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ORIGINAL ARTICLE

Targeting human glioma cells using HSV-1 amplicon peptide display vector

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Targeting cell infection using herpes simplex virus type 1 (HSV-1) vectors is a complicated issue as the process involves multiple interactions of viral envelope glycoproteins and cellular host surface proteins. In this study, we have inserted a human glioma-specific peptide sequence (denoted as MG11) into a peptide display HSV-1 amplicon vector replacing the heparan sulfate-binding domain of glycoprotein C (gC). The modified MG11:gC envelope recombinant vectors were subsequently packaged into virions in the presence of helper virus deleted for gC. Our results showed that the tropism of these HSV-1 recombinant virions was increased for human glioma cells in culture as compared with wild-type virions. The binding of these recombinant virions could also be blocked effectively by pre-incubating the cells with the glioma-specific peptide, indicating that MG11 peptide and the recombinant virions competed for the same or similar receptor-binding sites on the cell surface of human glioma cells. Furthermore, preferential homing of these virions was shown in xenograft glioma mouse model following intravascular delivery. Taken together, these results validated the hypothesis that HSV-1 binding to cells can be redirected to human gliomas through the incorporation of MG11 peptide sequence to the virions.

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Introduction

Targeting gene therapy of malignancies can be achieved through selective cell entry or use of a cell-specific promoter to regulate transgene expression.[1] Targeting can improve the therapeutic index of gene transfer by increasing the relative infectivity of tumor tissue, thereby reducing damage of healthy tissues and decreasing the risk of germline transduction. Natural herpes simplex virus type 1 (HSV-1) infection is initiated when the virus enters the epithelial cells of the skin or mucous membrane. Initial attachment of the enveloped particle is mediated by nonspecific charge interactions between the heparan sulfate (HS)-binding domains of glycoproteins (gC or gB or both) in the virion envelope and that of the HS glycosaminoglycan side chains of proteoglycans found on the host cell surface.[2] Subsequently, the conformation of the viral glycoprotein D (gD) becomes altered as a consequence of its interaction with one of three cell surface entry receptors, namely (i) the herpesvirus entry mediator A (HveA), a member of the tumor necrosis factor receptor family;[3] (ii) nectin-1 (HveC), and in some HSV-1 gD mutants, nectin-2 (HveB),[4] both of which belong to the immunoglobulin superfamily;[5,6] and (iii) the specific O-linked sulfated proteoglycan.[7] The choice of entry receptors varies among cell types. Thereafter, the interaction of gD, its receptors and three additional HSV glycoproteins gB, gH and gL leads to the fusion of the viral envelope and the plasma or endosomal membrane.[8–11] Recently, the paired immunoglobulin-like type 2 receptor (PILR)-α has been identified as the entry co-receptor for gB.[12] Thus, it appears that both HVEM and PILR are required for efficient HSV-1 infection. This complex multipartite entry-fusion system eventually leads to the entry of viral capsid with its surrounding tegument proteins into the cell.[13,14] After entry, the viral capsid is carried along the cellular cytoskeleton by active transport to the nucleus,[15,16] where the capsid opens and viral DNA is injected in through nuclear pores to initiate a productive infection or enter into latency.[17]

Native HSV-1 tropism can be altered by replacing the natural cell-recognition domains in the viral envelope glycoproteins with cell-specific ligands to allow for selective entry in cells that bear receptors for those ligands. For example, the gC has been fused with glial cell line-derived neurotrophic factor (GDNF) or brain-derived neurotrophic factors (BDNFs) as a mean of gene delivery to nigrostriatal neurons that expressed the GDNF receptor-α-1 or the high-affinity receptor for

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BDNF, *TrkB*.[18] The specificity of such targeted vectors could be further enhanced by co-transfecting the HSV-1 cosmid sets that contained the gC-GDNF or gC-BDNF with an amplicon plasmid, which contains the *lac* reporter gene under the regulation of neuronal-specific promoters.[19] The combination of targeted gene transfer with neuronal-specific promoters was shown to markedly improve the nigrostriatal neuron-specific expression, which may be useful for the treatment of Parkinson's and other neuronal diseases. Mutational analysis across the external N-terminal domain of gC has defined the major HS-binding determinant to lie between amino acids (aa) 33 and 123.[20] Modification of the HSV virion by replacement of the HS-binding domain in the gC gene with receptor ligands, for example, for erythropoietin, was found to enrich virion binding to cells bearing this receptor, but also decreased viral infectivity.[21]

In another study, the HS site was replaced with a His-tag sequence inserted in-frame within gC using an amplicon plasmid packaged with a gC-deleted helper virus.[22,23] The resulting HSV-1 virions containing the modified gC virion envelope were found to bind to cells expressing a pseudo-His-tag receptor at enhanced efficiency as compared with wild-type virions. This study provides a good strategy for introducing known ligands or small peptide epitopes into the viral envelope for targeting purposes. *In vitro* biopanning using phage display peptide libraries is a powerful tool to isolate and characterize small peptide ligands against viable glioma cells.[24] Using this technique, we have reported on the isolation and functional characterization of a novel glioma-specific peptide (MG11) that could target specifically both to a human glioma cell line in culture and glioma tumors *in vivo* in a xenograft model. Further, the MG11 peptide was also shown to bind to primary human glioma cells and not to normal human brain cells in culture.[25] At this moment, we have yet to confirm the identity of the receptor for the glioma-specific peptide, MG11; however, we have previously reported that this peptide sequence is similar to that of the semaphorin 4B precursor.[25] The semaphorins are a family of proteins that have been implicated not only in axon guidance, but also in the regulation of cell migration, angiogenesis and the immune response.[26,27] The class 4 semaphorins contain seven members, SEMA4A to G.[28] Other than SEMA4D, which acts as a repulsive signal that uses Plexin-B1 as its receptor, the rest of the class 4 semaphorins are not well studied.[29] Recently, SEMA4B has been shown to interact with CLCP1 (CUB, LCCL-homology, coagulation factor V/VII homology domains protein).[30] CLCP1 degradation after CLCP1–SEMA4B interaction may indicate an induction of CLCP1 endocytosis by SEMA4B. It is therefore of interest to study the functional relationship between CLCP1, SEMA4B and MG11 in future.

In this study, we evaluated whether the glioma-specific MG-11 peptide can be used as a targeting ligand for HSV-1 infection of human glioma cells. HSV-1 virions expressing the MG11-modified gC were evaluated for infection of human glioma cells versus non-glioma cells, as compared with virions with wild-type gC, both in culture and *in vivo*.

