

An E2F-responsive Replication-selective Adenovirus Targeted to the Defective Cell Cycle in Cancer Cells: Potent Antitumoral Efficacy but No Toxicity to Normal Cell¹

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ABSTRACT

To improve the transduction and distribution of adenovirus in a tumor mass, we generated an adenovirus to selectively replicate in tumors. We hypothesized that after infection the replicating adenovirus would spread throughout the tumor mass and cause direct oncolysis of tumor cells. E2F transcription factors are critical regulators of cell growth and are often overexpressed in cancer cells because of the frequent aberrations in the *pRb/E2F/p16^{INK4a}* pathway. As a result, a majority of tumor cells exist in a high proliferative state. E2F-1 is a transcription factor that activates its own transcription and that of other genes involved in the G₁ to S transition phase of the cell cycle. We constructed an adenovirus (Ad(E2F-1^{RC})) so that E1A expression and viral replication were under the control of the human E2F-1 promoter element. AdE2F-1^{RC} virus replicated as efficiently as the wild-type adenovirus and caused extensive cell killing in a panel of tumor cells *in vitro*. In contrast, nonproliferating normal epithelial, fibroblast, and endothelial cells, which express no E2F-1, were not able to support AdE2F-1^{RC} replication. In animal studies, different dosing regimens of AdE2F-1^{RC} administered to flank xenografts of ovarian and lung cancers led to a significant therapeutic advantage often surpassing that seen in animals treated with the wild-type adenovirus. This novel selectively replicating adenovirus offers a promising treatment platform for a variety of cancers of which the hallmark is uncontrolled cell growth.

INTRODUCTION

Replication-defective adenoviruses have been successfully exploited to deliver therapeutic genes for cancer gene therapy (1). Although adenovirus offers several advantages, progress in the clinic has been hampered by high viral immunogenicity, lack of cell specific infectivity, and poor viral distribution within the tumor-mass (2).

To confer specificity of infection and increase viral spread in the tumor mass, RSAds³ are now being actively developed as cancer therapeutic agents (3–6). These viruses have several advantages. First, viral replication amplifies the initial input dose allowing the use of a low dosing regimen. Second, lysis of the cell as part of the virus life cycle induces cell death. Third, the new virus progeny infects neighboring cells and continues to replicate and spread until the tumor is eradicated. Fourth, tumor antigens released from dead cells can enhance antitumor immunity. Fifth, these viruses can be additionally modified either to deliver cytotoxic transgenes (7, 8) or be enhanced

for increased infectivity (9). Finally, replication is restricted to tumor cells thus avoiding toxicity to normal tissues.

Two commonly used approaches to restrict adenoviral replication to tumor cells are: (a) deleting entire (*E1B*) or partial regions (*E1A*) of essential viral genes that normally disarm host-defense mechanisms; and (b) to regulate expression of viral genes (*E1A*) essential for replication with heterologous promoters that are specifically active in tumor or tissues. For example in the well-characterized Onyx-015 (C1–1042) adenovirus (10), the *E1B* 55 kDa gene is deleted so that the virus replicates in *p53*-deficient tumor cells and not in normal cells, which contain wild-type *p53*. However, evidence from many studies indicates that the proposed basis for this selective replication is not solely determined by *p53* deficiency (4, 11, 12). Nonetheless, its therapeutic potential often in conjunction with chemotherapy has been vigorously evaluated in >240 patients enrolled in Phase I/II/III clinical trials for a variety of carcinomas (13). Additional modifications of the Onyx-015 virus, including arming it with suicide genes (14, 15), deleting the *E1B* 19 kDa gene (16), and retargeting its receptor-mediated infection (17) have all provided increased therapeutic benefit. Another strategy used to prevent viral replication in normal cells is to delete the *E1A*-domain required to bind pRb and inhibit the S phase induction. However, viral replication will proceed in tumor cells, because the *pRb/E2F/p16^{INK4a}* pathway is often defective. Such *E1A* mutant adenoviruses have proved to be highly effective in treating many mouse xenograft tumor models (18, 19), including metastatic disease models (20), and have exhibited low toxicities in normal cells (18, 20).

In another approach, viral replication has been restricted to tumor cells by regulating the transcription of the *E1A* gene with a heterologous promoter, which is highly active in given tumor and inactive in normal cells. For example, prostate cancer has been targeted with the adenovirus CN706 in which the *E1A* gene is under the control of the prostate-specific antigen enhancer element. CN706 has demonstrated a high safety index, selective replication, and a direct oncolytic activity that correlated with prostate-specific antigen level in prostate tumors (21). Moreover, CN706 selectivity has been additionally improved by regulating multiple adenoviral genes either with single or different tumor-specific promoters. For example, in CV787 virus (22), two different prostate-specific promoters control *E1A* and *E1B* genes, respectively. Alternatively, in CV890 adenovirus, a single α -fetoprotein promoter is used to control the *E1A*-IRES-*E1B* cassette to target liver cancer (23).

Several other promoters including α -fetoprotein (24), osteocalcin (25), Muc-1 (26), L-plastin (27), secretory leukocyte protease inhibitor 1 (28), and carcinoembryonic antigen are being evaluated in other laboratories to restrict viral replication to their cognate tumors. Although all of these vectors are severely replication attenuated in normal cells and demonstrate high selectivity, their one drawback is that they are available for treatment of only a narrow range of tumors because only a limited number of tumors express the targeted tumor markers. However, targeting a rate-limiting gene of a key biochemical

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³ The abbreviations used are: RSAd, replication-selective adenovirus; pRb, retinoblastoma protein; NHLF, normal human lung fibroblast; SAEC, small airway epithelial cell; HMEC, human mammary epithelial cell; HUVEC, human umbilical vein endothelial cell; RSVtk, rous sarcoma virus promoter-driven thymidine kinase; TBST, Tris-buffered saline with Tween 20; NHDF, normal human dermal fibroblast-adult; CPE, cytopathic effect; pfu, plaque-forming unit(s); MOI, multiplicity of infection; VRI, virus replication index; FBS, fetal bovine serum; β -gal, β -galactosidase.

pathway that is defective in tumors can increase the range of application with a single RSAd.

E2F-1 is a transcription factor that binds to four E2F-1 binding sites, found in its own promoter, and it positively auto-regulates its own transcription during the G₁ to S phase transition (29). In non-proliferating, normal cells, E2F-1 exists as a pRb/E2F-1 complex, and it is released when pRb is phosphorylated. The unbound form of E2F-1 transactivates several genes of the DNA synthesis pathway to coordinate cell cycle transit through the S phase (30, 31). In contrast, in continuously cycling tumor cells, frequent disruptions in the pRB/p16^{INK4a}/cyclin D pathway (32, 33) keep E2F-1 in a constitutively unbound state. Consequently, E2F-1 is overexpressed in many tumor cells (34, 35) including lung cancers (36, 37). Studies have shown that expression of E2F-1 is increased in tumor cells either because of a deregulated pRb pathway or because of *E2F-1* gene amplification (38), and either mutation or allelic loss of *E2F-1* has rarely been detected in cancer cells. Thus it appears that overexpression of E2F-1 generally contributes to tumorigenesis, although experimental evidence indicates that forced overexpression of E2F-1 induces apoptosis in tumor and normal cells (39).

Taking advantage of this, a replication-defective adenovirus containing the *E2F-1* promoter-driven herpes simplex virus-thymidine kinase gene has been used to target E2F-1-expressing cancer cells (40). Although tumor cell-specific thymidine kinase expression was achieved and treatment with gancyclovir administration reduced tumor growth in a glioma mouse model, poor viral transduction still posed a problem. We have extended this observation to establish direct oncolysis using a RSAd to target E2F-1-expressing tumors without additional transgene delivery.

