

Adenoviral Gene Therapy for Renal Cancer Requires Retargeting to Alternative Cellular Receptors¹

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ABSTRACT

Metastatic renal cell carcinoma (RCC) is one of the most treatment-resistant malignancies in humans. Therefore, the identification of new agents with better antitumor activity merits a high priority in the treatment of advanced RCC. In this regard, gene therapy with adenoviral (Ad) vectors is a promising new modality for cancer. However, a primary limiting factor for the use of Ad vectors for cancer gene therapy is their critical dependence on cellular expression of the primary Ad receptor, the coxsackie and adenovirus receptor (CAR), known to be down-regulated in many cancer types. Following the identification of CAR deficiency in RCC lines, we have found abundant membrane expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and of the putative receptor to Ad serotype 3 (Ad3). As an alternative gene therapy approach for RCC that would circumvent CAR deficiency, we employed retargeting of replication-incompetent Ad vectors and replication-competent Ad viruses to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and to the putative Ad3 receptor. These strategies to genetically alter Ad tropism were based on either the insertion of a cysteine-aspartate-cysteine-arginine-glycine-aspartate-cysteine-phenylalanine-cysteine (RGD) motif into the HI loop of the Ad fiber knob domain or on generation of a chimeric Ad fiber composed of adenovirus serotype 5 shaft/Ad3 knob. Both strategies proved highly efficient to circumvent CAR deficiency and enhance gene delivery into RCC cells. Furthermore, in the context of replication-competent Ad, tropism alteration resulted in distinct capacity of the retargeted viruses to infect, replicate, and lyse RCC models *in vitro* and *in vivo*. The retargeting strategies were particularly beneficial in the context of replication-competent Ad. These findings underscore the importance of CAR-independent cellular entry mechanisms in RCC and are highly consequential for the development of viral antitumor agents for RCC and other CAR-negative tumors.

INTRODUCTION

Approximately 30,000 new cases of RCC³ are diagnosed each year in the United States and 12,000 die of their metastatic disease annually. Despite extensive evaluation of many different treatment modalities, metastatic RCC remains highly resistant to systemic therapy, and the 5-year survival is 5–10% (1–3). No single agent or combination therapy has consistently shown a response proportion of $\geq 20\%$. While interleukin-2- and IFN- α -based therapies are most commonly used to treat advanced disease, only low response rates are observed, with durable responses of $\leq 5\%$. Therefore, the identification of new

agents with better antitumor activity merits a high priority in the treatment of advanced RCC. In this regard, gene therapy is a promising new modality for cancer, whereby transfer of immunomodulatory, tumor suppressor or suicide genes may alter the natural course of tumors. Current gene therapy approaches for RCC are based on harnessing the immune system for therapeutic recognition of RCC tumor-associated antigens (4). In contrast, other emerging gene therapy approaches for resistant tumors involve the direct cancer cell killing by replicating viruses. This approach has been previously reported for RCC only in the context of the neurotrophic herpes virus, tested empirically for all tumors derived from the urinary tract (5). In this regard, because the efficacy of cancer gene therapy critically depends on the infection rate of target tumor cells (6), the mechanism of vector entry into RCC cells is of primary significance. The cellular tropism of Ad5, a principal viral vector for cancer gene therapy, is primarily dictated by CAR recognition (7).

After anchoring at the receptor site, following binding of the fiber knob domain to CAR (7), the virus achieves internalization via interaction of the RGD peptide, located in the capsid penton base, with cellular membrane α_v integrins (8). On the basis of this, relative deficiency of target cell receptors limits the spectrum of Ad infection. Therefore, CAR deficiency emerges as a limiting factor for the use of Ad vectors in the context of cancer gene therapy (6, 9). One means to circumvent this biological limitation is the redirection of Ad vectors to target cancer cells via alternative cellular receptors. We, and others, have previously reported two distinct genetic retargeting approaches to alter the tropism of replication-deficient Ad vectors. First, a chimeric Ad vector chimera was generated displaying a recombinant Ad5 shaft/Ad3 knob fiber (10, 11). Second, after the identification of specific binding of the peptide RGD4C to various integrins (12), this peptide has been incorporated into the fiber shaft (13) or the knob of Ad5 (14). Both these approaches to modify Ad tropism have proven highly efficient for CAR-independent cellular entry in the context of replication-deficient Ad. In this regard, these approaches are now highly consequential for cancer gene therapy in recognition of the nearly universal finding of CAR deficiency in epithelial neoplasms (8, 15, 16). Furthermore, the absence of CAR not only inhibits Ad uptake but is also associated with invasive cancer phenotypes (16, 17). In accordance with these observations, the efficacy of Ad-mediated cancer gene therapy has been limited in preclinical and clinical studies (18, 19). In addition to their use as gene delivery vehicles, Ad viruses have also been used as replicative agents to achieve direct tumor killing.

In this regard, CAR deficiency would not only limit the infection efficiency of the initial viral inoculum, but more importantly, the potential therapeutic advantages afforded by viral replication would be negated by poor intratumoral spread of the viral progeny. In accordance with this concept, Phase I and II clinical trials, where patients with recurrent head and neck cancer had received direct intratumoral injection of attenuated replicating Ad5 viruses (ONYX-015), have resulted in clinical benefit in $<15\%$ of cases. Furthermore,

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³ The abbreviations used are: RCC, renal cell carcinoma; Ad, adenovirus; Ad5, Ad serotype 5; Ad3, Ad serotype 3; Adwt, Ad wild-type; CAR, coxsackie and adenovirus receptor; RGD, cysteine-aspartate-cysteine-arginine-glycine-aspartate-cysteine-phenylalanine-cysteine; GFP, green fluorescent protein; MOI, multiplicity of infection; Ni-NTA, nickel-nitrilotriacetic acid; DC, detergent compatible; CPE, cytopathic effect; vp, viral particle; pfu, plaque-forming unit; mAb, monoclonal antibody.

in patients with pancreatic and ovarian tumors, ONYX-015 did not appear to replicate at all (20, 21).

On the basis of these considerations, we have studied CAR expression in RCC cell lines and have found a dramatically low cellular CAR expression. In contrast, alternative cellular receptors were identified in these RCC lines. Consequently, we have investigated in this study the hypothesis that genetic Ad retargeting to alternative cellular receptors would enhance both Ad-mediated gene delivery and oncolysis of RCC cells. This strategy allowed the first direct evaluation of the use of tropism-modified Ad vectors to enhance RCC transduction efficiency. Furthermore, this study establishes the concept of the use of tropism alteration in the context of replicative Ad viruses for cancer gene therapy.

