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Blockade of transforming growth factor-β signaling enhances oncolytic herpes simplex virus efficacy in patient-derived recurrent glioblastoma models

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Abstract

Despite the current standard of multimodal management, glioblastoma (GBM) inevitably recurs and effective therapy is not available for recurrent disease. A subset of tumor cells with stem-like properties, termed GBM stem-like cells (GSCs), are considered to play a role in tumor relapse. Although oncolytic herpes simplex virus (oHSV) is a promising therapeutic for GBM, its efficacy against recurrent GBM is incompletely characterized. Transforming growth factor beta (TGF-β) plays vital roles in maintaining GSC stemness and GBM pathogenesis. We hypothesized that oHSV and TGF-β inhibitors would synergistically exert anti-tumor effects for recurrent GBM. Here we established a panel of patient-derived recurrent tumor models from GBMs that relapsed after post-surgical radiation and chemotherapy, based on GSC-enriched tumor sphere cultures. These GSCs are resistant to the standard-of-care temozolomide but susceptible to oHSVs G47Δ and MG18L. Inhibition of TGF-β receptor kinase with selective targeted small molecules reduced clonogenic sphere formation in all tested recurrent GSCs. The combination of oHSV and TGF-βR inhibitor was synergistic in killing recurrent GSCs through, in part, an inhibitor-induced JNK-MAPK blockade and increase in oHSV replication. *In vivo*, systemic treatment with TGF-βR inhibitor greatly enhanced the anti-tumor effects of single intratumoral oHSV injections, resulting in cures in 60% of mice bearing orthotopic recurrent GBM. These results reveal a novel synergistic interaction of oHSV therapy and TGF-β signaling blockade, and warrant further investigations aimed at clinical translation of this combination strategy for GBM patients.

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Keywords

Oncolytic HSV; recurrent glioblastoma; TGF-β; JNK

Introduction

Glioblastoma (GBM) is the most common primary malignant brain tumor in adults[1]. Despite the current standard management including surgery followed by radiotherapy and temozolomide chemotherapy, GBM remains lethal with median survival of only 14.6 months[2]. The refractory nature of GBM is characterized by its inevitable recurrence after intensive multimodality therapy. Once GBM recurs, median survival is only 4–7 months[3, 4]. Thus far, exploratory clinical investigations of a variety of molecular targeted and anti-angiogenic agents have failed to achieve enduring effects or overall survival benefit[5]. The culprit may be a subpopulation of GBM cells termed GBM stem-like or initiating cells (GSCs) possessing stem-like properties and sustaining tumor growth[6]. GSCs may survive cytotoxic therapy due to their inherent resistant phenotype and propagate recurrent disease. Thus GSCs represent a critical therapeutic target. In addition, patient-derived GSCs maintain patient-specific genetic and phenotypic alterations, and provide clinically representative GBM models suitable for therapeutic development[7, 8]. However, there is a paucity of the preclinical models of recurrent GBM that are based on patient-derived GSCs.

Oncolytic herpes simplex virus (oHSV) is a genetically engineered HSV that selectively replicates and kills cancer cells, while leaving non-neoplastic cells unharmed[9]. oHSVs have been extensively studied in both preclinical and clinical settings as a potential therapeutic for GBM. Early clinical trials that assessed oHSVs for malignant gliomas demonstrated the safety and feasibility of inoculating oHSV into the human brain, but their efficacy remains anecdotal[9]. Maximizing the therapeutic potential of oHSV will likely require its use in conjunction with other anti-cancer modalities such as chemotherapeutics or molecular targeted agents[10]. In fact, our group has reported beneficial or synergistic anti-GBM effects by oHSV combinations with cytotoxic chemotherapeutics[11, 12], PI3kinase inhibitors[13] and anti-angiogenic agents[14, 15], via distinct mechanisms of action in orthotopic GSC GBM models. Although in the past recurrent GBM tended to be the primary target for oHSV clinical trials, the activity of oHSV against recurrent GBM has not been preclinically validated in detail. Selective targeting of signaling pathways critical for GSC maintenance or resistance might provide a means for novel combination strategies with oHSV to enhance efficacy in the treatment of refractory, recurrent GBM.

The transforming growth factor beta (TGF-β) family of cytokines, TGF-1, 2, and 3, have diverse functions in the pathogenesis of cancer including GBM[16–18]. TGF-β1 and 2 are highly expressed in malignant gliomas and association with poor prognosis has been reported[19, 20]. TGF-β interacts with its cognate cell surface receptor tyrosine kinase TGF-βR2, which forms heterodimers with TGF-βR1 (also termed ALK5) and activates both canonical signaling pathways through phosphorylating the signaling transducer Smads and non-canonical pathways such as MAP kinase pathways. TGF-β signaling drives a variety of malignant phenotypes of GBM, including invasion/migration, angiogenesis, drug/radiation

resistance and immune-suppression[17, 18]. Importantly, accumulating evidence indicates that TGF-β signaling plays a vital role in the maintenance of GSC stemness and promotes GBM oncogenesis[21, 22]. These observations support the TGF-β signaling pathway as a promising therapeutic target in GBM and its GSC subpopulation. As such, a number of small molecule inhibitors of TGF-βR kinases (hereafter abbreviated as TβR) are in preclinical and clinical development. TβR inhibition by the selective TβRI/II dual small molecule inhibitor LY2109761 radiosensitized GSCs and combination treatment of radiation and LY2109761 extended survival in orthotopic GSC-derived GBM in mice[23]. A first-in-human dose study of the TβR1 kinase inhibitor galunisertib (LY2157299) revealed its safety[24], and early phase clinical trials evaluating galunisertib for GBM are ongoing[25].

The current study was devised to address our hypothesis that oHSV and TβR inhibitors would synergistically exert anti-tumor effects for recurrent GBM. Using a panel of patient-derived recurrent GBM models based on GSC-enriched tumor sphere cultures isolated from recurrent GBM resections, we show anti-tumor effects of targeted TβR inhibition and oHSV. Furthermore, combination treatment of TβR inhibitor and oHSV is synergistic *in vitro*, and mediates markedly enhanced therapeutic effects for recurrent GSC GBM models *in vivo*.

Materials and methods

Cells and xenograft models

Eight recurrent GSC lines (MGG24R, MGG31, MGG45, MGG50, MGG85, MGG91. MGG111R, MGG123) were established from GBMs that recurred after standard adjuvant therapy including surgical resection, radiotherapy, and temozolomide chemotherapy, and these are referred to as recGSCs. Primary GSCs (MGG4, MGG8, and MGG64) were established from newly diagnosed GBMs[7, 8], and termed as newly diagnosed GSCs. Tissue collection was approved by the Institutional Review Board at Massachusetts General Hospital, and the method to initiate culture has been previously described[7]. All GSCs were cultured as neurospheres in defined serum free media supplemented with EGF and FGF-2[7]. Vero cells (African green monkey kidney cells) were obtained from the American Type Culture Collection, and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Mediatech) supplemented with 10% calf serum. GSCs were intracerebrally implanted in SCID mice (NCI) as described[7, 8] and mice were euthanized when they developed significant neurological deficits or general condition deterioration (e.g. >15% body weight loss) for brain collection and pathological studies. *EGFR* copy number was determined by FISH as described[26, 27]. Targeted sequencing using SNapShot genotyping was used to detect hotspot mutations in *TP53* and *PTEN*[27]. EGFRVIII mutant was detected by RT-PCR following the method described in[28].

