Dual Systemic Tumor Targeting with Ligand-Directed Phage and Grp78 Promoter Induces Tumor Regression

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Abstract
The tumor-specific Grp78 promoter is overexpressed in aggressive tumors. Cancer patients would benefit greatly from application of this promoter in gene therapy and molecular imaging; however, clinical benefit is limited by lack of strategies to target the systemic delivery of Grp78-driven transgenes to tumors. This study aims to assess the systemic efficacy of Grp78-guided expression of therapeutic and imaging transgenes relative to the standard cytomegalovirus (CMV) promoter. Combination of ligand and Grp78 transcriptional targeting into a single vector would facilitate systemic applications of the Grp78 promoter. We generated a dual tumor-targeted phage containing the arginine-glycine-aspartic acid tumor homing ligand and Grp78 promoter. Next, we combined flow cytometry, Western blot analysis, bioluminescence imaging of luciferase, and HSVtk/ganciclovir gene therapy and compared efficacy to conventional phage carrying the CMV promoter in vitro and in vivo in subcutaneous models of rat and human glioblastoma. We show that double-targeted phage provides persistent transgene expression in vitro and in tumors in vivo after systemic administration compared with conventional phage. Next, we showed significant tumor killing in vivo using the HSVtk/ganciclovir gene therapy and found a systemic antitumor effect of Grp78-driven HSVtk against therapy-resistant tumors. Finally, we uncovered a novel mechanism of Grp78 promoter activation whereby HSVtk/ganciclovir therapy upregulates Grp78 and transgene expression via the conserved unfolded protein response signaling cascade. These data validate the potential of Grp78 promoter in systemic cancer gene therapy and report the efficacy of a dual tumor targeting phage that may prove useful for translation into gene therapy and molecular imaging applications. Mol Cancer Ther; 11(12); 1–12. ©2012 AACR.

Introduction
Integration of both ligand-directed tropism and transcriptional targeting combined into a single platform could help to facilitate the clinical use of systemic gene therapy and molecular imaging of cancer. The promoter of the glucose-regulated protein 78 (Grp78) gene, which encodes for an endoplasmic reticulum (ER) chaperone protein, and the ligand arginine-glycine-aspartic acid (RGD) that targets αv integrin receptors overexpressed in tumors (1, 2), may offer excellent candidates. The Grp78 promoter is stress inducible and is strongly activated by conditions of glucose deprivation, chronic anoxia, and acidic pH that persist within aggressive and poorly perfused tumors (3). Moreover, the Grp78 promoter is induced in a wide variety of tumors and thus makes it an attractive candidate for use in gene therapy (4–8). Previous studies have clearly shown several advantages of this promoter over viral promoters (9, 10). The safety and tumor specificity of this promoter have also been elegantly reported in transgenic mice carrying a LacZ transgene (11). Furthermore, unlike viral promoters used in gene therapy vectors, mammalian promoters such as Grp78 are not silenced in eukaryotic cells (12). Despite these advantages, the clinical use of the Grp78 promoter in cancer gene therapy remains hindered. Indeed, transcriptional targeting alone is not sufficient to ensure gene expression in the target cell, but also requires efficient transcriptional targeting combined into a single vector in vivo.

