A Model System for the Design of Armed Replicating Adenoviruses Using *p53* as a Candidate Transgene[1]

Yosef S. Haviv, Koichi Takayama, Joel N. Glasgow, Jerry L. Blackwell, Minghui Wang, Xiaosheng Lei, and David T. Curiel[2]

Division of Human Gene Therapy, Departments of Medicine, Pathology, and Surgery, and the Gene Therapy Center, The University of Alabama at Birmingham, Birmingham, Alabama 35294

Abstract

Cancer gene therapy endeavors to overcome the low therapeutic index of currently available therapeutic modalities via the efficient and safe delivery of genetic material into tumor cells. However, despite promising preclinical results, replication-deficient viral vectors have demonstrated a limited efficacy in the clinical setting. To increase vector efficiency, replication-competent viruses have been proposed. Clinical trials have shown the safety of locally injected, conditionally replicative adenoviruses (Ads) but have underscored the need for improved potency. To further increase the therapeutic effect of replicating viral vectors, armed therapeutic viruses (ATVs) have recently been used for high-efficiency transgene expression. However, interference with cellular signaling and viral production by constitutive transgene expression may be counterproductive for ATV replication, thereby hindering the therapeutic outcome. Consequently, studies are equivocal with regard to the potential benefits of ATVs. To address this issue, we hypothesized that induction of replication of an Ad expressing *p53* may be a useful strategy in the context of ATV because *p53* does not interfere with Ad replication and may even increase its cytolytic effect. We show that in our *in vitro* ATV model system, *E1* transcomplementation of a replication-deficient Ad encoding *p53* resulted in dramatic augmentation of cell killing and circumvented resistance to apoptosis. Correlation was found between the degrees of cell killing and apoptosis induction, rather than with viral burst. Furthermore, both Ad5 *E1B 55kDa* and *E4 orf6* genes were required to enhance the cell killing. In conclusion, our *p53*-ATV model system demonstrates the potential utility of therapeutic transgene expression by a replicating Ad after a rational selection of a candidate transgene.

Introduction

Gene therapy has been suggested as a novel strategy to improve the therapeutic index of cancer therapy. Whereas replication-deficient viral vectors have demonstrated great promise as anticancer agents in preclinical studies, this has not been translated into patient benefit in the clinical setting (1). As a natural extension, replication-competent viruses have been suggested as a means to address the multidimensional biological aspects of tumors (2). To date, replication-competent viruses used in cancer clinical trials have included Ads[3] and, to a lesser extent, HSVs. Whereas replication-competent vectors have been shown to be safe and potentially beneficial for therapy of localized tumors, their potency clearly needs to be improved (3). Therefore, "armed" replicative viruses, incorporating therapeutic transgenes, have been introduced for cancer gene therapy (1, 4, 5). These ATVs embody two potential advantages. First, they exhibit a capacity for up to 3 orders of magnitude higher levels of transgene expression relative to their replication-defective vector counterparts, in selected instances (1) (6). Second, incorporated transgenes may provide a fail-safe mechanism to abolish viral replication by the induction of toxic cell death (6, 7). There are, however, potential limitations to the use of ATVs. In this regard, constitutive gene expression by Ad vectors may interfere with cellular signaling and result in premature cellular toxicity. Consequently, early apoptosis may impair viral replication (8), confounding the antitumor effects linked to oncolysis.

Accordingly, the utility of ATVs for cancer gene therapy is uncertain. In three studies, the inclusion of suicide/prodrug gene therapy with HSV*tk*/GCV in a replicating Ad did not augment antitumor efficacy *in vitro* or *in vivo* (9–11). In contrast, other studies have shown that combined oncolysis, caused by a replicating virus and suicide/prodrug gene therapy with HSV*tk*/GCV, is complementary in improving outcome *in vivo* (4, 5). Furthermore, a replicating Ad with double suicide gene therapy containing the cytosine deaminase/5-FC (*cd*/5FC) and HSV*tk* fusion gene markedly enhanced the CPE relative to the isolated viral effect (7, 12). Of note, the role of E1B 55kDa deletion in the context of ATV is also inconclusive (9, 12).

To address these inconsistencies, we have developed a strategy that induces replication of transgene-expressing, replication-deficient Ad vectors. As a proof of principle, we

Received 10/31/01; revised 12/31/01; accepted 1/28/02.

[1] Supported by the Israel-University of Alabama at Birmingham Medical Exchange Fund (Y. S. H.) and by grants from the United States Department of Defense (DAMD17-00-1-0002 and DAMD17-98-1-8571), the National Cancer Institute (R01 CA83821, IT32 CA75930, and P50 CA83591), the Lustgarten Foundation (LF043), and the CaPCURE Foundation (to D. T. C.).

[2] To whom requests for reprints should be addressed, Division of Human Gene Therapy, University of Alabama at Birmingham, WTI 620, 1824 6th Avenue South, Birmingham, AL 35294. Phone: (205) 934-8627; Fax: (205) 975-7476; E-mail: david.curiel@ccc.uab.edu.

[3] The abbreviations used are: Ad, adenovirus; ATV, armed therapeutic virus; HSV, herpes simplex virus; tk, thymidine kinase; GCV, ganciclovir; CMV, cytomegalovirus; MOI, multiplicity of infection; CPE, cytopathic effect.

have selected a replication-deficient Ad vector encoding *p53*. Because the inhibition of viral replication by HSV*tk*/GCV or *cd*/5FC may counterbalance the therapeutic effect of ATV (13), *p53* may be a useful therapeutic transgene in the context of ATV because it does not interfere with Ad replication (14) and may even increase its cytolytic effect (15).

Furthermore, selection of *p53* to indirectly induce apoptosis may circumvent transgene effects that induce apoptosis downstream of *p53* and thus do not allow effective production and lateralization of the Ad vector (16, 17).

To this end, we induced replication of Ad vector encoding *p53* by a variety of Ad mutants and evaluated cell killing and viral kinetics. Our studies show that ATV has a potential for higher cancer cell killing rates *in vitro* in the context of a transgene that is not counterproductive for viral replication.

We further observed that the burst of replicating Ad did not fully correlate with cell killing in the context of ATV. Finally, we found that both the Ad *E1B 55kDa* and *E4 orf6* genes were essential for the enhanced therapeutic effect of ATV encoding *p53*. These findings are highly consequential for an understanding of the efficacy of replicating Ad agents and for a rational design of ATV.