Results

Construction of retargeted virions for human glioma cells

The glioma-specific peptide (denoted as MG11) was isolated by screening the Ph. D-12 phage display library against several human glioma cell lines, including ΔGli36 (also known as Gli36-EGFR or ΔGli36), SF767, U87MG, U251MG and U373MG, and its specificity toward human glioma cells was shown *in vitro* and *in vivo*.[25] DNA sequences encoding the MG11 peptide were synthesized and subcloned into unique restriction enzyme sites in the nucleic acid sequence of pCONGA4, which encodes a chimeric interleukin-4 (IL4)-gC protein on viral envelope (Figure 1a). As a result, the nucleotide sequence encoding IL-4 C-terminal domain was substituted by the MG11 sequence for the generation of a potential targeting vector to human glioma. Amplicon pCONGA4 was retained as the control vector as we compared it to the same amplicon with the IL-4 sequences deleted (pCONGA4Δ-Luc), and observed no significance differences in infection of glioma cells (see Supplementary data 1). In contrast, the level of luciferase activities mediated by MG11-pCONGA-Luc is significantly higher in SF767 cells than those observed in pCONGA4Δ-Luc or pCONGA4-Luc. The presence of the MG11 sequence was confirmed by restriction enzyme mapping (Figure 1b) and DNA sequencing. When pCONGA4 DNA (10 177 bp) was digested with *Sac*II, five DNA fragments were generated with the predicted size of 7553, 1724, 687, 126 and 87 bp. In MG11-pCONGA plasmid, the replacement of IL-4 by MG11 resulted in a slightly smaller, linearized plasmid DNA size of 10 135 bp. Further, there was an additional *Sac*II site within the MG11 sequence that digested the 1724 bp into two DNA pieces of 1160 and 522 bp (as shown in Figure 1b). These amplicon plasmid constructs are subsequently purified by QIAgen maxi columns (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Characterization of MG11-pCONGA viruses

The amplicon plasmids, pCONGA4 and MG11-pCONGA, were transfected into VERO cells using lipofectamine. At 6 h post-transfection, these cells were infected with gC-deleted helper viruses (gCΔ2-3) and harvested when 100% cytopathic effect was evident.[2] The absence of gC in the helper virus allows the incorporation of only the recombinant MG11-gC into the viral envelope of both the resulting amplicon vectors and helper virus without competition from wild-type gC. This packaging procedure results in a mixed population of retargeted amplicon vectors (enhanced green fluorescence protein (eGFP)-positive, represented in Figure 1c (i)) and high-titer helper virus virions (*lac*Z+, Figure 1c (ii)). Typically, the retargeted amplicon and helper virus virions were present in the virus stock in a ratio ranging from 1:84 to 1:144, or every ~110 modified gC-containing HSV virions include one modified amplicon virion. The presence of recombinant MG11-gC in the virions was subsequently confirmed using western blot analysis. Viral protein extracts were prepared from purified viral stocks of gCΔ2-3, MG11-pCONGA and pCONGA4, and were analyzed using western blots with anti-gC polyclonal antibody (R47, kindly provided by Drs GH Cohen and RJ Eisenberg, University of Pennsylvania). We were able to detect the presence of a protein migrating at the expected size of ~41 kDa in viral extracts of pCONGA4 and MG11-pCONGA, but not gCΔ2-3, confirming that the presence of recombinant glycoprotein C in amplicon stocks (Figure 1d). Unfortunately, the protein product of