Animal viruses have potential for targeted transgene delivery but require elimination of native tropism for mammalian cells and retargeting them to alternative receptors (13). A major drawback of these approaches has been the reduced efficacy resulting from entry via a non-natural receptor (14). Also, incorporation of tumor-homing peptide ligands derived from in vitro phage display screenings into viral vectors has been attempted, but remains challenging because the strategy can ablate the function of the ligand or disrupt viral assembly and function (14, 15). Our previous work suggests that bacteriophage (phage)—the viruses that infect bacteria...
only—have the potential to be adapted as targeted delivery vehicles to tumors after systemic administration. We previously reported that the M13-derived phage displaying the double cyclic RGD (CDCRGDCFC and RGD4C) ligand, and carrying an eukaryotic transgene cassette flanked by genomic cis-elements of adenovirus-associated virus can selectively deliver transgenes to tumors in rodents after intravenous administration, whereas sparing the normal organs (14, 16–20). The targeted RGD4C/phage was also used to deliver the TNF-α cytokine to cancers diagnosed in dogs. Repeated therapy with RGD-TNF-α proved safe and resulted in complete tumor eradication in a few dogs (21). Consequently, we hypothesized that the RGD4C is a suitable ligand candidate to guide Grp78-driven transgene cassettes after systemic administration in vivo. We show here our generation of a dual tumor-targeting system by using the RGD4C tumor-homing ligand and the Grp78 phage for transcriptional targeting in the context of bacteriophage. We evaluated this double-targeted phage for gene delivery both in vitro and in vivo. More specifically, we compared gene expression levels and therapeutic efficacy of our dual-targeted phage to those obtained using the conventional phage carrying the cytomegalovirus promoter (CMV). We show that the double-targeted vector provides much longer transgene expression than the standard phage in vitro and in tumors in vivo after intravenous administration to tumor-bearing mice. In addition, we have identified that the double-targeted phage carrying the Herpes simplex virus-1 thymidine kinase (HSVtk) plus ganciclovir (GCV) enhances tumor cell killing in vitro and produces marked regression of large and therapy-resistant tumors in vivo when intravenously administered. Finally, we have uncovered a novel mechanism of Grp78 promoter induction by HSVtk and GCV suicide gene therapy.

Materials and Methods

Cell culture

Human embryonic kidney (HEK293) cell line was purchased from American Type Culture Collection. The U87 and MCF7 cell lines were from the Cancer Research UK and 9L was provided by Dr. Hrvoje Miletic, University of Bergen, Bergen, Norway (22). No authentication of cells and 9L was provided by Dr. Hrvoje Miletic, University of Bergen, Bergen, Norway (22). No authentication of cells was done by the authors. All cell lines were maintained in Dulbecco’s Modified Eagle Medium supplemented with FBS, L-glutamine, penicillin, and streptomycin. Stress experiments were conducted with either 300 nmol/L thapsigargin for 16 hours or 0.5 μmol/L A23187 for 5 hours. GCV was used at 20 μmol/L and renewed daily.

Vector construction and phage production

To generate the double-targeted phage displaying the RGD4C ligand and carrying the Grp78 promoter, the 689 bp fragment containing the rat Grp78 promoter was released from pDrive-rGRP78 plasmid (Invivogen) by PstI and NcoI digestion, then ligated to XbaI linkers and subsequently inserted into XbaI site of pBluescript II. The pBluescript II plasmid was then used to release the Grp78 promoter by SpeI and NotI followed by ligation to the phage vector plasmid digested with Nhel and NotI to replace the CMV promoter (SpeI can ligate to Nhel). Phage viral particles were amplified as described (18) then expressed as transducing units (TU/μL).

Western blot analysis

Whole cell lysates were prepared in radioimmunoprecipitation assay buffer and subjected to immunoblot. We used goat anti-Grp78 (C-20, 1:400) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 6C5, 1:1,000) from Santa Cruz Biotechnology, rabbit anti-HSVtk (1:100) from Dr. William Summers (Yale University, New Haven, CT), mouse anti-ATF6 (IMG-273, 5 μg/mL) from Imgenex-USA, and rabbit-anti-phosphor-eIF2α (1:1,000) from Cell Signalling. Each immunoblot was done 3 times, quantified by ImageJ software and normalized to GAPDH.

XBP1 splicing measurement

To detect unspliced and spliced forms of the x-box binding protein 1 (XBP1), semiquantitative reverse transcription (RT)-PCR was conducted as described (23).

