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Original Article

Transient fasting enhances replication of oncolytic herpes simplex virus in glioblastoma

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Abstract: Short-term nutritional restriction (fasting) has been shown to enhance the efficacy of chemotherapy by sensitizing cancer cells and protecting normal cells in a variety of cancer models, including glioblastoma (GBM). Cancer cells, unlike normal cells, respond to fasting by promoting oncogenic signaling and protein synthesis. We hypothesized that fasting would increase the replication of oncolytic herpes simplex virus (oHSV) in GBM. Patient-derived GBM cell lines were fasted by growth in glucose and fetal calf serum restricted culture medium. "Transient fasting", 24-hour fasting followed by 24-hour recovery in complete medium, increased late virus gene expression and G47Δ yields about 2-fold in GBM cells, but not in human astrocytes, and enhanced G47Δ killing of GBM cells. Mechanistically, "transient fasting" suppressed phosphorylation of the subunit of eukaryotic initiation factor 2α (eIF2α) and c-Jun N-terminal kinases (JNK) in GBM cells, but not in astrocytes. Pharmacological inhibition of JNK also increased G47Δ yield. In vivo, transient fasting (48-hour food restriction and 24-hour recovery) doubled luciferase activity after intratumoral G47Δ-US11fluc injection into orthotopic GBM xenografts. Thus, "transient fasting" increases G47Δ replication and oncolytic activity in human GBM cells. These results suggest that "transient fasting" may be effectively combined to enhance oncolytic HSV therapy of GBM.

Keywords: Oncolytic HSV, fasting, glioblastoma, JNK, eIF2α, mTOR

Introduction

Glioblastoma (GBM), classified as grade IV by the World Health Organization, is the most common and malignant primary brain tumor in adults [1]. Current treatment including maximum surgical resection, radiotherapy, and chemotherapy has not substantially improved the survival of GBM patients; with a median survival of 14.6 months [2]. Oncolytic viruses are genetically modified or naturally occurring viruses that, upon infection, selectively replicate in and kill neoplastic cells while sparing normal cells [3, 4]. A wide variety of virus species, including herpes simplex virus (HSV), adenovirus, vaccinia virus, reovirus, Newcastle disease virus, vesicular stomatitis virus, vaccinia virus, and measles virus, have been tested as oncolytic platforms for GBM [5]. Two genetically engineered HSVs, G207 and 1716, have completed early clinical trials for recurrent gliomas and demonstrated favorable safety profiles [6, 7]. Currently a clinical trial testing a newer generation of oncolytic HSV (oHSV), G47Δ is ongoing for recurrent malignant gliomas [8] [http://apps.who.int/trialsearch/Trial2.aspx?TrialID=JPRN-UMIN000002661]. G207 clinical trials for glioma found evidence of virus replication, but only to a limited degree [9], suggesting that enhancement of virus replication might be necessary to improve efficacy.

Dietary restriction triggers highly conserved survival mechanisms that enhance the protection of organisms ranging from bacteria, yeast, flies, to mice and non-human primates against various types of stress and/or disease [10]. This effect is mediated in part by the suppression of conserved nutrient-signaling pathways, especially the insulin-like growth factor 1 (IGF-I) receptor pathway, as shown in fasted humans [11]. Interestingly, short term exposure to a severely restricted diet or fasting has been shown to enhance the efficacy of chemotherapy by sensitizing tumor cells and protecting normal cells in a variety of cancer models including GBM [12, 13]. In a case series report, 10 patients with different cancers were safely fasted

for 48 hours prior and/or following chemotherapy with various drugs, warranting randomized clinical trials to determine the effect of fasting in combination with chemotherapy [14]. In a pilot study evaluating ketogenic diet in recurrent GBM patients, there was a trend for longer progression-free survival in the group who had achieved stable ketosis compared to the group who had not [15]. Currently several clinical trials have completed recruitment or are ongoing that aim to assess whether short term fasting or low calorie diet in combination with chemotherapy reduces adverse effects and increases anti-tumor effects in different types of cancers (NCT00757094, NCT00936364, NCT01175837, NCT01802346, NCT01954836, and NCT02126449).

Mechanistically, fasting down-regulates proliferation-associated genes in normal tissues, but upregulates or does not alter them in breast cancer allografts [12]. Overall, fasting protects normal cells by reallocating energy from reproduction and growth processes to maintenance pathways. In contrast, cancer cells respond to fasting by promoting oncogenic signaling and protein synthesis. This different response, termed "differential stress resistance" [14, 16, 17], is the basis for fasting-induced selective sensitization of cancer cells to cytotoxic anticancer agents.

HSV must control and interact with an array of host cell functions to drive productive growth. In particular, ensuring virus mRNA translation is critical for progeny production since HSV has no virus gene encoding protein synthesis apparatus, and is completely dependent on the translational machinery of the host cell. To attenuate pathogenicity, all oHSVs that have been tested clinically for GBM have deletions of γ34.5, a key gene to sustain protein synthesis by counteracting the host double stranded RNA-dependent protein kinase (PKR)-mediated phosphorylation of eukaryotic initiation factor 2α (eIF2α). Thus these HSV mutants may be susceptible to host anti-HSV defense mechanisms, potentially leading to compromised virus replication and efficacy. On the other hand, aberrantly active oncogenic signaling such as Ras pathway in cancer can enhance oHSV replication and cancer selectivity [18]. We hypothesized that short time fasting induces differential promotion of oncogenic signaling and protein synthesis in cancer cells and sets a stage for oHSV to replicate better and exert increased oncolytic activity, without compromising its safety profile in normal cells. In this study, we investigated the effect of fasting on gene expression, replication, and cytotoxicity of oHSV in patient-derived GBM cell-based models in vitro and in vivo.