Materials and Methods

Recombinant Ads. A replication-deficient Ad expression vector for the delivery of wild-type human *p53* cDNA has been reported previously (18). This vector expresses human wild-type *p53* under the transcriptional control of the CA promoter comprising a CMV enhancer and the chicken β-actin promoter (AdCA*p53*). Ad338 is an Ad mutant lacking 524 bp within the *E1B 55kDa* gene (19). Ad355 is deleted for the *E4 orf6* gene (20). As a control for AdCA*p53*, we used Ad5luc1, a replication-deficient Ad (*E1*/*E3* deleted) expressing the *luciferase* gene from the *E1* region. As a wild-type equivalent we used Ad5*luc*3, a replication-competent Ad (*E1* intact, *E3* deleted) expressing the *luciferase* gene from the *E3* region. Both these viruses were constructed and propagated in our laboratory.

Cells, Transfections, and Infections. A549 and H460, human lung cancer cell lines with intact *p53*, were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were grown at 37°C in RPMI 1640 with 2 mM L-glutamine, supplemented with 10% fetal bovine serum. Infections were performed 24 h after seeding 2×10^5 cells/well in 12-well plates. For infections, growth medium was replaced by serum-free medium with the index virus at the indicated MOI. An hour later, the infection medium was removed, cells were rinsed with PBS, and 5% fetal bovine serum growth medium was restored. The medium was not replaced thereafter during the experiment and was sampled daily for determination of Ad *E1* or *E4* gene copy numbers.

For transient transfections, cells were seeded on 12-well plates and transfected with the indicated plasmids (1 μg/well) at a confluence in the range of 70%, using the Superfect (Qiagen, Santa Clarita, CA) method, according to the instructions of the manufacturer. Expression vectors used for transfection were constructed as follows; pCMV*E1* was derived from the shuttle plasmid (pShuttle) of the "Adeasy" system (21) by cloning the consecutive Ad E1 region extending from position 489 to 5789 of the Ad genome into the multicloning site, thereby deleting the right arm of pShuttle. Next, the CMV promoter/enhancer was cloned into the *Xho*I and *Eco*RV restriction sites of the recombinant plasmid. pCMV*luc* was derived from cloning of the CMV promoter/enhancer into the mammalian expression vector pGL3 basic vector (Promega, Madison, WI) upstream of the *luciferase* gene.

Apoptosis Assay. To determine whether apoptosis or necrosis was the underlying mechanism of cellular death, we infected A549 cells with AdCA*p53* at a MOI of 20, and 48 h later, we coinfected the cells with either the replication-competent Ad5luc3 or the *E1B 55kDa*-deleted Ad338, at a MOI of 5. Control wells were initially infected with the replication-deficient control virus Ad5luc1 and coinfected 48 h later with Ad5luc3. Forty-eight h after the second infection, cells were harvested, washed in cold PBS, and adjusted for a cell density of $\sim 1 \times 10^6$ cells/ml in PBS.

To detect apoptosis or necrosis, cells were stained with the dyes of the Vybrant Apoptosis Assay (Molecular Probes, Eugene, OR). This assay allows the detection of three groups of cells under a fluorescence microscope equipped with the appropriate filters for fluorescein or rhodamine. Whereas live cells show only a low level of fluorescence, apoptotic cells show green fluorescence, and necrotic cells show both red and green fluorescence (and therefore show yellow fluorescence when merged).

TaqMan PCR Assay. The *E1a* copy number was determined for each medium sample obtained as of the first day after infection. Genomic DNA was isolated and cleaned using a Qiagen Tissue Kit (Qiagen), following the instructions of the manufacturer. The concentration of isolated DNA was determined by spectophotometry. TaqMan primers and probe design, the forward primer, the reverse primer, and the 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 AACCAGTTGCCGTGAGAGTTG (anneals between residues 966 and 986) and CTCGTTAAGCAAGTCCTCGATACAT (anneals between residues 1033 and 1009), respectively, whereas the TaqMan probe was CACAGCCTGGCGACGCCA (anneals between residues 988 and 1006). The sequences of the forward and the reverse *E4* primers were GGAGTGCGCCGAGACAAC (anneals between residues 816 and 833 of the E4 orf6 open reading frame) and ACTACGTCCGGCGTTCCAT (anneals between residues 883 and 865), respectively.

The sequence of the TaqMan probe was TGGCATGACACTACGACCAACACGATCT (anneals between residues 836 and 863). 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 Car-

ousel Centrifuge (Roche Molecular Biochemicals, 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.

Statistical Analysis. Data were initially tested for normality by the Shapiro-Wilk test. All abnormal tests were further tested for significance by the Wilcoxon scores test. Results are expressed as a mean of at least three samples. Results were considered statistically significant for $P < 0.05$.

Results

Induction of Replication of AdCA*p53* by *E1* Transfection to Determine Cell Killing of ATV. Expression of *p53* has been reported to be detrimental for Ad replication and cellular transformation (22), suggesting that Ad *E1B 55kDa* mutants may not grow in normal tissues (23). In contrast, others have shown that *p53* may in fact be essential for productive Ad infection (15) and that *p53* overexpression does not interfere with Ad replication (14). Based on these considerations, we first determined the effect on viral replication and cell killing of heterologous *p53* expression. In a previous study, we have confirmed that AdCA*p53* expresses *p53*, induces apoptosis, and inhibits the growth of selected lung cancer cell lines *in vitro* and *in vivo* (18).

To evaluate the effect of *E1* transfection on AdCA*p53* replication and cell killing, we first confirmed that at a MOI of 20 plaque-forming units/cell, AdCA*p53* does not replicate or cause significant cell killing. Specifically, A549 cells infected with either the replication-defective Ad5*luc*1 or with AdCA*p53* remained viable for more than 12 days after infection. After this period, cells began to degrade but did not manifest any overt signs of viral CPE. Additionally, Ad *E1a* gene copy levels, as determined by quantitative PCR, were at the background level (data not shown). These results indicate that AdCA*p53* does not replicate and does not cause a significant CPE in A549 cells. Next, we induced replication of AdCA*p53* by transcomplementation with an intact *E1* gene. For this study, A549 cells were plated in each well of 12-well plates. After reaching 70% confluence, cells were transfected in triplicates with either pCMV*E1* or pCMV*luc*.