Animal models and antitumor therapy of HSVtk/GCV

To establish tumors in mice, a total of $1 \times 10^6$ 9L or $1 \times 10^7$ U87 cells were subcutaneously implanted into immunodeficient nude mice. Mice were anesthetized by gas (2% isoflurane and 98% oxygen) inhalation. Tumor-bearing mice were intravenously administered through the tail vein with targeted or control insertless vectors carrying the HSVtk at a dose of $5 \times 10^{10}$ TU vector/mouse as we reported (17, 18). GCV (70 mg/kg/d), intraperitoneal, was given to mice as indicated in the figures. Tumor growth was monitored 3 times a week by caliper measurements and tumor volumes were calculated as described (7, 16, 17). All in vivo experiments were carried out according to the Institutional and Home Office Guidelines. We have used 6 mice per group and repeated the experiments 3 times. However, in some therapy experiments involving mice with large tumors, we have repeated the experiments 3 times with the minimal number of animals per group to reduce animal suffering and apply the 3Rs (Reduce, Refine, and Replace) in accordance with the Institutional and Home Office guidelines.

In vivo bioluminescence imaging

To monitor the firefly luciferase (Luc) expression, mice were anesthetized and administered with 100 mg/kg of β-luciferin (Gold Biotechnology), then imaged using the In Vivo Imaging System (IVIS 100; Caliper Life Sciences). A region of interest was defined manually over the tumors for measuring signal intensities recorded as total photon counts per second per cm² (p/sec/cm²/sr).

Statistical analyses

We used GraphPad Prism software (version 5.0). Error bars represent standard error of the mean (SEM). P values

Published OnlineFirst October 10, 2012; DOI: 10.1158/1535-7163.MCT-12-0587
were generated by ANOVA and denoted as: *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

Results

**Ligand-directed and transcriptional-targeted RGD4C/Grp78 particles are functional**

As a proof of concept, we generated a double-targeted phage viral particle consisting of the rat Grp78 promoter and phage displaying the RGD4C ligand. We used the Grp78 of rat because the promoter sequences of Grp78 are highly conserved among rodents and humans (24, 25), and this promoter is active in a wide range of species including human and murine (10, 11). To obtain the double-targeted phage (hereafter referred to as RGD4C/Grp78), we replaced the CMV promoter in the RGD4C phage (hereafter referred to as RGD4C/CMV) with the Grp78 promoter. As a control, we replaced the CMV promoter of the insertless phage (nontargeted) with the Grp78 (Supplementary Fig. S1). To show that the Grp78 promoter retains its inducible function in the context of the double-targeted phage, we transduced the αv integrin–expressing HEK293 cells with RGD4C/Grp78-GFP, and showed that treatment with thapsigargin, a standard inducer of Grp78, induced GFP expression relative to nontreated cells (Supplementary Fig. S2). We also confirmed these findings with calcium ionophore A23187, as an additional inducer of Grp78 promoter (Supplementary Fig. S2). No GFP expression was detected with control nontargeted Grp78-GFP and either thapsigargin or A23187. These data establish that (i) the ligand-directed transduction by RGD4C and (ii) the stress-inducible property of Grp78 promoter are intact and functional in the context of the dual-targeted phage.

**RGD4C/Grp78 provides long-term transgene expression in tumor cells**

We conducted transgene expression analyses and all subsequent studies in models of glioblastoma as this tumor remains a challenge to treat in patients. We used...
the rapidly growing 9L rat glioblastoma cells, because Grp78 is known to be highly active in aggressive tumors (26–28). In addition, we evaluated efficacy in the U87 human glioblastoma cells characterized by being moderately aggressive (29). We carried out an in vitro characterization of these cell lines and observed a higher proliferation rate in 9L cells relative to U87 (Supplementary Fig. S3A) and better survival in low serum conditions (Supplementary Fig. S3B). Moreover, Western blot analysis showed increased levels of endogenous Grp78 in 9L as compared with U87 cells (Supplementary Fig. S3C). RT-PCR experiments revealed the presence of the spliced form of the messenger RNA (mRNA) of the XBP1 in the 9L cells, whereas it was induced in U87 cells upon thapsigargin treatment only (Supplementary Fig. S3D). This spliced mRNA is translated into the functional XBP1 protein, which acts as a transcription factor for the Grp78 promoter.

