HHS Public Access

Author manuscript

Gene Ther. Author manuscript; available in PMC 2013 October 01.

Published in final edited form as:

Gene Ther. 2013 April ; 20(4): 444–449. doi:10.1038/gt.2012.62.

Oncolytic measles virus strains have significant antitumor activity against glioma stem cells

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Abstract

Glioblastoma (GBM) is the most common primary brain tumor in adults and has a dismal prognosis despite multimodality treatment. Given the resistance of glioma stem cells (GSC) to chemotherapy and radiation therapy, their eradication could prevent tumor recurrence. We sought to evaluate the antitumor activity of MV derivatives against GSC. We generated neurosphere cultures from patient derived primary tumor GBM xenografts, and we characterized them for the GSC markers CD133, SOX2, Nestin, ATF5 and OLIG2. Using the MV-strains MV-GFP, MV-CEA and MV-NIS we demonstrated infection, viral replication and significant cytopathic effect in vitro against GSC lines. In tumorigenicity experiments, GBM 44 GSC were infected with MV *in vitro* and subsequently implanted into the right caudate nucleus of nude mice: significant prolongation of survival in mice implanted with infected GSC was observed, compared to mock infected controls (p=0.0483). In therapy experiments in GBM6 and GBM12 GSC xenograft models, there was significant prolongation of survival in MV-GFP treated animals compared to inactivated virus treated controls (GBM6 p=0.0021, GBM12 p=0.0416). Abundant syncytia and viral replication was demonstrated in tumors of MV treated mice. Conclusion: Measles virus derivatives have significant antitumor activity against glioma derived stem cells *in vitro* and *in vivo*.

Keywords

measles virus; gliomas; stem cells; MV-CEA; MV-NIS

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary information is available at Gene Therapy's website.

Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults and has a dismal prognosis despite aggressive use of multimodality treatment including surgery, chemotherapy and radiation therapy.[1] One potentially significant hurdle to overcome in the treatment of GBM pertains to eradication of glioma cells with stem-cell like properties (GSC); these are cells with self-renewing properties, able to differentiate into neurons, oligodendrocytes and astrocytes, initiate brain tumors *in vivo*, and have been associated with the promotion of angiogenesis development of resistance to radiation and chemotherapy.[2–4] Our laboratory has previously demonstrated that derivatives of the measles virus (MV) Edmonston's strain have significant activity against glioma lines and orthotopic xenografts,[5–7] and a phase I trial of the measles derivative MV-CEA is currently ongoing in recurrent GBM patients. Assessing the ability of MV-strains to infect and eradicate glioma cells with stem-cell like properties represents an important parameter which could potentially affect the antitumor activity of this novel virotherapy platform and impact on the development of combinatorial strategies. Here we show that GSC overexpress the measles virus receptor CD46. Futhermore, MV-strains of the Edmonston vaccine lineage have significant antitumor activity against glioma stem cells *in vitro* and *in vivo*, and can decrease their tumorigenicity; these findings can have important translational implications in glioma treatment.

Results

Viral strains

Antitumor activity of the MV-strains MV-CEA, MV-NIS, and MV-GFP against GSC was examined. Their construction has been previously described.[8–10] A schematic representation of the strains used is included in Figure 1.

Characterization of stem cell marker expression in neurosphere lines

GBM xenografts were developed in BALB/c nude mice from GBM patients as previously described.[11, 12] Tumors were excised from mice bearing the GBM xenografts GBM 6, 10, 12, 14, 23, 34, 38, 39, 43, and 44, mechanically disrupted and grown in NeuroCult medium formation of neurospheres was observed within 2–6 weeks depending on the cell line. Neurosphere lines used in vitro and in vivo experiments were further characterized for the expression of the glioma stem cell markers CD133, Nestin, Sox 2, ATF5, and OLIG 2. All neurosphere lines tested expressed at least 3/5 stem cell markers. Representative results for three of the neurosphere lines are shown in Figure 2.

Expression of measles virus receptor CD46 in neurosphere lines

CD46 expression in neurosphere lines was tested by FACS and immunohistochemistry overexpression was confirmed in all 10 lines tested. Representative results are shown for GBM34 and GBM22 (Figure 3).

Assessment of cytopathic affect of MV strains against GSC

In order to access antitumor activity of measles virus strains against GSCs neurosphere lines were infected with the MV strains, MV-NIS, MV-GFP and MV-CEA at an MOI of 0.1 or 1.0 and trypan blue exclusion assays were performed at multiple timepoints. Results were presented as percentage of the uninfected controls. Measles virus derivatives had significant antitumor activity against GSCs *in vitro*, which was dependent on MV strain and MOI (Figure 4).

Assessment of viral replication

Virus replication was assessed using one-step viral growth curves as previously described.[7] Neurosphere cultures supported robust growth of the MV-NIS, MV-GFP and MV-CEA virus (Figure 5).

