig. 3B). There is no discernible difference between GSC and serum-differentiated CD133(–) cells or established glioma cell lines with respect to expression of human leucocyte antigen (HLA) class I and inter cellular adhesion molecule (ICAM) 1, ability to trigger degranulation and synthesis of cytokines by antigen-specific CTL and susceptibility to cytolysis *via* a perforin-mediated mechanism. GSC further display ability to process and present antigens as suggested by the observations that GSC can be eliminated by CTL in an antigen-specific mode when the antigen is endogenously expressed and GSC with tumour-initiating capacity can be effectively eliminated *in vivo* by antigen-specific CTL (Fig. 3B). SOX6 is a human antigen used for induction of specific CTL against glioma.[72] SOX6-derived peptides may be specific targets for T-cell-mediated immunotherapy targeting SOX6-positive GSC. *In vitro* stimulation with the HLA-A24-restricted peptide $SOX6_{628}$ was capable of inducing SOX6 peptide-specific CTL in peripheral blood mononuclear cells derived from healthy donors and glioma patients. These CTL were able to lyse a majority of glioma cell lines and a GSC line derived from human glioblastoma in an HLA class I-restricted and an antigen-dependent manner. In

particular, CTL-SOX6$_{628}$ efficiently lysed HLA-A24(+)/SOX6(+) GSC and the blockade of HLA class I with a specific monoclonal antibody inhibited the specific lysis of CTL-SOX6$_{628}$ against this GSC line. Hence, the SOX6 peptide is an immunogenic peptide that is naturally processed and expressed with HLA-A24 on GSC and its use may represent a possibility for T-cell-based targeting of GSC. GSC are susceptible to lysis by allogeneic and autologous natural killer (NK) cells activated by IL2 or IL15.[73] No protective HLA class I molecules have been found on GSC lines while ligands of activating NK receptors that trigger optimal NK cell cytotoxicity may be expressed. As aforementioned (see Section 3.1) growth factors-mediated signalling may sustain the self-renewal of GSC. As EGFR has been reported to affect the radiation response, two monoclonal antibodies (mAb) to EGFR, nimotuzumab and cetuximab have been used as radiosensitisers in a murine glioma model *in vivo*.[74] Both subcutaneous and intracranial tumours were studied. Subcutaneous tumour growth driven by U87 human glioma cells was significantly delayed after combined treatment with IR and both nimotuzumab and cetuximab. Brain tumour sizes and tumour cell invasion provoked by radiotherapy were reduced as well. Unlike cetuximab, nimotuzumab showed antiangiogenesis activity and both antibodies reduced the total number of radioresistant CD133(+) GSC. Hence, these mAbs against EGFR target radioresistant GSC and may possess potential utility as radiosensitisers in human GBM. It has been demonstrated that GSC promote angiogenesis *via* increased expression of vascular endothelial growth factor (VEGF) and stromal-derived factor 1.[75,76] Tumours enriched for GSC exhibited increased vessel density, increased endothelial cell proliferation and tubule formation and increased endothelial progenitor mobilisation. Bevacizumab, an antibody to VEGF, has shown transitory efficacy for the treatment of recurrent or progressive GBM with high radiographic response rates and a modest toxicity profile. Accordingly, the US Food and Drug Administration (FDA) has granted accelerated approval for the use of bevacizumab in the treatment of recurrent GBM.[77] Albeit the shadowy spectre of resistance regularly re-appears after antiangiogenic therapies as well with inevitable disease progression and patients' death within a few months, this area of research certainly deserves increased efforts.