Viruses

oHSV G47Δ contains deletions of both copies of γ34.5, α47, and a LacZ insertion inactivating ICP6[29]. G47Δ-mCherry and G47Δ-US11fluc are recombinant HSVs derived from G47Δ, and express mCherry driven by the HSV IE4/5 immediate-early promoter and firefly luciferase driven by the HSV US11 true late promoter of HSV-1, respectively[30].

oHSV MG18L contains a deletion of US3 and a LacZ insertion inactivating ICP6[13]. All viruses were grown, purified, and titered on Vero cells.

Reagents

SB431542 (Sigma-Aldrich), LY2109761 (Selleckchem, Houston, TX), and galunisertib (LY2157299, Selleckchem) are selective and reversible inhibitors of the TGF-β receptor type I kinase. Selective and reversible JNK inhibitor SP600125 and the alkylating agent temozolomide were from Sigma-Aldrich. All the regents were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Recombinant human TGF-β1 (Peprotech) was dissolved in 0.1 M citric acid.

Immunohistochemistry

Immunohistochemistry (IHC) for CD44 (Anti-CD44 from Cell Signaling Technology), human nestin (Santa Cruz) and YKL-40 (Quidel) was performed on formalin-fixed paraffin embedded sections using the protocol described previously[7] with slight modifications. For antigen retrieval, 0.1% saponin (Sigma) treatment for 5 minutes was used for CD44 and YKL-40, and microwave in citrate buffer for human nestin.

Western blot

Immunoblot was performed as described previously[12]. Briefly, lysates were separated by 4–15% SDS-PAGE, and electro-blotted to PVDF membranes. After blocking with 5% non-fat dry milk, membranes were incubated with primary antibodies, followed by appropriate peroxidase-conjugated secondary antibodies. Primary antibodies used were: anti-Smad 2/3, phosphor-Smad2/3, JNK, phospho-JNK (all from Cell Signaling Technology), and Vinculin (Thermo).

RT-PCR

Total RNA was isolated from GSCs using Trizol reagent (Invitrogen) and first strand DNA was synthesized using Superscript II (Invitrogen). Real time PCR was conducted using SYBR green master mix (Applied Biosystems) in a StepOnePlus Real-time PCR System (Applied Biosystems). PCR primer sequences are: *TGFB1* (TGF-β1, forward: GGCTACCATGCCAACTTCTG, reverse: CCGGGTTATGCTGGTTGTA), *TGFB2* (TGF-β2, forward: TTCAGACACTCAGCACAGCA, reverse: TTGGGTGTTTTGCCAATGTA), *TGFBR2* (TGF-β receptor II, forward: TGTGTCGAAAGCATGAAGGA, reverse: GGTCCCAGCACTCAGTCAAC) and GAPDH (forward: CAATGACCCCTTCATTGACC, reverse: GACAAGCTTCCCGTTCTCAG).

Sphere formation assay

To measure clonogenicity of GSCs, sphere formation assay was performed as described previously[7]. Briefly, 1 or 5 cells / well were seeded into 96 well plates with TβR inhibitor or DMSO control, and spheres (> 80 μm in diameter) were counted 12–14 days later.

Cell viability and cell count assay

Dissociated GSCs were plated in triplicate in 96-well plates and treated with drug or virus the next day and incubated at 37°C for 5 days. Cell viability was measured by incubating with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Sigma) for 2–4 hours and absorbance measurement at 490nm on a plate reader. For cell count assay, GSCs were plated in 24-well plates and treated with TβR inhibitor and virus for 5 days. Viable cells that exclude trypan blue were counted on a hemocytometer.

Virus spread and yield assay

Dissociated GSCs were plated with 4×10^4 cells/well in 24-well plates. The next day, cells were treated with indicated doses of TβR inhibitors and/or MG18L or G47Δ at MOI=1 in triplicate. Cells and media were harvested at indicated time points, and virus released by 3 cycles of freeze/thaw was titrated by plaque assay on Vero cells. Fluorescent and phase contrast microscope pictures were captured on Day 1, 3 and 5 post-infection of GSCs to assess spread of G47Δ-mCherry (MOI=0.1).

Luciferase assay

GSCs were plated into 96-well plates in triplicate and next day treated with drug and G47Δ-Us11fluc as indicated. Twenty four hours post infection, D-Luciferin (Gold Biotechnology) was added to cells to 1 mM and luminescence was immediately measured on a BioTek Synergy HT multipurpose plate reader.

Chou-Talalay synergy analysis

The interaction of TβR inhibitors and virus on GSCs *in vitro* was determined with Chou-Talalay analysis[31] as described in[12, 32].

Caspase 3/7 activity assay

GSCs were plated at 5000 cells per well into 96-well plates and treated with drug and virus. Twenty four hours later, activation of the execution caspases 3/7 was measured by the Caspase-Glo 3/7 assay kit (Promega), following the manufacturer's instructions.

Treatment studies in orthotopic GSC tumor models

One hundred thousand MGG31 GSCs (in 3 μl) were stereotactically implanted into the right cerebrum of anesthetized 7–8-week-old female SCID mice (2.3 mm lateral of bregma, 2.5 mm deep from dural surface). Mice were randomized and assigned to 4 treatment arms (N=6 / group): 1) control (vehicle and PBS), 2) vehicle and MG18L, 3) LY2157299 and PBS, and 4) combination of MG18L and galunisertib. Galunisertib (100 mg/kg) or vehicle was given by oral gavage daily from day 7 to 16. MG18L (1×10^6 pfu/3 μl) or PBS was injected stereotactically into the tumors on day 9. Mice were followed for health status with body weight measurement 2–3 times a week. Mice were euthanized when they developed significant neurological deficits or general condition deterioration (>15% body weight loss). Survival was analyzed by using the Kaplan-Meier method. All the animal procedures were approved by the IACUC at Massachusetts General Hospital.

Statistical analysis

Student *t* test (2-sided) was used to compare 2 groups. Statistical differences of animal survival were determined by log-rank test. *P* values <0.05 were considered significant. Statistical analyses were done with Excel and Graph Pad Prism.