Materials and methods

Cells

Patient-derived GBM cell lines, MGG8F and MGG29F, were isolated from surgical specimens of GBM. To establish these GBM lines, minced tissues were digested with 0.1% trypsin and 10 U/mL of DNaseI at 37°C for 45 min, washed with HBSS, and were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Mediatech, Manassas, VA) supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO_2. Vero cells (African green monkey kidney cells) were obtained from the American Type Culture Collection, and cultured in DMEM with 10% calf serum. Human astrocytes were obtained from ScienCell, maintained in DMEM with 10% FCS, and used for experiments before passage #10.

Viruses

G47Δ contains deletions of both copies of γ34.5 and α47, and a LacZ insertion inactivating ICP6 [19]. G47Δ-US11fluc is a recombinant HSV derived from G47Δ and expresses the firefly luciferase gene driven by the true late US11 gene promoter of HSV-1 [20]. All viruses were grown, purified, and titered on Vero cells.

Luciferase-based assay of late gene expression

MGG8F and MGG29F cells were seeded into 96-well plates at 5,000 cells/well. Next day, cells were subjected to fasting by switching culture medium from DMEM (4.5 g/L of glucose) supplemented with 10% FCS (termed as normal medium) to DMEM containing low glucose (0.5 g/L) and 1% FCS (termed as fasting medium) or not for indicated periods of time. Cells were infected with G47Δ-US11fluc at multiplicity of infection (MOI) of 1, and cultured for 24 hours. D-luciferin (Gold Biotechnology, St Lois, MO) was added to cells at 2 mM, and bioluminescence was immediately detected using a microplate reader (Synergy-HT; Bio-Tek, Vinooski, VT).

Viral yield after transient fasting

MGG8F and MGG29F cells, and human astrocytes were seeded into 24-well plates (25,000 cells/well). Next day, cells were subjected to fasting for 24 hours or not, followed by 24-hour normal media culture. Cells were then infected with oHSV at an MOI of 1, and cells and the media were harvested at 48 hours post-infection. After three freeze/thaw cycles and sonication, titers of infectious virus were determined by plaque assay on Vero cells.

G47Δ cytotoxicity after transient fasting

For cell killing assay, MGG8F and MGG29F cells were seeded into 24-well plates (25,000 cells/well). Next day, cells were cultured in fasting or normal media for 24 hours, followed by normal media culture for 24 hours, then were infected with mock or oHSV. After 4-day culture in normal media, cells were harvested, stained with trypan blue and viable cells were counted on a hemocytometer. For cell viability assay, MGG8F and MGG29F cells were seeded into 96-well plates (5,000 cells/well), and treated and cultured as above. MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega, Fitchburg, WI) was carried out according to manufacture's instructions after 2- or 4-day culture in normal media.

Treatment with JNK inhibitor

A selective and reversible JNK inhibitor, SP600125 (Sigma-Aldrich), was dissolved in DMSO. MGG8F and MGG29F cells were incubated with the indicated concentrations of SP600125 for 1 hour and were infected with G47Δ-US11fluc or G47Δ at an MOI of 1. Late gene expression assay and virus yield assay were performed as described above.

Immunoblot

After treatment, cells were washed once with PBS, and lysed in RIPA buffer (Boston Bioproducts, Ashland, MA) with protease inhibitors (Complete Mini; Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (PhosSTOP; Roche Diagnotics). Protein was separated by 5-15% SDS-PAGE and transferred to PVDF membranes by electroblotting. After blocking with 5% nonfat dry milk in TBS-Tween 20, membranes were incubated at 4°C overnight with primary antibodies followed by incubation with appropriate HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Bio-Rad Laboratories, Hercules, California) for 1 hour at room temperature. Protein-antibody complexes were detected using Clarity Western ECL Substrate (Bio-Rad Laboratories) and visualized on films or with ChemiDoc XRS system (Bio-Rad Laboratories). The primary antibodies used include: phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, eIF2α, phospho-4E-BP1 (Thr37/46), phospho-p70 S6 Kinase (Thr389) (from Cell Signaling Technology, Danvers, MA), EIF2S1 (phosphor S51) (Abcam, Cambridge, MA), or vinculin (Thermo Fisher Scientific, Waltham, MA).

Effects of transient fasting on oHSV late gene expression in vivo

GBM8F cells (500,000 cells/5 μL) were stereotactically implanted into the brain (right striatum, 2.5 mm lateral from bregma and 2.5 mm deep) of 6-week-old female SCID mice (n=8) under anesthesia with pentobarbital sodium, as described [21]. Twenty-three days after tumor implantation, mice in the fasting group (n=4) were fasted for 48 hours by food deprivation and free access to water, followed by a 24-hour ad libitum feeding. Food was provided throughout the course for the control group (n=4). On day 26, G47Δ-US11fluc (1.5×10^6 pfu/3 μL) was stereotactically injected into the tumor for both groups. At 24 and 96 hours after virus infection, mice were anesthetized with 2% isoflurane and bioluminescent imaging (BLI) was performed with intraperitoneal injection of 4.5 mg D-luciferin. Under the imaging condition used (binning, 16 s and exposure 60 s), the background signal levels were below 100 photons/s. These procedures were approved by the institutional animal care and use committee (IACUC) at Massachusetts General Hospital.

