Pharmacokinetic study of neural stem cell-based cell carrier for oncolytic virotherapy: targeted delivery of the therapeutic payload in an orthotopic brain tumor model

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INTRODUCTION

Glioblastoma (GBM) is the most common primary brain tumor and portends the worst prognosis among all central nervous system malignancies.¹ The mean overall survival has only slightly improved over the last 30 years.² The current standard of care relies on surgical resection, fractionated radiotherapy and chemotherapy.³ The therapeutic efficacy of most of these treatment modalities is limited owing to the invasive nature of the tumors. By the time gliomas are diagnosed they have already infiltrated diffusely and thus are extremely difficult for complete surgical resection therefore limiting the benefits of gross total removal. The low oxygen level in the glioma environment negatively affects radiotherapy;⁴ while cellular heterogeneity and glioma stem cells account for the emergence of resistance to therapeutic regimens.⁵,⁶ Therefore, there is an urgency to develop novel therapies capable of overcoming the common resistance mechanisms of gliomas.⁷

One such therapy involves harnessing oncolytic viruses to kill glioma cells. The concept of exploiting viruses, for their oncolytic potential, has a century-old history, but only within the last 20 years has this approach been considered for the treatment of brain tumors.⁸ Our lab has been focused on conditionally replicating adenoviruses (CRAds) because of their proven efficacy in preclinical glioma models.⁹ CRAd-S-pk7, the vector that we intend to bring into clinical trials, consists of two genetic modifications: (i) a fiber modification containing polylysine that binds with high affinity to heparan sulfate proteoglycans and (ii) E1A transcription under the control of survivin promoter.⁹–¹¹ These modifications provide CRAd-S-pk7 the necessary tumor specificity allowing for selective replication in glioma cells and minimal toxicity to normal brain tissue.⁹ Furthermore, CRAds have the capacity to kill different subsets of glioma cells without being confined to the resistance of conventional therapies.¹² Therefore, they hold a great promise in the armamentarium against gliomas.

There have been at least six formal clinical trials using oncolytic viruses to treat malignant brain tumors.¹³,¹⁴ Despite some degree of therapeutic efficacy shown in these clinical trials, overall, they have fallen short of expectations. Most oncolytic viral agents, including CRAd, face some challenges before they can be translated into therapies. Issues pertaining to the successful clinical translation of oncolytic virotherapy for gliomas are as follows: (i) the limited distribution of viral vectors after intratumoral injection, (ii) the immune clearance induced shortly after injection and (iii) the inability of the currently available vectors to target disseminated tumor burdens. To overcome these hurdles, cell carriers have been used to improve targeting and distribution while reducing the immune response toward viral vectors.¹⁵–¹⁷ Numerous carrier cell/oncolytic virus combinations are currently under investigation.¹⁸,¹⁹ Each represents a unique biotherapeutic system with different kinetics of therapeutic virus replication, in vivo tumor-homing ability and thus must be examined extensively.

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Our laboratory along with others is actively pursuing the development of a stem cell-based carrier system for anti-glioma oncolytic virotherapy. Recently, we have shown neural stem cell (NSC) carriers to be superior to mesenchymal stem cells in delivering CRAd-S-pk7 to orthotopic glioma models and therefore NSCs enhance the therapeutic potential of oncolytic virotherapy. To progress to a clinical trial, it is necessary to characterize and quantify the pharmacokinetic properties of NSCs as cell carriers. Here, we report a thorough characterization of a Food and Drug Administration (FDA) approved immobilized NSC line (HB1.F3-CD) as an effective cell carrier for CRAd-S-pk7. First, we provide evidence of the NSC's potential of replicating and releasing infectious progeny that can kill glioma cell lines. Then, we show that, in nude mice bearing orthotopic human glioma, NSCs do not alter their inherent tumor-tropic properties post infection and produce infectious virus progeny for more than a week after reaching the tumor site. Furthermore, we observed that the NSC-based cell carrier significantly reduced the non-specific therapeutic virus distribution in the animal brain. Finally, to better characterize the systemic biodistribution of adenovirus after intracranial (IC) injection of NSCs loaded with CRAd-S-pk7, we utilized cotton rats and hamsters, two pre-established semi-permissive animal models. We show that carrier cells do not disseminate to distant organs and high titers of infectious progeny are present only at the injected hemisphere. Thus, the unique tumor-tropic properties of NSCs combined with an improved safety profile of IC adenovirus injection brings this cell-carrier-based anti-glioma oncolytic virotherapy one step closer to clinical trials.

MATERIALS AND METHODS

Cell lines and vectors

HB1.F3-CD is a v-myc immortalized human NSC (hNSC) line, derived from the human fetal brain that constitutively expresses cytosine deaminase (CD). NSCs were maintained as adherent cultures in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2 mmol l⁻¹ L-glutamine, 100 units ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 0.25 μg ml⁻¹ amphotericin B (Invitrogen, Carlsbad, CA). U87MG, U251MG, U118MG and A549 carcinoma cell lines were purchased from the American Type Culture Collection (Invitrogen, Carlsbad, CA); while N10 glioma was purchased from the Japanese Tumor Bank (Tokyo, Japan). All cells were grown in a minimal essential medium (MEM; Biologicals, Lawrenceville, GA) while N10 glioma was purchased from the American Type Culture Collection (Manassas, VA); both cell lines were cultured in serum-free MEM medium supplemented with 2 mmol l⁻¹ L-glutamine, 100 units ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 0.25 μg ml⁻¹ amphotericin B (Invitrogen, Carlsbad, CA). U87MG, U251MG, U118MG and A549 carcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA); while N10 glioma was purchased from the Japanese Tumor Bank (Tokyo, Japan). All cells were grown in a minimal essential medium (MEM; Biologicals, Lawrenceville, GA) while N10 glioma was purchased from the American Type Culture Collection (Manassas, VA); both cell lines were cultured in serum-free MEM medium supplemented with 2 mmol l⁻¹ L-glutamine, 100 units ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 0.25 μg ml⁻¹ amphotericin B (Invitrogen, Carlsbad, CA). Thus, the unique tumor-tropic properties of NSCs combined with an improved safety profile of IC adenovirus injection brings this cell-carrier-based anti-glioma oncolytic virotherapy one step closer to clinical trials.