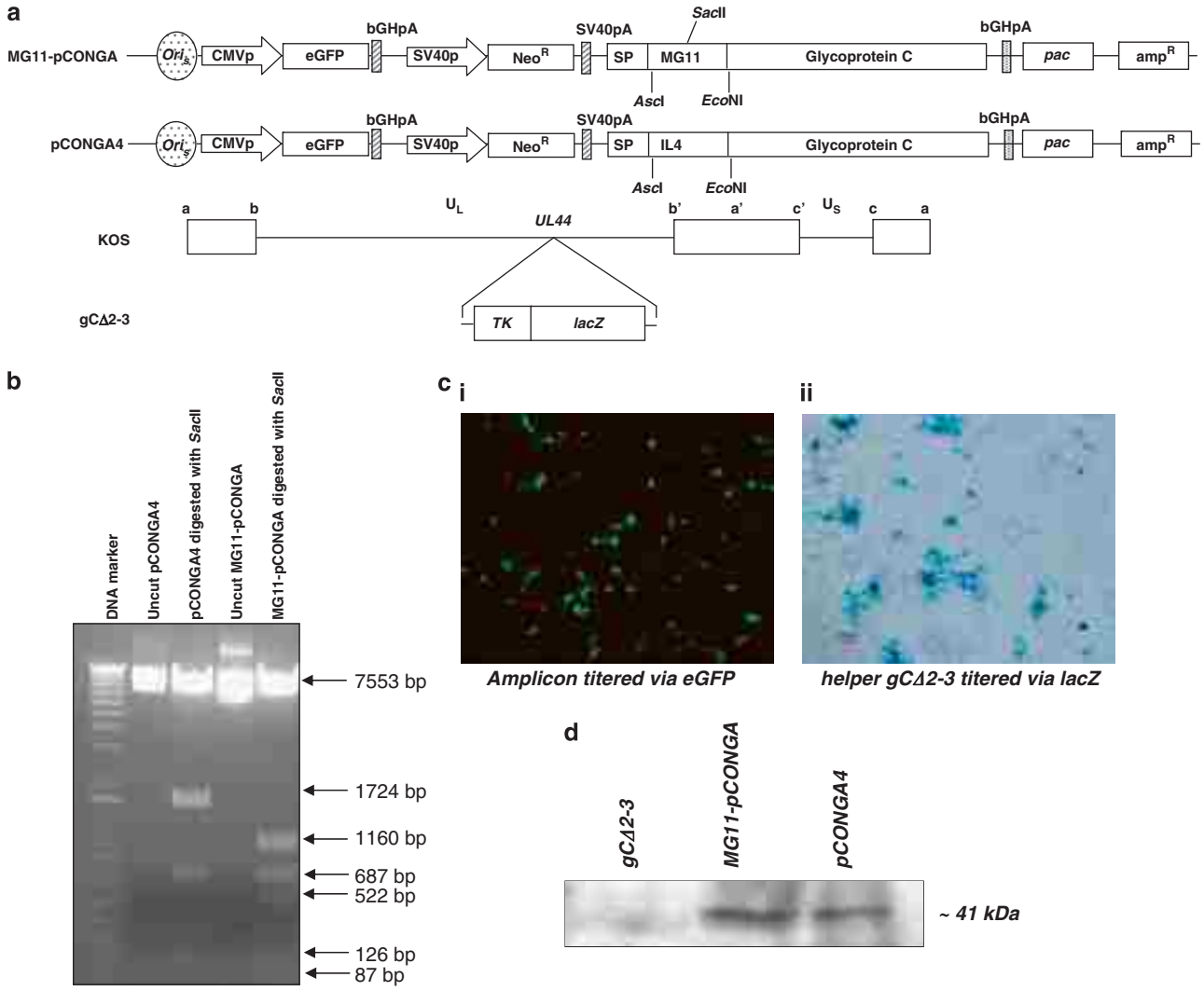

Figure 1 Characterization of MG11-pCONGA construct and viral vectors. (**a**) Schematic representation of MG11-pCONGA. Two sets of amplicon vectors have been generated for the various studies. The first set is the glioma-specific MG11-pCONGA and its backbone vector, pCONGA4 (as shown in Figure 1a). The chimeric MG11-glycoprotein C (gC) is driven by the gC endogenous promoter, which is located upstream of the signal peptide sequence. To visualize the targeting ability *in vivo*, the neomycin-resistance gene in MG11-pCONGA and pCONGA4 was substituted by the luciferase gene, thus generating MG11-pCONGA-Luc and pCONGA4-Luc. Details of vector construction can be found in the Materials and methods section. The lower panel is a schematic representation of the helper virus gCΔ2-3 that was derived from wild-type herpes simplex virus (HSV) KOS strain. In gCΔ2-3, majority of gC (encoded by *UL44*) was replaced by a lacZ cassette driven by the TK promoter (TK). *Ori*S, herpes simplex virus type-1 origin of replication; CMVp, cytomegalovirus promoter; eGFP, enhanced green fluorescence protein; SV40pA, simian virus 40 polyadenylation signal; SV40p, simian virus 40 promoter; Neo^R, neomycin-resistant gene; SP, signal peptide sequence; bGHpA, bovine growth hormone polyadenylation signal; pac, herpes simplex virus type-1 packaging signal; amp^R, ampicilin-resistant gene; TK, thymidine kinase promoter. (**b**) The insertion of MG11 sequence into pCONGA4 was confirmed with a *Sac*II diagnostic restriction enzyme digest. The MG11 sequence and the adjoining nucleotide sequence were confirmed by DNA sequencing. (**c**) MG11-pCONGA amplicons were propagated and packaged in the presence of gCΔ2-3 helper viruses. Amplicon vector titers of MG11-pCONGA were assessed by counting eGFP-positive VERO cells (1:1000 dilution) (i), whereas the helper virus was titered by counting *lac*Z+VERO cells after staining with X-gal (1:1 000 000 dilution) (ii). (**d**) 100 μg of protein extracted from virus pellets (gCΔ2-3; MG11-pCONGA and pCONGA4) were resolved on SDS–polyacrylamide gel electrophoresis and analyzed by western blotting using an anti-gC polyclonal antibody (R47).

MG11-pCONGA was indistinguishable from pCONGA4 because of the small difference of 12 amino acids.

Altered tropism of recombinant HSV-1 virions containing the glioma-specific peptide

To monitor the targeting abilities of MG11-pCONGA recombinant virions, the luciferase reporter gene was subcloned into MG11-pCONGA and pCONGA4 amplicons and the derivatives were designated as MG11-pCONGA-Luc and pCONGA4-Luc, respectively. To determine whether incorporation of glioma-specific targeting peptide MG11 could alter the tropism of the virions, the relative ability of MG11-pCONGA-Luc and pCONGA4-Luc to transduce glioma and other tumor cells was examined in a series of human tumor cell lines. To compare the transduction efficiency of these two vectors, each of the cell lines was synchronously infected with either of the two virus stocks at an multiplicity of infection (MOI) of 0.1 by using low-temperature (4 °C)

adsorption for 30 min, followed by removal of unbound virions by rinsing cells with cold phosphate-buffered saline (PBS) and initiation of infection by a temperature shift to 37 °C. Luciferase activity was measured in cell lysates 5 h after infection. Our results showed that MG11-pCONGA-Luc virus stocks produced an almost twofold higher level of luciferase expression for all human glioma cell lines tested (T98G, SF767, ΔGli36, U251MG, U373MG) in comparison with the parental vector (Figure 2a). In contrast, the level of luciferase activities mediated by either pCONGA4-Luc or MG11-pCONGA-Luc was similar in non-glioma cell lines, CNE2 and A549. Next, the preferential infectivity of MG11-pCONGA for human glioma cells was confirmed by counting the number of eGFP-positive glioma cells in the mixed cell populations. The glioma cells (ΔGli36 cells and U251MG) were distinguished by pre-labeling with CM-DiI dye (Figure 2b). The labeled glioma cells were co-cultured with non-glioma cells (A549) for 1 day before infection with either MG11-pCONGA or pCONGA4 virus stocks (MOI = 2). At 6 h after infection, images were acquired and scored for cells that were positive for both eGFP and CM-DiI staining (that is, glioma cells) versus eGFP alone (non-glioma cells). The number of infected glioma cells (eGFP and CM-DiI) was subsequently expressed as a percentage of all eGFP-positive cells. Our results showed that in a mixed cell population consisting of both glioma (either ΔGli36 or U251MG) and non-glioma (A549) cells; the percentage of eGFP-positive glioma cells transduced by MG11-pCONGA was significantly higher than those of pCONGA4. To normalize for possible variation in the labeling efficiency and differential infectivity exhibited by the different cell lines, similar experiments were carried out using different ratios of human glioma versus non-glioma cells (data not shown). In all experiments, we have consistently observed that the percentage of eGFP-positive human glioma cells transduced by MG11-pCONGA was higher than pCONGA4. Next, we examined whether MG11 amplicon display vectors could mediate a dose-dependent infectivity specifically in human glioma cells. In this experiment, SF767 glioma cells and CNE2 non-glioma cells were incubated with either MG11-pCONGA-Luc or pCONGA4-Luc at 4 °C for 30 min. The unbound viruses were subsequently removed by rinsing with PBS and the temperature was shifted to 37 °C to synchronize infection. After 5 h, the cells were harvested for luciferase activities. Our results showed that MG11-pCONGA-Luc amplicon virions could mediate enhanced transgene expression in the presence of increasing viral

