Enhanced Therapeutic Efficacy for Ovarian Cancer with a Serotype 3 Receptor-Targeted Oncolytic Adenovirus

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Oncolytic viruses that are replication competent in tumor but not in normal cells represent a novel approach for treating neoplastic diseases. However, the oncolytic potency of replicating agents is determined directly by their capability of infecting target cells. Most adenoviruses used for gene therapy or virotherapy have been based on serotype 5 (Ad5). Unfortunately, expression of the primary receptor for Ad5 (the coxsackie–adenovirus receptor, or CAR) is highly variable on ovarian and other cancer cells. By performing genetic fiber pseudotyping, we created Ad5/3-H9004, a conditionally replicating adenovirus that does not bind CAR but facilitates entry into and killing of ovarian cancer cells. We show replication of Ad5/3-H9004 and subsequent oncolysis of ovarian adenocarcinoma lines. Replication was also analyzed with quantitative PCR on three-dimensional primary tumor cell spheroids purified from patient samples. Moreover, in a therapeutic orthotopic model of peritoneal carcinomatosis, dramatically enhanced survival was noted. Finally, Ad5/3-H9004 achieved a significant antitumor effect as assessed by noninvasive, in vivo bioluminescence imaging. Therefore, the preclinical therapeutic efficacy of Ad5/3-H9004 is improved over the respective CAR- and integrin-binding controls. Taken together with promising biodistribution and toxicity data, this approach could translate into successful clinical interventions for ovarian cancer patients.

Key Words: gene therapy, ovarian neoplasms, adenovirus, virus replication, biological therapy

INTRODUCTION

Adenovirus-mediated gene therapy has been proposed as a treatment alternative for advanced cancers refractory to other therapies. Adenoviruses are attractive vectors for cancer due to their unparalleled capacity for gene transfer, stability in vivo, and easy high-titer production. Consequently, replication-deficient first-generation viruses have shown high preclinical transduction rates and good antitumor efficacy in animal models. Most completed clinical trials have been Phase I, and the safety data have been excellent. However, there is little evidence supporting significant clinical benefits [1]. Nevertheless, supporting the feasibility of adenoviral gene therapy, positive results were reported in the only randomized trial published to date, albeit a Phase I [2].

Recently, it has been suggested that the binding of adenovirus to its primary receptor may be an important rate-limiting step for gene transfer [reviewed in 3]. As most adenoviral gene therapy vectors are based on serotype 5 (Ad5), which binds to the coxsackie-adenovirus receptor (CAR), lack of CAR could make target tissues refractory [3,4]. Indeed, recent data suggest that CAR expression in tumors may be highly variable, resulting in resistance to Ad5 infection [3,5–9]. Specifically, resistance may be due to low expression levels or aberrant localization at the cellular or tissue level [10]. Consequently, various strategies have been evaluated to modify adeno-
virus tropism to circumvent CAR deficiency, including retargeting complexes or genetic capsid modifications [4].

In earlier studies, we explored substituting the receptor binding fiber knob domain of Ad5 with the serotype 3 (Ad3) knob. This resulted in CAR-independent virus attachment to ovarian and other cancer cells, as Ad3 has a distinct, but unidentified receptor [11]. Further, our previous studies suggest that, in comparison to CAR, the Ad3 receptor is expressed at higher levels on ovarian cancer cells and that 5/3 serotype chimeras bind to the Ad3 receptor but not to CAR as analyzed by competition with free Ad knob protein [5]. Importantly, Ad5/3 displayed higher transgene expression in primary ovarian cancer cells. Furthermore, the murine safety profile of Ad5/3 was evaluated by biodistribution, liver toxicity, and blood clearance analyses and was comparable to that of Ad5 [6].

The main reason for the mostly less than impressive clinical results obtained with replication-deficient agents may be related to inefficient transcription of solid tumor masses [12]. To help overcome this obstacle, selectively oncolytic adenoviruses, i.e., conditionally replicating adenoviruses (CRAds), have been constructed. Infection of tumor cells results in replication, oncolysis, and subsequent release of the virus progeny. In combination with chemotherapeutics, CRAds have recently displayed some clinical utility [13]. Normal tissue is spared due to abrogation of replication, which can be achieved by incorporation of mutations in immediately-early (E1A) or early (E1B) adenoviral genes resulting in mutant E1 proteins unable to bind cellular proteins necessary for viral replication in normal cells, but not in cancer cells [14–17]. Ad5-Δ24 contains a 24-bp deletion in constant region 2 (CR2) of E1A, and the expressed protein is unable to bind retinoblastoma (Rb) protein for induction of S phase [15]. Thus, Ad5-Δ24 is unable to replicate in nondividing normal cells, but replicates in cells inactive in the Rb/p16 pathway [15,16]. It has been suggested that all human cancers, including ovarian cancers, may be deficient in this crucial pathway [18].