Assessment of tumorigenicity of infected neurospheres

GSC cells obtained from GBM44 xenografts were infected with either MV-NIS (MOI of 10) or mock infected control and 3×10^5 cells were implanted into the right caudate nucleus of BALB/C nude mice. There was significant prolongation of survival in mice implanted with infected neurospheres as compared to mice implanted with mock infected neurospheres ($p=0.04$) indicating that infection of GSC *in vitro* can modify their aggressive biologic behavior following orthotopic implantation (Figure 6).

As Figure 6 illustrates, some of the mice implanted with infected neurospheres developed tumors. Examination of these tumors at the time of euthanasia showed lack of syncytia, indicating that the tumors likely derived from non-infected cells. Immunofluorescence for the natural killer cell lectin-like receptor (KLRG1) and the macrophage marker CD68, sohowed lack of a significant immune infiltrate (Supplemental Figure 1), suggesting a low likelihood that innate immune response driven viral inactivation was responsible for tumor cell repopulation in this model.

Assessment of therapeutic efficacy in vivo

In order to assess the antitumor activity of MV strains against neurosphere derived xenografts, GBM6 or GBM12 derived glioma stem cells were orthotopically implanted into the right caudate nucleus of nude mice. Animals were treated with a total dose of either 9×10^5 TCID50 (GBM12) or 1.8×10^6 TCID (GBM6) of MV-GFP. Control animals received UV-inactivated virus. Significant prolongation of survival of the treated animals was observed in both models ($p=0.0416$ and $p=0.0021$ respectively) (Figure 7). In a parallel experiment treated and control animals bearing orthotopic GBM6 GSC xenografts (a CD133 positive neurosphere line) were euthanized either on day 3 post treatment completion or when moribund. Abundant syncytia were observed in MV-GFP treated mice. Syncytia expressed CD133 (Figure 7D) confirming activity of the measles virus derivatives against GSC derived tumors in vivo. In situ hybridization for the measles virus N (nucleoprotein)-mRNA was strongly positive (Figure 7F) indicating viral replication in the GSC derived syncytia.

Discussion

Glioblastoma (GBM) remains incurable with a median survival of 15–16 months, despite the use of multimodality treatments including surgery, chemotherapy, and radiation therapy.[13] Brain tumor stem cells are believed to play a key role in mediating glioma resistance to chemotherapy and radiation therapy, in part due to their ability to more efficiently repair damaged DNA[3, 14–16] as compared to tumor cells without stem cell properties. Treatment of GBM with temozolomide, an agent commonly used in glioma treatment, leads to preferential survival of a cell population, enriched in the stem cell marker[16] CD133, and glioblastomas that recur following chemoradiation are characterized by a stem-cell related gene signature.[17] Stem cell resistance to chemotherapy agents such as temozolomide, carboplatin, paclitaxel and etoposide appears to be induced through elevated expression of antiapoptotic genes and 0–6 methylguanine-methyltransferase (MGMT), a DNA repair enzyme that inhibits the activity of DNA methylating agents such as nitrosoureas and temozolomide.[18] These cells have the capacity for self renewal and multilineage differentiation and efficiently initiate tumor xenografts in vivo.[3]

Development of approaches that can effectively eradicate stem cells has therefore the potential to significantly improve outcome for glioblastoma multiforme patients. Although small molecule inhibitors of key pathways in glioma stem cells survival, such as the notch pathway, are currently in early clinical testing;[19] their clinical efficacy to date has been modest and the development of additional approaches targeting glioma cells with stem cell-like properties is needed. Oncolytic viruses might represent such an approach. DNA viruses such as the Delta-24-RGD adenovirus[19] and oncolytic HSV strains[20, 21] have been reported to have antitumor activity against glioma stem cells. There is no information to date, however, regarding activity of oncolytic RNA viruses.

We have demonstrated that engineered strains of measles virus, a negative strain RNA virus of the paramyxovirus family, deriving from the Edmonston vaccine lineage have significant activity against glioma cells with stem-like properties. These cells allow efficient viral propagation as indicated by one-step viral growth curves and were effectively killed even in MOIs as low as 0.1. In addition, infection prior to orthotopic implantation of human GBM xenograft derived glioma stem cells modified the aggressive biologic behavior of these cells as demonstrated by the significant prolongation of survival of animals treated with infected cells. Treatment of orthotopic xenografts established by glioma stem cells using two different models, GBM6 and GBM12, also led to therapeutic benefit and significant prolongation of median survival. The characteristic cytopathic effect with formation of syncytia was observed in these CD133 positive orthotopic xenografts. In situ hybridization for measles virus mRNA confirmed in vivo replication of the virus.