MATERIALS AND METHODS

Viruses. Generation and characterization of Ad5luc1, Ad5/3luc1, and Ad5RGDluc1 have been described previously (10, 14). These replication-incompetent Ad vectors are isogenic and contain similar luciferase cassettes replacing the E1 locus, whereas their capsid has been modified genetically. Ad5/3luc1 was constructed to display a chimeric fiber, composed of Ad5 shaft and Ad3 knob. Tropism of Ad5RGDluc1 was altered via insertion of an RGD peptide into the HI loop of the Ad5 fiber knob. The vp:pfu titer ratio for these vectors was <25:1. Ad5GFP and Ad5RGDGFP both contain the jellyfish GFP cassettes but differ in RGD peptide incorporation into the Ad5 fiber knob. The vp:pfu titer ratio of both vectors was <35. Ad5luc3 is a replication-competent Ad5 virus, differing from Adwt by the replacement of the E3 region with the cytomegalovirus promoter-driven *luc* gene. Ad5RGDwt is an E1⁺, E3⁺ Ad5 incorporating an RGD as above. Ad5/3luc3 is an E1⁺, E3⁻ chimeric Ad, displaying Ad5 shaft/Ad3 knob.

Cells, Transfections, and Infections. The human RCC cell lines ACHN, A498, CaKi-1, and SW157 were purchased from the American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 growth media supplemented by 10% fetal bovine serum, and 2 mM L-glutamine, sodium pyruvate, sodium bicarbonate, glucose, and 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY). Cells were grown at 37°C in a 5% CO₂ humidified incubator. SN12C, a highly metastatic RCC line, was described previously (22) and grown as above.

Infections were performed 24 h after seeding 2 × 10⁵ cells/well in 12- or 24-well plates. For infections, growth medium was replaced by serum-free medium with the index virus at the indicated MOI. One h later, the infection media was removed, cells were rinsed with PBS, and 5% fetal bovine serum growth media was restored. The media was not replaced thereafter during the experiment. At the indicated time points, cells and media were collected and analyzed for transgene expression or Ad5 E1 gene copy numbers.

Determination of Receptor Expression by Flow Cytometry. RCC cells were rinsed with PBS, harvested by incubating with 0.53 mM EDTA in PBS, and resuspended in PBS containing 1% BSA (Sigma Chemical Co.). For antibody incubation, 2 × 10⁵ cells were incubated with RmcB (1:80), LM609 (1:100) or P1F6 (1:100) for 1 h at 4°C. An isotype-matched normal mouse IgG₁ (1:100) was used as a negative control. The cells were then rinsed with PBS-BSA, and incubated with 1:100 dilution of FITC-labeled goat antimouse IgG for 1 h at 4°C. After another PBS rinse, 2.6 μg/ml propidium iodide (Sigma Chemical Co.) was added to sort out dead cells from the sample. Next, 10⁴ live cells were analyzed immediately by flow cytometry. Relative mean fluorescence intensity was calculated as the ratio of the mean fluorescence intensity of the sample of the interest to the mean fluorescence intensity of the corresponding negative control for each cell line. Cell surface expression of CAR and the Ad3 receptor was measured by knob binding assay as described previously (11). Briefly, cells were incubated with 20 ng of 6× His-tagged recombinant Ad5 or Ad3 knob protein or with buffer only, followed by incubation with 1:125 dilution of Tetra-His-Antibody (Qiagen, Santa Clarita, CA).

Finally, cells were incubated with FITC-labeled goat antimouse IgG (Sigma Chemical Co.). All the incubations were performed for 1 h at 4°C. In the negative control, cells were incubated with primary and secondary antibodies without the knob protein. The FITC-positive (live) cell population for each cell

line was determined by gating cells incubated with buffer only (negative control) at 1%.

Antibodies. The anti-CAR mAb RmcB was produced using hybridoma purchased from American Type Culture Collection. Murine mAb LM609 to α_vβ₃ integrin and P1F6 to α_vβ₅ integrin were both purchased from Chemicon (Temecula, CA). Normal mouse IgG₁ and FITC-labeled goat antimouse IgG were from Sigma Chemical Co.

Recombinant Fiber Knob Proteins. Recombinant Ad5 and Ad3 fiber knob proteins were expressed in *Escherichia coli* using the pQE30 expression vector (Qiagen, Valencia, CA) and purified on nickel-nitrilotriacetic acid (Ni-NTA) agarose columns (Qiagen) as recommended by the manufacturer and described elsewhere (10). The concentration of the purified proteins was determined by Bio-Rad detergent compatible (DC) protein assay (Bio-Rad, Hercules, CA). The ability of each knob protein to form a homotrimer was verified by Western blot of unboiled samples.

Ad Gene Transfer Assays. To assess the efficiency of reporter gene transfer by Ad vectors into RCC or control cell lines, 1 × 10⁵ cells were plated in 24-well plates and allowed to adhere overnight at 37°C. On the next day, media were removed and cell monolayers were washed once with PBS. Cells were infected for 1 h at an MOI of either 10, 100, or 1000 vp/cell, with either no virus, Ad5luc1, or the tropism-modified Ad vectors AdRGDluc1 or Ad5/3luc1. Control infections and dilutions were performed in Opti-MEM (Life Technologies, Inc.).

One h later, cells were washed once with PBS and overlaid with growth medium. After 48 h at 37°C in a 5% CO₂ humidified incubator, cells were rinsed with PBS and lysed with 25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% glycerol, and 1% Triton X-100. Cell lysates were assayed for luciferase expression in a luminometer (Berthold, Bad Wildbad, Germany), using Luciferase assay system (Promega, Madison, WI), and total protein concentration was determined using the DC protein assay (Bio-Rad), according to the manufacturer's instructions. Light emission was detected in live CaKi-1 cells, 48 h after infection with either Ad5/3luc1 or Ad5luc1, as reported previously (23). GFP expression was detected with a Leica fluorescence stereo microscope equipped with a 50-W mercury lamp for high magnification imaging of GFP-expressing RCC cells. Selective excitation of GFP was produced through a D425/60 bandpass filter. Emitted fluorescence was collected through a long pass filter on a Hamamtsu cooled-charged-coupled device camera. Images were processed accordingly for contrast and brightness with Adobe Photoshop 6.0 software.

Ad Cell-Killing Assays. (Cells) 1 × 10⁵ of ACHN, CaKi 1, SW157, or SN12C were seeded in 12-well plates. Twenty-four h later, growth medium was replaced by serum-free medium with the index virus at the indicated MOI. One h later, the infection media were removed, cells were rinsed with PBS, and 5% fetal bovine serum growth media were restored. The media were not replaced thereafter during the experiment. Once an advanced CPE was observed for any of the wells, simultaneous crystal violet stains were performed.

TaqMan PCR Assay. E1a copy number was determined for each sample obtained from collected cells and media as of the first day after infection. Viral genomic DNA was isolated and cleaned using a Qiagen Tissue kit (Qiagen), following instructions of the manufacturer. Concentration of isolated DNA was determined by spectrophotometry. TaqMan and probe primers were designed as follows: the forward primer; reverse primer; and 6-carboxyfluorescein-labeled probe to amplify the *E1a* and *E4* genes were designed by the Primer Express 1.0 software (Perkin Elmer, Foster City, CA) following the recommendations of the manufacturer. The sequences of the forward and the reverse E1a primers were ACCAGTTGCCCTGAGAGTTG (anneals between 966 and 986) and CTCGTTAAGCAAGTCCTCGATACAT (anneals between residues 1033 and 1009), respectively, whereas the TaqMan probe was CACAGCCTGGCGACGCCA (anneals between residues 988 and 1006). With optimized concentration of primers and probe, the components of Real-Time PCR mixture were designed to result in a master mix with a final volume of 10 μl/reaction containing 1× Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 100 nM forward primer, 100 nM reverse primer, 1 nM probe, and 0.025% BSA. For the assay, 1 μl of extracted DNA sample was added to 10 μl of PCR mixture in each reaction capillary. A no-template control received 10 μl of reaction mixture with 1 μl of water. All capillaries were then sealed and centrifuged using LC Carousel Centrifuge (Roche Molecular Bio-

chemicals, Indianapolis, IN) to facilitate mixing. All PCR reactions were carried out using a LightCycler System (Roche Molecular Biochemicals).

