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Suppression of Primary and Disseminated Murine Tumor Growth with eIF5A1 Gene Therapy

Research Article

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Key words: eukaryotic translation initiation factor 5A (eIF5A), gene therapy, Adenovirus, DOTAP, melanoma, lung cancer, apoptosis

Abbreviations: Adenoviral vectors, (Adenovirus 5 serotype, E1 and E3-deleted) expressing eIF5A1, (Ad-eIF5A1); Adenoviral vectors, (Adenovirus 5 serotype, E1 and E3-deleted) expressing eIF5A2, (Ad-eIF5A2); adenovirus vector expressing the bacterial *lacZ* gene, (Ad-LacZ); deoxyhypusine hydroxylase, (DOHH); deoxyhypusine synthase, (DHS); Eukaryotic translation initiation factor 5A, (eIF5A); hemagluttin, (HA); Hematoxylin and eosin, (H&E); multiplicities of infection, (MOI)

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Summary

Eukaryotic translation initiation factor (eIF5A) is the only known protein that is post-translationally modified to contain hypusine. The purpose of this study was to establish whether eIF5A1 gene delivery might be an effective therapy against primary and disseminated tumors. The effects of adenoviral-mediated eIF5A1 gene transfer on tumor growth and animal survival were examined using a syngeneic murine melanoma (B16-F0) model and a human lung xenograft (A549) model. Significant suppression was observed in both primary melanoma ($p < 0.001$; B16-F0) and lung tumor ($p < 0.001$; A549) growth following intra-tumoral injections of Ad-eIF5A1. Increased incidence of apoptosis was evident in melanoma tumors following Ad-eIF5A1 treatment. Gene transfer of the second member of the eIF5A family, eIF5A2, also gave rise to significant delays in growth of primary melanoma tumors. Animal survival experiments revealed prolonged survival [median survival time: 25 days (treated), 7 days (control) for B16-F0; and 54 days (treated), 24 days (control) for A549]. Systemic administration of DOTAP:pCpG-eIF5A1 complexes into C57BL/6 mice suppressed tumor growth ($p < 0.05$) in a B16-F10 model of experimental disseminated metastases. Our findings suggest that eIF5A1 may be an important target in the development of treatments for primary and disseminated cancers.

I. Introduction

Despite advances in cancer treatment, statistics show that cancer deaths are still on the rise and that the 5-year survival rates for both lung cancer (Sörenson et al, 2001) and melanoma (Balch et al, 2001) remain low. Thus there is a growing need for new treatments.

Eukaryotic translation initiation factor 5A (eIF5A) is the only protein found in nature that contains the amino acid, hypusine. The formation of hypusine occurs post-translationally on a conserved lysine residue in a two-step process. The first step is the transfer of a butylamine group from spermidine and is catalyzed by deoxyhypusine synthase (DHS) (Wolff et al, 1990). A second hydroxylation reaction, catalyzed by deoxyhypusine hydroxylase (DOHH), results in the formation of the mature hypusine-containing eIF5A. Although the precise function of eIF5A remains elusive, numerous functions have been proposed, including roles in protein translation (Kang and Hershey, 1994; Zanelli et al, 2006), mRNA transport (Kang and Hershey, 1994; Hanauske-Abel et al, 1995; Liu et al, 1997; Xu et al, 2004), and mRNA stability (Zuk and Jacobson, 1998; Schrader et al, 2006). EIF5A has also been implicated in the regulation of cell proliferation (Schnier et al, 1991; Kang and Hershey, 1994) and apoptosis (Li et al, 2004; Taylor et al, 2007). There are two isoforms of eIF5A in the human genome, eIF5A1, which is abundant in all tissues and cancer cell lines, and eIF5A2, for which expression appears to be restricted to testis, parts of the brain, and certain cancers, such as colon cancer (Jenkins et al, 2001).

Over-expression of eIF5A1 has been found to induce apoptosis in lung (Li et al, 2004) and colon cancer cell

lines (Taylor et al, 2007), but no studies have yet determined whether eIF5A1 gene therapy provides therapeutic benefit in *in vivo* cancer models. In this study, we address this question by looking at survival of melanoma and lung tumor-bearing mice treated with an adenovirus expressing eIF5A1. The ability of eIF5A1 to treat disseminated tumors was also examined in an experimental metastasis lung cancer model.

II. Material and Methods

A. Cell lines

B16-F0 (CRL-6322) and B16-F10 (CRL-6475) murine melanoma cell lines were purchased from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Media containing 10 % fetal bovine serum. A549 lung carcinoma cells were obtained from Anita Antes (University of Medicine and Dentistry, New Jersey) and were cultured in RPMI 1640 supplemented with 1 mM sodium pyruvate and 10 % fetal bovine serum. Cell cultures were maintained at 37 °C in a humidified environment containing 5 % CO_2.

B. Construction of Adenoviral Vectors

Adenoviral vectors (Adenovirus 5 serotype, E1 and E3-deleted) expressing eIF5A1 (Ad-eIF5A1) or eIF5A2 (Ad-eIF5A2) were generated using the AdMax™ Hi-IQ system (Microbix Biosystems Inc., Toronto, Canada). The creation and propagation of Ad-eIF5A1 is described elsewhere (Taylor et al, 2007). Ad-eIF5A2 was constructed by subcloning a PCR-amplified cDNA of eIF5A2 into the *SmaI* site of the adenovirus shuttle vector pDC516(io) using primers having the following sequences: forward 5'-ATCAAGCTTGCCCACCATGGCAGACG-3 ; and reverse 5'-AACGAATTCCATGCCTGATGTTTCCG-3. An adenovirus vector expressing the bacterial *lacZ* gene (Ad-LacZ) was purchased from Qbiogene (California, USA) and used as a reporter gene and negative control. Pure, high-titer adenovirus stocks were prepared using the ViraBind™ Adenovirus Purification Mega Kit (Cell Biolabs, San Diego, CA, USA). The viral stocks were titered by plaque assay on 293-IQ cells (Microbix Biosystems Inc., Toronto, Ontario). Viral stocks were diluted with PBS/10% glycerol for injection into mice.

C. Construction of pCpG-eIF5A1 vector

An expression vector lacking immunostimulatory CpG dinucleotides and expressing LacZ (pCpG-LacZ) was purchased from Invivogen (San Diego, California, USA). In order to create a CpG-free expression vector expressing a hemagluttin (HA) epitope-tagged eIF5A1, pCpG-LacZ was digested with *NcoI* and *NheI*, and the vector backbone was isolated. The pCpG vector backbone was ligated to an eIF5A1 cDNA fragment generated by PCR using a pHM6 vector (Roche Molecular Biochemicals) containing eIF5A1 cDNA as a template. The following primers were used to generate the PCR fragment for subcloning: forward 5'-GCTCCATGGCAGATGATTTGGACTTCG-3'; and reverse 5'- CGCGCTAGCCAGTTATTTTGCCATCGCC-3'. The plasmids were propagated in *E. coli* GT115 and purified using the QIAGEN EndoFree Plasmid Giga kit.