Statistical analysis

Data are shown as mean ± standard error and were analyzed using Prism (GraphPad Software, San Diego, CA). Late gene expression was analyzed using one-way factorial analysis of variance followed by Dunnett's test for comparison. Two group comparisons were analyzed with unpaired Student t-test. Differences with a probability value of $P < 0.05$ were considered statistically significant.

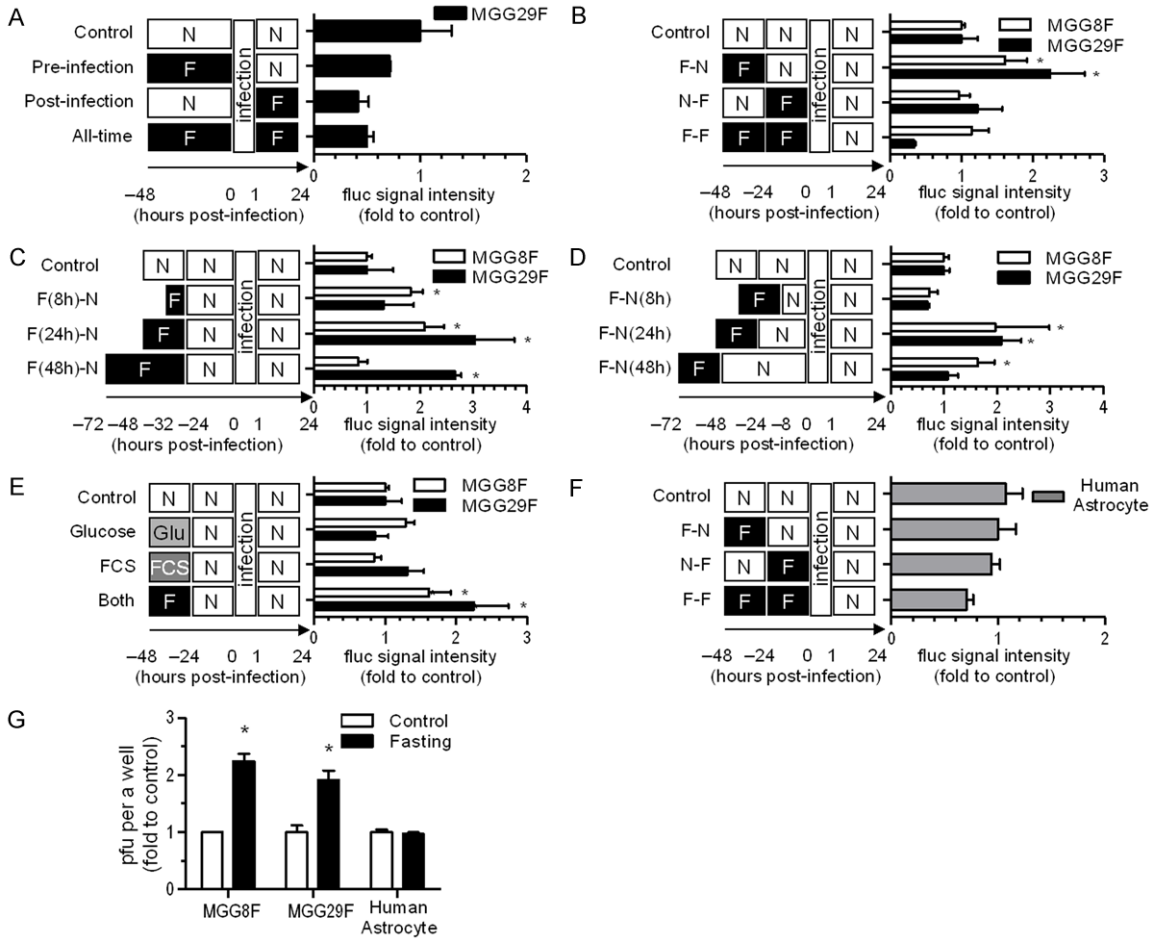
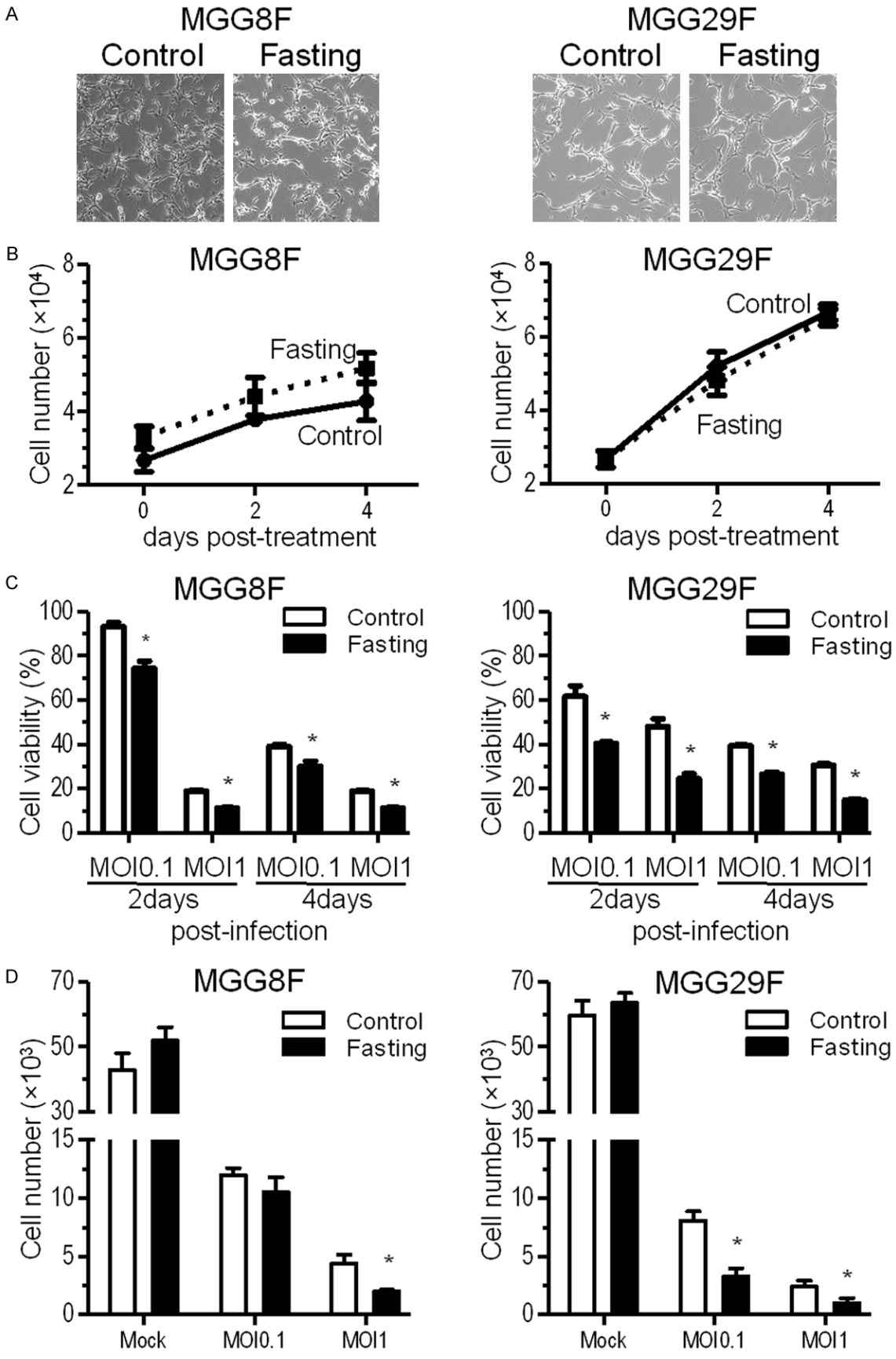