Recently, it has been demonstrated that the oncolytic potency of replicating agents is determined directly by their capability of infecting target cells [19,20]. Thus, variable CAR expression on cancer cells could hinder CRAd-mediated oncolysis. Therefore, methods to circumvent CAR deficiency and improve cell killing have been evaluated in the context of CRAds. Heretofore, these endeavors include infectivity enhancement of the agent by incorporating motifs in fiber, such as the integrin-binding arginine-glycine-aspartic acid (RGD-4C) modification into the HI loop of the knob [21,22] or the heparan sulfate-binding polysine residue at the COOH-terminus of the fiber [23]. These viruses bind both CAR and an alternative receptor. In this study, ablation of CAR binding was achieved by genetic pseudotyping of the fiber with the knob domain from Ad3 (Ad5/3-Δ24, Fig. 1). This may be a member of a new generation of agents that utilize a non-CAR pathway, which may be advantageous for enhanced tumor targeting. Further, the novel agent was compared to Ad5-Δ24RGD, a recently described promising oncolytic agent, which is currently in ovarian cancer clinical trials.

**RESULTS**

**Ad5/3-Δ24 Displays Efficient Killing of Ovarian Cancer Cells in Vitro**

We infected monolayers of SKOV3.ip1, OV-4, OV-3, Hey, and ES-2 cells (Fig. 2) with Ad5/3-Δ24, Ad5-Δ24E3, and AdCMVHSV-TK. In all cell lines, the quantitative cell killing assay showed oncolysis with Ad5/3-Δ24, while Ad5-Δ24E3 (the isogenic control with the Ad5 fiber) caused minimal (OV-4, OV-3, Hey) or no cell killing (SKOV3.ip1, ES-2). The percentage of viable cells remaining with Ad5/3-Δ24 was 0.5, 5.6, 15, 2.7, and 5.5% for SKOV3.ip1, OV-4, OV-3, Hey, and ES-2, respectively, compared to uninfected wells. With Ad5-Δ24E3, viability was 117, 91, 87, 85, and 109%, respectively. On all cell lines, oncolysis was significantly improved with fiber-modified Ad5/3-Δ24 in comparison to Ad5-Δ24E3 (P < 0.0001, P < 0.0001, P = 0.0033, P = 0.0011, and P < 0.0001 for SKOV3.ip1, OV-4, OV-3, Hey, and ES-2, respectively). AdCMVHSV-TK was included as an E1-deleted control, and it did not cause oncolysis (P = 0.0018 for SKOV3.ip1, P < 0.0001 for other cell lines, compared to Ad5/3-Δ24).

**Ad5/3-Δ24 Replicates in Ovarian Cancer Primary Cell Spheroids**

We analyzed four purified, unpassaged human ovarian cancer samples for adenovirus replication. To measure viral copy number, we collected spheroids and growth medium at indicated time points and performed quantitative PCR for the adenoviral E4 gene (Figs. 3A–3D). To determine the relative increase in copies, we normalized

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**FIG. 1.** Structure of Ad5/3-Δ24. Ad5/3-Δ24 has a 24-bp deletion in constant region 2 (CR2) of the E1A gene, corresponding to the region utilized for Rb protein binding. This results in an agent replication competent and oncolytic in cells defective in the Rb/p16 pathway, such as ovarian cancer cells. The fiber is modified to incorporate the serotype 3 knob, while retaining the Ad5 shaft and tail.
E4 copy number at each time point to the copy number obtained with AdCMVHSV-TK at 12 h. On day 4 after infection, Ad5/3-Δ24 copy number had increased 378-, 149-, 562-, and 212-fold in patient samples 1–4, respectively, compared to 27.3-, 71.1-, 11.6-, and 104-fold increases with Ad5-Δ24E3. On day 12, Ad5/3-Δ24 copy number had increased to 406-, 267-, 572-, and 73.8-fold, while these figures were 252-, 115-, 172-, and 131-fold for Ad5-Δ24E3. Thus, replication of Ad5/3-Δ24 occurred more rapidly than that of Ad5-Δ24E3 in all cases and to a higher degree in two of four patients (Figs. 3A and 3C).