Twenty-four h later, cells were infected with either Ad5*luc*1 or AdCA*p53* at a MOI of 20. An advanced CPE was observed 3 days after infection only for the *E1*-transfected, AdCA*p53*-infected cohort (Fig. 1*A*). Transfection with pCMV*luc* followed by infection with AdCA*p53* did not induce any CPE, whereas transfection with pCMV*E1* followed by infection with Ad5*luc*1 resulted in delayed and low CPE relative to *E1*-transcomplemented AdCA*p53*. These data suggest that in our *p53*-ATV model, cell killing by *p53* overexpression after induction of replication of AdCA*p53* is more efficient relative to Ad-mediated oncolysis or relative to cell killing of nonreplicating AdCA*p53*. Because previous studies have shown that transgene expression may impair viral oncolysis and that *E1* has an independent apoptotic effect (24), we next evaluated the kinetics of viral replication and burst relative to CPE. To this end, we assayed Ad *E4* gene copies in the medium and found that kinetics of Ad DNA accumulation in the medium of *E1*-transfected, AdCA*p53*-infected cells indicated that viral replication was similar to the viral replication of *E1*-transfected, Ad5*luc*1-infected cells (Fig. 1*B*). Therefore, *p53* overexpression by AdCA*p53* does not significantly inhibit or support Ad replication and burst. These patterns were corroborated by the corresponding intracellular Ad DNA levels (data not shown). To account for enhanced cell killing, a synergistic effect of E1 and p53 proteins, independent of Ad replication, is unlikely because it would be counterproductive for viral replication. Thus, it appears that the enhanced killing effect of *E1*-transcomplemented AdCA*p53*

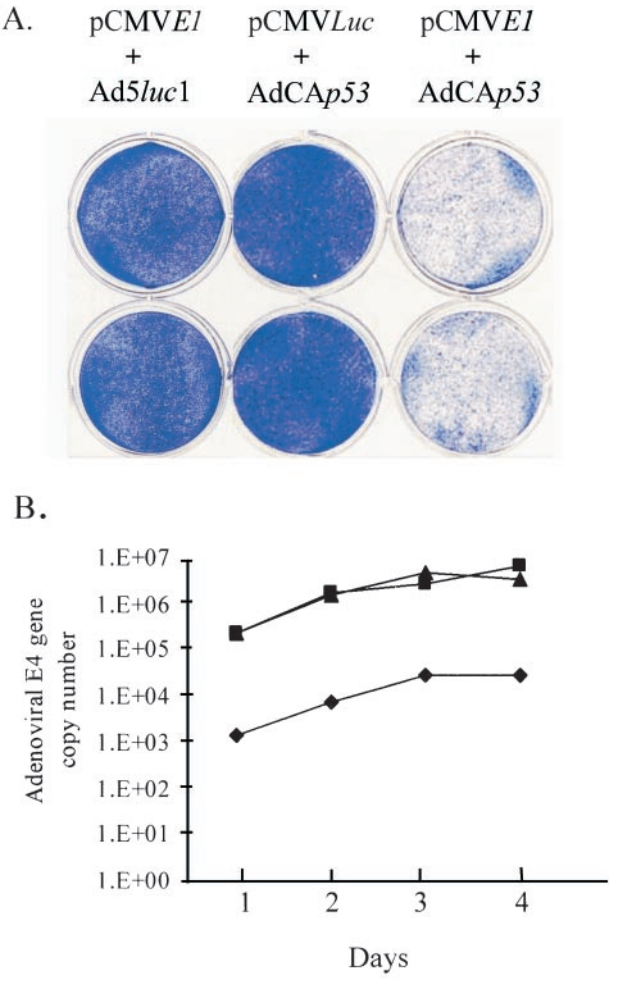

Fig. 1. Transfection of the adenoviral *E1* gene enhances the cell killing effect of AdCA*p53* in an oncolytic-independent fashion. *A,* the human lung adenocarcinoma cell line A549 was transfected in triplicates with the adenoviral *E1* expression vector pCMV*E1* or with a control plasmid expressing luciferase (pCMV/uc). Twenty-four h later, cells were infected with *E1*-deleted, replication-incompetent adenoviral vectors at a MOI of 20 plaque-forming units/cell. Vectors studied included a control vector expressing the luciferase reporter gene (Ad5*luc*1) and a *p53*-expressing vector (AdCA*p53*). Cells were stained with crystal violet after observation of advanced CPE. *B,* analysis of replication of Ad vectors induced by *E1* transcomplementation. A549 cells were transfected with pCMV*E1* or pCMV*luc* and infected 24 h later with Ad5*luc*1 or AdCA*p53*, exactly as described in *A*. Media samples were collected in triplicates from the different cohorts and subjected to quantitative PCR analysis of Ad *E4* copy number as an index of Ad replication and burst. Experimental groups included pCMV*E1* + Ad5*luc*1 (▲), pCMV*E1* + AdCA*p53* (■), and PCMV*luc* + AdCA*p53* (◆).

