Modulation of Coxsackie-Adenovirus Receptor Expression for Increased Adenoviral Transgene Expression

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ABSTRACT
An important determinant of gene transfer efficacy with the primary receptor, the coxsackie-adenovirus receptor. Unfortunately, expression may often be low in advanced clinical cancers, including ovarian, colorectal, lung, prostate, and breast cancer. In this study we investigated the feasibility of increasing transgene expression by incubating ovarian cancer cells with various agents and then performing transgene expression analysis. Fluorescence-activated cell sorting and quantitative reverse transcription-PCR were subsequently performed for correlation with receptor and mRNA up-regulation. Furthermore, the results were confirmed in purified clinical ovarian cancer specimens. Possible clinical application was tested using i.p. administration in an orthotopic ovarian cancer animal model. This approach could be useful for increasing adenoviral transgene expression in the context of clinical trials.

INTRODUCTION
Ad is a useful agent for cancer gene therapy because of its high potential for gene transfer, ease of high titer production, and demonstrated safety in trials (1, 2). Furthermore, many gene therapy approaches lack cross-resistance and may show synergy with chemotherapy or radiation therapy approaches for advanced cancers that currently lack effective treatments. Although there are some promising early clinical results, single-agent efficacy has been mostly unimpressive. Concurrently, it has become apparent that a major determinant of gene transfer efficacy with Ad is expression of its primary receptor, CAR, on target cells (1–9). CAR is ubiquitously expressed and this was followed by a 90-min infection with 10 pfu/cell Ad5luc1. CAR expression may have a tumor-suppressing effect, perhaps related to cell cycle phase and regulation (5, 6).

Considering that patients enrolled in adenoviral cancer gene therapy trials usually have advanced disease, these associations are ominous, if confirmed. Although targeting strategies are being vigorously investigated as a means to circumvent dependence on CAR, all clinical adenoviral gene therapy approaches reported heretofore have been CAR dependent. Therefore, variable CAR expression may have been a factor contributing to the disconnect between clinical and preclinical gene transfer rates. We hypothesized that CAR expression could be induced with biological or chemical agents predicted to affect the cell cycle or adhesion, which would lead to increased Ad-mediated transgene expression. Agents identified in vitro were tested in purified patient samples and by i.p. injection into tumor-bearing mice.

MATERIALS AND METHODS
Cell Lines and Agents. Ovarian cancer cell lines SKOV3.Ipl and Hey were gifts from Drs. Janet Price and Judy Wolf (both from M. D. Anderson Cancer Center, Houston, TX), respectively, and OV-4 cells were provided by Dr. Timothy J. Eberlein (Harvard Medical School, Boston, MA). OvCAR3 and OV-3 cells are from the American Type Culture Collection (Manassas, VA). Cells were cultured in recommended conditions. All ILs, EGF, TGF-β2, and stem cell factor were from Peprotech (Rocky Hill, NJ); 5-azacytidine, trichostatin A, and dexamethasone were from Sigma (St. Louis, MO); and U0126 was from Promega (Madison, WI). Chemotherapeutics and IFN-α (Intron A) were from the UAB Pharmacy (Birmingham, AL); PD98059 was from A.G. Scientific (San Diego, CA); and FR901228 was provided by Fujisawa Inc. (Osaka, Japan). Preliminary data have been reported for dexamethasone (12), IL-2 (13), U0126 (14), and FR901228 (15, 16). The concentrations were chosen to cover a wide range predicted to be biologically active but low enough to allow in vivo use, as reported for 5-azacytidine (17, 18), trichostatin A (18, 19), U0126 (20), PD98059 (21), cyclophosphamide (22, 23), gemcitabine (24, 25), paclitaxel (24, 26, 27), etoposide (24, 28), topotecan (24, 26), doxorubicin (24, 29), cisplatin (24, 26, 27, 30), and 5-fluorouracil (24, 31). Specifically, the tested concentrations were as follows: (a) 0.1–10 ng/ml, IL-1α, IL-1β, IL-4, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-15, and TGF-β2; (b) 0.1–1 ng/ml, IL-2 and IL-3; (c) 0.1–0.5 ng/ml, IL-5; (d) 25–150 ng/ml, IL-8 (77-amino acid form); (e) 0.2–20 ng/ml, IL-10, IL-16, and IL-17; (f) 2–40 ng/ml, EGF; (g) 200–10,000 IU/ml, IFN-α; (h) 10–100 ng/ml, stem cell factor; (i) 5–500 ng/ml, dexamethasone; (j) 12–1,200 ng/ml, 5-azacytidine; (k) 1–121 ng/ml, trichostatin A; (l) 1 ng/ml, FR901228; (m) 43–4,300 ng/ml, U0126; (n) 0.27–26.7 ng/ml, PD98059; (o) 3–300 μg/ml, cyclophosphamide; (p) 1–500 μg/ml gemcitabine; (q) 0.5–50 μg/ml, paclitaxel; (r) 1–500 μg/ml, etoposide; (s) 5–2,500 ng/ml, topotecan; (t) 0.1–10 μg/ml, doxorubicin; (u) 0.2–150 μg/ml, cisplatin; and (v) 10–300 μg/ml, 5-fluorouracil.