D. Animal Experimentation

Animal experiments were conducted in accordance with the guidelines set out by the University of Waterloo Animal Care Committee (Waterloo, Ontario, Canada). All animal experiments using murine melanoma cell lines B16-F0 and B16-F10 were performed using five- to seven-week-old female C57BL/6NCRL mice. A549 xenograft experiments were performed using five- to seven-week-old female BALB/c nude mice. All mice were obtained from Charles River Canada (Saint Constant, Quebec).

E. B16-F0 subcutaneous tumor model and therapy

B16-F0 tumors were established by subcutaneous injection of 500,000 cells into the right flank of C57BL/6 mice. When tumor size reached an average diameter of 5 mm the mice were randomized into three groups of nine to ten mice each and received the following treatments: (a) PBS/10% glycerol, (b) Ad-LacZ, or (c) Ad-eIF5A1. Adenovirus (1 x 10^9 pfu diluted in 100 microliters of PBS/10% glycerol) was injected intra-tumorally at multiple sites every other day (for a total of 3 injections/week) until the animals were sacrificed. Tumors were measured every day with calipers, and the mice were sacrificed when tumor size reached 10 % of body weight (tumor diameter exceeding 17 mm). The average weight of the mice at the initiation of treatment was 20 grams. Tumor volume was calculated using the equation: tumor volume (mm^3) = L * W^2 * 0.52. For TUNEL, H&E, and immunohistochemical analysis, mice bearing B16-F0 subcutaneous tumors were treated as above except that three mice per group were sacrificed 0, 3, and 6 days after the initiation of treatment. The tumors were cut in half, and one segment was frozen at - 80°C while the other was immediately fixed in 4 % formaldehyde/PBS, embedded in paraffin, and sectioned (10 mm).

F. B16-F10 lung tumor model and therapy

B16-F10 lung tumors were established by tail vein injection of 50,000 cells into C57BL/6 mice. The mice were randomized into three groups of five mice each and received the following treatments: (a) PBS, (b) pCpG-LacZ:DOTAP, and (c) pCpG-eIF5A1:DOTAP. Plasmid DNA was delivered to the lung by tail vein injection of DOTAP:DNA complexes. Immediately prior to injection, fifty micrograms of plasmid DNA was diluted in 100 microliters of PBS and mixed with 80 microliters (80 mg) of DOTAP (Roche Applied Science) diluted to 100 microliters in PBS. The complexes were incubated for 15 minutes at room temperature, and 200 microliters of complexed DNA was injected via tail vein into each mouse. Complexed DNA was injected on days 7, 14, and 21 after tumor seeding. Animals were sacrificed as soon as they showed signs of distress such as lethargy, ruffled fur, or difficulty breathing. Lungs were removed, weighed, and photographed.

G. A549 subcutaneous tumor model and therapy

Twenty-five BALB/c nude mice were injected subcutaneously with 1 million A549 cells on the right flank and randomized into the following three treatment groups (a) PBS/10% glycerol, (b) Ad-LacZ, or (c) Ad-eIF5A1. Treatment was initiated when the tumors reached approximately 4 mm in diameter. Mice received intra-tumoral injections of 1 x 10^9 pfu of adenovirus three times weekly (days 1, 3, 5, 8, 10, 12, 15, 17, 19, ect...). Tumors were measured three times per week, and mice were sacrificed when tumor size reached 10 % of body weight (tumor diameter exceeding 17 mm).

H. Immunohistochemistry and TUNEL

In order to detect expression of LacZ, tumor sections were deparaffinized in xylene and rehydrated. A beta-galactosidase antibody (ab616; Abcam, Cambridge, MA) was used at a dilution of 1:2000 in combination with a FITC-conjugated goat anti-rabbit antibody (Sigma). Apoptotic cells in tumor sections were detected using the DeadEnd™ Fluorometric TUNEL system (Promega) according to the manufacturer's instructions. The

labeled apoptotic cells were observed by fluorescence microscopy and photographed with an attached digital camera.

I. Hematoxylin and Eosin Staining

Hematoxylin and eosin (H&E) staining was used to observe tumor ultrastructure. B16-F0 tumor sections were prepared as described above. Sections were deparaffinized, rehydrated, and stained in Mayer's hematoxylin solution (15 minutes; Sigma). The sections were washed with water and stained with aqueous Eosin Y (30-60 minutes; Sigma). Following staining, the sections were dehydrated, mounted with resinous mounting media and photographed using light microscopy.

K. Western blotting

A549 cells were infected with adenovirus constructs at increasing multiplicities of infection (MOI). Forty-eight hours after infection, the cells were lysed [2 % SDS, 62.5 mM Tris-HCl (pH 7.4), 10 % glycerol]. The protein was fractionated by SDS-PAGE and western blotted using antibodies against eIF5A (BD Transduction Laboratories; 1:20,000), eIF5A2 (Novus Biologicals; 1:2000), p53 (Cell Signalling; 1:1000) or β-actin (Oncogene; 1:20,000).

L. Annexin/PI

A549 cells were infected with Ad-LacZ, Ad-eIF5A1, or Ad-eIF5A2 at an MOI of 80. Four hours later fresh media was added and the cells were incubated for seventy-two hours with media changes every 24 hours. Cells were labeled using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen) according to the manufacturer's instructions. The cells were sorted by flow cytometry (Becton Dickinson FACSVantage SE with a 488 nm argon laser) and the data were analyzed using WinMDI 2.8. The percentage of cells in early apoptosis (Ann+/PI-) was combined with the percentage of the cell population in late apoptosis (Ann+/PI+) to give the total percentage of cells in apoptosis.

M. Statistical analyses

Student's *t*-test was used for statistical analysis. Significance was deemed to be a confidence level above 95 % ($p < 0.05$).

III. Results

A. Intra-tumoral injection of Ad-eIF5A1 induces apoptosis and prolongs survival in a murine melanoma model

To determine whether eIF5A1 gene therapy could have therapeutic value in *in vivo* cancer models, we used adenovirus-mediated gene delivery in a murine melanoma model. B16-F0 cells were injected subcutaneously into the right flank of C57BL/6 mice. Tumors of 5 mm in diameter were given intra-tumoral injections of either Ad-LacZ or Ad-eIF5A1 every other day. Three mice per group were sacrificed on days 0, 3, and 6, and the tumors were paraffin-embedded for further analysis. Expression of β-galactosidase was observed after 3 days (supplemental data **Figure 1**) and 6 days (**Figure 1**, supplemental data **Figure 1**) in tumor sections from mice that had received intra-tumoral injections of Ad-LacZ, indicating efficient gene transfer.

In order to determine whether intra-tumoral injections of Ad-eIF5A1 resulted in the induction of apoptosis in tumors, paraffin-embedded tumor sections were examined for DNA fragmentation using TUNEL. A significant number of TUNEL-positive cells were observed 3 days (**Figure 1**) and 6 days (supplemental data **Figure 2**) after intra-tumoral injection with Ad-eIF5A1 but not after injection with Ad-LacZ or PBS. A significant number of apoptotic cells were also observed in Ad-eIF5A1-treated tumors sections from mice that were sacrificed on day 6 (supplementary data **Figure 2**). Sections derived from tumors treated with Ad-eIF5A1 for 6 days were also found to have many regions of decreased cell density compared to Ad-LacZ controls when examined by H&E staining (**Figure 1**, supplementary data **Figure 3**), indicating considerable cell loss due to apoptosis and/or necrosis.