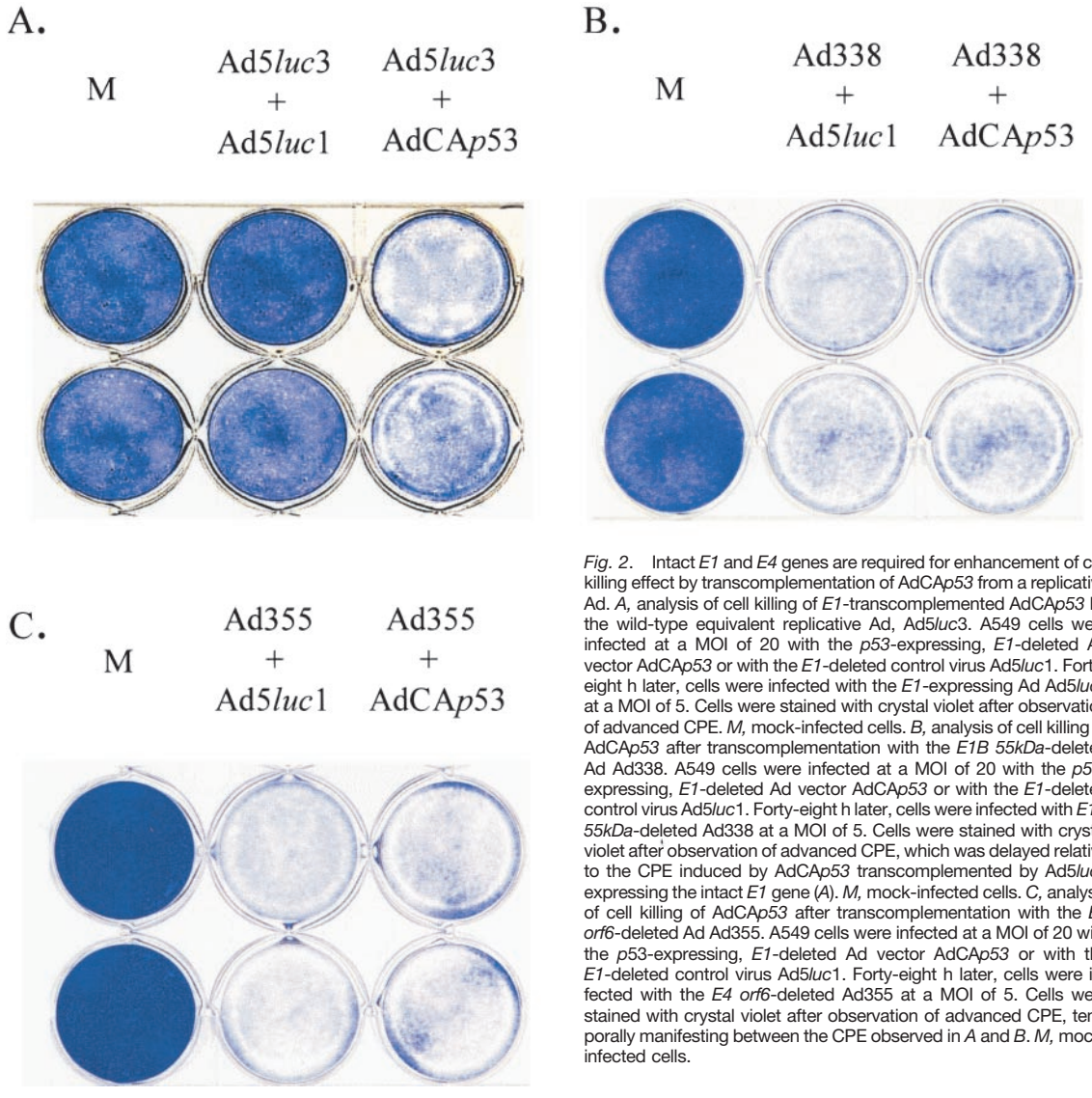

Fig. 2. Intact *E1* and *E4* genes are required for enhancement of cell killing effect by transcomplementation of AdCA*p53* from a replicative Ad. *A,* analysis of cell killing of *E1*-transcomplemented AdCA*p53* by the wild-type equivalent replicative Ad, Ad5*luc*3. A549 cells were infected at a MOI of 20 with the *p53*-expressing, *E1*-deleted Ad vector AdCA*p53* or with the *E1*-deleted control virus Ad5*luc*1. Forty-eight h later, cells were infected with the *E1*-expressing Ad Ad5*luc*3 at a MOI of 5. Cells were stained with crystal violet after observation of advanced CPE. *M,* mock-infected cells. *B,* analysis of cell killing of AdCA*p53* after transcomplementation with the *E1B 55kDa*-deleted Ad Ad338. A549 cells were infected at a MOI of 20 with the *p53*-expressing, *E1*-deleted Ad vector AdCA*p53* or with the *E1*-deleted control virus Ad5*luc*1. Forty-eight h later, cells were infected with *E1B 55kDa*-deleted Ad338 at a MOI of 5. Cells were stained with crystal violet after observation of advanced CPE, which was delayed relative to the CPE induced by AdCA*p53* transcomplemented by Ad5*luc*3 expressing the intact *E1* gene (*A*). *M,* mock-infected cells. *C,* analysis of cell killing of AdCA*p53* after transcomplementation with the *E4 orf6*-deleted Ad Ad355. A549 cells were infected at a MOI of 20 with the *p*53-expressing, *E1*-deleted Ad vector AdCA*p53* or with the *E1*-deleted control virus Ad5*luc*1. Forty-eight h later, cells were infected with the *E4 orf6*-deleted Ad355 at a MOI of 5. Cells were stained with crystal violet after observation of advanced CPE, temporally manifesting between the CPE observed in *A* and *B*. *M,* mock-infected cells.

is caused by induction of AdCA*p53* replication and efficient transgene expression, rather than by the isolated effects of Ad oncolysis or the toxic protein effect of the combination of *E1* and *p*53.

Intact E1 and E4 Transcomplementation Are Required to Enhance the Cell Killing Effect of AdCAp53. To further evaluate the enhancement of cell killing by AdCA*p53* in the context of a replicating Ad, we transcomplemented AdCA*p53* or Ad5*luc*1 with a wild-type equivalent Ad vector (Ad5*luc*3) or with Ad vectors deficient in either the *E1B 55kDa* or the *E4 orf6* genes. We reasoned that evaluation of distinct deletions of the Ad genome would allow the identification of Ad genes that are essential to achieve enhanced cell killing in the context of ATV. First, we validated that the fidelity of *E1* transcomplementation of AdCA*p53* is maintained at the viral level and that it results in augmentation of cell killing. To this end, A549 cells were infected with AdCA*p53* or Ad5*luc*1 at a MOI of 20. Forty-eight h later, cells were infected with Ad5*luc*3 at a MOI of 5. Three days after infection with Ad5*luc*3, CPE was evident only for cells coinfected with AdCA*p53* and Ad5*luc*3, whereas CPE for cells coinfected with Ad5*luc*1 and Ad5*luc*3 was delayed (Fig. 2*A*). To confirm the enhanced cell killing potency of *E1* transcomplementation of AdCA*p53* in the context of a replicating virus, we performed the same experiment with the human lung adenocarcinoma H460 cell line. These cells have a significantly lower infectivity rate by Ad, and because they express wild-type *p53*, they are also relatively resistant to apoptosis induced by the replication-deficient AdCA*p53* (18). We infected H460 exactly as described above for Fig. 2*A,* and we found that AdCA*p53* transcomplemented by *E1* from Ad5*luc*3 induced cell killing efficiently. As in A549 cells, transcomplementation of Ad5*luc*1 by Ad5*luc*3 resulted in delayed CPE, whereas AdCA*p53* alone had no effect at this MOI (data not shown).

A plausible interpretation of the capacity of *E1*-transcomplemented AdCA*p53* to circumvent the resistance

of A549 and H460 to AdCA*p53* would be that the therapeutic effect of the replication-deficient AdCA*p53* is limited due to the low efficiency of infection of the initial viral inoculum and the inherent resistance of these *p53*-positive cells to heterologous *p53* expression. In contrast, the potential therapeutic advantages afforded by *E1* transcomplementation involve augmented transgene expression, viral replication, and spread of the viral progeny to infect neighboring tumor cells. Thus, *E1* transcomplementation with an Ad vector encoding *p53* results in highly efficient killing of resistant cancer cells.

To further evaluate the indispensability of candidate Ad genes for the enhancement of the therapeutic effect of AdCA*p53*, we evaluated the interaction of several Ad mutants with AdCA*p53*. To this end, we used Ad338 and Ad355, Ad vectors with deleted *E1B 55kDa* or *E4 orf6* genes, respectively. First, we infected A549 cells with AdCA*p53* or Ad5*luc*1, as described above for Fig. 2*A*. After 48 h, cells were coinfected with the *E1B 55kDa*-deleted Ad338 at a MOI of 5. Plates were stained with crystal violet after the observation of advanced CPE (Fig. 2*B*). In this instance, transcomplemenation of AdCA*p53* by Ad338 was distinct from transcomplementation by the *E1*-intact Ad5*luc*3 in two ways. First, CPE induced by Ad338 transcomplementation of AdCA*p53* was delayed in comparison with the efficient CPE after Ad5*luc*3 transcomplementation of AdCA*p53*. Second, there was no difference in the cell killing patterns of AdCA*p53* or Ad5*luc*1 after coinfection with Ad338. These findings were corroborated by coinfecting AdCA*p53* with another *E1B 55kDa*-deleted Ad vector constructed in our laboratory.