The thermal cycling conditions were 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

Animal Models. To determine the effect of viral preinfection in RCC tumor growth, we infected CaKi-1 cells at a confluence of 60–70% with Ad5luc3 or Ad5/3luc3, at an MOI of 1 pfu/cell, for 1 h. Cells were allowed to grow for 24 h and then 5×10^6 CAKi-1 cells were injected s.c. into the flanks of athymic nude mice. To determine the effect of viral injection into pre-established RCC tumors, we followed the surgical implantation model for RCC (24). Briefly, 6–8-week-old female athymic *nu/nu* mice were injected s.c. with 5×10^6 CaKi-1 cells. The purpose of growing s.c. tumors was to generate a stock of histologically intact tumors for the surgical implantation model. When tumors were growing in the log phase, the animals were sacrificed and tumors harvested. The periphery of the tumor tissue was collected after removal of necrotic tissue near the center of the tumors. The tumor tissue was cut into small pieces of 1 mm³ each and implanted into athymic mice s.c. under anesthesia with xylazine/ketamine. This method allowed a relatively uniform growth of the RCC tumors. Tumor volume was calculated using the simplified formula for a rotational ellipsoid ($0.5 \times l \times w^2$) (25). On reaching ~ 80 mm³, tumors were injected with 3 weekly 50- μ l doses of either PBS (4 mice), Ad5luc3 in PBS (5 mice), or Ad5/3luc in PBS (7 mice). Bidimensional tumor measurements were taken twice weekly with calipers. Animals were sacrificed when tumor burden became excessive. Experiments were performed in accordance with federal guidelines for animal care and approved by the institutional animal care and use committee.

Immunohistochemistry. RCC xenografts were embedded in OCT compound, frozen, cut into 5 μ m-thick sections, and fixed in 4% paraformaldehyde. Tissues were blocked with 1% BSA in PBS for 30 min before each antibody incubation. Primary and secondary antibody incubations were for 30 min at 37°C. The hexon capsid protein of Ad5luc3 and Ad5/3luc3 was determined in infected CAKi-1 tumor sections by immunohistochemical analysis, using polyclonal goat anti-hexon (Chemicon) as the primary antibody and a FITC-labeled donkey anti-goat (Molecular Probes, Eugene, OR) as a secondary antibody. Nuclei were stained with Hoechst 33258 (Molecular Probes). Images were acquired on a Leitz orthoplan microscope (Leica, Inc., Wetzlar, Germany) and processed accordingly for contrast and brightness with the Adobe Photoshop 6.0 software.

Statistical Methods. Tumor volume (mm³) for the three injected RCC xenograft groups was recorded for 6 weeks. Descriptive statistics (mean, SD, and SE) on tumor volume were calculated for each measurement in each group. Using two approaches, tests of repeated measures were performed to compare the mean tumor volumes between the following groups: (a) Ad5/3luc3 and the control group Ad5luc3; (b) Ad5/3luc3 and PBS; and (c) PBS and Ad5luc3. In the first approach, time was assigned as a continuous variable. The second approach set time as a discrete variable. The model with time as a continuous variable provided a parsimonious mathematical form to characterize the evolution of response measure over time. The model with time discrete variables showed whether mean tumor volume for one group was larger or smaller than a second group at each time point.

$P < 0.05$ was considered statistically significant in all of the analyses. All tests were performed using SAS software (version 8.0; SAS Institute, Inc., Cary, NC).

RESULTS

RCC Lines Show Resistance to Infection by Ad5. To evaluate Ad5 vectors as gene delivery vehicles in the context of RCC, we tested their infection efficiency. We studied transgene delivery into RCC, using the Ad vector Ad5luc1, in several RCC lines compared with the lung cancer cell line A549, known to display an intermediate level of susceptibility to infection by Ad5 vectors (26). In these studies, whereas the control A549 cell line demonstrated high levels of Ad5-mediated gene delivery, the human RCC lines have shown relative resistance to Ad5 infection (Fig. 1A). These experiments questioned the adequacy of replication-deficient Ad5 for gene delivery into RCC lines. Next, we hypothesized that the relative resistance of RCC lines to Ad5 vectors may be further highlighted in the context

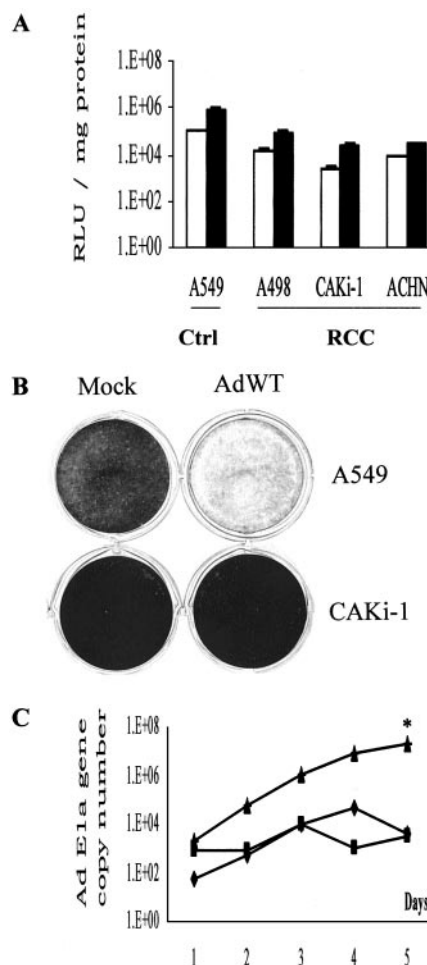


Fig. 1. Human RCC lines show resistance to infection with Ad5. **A**, analysis of the infection efficiency of replication-deficient Ad5 in RCC lines. The human RCC lines CaKi-1, ACHN, and A498 were infected in triplicates at MOIs of 100 (□) or 1000 (■) vp/cell, with the replication-deficient Ad vector Ad5luc1. The human lung adenocarcinoma cell line A549 served as a control cell line to evaluate the infection efficiency. **B**, analysis of the cell killing potency of replication-competent Ad5 viruses in the RCC CaKi-1 cell line and the lung cancer cell line A549. Cells were infected at an MOI of 1 with AdWT. Cells were stained with crystal violet 10 days after infection. Mock indicates mock infection. **C**, analysis of the replication kinetics of Ad5 viruses in RCC lines or the control A549 cell line. CaKi-1 cells were infected at an MOI of 10 with Ad5luc3 (◆) or AdWT (■), whereas A549 cells were infected with Ad5luc3 (▲). Cells and media were collected in triplicates from the different cohorts and subject to quantitative PCR analysis of Ad E1a copy number as an index of Ad replication. *, $P < 0.05$.