The therapeutic effects of delivering the eIF5A1 gene to subcutaneous murine tumors were also evaluated in the syngeneic B16-F0 model. Injections of Ad-LacZ or Ad-eIF5A1 were given intra-tumorally three times per week until sacrifice. Treatment with Ad-eIF5A1 over a course of several weeks resulted in a substantial increase in survival compared to control mice that received injections of PBS or Ad-LacZ (**Figure 2A**). Mice that received injections of

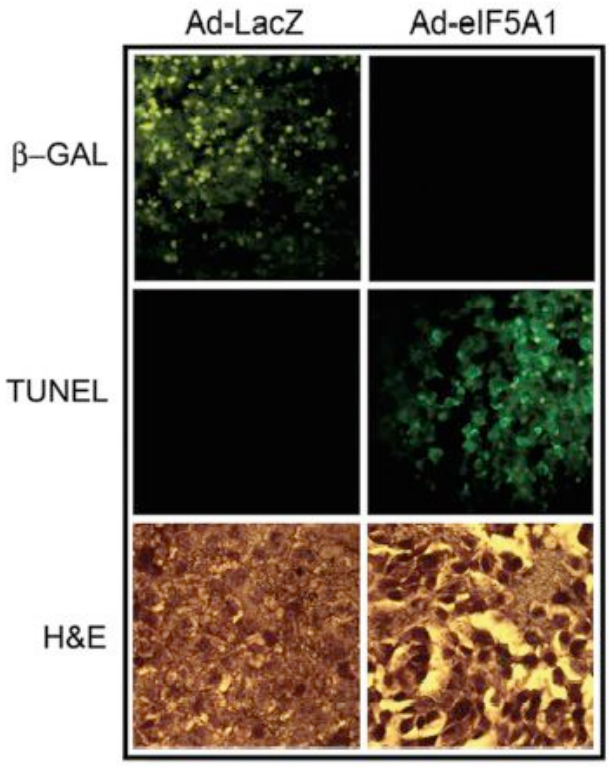
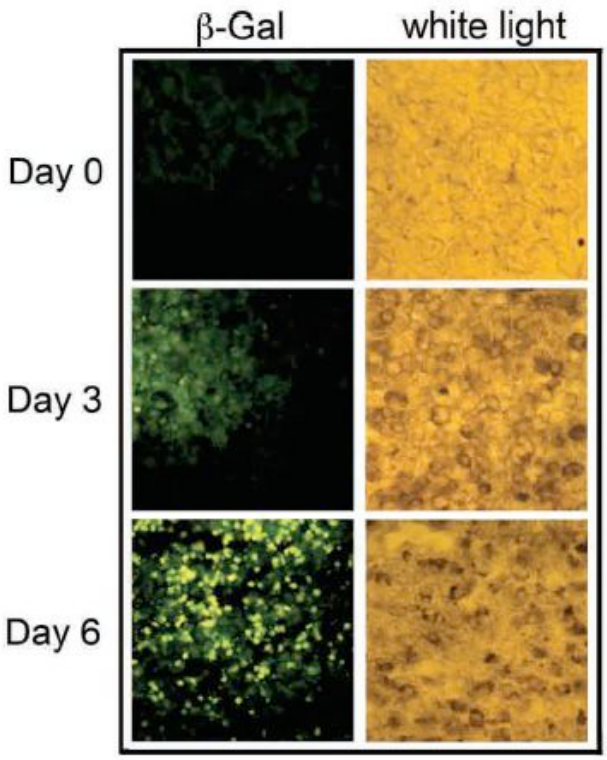


Figure 1. Subcutaneous B16-F0 tumors exhibit increased apoptosis following intra-tumoral injection of Ad-eIF5A1. C57BL/6 mice were injected subcutaneously on the flank with B16-F0 cells. Intra-tumoral injections of PBS, Ad-LacZ, or Ad-eIF5A1 were given on days 0, 2, and 4. Tumors were harvested on days 0, 3, and 6. Paraffin-embedded tumor sections were examined for LacZ expression (day 6) using an antibody against β-galactosidase. Apoptotic cells in tumor sections were labelled by TUNEL (day 3) and tumor structure was observed by H&E staining (day 6). Data shown are representative of results obtained from three different mice. All photographs were taken at 400x magnification.



Supplemental Figure 1. Subcutaneous B16-F0 tumors express β-galactosidase following intra-tumoral injection of Ad-LacZ. C57BL/6 mice were injected subcutaneously on the flank with B16-F0 cells. Intra-tumoral injections of Ad-LacZ were given on days 0, 2, and 4. Tumors were harvested on days 0, 3, and 6. Paraffin-embedded tumor sections were examined for β-galactosidase expression by immunohistochemistry using an antibody against β-galactosidase. Images of the same field of view under white light are also shown. Data shown are representative of results obtained from three different mice. All photographs were taken at 400x magnification.

PBS or Ad-LacZ had a median survival of 7 days following the initiation of treatment, whereas mice that received injections of Ad-eIF5A1 had a median survival of 25 days (**Figure 2A**). There was also a significant delay in tumor growth in mice that received intra-tumoral injections of Ad-eIF5A1 (**Figure 2B**). Regression of tumors was observed in 5 out of 10 (50%) mice in the Ad-eIF5A1 treatment group, and in one mouse the tumor completely regressed and did not return for the duration of the study. Additionally, no decrease in body weight or activity level was observed in any of the treatment groups indicating that the treatments were well tolerated.

B. Intra-tumoral injection of Ad-eIF5A2 delays growth of tumors in a murine melanoma model

A second isoform of eIF5A, eIF5A2, has been localized to a chromosomal region that is frequently amplified in ovarian cancer and has been identified as a potential oncogene (Guan et al, 2001, 2004). Although eIF5A1 and eIF5A2 share 82% homology at the amino acid level, it is not known whether they have conserved functions. In order to determine whether eIF5A2 may also have anti-cancer properties, we examined the effect of Ad-eIF5A2 intra-tumoral injections on tumor growth in the B16-F0 subcutaneous melanoma model. Ad-eIF5A2-treated mice also exhibited a significant delay in tumor growth compared to control mice, although it was not as great as that seen in mice that received Ad-eIF5A1 (**Figure 3**). The median survival for mice that received injections of PBS was 8 days while the median survival for mice injected with Ad-eIF5A1 and Ad-eIF5A2 was 16 and 14 days, respectively (data not shown). Thus eIF5A2 and eIF5A1 gene delivery have similar anti-cancer properties.