Generation of green fluorescent protein (GFP)- or firefly luciferase (Fluc)-expressing HB1.F3-CD

To detect the distribution of NSCs in vivo, we generated a GFP- or Fluc-expressing HB1.F3-CD. GFP expressing cells were infected with a replication-incompetent retroviral construct; whereas for Fluc we infected cells with a replication-incompetent lentiviral vector, as described in detail elsewhere. We used 4 μg ml⁻¹ puromycin in Dulbecco’s modified Eagle medium to isolate stable expressing clones. Antibodies and other reagents

For flow cytometry, cells were stained with mouse anti-human CAR (Abcam, Cambridge, MA), CD138, sFlt3, sFlt1 (Ebioscience, San Diego, CA) and rat anti-human perlecan; followed by AlexaFluor647 (Invitrogen)-conjugated secondary antibodies. Adenovirus-transduced cells were detected using a goat anti-hexon fluorescein isothiocyanate (FITC)-conjugated antibody (Millipore, Billerica, MA). For immunofluorescence, FITC-conjugated anti-GFP antibody, biotin-conjugated anti-hexon and FITC-conjugated immunoglobulin controls were purchased from Abcam; human CD44 rabbit monoclonal antibody purchased from Epitomics (Burlingame, CA); AlexaFluor555-streptavidin and Alexafluor350 donkey anti-rabbit were purchased from Invitrogen.

Flow cytometry

For detection of surface receptors, cells were detached using trypsin/EDTA and stained with primary antibodies for 1 h at 4 °C, followed by secondary antibodies for 30 min at 4 °C. For quantification of adenovirus transduction of HB1.F3-CD, 48 h after infection, cells were detached, washed with phosphate-buffered saline (PBS) and then permeabilized with a methanol/acetone solution (as per Millipore protocol) before staining with FITC-conjugated goat anti-hexon. Cells were analyzed using a BD FACSCanto cytometer (Becton Dickinson, Franklin Lakes, NJ) and graphs were rendered using the Flojo software (TreeStar, Ashland, OR).

Cell viability assays

NSC and glioma viability was determined using the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation kit (Roche Diagnostics, Mannheim, Germany). Briefly, in a 96-well plate we plated 3000 cells per well, the day before infection. NSCs were infected with different concentrations of CRAd-S-pk7; instead glioma cells were incubated with the supernatant of previously infected NSCs. The viability was determined 3 days later, as described by the manufacturer’s protocol. Viability of glioma cells after incubation with the supernatant of previously infected NSCs was also assessed by Crystals violet. NSCs were infected earlier with CRAd-S-pk7 at different concentrations 1, 10, 50 and 100 infectious units per ml (IU/ml). After five days, the supernatant was collected and used to infect glioma cell lines and A549 carcinoma plated in 24-well plates. After 3 days viability was determined. Shortly after aspirating the media the cell layer was covered with the Crystal violet solution (1%) and incubated for 20 min at room temperature. Then wells were washed carefully and let dry at room temperature. Images were taken with an inverted microscope.

Determination of adenoviral E1A copies via quantitative PCR

Total DNA from cultured cells or animal tissues was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Adenoviral E1A gene expression was quantified via quantitative real-time PCR using SYBR green supermix (Bio-Rad, Hercules, CA), using primers described elsewhere. For each animal model, we generated separate standard curves of E1A copies containing 100 ng of DNA. The sensitivity of this assay was set to detect as low as 5 E1A copies per 100 ng of DNA (Supplementary Figure S1). All samples were run in triplicates using an Opticon2 system (Bio-Rad). Results are expressed as E1A copy number per 100 ng of DNA.

Immunohistochemistry

For immunohistochemistry, brains were sectioned in 10-μm thick sections. After thawing, sections underwent fixation/permeabilization with a solution of 50/50 acetone–methanol at −20 °C for 5 min. Then, the slides were washed with ice-cold PBS and blocked with 10% bovine serum albumin for 30 min. We incubated overnight at 4°C with primary antibodies and 1 h at room temperature with the secondary antibody. After washing the excess antibody, slides were mounted with Prolong Gold antifade reagent with 4′-diamidino-2-phenylindole (Invitrogen). Fluorescent images were documented with an inverted Axiovert200 Zeiss microscope (Carl Zeiss Microscopy, Thornwood, NY).

In vivo tracking of NSCs with bioluminescence imaging

For in vivo tracking of NSC migration to the tumor, we relied upon photon flux imaging (PFI). Mice were imaged for Fluc activity following intraperitoneal injection of α-luciferin (4.5 mg per animal in 150 μl saline), and photon counts were recorded 10 min after α-luciferin administration by using a cryogenically cooled high-efficiency charged-coupled device camera system (Xenogen IVIS200 Optical Imaging System, Caliper Life Sciences, Mountain View, CA).
Pharmacokinetics of adenovirus-loaded carrier cells
B Thaci et al

RESULTS

Permissiveness of HB1. F3-CD NSC carrier for CRAd-S-pk7 infection

The ultimate goal of ex-vivo loading is to effectively infect as many carrier cells as possible. Thus, to evaluate the permissiveness of NSC to CRAd infection, we first examined the expression of adenovirus cell attachment and internalization receptors in NSCs (Figure 1a). Although NSCs expressed minimal levels of the adenovirus primary attachment receptor CAR, about 50% of NSCs expressed CD138, one of many heparan sulfate proteoglycans that express CD138, one of many heparan sulfate proteoglycans that 

![Image of results graph]

**Figure 1a:** Expression of adenovirus cell attachment and internalization receptors in NSCs. (A) Western blot analysis showing CAR expression levels in NSCs. **(B)** Real-time PCR analysis of Adenoviral E1A gene expression in NSCs. 

**Statistical analysis**

The statistical analysis presented was performed using the GraphPad Prism Software, v4.0. (GraphPad Software, La Jolla, CA). Where applicable a standard independent two-sample t-test was applied. A P-value < 0.05 was considered statistically significant (**P < 0.001; *P < 0.01 and *P < 0.05).
virus during this process (Figure 1b). At the low dose (1–10 i.u. per cell) CRAd-S-pk7 did not induce toxicity to NSCs. However, the viability was reduced about 25% at the dose of 50 i.u. per cell and about 50% when NSCs were infected with 100 i.u. per cell.