of replication-competent Ad5. To this end, we infected the RCC lines CaKi-1, SN12C, and SW157 with replication-competent Ad5 viruses either containing the E3 region (wild-type Ad5) or E3-deleted (Ad5luc3). We observed in these experiments that under the conditions we used, viral CPE could not be demonstrated even after prolonged periods. In contrast, these viruses efficiently killed cells of other lineages, such as the human lung cancer cell line A549, and the human cervical cancer cell line HeLa, both known to express CAR (Fig. 1B). Consequently, we hypothesized that the variations in the cell killing properties of the replication-competent Ad5 virus may be accounted for by different patterns of viral infection and replication in the various cell lines. To study the kinetics of Ad5 replication in RCC and control cell lines, we collected the cellular and the media fractions after infection with replicative Ad5 virus and measured Ad5 DNA over time.

Evaluation of the viral DNA copy number of the replication-competent Ad5 indicated that under these conditions, Ad5 replication in RCC lines was largely restricted relative to the control, well-

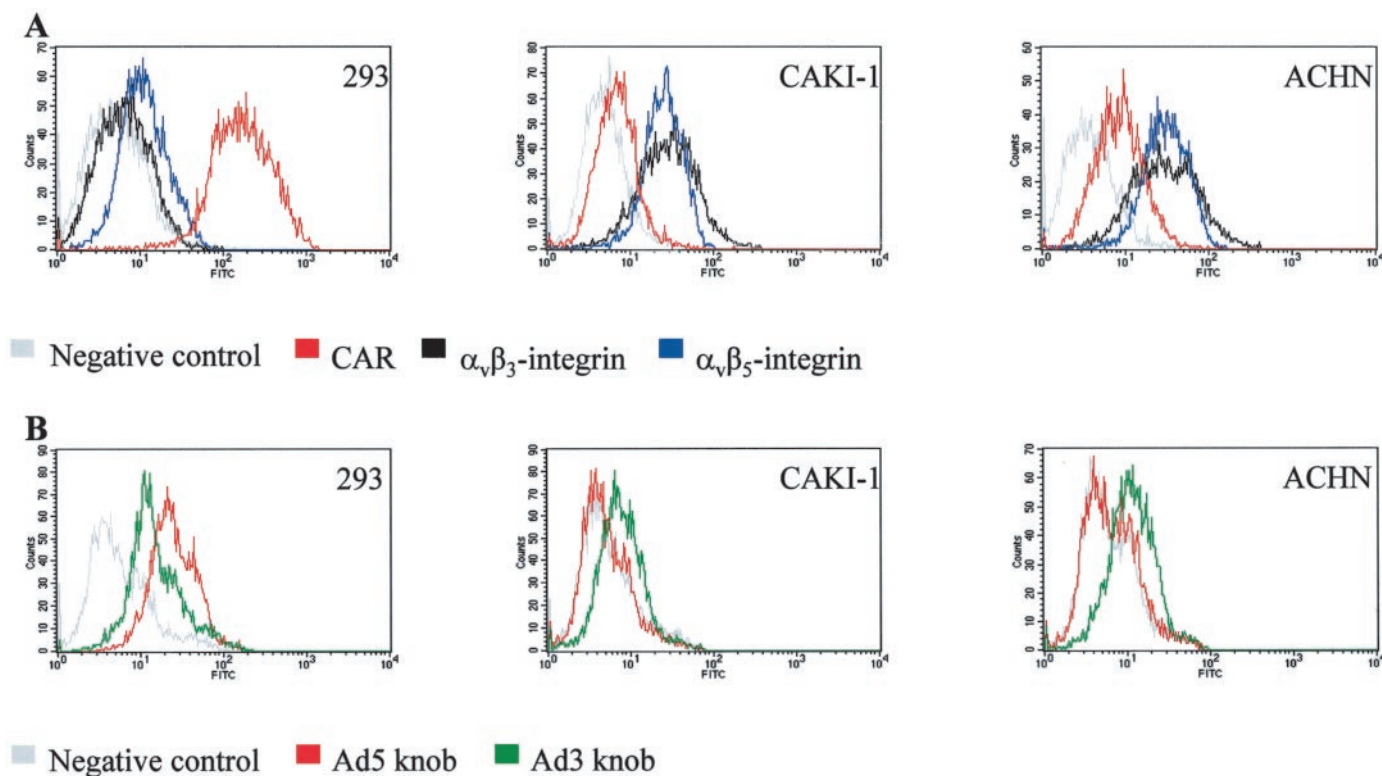


Fig. 2. Human RCC lines do not express CAR but express alternative Ad receptors. *A*, expression of CAR (red) and the integrins $\alpha_v\beta_3$ (black) and $\alpha_v\beta_5$ (blue) was measured in the human RCC lines CaKi-1 and ACHN or in 293 control cell line with indirect flow cytometry. Cells were incubated with the mAbs RmcB (CAR), LM609 ($\alpha_v\beta_3$), or P1F6 ($\alpha_v\beta_5$), followed by detection with FITC-labeled secondary antibody. *B*, the same cell lines were analyzed with modified, three step flow cytometry, whereby cells were first incubated with recombinant Ad5 knob (red) or Ad3 knob (green), then with primary antiknob antibodies, and finally with secondary FITC-labeled antibodies. Normal mouse serum was used as a control in both experiments (gray line).

infectible A549 cells (Fig. 1C). Because several Ad5 viruses, differing in transcriptional regulation but sharing the Ad5 capsid, could efficiently replicate in CAR-expressing cells but not in the RCC lines we tested, the underlying mechanism is compatible with distinct infection patterns stemming from variable expression of Ad receptors. Thus, these studies have demonstrated that although RCC lines are relatively resistant to replication-deficient Ad vectors based on the wild-type Ad5 capsid, their resistance to replication-competent Ad5 viruses is striking.

Resistance of RCC Lines to Ad5 is Because of CAR Deficiency. To establish the biological basis for the resistance of RCC lines to Ad5, we studied the expression of Ad receptors with indirect flow cytometry, using antibodies specific for CAR and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Because RCC typically derives from the renal proximal tubule, we selected the stably transformed embryonic renal tubular 293 cell line as a control. We found that although 293 cells expressed high levels of CAR, the renal cancer cells displayed dramatically reduced levels of CAR (Fig. 2A). We confirmed these findings using knob-binding assays as an alternative approach for CAR measurement. In these studies, functional CAR expression, as determined indirectly by Ad5 knob binding, was undetectable in RCC lines unlike in 293 cells (Fig. 2B). To determine the cell surface expression of alternative Ad receptors, we measured the expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and the putative Ad3 receptor in the same RCC and 293 cell lines. We found in 293 cells that the expression of $\alpha_v\beta_3$ is extremely low and of $\alpha_v\beta_5$ is moderately low.