Thus, deletion of the *E1B 55 kDa* gene prevents the enhancement of cell killing observed for transcomplementation of AdCA*p53* by an intact *E1* gene. Furthermore, these results support previous reports regarding the significance of *E1B 55kDa-p53* interaction for efficient Ad-mediated cell killing (15). Because the E1B 55kDa protein functions in concert with the *E4 orf6* gene product during the late Ad infection phase, we hypothesized that the latter is also essential to augment the effect of AdCA*p53* in the context of ATV. To this end, we infected A549 cells with AdCA*p53* or Ad5*luc*1, as described above for Fig. 2*A*. Forty-eight h later, we coinfected cells with the *E4 orf6*-deleted Ad355 at a MOI of 5. As for Ad338, transcomplementation of AdCA*p53* with Ad355 did not increase cell killing to a level greater than that achieved by Ad oncolysis alone (Fig. 2*C*). However, cell killing induced by Ad355 was observed before CPE was detected in Ad338-infected cells. Of note, whereas *E4 orf6* is intact in AdCA*p53*, coinfection with Ad355 and AdCA*p53* was comparable with coinfection with Ad355 and Ad5*luc*1, implying that the primary *E4 orf6* mutation in the complementing Ad negated its capacity to significantly enhance the therapeutic effect of AdCA*p53*. These studies, taken together with the important role of the *E1B 55kDa* and *E4 orf6* genes in the replicative life cycle of Ad (25, 26), suggest that transformation of the replication-deficient AdCA*p53* into an ATV may depend on intact *E1B 55kDa* and *E4 orf6* genes.

The Cell Killing Mechanism of E1-transcomplemented AdCAp53 Involves Augmented Apoptosis. To further delineate the mechanism of the augmentation in cell killing after *E1* transcomplementation of AdCA*p53*, we evaluated parameters of apoptosis and necrosis. Ads have developed distinct strategies to counteract cellular responses to viral infection by blocking cellular apoptosis at critical junctions in the death-signaling cascade (27). On the other hand, wild-type *p53* may enhance the ability of Ad to induce cell death (28). In the absence of *E1B 55kDa*, cell death is delayed similar to that observed for *p53*-deficient cells infected with a wild-type Ad (15). Based on these considerations, we hypothesized that the combination of heterologous *p53* and intact *E1* expression, in the context of our ATV model, would be optimal in terms of apoptosis induction. To this end, we infected A549 cells with AdCA*p53* at a MOI of 20, and 48 h later, we coinfected the cells with either the replication-competent Ad5*luc*3 or Ad338 at a MOI of 5. Control wells were initially infected with the replication-deficient vector Ad5*luc*1 and coinfected 48 h later with Ad5*luc*3. Forty-eight h after the second infection, cells were stained to detect apoptosis or necrosis. Whereas necrosis was detected in all cohorts, significant apoptosis was detected only in cells coinfected with AdCA*p53* and Ad5*luc*3 (Fig. 3). The *E1B 55kDa* deletion at Ad338 abolished the apoptosis-enhancing potency of *E1*-transcomplemented AdCA*p53*. In addition, under these conditions, we could not detect significant apoptosis for the replicating Ad5*luc*3 alone. Thus, these data indicate that the augmented cell killing induced by intact *E1* transcomplementation of AdCA*p53* is mediated by efficient apoptosis induction.

Enhancement of AdCAp53-induced Cell Killing Is Related to the Replication and Burst Kinetics of the Transcomplementing Ad Vectors. One interpretation of the above-mentioned studies would be that in this model system of *p53*-ATV, AdCA*p53* transcomplementation by *E1* results in dramatically higher cell killing by virtue of increased transgene expression.

Alternatively, it could be suggested that *p53* overexpression supports the oncolytic effect of Ad5*luc*3, thereby inducing Ad burst as the primary cause of cell death. To address this issue, we assayed the burst kinetics of Ad5*luc*3, Ad338 and Ad355. We selected the measurement of the Ad *E1a* gene as a specific indicator of the burst kinetics of the complementing viruses because it is absent from AdCA*p53*. To this end, we sampled daily the media from wells infected as described above for Fig. 2 and determined the Ad *E1a* gene copies for the relevant combination of viruses (Fig. 4*A*). Whereas media sampling is a direct method to evaluate viral burst of replication-competent viruses (2), it indicates replication only indirectly. Therefore we also evaluated intracellular Ad DNA parameters (Fig. 4*B*). When evaluating the kinetics of the transcomplementing vectors Ad5*luc*3, Ad338, and Ad355, without the effect of heterologous *p53* overexpression, the gene copy levels of the wild-type equivalent Ad5*luc*3 in the media were the highest as of the first day after infection, indicating efficient primary replication and burst of this vector. In contrast, Ad355 levels were lower than those of Ad5*luc*3, and Ad338 levels were the lowest. These patterns are in accord with the major role of *E1B 55kDa* in the Ad life cycle (25), and its role in supporting AdCA*p53*-mediated cell killing in the context of ATV. Likewise, the impaired