Here, we report a strategy for the development and preclinical testing of a novel RSAd named AdE2F-1^{RC}. In this virus the *E1A* gene is placed under the control of the human *E2F-1* promoter element to allow us to target a variety of tumors expressing *E2F-1*, thus exhibiting a deregulated G₁-S phase. The AdE2F-1^{RC} virus replicated in and killed a panel of tumor cells to the same extent as the wild-type adenovirus, dl309. More importantly, AdE2F-1^{RC} showed extremely low levels of replication and CPE in nonproliferating normal endothelial, epithelial, and fibroblast cells. In addition, AdE2F-1^{RC} was as potent as the wild-type adenovirus in inhibiting ovarian and lung tumor growth in mouse xenograft models. AdE2F-1^{RC} offers a new treatment platform applicable for any cancer cell of which the hallmark is a deregulated control of cell growth.

MATERIALS AND METHODS

Cell Lines. The human cell lines A549 (lung adenocarcinoma), 293 (transformed human embryonal kidney), SKOV3 (ovarian adenocarcinoma), HeLa (epithelioid cervical carcinoma), WI38 (diploid lung fibroblasts), 1MR90 (lung fibroblasts), and H661 (lung adenocarcinoma) were purchased from the American Type Culture Collection (Manassas, VA). The normal cells, NHDF, NHLF, SAEC, HMEC, and HUVEC were purchased from Clonetics (BioWhittaker, Walkersville, MD) and grown in culture medium recommended by the manufacturer. The malignant human mesothelioma cell line, REN, has been described elsewhere (41). HeLa, 293, 1MR90, H661, and WI38 cells were

maintained in DMEM plus 10% FBS. A549, SKOV3, and REN were maintained in RPMI 1640 with 10% FBS. Medium for all of the American Type Culture Collection cell lines was supplemented with 2 mM L-glutamine, and 1% penicillin (100 units/ml) and streptomycin (100 µg/ml). HUVEC cells were maintained in M199 medium containing 15% FBS, epidermal growth factor (20 ng/ml; Collaborative Biomedical Products, Bedford, MA), and heparin sulfate (50 µg/ml).

Cloning of the *E2F-1* Promoter. A 269-bp fragment of the human *E2F-1* promoter (GenBank accession no. S74230) was amplified by PCR from human genomic DNA using the forward (5'-GTT-TAA-TTA-ACT-CGA-GGG-TAC-CAT-CCG-GAC-AAA-GCC-TGC-G-3') and reverse (5'-ACT-TAA-TTA-AAA-GCT-TAG-ATC-TCG-AGG-GCT-CGA-TCC-CGC-TCC-GC-3') primers (29). *PacI* and *XhoI* (forward primer), and *PacI*, *HindIII*, and *BglII* sites (reverse primer) were incorporated into the primers to facilitate additional cloning of the *E2F-1* promoter. The amplification conditions were an initial step of 94°C for 5 min followed by 35 cycles, with each cycle of 94°C for 30 s and 70°C for 0.5 min, and a final extension step at 72°C for 10 min. The amplified product was subcloned into the pCRII-TOPO plasmid as per the manufacturer's instructions (Invitrogen, Carlsbad, CA). A positive clone pCRIIE2F-1 was confirmed to contain the *E2F-1* promoter by restriction enzyme digestion and DNA nucleotide sequencing. The *E2F-1* promoter was digested with *XhoI* and *HindIII* from pCRIIE2F-1, and cloned upstream of the luciferase gene into similarly cut pGL2-Basic plasmid (Promega, Madison, WI) to generate pGL2E2F-1.

Analysis of the *E2F-1* Promoter Activity. The activity of the *E2F-1* promoter was measured by transiently, cotransfecting 0.2 µg of pSVβ-gal plasmid (for transfection-control) with 1.0 µg of either pGL2E2F-1 or pGL2-promoter (positive control), or pGL2-Basic (negative control) into a panel of tumor cells using lipofectin (Life Technologies, Inc. Rockville, MD). Cell extracts were assayed 48 h later for luciferase and β-galactosidase activities using the Luciferase Assay System and β-Galactosidase Assay System, respectively, according to the manufacturer's instructions (Promega, Madison, WI). Luciferase values were normalized for β-galactosidase activity.

Construction of the AdE2F-1^{RC} Vector. The pXC1 plasmid has adenovirus 5 sequences from 22–5790 bp containing the *E1* gene (Microbix Biosystems Inc., Toronto, Ontario, Canada). A unique *PacI* site was introduced at nucleotide position 522, essentially as described by Rodriguez *et al.* (21) to generate the plasmid pXC1522. A *PacI* site at nucleotide position 522 was confirmed by restriction digestion with *PacI* and by DNA nucleotide sequencing. The *E2F-1* promoter was cut out of the pCRIIE2F-1 plasmid with *PacI* and ligated to pXC1522 plasmid (also cut with *PacI*) to obtain pXC1522E2F-1. The orientation of the *E2F-1* promoter was confirmed by DNA nucleotide sequencing.

To construct the AdE2F-1^{RC} virus, homologous recombination was performed between pXC1522E2F-1 plasmid and the right hand side of *ClaII* digested H5.010CMVGFP adenovirus DNA in 293 cells by standard techniques, including the green/white selection process (42). Individual white plaques, visible in 7–10 days, were grown in 293 cells until they exhibited CPE. Low molecular weight DNA isolated from these cells was analyzed for the *E2F-1* promoter by restriction digestion and PCR using the primers 7-S (5'-TCG-TGG-CTC-TTT-CGC-GGC-AA-3') and 8-AS (5'-ACC-CAA-GGC-TCT-CTG-CTC-CG-3'), which span the junction between the *E2F-1* promoter and the *E1A* gene as shown in Fig. 1. After three rounds of plaque purification on 293 cells, one clone was processed for large-scale AdE2F-1^{RC} preparation of and purified by two-step ultra-centrifugation on cesium chloride gradients. Serial dilutions of AdE2F-1^{RC} were plated on 293 cells for plaque assay, and the titer was expressed as pfu/ml. Ad5dl309 (dl309) is a typical wild-type virus but has the *14.7K* and *RID* genes in the *E3* region deleted. The *E1/E3*-deleted

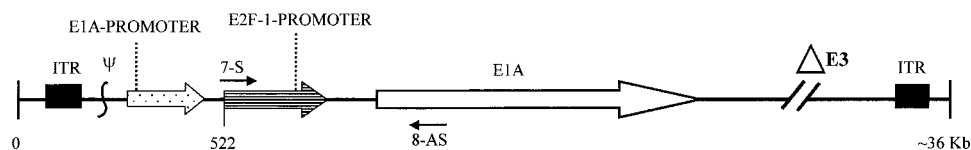


Fig. 1. A schematic diagram of the AdE2F-1^{RC} adenoviral construct. A 269-bp fragment of the human E2F-1 promoter was inserted downstream of the *E1A* promoter, into the viral backbone with the *E3* region deleted. The dark boxes represent the inverted terminal repeats (*ITR*) and ψ —the adenovirus 5 packaging signal. Primers, 7-S, and 8-AS used in a PCR assay to confirm the insertion of *E2F-1* promoter are shown.

AdRSVtk and AdLacZ viruses described elsewhere (41) are replication defective and contain RSV promoter-driven herpes simplex virus-thymidine kinase gene and cytomegalovirus-driven LacZ cassettes, respectively. All of these viruses were purchased from the Vector Core Facility (Wistar Institute, Philadelphia, PA).

Immunoblot Analysis. Nuclear protein extracts for E2F-1 analysis were prepared from log phase or confluent tumor cells and from growth-arrested normal cells (HMEC, SAEC, IMR90, NHLF, HDLF HMEC, and W138) essentially by the protocol described by Zumbansen and Stoffel (43).

To measure E1A protein levels, nonproliferating NHLF, HDLF, and W138 cells were infected with different viruses at MOIs of 2000 to achieve at least 10% infectivity, predetermined with the replication-defective AdLacZ virus. Later (48 h), cell extracts were prepared by scraping the cells into lysis buffer [2% SDS and 0.125 M Tris-HCl (pH 6.8)] and shearing the DNA with an insulin syringe. Total protein in the nuclear and cell extracts was measured using the BCA protein assay kit (Pierce, Rockford, IL).