In contrast, the cell surface expression of these integrins in RCC lines is up-regulated (Fig. 2A). The expression of the putative Ad3 receptor was not dramatically altered in RCC lines when compared with 293 cells. However, because the expression of Ad3 receptor was

maintained in RCC lines, its relative ratio to CAR was increased (Fig. 2B). Taken together, these findings provide a biological basis for the resistance of RCC to Ad5 and imply that the cell surface expression of alternative Ad receptors may provide potential targets for CAR-independent Ad infection.

Resistance of RCC to Ad5 Can Be Circumvented by Retargeting Ad Vectors to Alternative Cellular Receptors. The paucity of the primary Ad receptor CAR on RCC lines mandates the consideration of alternative pathways. To enhance Ad infection, we employed genetic approaches to generate two tropism-modified Ad vectors. Redirection strategies to α_v integrins and the putative Ad3 receptor were based on genetic modification of the viral fiber knob (10, 14). We employed two types of vectors with retargeting motifs. Specifically, one strategy comprised an RGD4C peptide insertion into the HI loop of the Ad5 fiber knob (Fig. 3, A and B). In the other retargeting strategy, a chimeric Ad5/3 vector was constructed composed of the Ad3 knob and the Ad5 shaft. For each approach, replication-deficient vectors and replication-competent viruses were developed. To evaluate the use of tropism-modified Ad vectors to infect RCC lines, we compared gene delivery by the replication-incompetent, tropism-modified vectors AdRGDluc1 and Ad5/3luc1, relative to the replication-incompetent, nontropism-modified Ad5luc1 (Fig. 3, C–F). In accordance with the observed CAR deficiency in RCC, gene delivery by the nontropism-modified Ad5luc1 was low.

In contrast, the RCC lines tested demonstrated the superiority of the retargeted vectors AdRGDluc1 and Ad5/3luc1. However, in the A498 cell line, AdRGDluc1 was not superior to Ad5luc1 (Fig. 3, C). We further confirmed the use of tropism-modified vectors for RCC with real-time determination of gene expression in live RCC cells (Fig. 3,