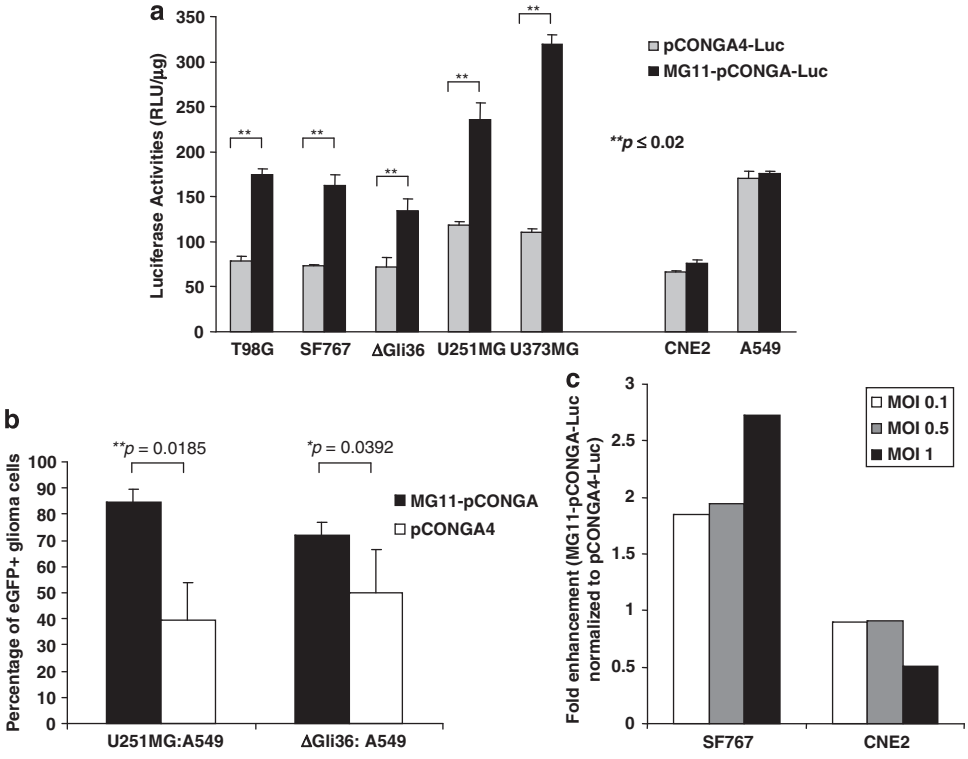

Figure 2 Altered tropism of HSV-1 amplicon containing glioma-specific peptides. (**a**) A panel of glioma and non-glioma cells (3×10^5 cells per well in 12-well plates) was infected with 3×10^4 transducing units (TUs) of pCONGA4-Luc or MG11-pCONGA-Luc virion stocks at 4 °C for 30 min, after which the unbound virus was removed by washing the cells with cold PBS. Cells were then shifted to 37 °C to allow virus penetration. At 5 h later, cells were harvested and assayed for luciferase activity. Activity is expressed as relative light units (RLUs) per μg cell protein. (**b**) CM-DiI-labeled ΔGli36 cells or U251MG cells were co-cultured with A549 cells 1 day before infection. The next day, cells were infected with multiplicity of infection (MOI) of 2.0 of either pCONGA4 or MG11-pCONGA viruses for 6 h. Images were captured using Nikon TE3000 equipped with DMX1200F CCD color digital camera using image acquisition software (ACT-1 v2.7; Nikon). In all the above experiments, data shown are averages of triplicates ± s.e.m. from representative experiment. The levels of significance for difference between the two vectors were determined using Student's *t*-test, and *P*-values <0.05 were considered statistically significant. (**c**) Human glioma and non-glioma cells were incubated with either MG11-pCONGA-Luc or pCONGA4-Luc at 4 °C for 30 min, and temperature was raised to 37 °C for 5 h to allow for synchronized infection. Data shown are averages of quadruplicates and repeated twice independently.

dose (Figure 2c). In contrast, similar trend was not observed in non-glioma cells. Taken together, these results clearly show that MG11-pCONGA virus stock preferentially infect human glioma cells in a mixed cell population.

Glioma-specific peptides block adsorption of MG11-gC recombinant virions to cells

We next investigated whether MG11 peptides could inhibit MG11-pCONGA-Luc adsorption. T98G or SF767 cells were incubated in either standard tissue culture medium or tissue culture medium containing 1 μg per well of MG11 peptide for 30 min at 4 °C The cells were rinsed with cold PBS followed by synchronized viral infections with either MG11-pCONGA-Luc or pCONGA4-Luc virus stocks at MOI = 0.1 at 4 °C for 30 min. After viral adsorption, the cells were rinsed with cold PBS and then incubated for 5 h at 37 °C before the measurement of luciferase activities. Our results showed that MG11 peptides could effectively block infection of glioma cells by MG11-pCONGA-Luc virions (Figures 3a and b). In contrast, treatment of glioma cells with the MG11 peptide has no significant effect on the infectivity of pCONGA4-Luc virions, indicating that MG11 peptides and MG11-pCONGA-Luc virions compete for the same or similar receptor-binding sites on the glioma cells.

In vivo *homing of MG11-pCONGA virions to glioma xenografts*

To determine whether MG11-pCONGA-Luc virions could enhance transgene expression in glioma tumors, MG11-pCONGA-Luc virions (a total of 3×10^6 transducing units (TU) per animal) were administered intratumorally into immunoincompetent mice bearing xenografts of either SF767 glioma cells or CNE2 nasopharyngeal carcinoma cells ($\sim 80\ mm^3$). The parental vectors, pCONGA4-Luc, were also administered as controls. At 24 h after injection, the *in vivo* bioluminescence signals in MG11-pCONGA-Luc-injected SF767 xenografts were robust, whereas those in CNE2 cells were undetectable (Figure 4a). Bioluminescence intensity observed in MG11-pCONGA-Luc-injected SF767 xenografts were above the background or nonspecific luminescence of 500 total luciferase count as represented in pCONGA4-Luc-injected SF767 or CNE2 xenografts. By comparing the ratio of activity in SF767 tumors with that in CNE2, MG11-pCONGA-Luc virions conferred approximately two-fold higher specific expression in glioma tumors than did pCONGA4-Luc virions (Figure 4b), which is similar to the difference seen in these same cell types in culture (Figure 2a). To further examine the targeting potential of MG11-pCONGA-Luc virions, they were introduced into the carotid artery in mice bearing intracranial human glioma tumors to achieve first-pass viral delivery[31] under previously optimized conditions. At 18 h post-viral injections, luciferase signals were acquired on both tumor and a normal part of the brain for mice injected with either MG11-pCONGA-Luc ($n = 4$) or pCONGA4-Luc ($n = 3$) virus stocks. The presence of the exogenous luciferase genes in the cerebral hemispheres were also confirmed using Hirt's DNA extraction of the brain extracts after the images were acquired (data not shown). Unfortunately, in the control group treated with pCONGA4-Luc, two of the three mice died

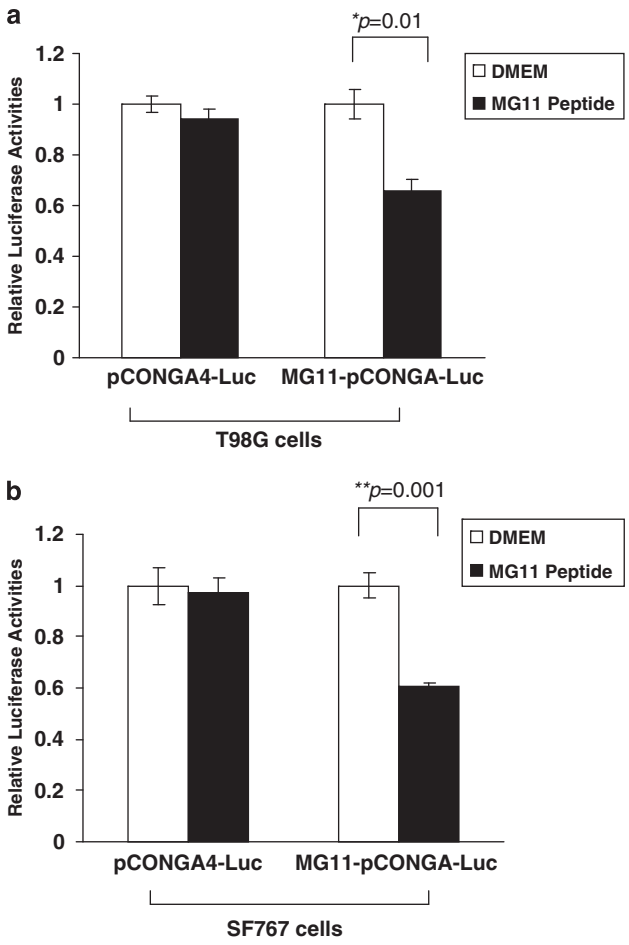

Figure 3 MG11 peptides block HSV-1 adsorption to glioma cells. (**a**) Monolayer of confluent T98G glioma cells (3×10^5 cells per well in 12-well plates) were incubated in Dulbecco's modified Eagle's medium with or without 1 μg of MG11 peptide at 4 °C for 30 min. Cells were then washed with cold PBS and infected with 3×10^4 TU of pCONGA4-Luc or MG11-pCONGA-Luc virions at 4 °C for 30 min. Cells were washed with cold PBS and shifted to 37 °C. At 5 h later, cells were harvested and assayed for luciferase activity. The relative luciferase activity was calculated by normalizing the activity of peptide treatment group to the mean of the nontreatment group. (**b**) Similar experiment was carried out as described above but in SF767 glioma cells. In all of above experiments, data shown are averages of triplicates ± s.e.m. from representative experiment.