Sensitivity of glioma cells to measles infection can at least in part be explained by overexpression of measles virus receptor CD46 in neurosphere lines. In contrast to the wild type MV strains which enter the cells predominantly via the SLAM receptor (expressed in B-, T-cells and macrophages)[22, 23], measles virus oncolytic vaccine strains of the Edmonston vaccine lineage have been shown to enter cells predominantly via the ubiquitous expressor CD46.[24, 25] CD46 or membrane cofactor protein is overexpressed in different

tumors, including gliomas[26] and protects them from complement mediated lysis.[27–29] We have tested levels of CD46 expression by both immunohistochemistry and FACS in ten different neurosphere lines deriving from patient originated xenografts and observed high expression in all ten neurosphere lines tested, indicating the glioma stem cells represent a good target for CD46 targeted therapeutics such as MV strains. In an ongoing trial of intratumoral and resection cavity administration of the measles derivative MV-CEA in recurrent GBM patients, we are in the process of investigating the correlation between CD46 expression in tumors and viral replication/response to oncolytic measles virotherapy.[30]

It is of note that recently a third measles receptor, the adherens junction protein nectin-4, has been identified.[31] The new receptor, the adherens junction protein nectin-4, is expressed in the respiratory epithelium.[31] It is also expressed in ovarian, breast, and lung cancer.[32–34] Although members of the nectin family, such as nectin-1 and -3 appear to play an important role in the function of brain synopses,[35] the role of nectin-4 is not well characterized in brain tumors or normal brain. FACS analysis in 4 different measles permissive glioma stem cell lines (GBM6, GBM12, GBM102, GBM143) showed no nectin-4 expression in GSC (Allen and Galanis, unpublished data), indicating a low likelihood that nectin-4 expression represent an important parameter determining the efficacy of MV based therapeutics against GSC. We are in the process of expanding this analysis in a larger panel of GSC, however.

Our results, demonstrating the efficacy of MV strains against glioma cells with stem cell like properties, suggest that combinatorial strategies of oncolytic measles strains with agents representing the standard of care in GBM treatment, such as RT or temozolomide, represent a direction worth exploiting since they have the potential to improve outcome in response to these conventional treatment strategies by eradicating resistance mediating cells with stem cell-like properties.

Methods

Viral construction

The construction of recombinant measles virus strains which contain the soluble extracellular domain of human CEA (MV-CEA), green fluorescent protein-eGFP (MV-GFP) or the sodium-iodide symporter gene (NIS, MV-NIS) has been described elsewhere[8–10] (Figure 7).

Cell lines

Subcutaneous xenografts were developed in BALB/c nude mice from GBM patients as previously described;[36] when orthotopically implanted these xenografts have been demonstrated to maintain the histologic characteristics and invasiveness of the primary tumor they derived from. Tumors were excised from mice bearing the GBM xenografts GBM6, 10, 12, 14, 22, 34, 38, 39, 43, and 44 and each was mechanically disrupted with a sterile blade and grown at 37°C in a humidified environment, 5% CO_2 in either regular media (DMEM with 10% FBS, 1X Penn/Strep) or NeuroCult® media (Stemcell Technologies, Vancouver, BC, CA).

Immunohistochemistry to assess expression of GSC markers, CD133, SOX2, Nestin, ATF5, OLIG2, and the MV receptor CD46 in neurospheres

Neurospheres from primary GBM xenograft cultures were harvested, expanded *in vitro*, fixed in acetone, and examined by IHC for the expression of: a) GSC markers, CD133, SOX2, Nestin, ATF5, OLIG2, and MV receptor CD46. Primary antibodies to test marker expression were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and IHC was performed as per manufacturer's instructions. In summary, slides were blocked in PBS containing 10% goat or donkey serum, 1% BSA and 0.025% Triton-X-100 for one hour and incubated overnight at 4°C with 1:100 dilution of primary antibody. Primary antibody was omitted in negative controls. The slides were rinsed thrice for 5 min in PBS and exposed to a 1:500 dilution of either biotinylated goat anti-mouse or donkey anti-goat for one hour and visualized with DAB substrate staining followed by VECTASTAIN Elite ABC kit (Vector Laboratories, INCl, Burlingame, CA) and counterstained with ACCUSTAIN (Sigma-Aldrich INC., St. Louis, MO).

CD46 flow cytometry

Primary GSC cultures deriving from GBM 6, 10, 12, 14, 22, 34, 38, 39, 43, and 44 xenografts (5×10^5 cells), were incubated for 45 minutes on ice with 10μg of FITC labeled mouse anti-human CD46 (BD Pharmingen, San Jose, CA) in DPBS containing 0.5% BSA, FITC labeled mouse IgG2a, κ serving as the isotype control. The cells were washed twice, fixed in DPBS containing 0.5% paraformaldehyde and were analyzed using a Becton Kickinson FACScan Plus cytometer with Cell Quest software.