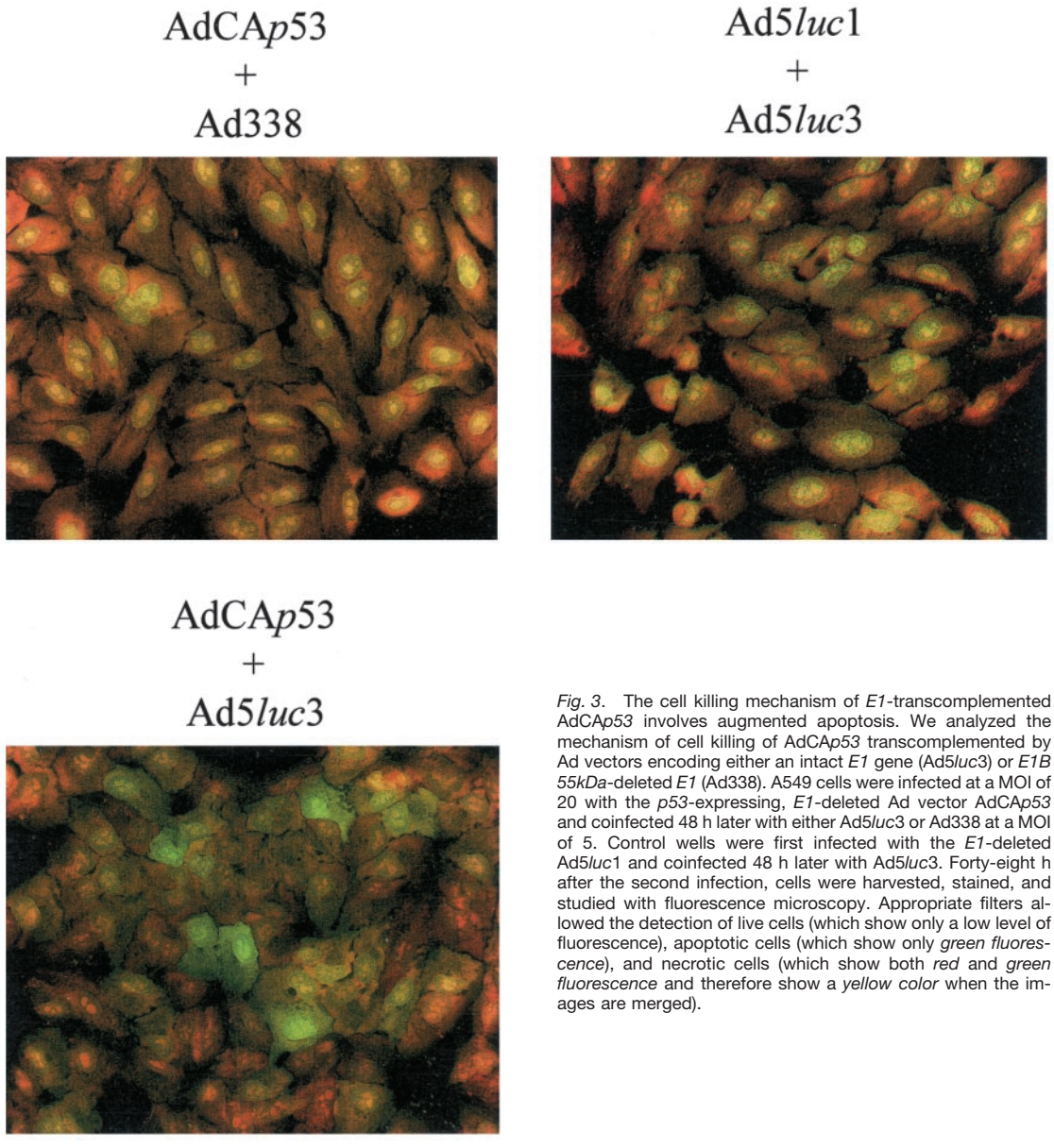

Fig. 3. The cell killing mechanism of *E1*-transcomplemented AdCA*p53* involves augmented apoptosis. We analyzed the mechanism of cell killing of AdCA*p53* transcomplemented by Ad vectors encoding either an intact *E1* gene (Ad5*luc*3) or *E1B 55kDa*-deleted *E1* (Ad338). A549 cells were infected at a MOI of 20 with the *p53*-expressing, *E1*-deleted Ad vector AdCA*p53* and coinfected 48 h later with either Ad5*luc*3 or Ad338 at a MOI of 5. Control wells were first infected with the *E1*-deleted Ad5*luc*1 and coinfected 48 h later with Ad5*luc*3. Forty-eight h after the second infection, cells were harvested, stained, and studied with fluorescence microscopy. Appropriate filters allowed the detection of live cells (which show only a low level of fluorescence), apoptotic cells (which show only *green fluorescence*), and necrotic cells (which show both *red* and *green fluorescence* and therefore show a *yellow color* when the images are merged).

replication and gene expression of *E4 orf6* mutants (26, 29) may have negated the effect of Ad355 on both AdCA*p53* replication and cell killing. Consequently, it appears that the functional *E4 orf6* from AdCA*p53* could not have complemented Ad355 to support viral replication and cell killing. Therefore, only the wild-type equivalent Ad5*luc*3 could achieve both high replication and burst rates and enhance the cell killing of AdCA*p53*.

A striking finding of the evaluation of Ad5*luc*3 burst after coinfection with AdCA*p53* was that despite the clear augmentation of cell killing upon coinfection with AdCA*p53*, the kinetics of the Ad5*luc*3 burst did not differ significantly from the burst kinetics assayed for coinfection of Ad5*luc*3 with Ad5*luc*1. A trend toward earlier burst of Ad5*luc*3 was observed after coinfection of Ad5*luc*3 with AdCA*p53*, possibly indicating the effect of *p53* overexpression and earlier cell death on viral burst. However, the slightly earlier burst is clearly distinct from the unequivocally higher cell killing effect induced by coinfection with these two viruses. Because the burst of the wild-type equivalent, Ad5*luc*3, was not significantly affected by coinfection with AdCAp53, an earlier Ad burst, possibly induced by transgene expression, cannot completely account for the enhanced cell killing potency after coinfection with a replicative Ad and the *p53*-expressing Ad. Rather, *E1* transcomplementation of AdCA*p53* may have resulted in an augmented effect of the toxic transgene, as has been recently demonstrated for HSV*tk*/GCV expressed by a replicative Ad (6). Thus, because differences in burst kinetics cannot explain the enhanced cell killing, timely and efficient transgene expression by the *E1*-transcomple-

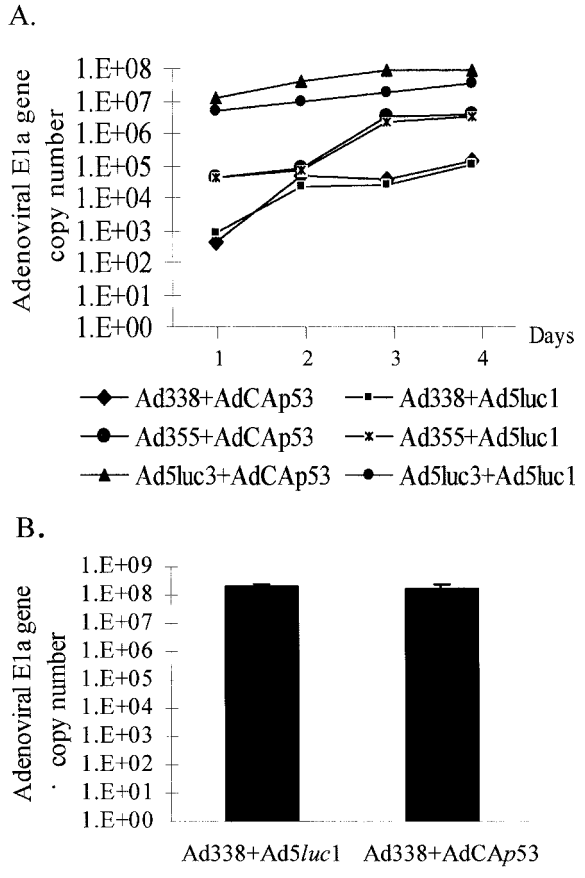

Fig. 4. Wild-type equivalent Ad and E1B and E4 mutant Ad vectors differ in their replication and burst kinetics. *A,* analysis of replication and burst of Ad vectors as a function of *E1* and *E4* status and as a function of *p53* overexpression. A549 cells were infected with AdCA*p53* or Ad5*luc*1 at a MOI of 20. Forty-eight h later, cells were infected with Ad5*luc*3, Ad338, or Ad355. Media samples were collected daily in triplicates from the different cohorts and analyzed with quantitative PCR for Ad *E1a* gene copy numbers. *B,* direct analysis of Ad338 DNA replication as a function of *p53* status. A549 cells were infected as described in Fig. 3*A* with AdCA*p53* or Ad5*luc*1 and coinfected 48 h later with Ad338. Seventy-two h after the second infection, the cellular fraction was harvested and analyzed for Ad DNA.

mented Ad vector AdCA*p53* may account for the augmented cell killing in this ATV model system.