Results

Establishment of recurrent GSCs based orthotopic xenograft models

To develop therapeutic strategies for recurrent GBM, we first established a panel of patient-derived GSC-based xenograft models of recurrent GBM that are based on neurosphere-forming stem-like GBM cells. By applying the stem cell enriching culture methodology that enabled us to establish a large panel of primary GSC lines from newly-diagnosed GBM[7], we were able to establish 8 orthotopic recurrent GBM (recGBM) PDX models (Fig. 1A). The recurrent GBM models demonstrated highly variable in vivo phenotypes: human specific nestin staining revealed that 4 lines (MGG24R, 45, 50, and 111R) exhibited extensive tumor invasiveness/migration and infiltration to brain parenchyma, 1 line (MGG91) showed semi-invasiveness, while the other 3 lines (MGG31, 85, and 123) showed a nodular phenotype with relatively discrete tumor-brain borders (Fig. 1A). One of the nodular recGSCs, MGG85, displayed a characteristic sarcomatous histology (Supplementary Fig. S1). MGG123 was previously described and contained pseudopalisading necrosis with a hypoxic microenvironment[33]. Genetically, MGG24R, 45, 50 and 111R had *EGFR* amplification, with MGG24R having the EGFRvIII mutant. All of these *EGFR*-amplified lines were invasive *in vivo*, in line with the reported function of EGFR in promoting invasiveness of GBM[34, 35]. MGG45 and 50 had *PTEN* mutations, and MGG85 gliosarcoma had a TP53 mutation (p.Arg248Trp).

Mesenchymal-like phenotype and activated TGF-β pathway in recurrent GSCs

CD44 has been shown to be a representative marker of the mesenchymal subtype of GBM[36–38]. We therefore used CD44 IHC as a surrogate of a mesenchymal phenotype of GBM to characterize our tumor models. Tumor cells were strongly immuno-positive for CD44 in all the recGBM models tested, while the newly diagnosed GSC xenograft of the proneural subtype (MGG8) was negative (Fig. 1B). Another mesenchymal marker YKL-40 was immuno-positive in the majority of the recGBM models, although staining was variable and typically patchy (Supplementary Fig. S1). TGF-β signaling pathway has been implicated in epithelial-to-mesenchymal transition (EMT) in cancer[16, 39, 40] and mesenchymal GBM[38]. RT-PCR analysis of TGF-β1, 2, and TβRII showed a tendency toward increased expression of these components of the TGF-β signaling pathway in our recGSC lines compared with newly diagnosed GSCs *in vitro* (Fig. 1C, Supplementary Fig. S2A). Furthermore, most of the recGSC lines had activation of the canonical TGF-β signaling *in vitro* as shown by phosphorylated forms of the TβR downstream signaling molecules Smad2 and Smad3 (p-Smad2/3) in western blots (Fig. 1D).

Since these recGSCs were isolated from recurrent GBM having failed the standard of care radiation and the alkylating agent temozolomide (TMZ), they were expected to be resistant to temozolomide *in vitro*. Indeed, *in vitro* cell viability assays of the recGSCs demonstrated

resistance to TMZ, with IC50 values typically >400 μM, as opposed to some of our previously characterized TMZ-sensitive primary GSC lines (MGG4 and MGG8) (Fig. 1E, Supplementary Table). Thus we established a set of orthotopic GBM models generated from recurrent GSCs that display a mesenchymal phenotype and have an activated TGF-β pathway.

Suppression of TGF-β pathway decreased clonogenicity and viability of recurrent GSCs

The TGF-β signaling pathway has been shown to play a role in the maintenance of GSC[21, 22], however its role in recGSCs is unknown. We therefore tested small molecule inhibitors that selectively target TβRI kinase for their impact on recGSC phenotypes *in vitro*. Supplementation of recombinant TGF-β1 to stem cell culture media potently activated p-Smad2/3 in recGSCs (Fig. 2A). Exposure of recGSCs to TβR inhibitors, SB431542, LY2109761, and galunisertib, almost completely shut down p-Smad2/3 in the presence or absence of TGF-β1 (Fig. 2A). TβR inhibitors reduced cell viability of recGSCs typically at relatively high doses (> 50μM), with SB431542 generally being the most potent (Fig. 2B, Supplementary Table). Clonogenic assays showed that SB431542 at non-cytotoxic doses consistently inhibited sphere formation of recGSCs (Fig. 2C, Supplementary Fig. S2B), supporting its activity on self-renewal of recGSCs. Thus, we show that TGF-β signaling promotes clonogenicity and viability of recGSCs.

oHSV infects, spreads, and kills recurrent GSCs in vitro

We next investigated oHSV as a therapeutic for recGSCs. Infection of 6 recGSC lines with G47Δ-mCherry at low MOI resulted in the spread of oHSV within the spheres over 3–5 days post infection (Fig. 3A). We then determined the cytotoxicity of two clinically translatable oHSVs, G47Δ and MG18L, in recGSCs. Cell viability assays revealed that all 8 recGSC lines tested were susceptible to both oHSVs, with MG18L more efficacious in 4 lines (MGG31, MGG45, MGG91 and MGG111R) (Fig. 3B, Supplementary Table).

Combination of oHSV and TGF-β receptor inhibitors synergistically kills recurrent GSCs

We then combined oHSV and TβR inhibitors and studied whether the two agents acted synergistically in killing recGSCs. Synergy was measured using the median-effect method of Chou-Talalay[12, 31, 32]. Across the different recGSC lines, the interaction of oHSV (G47Δ and MG18L) and TβR inhibitors (SB431542 and galunisertib) was synergistic at most fraction affected doses (Fig. 4AB, Supplementary Fig. S3). The beneficial impact of combination therapy was confirmed using viable cell count assays, where the oHSV/TβR inhibitor combination was superior to either monotherapy in mediating recGSC death (Fig. 4C).

TGF-β receptor inhibition potently synergizes with oHSV in an orthotopic recurrent GSC xenograft model in vivo

We next asked whether combination therapy with oHSV and TβR inhibitor improves therapeutic efficacy in orthotopic GBM models generated from recGSCs. We chose galunisertib as the TβR inhibitor to use as this is currently being investigated in clinical trials for recurrent GBM (NCT01582269, NCT02423343, NCT01220271). The MGG31 model

was selected because of this line's consistent tumorigenic ability and small variation in survival times. Systemic treatment of galunisertib alone had no effect on survival of mice bearing orthotopic MGG31 xenografts, although 1 animal lived an additional 200 days (Fig. 5A). Single intratumoral injection of MG18L significantly extended animal survival (approximately 75% prolongation of median survival over control), but all animals succumbed to tumor progression eventually. In contrast, combination treatment of systemic LY2157299 and local MG18L greatly extended survival, with 60% of the treated mice surviving long-term with no evidence of tumor at autopsy on day 400. We did not observe noticeable adverse events in any treatment group as evidenced by the lack of changes in body weight (Fig. 5B). Thus, TβR inhibition dramatically potentiated the therapeutic efficacy of oHSV in an orthotopic recurrent GSC GBM model *in vivo*.