Protein (20 μ g) was separated on 10% SDS-polyacrylamide gel and electrophoretically transferred onto polyvinylidene difluoride (NEN Life Sciences, Boston, MA) membrane, and blocked in 5% milk in TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20) for 30 min. The membrane was incubated with either monoclonal anti-E2F-1 or polyclonal anti-E1A (clone KH95 and clone 13S-5, respectively; Santa Cruz, Inc., Santa Cruz, CA), or monoclonal α -actin (Amersham Pharmacia Biotech, Piscataway, NJ) primary antibodies for 1 h and repeatedly washed in TBST. After incubation for 30 min with appropriate secondary horseradish peroxidase-conjugated antibodies and extensive washing with TBST, immunocomplexes on the membrane were detected with the enzyme chemiluminescence reagent substrate according to the manufacturer's instructions (NEN Life Sciences, Boston, MA).

In Vitro Cell Viability Assay. Tumor cells were plated at 60–70% confluence in six-well plates and 24 h later, infected with different adenoviral vectors at various MOIs. Normal, HUVEC, and W138 cells were plated in 96-well plates and cultured for another 2 and 4 days, respectively, before being infected. Tumor cells were trypsinized and plated at a density of 3000 cells/100 μ l/well in 96-well plates. After 6 (HUVEC) and 9 (W138) days, cell viability was measured by the MTS assay using the Nonradioactive Cell Proliferation Assay kit (Promega, Madison, WI) based on the dehydrogenase enzyme activity of metabolically active cells. The percentage of cell survival was calculated using the formula: % cell survival = A_{490nm} of infected cells/ A_{490nm} uninfected cells \times 100%.

In Vitro Cytopathic Assay. REN, HeLa, A549, and SKOV3 cells were plated at a density of $2\text{--}3 \times 10^5$ in six-well plates and 24 h later infected with dl309 or AdE2F-1^{RC} at various MOIs. Normal cells (W138, IMR90, NHLF, SAEC, and HMEC) were contact inhibited by growing them for 3–4 days in 10% serum, and infected with dl309 and AdE2F-1^{RC} at MOIs of 1000 (HMEC) and 2000 (W138, IMR90, NHLF, and SAEC). The assay was terminated once dl309-infected cells showed significant CPE (~90–95%). All of the cells were fixed in acetone:methanol (1:1 v/v) for 10 min at room temperature and stained with 1% crystal violet (Sigma, St. Louis, MO) for 1–2 min, the plates dried, and photographed.

Adenoviral Replication Assay. Contact-inhibited normal cells and log phase tumor cells (plated at 60–70% confluence) were infected with dl309 or AdE2F-1^{RC} at various MOI's. After infection (48 h), medium and cells were scraped into 1-ml medium, subjected to three freeze-thaw cycles, and centrifuged to collect the supernatant. Serial dilutions of the supernatant were assayed for live virus particles by standard plaque assays on 293 cells. For each cell line, the efficiency of replication of the AdE2F-1^{RC} virus was expressed as VRI, which was calculated using the formula: VRI = number of AdE2F-1^{RC} plaques per 48 h/number of dl309 plaques per 48 h.

Treatment of Established Tumors with AdE2F-1^{RC}. Female CB17-SCID (Taconic Laboratory, Germantown, NY) mice, aged 6–8 weeks, were acclimatized in a pathogen-free animal facility of the Wistar Institute for at least 1 week before each experiment. All of the animal protocols were in compliance with the Guide for the Care and Use of Laboratory Animals (NIH) and were approved by the Animal Use Committees of the Wistar Institute and the University of Pennsylvania. Four million A549 or 7×10^6 SKOV3 cells in 200 μ l of serum-free medium were injected s.c. into both flanks of 21 SCID mice. Tumor sizes were measured regularly, and the volume was calculated using the formula for an ellipsoid, *i.e.*, tumor volume (mm^3) = $\pi/6 \times W \times L^2$, where L = length of the tumor and W = width of the tumor. When the tumors

had reached approximately 50–100 mm^3 , mice ($n = 7$) were randomly divided into 3 treatment groups: (a) medium only; (b) dl309; and (c) AdE2F-1^{RC}. Viruses (10^9 pfu) in 150 μ l of serum-free medium were administered intratumorally for a total of three times every other day and the tumor size monitored. Animals were sacrificed ~3 weeks after viral injection and the tumors weighed.

In a second experiment, 3.5 million A549 cells were injected in the flanks of SCID mice and allowed to reach to a size of 30–50 mm^3 . Flank tumors in four groups of mice ($n = 7$) were injected directly with a single dose of 10^9 pfu of dl309, AdE2F-1^{RC}, AdRSVtk, or medium only. Tumor volumes were monitored regularly for 60 days. This experiment was repeated, but the tumors were allowed to grow to an average size of 900 mm^3 before they were injected with a single dose of 10^9 pfu of different viral vectors. Tumor size was monitored periodically for the following 50–55 days.

Statistical Analysis. The results of the MTS assay and tumor weights are presented as mean \pm SE (error bars). Comparisons were made using ANOVA with appropriate pot-hoc testing (Fisher's PLSD).

RESULTS

Construction of the AdE2F-1^{RC} Virus. We developed an RSAd in which the E1A gene was placed under the control of the human E2F-1 promoter (Fig. 1). Adenoviral DNA was extracted, and recombination was confirmed by restriction enzyme digestion and by PCR amplification of the region between the E2F-1 promoter and the E1A gene using the 7-S and 8-AS primers shown in Fig. 1. Absence of wild-type virus was confirmed by performing PCR for the E3 gene.

Rationale for Assessing the Selective Replication of the AdE2F-1^{RC} Virus. We reasoned that comparison of AdE2F-1^{RC} replication between tumor cells overexpressing E2F-1 protein and cells expressing reduced levels of E2F-1 protein would provide a reasonable assessment of the selectivity of this transcriptionally regulated adenovirus. Because, in the clinical setting, the adenovirus would normally be administered to tumor cells in the milieu of normal, nonproliferating cells (*i.e.*, endothelial cells, epithelial cells, and fibroblasts), we reasoned that these cells would be the best controls to assess the selectivity of the AdE2F-1^{RC} virus. Because adenovirus does not replicate in murine cells, assessment of toxicity in animal models is extremely limited. Thus, studying the effects of high doses of the AdE2F-1^{RC} virus on normal human cells provides the best approach for safety studies.

Tumor Cells Express High Levels of E2F-1 Protein. One consequence of a deregulated *pRb/E2F/p16^{INK4a}* pathway seen in many tumor cells is the dissociation of E2F-1 protein from *pRb/E2F* complex with an increased level of "free" E2F-1 protein (32, 33). To verify this, we measured the E2F-1 protein levels in nuclear extracts prepared from tumor and normal cells by immunoblot analysis. E2F-1 protein levels varied widely in lung, mesothelioma, cervical, and ovarian cancer cells as shown in Fig. 2A. However, in all of the tumor cells, a much higher level of E2F-1 was expressed compared with nonproliferating normal fibroblasts (Fig. 2B). In addition, in two tumor cell lines (REN and A549), E2F-1 protein levels in the log phase of cell growth (Fig. 2A, Lanes 1 and 4, respectively) did not change significantly when these cells were grown to confluence for 3 days before analysis (Fig. 2A, Lanes 2 and 5, respectively).

E2F-1 protein was not detected in 4 of the 6 (Fig. 2B, Lanes 1–3 and 4) nonproliferating normal epithelial and fibroblast cells. A low level was detected in two fibroblast cell lines (Fig. 2B, Lanes 4 and 5). A positive control (HeLa) is shown in Fig. 2B, Lane 7, and actin loading control is shown in the bottom panel of Fig. 2B. It is well known that E2F-1 activity is very tightly regulated during the cell cycle progression from G₁ to S phase in normal cells (44). This data shows that whereas E2F-1 protein was generally undetectable in a majority of growth-arrested normal cells, E2F-1 protein levels in tumor cells were unaffected by the growth state of the cell.