Discussion

A major limitation of cancer gene therapy is the inadequacy of replication-deficient Ad vectors to efficiently infect tumors (30). To address this problem, replicating vectors have been suggested as a means to amplify an initial infection event (31). However, despite the safety of these viruses deriving from the tumor selectivity of replication dynamics, their efficacy is limited (3). Although incorporation of a therapeutic transgene into a replicating Ad to form an ATV has the potential to enhance the potency of replication-competent vectors, recent studies could not resolve this issue. Specifically, expression of incorporated transgenes may directly compromise the goal of viral replication and thus indirectly interfere with antitumor oncolysis (32).

In this study, we hypothesized that alleviation of the replication restriction on AdCA*p53* would form a platform for studying the therapeutic effects and viral kinetics of ATV. First, we validated that intact *E1* induces replication of AdCA*p53* and that Ad replication is not affected by *p53* overexpression. These findings validated the recent documentation of Ad replication despite *p53* overexpression (14). Next, we showed that induction of replication of AdCA*p53*, mimicking an ATV, results in a higher therapeutic effect than the oncolytic effect of replicative Ad alone. However, this effect depended on Ad transcomplementation by intact *E1B 55kDa* and *E4 orf6*. In this regard, although one might expect faster killing of cells infected with an *E1B 55kDa*-deleted Ad because of unbalanced up-regulation of *p53* by *E1a*, this is not the case (15, 33, 34). Rather, both *E1B 55kDa* and *E4 orf6* genes are important for productive Ad infection and viral oncolysis (24, 35). Furthermore, the cell killing potency of both *E1B 55kDa* and *E4 orf6* may in fact depend on cellular expression of *p53* (15, 28, 36).

Our findings of the enhanced cancer cell killing potency after *p53* overexpression, in the context of intact *E1B 55kDa* and *E4 orf6* genes, question the utility of ATV derived from *E1B 55kDa*-deleted or *E4 orf6*-deleted Ad genomes, at least for vectors encoding *p53*, and require additional studies. Because a number of previous studies have shown that ATVs with HSV*tk*/GCV are not more oncolytic than replicating Ad alone, stringent selection of the therapeutic transgene is clearly essential for the design of ATV. In this regard, our ATV model allows screening of therapeutic transgenes *in vitro*. Careful scrutiny of transgenes according to this model may select for ATV expressing transgenes that are not counterproductive for Ad replication.

A pertinent finding of this study involves the mechanism of the induction of cell death by adenoviral vectors. In this ATV model, it appears that earlier viral burst may have been secondary to the primary transgene therapeutic effect, as indicated by the selective and extensive apoptosis in cells coinfected with Ad5*luc3* and AdCA*p53* and by the borderline induction of an earlier burst of Ad5*luc*3 by AdCA*p53*. In this regard, the role of intact Ad genes in the context of an ATV has been recently demonstrated by augmented transgene expression and oncolytic effect *in vitro* and *in vivo* (6). However, in the context of cancer cell killing by an ATV, earlier viral burst may act in concert with transgene overexpression to expedite infection of neighboring cells. Therefore, means to support burst and lateralization of replicative Ad are also warranted to achieve efficient infection of all tumor cells.

In conclusion, we have developed an *in vitro* model for the evaluation of therapeutic transgenes in the context of ATV. A replication-deficient Ad vector encoding *p53* was induced to function as an ATV and shown to be superior to replicative Ad alone by virtue of its enhanced apoptotic cell killing effect. In view of the need to improve vector efficacy for cancer gene therapy, we suggest this model system to allow for a rational design of ATV.

Acknowledgments

We thank Dr. Hikaru Ueno for AdCA*p53*, Tom Shenk and Trish Robinson for Ad338 and Ad355, Yasou Adachi for pCMV*E1*, Candace Coolidge for

pCMV*luc*, Albert Tousson for expert imaging, and Delicia Carey for biostatistical studies.

References

1. Hermiston, T. Gene delivery from replication-selective viruses: arming guided missiles in the war against cancer. J. Clin. Investig., *105:* 1169–1172, 2000.

2. Alemany, R., Balague, C., and Curiel, D. T. Replicative adenoviruses for cancer therapy. Nat. Biotechnol., *18:* 723–727, 2000.

3. Kirn, D. Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? Gene Ther., *8:* 89–98, 2001.

4. Wildner, O., Blaese, R. M., and Morris, J. C. Therapy of colon cancer with oncolytic adenovirus is enhanced by the addition of herpes simplex virus-thymidine kinase. Cancer Res., *59:* 410–413, 1999.

5. Wildner, O., Morris, J. C., Vahanian, N. N., Ford, H., Jr., Ramsey, W. J., and Blaese, R. M. Adenoviral vectors capable of replication improve the efficacy of HSVtk/GCV suicide gene therapy of cancer. Gene Ther., *6:* 57–62, 1999.

6. Nanda, D., Vogels, R., Havenga, M., Avezaat, C. J., Bout, A., and Smitt, P. S. Treatment of malignant gliomas with a replicating adenoviral vector expressing herpes simplex virus-thymidine kinase. Cancer Res., *61:* 8743–8750, 2001.

7. Freytag, S. O., Rogulski, K. R., Paielli, D. L., Gilbert, J. D., and Kim, J. H. A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. Hum. Gene Ther., *9:* 1323–1333, 1998.

8. Edholm, D., Molin, M., Bajak, E., and Akusjarvi, G. Adenovirus vector designed for expression of toxic proteins. J. Virol., *75:* 9579–9584, 2001.

9. Lambright, E. S., Amin, K., Wiewrodt, R., Force, S. D., Lanuti, M., Propert, K. J., Litzky, L., Kaiser, L. R., and Albelda, S. M. Inclusion of the herpes simplex thymidine kinase gene in a replicating adenovirus does not augment antitumor efficacy. Gene Ther., *8:* 946–953, 2001.

10. Morris, J. C., and Wildner, O. Therapy of head and neck squamous cell carcinoma with an oncolytic adenovirus expressing HSV-tk. Mol. Ther., *1:* 56–62, 2000.