To estimate total virus production and allow visual comparison of replication dynamics, we calculated cumulative virus copy numbers (Figs. 3E–3H). With patients 1–3 the highest cumulative amount of E4 copies was measured with Ad5/3-Δ24. When total cumulative virus production was compared, Ad5/3-Δ24 produced 241-, 63.1-, 450-, and 54.9-fold more virus than AdCMVHSV-TK (patients 1–4, respectively). Ad5-Δ24E3 achieved 124-, 35.5-, 116-, and 57.8-fold higher total virus production than AdCMVHSV-TK.

Ad5/3-Δ24 in an Orthotopic Murine Model of Ovarian Cancer
We tested the efficacy of Ad5/3-Δ24 in an ip model of disseminated ovarian cancer. We allowed advanced carci-
nomatosis to develop for 10 days and then treated the mice with a single injection of Ad5/3-Δ24, Ad5-Δ24E3 (non-fiber-modified isogenic control virus), or AdCMVHSV-TK (E1-deleted control virus). Spheroids and growth medium were harvested at indicated time points, and virus copy number was measured with quantitative PCR. (A-D) The increase in virus copy number with Ad5/3-Δ24 and Ad5-Δ24E3 in patient samples 1–4, respectively. (E–H) Cumulative values from patients 1–4 allow comparison of total virus production and replication kinetics. The background values (uninfected spheroids) were subtracted from the data at each time point.

FIG. 3. Ad5/3-Δ24 replicates in three-dimensional human ovarian primary cancer cell spheroids. Purified, unpasaged cancer cells were allowed to form spheroids, which were infected with 1000 vp/surface cell of Ad5/3-Δ24, Ad5-Δ24E3 (non-fiber-modified isogenic control virus), or AdCMVHSV-TK (E1-deleted control virus). Spheroids and growth medium were harvested at indicated time points, and virus copy number was measured with quantitative PCR. (A–D) The increase in virus copy number with Ad5/3-Δ24 and Ad5-Δ24E3 in patient samples 1–4, respectively. (E–H) Cumulative values from patients 1–4 allow comparison of total virus production and replication kinetics. The background values (uninfected spheroids) were subtracted from the data at each time point.

other groups, the overall survival of mice treated with Ad5/3-Δ24 was statistically significantly improved (log-rank test \( P < 0.0001 \)). In pair-wise comparisons survival with Ad5/3-Δ24 was significantly enhanced (versus Ad5-Δ24E3, \( P = 0.0001 \); versus AdCMVHSV-TK, \( P < 0.0001 \); versus no virus, \( P < 0.0001 \)).

Injection on 3 consecutive days was also tested (Fig. 4B). Again, the median survival of mice treated with Ad5/3-Δ24 was not reached, and 50% of mice were alive on day 135. For Ad5-Δ24E3, AdCMVHSV-TK, and no virus, the
median survivals were 41.5, 33, and 32 days, respectively. The overall survival was significantly better in mice treated with Ad5/3-Δ24 (log-rank test \( P < 0.0001 \)). \( \chi^2 \) testing confirmed the significantly improved survival with Ad5/3-Δ24 (versus Ad5-Δ24E3, AdCMVHSV-TK, or no virus, \( P < 0.0001 \)).

The survival of mice treated with a single injection and multiple injections of Ad5/3-Δ24 did not differ significantly (log-rank test \( P = 0.8095 \), \( \chi^2 \) test \( P = 0.8034 \)). The experiment was terminated on day 135, as surviving mice looked healthy, and all the control groups had expired. Surviving mice were autopsied and upon macroscopic examination of the peritoneal cavity, no sign of viable ip tumor tissue was found.

Interestingly, preliminary data from a survival experiment comparing Ad5/3-Δ24 to a previously described infectivity-enhanced CRAd, Ad5-Δ24RGD, suggest superior efficacy of Ad5/3-Δ24 over Ad5-Δ24RGD. We injected \( 5 \times 10^6 \) SKOV3.ip1 cells ip and administered a single injection of \( 1 \times 10^8 \) viral particles (vp) ip 10 days later. On day 120 after cell inoculation, the percentage of surviving mice was 82 and 55% for Ad5-3-Δ24 and Ad5-Δ24RGD, respectively. Nevertheless, longer follow-up is needed to confirm this preliminary observation.