Next, to establish the replication kinetics of CRAd-S-pk7 in NSCs, we infected/loaded them with varying concentrations of CRAd. As shown in Figure 1c, the viral DNA replication at day 3 was highest when loaded with 50 i.u. per cell, amounting to $1.6 \times 10^6$ E1A copies per ng of DNA. At this loading dose, the viral burst size was about 5 and 2 fold larger than the loading dose of 1 and 10 i.u. per cell, respectively (**$P = 0.002$ and *$P = 0.024$). When NSCs were loaded with 100 i.u. per cell, the CRAd-S-pk7 DNA replication did not improve significantly as compared with 50 i.u. per cell ($P = 0.58$), and NSC viability was significantly reduced at this level. Combining toxicity data along with DNA replication data, a loading dose of 50 i.u. per cell was selected to further evaluate NSCs as a carrier system for oncolytic virotherapy. At this loading dose, viral DNA replication continued to increase up to 7 days post infection (Figure 1d).

Our next objective was to establish an optimal exposure time to achieve maximum infectivity/loading, while minimizing oncolytic virus-mediated toxicity. We measured adenovirus transduction rate by analyzing Ad-hexon expression and viral DNA replication after varying CRAd-S-pk7 incubation times with both HB1.F3-CD adherent monolayer and suspension cells (1–4 h). After incubating NSCs in suspension at the loading dose of 50 i.u. per cell for 2 h, a maximum loading/infection of the CRAd-S-pk7 was achieved (***$P = 0.002$; Figures 1e and f). On the basis of these data, we decided to load/infect NSCs in suspension at the loading dose of 50 i.u. per cell for 2 h for our future experiments.

HB1.F3-CD loaded with CRAd-S-pk7 produces infectious progeny and induces glioma cell oncolysis

To assess the ability of this carrier system to produce infectious adenoviral progeny, we incubated tumor cells with the supernatant of previously infected HB1.F3-CD cells. At day 5 post infection the intracellular virus titer reached its maximum. At day 7 post infection, the intracellular viral titer decreased as the titer of the cell-free viral progeny reached its maximum level, indicating that it takes about 5–6 days for the CRAd-S-pk7 to complete its life cycle in the HB1.F3-CD carrier system (Figure 2a).

Next, to evaluate the oncolytic capacity of the viral progeny released from CRAd-S-pk7-infected NSCs, a panel of four human glioma cell lines was exposed to the supernatant of NSCs loaded/infection with various doses of CRAd-S-pk7 for 120 h. Figure 2b is a pictorial representation of tumor cell toxicity produced by the therapeutic viral progeny from infected HB1.F3-CD cells. Regardless of the loading dose, the released CRAd-S-pk7 viral progeny was able to induce tumor cell killing in all tested glioma cell lines at day 3 post incubation (Figure 2c). As compared with the tested glioma cell lines, NSC carrier cells were much more resistant to CRAd-S-pk7-mediated oncolysis (Figures 1b and 2c).

Distribution of the CRAd-S-pk7-loaded HB1.F3-CD carrier cells in nude mouse brains

The intrinsic tumor-homing properties of NSCs are the key attribute to their utility as a cell carrier for oncolytic virotherapy. Therefore, it is of paramount importance to examine how adenovirus loading affects the engraftment, distribution and survival of the implanted NSC in the animal brain. To monitor the implanted stem cell distribution effectively, the HB1.F3-CD cells were further modified to express GFP by using a replication-incompetent retroviral vector, as described in Materials and Methods. A total of $5 \times 10^5$ NSCs loaded with or without the CRAd-S-pk7 virus (50 i.u. per cell) were stereotactically implanted in the brains of nude mice. Viability and distribution of NSCs were assessed at the indicated time points (1, 5, 12 and 17 days post NSC implantation) via immunohistochemistry. On day 1 (Figure 3a), a majority of the implanted cells were localized and clumped together at the injection site. On day 5, the clumping was significantly reduced and most of the cells were located at the implanted site. Most importantly, CRAd-S-pk7 loading appeared to have had minimal or no effect on the viability of the carrier cell as measured by the number of GFP-positive HB1.F3-CD cells present in the section of animal brains at day 5 and day 12 post implantation (Figure 3b). Also to be noted: NSCs were not detectable in the contralateral hemisphere (NSCs were implanted on the right side). At day 17 of post implantation, the NSCs were undetectable in animal brains via immunohistochemical analysis.

Distribution of CRAD-S-pk7-loaded HB1.F3-CD in nude mouse brain bearing orthotopic U87 human glioma xenograft

Next, the adenovirus loading effects on the NSCs’ engraftment, distribution and survival were examined in the animal brain bearing a human glioma xenograft. On day 1, the distribution was very similar to the animal brain without any tumor as cells clumped at the injection site (Figure 4a). On day 5, implanted cells were distributed around the tumor (Figures 4a and b). Again, we did not observe any NSCs migrating to the hemisphere contralateral to the implanted hemisphere. The total number of NSC-GFP-positive cells that surrounded the U87 xenograft did not differ between infected vs non-infected NSC, showing no difference in cell survival (Figure 4c). After 12 days post implantation, the viability of NSCs drops to 1 NSC/high-power field for both infected and non-infected and became undetectable at 17 days post implantation. Taken together, we conclude that loading oncolytic virus into NSCs had a minimal effect on their viability and engraftment capacity in vivo.