Figure 1. Increased virus late gene expression and replication after transient fasting. A-F. Bioluminescence assay measuring firefly luciferase (fluc) expression 24 hours after infection with the reporter oHSV G47Δ US11fluc. A. MGG29F cells were fasted before and/or after virus infection. B. MGG8F and MGG29F cells were fasted or fed with normal medium 48-24 hours or 24-0 hours before infection. C. Different fasting durations were tested before 24-hour normal medium culture of GBM cells. D. Different pre-infection culture durations in normal medium were tested after 24-hour fasting of GBM cells. E. Restriction of FCS, glucose and both was applied during 48-24 hours before infection of GBM cells. F. Normal human astrocytes were fasted or fed with normal medium 48-24 hours or 24-0 hours before infection. N, normal medium; F, fasting medium. In A-F, black boxes indicate duration of fasting and white boxes duration of normal media. Fluc signal intensity relative to that of normal medium culture throughout the experiment is shown. G. Virus yield assay showing the effects of transient fasting (black bars) on virus yield in GBM cells and human astrocytes. Data were normalized to the virus yields obtained after normal medium culture (control). *, $P < 0.05$, compared with control. Data are shown as mean ± standard error.

Results

Transient fasting increases oHSV late gene expression in human GBM cells

We first sought to determine whether transient restriction of nutrition (fasting) enhances replication of oHSV in tumor cells. To this end, we used oHSV G47Δ US11fluc, which has the firefly luciferase reporter gene under the control of the true late US11 gene promoter, allowing assessment of oHSV replication via bioluminescence measurement [20]. Forty-eight-hour fasting before G47Δ-US11fluc infection and/or 24-hour fasting after infection decreased the levels of late gene expression in GBM cells MGG29F compared to normal nutritional culture control (**Figure 1A**). The decrease with post-infection fasting was more than 40%, suggestive of the necessity of post-infection normal nutrition for optimum oHSV replication. Next, fasting or normal medium was used sequentially 24 hours each (total 48 hours) before infection and late gene expression assayed. Surprisingly, 24-hour treatment with fasting medium followed by 24-hour normal medium


A
MGG8F
Control
Fasting
MGG29F
Control
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Mock
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MGG29F
Control
Fasting
Cell number (×10³)
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Figure 2. Transient fasting increases G47Δ cytotoxicity of GBM cells. (A) Microscopic photographs showing morphology of GBM cells grown under normal conditions (Control) and transient fasting conditions. (B) In vitro cell growth curves showing viable cell number measured after normal medium culture or transient fasting of MGG8F and MGG29F cells. (C, D) In vitro cytotoxicity assay. MGG8F and MGG29F cells were transiently fasted and infected with G47Δ at indicated MOIs. Cytotoxicity was measured by MTS cell viability assay at days 2 and 4 post-infection (C) and by cell counting assay at 4 days post-infection (D). Normal medium culture was used as control. Significantly decreased cell viability and cell number after G47Δ infection with transient fasting compared with G47Δ infection without fasting is indicated by *, $P < 0.05$, compared with control. Data are shown as mean ± standard error.