**Quantitative Measurement of Therapeutic Response of Ovarian Cancer with Ad5/3-Δ24 Using in Vivo Bioluminescence Imaging**

We also evaluated the efficacy of Ad5/3-Δ24 with quantitative, noninvasive in vivo imaging. We injected firefly luciferase-expressing SKOV3-luc cells ip (day 0), followed by a single injection of Ad5/3-Δ24 or no virus on day 8. Bioluminescence imaging was performed weekly starting from day 7 (Fig. 5). Ad5/3-Δ24-treated mice displayed slowly increasing light intensity up to day 35. In fact, the amount of emitted light decreased initially, and on day 28 the signal was only 2.4-fold higher than before virus administration, compared to a 21-fold increase in the untreated group (Fig. 5C). The increasing photon count on days 35 and 43 in the treatment group appeared to be from sc tumors growing in the needle track of ip tumor cell injection. When the abdominal wall was removed on day 43, light emitted from the peritoneal cavity of Ad5/3-Δ24-treated mice was only 4% of the signal of the untreated group and only 2.3-fold higher than before treatment, while the control group displayed an 86-fold increase in signal. Further, these imaging data correlated with actual tumor mass weighed after the experiment was terminated. The sc tumors in both groups were 1.3% of body mass, while ip tumors were 1.2 and 16% of body mass in Ad5/3-Δ24-treated and untreated mice, respectively.

**Ad5/3-Δ24 Displays Similar or Enhanced Oncolysis Compared to Ad5-Δ24RGD**

We compared Ad5/3-Δ24 to Ad5-Δ24RGD on SKOV3.ip1 cells. We analyzed oncolysis 5, 9, and 13 days after monolayers were infected by 1 and 100 vp/cell of Ad5/3-Δ24 and Ad5-Δ24RGD; we determined cell survival by MTS assay and expressed it as a fraction of the uninfected mock value at each time point (Fig. 6A). At the lower dose,
Ad5-Δ24RGD did not cause significant cell killing, while the percentage of viable cells remaining with Ad5/3-Δ24 was 76, 12, and 1.7% on days 5, 9, and 13 after infection, respectively, compared to uninfected wells. At the higher viral dose, the viability with Ad5/3-Δ24 was 17, 0.3, and 0.5%, while with Ad5-Δ24RGD these percentages were 92, 35, and 1.2%, respectively. At 1 vp/cell dose, the difference was significant at every time point (P = 0.0048, P < 0.0001, and P < 0.0001 for days 5, 9, and 13, respectively). When we compared the higher doses, statistically significant differences were found at the earlier time points (P < 0.0001, P = 0.0022) but not on day 13 (P = 0.2652).

We also evaluated oncolytic efficacy on two human primary ovarian cancer patient samples (Fig. 6B). We infected spheroids and measured cell viability at two time points and compared it to that of uninfected cells. With patient sample A, cell viability was 71 and 59% for Ad5/3-Δ24 and 60 and 47% for Ad5-Δ24RGD on days 9 and 13, respectively. There was no statistical significance (P = 0.4022 and P = 0.5312, respectively). On patient sample B, Ad5/3-Δ24 showed earlier cell killing than Ad5-Δ24RGD, and the viabilities were 66% versus 84% (P = 0.0020) on day 8. However, on day 14 the oncolysis was comparable (cell viability of 62 and 59% for Ad5/3-Δ24 and Ad5-Δ24RGD, respectively, P = 0.0904).

**DISCUSSION**

Despite exciting preclinical data, cancer gene therapy approaches utilizing various strategies have yet to display
difficulties in clinical translation. Even the most successful agents, such as Ad5/3-24, have shown variable transduction efficacy in clinical trials [24-27]. Consequently, efforts to enhance in vivo transduction or retarget Ad5 have been pursued. We demonstrated recently that swapping the primary receptor binding knob domain for a serotype 3 knob allows increased transgene expression in the most stringent available preclinical ovarian cancer substrates, purified human primary cancer cells, and in an animal model. This 5/3 chimeric virus binds to the distinct Ad3 receptor. In fact, Ad3 pseudotyping was superior to RGD-4C modification [6]. Also, with regard to preclinical evaluation of safety, we showed that the murine biodistribution, liver toxicity, and blood clearance of an Ad5/3 chimera were not significantly different from those of Ad5 [6]. As Kupffer cell uptake of adenovirus in liver is not CAR mediated, it is not surprising that liver uptake of chimeric and other retargeted viruses would not differ from the parental virus.

Replication-competent agents such as CRAds have emerged as a tool for overcoming low tumor transduction. Importantly, they have been tested in a number of clinical trials, demonstrating excellent safety with some preliminary evidence of efficacy. In a Phase II study of an intratumorally administered E1B 55K-deleted CRAd in 40 patients with head and neck cancer, three complete and two partial responses were reported [24]. Further, CRAds have been administered ip, iv, intra-arterially, and intratumorally in large doses without significant toxicity [25].