In vivo delivery of the therapeutic CRAd-S-pk7 virus by the HB1.F3-CD carrier cell

To assess the clinical relevancy of our carrier-based oncolytic virotherapy, we next investigated virus hand-off ability and the IC distribution of the therapeutic virus in vivo. The HB1.F3-CD cells infected with CRAd-S-pk7 virus were implanted in the brain of nude mice bearing U87 human xenograft tumor as described

| Tissue                  | Killed (day) | Nude mouse | Hamster | Cotton rat |
|-------------------------|-------------|------------|---------|------------|
| Serum                   | 1           | NA         | 0/6     | 0/6        |
|                         | 4           | NA         | 0/6     | 0/6        |
|                         | 7           | NA         | 0/6     | 0/6        |
|                         | 14          | NA         | 0/6     | 0/6        |
|                         | 30          | NA         | 0/6     | 0/6        |
| Right hemisphere (injected) | 1           | NA         | 4/6     | 5/6        |
|                         | 4           | 5/5        | NA      | NA         |
|                         | 7           | 5/5        | 0/6     | 0/6        |
|                         | 14          | 5/5        | 0/6     | 0/6        |
|                         | 30          | NA         | 0/6     | 0/6        |
| Left hemisphere (non-injected) | 1           | NA         | 0/6     | 0/6        |
|                         | 4           | 4/5        | NA      | NA         |
|                         | 7           | 2/5        | 0/6     | 0/6        |
|                         | 14          | 1/5        | 0/6     | 0/6        |
|                         | 30          | NA         | 0/6     | 0/6        |

Infectious adenoviral progeny was determined in the tissues of injected animals at the indicated time points. NA, means that such tissue was not available for the indicated animal at that specific time point.
previously. Mice were killed at days 1 and 5 post implantation, and the animal brains were subjected to immunohistochemical analysis for GFP (carrier cell specific) and adenoviral hexon protein. As shown in Figure 5a, implanted carrier cells were clumped together at the injected site at 24 h post implantation. At 5 days post implantation, the carrier cells surrounded the tumor and, most importantly, the GFP-negative tumor cells were positive for hexon staining (Figure 5a, right panel, arrows). Taken together, these data indicate that carrier cells loaded with CRAd-S-pk7 are able to hand off the therapeutic virus to their surrounding tumor cells.

Glioma foci/microsatellites can be located further away from the initial implanted site of the therapeutic NSC, and loaded NSCs will have to migrate longer distances before delivering the payload. To examine whether CRAd-loaded HB1.F3.CD can migrate to disseminated tumor foci and deliver the therapeutic payload effectively, we first established HB1.F3.CD cells stably expressing F-luciferase (Luc) gene by lentivirus-mediated transduction. We then loaded the HB1.F3.CD-Luc cells with 50 i.u. per cell of CRAd-S-pk7 and implanted the cells in the contralateral hemisphere of the U87 xenograft-containing animals (Figure 5b). As shown in Figure 5c with bioluminescence imaging, at 72 h post implantation adenovirus-loaded HB1.F3.CD.Fluc cells were able to cross the midline and migrate to the contralateral hemisphere. Animals were killed and the brain tissue was subjected to immunohistochemical analysis for adenovirus hexon-positive HB1.F3.CD cells in the U87 tumor foci stained with human-specific anti-CD44 antibody (Figure 5d-ii). We observed Ad hexon-positive cells in the xenograft tumor foci located in the contralateral hemisphere, indicating that implanted CRAd-loaded HB1.F3.CD cells were able to migrate to the distance tumor foci and deliver the therapeutic payload (Figure 5d i--iii).

Carrier cell delivery reduces off-site levels of adenoviral titers Next, we evaluated IC distribution of CRAd-S-pk7 post implantation, either of the naked virus- or virus-loaded HB1.F3.CD cell carrier, in a nude mice brain bearing human glioma xenografts. Five animals from each group were killed at 4, 7 and 14 days post implantation and were subjected to viral distribution analysis by examining the presence of the viral DNA as well as infectious progeny. As shown in Figure 6a, the amount of infectious viral particles recovered from both the injected and the contralateral hemisphere of the animal brains that received naked CRAd-S-pk7 virus was very similar. On the other hand, when the therapeutic virus was delivered loaded into carrier cells, the viral distribution was more robust (about 2-log greater) and localized at the injected hemisphere (Figure 6b) as compared with the contralateral hemisphere (**P < 0.01). As shown in Table 1, the infectious viral progeny was recovered in two out of five animal brains from the contralateral non-injected hemisphere (Figure 6d) as compared with all five animals in the virus alone group (*P < 0.05).
Evaluation of IC viral distribution in the semi-permissive cotton rat and hamster model

To examine the adenovirus replication in immunocompetent semi-permissive hosts, cotton rat and hamster animal models, a previously established quantitative real-time PCR protocol was utilized to monitor viral DNA copies over time. In both hamster and cotton rat models, adenoviral replication was predominately localized at the injection site (right hemisphere) and over time the viral replication gradually decreased to less than 100 copies of E1A (Figure 7a). The recovered adenoviral E1A copies on the non-injected left hemisphere were about 2-log lower as compared with the injected site. Moreover, we could detect infectious progeny only from the injected right hemisphere (Table 1). The liver was the only organ outside of the brain where viral replication was detectable up to 7 days post implantation. In other organs, viral DNA was only detectable at 24 h post implantation. In addition, we did not detect any infectious progeny in hamster or cotton rat sera (Table 1). To investigate whether loaded NSCs were migrating away from the original injection site, we relied on a highly sensitive, nested PCR-based method that could detect DNA from a single HB1.F3-CD cell in 100 ng of host DNA (Supplementary Figure S2). By using this method we were able to detect HB1.F3-
positive cells per HPF for each animal. Bars: 400 µm (H&E); 100 µm (immunohistochemistry). H&E, hematoxylin and eosin; ND, none detected; NS, not significant.

**Figure 3.** IC distribution of the HB1.F3-CD carrier cells loaded or not with CRAd-S-pk7 after injection in nude mouse brains. (a) Nine- to ten-week-old nude mice were injected in the right hemisphere, 3 mm deep, with 5 x 10⁵ HB1.F3-CD-GFP cells loaded or not with 50 i.u. of CRAd-S-pk7. IC distribution of GFP-positive cells was evaluated at the indicated time points for both loaded and non-loaded cells (n = 3 per time point per group). (b) Positive GFP cells were quantified based on the number of cells per high-power field (HPF; x 630). Values on the y axis represent the mean number of GFP-positive cells per HPF for each animal. Bars: 400 µm (H&E); 100 µm (immunohistochemistry). H&E, hematoxylin and eosin; ND, none detected; NS, not significant.