before bioluminescence image could be acquired. As the experimental design was based on intracarotic route of viral administration, the nontumor-bearing hemisphere of the brain could also serve as an internal control to the tumor-bearing hemisphere of the same animal. Our results showed that MG11-pCONGA-Luc virion preferentially infected the tumor-bearing region of the mouse brain as supported by the 20% increase in the luciferase reporter activities compared with the nontumor-bearing hemisphere of the mouse brain (Figure 5a). In contrast, we did not observe a differential level of infectivity with pCONGA4-Luc viruses. The systemic homing ability of MG11-pCONGA-Luc virions was further examined using similar pair of vector constructs and helper virus, but without the luciferase gene in an additional set of animal experiments. In this experiment, SF767 and CNE2

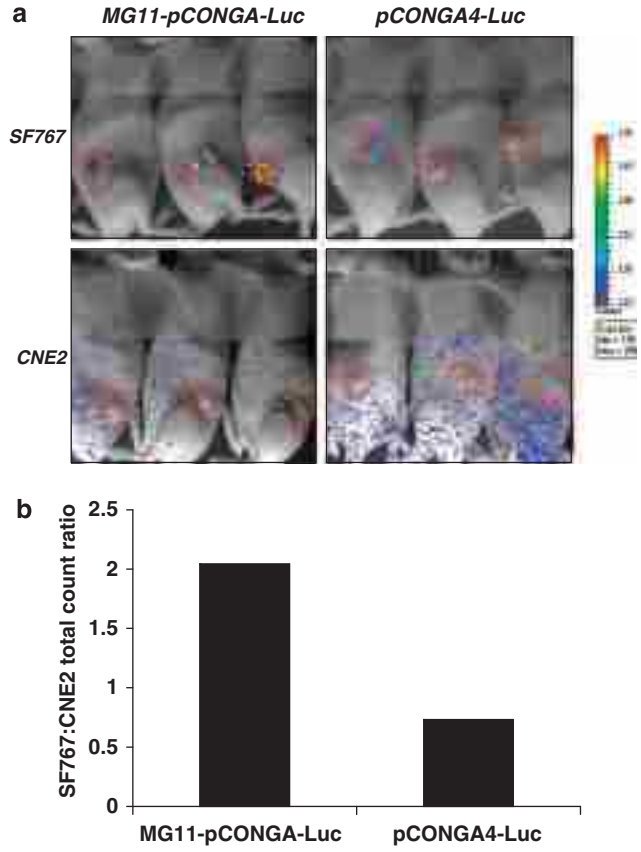

Figure 4 Intratumoral administrations of recombinant HSV-1-targeting vectors in mice bearing SF767 and CNE2 xenografts. (**a**) SF767 (glioma) and CNE2 (non-glioma) cells were injected subcutaneously into the 4 to 6-week-old female BALB/c nude mice as described in the Materials and methods section. In brief, 7 days after tumor cell inoculation, 1×10^6 TU of viral vectors were injected intratumorally. At 24 h later, the mice were imaged and bioluminescent images were overlaid on brightfield images using Living Image 3.0 software (Caliper Life Sciences). (**b**) The ratio of total photon counts were quantified in SF767 and CNE2 xenografts and presented as a ratio.

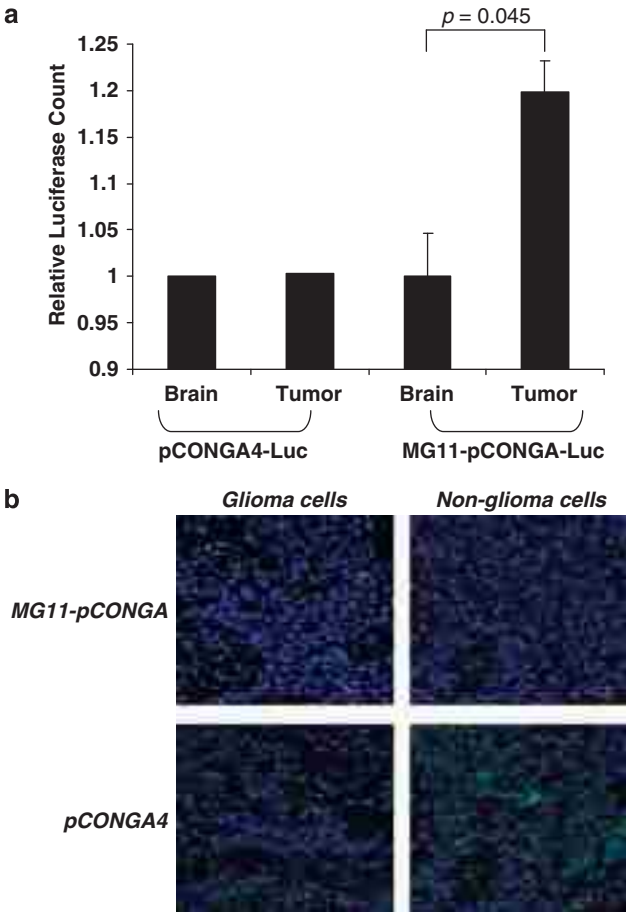

Figure 5 Systemic administrations of recombinant HSV-1-targeting vectors in orthotopic and heterotopic glioma mouse models. (**a**) Immunoincompetent female nude mice of 4–8 weeks old were stereotaxically implanted with ΔGli36 human glioma cells. In all, 1×10^6 TU of either pCONGA4-Luc or MG11-pCONGA-Luc viruses were administered intracarotically. On the following day, mice were injected intraperitoneally with D-luciferin and bioluminescence was measured as described in the Materials and methods sections. (**b**) Immunodeficient mice bearing SF767 glioma cells and CNE2 non-glioma cells were injected with 1×10^6 TU of either pCONGA4 or MG11-pCONGA virions through the tail vein. After 6 h, mice were perfused through the heart with cold PBS followed by 4% paraformaldehyde. Tumor were harvested, cryosectioned and mounted. Localization of eGFP-positive cells were visualized using either a $\times 20/0.75$ NA Plan-Fluor objective or a $\times 60/1.4$ NA Plan-Apochromat oil immersion objective and captured using LSM 510 Meta confocal microscope (Carl Zeiss).

were implanted on either flank of the same immunoincompetent mouse ($n = 6$). Owing to the differences in tumor cells growth rate, the number of tumor cells administered were pre-optimized accordingly such that only animals bearing similar tumor volume on both flanks ($\sim 80\ mm^3$) were used in the experiment. These animals were randomly divided into two groups in which similar viral dosage of either MG11-pCONGA or pCONGA4 (1×10^7 TU in 200 µl PBS per animal) was administered through the tail vein. The viruses were allowed to circulate in the blood for 6 h, the tumors were then harvested and cryosectioned. Our results showed that MG11-pCONGA viruses infected glioma xenograft apparently exclusively as represented in Figure 5b. In contrast, eGFP-positive cells were detectable in both SF767 glioma and CNE2 non-glioma xenograft in animals injected with pCONGA4 virions, supporting a lack of glioma specificity. Taken together, these results showed that MG11-pCONGA and MG11-pCONGA-Luc virions could target human glioma cells when administered through two different systemic routes.