C. Systemic administration of eIF5A1 plasmid DNA reduces tumor burden in a murine experimental metastasis model

The ability of eIF5A1 to control disseminated tumors was also examined using a metastatic lung cancer model. Lung tumors were established in C57BL/6 mice by tail vein injection of the highly metastatic melanoma cell line, B16-F10. Although viral delivery of genes can be very efficient, repeated dosing, particularly intravenously, can give rise to an immune response targeted against the viral vector, thereby limiting the effectiveness of subsequent doses (Bessis et al, 2004). Non-viral gene therapy can also elicit immune responses that inhibit expression from later doses (Tan et al, 1999). Consequently, in order to get the highest possible transgene expression in this lung tumor model, an expression plasmid (pCpG) that has been modified to remove all CpG dinucleotides was administered. CpG-reduced plasmids have been shown to decrease toxicity and increase the duration of transgene expression *in vivo* (Yew et al, 2000, 2002; Hodges et al, 2004). Gene delivery to the lung was accomplished by tail vein injection of plasmid DNA complexed with DOTAP, a combination that has been used previously to deliver genes to the lung (Li and Huang, 1997; Bragonzi et al, 1999, 2000). Plasmid DNA complexed with DOTAP was injected by tail vein once per week (days 7, 14, and 21 after injection of B16-F10 cells). A small, but statistically insignificant, decrease in lung weight was observed in mice treated with pCpG-LacZ compared to mice that only received injections of PBS (**Figure 4A**) and could be due to residual immune stimulation from the DOTAP:DNA complex. Injections with pCpG-eIF5A1 resulted in a 59 % decrease in average lung weight compared to mice that received pCpG-LacZ (**Figure 4A**) indicating a significant reduction in tumor burden. The lungs of mice treated with eIF5A1 plasmid DNA were noticeably smaller and had considerably less metastatic tumor growth than control groups (**Figure 4B**). Thus eIF5A1 treatment may also be feasible as a therapy for disseminated tumors.

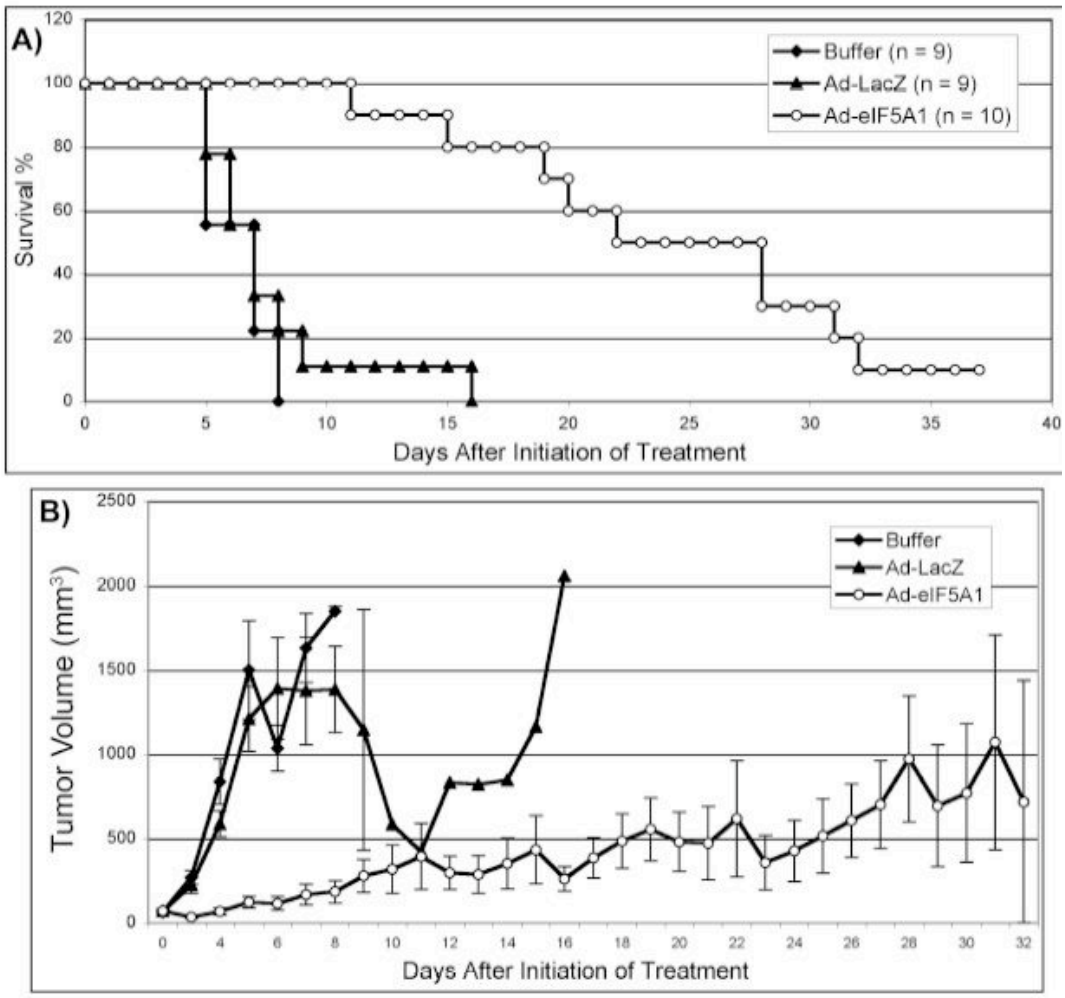
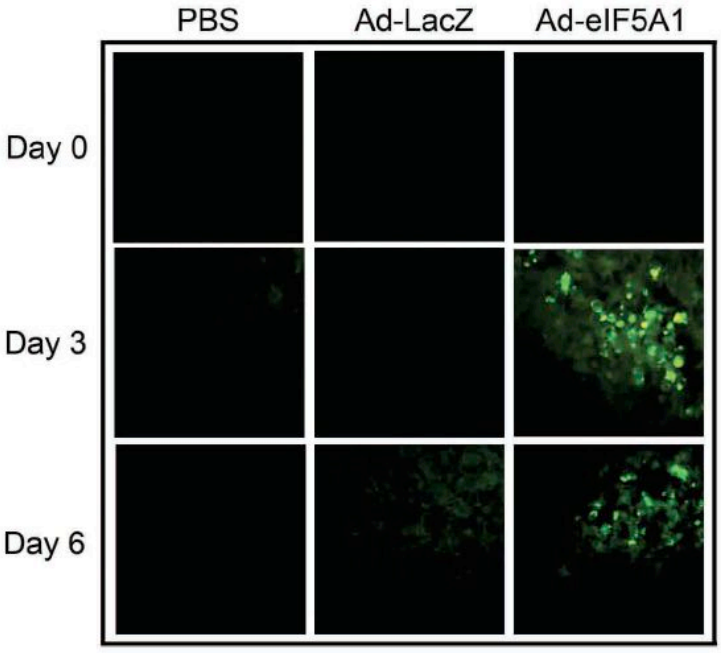


Figure 2. Mice bearing B16-F0 subcutaneous tumors exhibit prolonged survival following intra-tumoral injection of Ad-eIF5A1. C57BL/6 mice were injected subcutaneously on the flank with B16-F0 cells. Intra-tumoral injections of PBS (buffer), Ad-LacZ, or Ad-eIF5A1 were given three times per week until sacrifice. Survival of mice (**A**) and mean tumor volumes ± SE of surviving mice (**B**) are shown.