We next set out to determine efficacy of transgene expression mediated by the RGD4C/Grp78 in 9L and U87 tumor cells. Stably transduced cells were generated by using vectors carrying puro\(^\text{\textregistered}\) that confers resistance to puromycin. Either RGD4C/Grp78-GFP-puro\(^\text{\textregistered}\) or RGD4C/CMV-GFP-puro\(^\text{\textregistered}\) were used to transduce 9L and U87 cells and clones were isolated under puromycin selection. First, we counted and monitored GFP-positive cell clones. Although cell clones transduced with RGD4C/CMV-GFP-puro\(^\text{\textregistered}\) retained resistance to puromycin, GFP expression was generally weaker over time than that of the RGD4C/Grp78-GFP-puro\(^\text{\textregistered}\) clones. At day 14 postvector transduction (Fig. 1A), we observed GFP expression in 92% of the 9L clones transduced by the RGD4C/Grp78-GFP-puro\(^\text{\textregistered}\) construct (\(n = 846\) of 959 clones) compared with 83% of clones transduced by RGD4C/CMV-GFP-puro\(^\text{\textregistered}\) (\(n = 845\) of 1,258 clones). At day 28 posttransduction, we detected GFP in 83% of RGD4C/Grp78-GFP-puro\(^\text{\textregistered}\) 9L clones, whereas only 59% of the clones transduced by RGD4C/CMV-GFP-puro\(^\text{\textregistered}\) had GFP-positive cells (Fig. 1A). We also observed 67.4% of the U87 clones transduced by RGD4C/Grp78-GFP-puro\(^\text{\textregistered}\) contained GFP-positive cells (\(n = 177\) of 276 clones) compared with only 47.3% (\(n = 126\) of 300 clones) of clones transduced with RGD4C/CMV-GFP-puro\(^\text{\textregistered}\) at day 17 posttransduction (Fig. 1A). No further significant change in GFP was observed in U87 cell clones at day 31 posttransduction with either vector.

To confirm persistence of transgene expression by RGD4C/Grp78-GFP, all cell clones were pooled and GFP expression analyzed by flow cytometry. At day 39 postvector transduction, 57% of 9L clones transduced by RGD4C/Grp78-GFP were positive for GFP expression and this number increased drastically to 85% at day 97 (Fig. 1B). In contrast, only 37% of 9L cells transduced by RGD4C/CMV-GFP were positive for GFP expression and this number increased drastically to 85% at day 97 (Fig. 1B). Furthermore, we observed approximately 10-fold drop of the mean fluorescent intensity (MFI) of RGD4C/CMV-GFP cells, whereas the MFI remained stable in cells transduced with RGD4C/Grp78-GFP.

Similarly, fluorescence-activated cell sorting (FACS) analyses showed that GFP expression in U87 cells
transduced by RGD4C/Grp78-GFP increased from 37% at day 39 to 66.3% at day 75 posttransduction (Fig. 1C). In contrast, although cells transduced with RGD4C/CMV-GFP had 59% GFP-positive cells at day 39, this number decreased remarkably to 5.6% at day 75 posttransduction (Fig. 1C). Moreover, while U87 cells transduced with RGD4C/CMV-GFP presented significantly higher MFI than RGD4C/Grp78-GFP cells at day 39, their MFI decreased by approximately 18 fold at day 75 posttransduction.

Next, we sought to confirm the improved transgene expression in RGD4C/Grp78-transduced cells over time in an in vivo context. 9L cells stably transduced with RGD4C/Grp78 or RGD4C/CMV vectors expressing the firefly luciferase reporter transgene were subcutaneously implanted into immunodeficient nude mice. Luc expression was monitored in tumors by repetitive bioluminescence imaging (BLI) of tumor-bearing mice (Supplementary Fig. S4A). While Luc expression was comparable between the 2 groups within the initial tumor growth phase, it increased significantly over time in tumors derived from RGD4C/Grp78-transduced cells compared with RGD4C/CMV-derived tumors (Supplementary Fig. S4B).