Transgene expression analysis was performed with Ad5Luc1, an E1E3-deleted Ad expressing the luciferase marker gene, for which construction and propagation have been described previously (32). Cells were incubated in growth medium containing the indicated concentrations of each agent for 24 h, and this was followed by a 90-min infection with 10 pfu/cell Ad5Luc1. Twenty-four h later, luciferase assay was done (Luciferase Assay System; Promega). Normalization for the amount of cells was performed by measuring total protein (33). For studying combinations, the concentrations were as follows: (a) 5 μg/ml, etoposide; (b) 36.2 ng/ml, trichostatin A; (c) 20 ng/ml, topotecan; and (d) 1 ng/ml, FR901228.
**CAR Expression by FACS.** Cells were incubated with agents for 24 h (150 μg/ml cisplatin, 5 μg/ml etoposide, 1 μg/ml gemcitabine, 100 ng/ml topotecan, 1 ng/ml FR901228, 121 ng/ml trichostatin A, 15 ng/ml dexamethasone, 10 ng/ml IL-1β, and 0.1 ng/ml IL-16). Cells were washed with PBS, harvested with 0.53 mM EDTA in PBS, and resuspended in PBS containing 1% BSA (Sigma). Cells (2 × 10⁶) were incubated in a 1:80 dilution of anti-CAR monoclonal antibody RmcB (hybridoma from American Type Culture Collection) or with buffer only for 1 h at 4°C. After being washed with PBS-BSA, cells were incubated in a 1:100 dilution of FITC-labeled goat antimouse IgG (Sigma) for 1 h at 4°C. Propidium iodide (2.6 μg/ml; Sigma) was added to exclude dead cells, and 10⁴ cells were analyzed immediately by FACS.

**Quantitative RT-PCR for CAR.** Cells were incubated in the presence of agents (concentrations were as described for FACS). Twenty-four h later, total RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Santa Clarita, CA). For creation of the standard curve, a PCR product of CAR gene was subcloned into pCR 2.1-TOPO (TOPO TA Cloning Kit; Invitrogen, Carlsbad, CA). After confirmation of the insert and presence of the T7 promoter by restriction enzyme digestion, in vitro transcription was performed (AMBION MAXIscript; Ambion, Austin, TX). The resulting CAR cRNA was confirmed and purified from a 5% acrylamide/8 M urea gel, and the concentration was estimated with absorbance at 260 nm, which was then converted to copy number using coefficients validated previously for E1A (33). Primers for

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Fig. 1. Modulation of adenovirus-mediated transgene expression in ovarian cancer cells. Cells were incubated with or without agents at indicated concentrations for 24 h and infected with a luciferase-expressing virus. Results indicate luciferase expression as a percentage of expression with virus only. A–D, chemotherapeutics; E and F, histone deacetylators; G, RAS inhibitor; H and I, other agents. Error bars indicate 1 SD. Data were normalized to the number of cells. *, P < 0.05 versus no agent; **, P < 0.01; ***, P < 0.001.

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determination of the CAR copy number were as follows: forward, AAATT-TACGCTTAGTCCGAAAGAC; reverse, CCTCTGATTATCGCTGGTAGATATC; and probe, CGACTCGATGTCACGCGTCCCC-TAMRA. Normalization to glyceraldehyde-3-phosphate dehydrogenase was done as reported previously (33). Quantitative real-time RT-PCR was performed with the LightCycler (Roche Molecular Biochemicals).