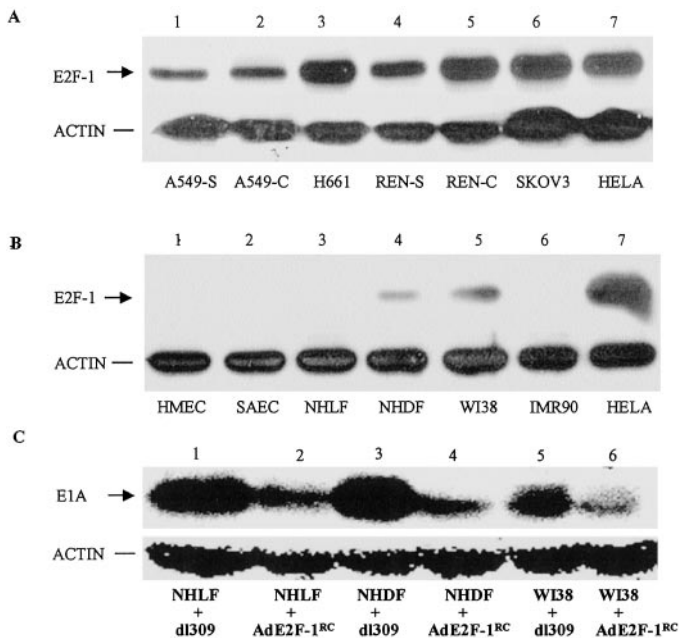


Fig. 2. Immunoblot analysis. **A**, E2F-1 expression analyzed was from Lane 1, subconfluent A549 cells (lung adenocarcinoma); Lane 2, confluent A549 cells; Lane 3, subconfluent H661 cells (lung adenocarcinoma); Lane 4, subconfluent REN cells (mesothelioma); Lane 5, confluent REN cells; Lane 6, subconfluent SKOV3 cells (ovarian cancer); and Lane 7, subconfluent HeLa (cervical cancer) cells. Nuclear extract (20 μ g) was separated on a 10% SDS-PAGE gel and immunoblotted with E2F-1 antibody. Arrow in the top panel shows the E2F-1 protein band position (60 kDa), and the actin control is shown in the bottom panel. **B**, E2F-1 protein in nonproliferating normal cells; Lane 1, HMECs; Lane 2, SAECs; Lane 3, NHLFs; Lane 4, NHDFs; Lane 5, WI38 cells (normal lung fibroblast); and Lane 6, IMR90 cells (human lung fibroblasts). A positive control HeLa is shown in Lane 7. Bottom panel shows the actin control. **C**, E1A protein expression is reduced in nonproliferating normal cells infected with the AdE2F-1^{RC} virus. NHLF, NHDF, and WI38 cells were grown until contact inhibited and infected with dI309 (Lanes 1, 3, and 5) and AdE2F-1^{RC} (Lanes 2, 4, and 6) viruses at MOI of 2000. Forty-eight h later, E1A protein was detected by immunoblot analysis. Arrow in the top panel indicates the E1A (43–45 kDa) protein, and actin control is shown in the bottom panel.

E2F-1 Promoter Is Activated in Tumor Cells. To assess the transcriptional activity of the *E2F-1* promoter, the human *E2F-1* promoter was subcloned upstream of the luciferase gene in the pGL2-Basic plasmid. Using the luciferase reporter assay, the *E2F-1* promoter activity was compared with the ubiquitous SV40 promoter in a panel of tumor cells and normalized with the cotransfected, pSV β -gal plasmid. In REN cells, with low levels of *E2F-1*, the promoter showed 8% of the control SV40 promoter activity. In log phase A549 cells the *E2F-1* promoter activity was 23% of control. In H661 cells, with a higher E2F-1 protein expression than A549 cells (Fig. 2A, Lane 3), the promoter activity was 165% of control. The *E2F-1* promoter element demonstrated the capability of directing the transcription of the luciferase gene, and its activity correlated well with E2F-1 protein levels. It was not possible to measure the promoter activity in nonproliferating normal cells, as they could not be easily transfected with the plasmids.

E2F-1 Promoter Has Negligible Activity in Normal Quiescent Cells. To test the *E2F-1* promoter activity in an adenoviral context, we infected nonproliferating normal fibroblasts with dI309 or AdE2F-1^{RC} at MOI of 2000. After infection (48 h), E1A protein was measured by semiquantitative immunoblot analysis, and this is shown in Fig. 2C. Clearly, dI309 containing the intact E1A promoter expressed 3–4-fold more E1A protein than the AdE2F-1^{RC} virus containing the *E2F-1* promoter with a displaced *E1A* promoter. It is not clear if the low background activity seen in AdE2F-1^{RC}-infected cells is because of a low level of activity from the displaced *E1A* promoter or is

because of the leakiness of the *E2F-1* promoter. We would require a construct with no *E1A* promoter sequences.

Transcriptionally Targeted AdE2F-1^{RC} Has a Potent Antiproliferative Effect in Tumor Cells but not in Normal Cells. The extent of the antiproliferative effect of AdE2F-1^{RC} was determined by comparing the growth rates of AdE2F-1^{RC} and dI309 infected cells. Normal endothelial (HUVEC) and fibroblast (WI38) cells were infected with AdRSVtk, dI309, or AdE2F-1^{RC}, at MOIs of 10 and 2000, respectively. Cell viability of endothelial cells and fibroblasts was measured 6 and 9 days, respectively, by the MTS assay. This is shown in Fig. 3. The dI309 virus exhibited a strong antiproliferative effect in both cell lines with 60–80% of the cells dead at the end of the experiment. In contrast, AdE2F-1^{RC} had minimum effect on the growth of cells and appeared to behave in the same manner as the replication defective adenovirus, AdRSVtk.

To test the anticancer effects of AdE2F-1^{RC} virus, different cancer cells in log phase of growth were infected with AdRSVtk, dI309, or AdE2F-1^{RC} at various MOIs, and cell viability was measured within 9 days. Because it is difficult to grow tumor cells until they are contact inhibited, as they continue to grow in other planes often forming