**RGD4C/Grp78-HSVtk plus GCV treatment produces strong and constant tumor cell killing**

To test tumor cell killing efficacy, we constructed the RGD4C/Grp78-HSVtk encoding the HSV1-tk mutant SR39 (30) and compared with the RGD4C/CMV-HSVtk construct. The HSVtk gene can serve as a suicide gene when combined with GCV. Tumor cells were transduced with RGD4C/Grp78-HSVtk-Puro® or RGD4C/CMV-HSVtk-Puro® to generate stably transduced cells. HSVtk expression was assessed over a time course by Western blot analysis. Consistent with FACS experiments, HSVtk expression was assessed over a time course by Western blot analysis. Consistent with FACS experiments, HSVtk expression was initially stronger in cells transduced with RGD4C/CMV-HSVtk, it decreased gradually overtime, whereas HSVtk remained constant in cells transduced with RGD4C/Grp78-HSVtk (Fig. 2B).

We next set out to assess tumor cell killing in vitro by vectors for days 47 (t1) and 109 (t2) posttransduction. HSVtk gene therapy was induced at each time point by GCV treatment for 24, 48, 72, or 96 hours. Addition of GCV at t1 resulted in significantly higher death of 9L cells by RGD4C/Grp78-HSVtk than RGD4C/CMV-HSVtk (Fig. 2C and D). Importantly, we observed a rapid decrease in the efficacy of tumor cell killing by RGD4C/CMV-HSVtk at t2, whereas efficacy of RGD4C/Grp78-HSVtk remained stable overtime consistent with Western blot analysis of HSVtk expression (Fig. 2A).

Surprisingly, in U87 cells, there was no significant difference between RGD4C/Grp78-HSVtk and RGD4C/CMV-HSVtk vectors in cell death induction by GCV at day 66 (t1) posttransduction (Supplementary Fig. S5), although HSVtk expression was higher in cells transduced by the RGD4C/CMV-HSVtk as shown in Fig. 2B.

Because RGD4C/Grp78 vector has been designed for systemic gene therapy in vivo, we sought to examine whether the persistence of transgene expression by RGD4C/Grp78 occurs in unselected transduced cells. Thus, transduction in the absence of puromycin selection showed better persistence over time of Luc transgene expression by the RGD4C/Grp78-Luc compared with RGD4C/CMV-Luc (Supplementary Fig. S6A). Furthermore, the tumor cell killing effect of the RGD4C/Grp78-HSVtk in both 9L and U87 cells proved more efficient than that of the RGD4C/CMV-HSVtk as measured at day 9 posttransduction (Supplementary Fig. S6B and S6C).

To rule out the possibility that the activity of Grp78 promoter is tumor cell specific, we assessed efficiency of RGD4C/Grp78 in the MCF7 human breast cancer cells in the absence of puromycin selection. The basal Grp78 promoter activity was initially low following transduction of MCF7 cells; importantly, however, this activity increased overtime and was further induced by stressing the cells with thapsigargin treatment that resulted in 2.2-fold increase toward day 10 posttransduction (Supplementary Fig. S7A). Consistently, Western blot analysis confirmed induction of the endogenous Grp78 gene by thapsigargin treatment (Supplementary Fig. S7B).

**Systemic tumor targeting of gene delivery by the RGD4C/Grp78**

Next, we evaluated the specificity and efficacy of gene delivery in tumors after systemic administration of RGD4C/Grp78 and compared this alongside RGD4C/CMV. As an initial preclinical model, we used immunodeficient nude mice bearing subcutaneous tumors derived from 9L cells. We first used in vivo BLI of the Luc transgene reporter (31, 32) after a single intravenous administration of RGD4C/Grp78-Luc, RGD4C/CMV-Luc, or control insertless vectors in tumor-bearing mice. Luc expression within 9L tumors was detectable at day 3 after RGD4C/CMV-Luc administration (Fig. 3A and B) with a linear increase to reach maximum transgene expression at day 5 that lasted only briefly, followed by a rapid decrease. In sharp contrast, a significantly higher tumor expression of Luc was achieved at day 3 by RGD4C/Grp78-Luc, followed by gradual and much longer stabilization of Luc expression toward day 7 (Fig. 3A and B). Finally, no tumor-associated bioluminescent signals were observed with control vectors, and no bioluminescence was observed in normal organs from all experimental groups.