Assessment of cytotoxicity of MV strains against GSC

Neurospheres were enzymatically and mechanically disrupted in TrypleE (Invitrogen, Carlsbad, CA) with a 10 ml pipette, 5×10^6 cells were plated into 6-well dishes and infected 24 hours later at an MOI of 0.1 or 1.0 with MV-GFP, MV-CEA, or MV-NIS, with uninfected cells serving as controls. Cytotoxicity of MV-strains against GSC was evaluated by counting the number of viable cells by trypan blue exclusion assay as previously described[7] and compared against uninfected controls.

Assessment of viral growth in GSC

Viral growth was assessed by one step viral growth curves as previously described.[10] In summary, neurospheres were infected with MV-NIS, MV-GFP or MV-CEA at an MOI of 1. Cells were harvested at different time points and viral titer was determined by 50% end point dilution assay on Vero cells, as previously described.[37]

Tumorigenicity experiments

Neurospheres derived from GBM44 xenografts were infected with MV-NIS *in vitro* at a MOI of 10 TCID50. Mock infected neurospheres were used as controls. Six hours later the cells were harvested, mechanically disrupted and subsequently 3×10^5 cells were orthotopically implanted into the right caudate nucleus of 5–6 week old BALB/c mice (n=5 per group). These mice were followed daily and euthanized when neurologic

symptomatology or >10% weight loss was observed. All animal experimental protocols were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Assessment of therapeutic efficacy *in vivo*

GBM6 and GBM12 orthotopic models were established by implantation of 3×10^6 GSC into the right caudate nucleus of five week old BALB/c mice, using the small animal stereotactic frame (ASI Instruments, Warren, MI) and a 26-gauge Hamilton syringe. Treatment was initiated at either 1 wk or 2 wks post implantation respectively. Mice received either MV-GFP (N=5) or UV-inactivated virus as control (N=5) using the same coordinates as for implantation. MV-GFP treated mice received 1×10^5 (GBM12 GSC model) or 2×10^5 (GBM6 GSC model) TCID50 per dose three times per week over a three-week period for a total dose of 9×10^5 or 1.8×10^6 TCID50 respectively. Mice were observed daily and were euthanized when neurologic symptomatology or >10% weight loss was observed.

In a parallel experiment, designed to perform correlative studies in treated tumors, 3×10^6 GBM6 derived GSC (a line expressing the stem cell marker CD133) were implanted into the right caudate nucleus of 5–6 weeks old BALB/c mice as previously described. One group of animals (n=10) received MV-GFP, 1.5×10^5 TCID50 per dose every second day for a total of 3 doses starting at 2 weeks following implantation and a second group treated with UV inactivated virus served as the control (n=5). Treated mice and corresponding controls were euthanized either at 3 days following completion of viral treatment (n=5) or when mice became moribund (n=5). Animal brains were formalin fixed, stained with H and E and examined by immunofluorescence for CD133 expression and in situ hybridization, for viral N mRNA.

Immunofluorescence for CD133 expression in vivo

Formalin fixed brains were examined for CD133 expression by immunofluorescence: following heat mediated sodium citrate antigen retrieval, slides were blocked in PBS containing 10% goat serum, 1% BSA and 0.025% Triton-X-100 for one hour and incubated overnight at 4°C with primary antibody (AbCam, Inc., Cambridge, MA, 1:200 dilution) or without primary antibody control. The slides were rinsed three times for 5 min in PBS and exposed to Alexa Fluor 647 Goat Anti-Rabbit (Invitrogen, Eugene, OR). Slides were counterstained with DAPI and examined with a Zeiss LSM510 confocal microscope.

In situ hybridization for detection of measles virus N (nucleoprotein)-mRNA

A custom designed digoxigenin (DIG)-labeled oligonucleotide probe (GeneDetect) was used for ISH. The samples were treated with Proteinase K (20 mg/mL) for 20 minutes at 37°C, washed twice with phosphate-buffered saline/Glycine and prehybridized for 2 hours at 39.4°C. The sections were then washed and incubated overnight with the DIG-labeled oligonucleotide probe (250 ng) at 39.4°C in a humidified chamber. The next day the slides were washed in SSC/DTT followed by the detection step performed using Anti-DIG-AP, Fab fragments (Roche Diagnostics, Indianapolis, IN) at 4°C in a humidified chamber, followed by serial washes, NBT/BCIP detection and counterstaining with Nuclear fast red (Sigma).