CD-specific v-myc PCR signal in the implanted right hemisphere of the brain in both of the animal models (Figures 7b and c and Supplementary Figures 3a, b). The HB1.F3-CD signals were only detectable at 24 h post implantation. HB1.F3-CD distribution in the tissue of implanted brains was very similar to what we observed in the nude mice experiments. Both CRAd-loaded and -unloaded HB1.F3-CD cells were predominately found clumping together at the injected sites after 1 day of implantation (Figure 7d). However, we could not identify any viable HB1.F3-CD cells at 7 days post implantation. Taken together, we observed that carrier cells were only detectable in the implanted hemisphere of the animal brains at 24 h post implantation.

**DISCUSSION**

Virus-infected cells can serve as delivery vehicles to improve adenovirus distribution in tumors, hide the virus from the host immune system and function as in situ virus-producing factories that generate oncolytic virus progeny at the tumor beds. Specifically, cells with inherent tumor-tropic properties are a very attractive candidate for the anti-glioma oncolytic virotherapy carrier system. NSCs constitute one such carrier system that has demonstrated unique tropism toward brain neoplasia in animal models. This phenomenon was shown first by Aboody et al., who used engrafted immortalized NSCs, HB1.F3-CD, to selectively deliver therapeutic agents to invasive satellite tumors. In the last decade many studies have shown that NSCs expressing/carrying the therapeutic payload have anti-glioma activity and based on these promising results, the FDA has recently approved the HB1.F3-CD immortalized stem cell line for a clinical trial.

Our lab was one of the first to study the advantages of using stem cells as carriers for oncolytic adenoviruses. In that regard, we have studied the carrier properties of both mesenchymal stem cells and NSCs. We found that both of these carrier systems can be loaded with adenovirus and increase its distribution to the tumor site by acting as microfactories for virus replication. Also, we have shown that stem cell carrier systems not only hide the payload from the immune system but also have the capability to suppress anti-viral innate immune responses. This allows for enhanced dissemination, increased persistence of adenovirus and can result in enhanced therapeutic benefits. Moreover, we recently demonstrated that delivery of oncolytic adenovirus in the orthotopic human glioma xenograft model via NSCs can improve the median animal survival by ~50%. Nevertheless, such carrier systems need to be optimized before undergoing clinical testing.

Any new therapeutic intervention must go through rigorous pharmacological evaluation before it can translate into a clinical setting. Accordingly, we set out to characterize the pharmacokinetic properties of glioma-tropic oncolytic adenovirus-loaded HB1.F3-CD carrier system in three different animal models: nude mouse, hamster and cotton rat. We show that NSCs can be loaded with CRAd-S-pk7 and release new infectious progeny that can effectively lyse glioma cells. We observed that when injected in mice brains bearing human glioma xenograft, NSCs loaded with CRAd-S-pk7 home to glioma and hand off therapeutic adenoviral payload to tumor cells. In models permissive to adenovirus replication, hamster and cotton rat, we detect high adenoviral E1A replication only at the injection site. Furthermore, adenoviral replication declines and becomes barely detectable over 30 days. On the other hand, we detect implanted NSCs only at the injected hemisphere for less than a week. Thus, data presented in this report argue in favor of the possible future utilization of a NSC-based carrier to enhance the therapeutic potential of the anti-glioma oncolytic virotherapy.

The clinical outcome of any cell carrier system for oncolytic virotherapy relies on proper synchronization of three critical steps in both space and time. The ideal carrier cell should (i) be infected with the therapeutic virus; (ii) produce high levels of progeny that can infect target tumor cells and (iii) be relatively resistant to oncolytic virus-mediated toxicity. Even though the HB1.F3-CD cell carrier system express a very minimal amount of the primary adenovirus attachment receptors (CAR; Figure 1a), they express high levels (50%) of CD138 (syndecan), a heparan sulfate proteoglycan receptor that binds to the polysuline residues (pk7) of modified CRAd-S-pk7 fibers. The adenoviral internalization into target cells is mediated by the integrin family receptors αvβ3 and αvβ5, and almost 91.2% of HB1.F3-CD cells express αvβ5 on their surface. Once internalized, the virus starts replicating its genome within 24 h and gradually increases over time (Figures 1d and 2a). The cell associated infectious progeny reached its
maximum at day 5 post infection, whereas the cell-free virus titer reached its peak at $1 \times 10^{11} \text{i.u. ml}^{-1}$ after 7 days of infection (Figure 2a). The fundamental objective of the ex-vivo loading phase is to productively load/infect as many cell carriers as possible with a minimal exposure to the therapeutic virus. With the loading dose of 50 i.u. per cell, we observed the maximum amount of viral DNA replication and only about a 25% decrease in the carrier cell viability at 120 h post loading (Figures 1b and c). On the basis of this data, we selected a loading dose of 50 i.u. per cell to use in all our future experiments. For our system, the infection of HB1.F3-CD cells in suspension with the CRAd-S-pk7 at a loading dose of 50 i.u. per cell lead to the infection of almost 100% of carrier cells after a period of 2 h (Figures 1e and f). It is probably necessary to optimize the ex-vivo loading capacity of each carrier cell/oncolytic virus pair system as it is governed by the oncolytic virus life cycle within the particular cell type used for delivery; for example, the standard infection protocol for vesicular stomatitis virus infection specify a shorter (1 h) loading time for vaccinia virus.\textsuperscript{25}

An effective cell carrier system must be able to produce high quantities of infectious progeny upon arrival at the tumor site. HB1.F3-CD cells loaded/infected with 1 i.u. per cell of CRAd-S-pk7 virus produced sufficient amounts of infectious progeny to induce oncolysis on human glioma cell lines (Figures 2b and c). Moreover, loaded cells produced high intratumoral levels of progeny at the tumor site that were similar to naked CRAd injection (Figures 2d and e). Most importantly, IC delivery of the oncolytic adenovirus loaded into HB1.F3-CD significantly decreased the unwanted distribution of therapeutic virus out of the animal brain ($**P < 0.001$), thus reducing the vector-related toxicity (Figure 7a). Our lab has previously shown that cell carriers can deliver CRAd-S-pk7 to the tumor site when injected at a distance from the tumor.$^{15,16}$ We have further shown that NSCs tumortropic properties are not diminished by the loading/infection of oncolytic adenovirus.$^{17}$

The timing of the oncolytic viral life cycle is a crucial determinant of maintaining the tumor-homing ability of the carrier cells. To achieve true ‘targeted-delivery’ of the therapeutic virus, the carrier cell must accumulate in tumor beds before the viral progeny are released. According to our in vitro observation, the viral progeny released from the HB1.F3-CD carrier cell peaked at day 7 post loading (Figure 2a), therefore carrier cells should ideally reach the tumor site before this time. Based on our previous study, a majority of the tumor-specific migration of NSCs occurred within 24–48 h post implantation.$^{17}$ Therefore, the replication cycle of CRAd-S-pk7 oncolytic virus in the HB1.F3-CD should accommodate the tumor-homing ability of this carrier system.