3.3. *Viral targeting*

Virotherapy, a therapeutic approach utilising conditionally replicative viruses may have a potential role to directly target self-renewing GSC (Table 2).[78] Yet, although some vectors for

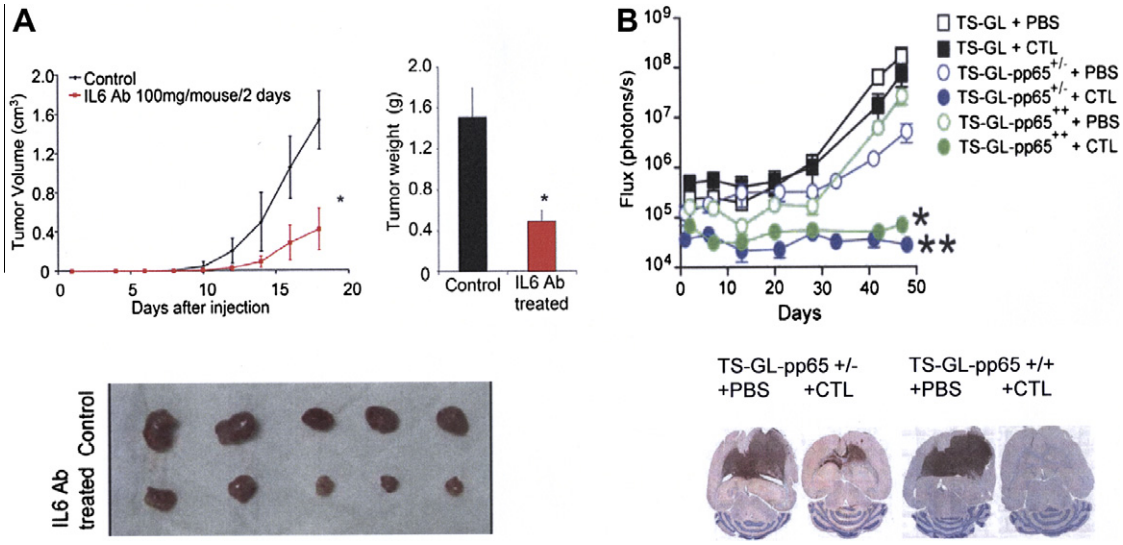
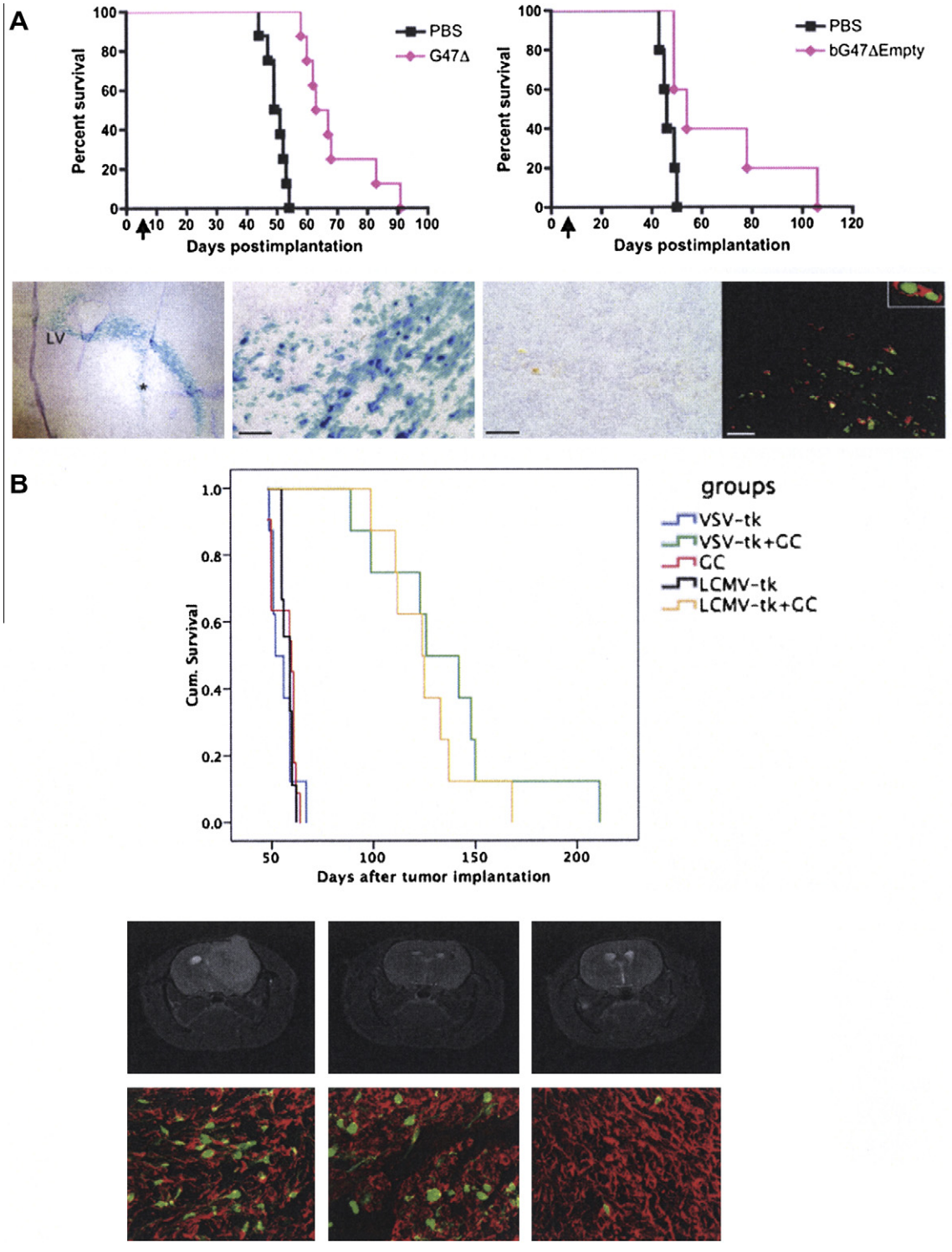