Immunofluorescence for natural killer cell lectin-like receptor (KLRG1) and CD68

Five –micron sections were cut from formalin fixed, paraffin embedded mouse brains, deparaffinized with Citrisolv (ThermoFisher Scientific) and then rehydrated in graded ethanol baths with a final three minute immersion in water. Antigen retrieval was performed with Antigen Retrieval Citra (Biogenex, San Ramon, CA) by boiling for twenty minutes. Sections were subsequently rinsed in water and then PBS. Blocking was performed in PBS containing 10% goat serum, 1% BSA and 0.025% Triton X-100 for two hours. Sections were incubated with a 1:100 dilution of either anti-KLRG1 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-CD68 (AbD serotec, Raleigh, NC) for one hour, primary antibody being omitted in the negative control sections. Mouse spleen sections were used as positive control. Sections were washed three times, five minutes each, in PBS and exposed to goat anti-rabbit or goat anti-rat alexa-fluor-647 respectively (Life Technologies, Grand Island, NY) for 45 minutes and subsequently washed in PBS for 35 minutes. Slides were dehydrated in graded ethanol baths, dried and mounted with UltraCruz mounting media (Santa Cruz Biotechnology, Santa Cruz, CA) and examined using a Zeiss LSM510 confocal microscope using a 42X objective (Carl Zeiss Incorporated, North America).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding support provided by NIH P50 CA 108961 and R01 CA 154348.

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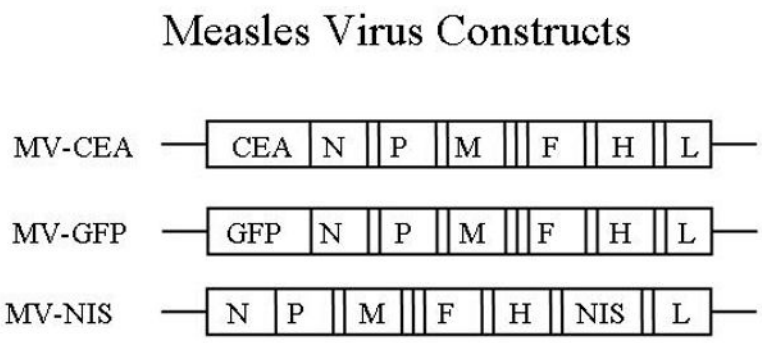

Figure 1.

Schematic representation of the viral strains: MV-CEA: MV strain expressing the human carcinoembryonic antigen; MV-NIS: MV strain expressing the sodium iodine symporter. N, nucleoprotein gene; MV-GFP: MV strain expressing the enhanced Green Fluorescent Protein; L, large protein gene; P, phosphoprotein gene; M, matrix protein gene; F, fusion protein gene; H, hemagglutinin gene; CEA, human carcinoembryonic antigen gene; NIS, sodium iodine symporter gene.

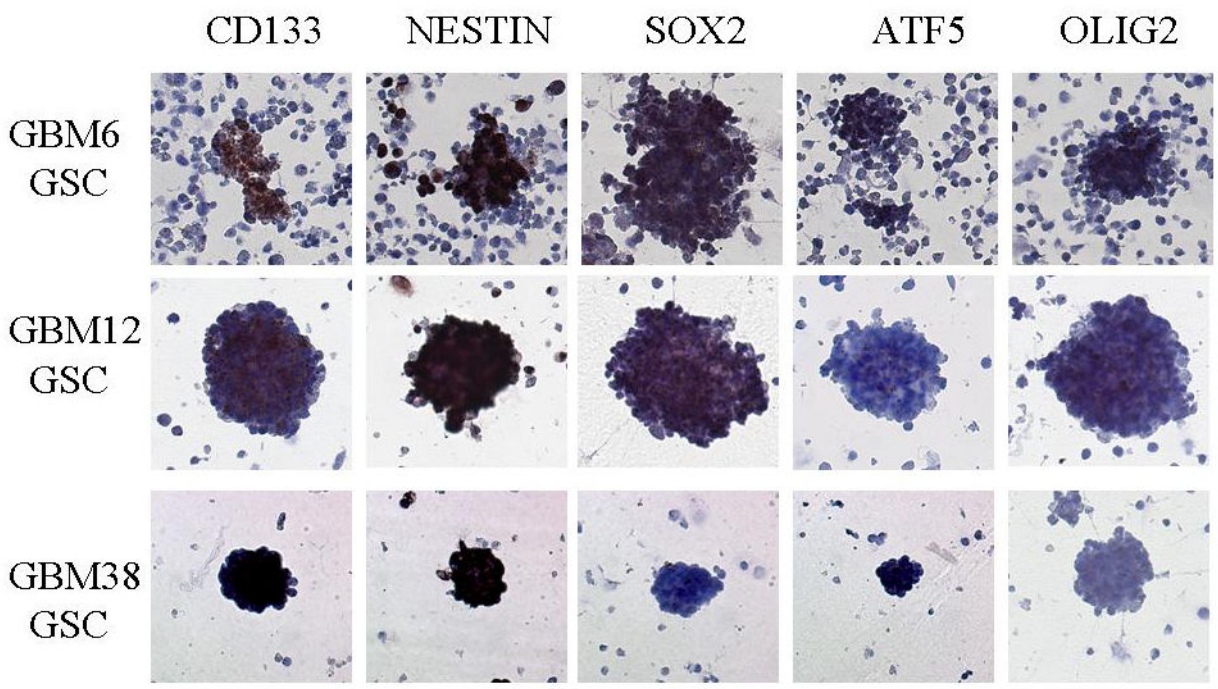

Figure 2.