(termed normal recovery medium) doubled late gene expression in MGG8F and MGG29F GBM cells (**Figure 1B**). Testing different fasting conditioning periods followed by 24-hour normal recovery medium revealed that 24-hour fasting resulted in the greatest increase in late gene expression (**Figure 1C**). When different recovery periods were tested after 24-hour fasting, 24-hour recovery provided the greatest late gene expression in GBM cells (**Figure 1D**).

We next examined whether restricting glucose or FCS is sufficient to induce the observed effect. Significant increases in late gene expression were only seen when both glucose and FCS were deprived (**Figure 1E**). In normal human astrocytes, transient fasting with or without combination with normal medium did not increase fluc expression of G47Δ-US11fluc (**Figure 1F**). Collectively, pretreatment of GBM cells with the 24-hour fasting medium and the subsequent 24-hour normal recovery medium increased late gene expression following infection with G47Δ-US11fluc. Hereafter we term this conditioning of 24-hour fasting plus 24-hour normal nutrition as "transient fasting".

Transient fasting increases G47Δ replication in GBM cells

We previously observed that Us11 promoter driven luciferase activity and virus yield linearly correlate after G47Δ-US11fluc infection [20]. We wanted to confirm that fasting also induced increases in virus replication and yield. Indeed, we found that "transient fasting" increased G47Δ yield about two-fold in MGG8F and MGG29F GBM cells at 48 hours post-infection; however, "transient fasting" did not alter G47Δ yield in normal human astrocytes (**Figure 1G**).

Transient fasting increases G47Δ cytotoxicity

Next, we determined whether the fasting-induced increases in oHSV replication translated into enhanced cytotoxicity of tumor cells. "Transient fasting" alone did not induce any apparent cell morphological changes (**Figure 2A**), and did not significantly alter the proliferation rate of MGG8F and MGG29F in vitro (**Figure 2B**). G47Δ infection decreased the viability of GBM cells in a time and dose dependent fashion when cultured in normal medium (**Figure 2C**; MTS assay). Cytotoxicity of G47Δ was further increased when G47Δ was infected at different MOIs following "transient fasting" (**Figure 2C**). Viable cell count assays confirmed these results that G47Δ in conjunction with "transient fasting" further decreased the number of viable GBM cells at 4 days post-infection (**Figure 2D**). Thus, "transient fasting" enhanced G47Δ cytotoxicity of GBM cells.

Transient fasting attenuates phosphorylation of and eIF2α and JNK

We next sought to identify possible molecular mechanisms that underlie "transient fasting"-mediated enhancement of oHSV replication. Fasting was shown to enhance mRNA translation in murine breast cancer cells through promoting dephosphorylation of eIF2α and phosphorylation of S6 kinase [12]. In human GBM cells, but not in normal astrocytes, we observed a modest reduction of phosphorylated eIF2α (p-eIF2α) levels after "transient fasting" (**Figure 3A**). G47Δ lacks γ34.5 that induces eIF2α dephosphorylation, but expresses Us11 under control of the α47 immediate early promoter, thereby blocking eIF2α phosphorylation [19]. Infection with G47Δ did not alter p-eIF2α levels in GBM cells, perhaps reflecting Us11 expression at an early phase of infection. However, G47Δ increased p-eIF2α levels in normal astrocytes, which was further boosted by "transient fasting" preconditioning (**Figure 3A**).

The mammalian target of rapamycin (mTOR) pathway is a major regulator of protein synthesis and cellular proliferation [22]. Phosphorylation of the mTOR1 downstream proteins, 4E-BP1 and S6 kinase (S6K), was not altered in MGG8F and MGG29F GBM cells after "transient fasting" (**Figure 3B**). In stark contrast, transient fasting dramatically decreased levels