In a second set of experiments, we used the RGD4C/Grp78 encoding the HSVtk to determine therapeutic efficacy. Cohorts of mice bearing 9L tumors (~100 mm³) received a single intravenous dose of RGD4C/Grp78-
HSVtk, RGD4C/CMV-HSVtk, or control vectors followed by GCV treatment in all groups at day 3 postvector delivery. Tumor size (Fig. 3C) and tumor viability (Fig. 3D) were evaluated. Similar tumor growth suppressive effects were initially observed with GCV treatment of mice receiving RGD4C/Grp78-HSVtk or RGD4C/CMV-HSVtk as compared with mice treated with control vectors (Fig. 3C). However, when tumors grew back after therapy, repeated treatment with GCV resulted in tumor growth inhibition in mice that received RGD4C/Grp78-HSVtk (Fig. 3C). In contrast, GCV had little to no effect on the large tumors treated with RGD4C/CMV-HSVtk therapy. Moreover, RGD4C/Grp78-HSVtk was associated with superior suppressive effects on tumor viability compared with RGD4C/CMV-HSVtk as assessed with BLI of Luc expression in tumors (Fig. 3D).

To rule out the possibility that the observed antitumor effects were tumor specific, we analyzed efficacy of RGD4C/Grp78-HSVtk on the U87-derived xenografts. Similar antitumor effects seen in the 9L tumors were initially detected in the U87 xenografts following GCV treatment of mice administered with single doses of either RGD4C/Grp78-HSVtk or RGD4C/CMV-HSVtk as compared with control mice injected with control vectors (Fig. 4A and B). Yet again, when tumors grew back after therapy, repeated GCV treatment resulted in a sharp regression of tumor volumes in mice that received the RGD4C/Grp78-HSVtk as compared with mice receiving...
RGD4C/CMV-HSVtk (Fig. 4A and B). In addition, tumor viability was markedly suppressed in tumors from mice injected with the RGD4C/Grp78-HSVtk vector as evidenced by loss of Luc activity (Fig. 4C).

**Systemic administration of RGD4C/Grp78-HSVtk and GCV induces regression of large tumors**

To confirm the therapeutic advantage of RGD4C/Grp78 in treating large and rapidly growing tumors, we evaluated RGD4C/Grp78-HSVtk in large 9L tumors. First, to monitor Grp78 activity during tumor growth before GCV treatment, we generated 9L cells stably expressing the Luc transgene under the Grp78 promoter. Next, 9L/Grp78-Luc cells were implanted subcutaneously into nude mice, then RGD4C/Grp78-HSVtk, RGD4C/CMV-HSVtk, or control vectors were intravenously injected when tumors reached large volumes (~250–300 mm³). At day 3 postvector injection, tumors grew rapidly to reach large size (~400–450 mm³) and BLI of Luc expression revealed high activity of the Grp78 promoter within tumors (Fig. 5A). We therefore initiated GCV treatment at day 3 postvector injection and after 5 days, the double-targeted RGD4C/Grp78-HSVtk vector induced a marked regression of the large tumors compared with mice injected with RGD4C/CMV-HSVtk (Fig. 5A and B). Moreover, no effect on tumor growth was observed in mice treated with control vectors. Hematoxylin and eosin (H&E) staining revealed extensive cell death in tumors by RGD4C/Grp78-HSVtk plus GCV (Fig. 5C). In contrast, RGD4C/CMV-HSVtk and control vectors had no such effect.