Neurospheres derived from GSC xenograft cultures express stem-cell markers (CD133, Nestin, SOX2, ATF5 and OLIG2). Representative data for GBM6, GBM12 and GBM38 glioma stem cell lines are shown.

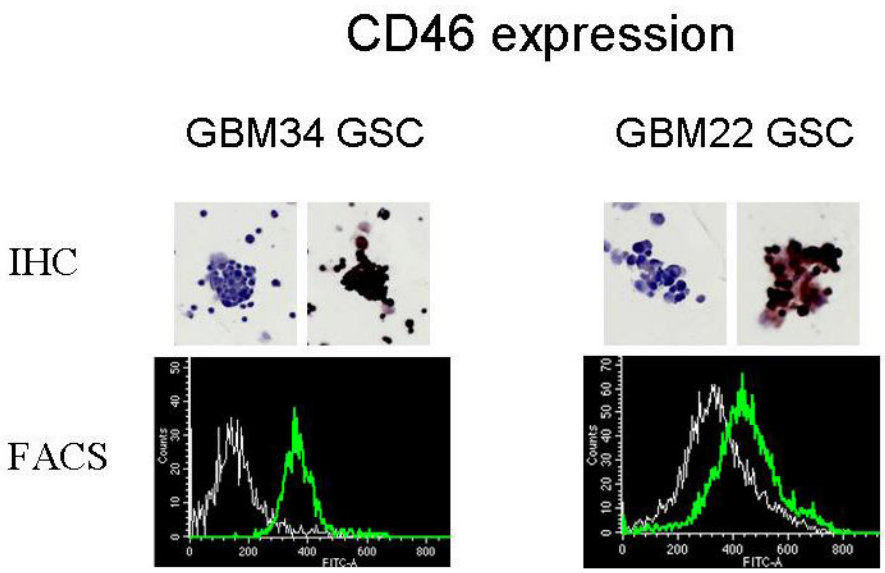

Figure 3.

Expression of measles virus receptor CD46 was examined by IHC and flow cytometry. Significant expression of CD46 was observed in all neurosphere lines tested. Representative ICH and FACS data in GBM34 and GBM22 GSC lines are presented.