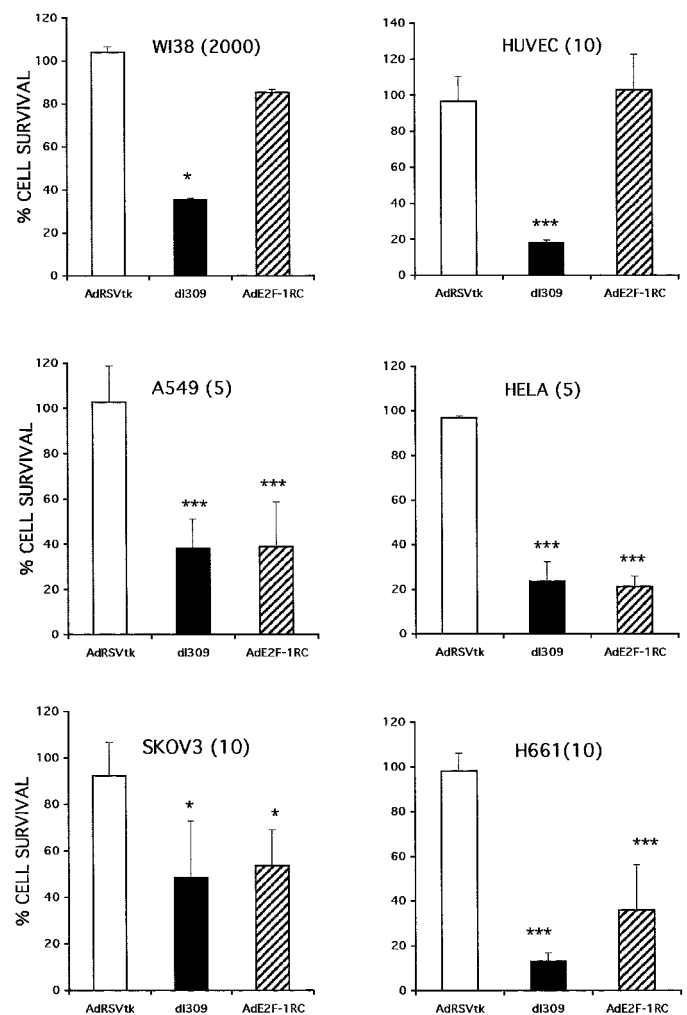


Fig. 3. AdE2F-1^{RC} has potent antiproliferative effect in tumor cells but not in normal cells. WI38 and HUVEC cells (grown to confluence for 4 and 2 days, respectively), and subconfluent tumor cells were infected either with AdRSVtk (control replication defective), dI309 (wild-type adenovirus), or AdE2F-1^{RC} (replication-selective) viruses. The MOIs used are shown in parentheses, next to the name of the cell line. Six (for HUVEC) to 9 (all other cells) days later cell viability was measured with the MTS assay. Cell survival is expressed as a percentage of uninfected control cells. The data represent mean, $n = 3$; bars, \pm SE. Statistical significance when compared with control was seen (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

colonies, we tested our viruses on subconfluent tumor cell monolayers. Tumor cells infected with the dl309 showed a marked decrease in cell viability by day 6, and >45–90% of cells were dead by day 9 (Fig. 3). Cell death was a result of viral replicative oncolysis because the replication-defective adenovirus, AdRSVtk, showed minimum effect on tumor cell viability. The cell killing effects of AdE2F-1^{RC} and dl309 viruses on SKOV3, A549, and HeLa cells were comparable. Lowest cell-killing activity was seen in SKOV3 cells with both viruses, suggesting that at the MOIs used the rate of virus production and spread may be a limiting factor. In H661 cells, the antiproliferative effect of AdE2F-1^{RC} was less than that for the dl309, but overall cell viabilities were reduced to 15–38% of uninfected control.

Replication of AdE2F-1^{RC} Is Abrogated in Nonproliferating Normal Cells but not in Tumor Cells. To assess the replicative ability of AdE2F-1^{RC}, we compared the replication of AdE2F-1^{RC} with that of dl309 in different types of normal and tumor cells. Contact-inhibited normal cells (WI38, IMR90, NHLF, NHDF, HUVEC, HMEC, and SAEC) were infected with AdE2F-1^{RC} and dl309 at MOIs of 10 for HUVEC, 1000 for HMEC, and 2000 for the remainder of the cell lines. Cell lysates were titered on 293 cells 48 h after infection, and the VRI (calculated as a ratio AdE2F-1^{RC} pfu/48 h:dl309 pfu/48 h) calculated is shown in Fig. 4.

Although high MOIs were required, we were able to infect all of the normal cells with dl309. This was evident from the robust replication of dl309 in all of the normal cell lines except in SAEC, which displayed a markedly lower dl309 titer ($\sim 2 \times 10^6$ pfu/ml) than seen with all of the other cell lines (range of $2\text{--}9 \times 10^{10}$ pfu/ml). In contrast, AdE2F-1^{RC} replication was severely attenuated in WI38, IMR90, NHDF, and NHLF cells with low to undetectable levels in the remainder of the cell lines. For example, in WI38, the virus replicated 80–100-fold less than dl309 (Fig. 4, Lane 1). The somewhat limited extent of viral replication seen in normal cells may be a result of a portion of cells that remain permissive to viral replication, because they may not be growth arrested and may support low E2F-1 activity. We also detected low levels of E1A production in nonproliferating normal fibroblast cells (Fig. 2C), although most of these cells do not express E2F-1 protein (Fig. 2B).

On the other hand, AdE2F-1^{RC} virus replicated as well as the dl309 in tumor cells (SKOV3, REN, HeLa, and A549) infected at 60–70% confluence with different viral vectors and processed for plaque assay on 293 cells, 48 h later (Fig. 4). In A549, AdE2F-1^{RC} replication was reduced 2.5–3.0-fold compared with the dl309 (Fig. 4, Lane 11) -infected cells.

Transcriptionally Targeted, AdE2F-1^{RC} Causes Extensive CPE in Tumor Cells but not in Normal Cells *in Vitro*. To test the selectivity and safety feature of AdE2F-1^{RC} we determined its CPE in several normal cells. We tested the virus on epithelial and fibroblast cells that were contact inhibited, and in a nonproliferative state. Normal cells grown to contact inhibition were infected in parallel with either AdE2F-1^{RC} or dl309, or remained uninfected. Cells were examined regularly for CPE, and when the dl309-infected cells showed almost complete cell death the experiment was halted, and cells were fixed and stained. All of the normal cells were susceptible to dl309 infection and succumbed to its lytic effect within 8–12 days after infection (Fig. 5A). In contrast, the morphology of the AdE2F-1^{RC}-infected cells appeared to resemble that of uninfected cells. In fact, remarkable integrity of the monolayer was seen in some cells for up to 12–15 days. The SAEC cell line did not appear as healthy as the uninfected cells, although it did not have any cells that were floating as seen in the dl309-infected wells. Overall, no significant CPE was observed in any of the AdE2F-1^{RC}-infected normal cells (Fig. 5A).

To monitor the cytolytic activity of AdE2F-1^{RC}, different tumor cells (HeLa, A549, and REN) were infected in parallel with either AdE2F-1^{RC} or dl309, or remained uninfected. Cells were monitored for CPE. Complete CPE was observed in all of the tumor cells (Fig. 5B). In REN cells, a dose-dependent CPE was observed (Fig. 5B). Moreover, REN cells appeared to be more sensitive, as equivalent killing was observed in A549 and REN cells (by day 8) even though REN cells were infected at a 50-fold lower MOI. In addition, the AdE2F-1^{RC} virus was just as effective in killing tumor cells as the dl309.

Replication-selective Virus AdE2F-1^{RC} Suppresses Growth of Tumors *in Vivo*. To evaluate the antitumoral effect of the AdE2F-1^{RC}, subcutaneous tumors were established in flanks of SCID mice using lung (A549) and ovarian cancer (SKOV3) cells. Fourteen (A549) and 17 (SKOV3) days later, macroscopic tumors with a volume of approximately 50–100 mm³ were injected intratumorally with medium only, dl309, or AdE2F-1^{RC} every other day for a total of three injections. Tumor size was measured with calipers every 3–4 days for 34 days. Tumor growths in dl309- and AdE2F-1^{RC}-treated groups were compared with medium only treated groups. As shown in Fig. 6, by 31–34 days, there were significant reductions in the tumor size in dl309 and AdE2F-1^{RC} versus medium alone-treated groups for both A549 and SKOV3 tumor models. At the end of the experiment, in dl309- and AdE2F-1^{RC}-treated groups, tumor sizes were significantly reduced by 50% ($P < 0.05$ for both treatments) and 75–85% ($P < 0.01$ for both treatments) of control values in A549 (Fig. 6A) and SKOV3 (Fig. 6B) tumor models, respectively. Because *in vivo* efficacy in dl309- and AdE2F-1^{RC}-treated groups were very similar, comparisons between these groups were not made. Although not significant, AdE2F-1^{RC} treatment provided a slightly improved therapeutic benefit compared with dl309 in the SKOV3 tumor model (Fig. 6B).