Discussion

The development of viral vectors that can be targeted to infect specific cell types is important both in achieving maximal therapeutic effect and limiting potential adverse effects. At present, surgery combined with radiation and chemotherapy remained the mode of clinical treatment for the central nervous system neoplasms. However, not all brain tumors are surgically accessible, and the mean survival rate with these treatments is about 1 year for glioblastoma multiforme.[32] Hence, the construction of glioma-targeted HSV-1 vectors could have potential clinical relevance as an adjunct therapy. In this

report, experiments were designed to generate recombinant HSV-1 virions that could specifically bind to and infect human glioma cells, as compared with normal cells, in culture and *in vivo*.

In general, the tropism of HSV-1 infection can be redirected in two different ways. The first is to tag virion surface proteins with a single-chain antibody (scFv) that recognizes a novel receptor on the cell surface. Menotti *et al*.[33] inserted a scFv against the human epidermal growth factor receptor 2 (HER2) into the gD of HSV-1. The insertion of gD with scFv to HER2 (scHER2) disrupted the ability of the virus to enter cells through the HVEM receptor, but maintained the ability to enter through the nectin 1 receptor. Interestingly, despite the fact that this insertion has almost doubled the relative molecular mass of gD, the mutant virus was capable of infecting HER2-expressing cells but not in the parental receptor-negative cells, indicating that the recombinant mutant virus has been redirected to HER2/neu receptor. Even so, it has been a challenging task for retargeted viruses to achieve detargeting from their natural receptors of HVEM and nectin-1. The same group of researchers has extended their study and reported the successful construction of an oncolytic HSV vector that could be retargeted to the HER2 receptor and detargeted from both natural receptors.[34] The approach taken relied primarily on the insertion of scHER2 at a different site in gD while removing the amino-acid residues known to be important for viral entry through HVEM. Likewise, the incorporation of a tumor antigen scFV into the amino acids of gD in oncolytic HSV1716 viruses have been shown to allow effective tumor-targeted therapy through a systemic route of administration.[35] The second approach is to incorporate into the virion surface natural ligands, such as IL-13 or the N-terminal fragment of urokinase-type plasminogen activator, that use the ligands' receptors enriched on the tumor cell surface as portals of viral entry.[36,37] An example is the interleukin-13 receptor α2 chain (IL-13Rα2), which is frequently overexpressed in human gliomas.[38] In normal tissues, the receptors have been reported to be present in human testes but not in other tissues. Zhou *et al*.[36] has generated a recombinant HSV-1 virus in which the polylysine tract in gB was removed and the coding sequence of IL-13 was inserted into the first 132 residues of gC, which were also removed, as well as sequences encoding amino acids 24 and 25 of gD to enhance the affinity of the virus particle to the surface of cells expressing IL-13Rα2. Although these mutant viruses could enter IL-13Rα2-expressing cells, they were also capable of entering cells without IL-13Rα2 receptors through other gD receptors, such as HveA or nectin-1.[36] At a later time, the same group constructed a recombinant virus containing mutations in gD that have been reported to be important for gD to interact with HveA and nectin. Indeed, they were successful in the generation of mutant viruses that could only enter IL-13Rα2-positive cells and could no longer bind through the HveA nor nectin-1 receptors.[39] These findings provide support that the host range of herpes simplex viruses can be genetically manipulated to target-specific cell types.

In this study, we provide additional evidence in support of the hypothesis that HSV-1 binding to the cells can be redirected to human glioma through the incorporation of a glioma-specific peptide sequence within gC of the HSV-1 virion. This was facilitated by combination of an HSV-1 amplicon platform vector allowing replacement of the HS-binding site in gC with a specific peptide ligand and use of a gC-deleted helper virus. We chose the MG11 peptide as the ligand because it was shown to bind specifically to human glioma cells, both primary GBM cells derived from human patients and established cell lines, whereas binding could not be detected with normal primary human astrocytes under similar culture conditions.[25] As the MG11 peptide sequence is relatively small (12 aa), it should not perturb actions of other HSV glycoproteins on the viral envelope. The MG11 peptide was used to replace the HS-binding sequence in gC, as gC is not essential for the viral life cycle.[40] However, gC can confer protection to HSV-infected cells and cell-free virus against complement-mediated lysis and neutralization.[41,42] The gC-null virus has also been shown to be less virulent than the wild-type virus in animals.[43] It would be interesting to examine the immune responses after the MG11-pCONGA-Luc and its equivalent control amplicon viral vectors in the tumor microenvironment. Depending on the findings, it may be possible to further manipulate the MG11-gC fusion for improved retention of the viruses in glioma cells after infection. Although this has yet to be confirmed, we have nevertheless shown the successful infection of MG11-pCONGA recombinant virions in glioma cells in culture and *in vivo*. The preferential infectivity of MG11-pCONGA-Luc virions was shown after a short period of infection of 5 h before the measurement of luciferase expression (Figure 2a). With longer periods after infection, the differential infectivity between MG11-pCONGA-Luc and pCONGA4-Luc in human glioma cells was less prominent (data not shown), as other glycoproteins, for example, gB, can compensate for the defective gC in gaining entrance into the cells. In another experimental design, human glioma (pre-labeled) and non-glioma cells were co-cultured and infected with one or the other of the viruses. Positively infected cells were subsequently scored by counting the eGFP-expressing cells. The results showed that MG11-pCONGA virions were more efficient at transducing human glioma cells compared with pCONGA virions (Figure 2b). Taken together, these data showed that MG11-pCONGA virions preferentially infect human glioma cells *in vitro*.

The supporting evidence for preferential infection of glioma tumors by MG11-pCONGA virions was also shown *in vivo* using three different routes of viral delivery. Both MG11 and control virions were administered either intratumorally (Figures 4a and b) or systemically by intracarotid injection (Figure 5a) and tail vein injection (Figure 5b) into different glioma xenograft mouse models. In all the three experimental set ups, preferential targeting of MG11-pCONGA virions to human glioma cells versus controls was clearly observed using either eGFP or luciferase as reporters. As gB is able to complement missing function of gC, and that neither gB or gC alone is essential for viral entry,[2,21,44] the targeted viral binding *in vivo* may be further improved if the viruses could be engineered to contain either a double deletion of the HS-binding domains of both gC and gB, or modification of one and deletion of the other. It is also possible that apart from the complex mechanism of HSV-1 adsorption and entry, enhanced targeting

could be achieved through other molecular determinants of the human glioma genotypes as they are elucidated. For example, it was shown recently that the MAPK status of the tumor cells may determine the efficacy of HSV-1 oncolytic therapy.[45]

In summary, we have shown the effectiveness of inserting a glioma-specific peptide sequence into an amplicon engineered to allow efficient replacement of the HS-binding site in gC with other ligand sequences so as to generate virions with recombinant gC. We further showed the targeted homing potential of these chimeric virions to human glioma cells both *in vitro* and *in vivo*. By replacing the reporter gene with a therapeutic gene, these modified virions are potentially useful tools for targeting gene therapy to human glioma tumors when administered systemically.