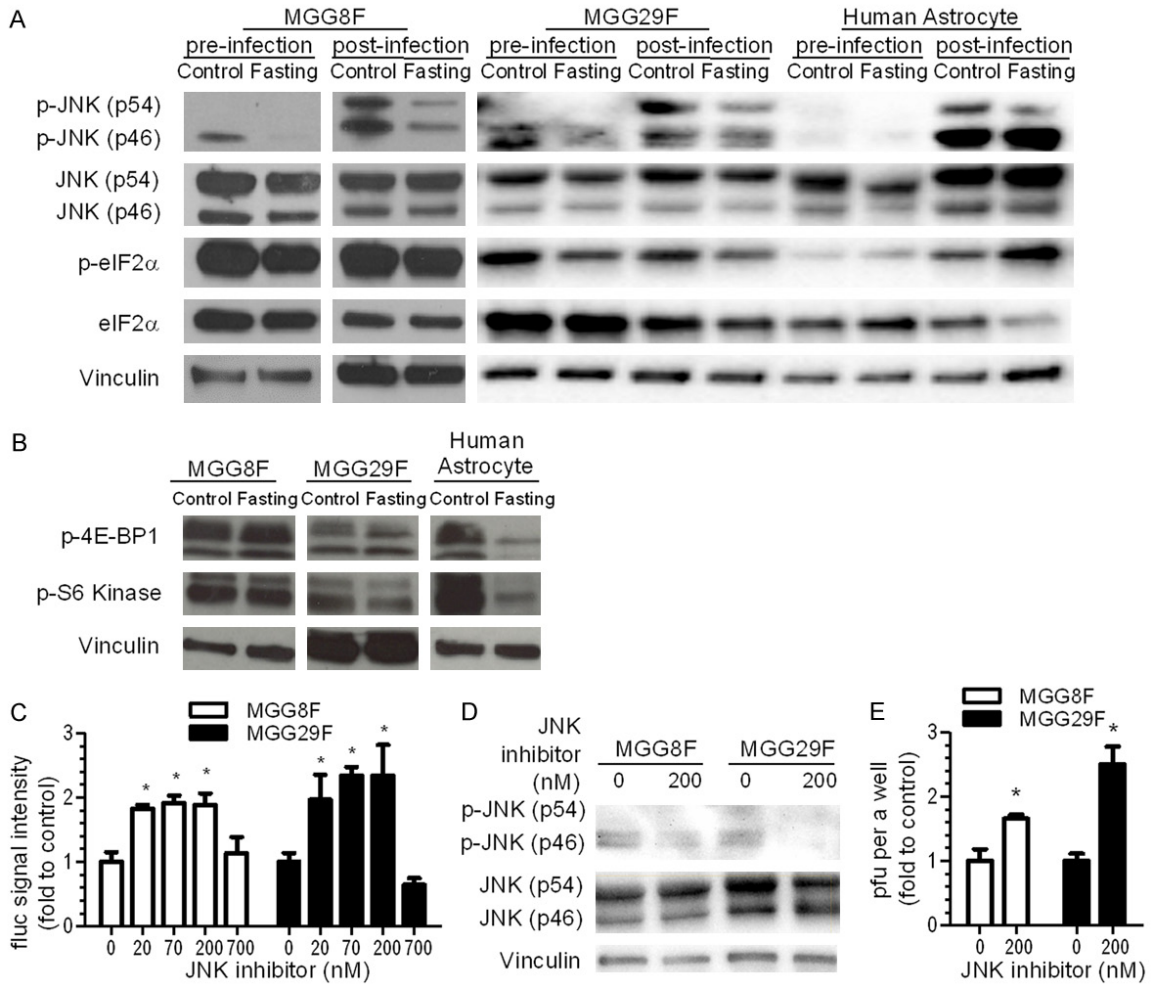

Figure 3. Transient fasting attenuates phosphorylation of JNK and eIF2α in GBM cells. A. Immunoblot showing the effects of transient fasting on phosphorylated-JNK (p-JNK) and phosphorylated-eIF2α (p-eIF2α) levels in GBM cells (MGG8F and MGG29F) and human astrocytes before and 24 hours after infection with G47Δ. Vinculin was used as loading control. B. Immunoblot showing the effects of fasting of GBM cells and human astrocytes on downstream targets of mTOR, 4E-BP1 and S6 kinase. C. Bioluminescence assay showing relative firefly luciferase activity 24 hours after infection with the reporter oHSV G47Δ US11fluc in the presence of indicated concentrations of JNK inhibitor, SP600125. Fluc signal intensity without SP600125 was used as reference. D. Immunoblot showing suppression of p-JNK by SP600125 (at 200 nM) in GBM cells. E. Virus yield assay showing G47Δ yields at 48 hours post-infection of GBM cells in the presence of SP600125 (200 nM). Virus yield without SP600125 was used as reference. In C and E, data are shown as mean ± standard error. *, $P < 0.05$, compared with control.

of phosphorylated 4E-BP1 and S6K in normal astrocytes, reflecting cell protection mechanisms driven by translation suppression (**Figure 3B**).

c-Jun N-terminal kinases (JNK) is one of the mitogen-activated protein kinases and primarily is activated in response to stress [23]. Since oHSV infection induces JNK activation in human GBM cells [24], we examined whether JNK signaling is involved in the "transient fasting" modulation of oHSV replication. We found that "transient fasting" decreased the levels of phosphorylated JNK (p-JNK), an active form of JNK, in MGG8F and MGG29F GBM cells (**Figure 3A**). While G47Δ infection clearly activated JNK phosphorylation under normal nutritional conditions, as expected, G47Δ-induced phosphorylation of JNK was attenuated when GBM cells were infected after "transient fasting" (**Figure 3A**). Notably, G47Δ mediated robust induction of p-JNK in normal astrocytes in both fasted and non-fasted conditions (**Figure 3A**).

Pharmacological JNK inhibition increases oHSV yield

To assess whether the JNK signaling plays a role in oHSV replication, we used a highly selec-