The oncolytic potency of replicating agents is determined directly by the capability of infecting target cells [19,20]. Consequently, infectivity-enhanced CRAds have been constructed, and in preclinical studies, their oncolytic potency has been increased, when additional cellular receptors have been used [23]. Ad5-Δ24RGD features an RGD-4C modification of the HI loop of the knob, which allows binding to αvβ integrins, which are regularly expressed and often overexpressed on ovarian cancer cells [26] and tumor vasculature [27]. In the context of ovarian cancer, Ad5-Δ24RGD demonstrated impressive oncolytic potential and significantly increased survival in an animal model [22]. Consequently, clinical trials utilizing this virus for ovarian cancer and glioma are in development. Nevertheless, Ad5-Δ24RGD continues to bind CAR and is therefore not retargeted but infectivity enhanced instead.

In this study, we used fiber chimerism as a retargeting strategy. The Ad5/3-Δ24 fiber features the knob from Ad3, and this chimerism results also in enhanced infectivity, which translated into increased oncolysis of target cells (Fig. 1). While the isogenic agent without the fiber modification displayed minimal or no cell killing, Ad5/3-Δ24 achieved dose-dependent cell lysis. Further, this virus was able to replicate in ovarian cancer primary cell spheroids as measured with quantitative PCR (Fig. 1). The in vitro assay used here is probably not very sensitive to the effects of infectivity enhancement, as in vitro all viruses present in the supernatant are expected to enter cells. Nevertheless, Ad5/3-Δ24 was superior to control viruses in all patient samples. Therefore, the novel CRAd effectively replicates in primary cancer cells and the replication kinetics are not adversely affected by the genetic fiber modification.
All ovarian cancer gene therapy trials published so far have relied on ip administration of the agent to patients with peritoneally disseminated disease [4]. To mimic a clinical situation, we inoculated advanced ip carcinomas, followed by ip administration of viruses (Fig. 4). With both doses, Ad5/3-Δ24 demonstrated significantly improved survival. In fact, with a single injection, Ad5/3-Δ24 allowed 80% survival on day 114, when all the control mice were dead. Direct comparisons with other studies utilizing this model are difficult due to differences in experimental setup. Nevertheless, other studies report smaller cell inoculum and earlier or more numerous injections of virus [22,28,29]. Therefore, the results obtained here could compare favorably to other reports. Interestingly, the survival with Ad5/3-Δ24 using single injection versus multiple injections did not differ significantly. However, the larger dose of Ad5-Δ24E3 seemed to result in less antitumor efficacy in addition to giving increased toxicity (Fig. 4B). This might be due to liver toxicity, as we have experienced same phenomenon with a wild-type Ad5 (A. Kanerva, work in progress).

There are possible obstacles to ip administration of CRAds. First, most ovarian cancer patients have malignant ascites fluid, which has neutralizing anti-adenovirus antibodies [26,30]. These antibodies could hamper the initial infection of tumor cells and spreading of new virions. Fortunately, previous studies have suggested partial escape of Ad5/3 chimeric viruses from preexisting neutralizing anti-adenovirus antibodies, perhaps due to the chimeric nature of the fiber [6]. Another possible problem could be related to local toxicity, specifically, replication in the peritoneal lining. An interesting finding in murine biodistribution studies was a lower peritoneal uptake of Ad5/3 [6]. This could be beneficial in the context of ip administration of Ad5/3-Δ24. Converted weight/weight into humans, the smaller dose used here would equal ca. 9 × 10^{10} vp. This is well below the 2 × 10^{12} vp daily for 5 consecutive days used in a CRAd trial in which the maximal tolerated dose was not reached [31]. In vector trials up to 7.5 × 10^{13} vp have been administrated daily for 5 consecutive days, without dose-limiting toxicity [32].

Imaging techniques can provide fundamental safety and efficacy information on experimental therapy approaches [33]. Specifically, utilizing an orthotopic animal model for monitoring CRAd efficacy is advantageous as it may resemble the clinical picture more closely than sc tumors, but this approach is also problematic as tumors are not accessible to diameter measurements. Further, another important feature of noninvasive imaging is the possibility of performing repeated measurements. Therefore, various noninvasive imaging systems have been evaluated [33]. For example, expression of somatostatin receptor subtype 2, coded by an adenovirus vector, can be imaged with radioisotope gamma camera after administration of the somatostatin analogue 99mTc-P2045 [29]. Also, optical charge-coupled device (CCD) imaging has been used to detect bioluminescence emitted from β-luciferin reacting with firefly luciferase, coded by an adenovirus vector [34]. Other approaches include magnetic resonance and positron-emission tomography imaging of positron-emitting ligands [33]. Cancer cells expressing reporter genes such as firefly luciferase and green fluorescence protein are useful means of following tumor growth [35,36].