TGF-β receptor inhibition increases oHSV replication

We next asked whether increases in oHSV replication played a role in the benefit of combination therapy. We first used G47Δ-US11fluc since the activity of its reporter, firefly luciferase, reflects late gene expression, which serves as a surrogate for virus replication[30]. There was a consistent increase in G47Δ-US11fluc-induced luciferase bioluminescence when infected recGSC lines were treated with TβR inhibitors SB431542 or galunisertib (Fig. 6A). We next determined virus yields after infecting recGSC lines with G47Δ or MG18L with or without TβR inhibitors. At 48 hours post infection, there was about a 1.5–2-fold and 2–6-fold increase in oHSV yields with G47Δ and MG18L, respectively, when co-treated with SB431542 or galunisertib (Fig. 6B, Supplementary Fig. S4). Addition of recombinant TGF-β did not alter the replication of oHSV at the doses tested (Supplementary Fig. S5). These data indicate that TGF-β signaling inhibition leads to increased oHSV replication, which may underlie the synergistic interaction between oHSV and TβR inhibitors in recGSCs.

TGF-β receptor inhibition attenuates JNK, and JNK inhibition increases oHSV replication

HSV infection activates the JNK-MAPK signaling pathway, which could regulate the survival of infected cells and induce apoptosis in some contexts[41–43]. Given an involvement of JNK signaling pathway in non-canonical signaling pathways triggered by TGF-β receptor activation[44], we tested whether the JNK pathway played a role in the increased oHSV replication induced by TβR blockade. Exogenous recombinant TGF-β activated JNK phosphorylation in recGSCs, which was suppressed by TβR inhibitor SB431542 *in vitro* (Fig. 6C). This result led us to test SP600125, a small molecule compound selectively inhibiting JNK, to determine the function of the JNK pathway in oHSV therapy of recGSCs. A non-cytotoxic dose (600 nM) of SP600125 (Supplementary Fig. S6A) blocked phosphorylation of JNK (Fig. 6D), and increased luciferase expression upon G47Δ-US11fluc infection of recGSCs (Supplementary Fig. S6B). Virus yield assays confirmed that pharmacological inhibition of JNK enhanced yields of G47Δ and MG18L at 48 hours post-infection (Fig. 6E, Supplementary Fig. S5C). SP600125 attenuated oHSV-induced caspase 3/7 activation in recGSCs, to a larger extent with MG18L, suggesting that SP600125 blockade of apoptosis in infected cells may contribute to increases in oHSV yields (Fig. 6F).

Discussion

The current standard of care for newly diagnosed GBM is unable to prevent tumor recurrence, and no effective therapy exists for recurrent GBM. Recurrent GBM is characterized by resistance to therapy, dissemination to distal regions of the CNS, and aggressive behavior[45]. Such phenotypic traits of recurrent GBM are accompanied by molecular alterations, such as increased stemness marker expression, a hypermutator genotype caused primarily by TMZ, and clonal evolution from the primary GBM[45, 46]. Preclinical models that represent clinical recurrent GBM will be useful for a better understanding of its unique biology and development of novel therapeutic approaches, which are sorely needed. However, infrequent biopsy for recurrent disease and the mixed presence of treatment effects (i.e., radiation necrosis), and low viable tumor cells limit the utility of patient specimens. Herein, we characterized a set of orthotopic recurrent GBM models that are based on GSCs derived from post-standard-of-care recurrent GBM. These models are variable in both *in vivo* phenotypes (invasive vs nodular phenotypes) and genotypes (e.g., EGFR and p53 status), likely reflecting the diverse molecular alterations in recGBM between patients[46]. Despite this heterogeneity, we observed consistent expression of the mesenchymal marker CD44 in our recGBM models. While EMT is a well-documented process that is involved in malignant progression in cancer, EMT or an analogous phenomenon (mesenchymal shift) seems to occur in GBM recurrence[36, 47, 48]. Recently, this was confirmed by Wood et al, who, through analysis of paired primary and recurrent GBM samples, observed upregulated expression of mesenchymal proteins including CD44 and YKL-40 upon recurrence when initial tumors have lower levels[49].

TGF-β has diverse functions in the pathogenesis of cancer including GBM[17] and plays a pivotal role in proliferation, invasion, therapy resistance, immune suppression and GSC maintenance in GBM[17, 18]. In addition, the TGF-β/Smad signaling pathway has been shown to promote EMT in cancer[16, 40] and *TGFBR2* overexpression marks the mesenchymal subtype of GBM[37]. In our recurrent GBM models, TGFβ signaling was active as TGF-β1, TGF-β2 and TβR were highly expressed and phosphorylation of canonical signaling molecules Smad 2 and 3 was present. Small molecule inhibition of TβR blocked Smad2/3 phosphorylation and potently inhibited sphere formation of our recGSC, consistent with prior reports showing the critical role of TGF-β in GSC self-renewal[21, 22, 50]. The TβR inhibitor LY2109761 has previously been demonstrated to block both TβR1 and TβR2 and have anti-GBM effects in GSC-derived orthotopic models[23, 50]. In our orthotopic recGSC-derived model, MGG31, we did not observe significant survival benefits with TβR inhibitor monotherapy.

The recGSC lines we tested were permissive to oHSV, as both G47Δ and MG18L exhibited the ability to infect, spread and kill our recGSCs in vitro. MG18L was more potent than G47Δ against 4 recGSCs (i.e., MGG31, 45, 91, and 111R), and single intratumoral injection showed significant monotherapy efficacy in an intracranial recurrent GBM model. Given that our recGSCs are resistant to the standard-or-care agent TMZ, the consistent potency of oHSV is particularly meaningful, and underscores the distinct cytotoxic mechanism employed by oHSV. Importantly, combination of oHSVs (G47Δ and MG18L) and TβR inhibitors (SB431542 and galunisertib) was synergistic in the treatment of multiple recGSCs

in vitro. We found that inhibition of the TGF-β signaling significantly increased virus yield in multiple recGSCs, which may have contributed to synergy. Mechanistically, we observed that TβR inhibitor-mediated blockade of the JNK-MAPK pathway, one of the SMAD-independent pathways that TGF-β activates[44], mediated viral replication enhancement. JNK signaling activation in HSV-infected cells is considered to be an innate defense mechanism, leading to caspase cascade activation and apoptosis of infected cells[41, 42]. A selective inhibitor of JNK (SP600125) similarly blocked JNK phosphorylation and increased oHSV amplification in recGSCs, suggesting a role for non-canonical TGF-β/JNK signaling in regulating oHSV replication in GBM. However, it is possible that SMAD-mediated transcriptional activation is also involved in modulating oHSV replication.