The preclinical characterization of most oncolytic adenovirus vectors has thus far been restricted to human xenograft models in immunodeficient mice. Although these immunodeficient mice can serve as valuable models to evaluate the therapeutic efficacy of adenovirus-based oncolytic vectors, the virus does not replicate in mouse tissue, and therefore prevents us from being able to vigorously evaluate safety and vector-related toxicity. On the other hand, semi-permissive animal models to adenovirus replication, such as cotton rat and hamster, have proven to be very useful for studying oncolytic adenovirus safety profiles.$^{26–28}$ Accordingly, we characterized the CRAd-S-pk7 biodistribution and safety profile after IC delivery of loaded NSCs in these immunocompetent animal models. To assess the distribution of both the adenovirus and carrier cell we relied on very sensitive PCR methods (Supplementary Figures S1 and 2). Both models show similar distribution of NSCs and CRAd-S-pk7. NSCs are found only at the injection site...
without any non-specific migration. However, we observed a decreased amount of the non-specific spread of CRAd when delivered with the carrier cell system, as evidenced by the 2-log lower adenoviral titers in the contralateral hemisphere (Figures 6b, c and 7a). It has been previously reported that after IC delivery the oncolytic virus can spread throughout the brain because of the presence of media such as cerebro-spinal fluid.29 However, this may be less of a concern as compared with other target tissue, such as the liver, as the effect of neutralizing antibody appears to be far weaker in the brain due to the distinct nature of the immune system in the central nervous system.30,31 Moreover, reports from early clinical trials with adenovirus vector-based anti-glioma gene therapy have uniformly reported sufficient tolerability and the absence of serious adverse events.32 In our study, after the delivery via carrier cells, adenovirus replication in the brain decreased to barely detectable levels over 30 days. Also, very low levels of adenovirus E1A copies were detected in the other harvested organs (Figure 7a) and none of the animals implanted with NSC-loaded CRAd-S-pk7 showed any sign of systemic toxicity.

Taken together, we conclude the IC injection of adenovirus-loaded NSCs appears safe in all three tested animal models with no adverse side effects observed. In our experience, even though cotton rats were able to support oncolytic adenovirus replication more effectively than hamsters,21 they are more aggressive and difficult to handle during any surgical procedure.33,34

Most of the preclinical studies on the efficacy of NSC-based anti-glioma therapeutics have been evaluated to target disseminated tumor sites beyond the primary tumor in small animal models. However, can NSCs withstand the test of distance and deliver to metastatic sites far away from the site of injection in a human brain? The failures of gene therapy can be undoubtedly linked to inaccessibility of optimal animal models that recapitulate human GBM and therefore we should be cautious when attempting to translate our findings into clinical trials. For example, it becomes imperative to study the efficacy of virus-loaded cell carriers in animals with larger brains and therefore larger-sized tumors than nude mice. As one of the possible options, a spontaneous GBM model in the brachycephalic canine breeds has been reported.35

Figure 5. In vivo hand off of CRAd-S-pk7 from NSCs to glioma cells. (a) Nude mice harboring orthotopic U87 malignant glioma in the right hemisphere were injected with HB1.F3-CD-GFP cells loaded or not with CRAd-S-pk7, using the same burr hole. In vivo CRAd-S-pk7 hand out from infected GFP-labeled HB1.F3-CD to U87 glioma cells was detected via immunohistochemistry. A representative area within the tumor where hand out of CRAd-S-pk7 is evident (*) was magnified and shown in the right panel. Cells that are positive for both GFP and hexon (arrowheads) represent infected HB1.F3-CD that are releasing adenovirus; whereas hexon-positive, GFP-negative, 46-diamidino-2-phenyl indole (DAPI) positive (arrow) represent glioma cells infected with adenovirus. To show that loaded NSCs can travel longer distances and still successfully deliver adenovirus, we injected CRAd-S-pk7-loaded HB1.F3-CD.Fluc in the contralateral hemisphere (b). Migration of NSCs was visualized via photonflux imaging at 72 h (c). (d) To show that migrating NSCs were still replicating and releasing adenovirus animals were killed at 72 h post NSC injection; we stained tumor-containing sections with antibodies for adenovirus hexon (i) and human CD44 (ii) to evidentiate human glioma cells. Bars: 100 μm. Tu, tumor.
Canine GBM is highly invasive and mimics human GBM characteristics such as necrosis with pseudopalisading, neovascularization, and endothelial proliferation. The most important aspect of the canine model is its comparable brain size to humans. This characteristic is essential for a good preclinical model to precisely assess such pharmacokinetic properties as toxicity, dosage, side effects, as well as more accurately measure delivery strategies.