We used an orthotopic ovarian cancer model of SKOV3-luc cells, which emit light after ip administration of β-luciferin. Therefore, oncolytic killing of tumor cells corresponds with reduction of signal in comparison to control animals. Kinetics of tumor growth with or without virus injection was followed weekly. Ad5/3-Δ24-treated mice initially responded, but then relapsed with sc tumors in the needle tract, an intrinsic defect of this animal model [22] (Fig. 5C). This has the potential for confounding ip imaging data as even small sc tumors can give a strong signal due to their proximity to the camera. Nevertheless, the sensitivity of the assays was sufficient to allow detection of ip cell killing.

A further round of imaging was performed without the abdominal wall to distinguish the antitumor without the confounding sc tumors, and we found that light emitted from the peritoneal cavity was only 4% of the untreated group, suggesting effective killing of tumor cells by the virus. Of note, the survival of the untreated mice was better than in Fig. 4. This might be due to the more malignant version of SKOV3 cells, i.e., SKOV3.ip1, in the survival experiment [37]. Interestingly, we could not find sign of viable ip tumor tissue in the treated mice in the survival experiment, though there was some signal left in the imaging. The susceptibility of the cells to the treatment agent could be different, or their viability during the injection procedure could be different. Also, it is possible that SKOV3-luc cells allow less virus production than SKOV3.ip1 cells, due to concomitant luciferase production. Further, although genetically similar in theory, different sets of mice were used and could contribute to the results.

We have previously reported promising results with another CRAd (Ad5-Δ24RGD) for ovarian cancer gene therapy [22]. While it is possible that a number of CRAds could be useful for treatment of patients with advanced disease, it may be of interest to obtain comparative preclinical efficacy data. Therefore, we compared Ad5-Δ24RGD to Ad5/3-Δ24 in cell lines, in clinical tumor specimens, and in an orthotopic murine model of peritoneally disseminated ovarian cancer. Preliminary evidence suggests superiority of the latter in each of the test systems.

Nevertheless, there are a number of applications in which it would be important to have effective oncolytic agents with distinct capsids. For example, if neutralizing antibodies prove to be limiting to clinical efficacy, “seroswitching” could be a useful way to retain antitumor efficacy. Preliminary studies have indicated that modifica-
tion of the fiber knob is sufficient to allow escape from preexisting neutralizing antibodies [6,30]. Further, sequential utilization of a number of alternative binding routes (CAR, integrins, serotype 3 receptor) could be helpful in avoiding selection-mediated resistance to treatment. This could be tested by randomizing animals (or patients) into treatment arms receiving either Ad5/3-Δ24 or sequentially alternating Ad5/3-Δ24, Ad5-Δ24RGD or Ad5-Δ24E3, all of which have antitumor efficacy but feature distinct capsids and entry routes.

In conclusion, we have demonstrated that retargeting a selectively oncolytic adenovirus to the Ad3 receptor results in improved infectivity of ovarian cancer cells and overcomes the CAR deficiency on primary cancer cells. Consequently, therapeutic efficacy was dramatically increased in vitro and in vivo. Further, we have demonstrated that Ad5/3-Δ24 allows cell killing comparable or superior to previously described Ad5-Δ24RGD. Thus, Ad5/3-Δ24 could be an effective agent for treatment of ovarian cancer and other tumors with an inactive Rb/p16 pathway and high expression of the Ad3 receptor. Clinical trials will ultimately show if preclinical advances such as reported here can be translated in to similar progress in cancer gene therapy.

**MATERIALS AND METHODS**

**Cell culture.** 293 cells were purchased from Microbix (Toronto, Canada) while 911 were courtesy of Dr. van der Eb (University of Leiden, The Netherlands). Lung adenocarcinoma cell line A549 and ovarian adenocarcinoma cell lines ES-2 and OV-3 were obtained from ATCC (Manassas, VA). Ovarian adenocarcinoma cell lines SKOV3.ip1, Hey, and OV-4 were obtained from Dr. Price, Dr. Wolf (both M. D. Anderson Cancer Center, Houston, TX), and Dr. Eberlein (Harvard Medical School, Boston, MA). Firefly luciferase-expressing ovarian adenocarcinoma cell line SKOV3-luc was kindly provided by Dr. Negrin (Stanford Medical School, Stanford, CA). All cell lines were cultured under recommended conditions.