Supplemental Figure 2: Subcutaneous B16-F0 tumors exhibit increased apoptosis following intra-tumoral injection of Ad-eIF5A1. C57BL/6 mice were injected subcutaneously on the flank with B16-F0 cells. Intra-tumoral injections of PBS, Ad-LacZ or Ad-eIF5A1 were given on days 0, 2, and 4. Tumors were harvested on days 0, 3, and 6. Apoptotic cells in paraffin-embedded tumor sections were labelled by TUNEL. Data shown are representative of results obtained from three different mice. All photographs were taken at 400x magnification.

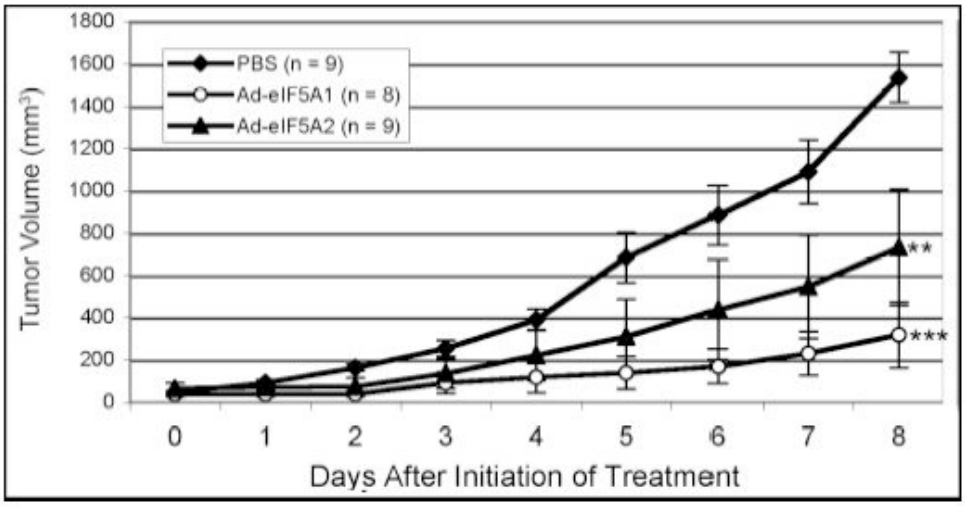
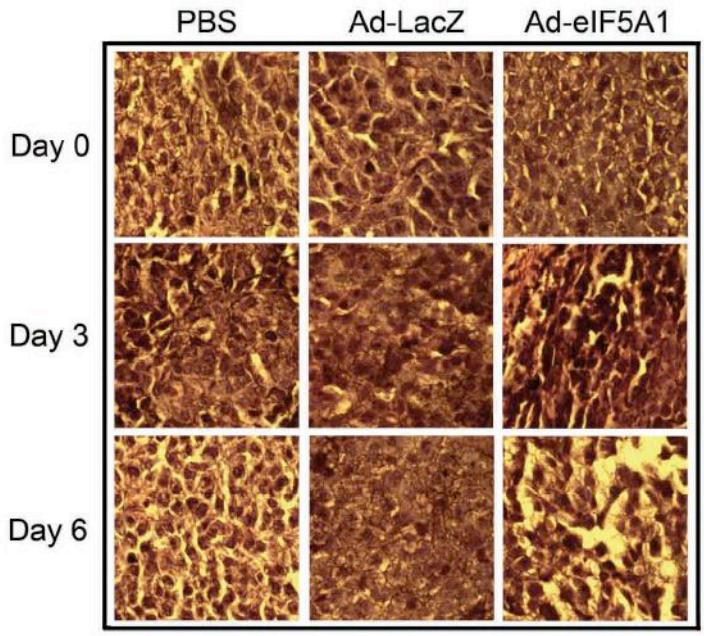


Figure 3: Mice bearing B16-F0 subcutaneous tumors exhibit delayed tumor growth following intra-tumoral injections of either Ad-eIF5A1 or Ad-eIF5A2. C57BL/6 mice were injected subcutaneously on the flank with B16-F0 cells. Intra-tumoral injections of PBS, Ad-eIF5A1 or Ad-eIF5A2 were given three times per week until sacrifice. The graph depicts mean tumor volume ± SE for each treatment group (** $p<0.01$, $n = 9$; *** $p <0.001$, $n = 8$).



Supplemental Figure 3: H&E staining of B16-F0 tumor sections following intra-tumoral injection of Ad-eIF5A1. C57BL/6 mice were injected subcutaneously on the flank with B16-F0 cells. Intra-tumoral injections of PBS, Ad-LacZ, or Ad-eIF5A1 were given on days 0, 2, and 4. Tumors were harvested on days 0, 3, and 6. Paraffin-embedded tumor sections were examined by H&E staining. Data shown are representative of results obtained from three different mice. All photographs were taken at 400x magnification

D. Intra-tumoral injection of Ad-eIF5A1 prolongs survival in a murine lung cancer model

In order to determine whether use of eIF5A1 to delay tumor growth is feasible in cancer models other than melanoma, the ability of Ad-eIF5A1 and Ad-eIF5A2 to infect and kill A549 cells was confirmed *in vitro* (**Figure 5**). A dose-dependent increase in p53 expression was observed in A549 cells infected with increasing amounts of Ad-eIF5A1 (**Figure 5A**). Over-expression of eIF5A1 has been correlated with increased p53 expression in lung cancer cells (Li et al, 2004) and that result is confirmed in this study. An increase in p53 expression was also observed in response to increasing expression of eIF5A2 (**Figure 5A**), although the upregulation of p53 was not as strong as that seen in response to Ad-eIF5A1 infection. These data suggest that eIF5A1 and eIF5A2 over-expression may activate similar apoptotic pathways. A significant increase in apoptosis was observed in A549 cells infected with either Ad-eIF5A1 or Ad-eIF5A2 (**Figure 5B, 5C**). It will be interesting to determine in future experiments the extent to which p53 up-regulation may contribute to apoptosis resulting from eIF5A1 and eIF5A2 over-expression in cell lines, such as A549, in which p53 is not mutated.