Despite the lack of *in vivo* activity of TβR inhibitor monotherapy in our recurrent GBM GSC model, galunisertib greatly potentiated the anti-GBM activity of oHSV MG18L in an orthotopic recurrent GBM GSC model, resulting in >60% long-term survivors compared with none in the single treatment groups. *In vivo* models in the current work are orthotopically transplanted xenografts in SCID mice that lack both T and B lymphocytes. Innate immune cells, particularly NK cells, have been shown to affect the outcome of oHSV therapy in xenografts by, in part, promoting the clearance of virus-infected cells and inhibiting intratumoral virus spread (reviewed in[51, 52]). TGF-β regulates the function of NK cells[53]. Han et al reported that pre-treatment of animals with TGF-β augmented the therapeutic effects of oHSV in both xenograft (human GBM) and syngeneic GBM models *in vivo*, phenocopying the outcomes of innate immune NK cell depletion[54]. On the other hand, Ilkow et al demonstrated that TGF-β produced by tumor cells reprogrammed nearby cancer-associated fibroblasts and rendered them sensitive to oncolytic rhabdovirus infection, leading to enhanced overall virus replication[55]. Thus, GBM-produced TGF-β acts on different cell types and impacts the complex cellular cross-talk between GBM and stromal/immune cells within the tumor microenvironment, which likely affects oncolytic virus activity. Although these published reports and our current work seem discordant, the role of TGF-β in oHSV virotherapy may be context- and schedule-dependent. It is possible that both exogenous TGF-β and TGF-β inhibition could enhance oHSV therapy via different mechanisms.

Using clinically relevant orthotopic patient-derived GSC recurrent GBM models, we observed a potent, synergistic anti-tumor interaction between TβR inhibition and oHSV therapy that warrants further investigation as a treatment for refractory GBM. However, the limitations of our animal study include: 1) treatment at an early time-point when tumors are perhaps only initiating, and 2) the use of a nodular (poorly invasive) GBM model. Further research, such as evaluation in additional models including invasive xenografts and immune-competent GBM models, is necessary to define the role of TGF-β and optimize the use of TβR inhibitor toward maximizing oHSV therapy for recurrent GBM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty and Impact

No effective treatment exists for glioblastoma once it recurs after standard of care. This work demonstrates that blockade of TGF-β signaling pathway enhances the therapeutic efficacy of oncolytic herpes simplex virus in patient-derived cancer stem cell-based recurrent glioblastoma models, supporting a clinical trial testing this combination approach.

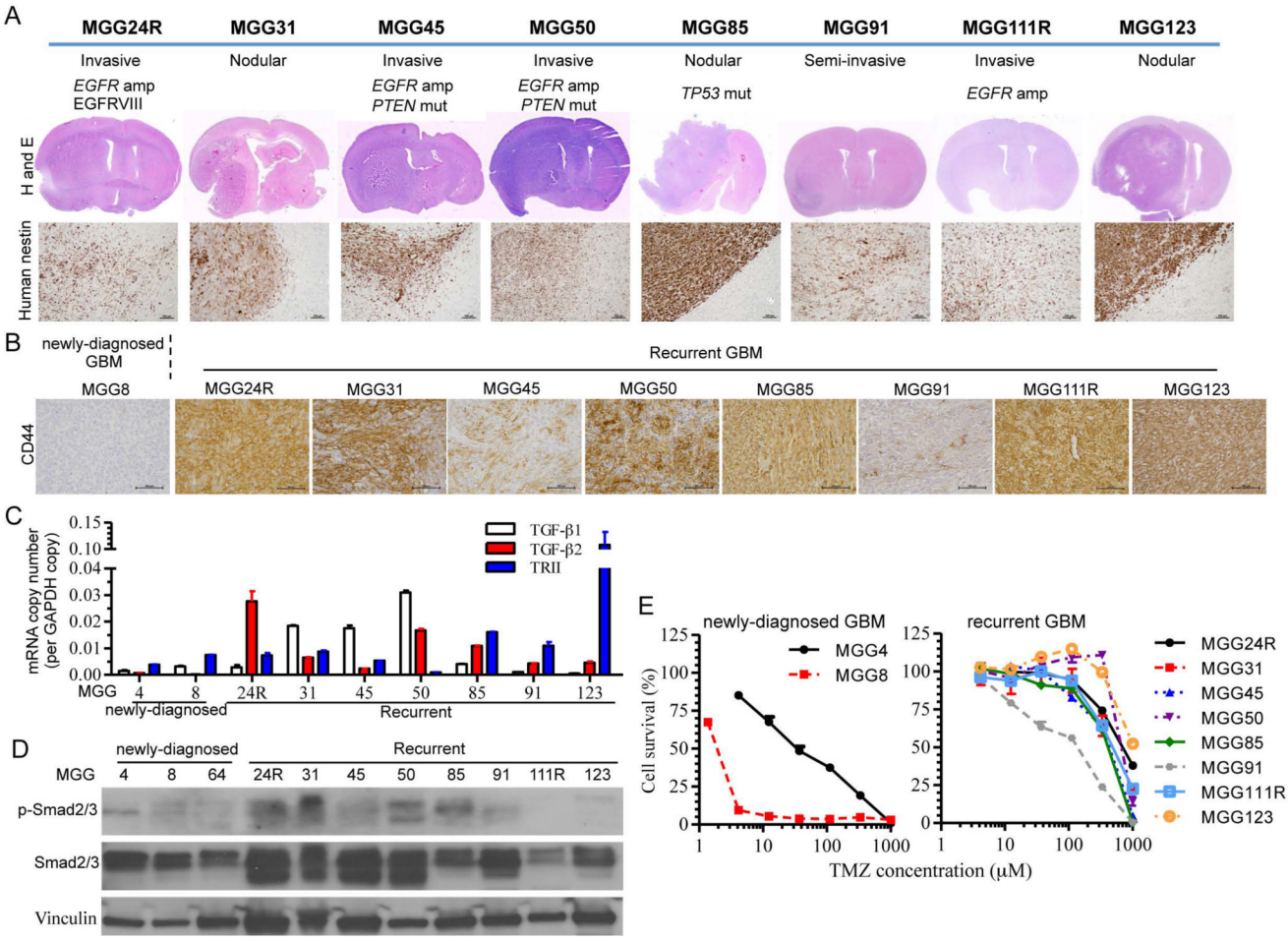

Figure 1. Generation of recurrent GSCs-derived orthotopic xenografts and characterization of their mesenchymal phenotype

(A) Histopathological and molecular characterization of orthotopic xenografts generated from recurrent GSCs established from 8 patients. Molecular (EGFR amplification, P53 and PTEN status) and phenotypic features (invasive vs nodular)(upper). Hematoxylin and eosin (H&E) stain of coronal sections of xenografts (middle) and microscopic images of human nestin immunohistochemistry (lower, original magnification, 100×, scale bars: 100 μm). (B) CD44 immunohistochemistry of GSC xenografts. All the xenografts generated from recurrent GBM expressed CD44 (brown, original magnification, 200×, scale bars: 100 μm). Newly diagnosed GBM-derived model, MGG8, served as negative control. (C) Quantitative RT-PCR analysis of TGF-β1, TGF-β2 and TGF-β receptor type II (TRII) mRNA levels in newly diagnosed and recurrent GSCs. GAPDH was used as reference. Data are shown as mean ± SD. (D) Western blot analysis of TGF-β receptor signal transducer Smad2/3 and its phosphorylation in newly diagnosed and recurrent GSCs. Vinculin is loading control. (E) Cell viability assay showing temozolomide (TMZ) dose response curves of newly diagnosed (left) and recurrent GSCs (right) *in vitro*. Cells were exposed to TMZ for 5 days. Data are shown as mean ± SD.