Fig. 3 – Immunological targeting of GSC. (A) Systemic treatment with an anti-IL6 antibody inhibits the growth of human glioma xenografts *in vivo*. (Top left) Subcutaneous tumour volume was significantly decreased with IL6 antibody treatment. Animals subcutaneously injected with GSC were intraperitoneally injected 24 h later with IL6 antibody at 100 mg every 2 d or phosphate-buffered saline (PBS) as a vehicle control. (Top right) Total tumour burden was reduced with IL6 antibody treatment as measured by tumour weight. *P < 0.05 as compared to non-targeting control. (Bottom) Images of xenografts measured in top right (from Ref. 70, with permission). (B) Cytomegalovirus (CMV)-specific CTL ablate the tumour initiation potential of pp65-expressing tumour spheres (TS). A patient brain tumour was passaged four times subcutaneously in the flank of immunocompromised mice before it was dissociated and expanded *in vitro*. TS expressing either EGFP:ffLuc [an engineered fusion between EGFP and firefly luciferase (ffLuc)] (TS-GL) or EGFP:ffLuc and the CMV pp65 antigen (TS-GL-pp65) were coinjected intracranial with pp65-specific CTL or PBS into NOD-scid mice. A biophotonic luciferase assay was used for assessment of cell-mediated cytotoxicity. (Top) Mean biophotonic flux of the intracranial tumours over time was determined by Xenogen imaging. *P = 0.017, when comparing TS-GL-pp65+/+ tumours that had been coinjected with CTL versus PBS at day 47; **P = 0.036, when comparing TS-GL-pp65+/– tumours that had been coinjected with CTL versus PBS at day 48. Bottom, images of horizontal brain sections from representative mice that had received TS-GL-pp65 +/– (left) or +/+ (right) cells with PBS or CTL and were stained with the B23 human cell marker (from Ref. 71, with permission). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gene therapy protocols have been successful in delivering genes *in vitro* and *in vivo* to specific cell types,[79] so far little has been achieved with human gliomas.[80] Major limits of this approach still reside in poor tumour selectivity and transduction efficiency as well as the still impending risk of iatrogenic tumours as the consequence of oncogene reactivation after random viral insertion. As a novel attempt against GSC, oncolytic herpes simplex virus (oHSV) vectors have been explored.[81] Infection with the oHSV G47delta strain that is genetically engineered for selective replication competence in cancer cells[82] impaired self-renewal of GSC as shown by their lost capacity to grow as neurospheres under stem culture conditions. Intratumoural injection of G47delta or a bacterial artificial chromosome (BAC) – derivative of it significantly improved survival of mice bearing GSC-driven orthotopic tumours (Fig. 4A).[81] Still using the rodent xenograft model of human GBM, the potential therapeutic effect of lentiviral pseudotyped vectors has been analysed by Huszthy