Primary ovarian adenocarcinoma cells were purified by a previously described immunomagnetic-based method [38] from malignant ascites fluid samples from patients undergoing a procedure for ovarian cancer at the University of Alabama at Birmingham Hospital. Briefly, ovarian cancer cells were bound with a murine anti-TAG-72 antibody and then collected with magnetic beads coated with anti-mouse IgG. To create three-dimensional spheroids, cells were suspended in growth medium in 3% agar-coated flasks, and reverse, 5′-ACTACGTCCGGCGTTCCAT-3′; knob3(AS), 5′-TCATTATGGTCTGGCCGATT-3′).

**Construction of Ad5/3-Δ24.** A fiber shuttle vector, pNEB.PK.E5/3, containing an Ad5 tail and shaft and Ad3 knob was digested with PacI and RndI, followed by cotransformation into Escherichia coli for homologous recombination with a Swat-linearized rescue plasmid containing an E1 deletion. This resulted in a plasmid with the E1-deleted Ad5 genome with the chimeric fiber domain (pTU.5/3) [40]. To create Ad5/3-Δ24, a shuttle vector containing a 24-bp deletion in CR2 of E1A (pShuttleΔ24) was used [41]. pShuttleΔ24 was linearized with PacI/Pmel and cotransfected into 911 cells with PacI-linearized pTU.5/3 to rescue Ad5/3-Δ24 (Fig. 1). Propagation was performed on A549 cells.

The presence of the 24-bp deletion in E1A and the absence of the wild-type E1A was confirmed with PCR (Δ24L1, 5′-GTCGGTTTCTATGGCGGACAC-3′; Δ24R1, 5′-TCACCTCTTCATCCTCGTC-3′). The presence of the E3 region and the Ad3 knob in the fiber was analyzed with PCR (E3L2, 5′-CCTGAAACACCTGTGCACCAC-3′; E3R2, 5′-GCCAACC-TAGGGGCTTCTGA-3′; knob3(AS), 5′-GCCAGGTGCCATACAGTAG-3′; knob3(AS), 5′-TCATTATGGTCTGGCCGATT-3′). Other adenoviruses. Ad5-Δ24E3 was created by homologous recombination between pShuttleΔ24 linearized by Pmel/EcoRI and PacI-linearized pTGT3602, resulting in an otherwise wild-type Ad5 virus but with the 24-bp deletion in E1A [41]. The presence of a 24-bp deletion and the E3 region was confirmed as described. AdCMVHSV-TK is an E1Δ/E3-deleted adenovirus expressing herpes simplex virus thymidine kinase [42]. The absence of wild-type E1 was confirmed with PCR (CRADTrIS, 5′-GATAAT-GAGGGGGTTAGTTTGTG3′; CRAdWT(AS), 5′-GAAAACTCTACGCTGGCAGCACTCA3′). Ad5-Δ24RGD [21] has been described previously. All viruses were purified on cesium chloride gradients. The vp concentration was determined at 600 nm, and standard plaque assay on 293 cells was performed to determine infectious particles. The ratio of vp/infectious particles was 4.7, 3.2, 9.8, and 50 for Ad5/3-Δ24, Ad5-Δ24E3, AdCMVHSV-TK, and Ad5-Δ24RGD, respectively.

**In vitro cytotoxicity assay.** Cells in quadruplicate were infected for 1 h at 37°C in 50 μl of growth medium with 2% FBS. Thereafter, cells were incubated with 5% growth medium. For Fig. 2, cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay; Promega, Madison, WI) on day 6 (SKOV3.ip1), OV-3, day 10 (Hey, ES-2), or day 13 (OV-4). The results with the Ad5/3-Δ24 group were compared to those of the other groups using two-tailed t test (SAS v8.2, SAS Institute, Cary, NY). For Fig. 6, SKOV3.ip1 monolayers were infected with no virus, Ad5/3-Δ24 (1 and 100 vp/cell) or Ad5-Δ24RGD (1 and 100 vp/cell). On days 5, 9, and 13, cell viability was measured as above. Primary ovarian adenocarcinoma cells were purified and cultured as spheroids overnight. After overnight incubation, spheroids were infected with 1 vp/cell (patient A) or 10,000 vp/cell (patient B) of Ad5/3-Δ24 or Ad5-Δ24RGD or no virus in 2% growth medium for 1 h at 37°C [39]. Then, FBS was added in cell suspension to achieve 5% concentration, and the spheroids were divided into aliquots of 105 cells in Costar 96-well ultralow attachment plates (Corning, Inc., Coming, NY). MTS assay was performed on days 8 and 13 (Patient A) or days 9 and 14 (Patient B). All cell viability results are expressed as percentage of viable cells compared to uninfected control at each time point.