To verify the tumor size measured by calipers, tumors were excised on day 31 (A549) and 34 (SKOV3), and weighed. Tumor weights in the dl309- and AdE2F-1^{RC}-treated groups were significantly smaller than in the medium alone group for both tumor models (Fig. 6, A and B, right panels). In the dl309-treated group, A549 and SKOV3 tumor weights were reduced significantly by 60% and 85%, respectively (both comparisons, $P < 0.001$). Similarly, significant reductions in

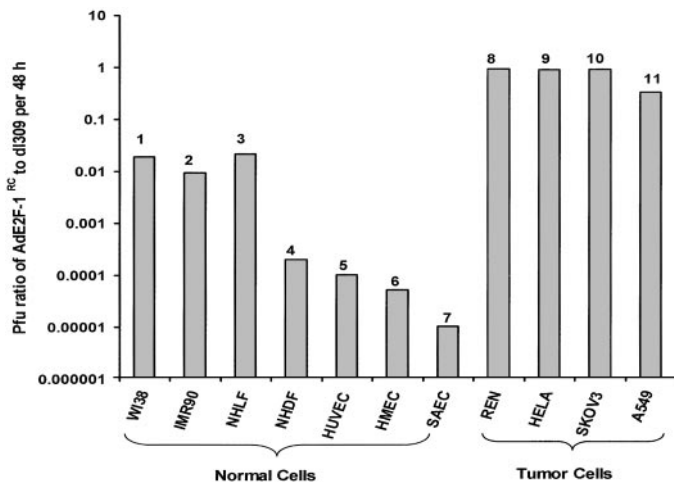


Fig. 4. AdE2F-1^{RC} virus replication is severely restricted in nonproliferating normal cells but not in tumor cells. Seven quiescent human cell lines (Lanes 1–7) and four tumor cell lines (Lanes 8–11) were infected with dl309 or AdE2F-1^{RC}. Normal HMEC cells were infected at MOI of 1000 and all other cells at MOI of 2000. Tumor cells were infected at various MOIs, chosen to infect $\sim 10\%$ of cells. Forty-eight h after infection cells were scraped into 1.0-ml culture medium, subjected to three cycles of freeze/thaw and assayed for pfu on 293 cells. Viral replication is reported as a ratio of pfu/ml for AdE2F-1^{RC} to dl309 per 48 h. AdE2F-1^{RC} replicated in normal cells 2–5 logs less well than dl309. In contrast, AdE2F-1^{RC} replicated almost as well as dl309 in four tumor cell lines. Results are of triplicate assays.

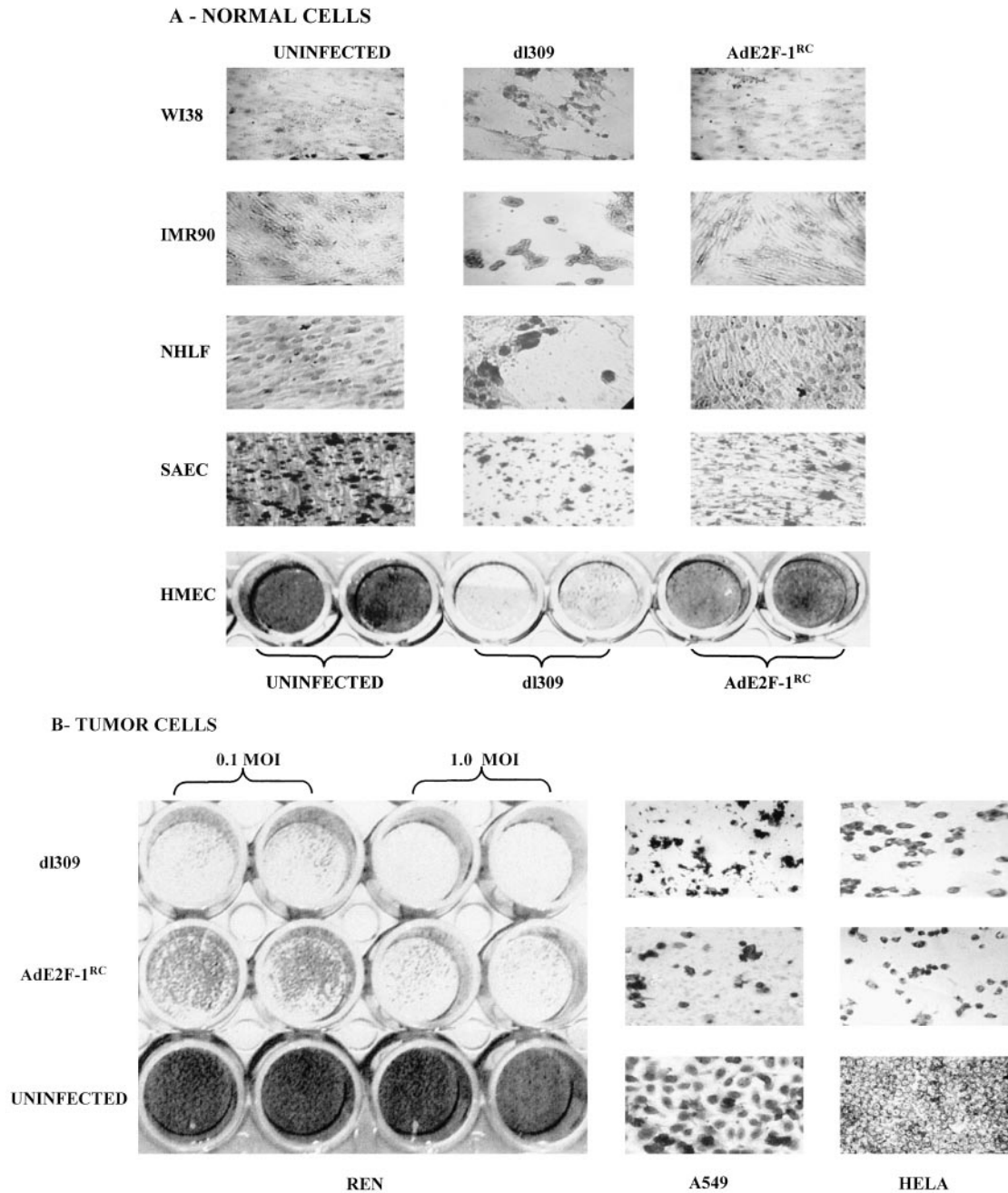


Fig. 5. *AdE2F-1^{RC}* virus exhibits a potent CPE in tumor cells but not in normal cells. A, monolayers of nonproliferating normal cells (WI38, IMR90, NHLF, SAEC, and HMEC) were infected with either dl309 or *AdE2F-1^{RC}* virus at MOI of 2000 except for HMEC (1000). Cells were monitored for CPE and compared with uninfected cells. CPE was almost complete with dl309 within 8–12 days, and cells were fixed and stained with 1% crystal violet, and documented as photographs. dl309 caused extensive CPE in all normal cells tested. In contrast, *AdE2F-1^{RC}* virus induced no CPE in any of the normal cell lines; B, subconfluent tumor cells (REN, A549, and HeLa) were infected at MOI of 5 (A549 and HeLa), and 0.1 and 1.0 (REN). dl309 caused extensive CPE in all tumor cells. Interestingly, *AdE2F-1^{RC}* also caused an equivalent CPE in all tumor cells with a dose-dependent CPE in REN cells.

the tumor weights of A549 (55%) and SKOV3 (90%) were obtained in *AdE2F-1^{RC}*-treated groups (both comparisons, $P < 0.001$).

To confirm intratumoral viral replication, we harvested tumors at an early and at a late time point from both tumor models. Tumor sections were subjected to immunohistochemistry for the detection of adenoviral hexon protein using the antibody described previously (42). Tumors excised from the dl309- or *AdE2F-1^{RC}*-treated groups demonstrated a strong presence of hexon protein in the tumor mass. Interestingly, tumors harvested 12 days after viral injection also showed marked hexon staining suggesting viral replication persisting for a prolonged period after injection, *in vivo* of tumors (data not shown).