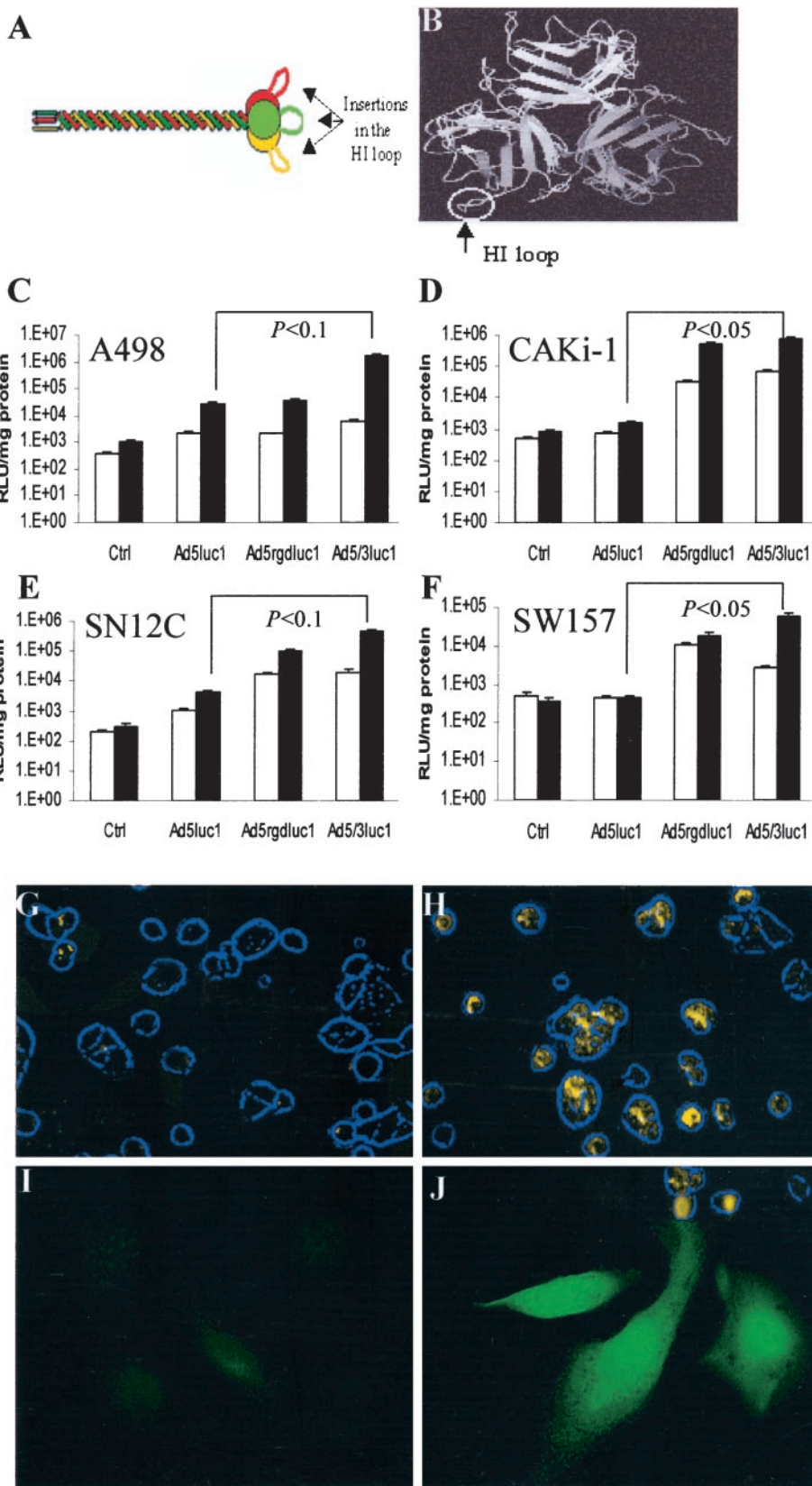


Fig. 3. Tropism modification of replication-deficient Ad vectors achieves CAR-independent RCC infection. *A* and *B*, structure of Ad fiber protein inclusive of its knob domain and the strategy to alter adenoviral tropism. *A*, the fiber protein is a homotrimeric molecule, which consists of three distinct structural domains: the tail; the shaft; and the knob. The knob domain fulfills double duties by maintaining trimerization of the fiber and binding to CAR. *B*, three-dimensional model of the fiber knob domain. The flexible HI loop (circle), which connects strands H and I, is exposed outside the knob, thereby providing a convenient locale for incorporation of the RGD-targeting ligand. *C–F*, analysis of RCC lines infection by Ad vectors expressing luciferase. RCC monolayers were infected with 10 MOI (□) or 100 MOI (■) of the replication-deficient vectors, either unmodified vector Ad5luc1 or the tropism-modified vectors Ad5/3luc1 or AdRGDluc1. Ctrl represents mock-infected cells. The relative light units of luciferase/milligram of total cellular protein are shown graphically as the mean of multiple assays. RLU, relative light unit. *G–J*, real-time detection of gene expression in live RCC cells infected with replication-deficient Ad vectors. *G* and *H*, CaKi-1 monolayers were infected at an MOI of 10 with the luciferase-expressing vectors, either the unmodified Ad5luc1 (*G*) or the tropism-modified Ad5/3luc1 (*H*). Twenty-four h later, luciferin in aqueous solution was added to the medium, followed immediately with light detection by a cooled-charge-coupled device camera connected to an Olympus IX70 inverted epifluorescence microscope. The light signals were merged with photomicrographs of cell membranes, captured with bright field microscopy. Light signals were pseudocolored into yellow, and cell membranes were pseudocolored to blue. All light sources were exclusively detected in cells. *I* and *J*, real-time detection of fluorescence from intact RCC cells infected with replication-deficient Ad vectors. CaKi-1 monolayers were infected at an MOI of 10 with the GFP-expressing vectors, either the unmodified Ad5GFP (*I*) or the tropism-modified AdGFPRGD (*J*). Twenty-four h later, cells were captured for selective excitation of GFP.

G–J). Thus, tropism-modified Ad vectors can achieve CAR-independent cellular entry in RCC lines.

Replication-competent, Tropism-modified Ad Viruses Efficiently Infect, Replicate in, and Kill RCC Lines *in Vitro*. To evaluate the use of tropism modification for RCC killing, we em-

ployed replication-competent Ad viruses. Replication-competent Ad viruses have been suggested as a means to overcome the multidimensional structure of tumors that impairs the use of replication-incompetent vectors for cancer (27, 28). Consequently, we hypothesized that viral replication may further increase the therapeutic effect of tropism

modification in RCC. To this end, we tested RCC lines killing *in vitro* with crystal violet assays. Specifically, the RCC lines CaKi-1, SW157, and SN12C were infected with replication-competent Ad viruses, either tropism modified or based on the Ad5 capsid. The tropism-modified Ad viruses included either Ad5/3luc3 or AdwtRGD. These replicating viruses differ by the deletion of the E3 region (E3 is replaced in Ad5/3luc3 by cytomegalovirus-driven luciferase). As controls, we infected RCC lines with the matching, noncapsid-modified, replication-competent Ad5 viruses Ad5luc3 and Adwt, respectively. Under the conditions we tested, we observed that only the tropism-modified viruses could kill the RCC lines, whereas viral CPE could not be demonstrated after infection with the unmodified replicative Ad5 viruses (Fig. 4, A, B).

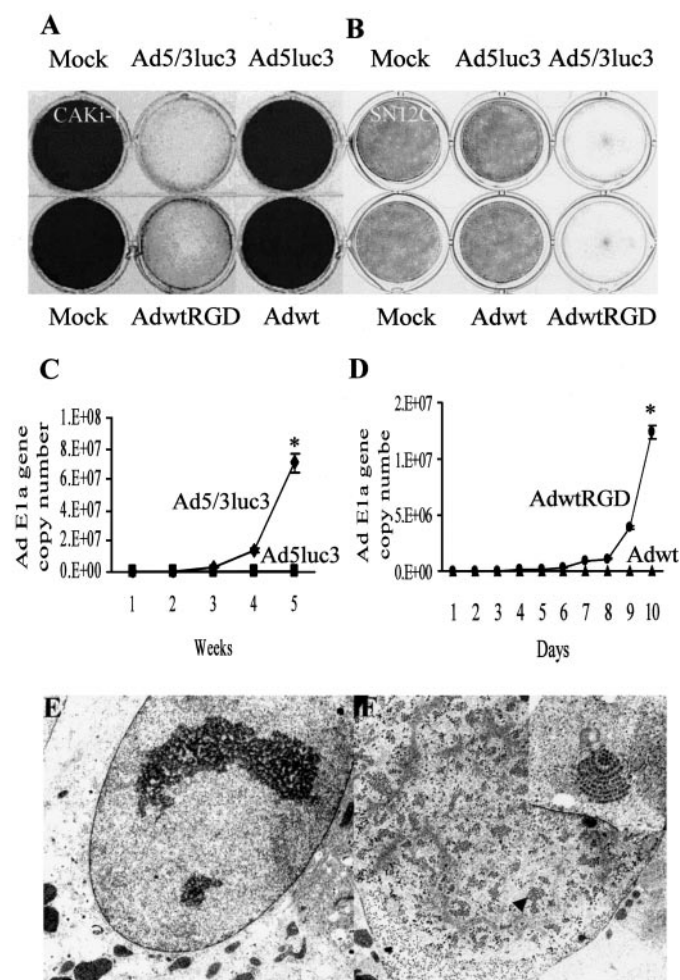


Fig. 4. Replication-competent, tropism-modified Ads circumvent RCC resistance to Ad5-mediated cell killing. **A** and **B**, direct analysis of RCC cell killing by replication-competent Ad viruses. Monolayers of the RCC lines CaKi-1 (**A**) or SN12C (**B**) were infected at an MOI of 1 in two different sets of experiments. The tropism-modified Ad5/3luc3 ($E1^+$, $E3^-$) was tested *versus* its control Ad5luc3 ($E1^+$, $E3^-$), and the tropism-modified AdRGDwt ($E1^+$, $E3^+$) was tested *versus* its control Adwt ($E1^+$, $E3^+$). Mock represents mock-infected cells. Crystal violet staining was performed once advanced CPE was observed for any of the wells. **C** and **D**, analysis of viral replication kinetics as a function of tropism modification. **C**, monolayers of the RCC line CaKi-1 were infected at an MOI of 1 with either the replicative, tropism-modified Ad5/3luc3 (●) or the replicative, nontropism-modified Ad5luc3 (■). Ad DNA was measured after collection of the cellular and media fractions. The E1a gene copy numbers are shown graphically as the mean of multiple assays. **D**, monolayers of the RCC line CaKi-1 were infected at an MOI of 0.1 with either the replicative, tropism-modified AdwtRGD (●) or the replicative, nontropism-modified Adwt (▲). **E** and **F**, transmission electron microscopy showing nucleus devoid of viral particles in CaKi-1 cells infected with the replicative, unmodified Ad5luc3 (**E**), or nuclei containing *de novo* synthesized virions in CaKi-1 cells infected with the replicative retargeted Ad5/3luc3 (arrowhead) (**F**). Magnification $\times 7000$. Inset in **F**, assembly of *de novo* Ad5/3luc3 virions in CaKi-1 cells, $\times 20,000$. *, $P < 0.05$.

Next, we hypothesized that the selective RCC killing capacity of Ad5/3luc3 and AdwtRGD stems from their selective infection and replication in RCC lines. To evaluate viral kinetics, we measured Ad DNA in cells and media collected from RCC lines infected with either replication-competent, tropism-modified Ad viruses or the matching replicative Ad5 viruses as controls. While the tropism-modified viruses replicated efficiently in RCC lines, these cells restricted the replication of nonmodified Ad5 viruses, indicating cellular resistance to viral infection (Fig. 4, **C** and **D**). Electron microscopy confirmed that *de novo* virion formation in Ad-infected RCC lines was restricted to the tropism-modified Ad viruses (Fig. 4, **E** and **F**). Thus, these studies have established that the mechanism of CAR-independent RCC killing, using the strategy of retargeting replication-competent Ad viruses to alternative cellular receptors, involves selective viral infection and replication.