Furthermore, the therapeutic efficacy of most anti-glioma gene therapeutic approaches are commonly evaluated in immunocompromised animal models using xenogenic cell lines post transplantation with only a short interval of time between engraftment and treatment. The circumstances in human GBM completely differ as tumor initiation is usually sporadic and clinical symptoms can be observed months to years after initial establishment of tumor, resulting in increased heterogeneity. Moreover, when a carrier cell system is injected into animal models, such as the one used in our study, it becomes vulnerable to the immune response generated toward any foreign antigen. We observed the effects of such an immune response when we noticed a rapid decline of implanted stem cell numbers in the immunocompetent cotton rat and hamster models over time (Figures 7b–d). As a result, the NSC viability is even more affected in immunocompetent animal models as compared with immunocompromised nude mice (Figures 3 and 4). In the clinical setting, we do expect to encounter some degree of immune response generated toward any foreign antigen. We observed the effects of such an immune response when we noticed a rapid decline of implanted stem cell numbers in the immunocompetent cotton rat and hamster models over time (Figures 7b–d). As a result, the NSC viability is even more affected in immunocompetent animal models as compared with immunocompromised nude mice (Figures 3 and 4). In the clinical setting, we do expect to encounter some degree of immune response generated toward the stem cell-based carrier as the immortalized HB1.F3-CD cell line will be mismatched to human leukocyte antigens and thus will be allogeneic to glioma patients. Despite low expression of major histocompatibility complex class II and co-stimulatory molecules, in vitro allore cognition of NSCs by peripheral blood lymphocytes has been reported. These observations do argue in favor of the use of readily available autologous NSC sources. However, there are some serious limitations to currently available technologies for isolating and expanding autologous NSCs in culture to produce a sufficient number of viable cells for a successful transplantation. If grafting of the carrier cell becomes an issue in the clinical setting, we may have to consider utilization of immunosuppressive drugs to prolong the half-life of the therapeutic NSCs.

Although, the HB1.F3-CD cell line per se has been approved by the FDA for clinical trials in recurrent glioma (NCT01172964), nevertheless the human immune system may impact the viability of adenovirus-loaded cells as patients will have antibodies toward adenoviral antigens. Taking this into consideration, our study has led us to expect such an immune response to be less robust as compared with direct adenovirus injection. Most currently available cancer gene therapies have failed to sustain anti-tumor effects in the tumor microenvironment long enough to achieve clinically relevant therapeutic efficacy. This is partly due to the mounting of a host immune response against the administered therapeutic agents. A wealth of preclinical data suggest that in vivo transplanted NSCs can function as immunosuppressants. Results from several studies in both rodent and non-human primate models of experimental autoimmune encephalomyelitis indicate that NSCs transplanted by either intrathecal or intravenous injection promote bystander immunomodulation within the central nervous system via the release of various soluble molecules. We reported that CRAd-loaded NSCs transiently secret immunosuppressive cytokines interleukin-10 and significantly reduced CRAd-mediated central nervous system injury. This immunosuppressive quality of NSCs is a very attractive attribute for a cell carrier given that it will allow therapeutic payloads such as oncolytic viruses to be shielded from host immunosurveillance.
Therefore, we propose that our delivery system would prove safer than the direct virus injection into the tumor.

In summary, we have demonstrated that a NSC-based cell carrier can significantly improve the safety and biodistribution profile of the anti-glioma oncolytic virotherapy in an animal model. Such a carrier system has shown the ability to support the delivery of a similar dose of therapeutic virus at the implanted site, as compared with a naked virus, and also reduce the leaky distribution of the virus throughout the animal brain. Moreover, we compare nude mouse, cotton rat and hamster animal models to evaluate the pharmacological and safety profiles of the cell-based oncolytic virotherapy. This information will be useful to other investigators who are interested in utilizing the cell carrier approach to achieve targeted delivery of oncolytic virus to metastatic tumor burdens in cancer patients and translating cell carrier-based oncolytic virotherapy to the clinic.

CONFLICT OF INTEREST
Karen Abboody is CSO and Director of TheraBiologics, Inc. The authors declare no conflict of interest.

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REFERENCES

1 Deoras S, Lynch CF, Sibenaller ZA, Ryken TC. Trends in brain cancer incidence and survival in the United States: Surveillance, Epidemiology, and End Results Program, 1973 to 2001. Neurology 2006; 18: E1.

2 Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 2009; 10: 459–466.

3 Wen PY, Kesari S. Malignant gliomas in adults. N Engl J Med 2008; 359: 492–507.

4 Sheehan JP, Shaffrey ME, Gupta B, Larner J, Rich JN, Park DM. Improving the radiosensitivity of radioresistant and hypoxic glioblastoma. Future Oncol 2010; 6: 1591–1601.

5 Sampson JH, Heimbager BB, Archer GE, Aldape KD, Friedman AH, Friedman MS et al. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. J Clin Oncol 2010; 28: 4722–4729.

6 Bao S, Wu Q, Sathornsumetee S, Hao Y, Li Z, Hjelmeland AB et al. Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. Cancer Res 2008; 68: 7843–7848.

7 Dey M, Ulasov IV, Tyler MA, Sonabend AM, Lesniak MS. Cancer stem cells: the final frontier for glioma virotherapy. Stem Cell Rev 2011; 7: 119–129.

8 Kelly E, Russell SJ. History of oncolytic viruses: genesis to genetic engineering. Mol Ther 2007; 15: 651–659.

9 Ulasov IV, Zhu ZB, Tyler MA, Han Y, Rivera AA, Khramtsov A et al. Survivin-driven and fiber-modified oncolytic adenovirus exhibits potent antitumor activity in established intracranial glioma. Hum Gene Ther 2007; 18: 589–602.

10 Zhu ZB, Makhiya SK, Lu B, Wang M, Rivera AA, Kim-Park S et al. Incorporating the survivin promoter in an infectivity enhanced CRAd-analysis of oncolysis and anti-tumor effects in vitro and in vivo. Int J Oncol 2005; 27: 237–246.

11 Ulasov IV, Rivera AA, Sonabend AM, Rivera LB, Wang M, Zhu ZB et al. Comparative evaluation of survivin, midkine and CXC4 promoters for transcriptional targeting of glioma gene therapy. Cancer Biol Ther 2007; 6: 679–685.

12 Jiang H, Gomez-Manzano C, Aoki H, Alonso MM, Kondo S, McCormick F et al. Examination of the therapeutic potential of Delta-24-RGD in brain tumor stem cells: role of autophagic cell death. J Natl Cancer Inst 2007; 99: 1410–1414.

13 Selznick LA, Shamji MF, Fecci P, Gromeier M, Friedman AH, Sampson J. Molecular strategies for the treatment of malignant glioma—genes, viruses, and vaccines. Neurosurg Rev 2008; 31: 141–155.

14 Chiocca EA, Aguilar LX, Bell SD, Kaur B, Hardcastle J, Cavaliere R et al. Phase IB Study of gene-mediated cytotoxic immunotherapy adjuvant to up-front surgery and intensive timing radiation for malignant glioma. J Clin Oncol 2011; 29: 3611–3619.