Quantitation of Virus Internalization. Cells were incubated in the presence of agents (1 ng/ml FR901228, 121 ng/ml trichostatin A, 100 ng/ml topotecan, and 5 μg/ml etoposide) in 24-well plates for 24 h. The cells were then incubated with 100 pfu/cell Ad5Luc1 for 60 min at 37°C to allow attachment of virus. Afterward, monolayers were washed three times, and this was followed by a 4-h incubation at 37°C to allow internalization of virus. Cells were harvested, DNA was purified using a DNeasy Tissue Kit (Qiagen, Valencia, CA), and virus copies (E4 gene) were detected with quantitative PCR and normalized to cellular β-actin as described previously (32, 33). All experiments were performed in quadruplicates. The background values (uninfected cells) were subtracted from each sample.

Analysis of Clinical Tumor Specimens. Fresh malignant ascites fluid samples were obtained with informed consent from patients with pathologically confirmed ovarian adenocarcinoma. Cancer cells were purified by an immunomagnetic-based method described previously (34). Briefly, cancer cells were initially bound with a murine anti-TAG-72-antibody (CC49) and then collected with magnetic beads coated with antimouse IgG. For formation of spheroids, cells were incubated on Costar 24-well ultra-low attachment plates (Corning Inc., Corning, NY) overnight at 37°C to allow internalization of virus. The following day, etoposide (5 μg/ml), topotecan (20 ng/ml), FR901228 (1 ng/ml), or no agent was added. After a 24-h incubation, cell suspensions were washed and infected with 100 pfu/cell Ad5Luc1 for 90 min at 37°C with rocking. Luciferase and protein assays were performed 24 h later, as described above. All experiments were performed in quadruplicates.

**Animal Model of Ovarian Cancer.** Hey cells (5 × 10⁶) were injected i.p. into CD1- nu athymic mice (Charles River Laboratories, Wilmington, MA) to induce peritoneal carcinomatosis. Fourteen days later, agents (200 μg of etoposide, 10 μg of topotecan, 1 μg of FR901228, or combinations of these) were injected i.p. twice, 8 h apart. Sixteen h after the latter injection, 10³ pfu of Ad5Luc1 were injected i.p. in a volume of 0.5 ml of OptiMEM. Twenty-four h later, the tumors were collected, and half of each tumor was subjected to luciferase analysis (32), whereas the other half was analyzed histologically to assess tumor content (N = 4 mice/group; N = 2 tumor nodules collected/mouse). Another model of disseminated ovarian cancer, based on SKOV3.ip1 cells in SCID mice (32), was also investigated.

**Statistics.** Statistical assessment of in vitro experiments was performed with a t test. In each case, pairwise comparisons were between the mean luciferase reading from samples treated with an agent and the mean from samples treated with virus only. In the animal experiment, statistical comparisons were performed with the nonparametric Wilcoxon two-sample test (SAS Institute, Cary, NC).

**RESULTS**

**Effect of Agents on Adenovirus-mediated Transgene Expression.** We used ovarian cancer cell lines as a primary screen for various agents that could have an effect on the CAR expression and subsequent transgene expression. The concentrations were chosen to cover a wide range predicted to be biologically active but low enough to probably lack toxicity in vivo. Adenoviral transgene expression was increased by cisplatin and etoposide in two of five lines, by gemcitabine in four of five lines, and by topotecan in three of five lines (Fig. 1, A–D). Cyclophosphamide, 5-fluorouracil, doxorubicin, and pacli-