**Quantitating virus replication.** Primary ovarian adenocarcinoma cell spheroids were cultured as above. Next day, spheroids were infected with 1000 vp/surface cell of Ad5/3-Δ24, Ad5-Δ24E3, AdCMVHSV-TK, or no virus as above [39]. After 1 h infection, FBS was added in to achieve 10% concentration, and aliquots of 105 cells were seeded in Costar 96-well ultralow attachment plates. Cells and growth medium were harvested and frozen at 1/2, 1, 2, 4, 6, 8, 12, and 16 days after infection. To quantify the total E4 copy number, DNA was purified from spheroid suspension (cellular and growth medium fractions together) using a DNeasy Tissue Kit (Qiagen, Valencia, CA). The primers used for amplifying the E4 were forward, 5′-GGAGT-GGCGCGCAGAACAC-3′, and reverse, 5′-ACTAGTCCGGCGTTTCCT-3′, and detected with the probe 5′-TGCGATGACACTAGCACCACAC-GATCT-3′. Human β-actin was amplified to control for the amount of cells as described [26]. The background values (uninfected spheroids) were subtracted at each time point. To estimate total virus production by the spheroids, cumulative virus copy number was calculated.

**Animals.** Mice were obtained at 3–4 weeks age and quarantined for 2 weeks. Mice were kept under pathogen-free conditions according to the American Association for Accreditation of Laboratory Animal Care guidelines. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of UAB.

**Therapeutic ovarian cancer model.** Female C17 SCID mice (University of Alabama at Birmingham CFAR SCID Mouse Core Facility) were injected ip with 1 × 105 SKOV3.ip1 cells on day 0. On day 10 mice were injected ip with 3 × 105 vp of Ad5/3-Δ24 (n = 10 mice), Ad5-Δ24E3 (n = 10), or AdCMVHSV-TK (n = 9) in 1 ml of Opti-MEM (Mediatech, Herndon, VA). In another experiment, mice were injected ip on days 10, 11, and 12 with 1 × 105 vp of Ad5/3-Δ24 (n = 10 mice), Ad5-Δ24E3 (n = 10), AdCMVHSV-TK (n = 9), or no virus (n = 9) in 1 ml of Opti-MEM. Mice were followed daily and killed when there was any evidence of pain or distress. Survival data were plotted on a Kaplan–Meier curve, and the Ad5/3-Δ24 group was compared with the other.
groups with log-rank analysis and χ² testing (LIFETEST procedure in SAS v8.2, the Weibull model of distribution).

**Bioluminescence imaging to detect in vivo luciferase expression.** The ip tumors were established by injecting 10⁷ SKOV3-SCID cells ip into female CB17 SCID mice (Charles River Laboratories, Wilmington, MA; n = 4/group) on day 0. On day 8, 3 ip tumors that contained sc tumors. Bioluminescent imaging was performed before treatment on day 7 and then weekly until untreated mice showed signs of distress (day 45). On day 45, images were also collected after removing the abdominal wall that contained sc tumors. Briefly, 150 mg/kg D-luciferin was injected ip, and mice were anesthetized with enflurane gas anesthesia and placed in a light-tight chamber. The photographic (gray-scale) reference image was obtained at 15 min after D-luciferin injection, and the bioluminescent image was collected immediately thereafter. Images were obtained with a CCD cooled to −120°C, using the IVIS Imaging System (Xenogen Corp., Alameda, CA), with the field of view set at 25 cm height. The photographic images used a 0.25-s exposure, 8 fstop, 2 binning (resolution), and open filter. The bioluminescent images used exposures ranging from 0.5 to 10 s, 1 fstop, 2 binning, and open filter. The bioluminescent and gray-scale images were overlaid using LivingImage software (Xenogen Corp.). Image analyses software (WaveMetrics, Lake Oswego, OR) was also used to obtain a pseudocolor image representing bioluminescence intensity (blue, least intense, and red, most intense). Regions of interest were drawn around the ip tumors, and the total counts (photons) were summed in the entire tumor areas. The total counts were normalized to image acquisition time.

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