Materials and methods

Cell culture

Human cell lines, A549 (human lung adenocarcinoma), VERO (African green monkey kidney cells), T98G and U373MG (human glioma), were obtained from American Type Culture Collection (Rockville, MD, USA). CNE2 cells were derived from advanced undifferentiated carcinoma of human nasopharyngeal carcinoma (gift from Professor HM Wang, Cancer Institute, Guangzhou, PR China). Human glioma lines, U251MG and SF767, were kindly provided by Dr DF Deen (Brain Tumor Research Centre, UCSF School of Medicine, CA, USA). All these cells were grown as monolayer in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), penicillin (100 U ml^{-1}; Invitrogen), streptomycin (100 U ml^{-1}; Invitrogen) and L-glutamine (2 mM; Sigma-Aldrich Corp., St Louis, MO, USA) at 37 °C in a 5% CO_2 and 95% air atmosphere. ΔGli36 human glioma cells were generated by retroviral transduction with a cDNA coding for a mutant epidermal growth factor receptor[46,47] and subcutaneously passaged in nude mice. These cells were cultured in growth medium in the presence of 1 μg ml^{-1} of puromycin (Sigma-Aldrich).

The generation of various HSV-1 amplicon constructs

The pCONGA4 amplicon was derived from pCONGA, which contains a gC gene that has been modified to contain unique *Asc*I and *Eco*NI sites flanking the sequence encoding gC HS-binding domain (aa 33–123), as well as an eGFP expression cassette.[48] In pCONGA4, the HS-binding domain sequence was excised and replaced by the sequence encoding the C-terminal domain of IL-4 (26 aa). The MG11-pCONGA amplicon was derived from pCONGA4 by replacing the IL-4 sequence with those for MG11. Two oligonucleotides containing the reverse and forward sequences of MG11 flanked with *Asc*I and *Eco*NI restriction sites were synthesized as follows:

5′-TTGGCGCGCCCCTGTGGGCTACTTTTCCTCCGC GGCCTCCGTGGCTGGGCCTAGAGGAGGTTTT-3′ and 5′-AAAACCTCCTCTAGGCCAGCCACGGAGGCCGCG GAGGAAAAGTAGCCCACAGGGGCGCGCCAA-3′.

Equal molar concentrations of the two oligomers were denatured at 94 °C for 10 min in reaction buffer (50 mM Tris–HCl pH 7.9, 10 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT) and allowed to anneal gradually as the reaction mixture reached room temperature to generate double-stranded MG11. The annealed oligomers were digested with *Asc*I and *Eco*NI at 37 °C for 2 h, followed by purification using the nucleotide removal kit (Qiagen) and cloned into the pCONGA4 plasmid digested with the same enzymes.

For generation of pCONGA4-Luc and MG11-pCONGA-Luc, overlap PCR strategy was used because of the lack of appropriate restriction enzyme sites. The plasmid pCONGA4 was digested with *Afl*II and *Msc*I to release a 1360-bp DNA fragment. This DNA was purified and used as template for PCR with primers 1 and 2. The resultant PCR product (~410 bp) contained a partial SV40 promoter sequence with an *Avr*II site for the subsequent cloning step. The remainder promoter sequence and the luciferase gene were amplified from 8GalLuc[49] using primers 3 and 4. Primer 4 contained a unique restriction enzyme site of *BstB*I as shown in bold fonts below:

Primer 1: 5′-GGAATGTGTGTCAGTTAGGGTGTGGA AAG-3′
Primer 2: 5′-GGTGGCTTTACCAACGCGAAACGAT CCTC-3′
Primer 3: 5′-GAGGATCGTTTCGCGTTGGTAAAGCC ACC-3′
Primer 4: 5′-GATCGCCGTGTAA**TTCGAA**CCCA-3′

Amplicons derived from both sets of PCR reactions then served as DNA templates for the reconstitution of SV40 promoter-driven luciferase gene using primers 1 and 4. This desired transgene cassette was digested using *Avr*II and *BstB*I sites and replaced the original SV40 promoter-driven neomycin cassette in either pCONGA4 or MG11-pCONGA. The recombinant plasmids were denoted as pCONGA4-Luc and MG11-pCONGA-Luc, respectively. All newly generated plasmids were confirmed by restriction mapping and DNA sequencing.

Production and titration of modified amplicon and virus vectors

VERO cells (3×10^6 cells in 100 mm dish) were transfected with pCONGA4 or pCONGA-MG11 (16 μg) using Lipofectamine (45 μl) (Invitrogen) according to the manufacturer's protocol. After 6 h, cells were infected with gCΔ2-3 at MOI = 0.1 in 8 ml Dulbecco's modified Eagle's medium (Invitrogen). After 2–3 days when 100% of cytopathic effect was observed, viruses in the cell lysates and supernatants were harvested through three cycles of freeze–thaw and purified through a 25% sucrose gradient using centrifugation. Virus pellet was resuspended in Hank's buffered salt solution and kept at −80 °C as stock.

Titers of amplicon vectors in virus stocks were determined in VERO cells by counting eGFP-positive cells at 12–24 h after infection and expressed as TU ml^{-1}. eGFP-positive cells were counted at an appropriate dilution using fluorescence microscopy (absorbance 480 nm, emission 507 nm). For titering the helper viruses in virus stocks, VERO cells were infected with virus stocks at the appropriate dilution. At 48 h after infection, the cells were fixed in 4% paraformaldehyde in PBS for 30 min and staining with 1 mg ml^{-1} X-gal in 5 mM $K_3[Fe(CN_6)]$, 5 mM $K_4[Fe(CN_6)]$ and 2 mM $MgCl_2$ in PBS

(Invitrogen) overnight; helper virus titers were evaluated by determining the numbers of *lac*Z-positive blue foci using light microscopy and the titers were expressed as blue-forming units per ml (BFU ml^{-1}). Typical titers were 10^8 TU ml^{-1} for amplicon vector and 10^{10} BFU ml^{-1} for helper virus in the same stock.

Western blot analysis

Amplicon vectors propagated in the presence of gCΔ2-3 (MOI = 0.1) was purified using a 25% sucrose gradient as described above. The purified virion pellet was resuspended in a volume of 500–700 μl of lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Triton X-100, and protease inhibitor cocktail diluted to 1 × concentration (25 ×; Roche Diagnostics, Indianapolis, IN, USA)). Viral proteins (100 μg) were resolved electrophoretically in 10% SDS–polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Roche Diagnostics) and immunoblotted with a polyclonal antibody specific against gC (1:500; rabbit 47 polyclonal antibody, kindly provided Drs GH Cohen and RJ Eisenberg, University of Pennsylvania)[50] in 5% milk with TBS/0.1% Tween 20 (TBS-T) at room temperature for 2 h. After immunobloting with primary antibodies, the membranes were rinsed three times with TBS-T and incubated in anti-rabbit horseradish peroxidase-conjugated antibody (1:2000 dilution, Sigma) for 60 min at room temperature in 5% milk/TBS-T solution. After incubation, the blots were washed again and antibody signal was revealed by ECL (Pierce, Rockford, IL, USA).