![Fig. 2. Effect of agents on CAR expression and transcription. A, FACS detection of CAR expression. Results are presented as the mean fluorescence intensity obtained with the agent versus no agent (%). The dashed line indicates 100%. +, positive concordance with transgene expression data; −, negative concordance. B, effect of agents on CAR mRNA transcription. Quantitative RT-PCR was used to determine the copy number of CAR mRNA. Results were normalized for the number of cells by determination of glyceraldehyde-3-phosphate dehydrogenase mRNA copy number. Differences are not statistically significant. C, effect of combinations of agents on transgene expression. Results are displayed as percentage increase in comparison to no agent. Error bars indicate 1 SD. All data were normalized to the number of cells. *, P < 0.05 versus no agent; **, P < 0.01; ***, P < 0.001.
taxol did not have significant effects. Histone deacetylase inhibitors are a new group of anticancer drugs with various gene expression-repressing and -activating functions. FR901228 dramatically induced transgene expression in two of five lines, whereas trichostatin A was active in four of five lines (Fig. 1, E and F). 5-Azacytidine had less effect. High activity of the RAS/MAPK pathway has been suggested as a reason for down-regulation of CAR (14). In our experiments, RAS/MAPK pathway inhibitor U0126 had little effect, whereas PD98059 was effective in two of five lines (Fig. 1G). Dexamethasone increased transgene expression in four of five lines (Fig. 1H). IL-16 was effective in one of five lines (Fig. 1I), IL-1β was effective in one of five lines, IL-5 was effective in two of five lines, and EGF was effective in two of five lines (data not shown). IL-1α, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-17, IFN-α, stem cell factor, and TGF-β2 did not have significant activity (data not shown).

Although the agents were used at concentrations that do not typically kill ovarian cancer cells in vitro, the sensitivity of ovarian cancer lines varies. Therefore, in some cases, we did see a reduction of cell number, in particular with cisplatin. It is unlikely that this would have significantly impacted virus concentrations and subsequent transgene expression because CAR levels are the main factor determining Ad entry in stringent conditions such as those used in this study. Nevertheless, we sought to use further assays not sensitive to virus concentration.

**Effect of Agents on CAR Detection by FACS.** In general, FACS data corresponded well with transgene expression (Fig. 2A). Specifically, cisplatin, etoposide, topotecan, FR901228, and trichostatin A treatment increased both marker gene expression and CAR expression (correlation between experiments is indicated by a + in Fig. 2A), as detected by FACS, for all analyzed cell lines. Dexamethasone had no detectable effect on CAR on the cell surface, but it increased luciferase expression. The same was true for gemcitabine in the case of SKOV3.ip1 cells. The most interesting agents identified in this initial screen were subjected to closer scrutiny, in which FR901228, trichostatin A, topotecan, and etoposide all caused dramatic induction of cell surface CAR expression in OV-4, SKOV3.ip1, and Hey cells (Fig. 3).

**Effect of Agents on CAR mRNA Expression.** The function or regulation of CAR is not well understood, and thus it is unclear how
important transcriptional regulation is in controlling CAR expression. Therefore, we developed a quantitative RT-PCR assay for CAR. Interestingly, the effect of the agents on CAR mRNA was variable (Fig. 2B), and significant increases were not seen (P > 0.05). Furthermore, no clear correlation between CAR mRNA levels and transgene expression or CAR FACS could be detected.

**Effect of Combinations of Agents on Transgene Expression.** Various combination treatments resulted in dramatic improvement in transgene expression in ovarian cancer cells (Fig. 2C). For Hey cells, luciferase expression was increased 4.9-fold (P = 0.00028) with etoposide and topotecan, 19.4-fold (P < 0.0001) with etoposide and trichostatin A, and 15-fold (P < 0.0001) with topotecan and trichostatin A. The combination of topotecan and FR901228 was effective in all tested cell lines, increasing transgene expression 52-fold (P = 0.0043), 4.3-fold (P = 0.014), and 41-fold (P = 0.0077) on OV-4, SKOV3.ip1, and Hey cells, respectively. Furthermore, etoposide and FR901228 increased marker expression on OV-4 cells 7.8-fold (P = 0.028), whereas etoposide and topotecan and trichostatin A were effective on OV-4 (1.9-fold increase; P = 0.032) and Hey cells (17-fold increase; P < 0.0001).

**Effect of Agents on Adenoviral Entry into Cells.** The previous experiments demonstrated the increase of cell surface CAR, as detected by FACS, which led to increases in transgene expression and subsequent protein production. Nevertheless, we sought to confirm that the increased CAR allowed increased virus entry into cells. Cells were incubated in the presence of the agents, and virus internalization was allowed to occur, followed by careful washing of the unbound virus. In all cases, the agents were found to mediate increased entry of the virus into the cells (Fig. 4).