Subcutaneous A549 tumors were established in SCID mice, and treatment was initiated when the tumors reached 4 mm in diameter. Mice were then divided into

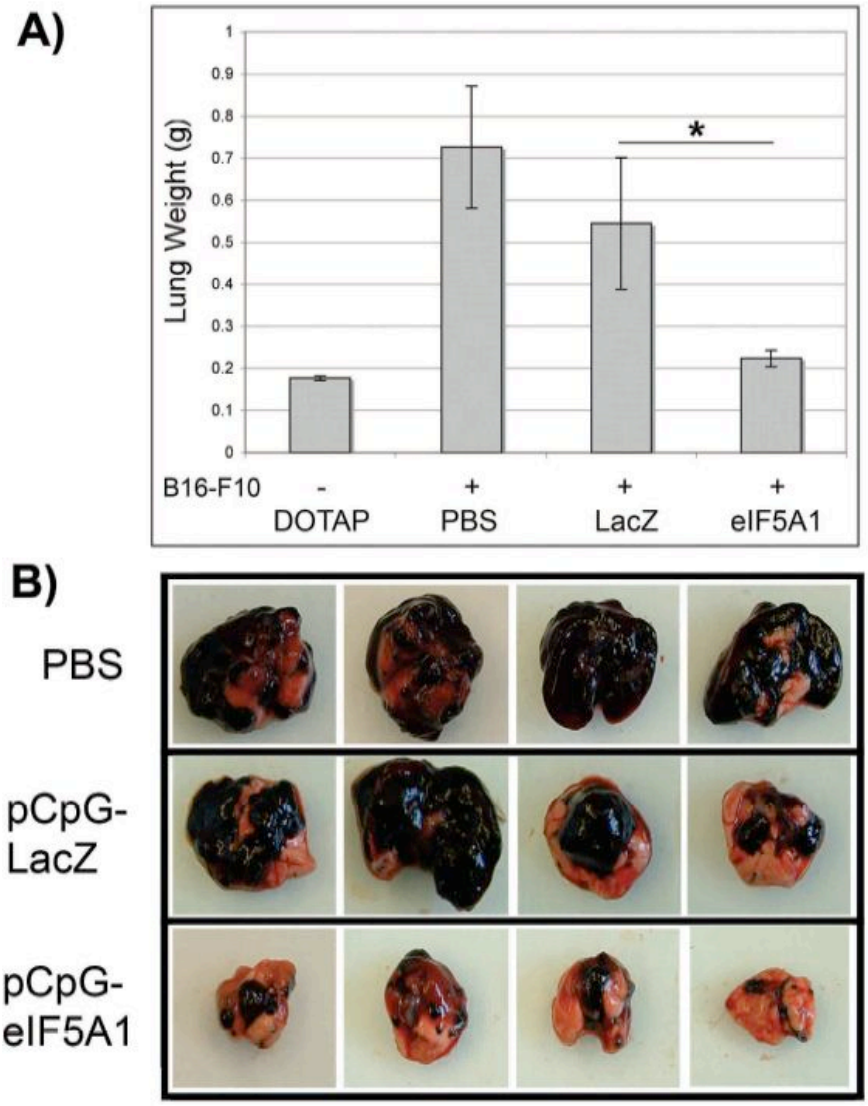


Figure 4: Mice bearing B16-F10 lung tumors exhibit reduced tumor burden following systemic treatment with pCpG-eIF5A1:DOTAP complexes. B16-F10 cells were injected into the tail vein of C57BL/6 mice. Fifty micrograms of plasmid DNA was complexed with DOTAP and injected via the tail vein on days 7, 14, and 21 following tumor seeding. (**A**) Mean lung weights ± SE for each treatment group (* $p<0.05$; $n = 5$) and (**B**) photographs of lungs for each treatment group.

three treatment groups and received injections of PBS, Ad-LacZ, or Ad-eIF5A1 three times weekly until sacrifice. As with the melanoma tumor models, Ad-eIF5A1 treatment provided a significant survival advantage in challenged mice (**Figure 6A**). The median survival of mice receiving injections of PBS or Ad-LacZ was 24 and 28 days, respectively. A549-bearing mice that were treated with Ad-eIF5A1 had a median survival of 54 days, and tumor growth was considerably delayed ($p<0.001$; **Figure 6B**). These results indicate that eIF5A1 has potent anti-tumor activity against both murine and human tumors.

IV. Discussion

Gene therapy has shown promise as a therapeutic strategy in the treatment of cancer. Numerous clinical trials using adenovirus to deliver therapeutic genes to tumors have been performed, and an adenovirus expressing p53 has been approved for use in malignant cancers in China (Peng, 2005). The major finding of the present study is that either local or systemic delivery of the eIF5A1 gene suppresses tumor growth *in vivo* and that this therapy does not result in any apparent toxic side effects. Although the precise function of eIF5A1 has yet to be clearly defined, it has been proposed that hypusine-modified eIF5A1 is necessary for cell growth based on the finding that depletion of hypusine-containing eIF5A1 by genetic or biochemical means induces cell cycle arrest (Park et al, 1993, 1994). A growing body of evidence indicates that eIF5A1 may also be an important component of the apoptotic process. EIF5A1 was recently identified as a gene target of p53 and demonstrated p53-dependent up-regulation in response to mitomycin C, a DNA damaging agent (Rahman-Roblick et al, 2007)