Assay for luciferase activity

Cell pellets were lysed with protein lysis buffer (50 mM Tris, 10 mM EDTA, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 × protease inhibitor cocktail) for 10 min on ice. Cell debris was discarded after centrifugation at 14 000 *g* at 4 °C for 10 min. A total of 100 μl of the collected supernatant was subjected to luciferase assay within the range of linearity for time and protein with an AutoLumat LB 9507 luminometer (Berthold Techologies, Bad Wildbad, Germany). In all, 2 μl of the supernatant was used to determine protein concentration using Bio-Rad (Hercules, CA, USA) protein assay dye reagent with an Ultrospec 3000 UV/visible spectrophotometer (GE Healthcare Life Sciences, Uppsala, Sweden).

Peptide competition assay and in vitro *binding experiments*

Monolayer of confluent T98G and SF767 cells (3×10^5 cells per well in 12-well plates) were incubated in Dulbecco's modified Eagle's medium with or without 1 μg of MG11 peptide at 4 °C for 30 min. Cells were then washed with cold PBS and infected with 3×10^4 TU of pCONGA4 or MG11-pCONGA virions at 4 °C for 30 min to allow viral adsorption. After the designated time point, the virions were removed by rinsing the cells with cold PBS. Cells were then shifted to 37 °C to allow virus entry to occur. After 5 h, the cells were harvested and luciferase activities in the various tumor cell lines were measured. The relative luciferase activity was calculated by dividing the activity of each sample by the mean of the nontreatment group, which was set as 100%.

For *in vitro* binding and penetration experiments, monolayer of confluent human glioma (ΔGli36 and SF767) and non-glioma cells (A549 and CNE2) were seeded at 3×10^5 cells per well in 12-well plates. Cells were washed with ice-cold PBS twice and incubated with either MG11-pCONGA-Luc or pCONGA4-Luc amplicon viruses at various MOI for 30 min at 4 °C. Unbound viruses were removed by washing the cells with ice-cold PBS twice. The cells were then fed with fresh complete medium and incubated at 37 °C to allow virus entry to occur. At 5 h later, cells were harvested and luciferase activities were measured.

Cell mixing experiment

Equal ratio of ΔGli36 or U251MG human glioma cells were co-cultured with A549 non-glioma cells 1 day before viral infection. The human glioma cells, ΔGli36 and U251MG, were pre-labeled with CM-DiI vital dye, whereas A549 was pre-labeled with DAPI before co-culture. On the day of the experiment, both cells mixture (ΔGli36/A549 and U251MG/A549) were infected with either MG11-pCONGA or pCONGA4 virus stock at MOI of 2.0. As both vectors contain the eGFP reporter gene, the percentage of infectivity can be captured and quantified using fluorescence microscopy. Images were visualized after 6 h using wide-field microscopy with an inverted microscope (TE300; Nikon, Tokyo, Japan), and images were acquired on a CCD color digital camera (DXM1200F) using image acquisition software (ACT-1 v2.7; Nikon). The number of glioma cells that were both positive for eGFP (infected cells) and CM-DiI was counted and expressed as a percentage of all eGFP-positive cells.

Intratumoral administration of glioma-specific viral vectors into glioma and non-glioma xenografts

To establish the xenografts model of glioma and non-glioma in nude mice, SF767 (glioma) and CNE2 (non-glioma) cells (5×10^6 cells) were injected subcutaneously into the right and left flank of the 4 to 6-week-old female BALB/c nude mice (Animal Resource Centre, Canningvale, Western Australia), respectively. At 7 days after inoculation, 1×10^6 TU of viruses (pCONGA4-Luc or MG11-pCONGA-Luc) were administered in 100 μl of Hank's buffered salt solution intratumorally ($n = 3$). The following day, mice were intraperitoneally injected with D-luciferin in PBS (0.17 mg per g body weight; Xenogen, Caliper Life Sciences, Hopkinton, MA, USA). After 5 min, the mice were anesthesized with 6 ml per kg body weight fentanyl/fluanison (Hypnorm, VetaPharma, Leeds, UK)/midazolam (Dormicum, Roche, Basel, Switzerland) in distilled water in a 1:1:2 (Hypnorm/Dormicum/water) ratio. Bioluminescence was measured noninvasively using a cryogenically cooled high efficiency CCD camera system, VersArray: 512B (Roper Scientific, Trenton, NJ, USA). Images were taken 20 min after intraperitoneal injection of D-luciferin at 10 min of exposure time, bin2 and readout speed at 50 kHz. Image analysis and bioluminescent quantification was performed using Metavue software (Universal Imaging, Downingtown, PA, USA). Images were overlaid with brightfield image using Living Image 3.0 software (Caliper Life Sciences, Hopkinton, MA, USA). All animal experiments were carried out according to the guidelines and protocols approved by the Institutional Animal Care and Use Committee at the Singapore General Hospital.

In vivo *targeting of MG11-pCONGA and pCONGA4 through intracarotid and tail vein routes*

(i) Intracarotid route. Immunoincompetent female nude mice of 4–8 weeks old were stereotaxically implanted with ΔGli36 human glioma cells at 2×10^5 cells (2-mm lateral and 2-mm depth from bregma). Seven days after tumor cells implantation, vectors (either MG11-pCONGA or pCONGA4, 5×10^6 TU per animal) were injected into the internal carotid artery of glioma xenograft-bearing nude mice according to the previously described method.[51] Injections were performed manually over 3–5 min. Animals were monitored closely for possible neurological deterioration related to the injections. At 18 h after injection, bioluminescence images of the mice were acquired as described above.

(ii) Tail vein route. Immunodeficient nude mice harboring SF767 human glioma cells (2.5×10^6 cells) and CNE2 non-glioma cells (5×10^5 cells) at its right and left flank, respectively, were injected with 1×10^6 TU of either MG11-pCONGA or pCONGA4 through the tail vein. The cell numbers have been pre-optimized for difference in growth rate *in vivo*. At 6 h after viral inoculation, mice were perfused through the heart with cold PBS followed by 4% paraformaldehyde in PBS to fix the tissues. Tumors were meticulously harvested and kept at 4 °C in 4% paraformaldehyde/PBS overnight. The next day, the tumors were transferred to PBS containing 30% sucrose to prevent freezing artifacts. The tumors were sectioned (10 μm) and counterstained with DAPI. The sections were visualized using confocal microscope (LSM 510 Meta; Carl Zeiss, Göttingen, Germany). Images were captured using a ×20/0.75 NA Plan-Fluor objective (Carl Zeiss).

Statistical analysis

Data are presented throughout this study as mean ± s.e.m. Statistical significance was evaluated by Student *t*-test and $P < 0.05$ was considered significant.

Conflict of Interest

The authors declared no conflict of interest.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)