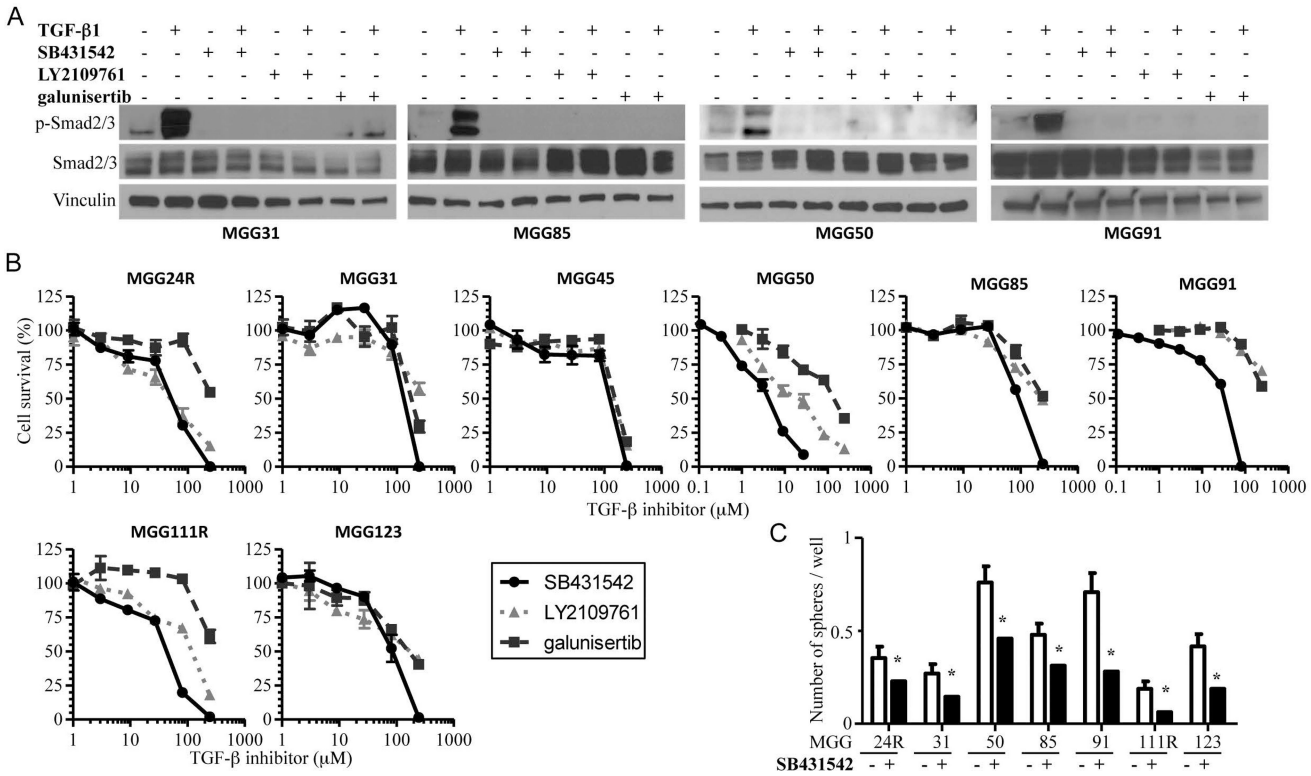

Figure 2. Suppression of TGF-β receptor signaling decreases clonogenicity and viability of recurrent GSCs

(A) Western blot analysis of p-Smad2/3 and Smad2/3 in recurrent GSCs after treatment with TGF-β1 (1 nM) and TGF-β receptor inhibitors, SB431542 (1 μM for MGG50, 10μM for the others), LY2109761 (1 μM for MGG50, 10μM for the others), and galunisertib (10 μM) for 24 hours. Vinculin is loading control. (B) Cell viability assay showing TGF-β receptor inhibitors, SB431542, LY2109761, and galunisertib, dose response curves in a cohort of recurrent GSCs *in vitro*. Cells were exposed to inhibitors for 5 days. Data are shown as mean ± SD. (C) *In vitro* sphere formation assay showing the inhibitory effect of TGF-β receptor inhibitor SB431542 (0.5 μM for MGG50, 10μM for the others) in a cohort of recurrent GSCs. Experiments were done with 1 cell per well. Data are shown as mean ± SD. *, $p<0.05$.