11. Wildner, O., and Morris, J. C. Therapy of peritoneal carcinomatosis from colon cancer with oncolytic adenoviruses. J. Gene Med., *2:* 353–360, 2000.

12. Rogulski, K. R., Wing, M. S., Paielli, D. L., Gilbert, J. D., Kim, J. H., and Freytag, S. O. Double suicide gene therapy augments the antitumor activity of a replication-competent lytic adenovirus through enhanced cytotoxicity and radiosensitization. Hum. Gene Ther., *11:* 67–76, 2000.

13. Wildner, O., and Morris, J. C. The role of the E1B 55 kDa gene product in oncolytic adenoviral vectors expressing herpes simplex virus-tk: assessment of antitumor efficacy and toxicity. Cancer Res., *60:* 4167–4174, 2000.

14. Koch, P., Gatfield, J., Lober, C., Hobom, U., Lenz-Stoppler, C., Roth, J., and Dobbelstein, M. Efficient replication of adenovirus despite the overexpression of active and nondegradable p53. Cancer Res., *61:* 5941–5947, 2001.

15. Dix, B. R., O'Carroll, S. J., Myers, C. J., Edwards, S. J., and Braithwaite, A. W. Efficient induction of cell death by adenoviruses requires binding of E1B55k and p53. Cancer Res., *60:* 2666–2672, 2000.

16. Arafat, W. O., Gomez-Navarro, J., Xiang, J., Barnes, M. N., Mahasreshti, P., Alvarez, R. D., Siegal, G. P., Badib, A. O., Buchsbaum, D., Curiel, D. T., and Stackhouse, M. A. An adenovirus encoding proapoptotic Bax induces apoptosis and enhances the radiation effect in human ovarian cancer. Mol. Ther., *1:* 545–554, 2000.

17. Zhang, H. G., Bilbao, G., Zhou, T., Contreras, J. L., Gomez-Navarro, J., Feng, M., Saito, I., Mountz, J. D., and Curiel, D. T. Application of a Fas ligand encoding a recombinant adenovirus vector for prolongation of transgene expression. J. Virol., *72:* 2483–2490, 1998.

18. Takayama, K., Ueno, H., Pei, X. H., Nakanishi, Y., Yatsunami, J., and Hara, N. The levels of integrin $\alpha_v\beta_5$ may predict the susceptibility to adenovirus-mediated gene transfer in human lung cancer cells. Gene Ther., *5:* 361–368, 1998.

19. Pilder, S., Moore, M., Logan, J., and Shenk, T. The adenovirus E1B–55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. Mol. Cell. Biol., *6:* 470–476, 1986.

20. Cutt, J. R., Shenk, T., and Hearing, P. Analysis of adenovirus early region 4-encoded polypeptides synthesized in productively infected cells. J. Virol., *61:* 543–552, 1987.

21. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. A simplified system for generating recombinant adenoviruses. Proc. Natl. Acad. Sci. USA, *95:* 2509–2514, 1998.

22. Yew, P. R., and Berk, A. J. Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. Nature (Lond.), *357:* 82–85, 1992.

23. Bischoff, J. R., Kirn, D. H., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., Nye, J. A., Sampson-Johannes, A., Fattaey, A., and McCormick, F. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science (Wash. DC), *274:* 373–376, 1996.

24. Braithwaite, A. W., and Russell, I. A. Induction of cell death by adenoviruses. Apoptosis, *6:* 359–370, 2001.

25. Goodrum, F. D., and Ornelles, D. A. The early region 1B 55-kilodalton oncoprotein of adenovirus relieves growth restrictions imposed on viral replication by the cell cycle. J. Virol., *71:* 548–561, 1997.

26. Leppard, K. N. E4 gene function in adenovirus, adenovirus vector and adeno-associated virus infections. J. Gen. Virol., *78:* 2131–2138, 1997.

27. Benedict, C. A., Norris, P. S., Prigozy, T. I., Bodmer, J. L., Mahr, J. A., Garnett, C. T., Martinon, F., Tschopp, J., Gooding, L. R., and Ware, C. F. Three adenovirus E3 proteins cooperate to evade apoptosis by tumor necrosis factor-related apoptosis-inducing ligand receptor-1 and -2. J. Biol. Chem., *276:* 3270–3278, 2001.

28. Hall, A. R., Dix, B. R., O'Carroll, S. J., and Braithwaite, A. W. p53-dependent cell death/apoptosis is required for a productive adenovirus infection. Nat. Med., *4:* 1068–1072, 1998.

29. O'Connor, R. J., and Hearing, P. The E4–6/7 protein functionally compensates for the loss of E1A expression in adenovirus infection. J. Virol., *74:* 5819–5824, 2000.

30. Schuler, M., Herrmann, R., De Greve, J. L., Stewart, A. K., Gatzemeier, U., Stewart, D. J., Laufman, L., Gralla, R., Kuball, J., Buhl, R., Heussel, C. P., Kommoss, F., Perruchoud, A. P., Shepherd, F. A., Fritz, M. A., Horowitz, J. A., Huber, C., and Rochlitz, C. Adenovirus-mediated wild-type p53 gene transfer in patients receiving chemotherapy for advanced non-small-cell lung cancer: results of a multicenter Phase II study. J. Clin. Oncol., *19:* 1750–1758, 2001.

31. Heise, C., Sampson-Johannes, A., Williams, A., McCormick, F., Von Hoff, D. D., and Kirn, D. H. ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. Nat. Med., *3:* 639–645, 1997.

32. Mi, J., Li, Z. Y., Ni, S., Steinwaerder, D., and Lieber, A. Induced apoptosis supports spread of adenovirus vectors in tumors. Hum. Gene Ther., *12:* 1343–1352, 2001.

33. Turnell, A. S., Grand, R. J., and Gallimore, P. H. The replicative capacities of large E1B-null group A and group C adenoviruses are independent of host cell p53 status. J. Virol., *73:* 2074–2083, 1999.

34. Goodrum, F. D., and Ornelles, D. A. p53 status does not determine outcome of E1B 55-kilodalton mutant adenovirus lytic infection. J. Virol., *72:* 9479–9490, 1998.

35. Orlando, J. S., and Ornelles, D. A. E4orf6 variants with separate abilities to augment adenovirus replication and direct nuclear localization of the E1B 55-kilodalton protein. J. Virol., *76:* 1475–1487, 2002.

36. Yamano, S., Tokino, T., Yasuda, M., Kaneuchi, M., Takahashi, M., Niitsu, Y., Fujinaga, K., and Yamashita, T. Induction of transformation and p53-dependent apoptosis by adenovirus type 5 E4orf6/7 cDNA. J. Virol., *73:* 10095–10103, 1999.