15 Tyler MA, Ulasov IV, Sonabend AM, Nandi S, Han Y, Marler S et al. Neural stem cells target intracranial glioma to deliver an oncolytic adenovirus. In: Gene Ther 2009; 16: 262–278.

16 Sonabend AM, Ulasov IV, Tyler MA, Rivera AA, Mathis JM, Lesniak MS. Mesenchymal stem cells effectively deliver an oncolytic adenovirus to intracranial glioma. Stem Cells 2008; 26: 831–841.

17 Ahmed AU, Thaci B, Alexiades NG, Han Y, Qian S, Liu F et al. Neural stem cell-based carriers enhance therapeutic efficacy of an oncolytic adenovirus in an orthotopic mouse model of human glioblastoma. Mol Ther 2011; 19: 1714–1726.

18 Ahmed AU, Tyler MA, Thaci B, Alexiades NG, Han Y, Ulasov IV et al. A comparative study of neural and mesenchymal stem cell-based carriers for oncolytic adenovirus in a model of malignant glioma. Mol Pharm 2011; 8: 1559–1572.

19 Dembinski JL, Spaeth EL, Fuego J, Gomez-Manzano C, Stedny M, Andreff M et al. Reduction of nontarget infection and systemic toxicity by targeted delivery of conditionally replicating viruses transported in mesenchymal stem cells. Cancer Gene Ther 2010; 17: 289–297.

20 Kim SK, Kim SJ, Park IH, Bang JH, Abboody KS, Wang KC et al. Human neural stem cells target experimental intracranial medulloblastoma and deliver a therapeutic gene leading to tumor regression. Clin Cancer Res 2006; 12: 5550–5556.

21 Sonabend AM, Ulasov IV, Han Y, Rolle CE, Nandi S, Cao D et al. Biodistribution of an oncolytic adenovirus after intracranial injection in permissive animals: a comparative study of Syrian hamsters and cotton rats. Cancer Gene Ther 2009; 16: 362–372.

22 Abboody KS, Najbauer J, Danks MK. Stem and progenitor cell-mediated tumor selective gene therapy. Gene Ther 2008; 15: 739–752.

23 Thu MS, Najbauer J, Kendall SE, Harutyunyan I, Sangalang N, Gutova M et al. Iron labeling and pre-clinical MRI visualization of therapeutic human neural stem cells in a murine glioma model. PLoS One 2009; 4: e7218.

24 Ahmed AU, Rolle CE, Tyler MA, Han Y, Sengupta S, Wainwright DA et al. Bone marrow mesenchymal stem cells loaded with an oncolytic adenovirus suppress the anti-adenoviral immune response in the cotton rat model. Mol Ther 2010; 18: 1846–1856.

25 Power AT, Bell JC. Taming the Trojan horse: optimizing dynamic carrier cell/ oncolytic virus systems for cancer biotherapy. Gene Ther 2008; 15: 772–779.

26 Toth K, Spencer JF, Tollefsen AE, Kuppuswamy M, Doronin K, Lichtenstein DL et al. Cotransplantation of neural and mesenchymal stem cells effectively delivers an oncolytic adenovirus to intracranial gliomas. Hum Gene Ther 2005; 16: 139–146.

27 Toth K, Spencer JF, Wald WS. Immunocompetent, semi-permissive cotton rat tumor model for the evaluation of oncolytic adenoviruses. Methods Mol Med 2007; 130: 157–168.

28 Thomas MA, Spencer JF, Wald WS. Use of the Syrian hamster as an animal model for oncolytic adenovirus vectors. Methods Mol Med 2007; 130: 169–183.

29 Studebaker AW, Hutzen B, Pierson CR, Russell SJ, Galanis E, Raffel C. Oncolytic measles virus prolongs survival in a murine model of cerebral spinal fluid-disseminated medulloblastoma. Neuro Oncol 2012; 14: 459–470.

30 Lowenstein PR. Immunology of viral-vector-mediated gene transfer into the brain: an evolutionary and developmental perspective. Trends Immunol 2002; 23: 23–30.

31 Bessis N, GarciaCazor FJ, Boissier MC. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. Gene Ther 2004; 11(Suppl 1): S10–S17.

32 Pulkkanen KJ, Yla-Herttuala S. Gene therapy for malignant glioma: current clinical status. Mol Ther 2005; 12: 585–598.

33 Niewiesk S, Prince G. Differentiating animal models: the use of hispid cotton rat (Sigmodon hispidus) in infectious diseases. Lab Anim 2002; 36: 357–372.

34 Niewiesk S. Cotton rats (Sigmodon hispidus): an animal model to study the pathogenesis of measles virus infection. Immunol Lett 1999; 65: 47–50.

35 Cardolfi M, Curtin JF, Nichols WS, Muhammad AG, King GD, Pluhar GE et al. Intracranial glioblastoma models in preclinical neuro-oncology: neuropathological characterization and tumor progression. J Neurooncol 2007; 83: 133–148.

36 Stoica G, Lungu G, Martini-Stoica H, Waghela S, Levine J, Smith III R. Identification of cancer stem cells in dog glioblastoma. Vet Pathol 2009; 46: 391–406.

37 Ubalii F, Nava S, Nessi V, Frigerio S, Parati E, Bernasconi P et al. Allorecognition of human neural stem cells by peripheral blood lymphocytes despite low expression of MHC molecules: role of TGF-beta in modulating proliferation. Int Immunol 2007; 19: 1063–1074.

38 Ahmed AU, Alexiades NG, Lesniak MS. The use of neural stem cells in cancer gene therapy; predicting the path to the clinic. Curr Opin Mol Ther 2010; 12: 546–552.

39 Cattaneo R, Miest T, Shashkova EV, Barry MA. Reprogrammed viruses as cancer therapeutics: targeted, armed and shielded. Nat Rev Microbiol 2008; 6: 529–540.

40 Einstein O, Ben-Hur T. The changing face of neural stem cell therapy in neurologic diseases. Arch Neurol 2008; 65: 452–456.

41 Pluchino S, Quattrini A, Brambilla E, Gritti A, Salani G, Dina G et al. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. Nature 2003; 422: 688–694.

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