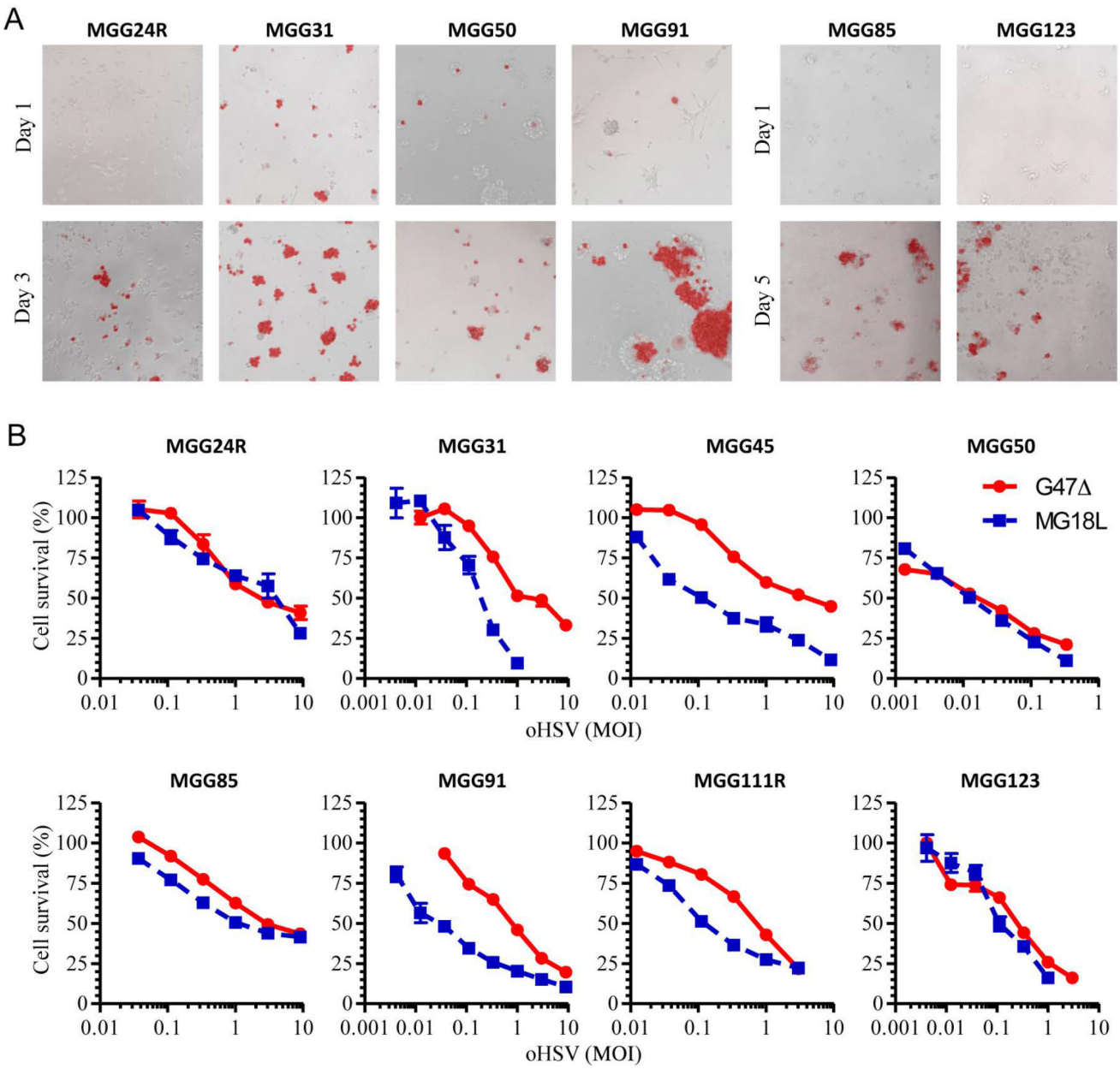

Figure 3. Oncolytic activity of oHSVs against recurrent GSCs *in vitro*

(A) Merged phase contrast and fluorescence microscopy images showing spread of G47Δ-mCherry within the spheres of recurrent GSCs. Images captured on Day 1 (upper), 3 or 5 (lower) post-infection. (B) Cell viability assay showing dose response curves of oHSVs, G47Δ and MG18L, in a cohort of recurrent GSCs *in vitro*. Assays were done 5 days post infection. Data are shown as mean ± SD.

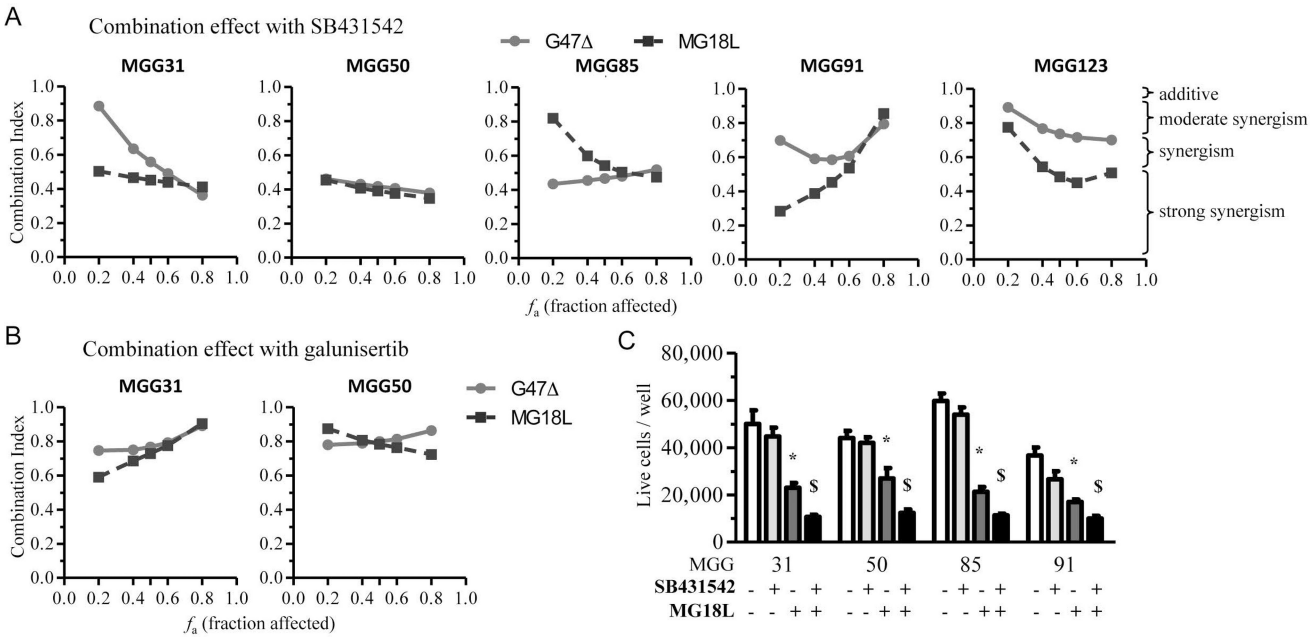

Figure 4. TGF-β receptor inhibitors synergize with oHSVs in the treatment of recurrent GSC models *in vitro*

(A and B) Chou-Talalay analysis to examine the interaction between oHSVs, G47Δ or MG18L, and TGF-β inhibitor, SB431542 (A) or galunisertib (B), in the treatment of recurrent GSCs. Cells were treated for 5 days. Data are plotted as fraction affected (Fa) versus combination index (CI). (C) Viable cell count assay in recurrent GSCs treated with TGF-β inhibitor SB431542 (1 μM for MGG50, 10 μM for the others) and MG18L (MOI=0.4) for 5 days. Data are shown as mean ± SD. *, $P< 0.05$; $, $P<0.01$.

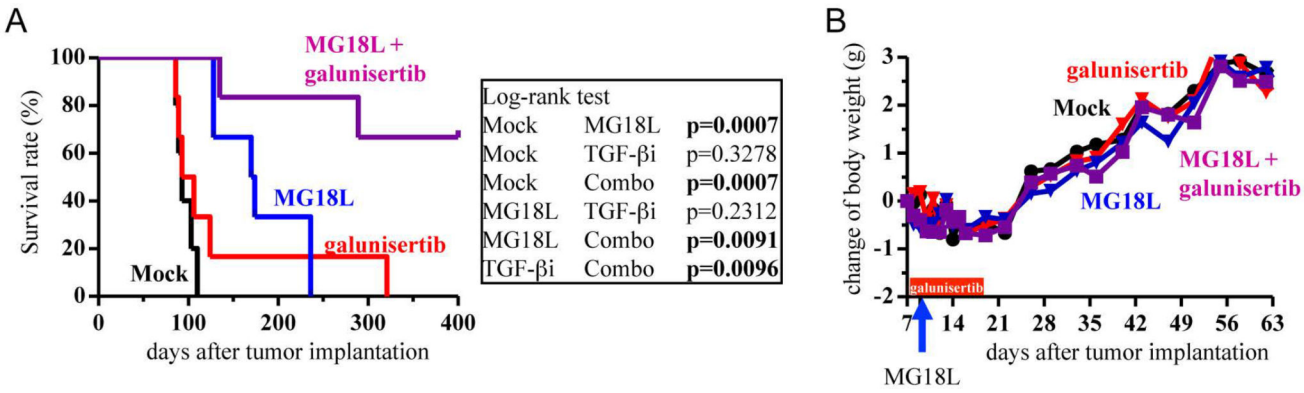

Figure 5. TGF-β receptor inhibitor augments therapeutic efficacy of oHSV, prolonging survival in a recurrent GSC orthotopic xenograft model

(A) Kaplan-Meier survival analysis of mice bearing MGG31 GSC brain tumors treated with mock, MG18L (on day 9), galunisertib (TGF-βi, day 7 through 16) and the combination. (B) Body weight of the mice during the course of treatment. No difference was observed between the groups.