and colleagues.[83] Human glioma cells were transduced by both lymphocytic choriomeningitis virus glycoprotein (LCMV-GP)- and vesicular stomatitis virus glycoprotein (VSV-G)- pseudotyped lentiviral vectors while poor delivery was obtained using pseudotyped gammaretroviral vectors, similar to some previously used with negative results in clinical trials. GSC cells, characterised for their expression of CD133, nestin and SOX2 and their ability to grow in neurospheres and to differentiate in the presence of serum, could be transduced as well with pseudotyped lentiviral vectors. Insertion of the suicide gene herpes simplex virus thymidine kinase (*HSV-1-tk*) fused to enhanced green fluorescent protein (*EGFP*) made both types of lentiviral vectors able to mediate remission of orthotopic tumours in the presence of ganciclovir (GCV) as observed by magnetic resonance imaging (MRI). Accordingly, overall survival increased in comparison to controls (Fig. 4B). Surviving EGFP-positive tumour cells could be detected in relapses, indicating a need for repeated and prolonged treatments to eradicate the tumour-driving cells (Fig. 4B).[83] In a further attempt performed with the intracranial glioma tumour model in nude rats, the therapeutic effect of lentivirus-based expression of doublecortin (DCX) was investigated.[84] *DCX* is a brain-specific gene that is expressed specifically in neural stem/progenitor cells (NSPC) and newborn neurons but is absent in other brain cells such as astrocytes and oligodendrocytes.[85] DCX is induced by the glioma gene suppressor *PTEN* thus suggesting that tumour suppression by PTEN may be in part DCX-mediated.[85] Further, DCX expression in U87 glioma cells causes loss of malignant phenotype with proliferation arrest, loss of migratory capacity and inability to induce intracranial tumour development in immunodeficient animals.[85] DCX-expressing lentivirus was injected directly into orthotopic tumours developed by U87 human glioma cells. The tumour volume was reduced by 60% after DCX treatment and overall survival of animals was significantly improved. The neuronal markers MAP2, TUJ1 and PSA-NCAM and the glial marker glial fibrillary acidic protein (GFAP) were induced in the U87 tumour after DCX treatment and mitoses were blocked. No increased apoptosis was observed under DCX lentivirus infection but the expression of the proliferation marker Ki-67 and the blood vessel marker von-Willebrand factor (vWF) were inhibited. Hence, local delivery of DCX-expressing lentivirus may be a differentiation-based therapeutic possibility against glioma.[84] Expression of the *Prkg2* gene encoding cyclic guanosine monophosphate (cGMP)-dependent protein kinase II (cGKII) causes reduced colony forming ability and proliferation capacity in human glioma cells as compared to controls transfected with truncated Prkg2 (lacking the kinase domain) or empty vector.[86] pcDNA3.1 plasmid vector-mediated stable transfection of *Prkg2* caused *per se* reduced expression of the stem cell marker Sox9. When cGKII was activated by cGMP analogue administration, Sox9 expression was suppressed and glioma cells displayed loss of Akt phosphorylation and proliferation capacity. Similar effects were observed after Sox9 repression by small interfering RNA (siRNA). The analysis of stem and glial markers indicated that reduced cell proliferation could be linked to glial differentiation induced by