**Transgene Expression in Unpassaged Primary Human Ovarian Cancer Samples.** Established cell lines may differ from unpassaged primary tumors with regard to receptor expression (35). Thus, clinical samples were analyzed (Fig. 5). FR901228 significantly increased transgene expression in all patient samples [4.7-fold (P = 0.0021), 5.0-fold (P = 0.0085), 2.3-fold (P = 0.003), and 2.5-fold (P = 0.0046)]. Topotecan increased transgene expression in two of four patient samples [2.5-fold (P = 0.025) and 1.5-fold (P = 0.013)]. The combination of etoposide and FR901228 increased transgene expression in two of four patient samples [3.4-fold (P = 0.032) and 2.5-fold (P = 0.001)]]. Topotecan and FR901228 increased transgene expression in three of four patient samples [3.2-fold (P = 0.0004), 2.3-fold (P = 0.017), and 2.7-fold (P = 0.031)].

**Transgene Expression in Animal Models of Ovarian Cancer.** To validate the capability of the agents to increase transgene expression in vivo, carcinomatosis was established in nude mice by i.p. injection of human ovarian cancer cells, followed by i.p. injection of the agents and Ad. All agents increased the mean transgene expression in the tumors (Fig. 6), but variability was larger than in vitro. FR901228 treatment increased transgene expression 11.2-fold (P = 0.037), whereas the combination of FR901228 and etoposide resulted in a 5.1-fold increase (P = 0.086). Mean transgene expression was in-
increased 1.2-fold, 3.0-fold, and 3.3-fold with etoposide, topotecan, and the combination of topotecan with FR901228, respectively (all \( Ps > 0.1 \)). In the histological analysis, the viable tumor content was \( >50\% \) in 100% of the samples and \( >76\% \) in 79% of the samples. When a similar experiment was performed in a model based on SKOV3.ip1 cells in SCID mice, using smaller doses of agents, an increase in transgene expression was not seen.

**DISCUSSION**

Adenoviral cancer gene therapy approaches have been hampered by variable expression of CAR in clinical cancer specimens (1–3, 10, 11). Especially disconcerting are reports suggesting the association of CAR down-regulation with advanced disease because most cancer gene therapy trials are performed in this patient population. Thus, effective transduction of tumor cells continues to be a formidable obstacle with currently available technologies. Therefore, if expression of CAR on target cells could be increased, this could potentially yield improved clinical efficacy. Although the function of CAR is not well understood, there is increasing evidence suggesting a role in adhesion and cell cycle control. Thus, agents potentially affecting these attributes were selected for testing. The objective of the study was to develop a strategy that could be used in humans for increasing the efficacy of adenoviral transgene expression. The chemotherapeutics that were included have been investigated for treatment of ovarian cancer. Furthermore, all agents were tested at concentrations that are unlikely to cause significant side effects when administered i.p. For instance, etoposide, topotecan, and FR901228 have been administered i.p. to mice at 100, 10, and 0.5 mg/kg, respectively. We used 5–10% of these doses.

The agents most effective in increasing transgene expression were etoposide, gemcitabine, topotecan, FR901228, trichostatin A, PD980159, dexamethasone, IL-5, and EGF (Fig. 1). Marker gene analysis is a useful end point when investigating factors that may affect efficacy of transgene expression because it allows for transductional efficacy but also for transcriptional, translational, and posttranslational aspects. However, to investigate the actual effect the agents have on cell surface expression of CAR, FACS was performed (Figs. 2A and 3). In general, good correlation with transgene expression analysis was seen. Thus, increased expression of CAR on the cell surface was probably a major factor contributing to the increased transgene expression. This is well in accord with a number of previous reports suggesting that CAR is a major factor contributing to Ad transduction efficacy and transgene expression (1–9). This was confirmed by detection of increased virus numbers inside cells treated with the agents that increased cell surface CAR (Fig. 4).

One possible mechanism for up-regulation of CAR could be increased CAR mRNA transcription. However, quantitative RT-PCR did not support this as a common mechanism (Fig. 2B). Instead, posttranscriptional events may be more important in this regard. Conceivably, the effect of some of the agents could be additive or synergistic. Interestingly, many combinations resulted in dramatically increased transgene expression (Fig. 2C). Particularly useful was FR901228 with topotecan, which increased transgene expression in all cell lines tested.