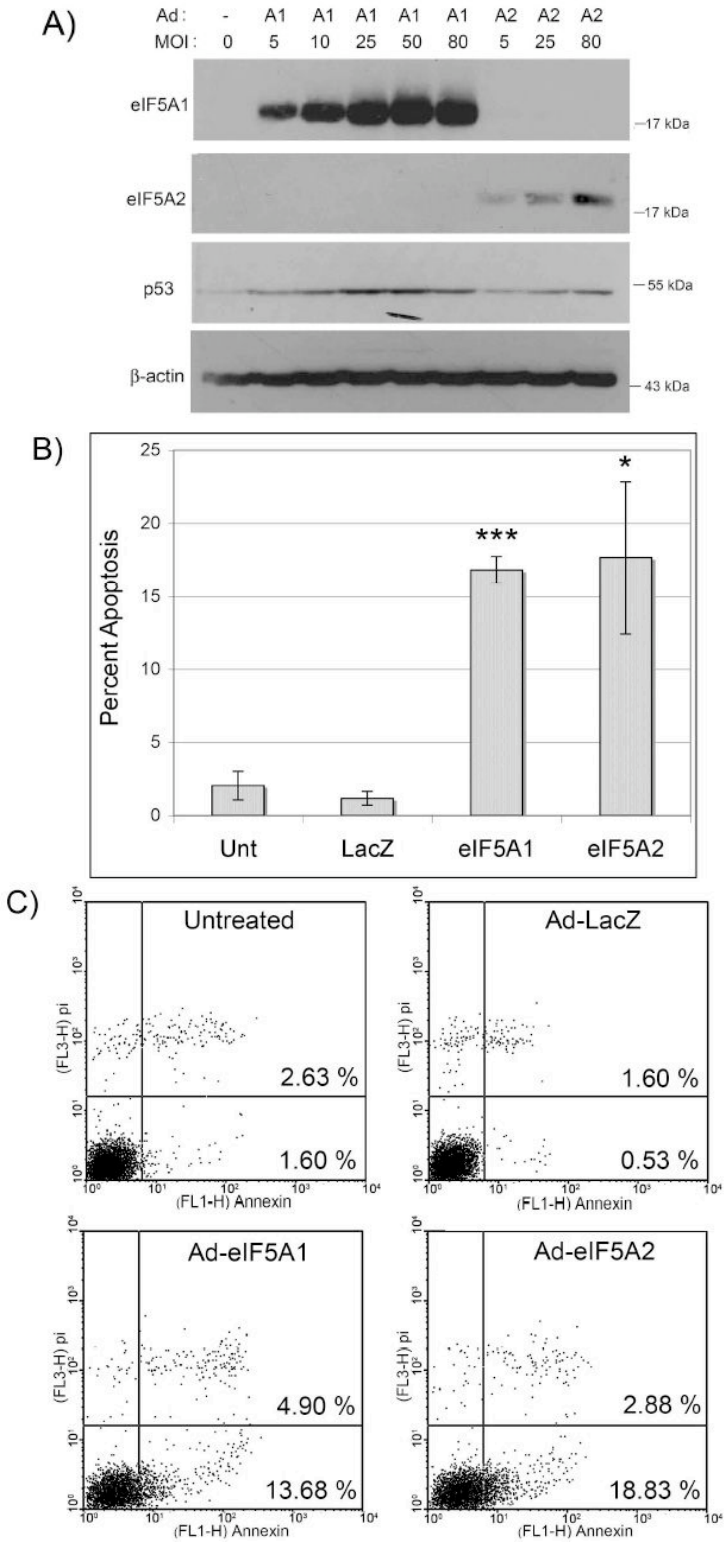


Figure 5: Ad-eIF5A1 and Ad-eIF5A2 infection up-regulate p53 and induce apoptosis in A549 cells. (**A**) A549 cells were infected with increasing multiplicity of infection (MOI) of adenovirus expressing either eIF5A1 or eIF5A2, and cell lysate was harvested forty-eight hours later. Western blot analysis of cell lysate using antibodies against eIF5A1, eIF5A2, p53 or β-actin is shown. Densitometry analysis of p53 and actin expression levels was performed using TotalLab TL100 v2006 software and the results expressed as a ratio of p53: actin. (**B**) A549 cells were infected with Ad-LacZ, Ad-eIF5A1, or Ad-eIF5A2 (MOI 80). Seventy-two hours later, the cells were labelled with Annexin/PI to identify apoptotic cells and analyzed by FACS. Data are means ± SE (* $p<0.05$; *** $p<0.0001$; n = 3). (**C**) Dot-plots of Annexin-FITC (x-axis) and PI (y-axis) labelling following adenovirus infection (without pifithrin) and Annexin/PI labelling as in B.

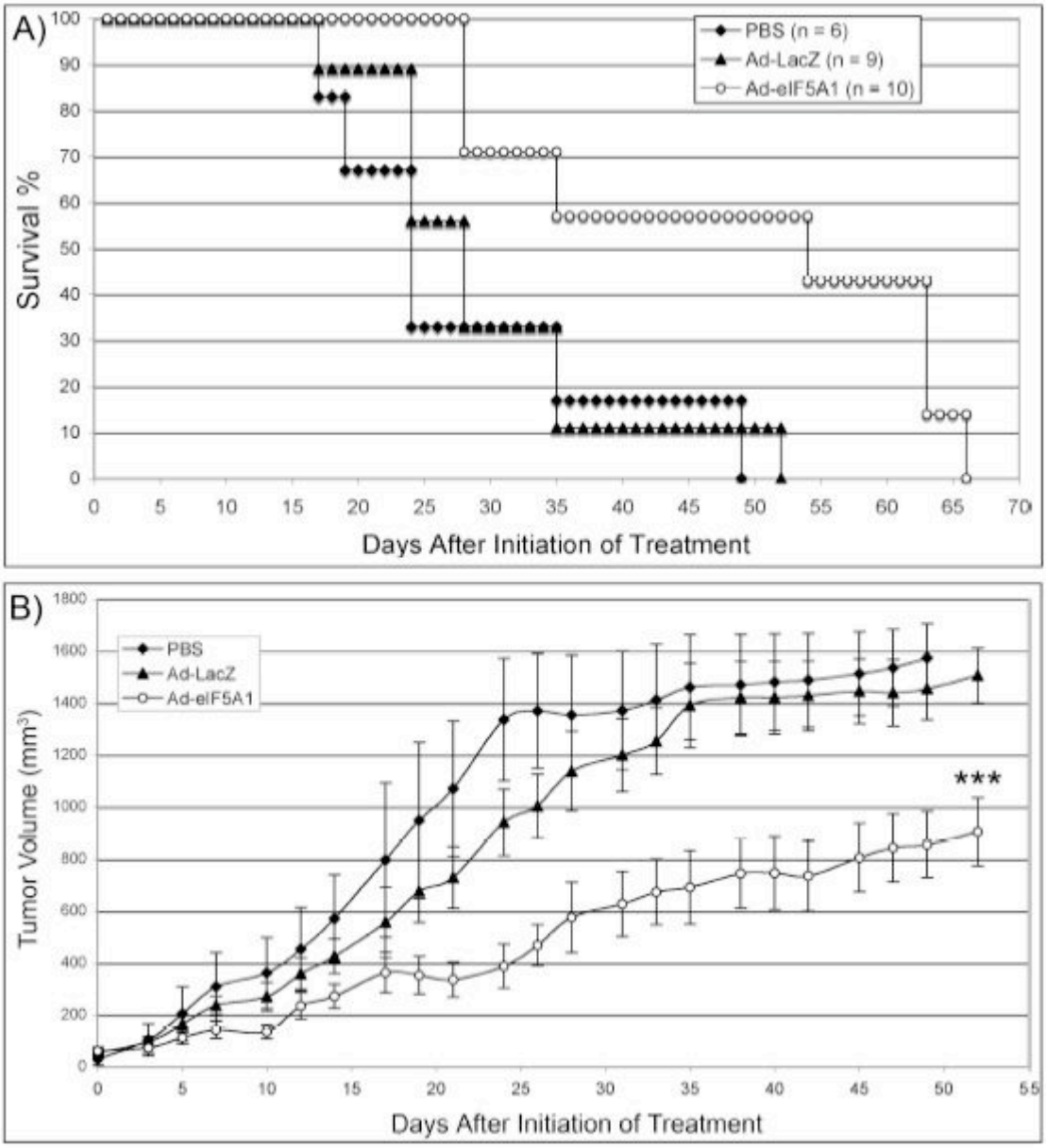


Figure 6. Mice bearing A549 subcutaneous tumors exhibit prolonged survival following intra-tumoral injection of Ad-eIF5A1. C17-SCID mice were injected subcutaneously on the flank with A549 cells. Intra-tumoral injections of PBS, Ad-LacZ, or Ad-eIF5A1 were given three times per week until sacrifice. A) Survival of mice and (B) mean tumor volumes $\pm$ SE are shown (*** $p < 0.001$; $n = 10$).

Studies with cell lines have indicated that up-regulation of eIF5A1 stimulates the expression of p53 and induces both p53-dependent and p53-independent apoptosis (Li et al, 2004; Taylor et al, 2007), while suppression of eIF5A1 using an siRNA protected lamina cribrosa cells from TNF-a-mediated apoptosis (Taylor et al, 2004). The present study confirmed these findings by demonstrating induction of apoptosis as well as a dose-dependent increase in p53 expression in A549 cells infected with Ad-eIF5A1. In addition, A549 cells exhibited a similar increase in apoptosis and p53 expression in response to eIF5A2 over-expression, indicating that eIF5A1 and eIF5A2 may activate similar pathways.