**Suicide gene therapy with HSVtk and GCV increases endogenous Grp78 promoter activity and boosts transgene expression mediated by RGD4C/Grp78**

To gain further insight into the efficacy of the double-targeted RGD4C/Grp78-HSVtk, we investigated the effect of the HSVtk plus GCV therapy on the activity of Grp78 promoter at both levels of endogenous Grp78 and RGD4C/Grp78 vector. 9L cells stably transduced with RGD4C/Grp78-HSVtk-puro⁸ or RGD4C/CMV-HSVtk-puro⁸ were treated with GCV for 1 to 12 hours. Western blot analyses showed a gradual increase of endogenous Grp78 levels, approximately 3-fold increase by 12 hours (Fig. 6A), and of the RGD4C/Grp78-HSVtk promoter (Fig. 6B). In contrast, HSVtk levels in cells transduced with RGD4C/Grp78-HSVtk-puro were only increased by GCV in cells transduced with RGD4C/Grp78-HSVtk-puro (Fig. 6A), whereas no effect of GCV on HSVtk expression was observed in cells transduced with RGD4C/CMV-HSVtk-puro (Fig. 6B). These data confirm that HSVtk/GCV therapy activates both promoters of endogenous Grp78 and of the RGD4C/Grp78 vector.

We then checked whether Grp78 activation by HSVtk/GCV is mediated through the conserved signaling cascade termed unfolded protein response (UPR) pathway that...
regulates the Grp78 (33–35). Thus, we investigated 3 ER transmembrane proteins representing the 3 arms of the UPR pathway (33–35). We first assessed the protein kinase-like ER kinase (PERK). Upon dissociation of Grp78, PERK homooligomerizes and phosphorylates the eukaryotic initiation factor 2α (eIF2α) that selectively promotes translation of the transcription factor ATF4, which induces the Grp78 promoter (35). Western blot analyses showed that GCV induces the phospho-eIF2α that is detectable at 9-hour post-GCV treatment (Fig. 6C).

Next, we found that GCV increases the 90-kDa form of the activating transcription factor 6 (ATF6), which became evident at 6-hour post-GCV treatment (Fig. 6D). Previous studies have shown that the PERK/phosphorylated-eIF2α pathway can activate and control ATF6 expression in response to ER stress (35). An increase in the 90-kDa ATF6 level after GCV treatment further shows initiation of the ER stress pathway and subsequently activation of UPR.

Finally, upon dissociation of Grp78, activated inositol requiring enzyme 1 splices the mRNA of XBP1 (33). RT-PCR analyses show that this mRNA was constitutively spliced in 9L cells (Supplementary Fig. S8) and that GCV treatment had no further effect.

Discussion

The RGD4C/Grp78 phage is a promising gene transfer tool that implements ligand-directed and transcriptional targeting into a single vector. Our studies here are the first to report efficacy of systemic gene delivery with the Grp78 promoter, and establish the double-targeted RGD4C/Grp78 as superior to the conventional RGD4C/CMV phage in providing striking persistence of gene expression in vitro and in vivo and a clear advantage in HSVtk systemic cancer gene therapy. It has been reported that in comparison with the murine leukemia long terminal repeat viral promoter, Grp78-regulated suicide cancer gene therapy is stronger (9). Our studies are the first to compare Grp78 to the commonly used CMV viral promoter in vitro and in vivo, and further prove the superiority of Grp78 to a viral promoter in cancer gene therapy, in particular when both promoters are systemically targeted.
to tumors. The in vitro cell killing advantage of RGD4C/Grp78 is supported by our reporter transgene expression studies and Western blot analysis of HSVtk showing persistence of gene expression by Grp78 and silencing of the CMV promoter. These data are consistent with numerous reports showing that the CMV promoter undergoes silencing in vitro and in vivo by mammalian host cells (12, 36, 37). Our observations following analysis of HSVtk/GCV killing of U87 cells merit further discussion. It is important to note that overall activity of the Grp78 promoter is low in this cell line, these cells are under less ER stress, and the proliferation rate of U87 cells is lower than that of 9L cells. A positive correlation between cell proliferation rate and Grp78 activity has been reported (28). Interestingly, similar cell death was induced by RGD4C/Grp78-HSVtk than RGD4C/CMV-HSVtk. One possible explanation is while U87 cells were not subjected to stress in gene expression analysis, those expressing HSVtk may have experienced ER stress following GCV treatment. Finally, we confirmed the efficiency of RGD4C/Grp78 in another model of the MCF7 human breast cancer. Overexpression of Grp78 gene was
previously reported in human malignant breast cancer but not in benign breast lesions (27). Consistently, the activity in MCF7 was increased upon stress induction with thapsigargin treatment, indicating that the stress-inducible Grp78 promoter of RGD4C/Grp78 is active in different tumor types and is induced under conditions that persist within aggressive tumors (3).