Primary cancer cells from patient samples may display various genotypic and phenotypic differences as compared with established cell lines. Therefore, the former may be a more stringent substrate for estimating potential clinical efficacy. We used a purification method for patient ascites-derived ovarian cancer cells, which yields highly pure tumor cell populations. These cells were then suspended as spheroids, which may resemble clinical tumor masses more closely than monolayers because of their three-dimensional structure (35).

Treatment with FR901228 resulted in increased transgene expression in all primary tumor specimens tested, whereas the combination of topotecan and FR901228 was effective in three of four specimens tested (Fig. 5).

The purpose of this study was to develop a clinically usable strategy for increasing adenoviral transgene expression in the context of clinical trials. All published ovarian cancer gene therapy trials have been performed with patients with peritoneally disseminated disease and have used i.p. administration of treatment via a catheter (1). Furthermore, such catheters have been used for i.p. administration of chemotherapy and could also be used for i.p. administration of the agents identified here. To mimic the clinical scenario, we used an orthotopic murine model of disseminated ovarian cancer and delivered the agents i.p., followed by Ad through the same route. An additional benefit of this model is that it allows for features present only in vivo, such as stromal cells, extracellular matrix, and vasculature. However, in vivo experiments feature less standardized conditions, such as variable tumor load and heterogeneous tumor nodule size and location. Nevertheless, increased transgene expression was seen (Fig. 6). In additional studies, it may be of interest to investigate how the agents affect the localization of CAR on tissue and cellular levels. This could perhaps be achieved using sophisticated immunohistochemistry techniques (7).

The data presented here identify FR901228, trichostatin A, topotecan, and etoposide as promising agents, alone or in combination, for increasing adenoviral transgene expression in ovarian cancer cells. FR901228 has been investigated in previous studies on murine cells and in a panel on human cell lines (15, 16). Heretofore, there have been no data reported on clinical samples or in animal models of human cancer. However, our results are well in accord with these previous reports, which also suggested increases in Ad-mediated transgene expression and CAR. FR901228 and trichostatin A are members of a new group of anticancer agents, the histone deacetylases, which alter the expression of a variety of genes in a poorly understood manner. The mechanism by which topotecan increases transgene and CAR expression may be related to its inhibition of topoisomerase I, an enzyme that regulates interconversion of DNA topoisomers, a process crucial for DNA replication, recombination, and transcription. Etoposide inhibits topoisomerase II and may thus have a similar mode of action. Clearly, additional studies are needed to clarify the exact mechanisms whereby these agents increase transgene expression and CAR. However, a fascinating common feature is that all four compounds seem to affect the cell cycle (36–38). Interestingly, one previous report studied the effect of cell cycle phase on transgene expression. When M phase was induced in lung cancer cells with paclitaxel, a modest in vitro increase in transgene expression and cell surface CAR was seen (5). In our experiments, paclitaxel did not increase transgene expression in ovarian cancer cells. Nonetheless, these preliminary findings become intriguing when taken together with reports suggesting the association of CAR with cell cycle control (6). Inhibition of the RAS/MAPK pathway had little effect on the tested ovarian cancer substrates, in contrast to a preliminary report on breast cancer (14).

In conclusion, we have identified a number of agents that could be useful for increasing adenoviral gene expression in the context of gene therapy for ovarian cancer. The feasibility of the approach in humans can only be confirmed in human trials. This could be accomplished by randomizing patients into groups receiving or not receiving the agent before Ad gene therapy. In contrast to the relatively modest results obtained in cancer trials, a number of clinical successes have been reported in gene therapy approaches for other diseases, including hemophilia (39), two forms of severe combined immune deficiency (40, 41), and various cardiovascular diseases (42). In fact, these...
studies demonstrated that viral vectors can achieve clinically significant gene transfer to severely ill patients, with subsequent correction of the disease phenotype. The common feature of these approaches is the rational manner in which the means for gene delivery and subsequent transgene expression were improved. Ultimately, phenotype correction was seen in a predictable, albeit dramatic, fashion when gene transfer was successful. The approach reported here, if confirmed in clinical trials, could help adenoaviral cancer gene therapy move toward similar goals.

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