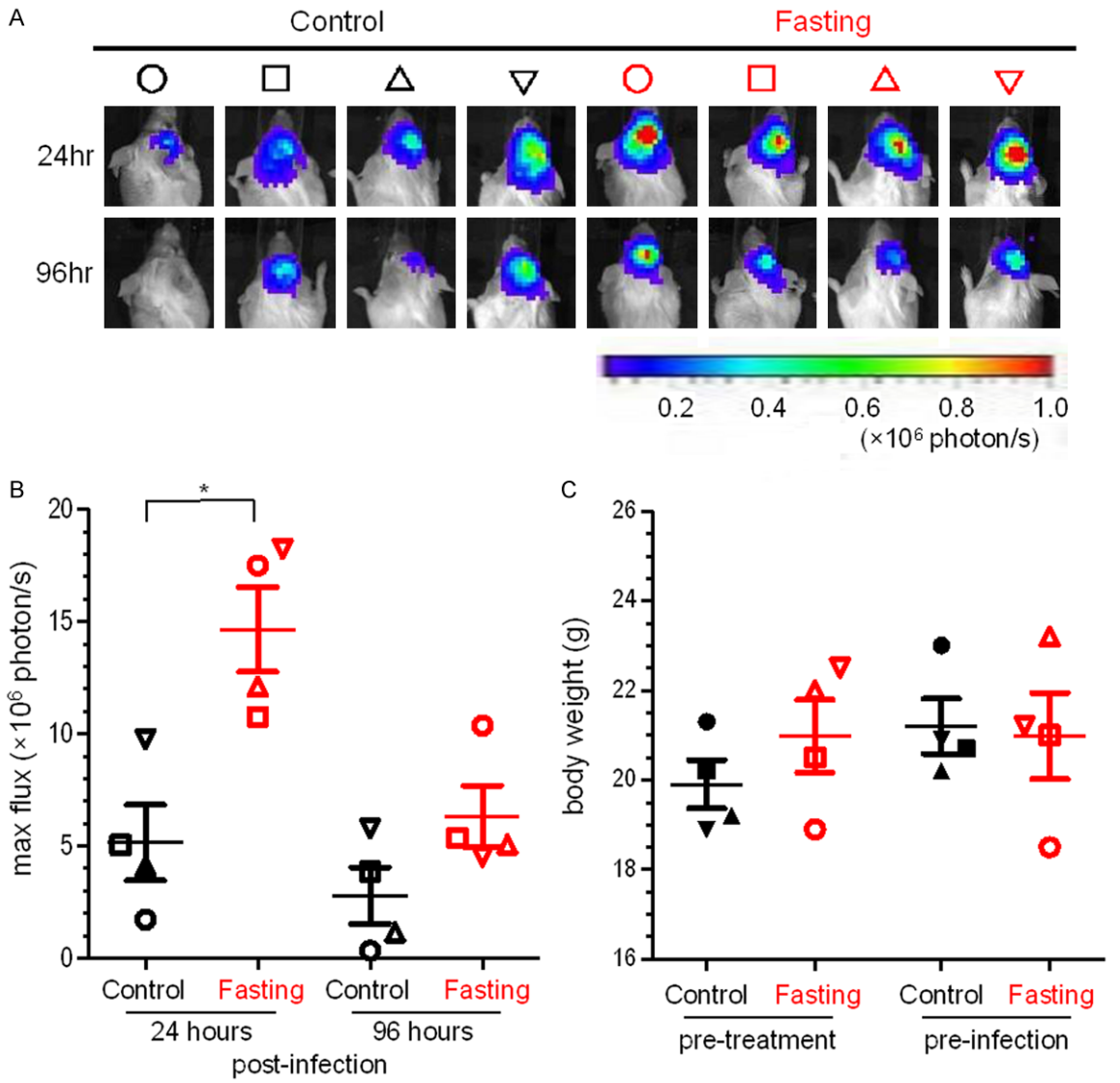

Figure 4. Transient fasting increases intratumoral oHSV replication in vivo. (A, B) Bioluminescence imaging of mice bearing intracerebral MGG8F xenografts that received intratumoral injection of G47Δ US11fluc. (A) Representative bioluminescence images of non-fasted mice (Control, left, N=4) and fasted mice (right, N=4), collected at 24 and 96 hours post-injection. (B) Bioluminescence signal intensity (max flux) for individual mice is plotted. Mice treated with transient fasting showed significantly higher signals at 24 hours post-infection compared with mice without fasting (*, $P < 0.05$). (C) Animal body weight before and after fasting of mice bearing MGG8F xenografts. Body weight was comparable between control and fasting groups when transient fasting (48-hour fasting and 24-hour feeding) was completed. In (B and C), symbols (circles, squares, triangles, reverse triangles) correspond to the mice shown in (A). Data are shown as mean ± standard error.

tive small molecule inhibitor of JNK, SP600125. In the presence of SP600125 at a dose range of 20-200 nM, there was significantly increased late gene expression upon G47Δ-US11fluc infection of GBM cells (**Figure 3C**). 200 nM of SP600125 suppressed phosphorylation of JNK in GBM cells (**Figure 3D**), and this pharmacological blockade of JNK significantly increased G47Δ yield at 48 hours post-infection (**Figure 3E**). These results suggest the involvement of JNK inhibition in "transient fasting"-induced enhancement of oHSV replication.

Transient fasting increased oHSV gene expression in GBM xenografts

Finally we determined whether "transient fasting" could enhance oHSV replication in vivo. Orthotopic GBM xenografts were established by intracerebral implantation of MGG8F cells in

8 SCID mice. Four mice (fasting group) were fasted for 48 hours followed by ad lib feeding for 24 hours when G47Δ-US11fluc oHSV was inoculated into the tumor. The control group was fed ad lib and was given the same amount of G47Δ-US11fluc. Bioluminescence imaging of luciferase activity showed that at 24 hours post virus injection, mice preconditioned with "transient fasting" had significantly higher luciferase signals, almost 3-fold, compared with the mice without fasting (**Figure 4A**, **4B**). By 96 hours after infection, luciferase signals were detectable but had dropped in all mice, so there was no significant difference between control and fasting mice (**Figure 4A**, **4B**). Body weight was comparable between the groups at the time of virus injection when transient fasting was completed (**Figure 4C**). These results indicate that short term fasting of mice transiently enhances replication of oHSV within brain tumors.

Discussion

Under conditions of nutritional depletion, normal cells respond by shutting down translational activities to protect themselves from stress whereas neoplastic cells promote signaling pathways in an attempt to sustain cellular metabolism and proliferation. This differential stress response is the rationale for using anti-cancer chemotherapy in combination with short term fasting to alleviate drug-induced toxicity to normal organs as well as to enhance cytotoxicity of cancer cells [10, 25]. In the current study, we showed that short term fasting enhances replication of oHSV and therapeutic effect in experimental therapy of GBM.