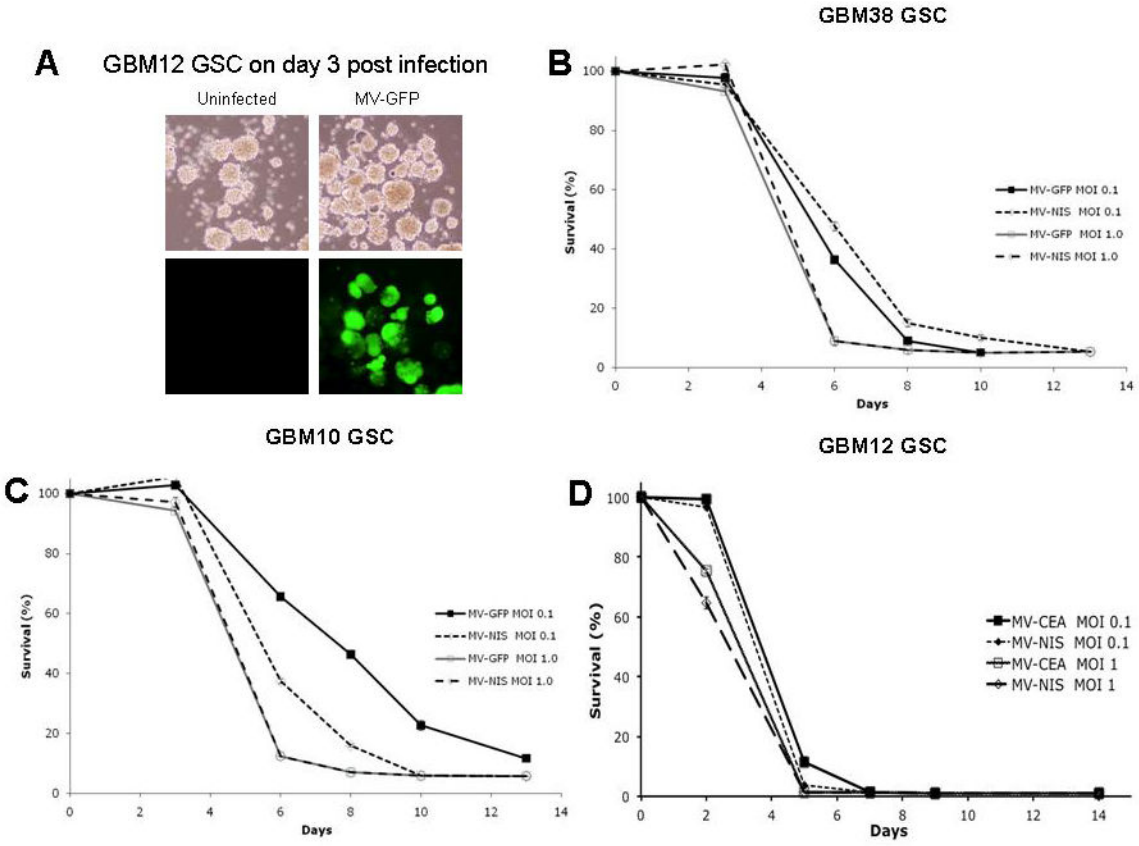

Figure 4.

A Infection with MV-GFP resulted in abundant GFP expression in infected GBM12 neurospheres. B, C, D: The cytopathic effect of MV-GFP, MV-NIS and MV-CEA was assessed in GSC neurosphere lines using trypan blue exclusion assays. Viral MOIs of 0.1 and 1 were used. Results are presented as percentage of uninfected controls. Representative results are shown for GBM 38 (B), GBM10 (C) and GBM12 (D). There was MOI dependent cytotoxicity with almost complete eradication of neurosphere cultures by day 6 post infection.

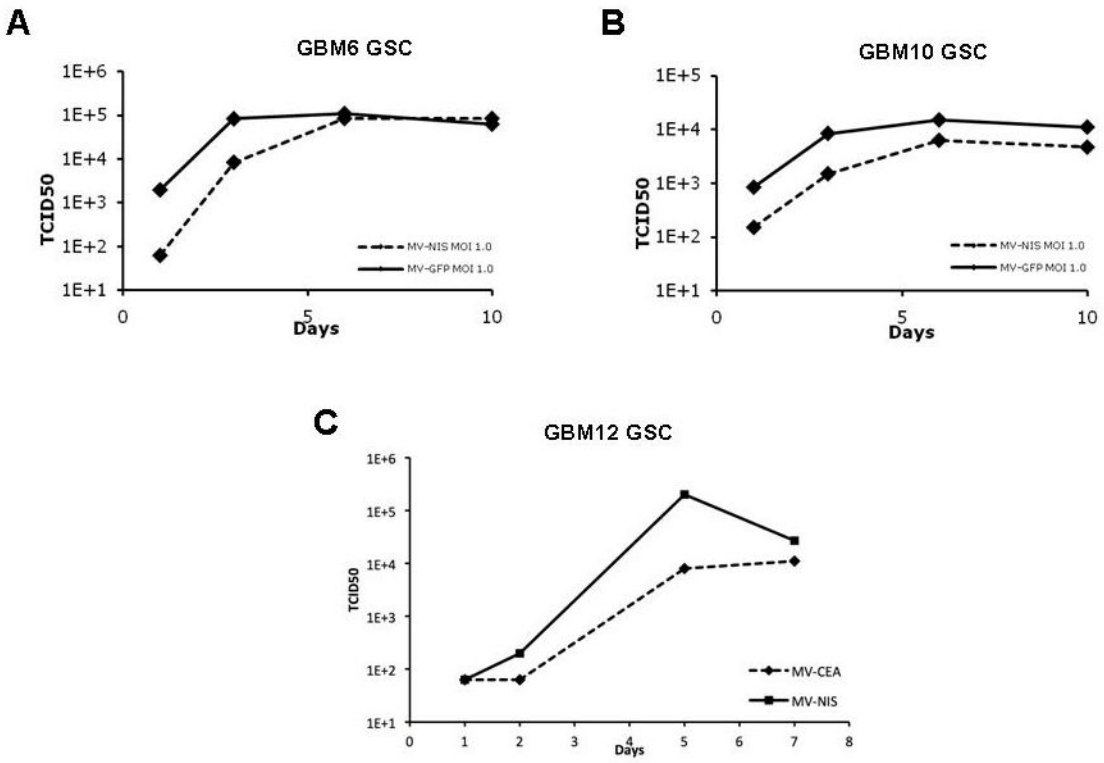

Figure 5.

Viral replication was assessed by one-step viral growth curves. Representative results in GSC deriving from GBM 6, 10 and 12 are shown. Cells were infected with MV-NIS, MV-GFP or MV-CEA at an MOI of 1, harvested at different time points and titered in Vero cells. Results are expressed as TCID50 of harvested virus. All glioma stem cell lines effectively supported viral replication.