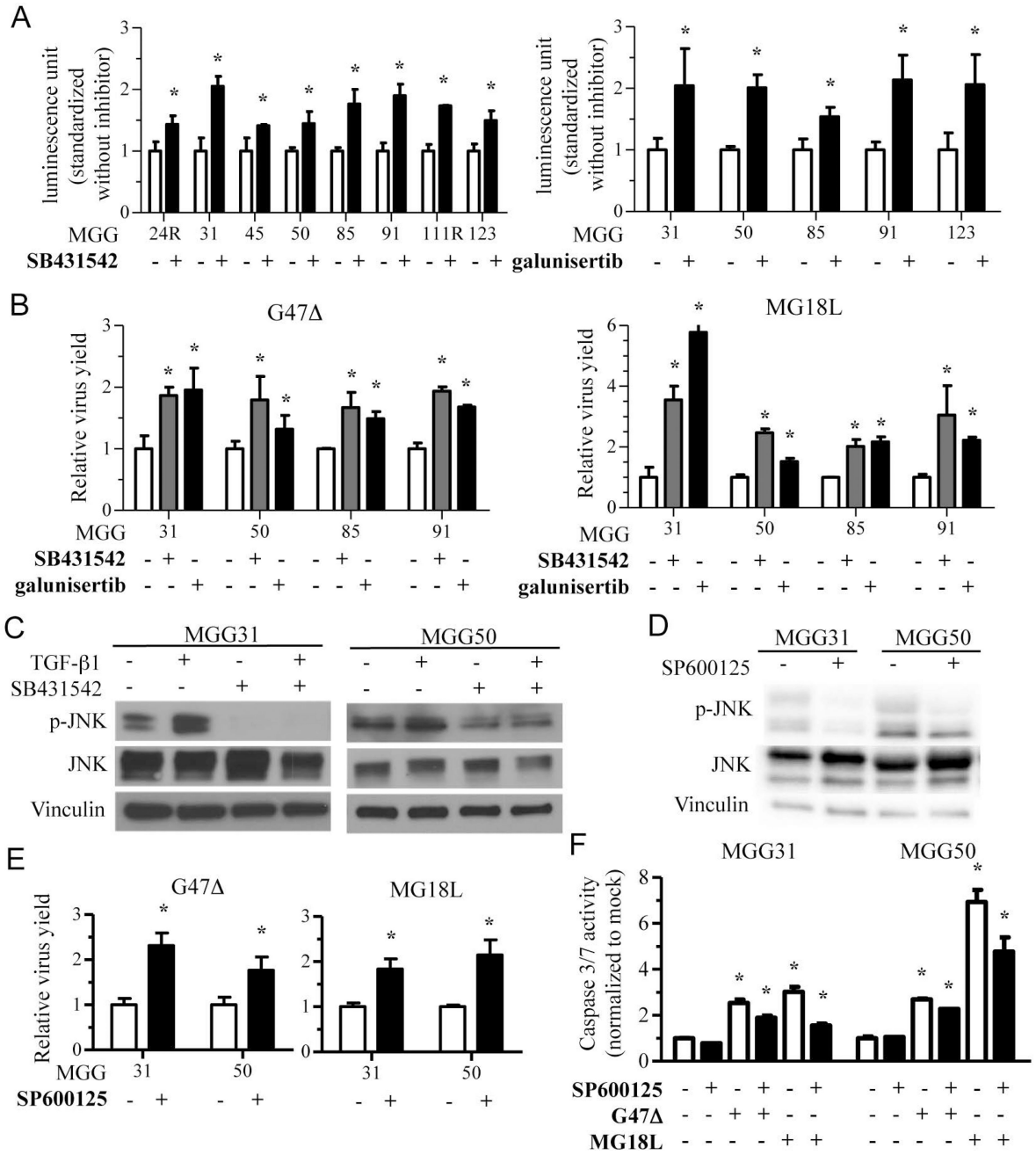

Figure 6. TGF-β inhibition increases oHSV yields via inhibition of JNK signaling in recurrent GSCs

(A) Measurement of firefly luciferase luminescence after infection of recurrent GSCs with oHSV G47Δ-Us11fluc (MOI=1) with and without TGF-β receptor inhibitor, SB431542 (left, 10 μM except for 1 μM for MGG50) or galunisertib (right, 10 μM). Data are normalized to the values without TGF-β inhibitor, and are shown as mean ± SD. *, $P< 0.05$. (B) Relative virus yields 48 hours post oHSV (G47Δ, left panel; MG18L, right panel) infection at MOI=1 of recurrent GSCs in the presence of DMSO, TGF-β inhibitor SB431542 (10 μM except for 1 μM for MGG50) or galunisertib (10 μM). Data were normalized to the values with DMSO treatment, and are shown as mean ± SD. *, $P< 0.05$. (C) Western blot showing inhibition of

JNK phosphorylation (p-JNK, an activated form of JNK) in recurrent GSCs with or without exogenous TGF-β (1 nM) after TGF-β receptor inhibitor SB431542 (10 μM for MGG31 and 1 μM for MGG50) treatment for 24 hours. Vinculin is loading control. (D) Western blot showing JNK phosphorylation (p-JNK) in recurrent GSCs treated with JNK inhibitor SP600125 (600 nM) for 24 hours. Vinculin is loading control. (E) Virus growth assay showing relative virus yields recovered from recurrent GSCs at 48 hours after infection with oHSV G47Δ or MG18L at MOI=1 in the presence of DMSO or JNK inhibitor SP600125 (600 nM). Data is normalized to the values with DMSO treatment. Data are shown as mean ± SD. *, $P< 0.05$. (F) Caspase 3/7 activity assay after 24-hour treatment of recurrent GSCs with oHSV G47Δ or MG18L (MOI=1) in the presence of DMSO or JNK inhibitor SP600125 (600 nM). Data were normalized to the values with mock (control) treatment and shown as mean ± SD. *, $P< 0.05$.