Superiority of Retargeted Ad Viruses in a Human RCC Model *in Vivo*. To study the potency of retargeted Ad viruses in the context of human RCC tumors *in vivo*, we used two different methods. First, we preinfected the RCC CaKi-1 cells with either the replicative control virus Ad5luc3 or the replicative tropism-modified chimeric virus Ad5/3luc3. Next, we injected the cells *s.c.* into the flanks of athymic nude mice. While Ad5luc3-treated CaKi-1 cells formed progressive tumors, the Ad5/3luc3 treated-CaKi-1 cells did not form tumors (Fig. 5, **A–C**). To further evaluate the usage of replication-competent tropism-modified Ad viruses for established RCC tumors *in vivo*, we used on a previously reported model for a human RCC in athymic mice (24).

This model allows a relatively uniform tumor growth after surgical implantation of comparable pieces of pre-established, histologically intact tumors. These tumor fragments include matrix and vascular supply, thereby quickly forming *s.c.* tumors. When reaching a volume of $\sim 80 \text{ mm}^3$, the tumors were injected with Ad5luc3 or the chimeric Ad5/3luc3 in three divided doses of 1×10^9 viral particles. Partition of the viral dose was required to improve the therapeutic outcome relative to a single injection (29). After three weekly injections, only the replicative chimera Ad5/3luc3 could significantly limit the growth rate of pre-established CaKi-1 tumors (Fig. 5**D**). With time as a continuous variable, it was shown that the difference in tumor size at baseline between groups injected with Ad5/3luc3 or Ad5luc3 was not significant ($P = 0.0952$). However, the difference over time (slope) in tumor growth between these two groups was highly significant ($P < 0.0001$). There was also a highly significant difference in growth slopes between Ad5/3luc3 and PBS-injected xenografts ($P < 0.0001$). There was no significant difference in baseline or growth slope when we compared Ad5luc3 to PBS-injected tumors ($P = 0.3475$, $P = 0.1122$, respectively). Of note, even the replicating tropism-modified Ad virus could not completely eradicate pre-established RCC tumors. This finding should be probably interpreted in the context of RCC xenograft invasion by host mouse fibroblasts building up solid strands of connective tissue (30, 31), which are likely to interfere with intratumoral viral propagation.

To confirm that the RCC xenograft growth inhibition induced by Ad5/3luc3 was because of intratumoral spread of the progeny of Ad viruses, tumor sections were analyzed for *de novo* synthesized Ad capsid proteins. Immunohistochemical staining for *de novo* Ad capsid protein hexon indicated that the chimeric Ad5/3luc3, but not the unmodified Ad5luc3, replicated and disseminated throughout the RCC xenograft (Fig. 5, **E** and **F**). Hexon staining was predominantly either nuclear, possibly indicating virion assembly, or perinuclear, compatible with hexon localization to the rough endoplasmic reticulum (Fig. 5**G**). Taken together, these studies have shown that the enhanced oncolytic potency of the replicating Ad5/3luc3 was because of intratumoral viral replication and spread.