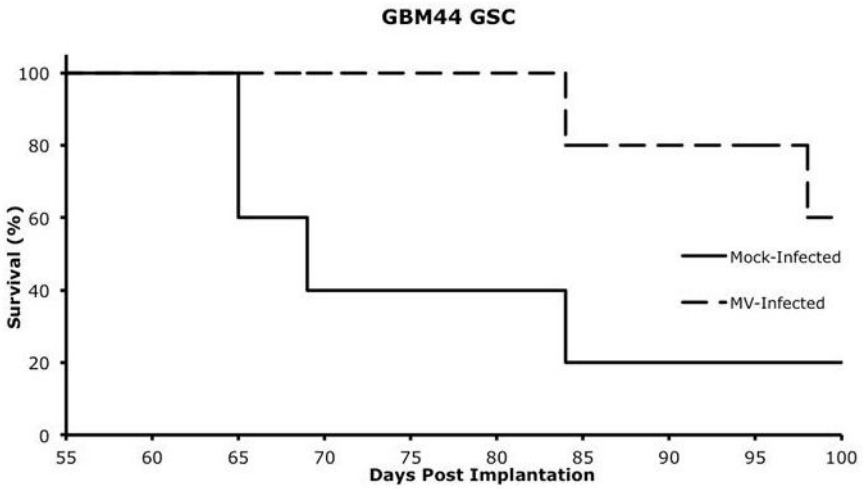

Figure 6.

GBM44 GSC were either infected with MV-NIS (MOI 10) or mock-infected, and 3×10^5 GSC cells were implanted into the right caudate nucleus of nude mice. Animals were followed for survival. There was significant prolongation of survival in mice which were implanted with cells infected with MV-NIS ($p=0.04$) with median survival of 68 days in the control group versus not having been reached at 100 days in mice implanted with infected GSC.

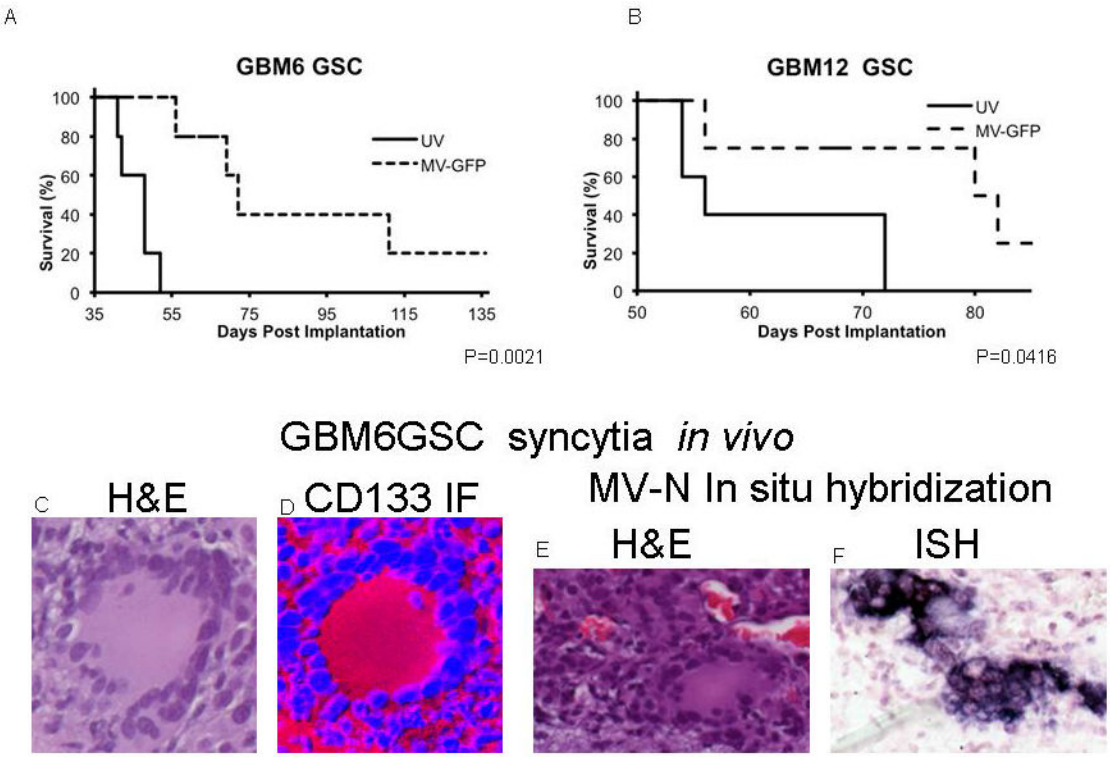

Figure 7.

GBM6 (A) or GBM12 (B) derived GSC were orthotopically implanted into the right caudate nucleus of nude mice. Animals were treated with a total dose of 1.8×10^6or 9×10^5 TCID50 MV-GFP, respectively, or UV inactivated virus. In both orthotopic treatment models, significant prolongation in survival was observed (p=0.0021 and p=0.0416 respectively). Abundant syncytia were observed in MV-GFP treated mice in H and E stain (C, E). Syncytia expressed the stem cell marker CD133 (D). Viral replication was confirmed by in situ hybridization for measles virus N mRNA (F).