EIF5A1 and eIF5A2 share 82 % amino acid identity, including the minimum domain and lysine residue required for the hypusine modification. While eIF5A1 is abundant in all tissues, expression of eIF5A2 is weak except in testis and parts of the brain, although it has been found to be over-expressed in colon and ovarian cancer. A growing body of evidence has linked eIF5A2 over-expression in cancers to tumor growth and progression. EIF5A2 has been mapped to 3q26, a chromosomal region that is frequently amplified in human ovarian cancer (Guan et al, 2001; Clement et al, 2003) and colorectal carcinomas (Xie et al, 2008). Over-expression of eIF5A2 in a liver cell line was found to increase colony formation and tumor growth in nude mice (Guan et al, 2004), while over-expression of eIF5A2 in colorectal carcinomas was positively correlated to tumor stage and tumor cell proliferation (Xie et al, 2008). Over-expresson of eIF5A2

has also been linked to advanced stages of ovarian cancer (Guan et al, 2004), and to a higher risk of lymph node metastasis in human gastric cancer (Marchet et al, 2007). Higher eIF5A1 expression has been correlated to K-*ras* mutations and shorter survival in lung cancer patients (Chen et al, 2003) and was identified as a marker of aberrant proliferation in neoplasia of the vulva (Cracchiolo et al, 2004), suggesting that it may contribute to tumor progression as well. In the present study both eIF5A1 and eIF5A2 gene delivery significantly inhibited melanoma tumor growth *in vivo*. While the anti-cancer effect of eIF5A1 and eIF5A2 over-expression may appear contradictory to their suspected roles in oncogenesis, we believe this apparent contradiction can be explained by distinct functions of the unhypusinated and hypusinated forms of eIF5A. The oncogenic activity of eIF5A can be attributed to the hypusinated form of the protein (Cracchiolo et al, 2004), particularly since DHS has also been observed to be upregulated in cancers (Ramaswamy et al, 2003; Clement et al, 2006) and inhibition of DHS inhibited the growth of melanoma tumors in mice (Jasiulionis et al, 2007). Furthermore, the *in vitro* formation of hypusine is responsive to the addition of serum and is greatly increased in Ras transformed NIH3T3 cells (Chen and Chen, 1997). Although the hypusination status of eIF5A1 and eIF5A2 that accumulate *in vivo* following adenovirus treatment were not identified in this study, it is likely to be the unhypusinated forms due to limiting amounts of DHS and DOHH (Clement et al, 2006). Indeed, we previously demonstrated that adenovirus-mediated over-expression of eIF5A1 in colon cancer cells resulted in the accumulation of the unmodified rather than hypusinated form of the protein and that this accumulation resulted in the induction of apoptosis (Taylor et al, 2007). Over-expression of both DHS and DOHH in the presence of eIF5A1 precursor has been found necessary to obtain an accumulation of hypusinated exogenous eIF5A1 in mammalian cells (Park et al, 2006). Thus, it is not surprising that the data presented here, which results from over-expression of exogenous eIF5A that is likely very inefficiently hypusinated, should be very different from findings resulting from studies looking at over-expression of the endogenous hypusinated protein. In contrast to the proliferative function of the hypusinated form of the protein, previous studies have indicated that the unhypusinated form of eIF5A is involved in apoptosis (Jin et al, 2003). Adenovirus mediated expression of an eIF5A mutant that is incapable of being hypusinated, induced apoptosis in colon cancer cells (Taylor et al, 2007). Furthermore, the use of inhibitors of DHS results in an accumulation of unmodified eIF5A and the induction of apoptosis (Tome et al, 1997; Caraglia et al, 2003; Jin et al, 2003). Since DHS is highly expressed in certain cancer cell lines (Clement et al, 2006) and has been identified as a marker for metastatic disease (Ramaswamy et al, 2003), these studies suggest the possibility that DHS over-expression in cancer cells not only enhances growth of tumors by increasing levels of hypusinated eIF5A but also enhances survival by preventing the accumulation of the unhypusinated, apoptosis-inducing form of eIF5A1.

In this study we demonstrate for the first time the anti-tumor effects of eIF5A1 and eIF5A2 in syngeneic and xenograft cancer models. Treatment of both melanoma and lung subcutaneous tumors with intra-tumoral injections of Ad-eIF5A1 resulted in delayed tumor growth and a significant survival benefit. In the murine melanoma subcutaneous model, regression of tumors was observed in 50% of the Ad-eIF5A1-treated mice, indicating that eIF5A1 treatment may be effective against established tumors. Evidence of nuclear fragmentation and cell loss *in situ* indicated that apoptotic cell death accounts, at least in part, for the significant inhibition of tumor cell growth observed in the murine melanoma model. The reduction in tumor growth following treatment with Ad-eIF5A1 was more pronounced for murine B16 tumors than for human A549 tumors. This difference could reflect involvement of the immune system in eIF5A-mediated reduction in tumor growth since the A549 tumors were formed in immuno-compromised mice. However, the possibility that the difference is attributable to the slower growth of A549 tumors is not ruled out. Indeed, the longer Ad-eIF5A1 treatment required for A549 tumor-bearing mice, 66 days compared with only 37 days for B16 tumor-bearing mice, means that there was a higher prospect of antibody production against the adenovirus in the A549 tumor-bearing mice. This would result in more rapid clearing of Ad-eIF5A1 in the A549 tumor-bearing mice and a less pronounced reduction in tumor growth in comparison with B16 tumors.

The systemic therapeutic effects of eIF5A1 in the treatment of disseminated tumors were also examined in an experimental metastatic lung cancer model. Intravenous administration of cationic liposomes complexed with bacterially derived plasmid DNA elicits pro-inflammatory cytokine production resulting in toxicity (Tousignant et al, 2000; Zhao et al, 2004) and ensuing loss of transgene expression (Tan et al, 1999; Yew et al, 1999). Bacterial DNA differs from mammalian DNA in that CpG dinucleotides are more frequent and remain unmethylated, resulting in elicitation of a pro-inflammatory response (Krieg et al, 1995). Methylation of bacterial plasmids or removal of their CpG motifs results in a reduced inflammatory response (Yew et al, 2000; Reyes-Sandoval and Ertl, 2004) and higher levels of transgene expression (Tan et al, 1999; Yew et al, 2002; Hodges et al, 2004). The immune response elicited by bacterially derived plasmid DNA limits the effectiveness of repeated doses (Song et al, 1997; Li et al, 1999) and thus removal of CpG motifs permits more effective redosing (Tan et al, 1999). Therefore, in order to maximize transgene expression, a CpG-free expression plasmid complexed with DOTAP was used to deliver eIF5A1 to lung tumors in mice. Treatment with pCpG-eIF5A1 resulted in a 60 % reduction in lung weight compared to pCpG-LacZ vector control indicating that systemic delivery of eIF5A1 has a significant anti-tumor effect on experimental lung metastasis.

The present study demonstrates that the pro-apoptotic effects of eIF5A1 extend to tumors in mice and suggests that up-regulated expression of this protein could have therapeutic value in the treatment of both primary

and disseminated cancers.

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