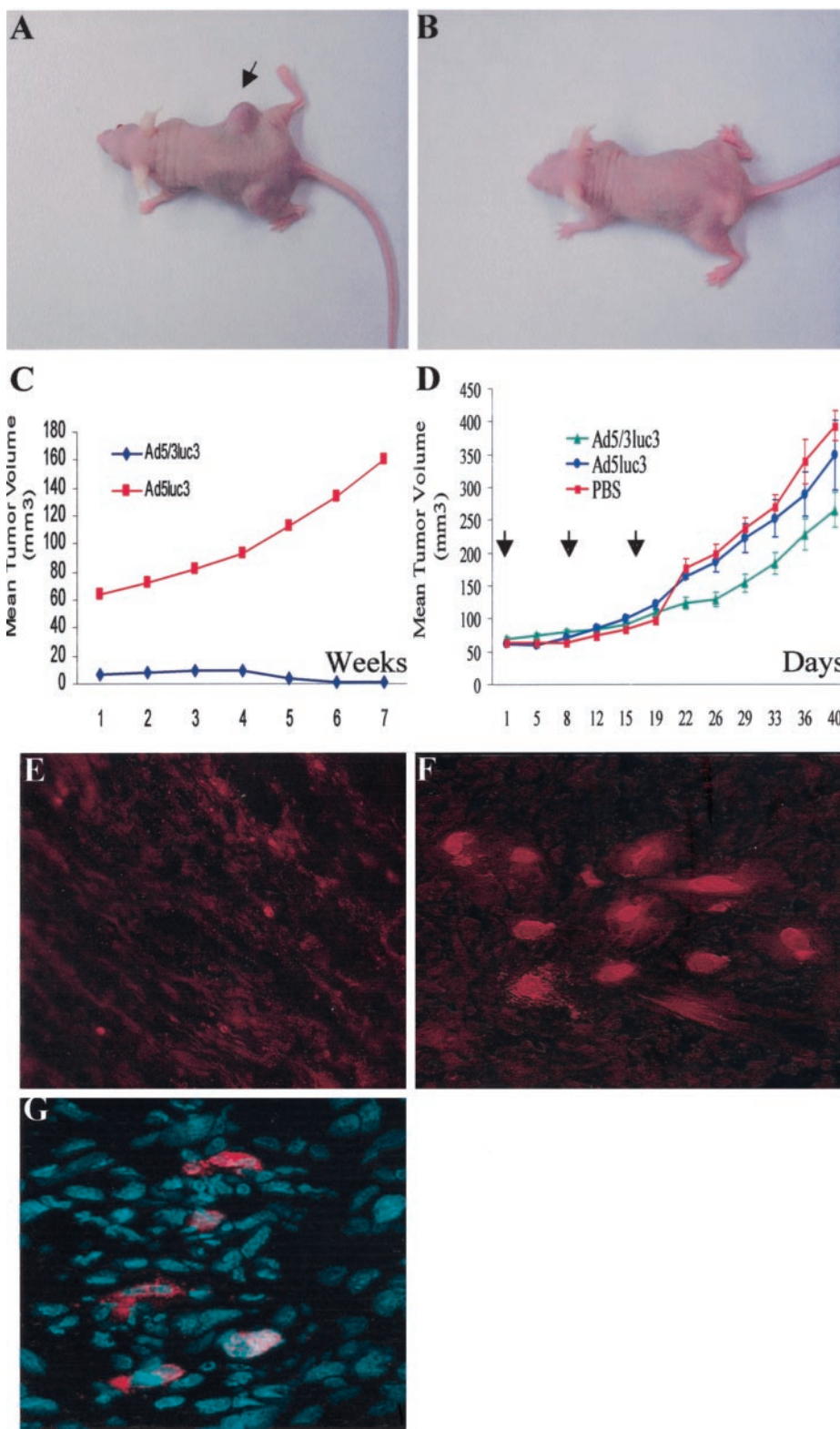


Fig. 5. *In vivo* superiority of replicative tropism-modified Ad in RCC tumor. A–C, athymic *nu/nu* mice were injected with CaKi-1 cells, preinfected at an MOI of 5 with either the unmodified replicative Ad5luc3 (A) or the tropism-modified Ad5/3luc3 (B), and monitored for tumor growth (C). D, intratumoral injection of Ad5luc3 or Ad5/3luc3 into pre-established RCC tumors. Tumors reaching a volume ~80 mm³ were injected with either PBS (*n* = 4), the replicative unmodified Ad5luc3 (*n* = 5), or the replicative tropism-modified Ad5/3luc3 (*n* = 7). Tumors were injected in three divided doses at the indicated times (arrows). Means ± SE of tumor size are shown. E and F, immunohistochemical staining of the Ad capsid protein hexon in sections from RCC xenografts infected with Ad5luc3 (E) or Ad5/3luc3 (F). F represents a composite of several foci of hexon-positive cells, identified only in Ad5/3luc3-infected tumors. G, double staining for hexon and nuclei in Ad5/3-infected CAKi-1 xenografts with disorganized cellular growth. Hexon stain was predominantly nuclear (suggesting virion assembly) or perinuclear (suggesting hexon localization to the rough endoplasmic reticulum). *, *P* < 0.05.

DISCUSSION

A major limitation of current cancer gene therapy strategies is the inability of replication-defective Ad vectors to efficiently infect a solid tumor (29). Consequently, a novel class of Ad viruses has been proposed to selectively replicate within cancer cells, thereby releasing the viral progeny to spread and infect neighboring tumor cells (32). However, the common finding of CAR deficiency in primary tumors (9,

15, 16) may not only limit the initial infection event but would also restrict the potential therapeutic benefits afforded by viral replication within the tumor cells. Thus, CAR deficiency may account for the insufficient therapeutic outcome in clinical trials with replication-selective Ad viruses (19).

In this study, we have investigated the hypothesis that Ad retargeting to alternative cellular receptors could circumvent the natural

resistance of RCC to infection by Ad5. To address this problem, we employed tropism-modified Ad vectors and viruses that achieved CAR-independent cellular infection in RCC models, after the identification of integrins of the ν class and the putative Ad3 receptor as potential receptors. Importantly, in the context of cell killing, the usage of CAR-independent infection was most prominent for replication-competent Ad viruses predicated on their capacity to replicate and spread the viral progeny *in vitro* and *in vivo*.

We and others have previously reported genetic retargeting approaches for replication-deficient Ad vectors (10, 13, 14, 33). Consequently, a number of studies have reported that replication-incompetent Ad vectors modified by insertion of a RGD peptide into the HI loop may be superior to nonmodified Ad vectors to transduce glioma cells (34, 35), ovarian cancer cells (36), and head and neck tumor cells (37).

Similarly, a chimeric replication-deficient Ad vector, displaying Ad shaft/Ad3 knob, could also overcome the resistance of ovarian cancer cells to infection with an Ad5 vector (11). In this study, we employed a novel approach of tropism modification of replication-competent Ad viruses to develop an experimental gene therapy approach for RCC, a disease with an extremely poor prognosis. We have shown here that replication-competent, tropism-modified Ad viruses can efficiently infect, replicate, and kill RCC tumor cells that are resistant to Ad based on the capsid of serotype 5. However, despite the beneficial therapeutic effect of the replicative tropism-modified Ad viruses *in vivo*, complete eradication of a mouse RCC xenograft could not be achieved. Consequently, it appears that retargeted replicative Ad viruses should be complemented by means to overcome the intratumoral physical barriers limiting viral dissemination throughout the tumor.

Because tumor cells abundantly express α_{ν} integrins and the putative receptor for Ad3, these alternative receptors may be of use for CAR-independent cancer gene therapy. In the context of renal cancer, $\alpha_{\nu}\beta_3$, which has an important function in tumor angiogenesis (38), is abundantly expressed by RCC in humans (39, 40). As well, the expression of $\alpha_{\nu}\beta_5$ is also selectively up-regulated in RCC. Furthermore, increased expression of α_{ν} integrins correlates with the histologic grade of RCC (40–42), and the integrin profile of RCC *in vitro* is maintained *in vivo* (31). Therefore, the significance of these alternative Ad receptors is underscored in the context of RCC.

Although the concept of tropism modification of replicative Ad viruses for cancer therapy holds great promise, its direct benefit in the context of CAR deficiency is yet to be confirmed. In this regard, Shinoura *et al.* (43) have reported that the potency of a replicating Ad virus in glioma cell lines *in vitro* and *in vivo* could be improved 30-fold by the addition of a stretch of 20 lysine residues to the COOH-terminal of the fiber protein, allowing the virus to bind to cellular heparan sulfate receptors. Similarly, Suzuki *et al.* (46) have shown in a CAR-positive cell line that the efficacy of a replicating Ad can be enhanced by incorporating a RGD peptide motif into the fiber protein. However, because in both these studies the unmodified Ad5 viruses achieved significant infection, replication, and cancer cell killing, it appears that these models may not completely represent the CAR-deficiency status of primary tumors. Additionally, when tested *in vitro* in the context of a CAR-negative rhabdomyosarcoma cell line, a replicative tropism-modified Ad virus was relatively inefficient in killing cancer cells (45), indicating the need to tailor the retargeting strategy to the tumor receptor profile. Taken together, these earlier studies imply that the use of CAR-independent Ad viral infection and replication, in the context of CAR-negative tumors, merits intensive investigation.

In this study, we have demonstrated relative resistance of RCC to infection with a variety of Ad vectors and viruses based on the Ad5

capsid. Furthermore, we have characterized the distinctive superiority of tropism modification for Ad vectors and, particularly for replicating Ad viruses, in the context of RCC. Although the RGD4C fiber knob modification of a selectively replicating Ad virus has been previously reported to reduce tumor size *in vivo* (44), this is the first report of enhanced cancer cell killing and tumor growth inhibition by a replicative Ad retargeted to the putative Ad3 receptor.

Because these approaches are expected to increase the transduction efficiency of other organs as well, thereby raising concerns regarding their safety profile, restriction of replication of these infectivity-enhanced Ad viruses to tumor cells appears crucial. To this end, transcriptional regulation may derive from placing the expression of Ad viral genes, most commonly the *E1A* gene, under the control of tumor- or tissue-specific promoters or from the complete or partial deletion of viral genes required for replication in normal cells but not in tumor cells.

In conclusion, we have shown in this RCC model that CAR deficiency restricts the use of Ad5 for RCC. To achieve a significant therapeutic outcome, CAR-independent infection appears to be required, mostly in the context of replication-competent Ad viruses. These findings may be highly consequential for the development of cancer gene therapy strategies in general and for RCC in particular.

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Adenoviral Gene Therapy for Renal Cancer Requires Retargeting to Alternative Cellular Receptors

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