Using the reporter oHSV G47Δ-US11fluc, we initially tested nutrient restriction (fasting) and normal medium either before or after infection to identify the nutritional conditions that maximize virus late gene expression. When GBM cells were fasted after G47Δ-US11fluc infection, fluc expression decreased by about 50%, indicating that optimal replication of G47Δ US11fluc requires normal medium conditions. 48-hour-pre-incubation of cells in fasting medium before G47Δ-US11fluc infection decreased fluc expression as well. However, when fasting and normal conditions were sequentially applied before infection, i.e., 24-hour fasting followed by 24-hour non-fasting: "transient fasting", late HSV gene expression was increased by 2-3 fold in 2 GBM cell lines. As expected, the increased fluc expression correlated with an increase in oHSV yield. Restriction of both glucose and proteins was necessary for this effect, which appeared specific in cancer cells as there was no increase in fluc expression or virus yield in normal astrocytes.

Short term fasting sensitizes cancer cells to chemotherapeutics such as doxorubicin when the agents are applied during or immediately after fasting [12]. It is not known whether the sequential "transient fasting" preconditioning we describe alters cancer cell response to chemotherapy. Given the distinct mechanisms of action mediated by oncolytic viruses and chemotherapeutic agents, it is possible that the nutritional environments of cancer most suitable for these treatments differ. We speculate that the switch from fasting to a normal nutritional condition evokes GBM cells to modulate an intracellular environment, setting the stage for optimal oHSV gene expression and replication to occur.

Mechanistically, the current study revealed that fasting-mediated decreases in the levels of phosphorylated form of eIF2α and JNK, likely contributing to the observed enhancement of oHSV replication. The eIF2α protein plays a pivotal role in regulating translation of mRNA, and for virus to produce progeny it is critical to maintain protein synthesis by suppressing phosphorylation of eIF2α. G47Δ and its derivative G47Δ-US11fluc lack γ34.5 that dephosphorylates eIF2α, but express US11 at an early stage of virus life cycle that inhibits the functions of PKR and eIF2α phosphorylation [26]. In accord with a report showing fasting-induced reduction of phospho-eIF2α in murine breast cancer cells [12], we show that "transient fasting" diminishes phospho-eIF2α levels in GBM cells. In normal astrocytes, however, "transient fasting" slightly elevated phospho-eIF2α levels, which was robustly upregulated 24 hours after oHSV infection, suggestive of induction of protection mechanisms via translation inhibition. In contrast, in GBM cells phospho-eIF2α levels remained relatively low even after oHSV infection, which would promote sustained translation of virus proteins.

mTOR complex 1 (mTORC1) regulates cell survival, proliferation and metabolism through phosphorylating 4E-BP1 and S6K [27]. Although fasting elevates phosphorylated S6K in murine breast cancer cells [12], we did not observe changes in phospho-4E-BP1 and S6K levels

after "transient fasting" of GBM cells. In astrocytes, however, the same preconditioning potently repressed phospho-4E-BP1 and S6K, reflecting a robust cellular response toward lowering translation and cellular proliferation.

The role of the JNK signaling pathway in HSV infection and replication is controversial. HSV-1 infection activates JNK which enhances viral replication, and prevention of JNK from activating its nuclear targets decreases HSV-1 replication [28]. On the other hand, HSV-1 infection phosphorylates JNK and triggers apoptosis in rat hippocampal cultures and in human brain tissues in HSV encephalitis [29]. Pharmacological inhibition of JNK reduces apoptosis in hippocampal cultures, supporting a role for JNK in inducing apoptosis in the HSV-infected brain [29]. This perhaps represents a host defense mechanism against virus infection since apoptosis of infected cells restricts viral replication and spread [30-32]. Consistent with previous observations [24], we show that G47Δ infection induces phosphorylation of JNK. The "transient fasting" conditioning decreased phospho-JNK, and suppressed G47Δ-induced phosphorylation of JNK in GBM cells. Since small molecule inhibition of JNK increased G47Δ replication in GBM cells, we reason that attenuation of JNK signaling contributes to the observed amplified oHSV replication after "transient fasting". Fasting-induced suppression of JNK pathway might impair apoptosis, leading to more efficient virus multiplication. Although JNK is well known as a pro-apoptotic kinase, recent studies have demonstrated anti-apoptotic functions of JNK [33, 34]. The distinct roles of JNK seem to depend on the cell type, the nature of the death stimulus, the duration of its activation and the activity of other signaling pathways [23].

We show that "transient fasting"-induced increased oHSV replication translates into enhanced cell killing. Using an orthotopic GBM xenograft model, we further demonstrate that "transient fasting" augments intratumoral replication of oHSV in vivo. The effects we saw were only transient, but this could be due to oHSV-mediated tumor destruction. Studies are currently ongoing to optimize the beneficial usage of dietary restriction in oHSV therapy in different murine models of GBM. In addition, it is of interest to study whether the transient fasting sensitizes cancer cells to other oncolytic viruses. The differential responses that GBM cells and normal astrocytes display upon "transient fasting" suggest that the nutritional conditioning can augment oHSV potency, allowing dose reduction to achieve the same anti-tumor effects without fasting. We expect to learn more about the safety and feasibility of short term fasting in cancer patients as clinical trials testing fasting-chemotherapy combination are currently ongoing. Results of Japanese clinical trials investigating G47Δ inoculation in GBMs appear encouraging in terms of safety and efficacy [8]. [9th international conference on oncolytic virus therapeutics]. Our results suggest that "transient fasting" might be safely and effectively combined to enhance oncolytic HSV therapy in patients with GBM, and should pave a way to a clinical trial testing this rationale.

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Disclosure of conflict of interest

None.

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