In vivo, systemic administration of the RGD4C/Grp78 initiates higher transgene expression with a more stable pattern than the RGD4C/CMV. The drop of Luc expression observed with both RGD4C/CMV-Luc and, at a later time point, with RGD4C/Grp78-Luc, could be explained by dilution effects of proliferating transduced cells within a larger population of proliferating non-transduced cells. However, comparable antitumor effects were obtained at early time points on small tumors, which could be because of the bystander effect of the HSVtk/GCV (20). Importantly, we have shown the antitumor effect of RGD4C/Grp78 on large 9L tumors consistently with specific activation of the Grp78 promoter in poorly perfused malignant tissues, including tumor-associated vasculature and tumor cells (38). Moreover, we report an antitumor effect of RGD4C/Grp78 on recurrent tumors, which can be explained by (i) Grp78 being a stress-inducible gene that encodes for a potent antiapoptotic protein that plays a critical role in tumor survival and resistance to therapy, (ii) specific overactivation of Grp78 in aggressive cancers, and (iii) our findings that HSVtk/GCV stimulates the UPR stress pathway leading to increased Grp78 activity. Recent studies have shown that chemotherapeutic drugs stimulate Grp78 expression (26). Yet, this is the first report to show that HSVtk/GCV gene therapy activates Grp78 at both endogenous and vector levels. In this manner, cells transduced by RGD4C/Grp78-HSVtk unintentionally facilitate their own death upon GCV treatment by activating the UPR stress pathway and ultimately Grp78. These data are also consistent with previous work reporting the stronger tumor cell killing efficacy of HSVtk under the Grp78 promoter in comparison to viral promoters. Accordingly, our studies provide the proof-of-concept for taking advantage of a cancer cell’s own resistant mechanisms to enhance gene therapy against therapy-resistant tumors.

Figure 6. Combination of HSVtk expression and GCV induces Grp78 promoter. The 9L cells stably transduced with RGD4C/Grp78-HSVtk (A) or RGD4C/CMV-HSVtk (B) were treated with GCV for the indicated times, then subjected to Western blot analyses with antibodies against Grp78, HSVtk, and GAPDH as control. Endogenous Grp78 and HSVtk levels were measured, normalized to GAPDH, and fold increase over time zero-control was calculated for each sample ($n = 3$). C and D, Western blot analysis of phospho-eIF2α and ATF6 expression, respectively.
In conclusion, RGD4C/Grp78 holds the potential to treat large and therapy resistant tumors after systemic administration. Our dual targeting platform further ensures selective transgene transfer to tumors. Moreover, our studies show the efficacy of combining homing ligands and a mammalian tumor-specific promoter in the context of bacteriophage. This vector could also enhance the effectiveness of molecular-genetic imaging. The translation of the double-targeted RGD4C/Grp78 particle may lead to clinical applications of the Grp78 promoter.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Georges Smith and Hrvoje Miletic for reagents. The authors thank Anna Ettorre for assistance with the FACS experiments and Harkila Elefterhorinou for statistical analyses. The authors also thank Elizabeth Hileman for editing and reading the manuscript, and Teerapong Yata for assistance with the figures.

Grant Support
Financial support was provided by Grant G0701159/1 of the UK Medical Research Council (A. Kia and A. Hajitou) and grant of the Brain Tumour Research Campaign (J.M. Przystal and A. Hajitou).

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Received June 11, 2012; revised August 24, 2012; accepted September 26, 2012; published OnlineFirst October 10, 2012.

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www.aacrjournals.org Mol Cancer Ther; 11(12) December 2012 OF11

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Molecular Cancer Therapeutics

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Mol Cancer Ther  Published OnlineFirst October 10, 2012.

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