Fig. 4 – Virus-mediated targeting of GSC. (A) Intratumoural injection of oncolytic Herpes simplex virus vector G47Δ prolongs survival of mice bearing GSC xenografts. (Top) Kaplan–Meier survival curves of the mice bearing GSC xenografts treated with Herpes simplex virus vectors. Forty thousand GBM8EF cells (left) or 2×10^4 BT74 cells (right) (two CD133(+) GSC cell lines grown under NSC conditions) were implanted into the brains of athymic (left) or severe combined immunodeficient (right) mice. Six (left) or 7 (right) days later, G47Δ (left) or its BAC-derived equivalent bG47Δ-Empty (right; pink diamond) or PBS (black square) was stereotactically injected at the same coordinates as the tumour cells. Treatment with G47Δ resulted in significantly prolonged survival compared with mock treatment. Arrows, time of virus injection. Bottom. G47Δ infects GSC tumours *in vivo*. Twenty-four hours after injection of G47Δ (first and second from left) or PBS (third from left) into GBM xenografts, the brains were collected. X-gal staining of the sections (first, second and third from left) revealed an extensive infection of tumour tissue that displays a progression along white matter tracts (first from left). LV, lateral ventricle. *, injection track. Scale bar, 200 μm. Efficient *in vivo* infection by G47Δ is shown at higher power magnification (second from left), whereas no lacZ positivity is seen in a PBS-treated section (third from left). Scale bars, 50 μm. Fourth from left. Immunofluorescent staining showing colocalised detection of β-galactosidase (red) and human nuclei (green) on a G47Δ-infected brain section. Immunofluorescent colocalisation of β-galactosidase and human-specific nuclei antigen show that the majority of LacZ-expressing cells are of human origin. Scale bar, 50 μm (from Ref. 81, with permission). (B). Therapeutic efficiency of LCMV-GP and VSV-G pseudotyped lentiviral vectors *in vivo*. Intracranial gliomas were injected with LCMV-GP or VSV-G pseudotyped lentiviral vectors expressing HSV-1-tk fused to EGFP three weeks after tumour implantation. Seven days after vector infection, animals in both treated groups and in one control group were treated with GCV for 30 d. Top. Kaplan–Meier survival curves. The survival benefit for both treatment groups compared to control groups was statistically significant. There was no significant difference in survival between the two treatment groups. (Middle) Tumours treated with lentiviral vectors and GCV show complete remission on MRI. Representative three-dimensional MRI. First from left: lentiviral LCMV-GP vectors without GCV treatment. Second from left: lentiviral LCMV-GP vectors with 4-week GCV treatment. Third from left: lentiviral VSV-G vectors with 4-week GCV treatment. Bottom. Sections of recurrent tumours were stained with antibodies against human-specific nestin and analysed by confocal microscopy. Pictures show overlay of nestin (red) and EGFP transgene (green). First from left: recurrent tumour of animals treated with VSV-G pseudotyped lentiviral vectors with EGFP-positive cells in the invasive area. Second from left: recurrent tumour of animals treated with LCMV-GP pseudotyped lentiviral vectors. EGFP-positive tumour cells in residual small lesion from the primary tumour. Third from left: few EGFP-positive cells in a contralateral recurrent tumour (from Ref. 83, with permission). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cGKII. The embryonic stem cell gene *SirT1* is expressed in CD133(+) GSC from some tumours. *SirT1* expression has been knocked-down in CD133(+) GSC using a lentiviral vector expressing a short hairpin RNA (shRNA).[87] Under conditions of *SirT1* silencing, CD133(+) cells were sensitised to IR displaying increased IR-induced-apoptosis. Consistently, the effectiveness of radiotherapy to inhibit GSC-driven orthotopic tumour growth in nude mice was improved and overall survival of animals increased. Hence, virus-mediated modulation of *SirT1* expression may be a potential mode to increase the sensitivity of GSC to radiotherapy.

4. Conclusions

The identification of GSC has prompted a number of novel approaches using small molecules, immunomodulators and viruses to attack these cells in order to eradicate GBM. Small molecules are easier to use than biologicals and our feeling is that the pharmacological targeting approach will pave the way for novel and effective treatments. Although to date most of the proposed protocols are still far from the clinical settings and conclusions about their efficacy often are over-stated, it is more than a hope that intensive research on GSC targets will yield useful information to improve glioma patients' survival.

Conflict of interest statement

None declared.

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