Effect of a Single Dose Treatment of *AdE2F-1^{RC}* on Tumor Growth. To determine the *in vivo* efficacy of a low dose of *AdE2F-1^{RC}* treatment on tumors, at different stages of growth, the experiment was repeated with A549 cells. A single dose (instead of 3×10^9 pfu of viral vectors given previously) of vectors or medium alone was administered intratumorally into very small tumors (30–35 mm³). In another similar experiment the tumors were allowed to grow to a larger size (~900 mm³) before being treated with virus. Changes in tumor size of the groups with microscopic tumor treatment and groups with larger tumor treatment are shown in Fig. 7, A and B, respectively. Microscopic tumors treated with 10^9 pfu of *AdE2F-1^{RC}* and dl309 were significantly smaller from the medium alone-treated groups by

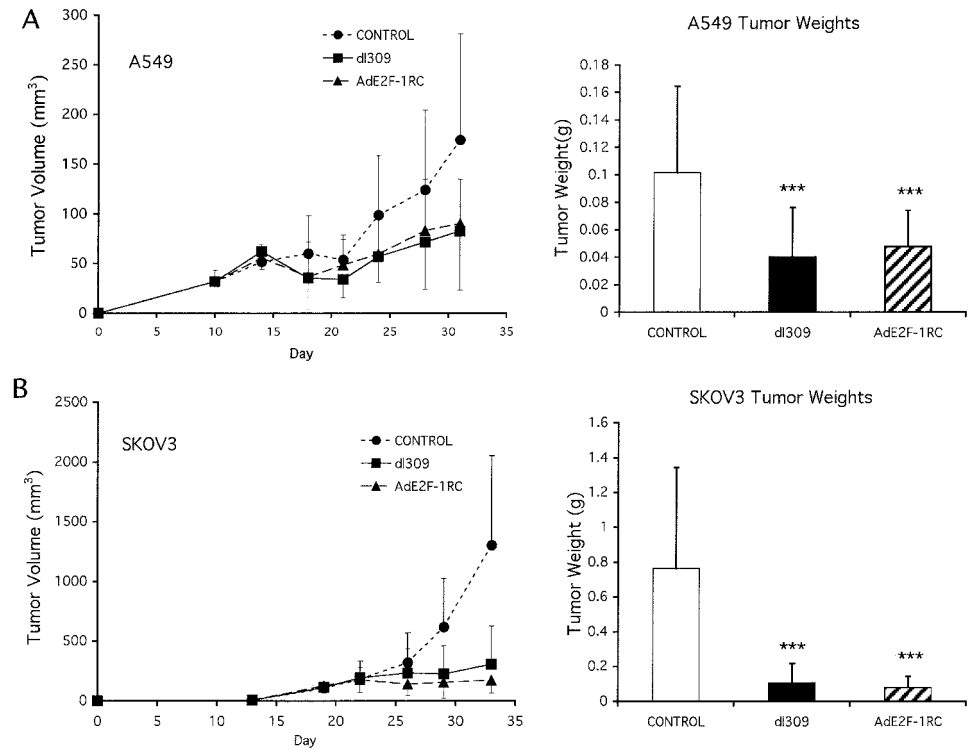


Fig. 6. Established flank xenograft tumors in SCID mice treated with multiple doses AdE2F-1^{RC}. A, subcutaneous tumors (50–100 mm³) growing in the flanks of SCID mice injected with 4×10^6 A549 cells were injected with 10^9 pfu of AdE2F-1^{RC} or dl309 intratumorally, three times every other day. B, the experiment was repeated with s.c. tumors grown with ovarian cancer cells, SKOV3. Average relative tumor volume sizes measured externally with calipers are shown in A (A549) and B (SKOV3). Right panels show tumors weights at the end of experiment (virus-treated groups versus control, ***, $P < 0.001$; tumor weight is mean; $n = 10$; bars, \pm SE).

day 60 ($P < 0.05$, both comparisons). Injection of a nonreplicating virus AdRSVtk, without administering gancyclovir, had no effect. In addition, compared with the dl309-treated group, a slightly improved efficacy, although not significant, was seen in the AdE2F-1^{RC}-treated group. Surprisingly, animals with a larger tumor burden were also very responsive to treatment with a single dose of vectors. As shown in Fig. 7B, by day 110, the tumor sizes in both dl309- and AdE2F-1^{RC}-treated groups were significantly smaller than the AdRSVtk (no gancyclovir)-treated group ($P < 0.01$, both comparisons). The control group was terminated by day 110 as tumors were reaching too large a size.

DISCUSSION

Our study is the first report of an RSAd, in which the “promoter-based regulation of E1A,” approach is used to target the deregulated G₁ to S phase in tumor cells. We demonstrated that AdE2F-1^{RC} replicated selectively in tumor cells and not in normal cells expressing high and low levels of E2F-1 protein, respectively. Additionally, in two mouse xenograft models, AdE2F-1^{RC} exhibited significant *in vivo* therapeutic benefit often equivalent to wild-type adenovirus treatment. These studies validate several design features of AdE2F-1^{RC}.

The wild-type adenovirus dl309 replicated in all of the normal cells tested. We reasoned that normal resting cells would be a good model for AdE2F-1^{RC} toxicity tests, because these cells do not express E2F-1 (44, 45) and are found in the tumor environment. In contrast to dl309, the replication and CPE of AdE2F-1^{RC} was significantly attenuated in normal cells suggesting that the E2F-1 promoter was not optimally activated. One reason is that the presence of pRb/E2F-1 complex in nonproliferating normal cells renders E2F-1 inactive. Our results agree with studies carried out by Fueyo *et al.* (18) who showed that an E1A mutant virus, Ad Δ 24 (unable to bind pRb), did not replicate in growth-arrested CCD32-Luc lung fibroblasts. Nonetheless, we were concerned that E1A expression was not completely abolished in AdE2F-1^{RC}-infected normal fibroblasts (Fig. 2C). This

may represent infection of a small population of proliferating cells, which had escaped contact inhibition. However, E1A immunostaining in normal AdE2F-1^{RC}-infected NHLF resting cells was not informative (data not shown). Although we formally did not test viral replication in proliferating normal cells, Heise *et al.* (20) have demonstrated that their dl922–947 E1A-mutant virus (unable to bind pRb) induced S phase, and replicated in proliferating epithelial and endothelial cells. Howe *et al.* (19) also showed that another E1A-mutant virus dl1107 (unable to bind pRb) replicated in proliferating MRC9 fibroblasts. Mechanistically, this may reflect the activity of increased levels of free E2F-1 in S phase cells. The extent to which either a leaky E2F-1 promoter, endogenously activated E1A promoter, or interference from neighboring adenoviral transcriptional elements contributed toward E1A induction in AdE2F-1^{RC}-infected resting normal cells is unclear.

In one respect, AdE2F-1^{RC} and the adenovirus CV890 (23) have similar construction designs in that both have an intact E1A promoter upstream of their respective heterologous promoters. In CV890, the α -fetoprotein promoter drives the E1A-IRES-E1B cassette. When hepatoma and normal HBL100 fibroblasts were infected with CV890, E1A and E1B genes were expressed only in hepatoma cells. In our study, E1B 55kDa and E1B 19kDa proteins were expressed in dl309-infected quiescent normal NHLF fibroblasts and in A549 cells. In comparison, when cells were infected with AdE2F-1^{RC}, both E1B proteins were expressed in A549 cells, and a low level of only E1B 55kDa was expressed in NHLF (data not shown). This may reflect the differential activation of the E1B promoter by E1A protein (46). With a view to improving selectivity we are currently examining the effect of removing the E1A-TATA box from AdE2F-1^{RC}.

We found that AdE2F-1^{RC} was as effective as dl309 in killing different tumor cells (Fig. 5B) with various levels of E2F-1 (Fig. 2A). Also, the high selective replication index, seen in tumor cells, correlated reasonably well with deregulated E2F-1 levels in tumor cells. These results suggest that AdE2F-1^{RC} potency in tumor cells corre-

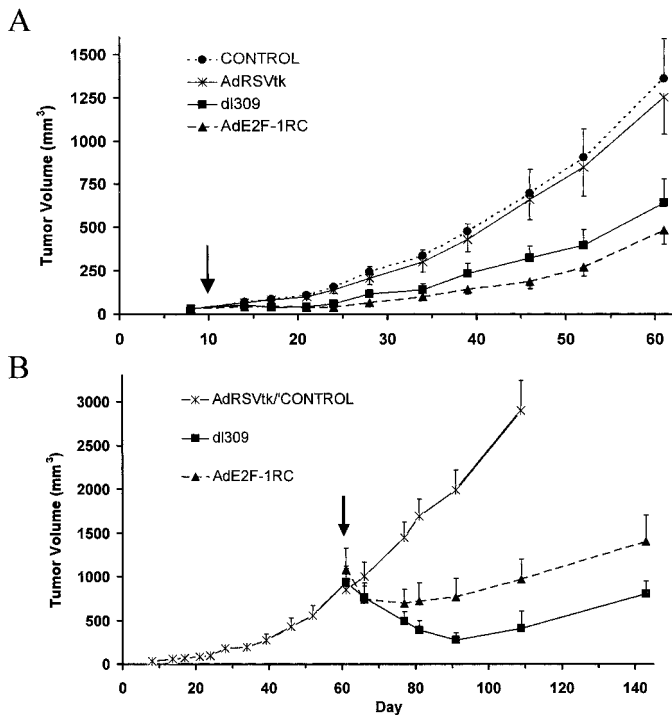


Fig. 7. Treatment of a single dose of AdE2F-1^{RC} on tumor growth. *A*, subcutaneous tumors established from 3.5 million A549 cells in SCID mice ($n = 7$) were injected intratumorally (average tumor size 30–35 mm³) with a single dose of 10⁹ pfu of either AdE2F-1^{RC} or dl309 virus, or AdRSVtk or medium alone (control) by day 10 (vertical arrow). By day 60, tumor size in AdE2F-1^{RC} and dl309 virus-treated groups was significantly ($P < 0.05$, both compared with medium alone) smaller than the medium alone-treated group. AdRSVtk without gancyclovir had no effect on tumor growth. The tumor volumes shown are mean; $n = 7$; bars, \pm SE. *B*, the experiment in *A* was repeated, but tumors were allowed to grow to an average size of 900 mm³ before being injected with a single dose 10⁹ pfu of viral vectors. Mean tumor volume is shown for the AdRSVtk/control ($n = 3$), the dl309 virus ($n = 2$), and the AdE2F-1^{RC} ($n = 2$)-treated groups; bars, \pm SE. By day 110 the tumor sizes for the AdE2F-1^{RC} and the dl309 virus-treated groups were significantly smaller than the AdRSVtk/control-treated group ($P < 0.01$, both comparisons). The AdRSVtk/control-treated experiment was terminated by day 110, as the tumors were growing too large.

lates with the transactivation of the *E2F-1* promoter by E2F-1 protein and it appears not be affected by other genetic lesions, particularly *pRb*. For example, the virus demonstrated equivalent potency in SKOV3 (*pRb*+/*p53*-), A549 (*pRb*-/*p53*+), REN (*pRb*+/*p53*), and HeLa (*pRb*-/*p53*-) cells. The potency effects of AdE2F-1^{RC} may be additionally enhanced by the differential regulation of the *E2* promoter in quiescent normal and tumor cells. Although *E2F-1* binding sites are present in the promoters of *E1A* and *E2* (Ref. 46; essential for DNA synthesis) genes, these promoters, because of the free *E2F-1*, are active only in AdE2F-1^{RC}-infected tumors cells and not in normal quiescent cells. This is because free E2F-1 is recruited to the *E2* promoter by the E4-6/7 protein (47). Thus, the dual regulation of *E1A* and *E2* in tumor cells most likely improves the viral replication kinetics. This strategy has been used to target colon cancer by using a colon cancer-specific promoter to drive the *E2* gene (48).

Data from our animal experiments showed that AdE2F-1^{RC} had a significant therapeutic effect *in vivo* in two xenograft mouse tumor models of ovarian and lung carcinomas after repeated intratumoral viral delivery. The therapeutic effect of AdE2F-1^{RC} was associated with viral replication (we detected viral replication in tumors 10–12 days after virus instillation; data not shown), cell lyses, and viral spread because replication-incompetent virus (AdRSVtk) had minimum effect. In the experiment with A549 cells, a single injection of AdE2F-1^{RC} into small flank tumors significantly inhibited tumor growth. Interestingly, on reinjecting AdE2F-1^{RC} (1×10^9 pfu) into

these tumors 60 days after the first treatment, tumor growth continued to regress compared with the control group (data not shown). Why cells that had not responded to the initial treatment and continued to grow for a long time would respond to a second treatment is unclear. Moreover, tumor growth inhibition was maintained over a prolonged time period although not totally eradicated. Our data agrees with that of Harrison *et al.* (49), who demonstrated that the growth of A549 tumors could be effectively inhibited by treatment with dl309. However, tumors could never be completely eradicated. Surprisingly, they were able to isolate dl309 virus from tumors 100 days after the first injection. Additional tumor growth retardation was achieved when the *E1B 19 kDa* gene was deleted, but again tumors did not disappear completely (50). They attributed tumor persistence to mouse cells growing within the tumors and forming barriers that prevented human adenoviral replication and spread.

The overall therapeutic index for a treatment is determined by the safety and the efficacy of the treatment. A major safety concern for the transition of AdE2F-1^{RC} into the clinic as a therapeutic agent is its replication in normal tissues. A common difficulty in testing replication-selective viruses is that there are no good laboratory animal models. However, the clinical experience with replicating vectors has indicated them to be generally nontoxic and nonimmunogenic (51, 52). Although not directly comparable, Parr *et al.* (40) developed a replication-incompetent AdE2F-1 β -gal vector in which the β -galactosidase gene was placed under the control of the *E2F-1* promoter. They showed selective expression and low toxicity in normal tissues. After AdE2F-1 β -gal injection into rat glioma tumors, β -galactosidase expression was observed only in tumor cells with minimal expression in normal brain tissues.

Other RSAds developed to target the G₁-S transition phase (18–20) have all used the “partial *E1A* gene deletion” approach. All of these vectors have demonstrated high therapeutic benefits in preclinical studies, although the effects, including immunological, of *E1A* mutant proteins in normal cells and any deleterious loss of radio or chemosensitization have not been studied. In this regard our vector expresses wild-type *E1A*, which has many other beneficial antitumoral activities as shown by its use in clinical trials for breast cancer (53). To reduce *E1A*-mutant protein toxicity in normal tissues, modifications that have been made include transcriptional control with the *E2F-1* promoter (ONYX-838; Ref. 54) and retargeting infectivity by genetically altering the Ad Δ 24 virus fiber-knob (55). A different modification describes the transcriptional control of a fusion protein, *E1A*-pRb, which is unable to destabilize the pRb/E2F-1 complex in normal cells with a multicopy *p53* response element (56).

AdE2F-1^{RC} and other RSAds targeting the G₁-S phase pathways may prove to be more effective in treating a broad range of malignancies than vectors with tumor-specific promoters, which have a narrow range of application as they rely on overexpression of a specific tumor or tissue protein (21–28). However appealing the notion of an agent that is applicable to a broad range of tumors, caution has to be exercised in assuming that the proposed mechanism of selective replication will hold true in the many different genetic backgrounds in tumors. For example, ONYX-015, which was engineered to target a broad range of tumors with *p53* defect has in practice proven to be somewhat controversial in treating every tumor with *p53* defect (4, 5, 11, 12). Nonetheless, ONYX-015 has proven to be beneficial, especially when it is genetically modified to improve viral spread (57–59), viral release (16, 50), and cell killing (14, 15), or is combined with chemotherapeutic agents (4, 5, 13).

The strategy of using RSAds as oncolytic agents has many advantages for cancer therapy. As shown in this study, even if only a small portion of cells is infected (single dose injection), viruses replicate and kill the cells and replicating viruses spread to adjacent cells. Our

approach of targeting E2F-1, a critical cell cycle pathway protein, with a tumor-specific promoter controlling the *E1A* gene allows the targeting of a wide variety of cancers, of which the hallmark is a deregulated cell cycle.

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