Efficient Replication of Adenovirus Despite the Overexpression of Active and Nondegradable p53

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ABSTRACT

The adenoviral oncoproteins E1B-55 kDa and E4orf6 inactivate and destabilize the tumor suppressor protein p53, thereby contributing to malignant transformation. However, it is unclear whether the elimination of p53 also contributes to the efficiency of viral replication. Furthermore, it is controversial whether adenoviruses with a deletion in the E1B-55 kDa-coding region might selectively replicate in cells with a mutation or deletion of the p53 gene and, therefore, represent a tool in cancer therapy. To address the role of p53 in virus replication, amino acid substitutions were introduced into the NH2-terminal portion of p53, replacing residues 24–28 with the corresponding sequence of the human p53-homologue p73. This replacement leaves p53 transcriptionally active but renders the modified protein, termed p53mt24-28, completely resistant to inhibition and degradation by adenoviral oncoproteins. Surprisingly, even strong overexpression of p53 or p53mt24-28 allowed the virus to replicate as efficiently as in the absence of p53 proteins, both in tumor cells and in primary endothelial cells. Also, p53 or p53mt24-28 did not reduce the amount of virus released from infected cells. These observations were made in primary cells or in cell lines that were capable of expressing the p53-agonist p14ARF. Thus, active p53 does not inhibit the growth of adenovirus. Alternative strategies should be used to improve the utility of adenoviruses in cancer therapy.

INTRODUCTION

Adenovirus type 5 expresses proteins that regulate the activity and stability of the tumor suppressor p53. The adenovirus E1B-55 kDa protein binds to the NH2-terminal portion of p53 and inhibits the transactivation function that is normally carried out by this domain, thereby contributing to malignant cell transformation (1–6). The adenoviral E4orf6 (E4–34 kDa) protein also regulates p53 (7), E4orf6 and E1B-55 kDa form a complex, and the two factors cooperate to destabilize p53. Therefore, p53 is eliminated from cells that are infected with adenovirus (8–13).

p53 has at least two homologues in human cells (14–21). These homologues, termed p73 (14) and p63 (22)/p51 (17)/KET (21), are capable of binding p53-responsive promoter DNA, and at least some splice variants of them can activate promoters much like p53. E1B-55 kDa specifically targets p53 but not its homologues p73 or p63 (12, 13, 23–25). Furthermore, p53 but not p73 is destabilized by the combination of E1B-55 kDa and E4orf6 (12, 13, 23, 24). On the basis of these findings, a chimeric version of p53 was created that has the five amino acid residues at positions 24–28 substituted by the homologous residues from p73. This chimera, termed p53mt24-28, transactivates p53-responsive promoters in a manner that is qualitatively and quantitatively indistinguishable from wild-type p53, but it is not detectably influenced in its activity by adenoviral oncoproteins (12).

It is well established that proteins from adenovirus as well as other small DNA tumor viruses antagonize p53 (26). However, there is little information on whether p53 inactivation contributes to viral replication and whether nonantagonized p53 has an inhibitory effect on the viral life cycle. It has been proposed that p53, in the absence of antagonists, might induce cell cycle arrest or apoptosis in infected cells, thereby preventing viral replication (27, 28). However, no direct proof was established for this hypothesis.

It has been reported that an adenovirus that lacks the E1B-55 kDa-coding region [dl1520 (29), renamed ONYX 015 (28)] replicates poorly in cells that express wild-type p53, whereas it does replicate efficiently in cells with mutant or absent p53 (28). These observations led to the use of dl1520/ONYX 015 for cancer therapy. Because the virus was expected to replicate in tumor cells that have a mutated or deleted p53 gene but not in normal tissue, it was injected into tumors with the aim of destroying them. This concept had preliminary success in animals and patients (30–38). Moreover, the application of dl1520/ONYX 015 in combination with cytostatic drugs was found superior to chemotherapy alone in Phase II trials to treat head and neck cancer (39–41). However, the theoretical basis of this approach has been challenged by a number of groups who all found that replication of this virus does not correlate with the p53 status of infected cells (34, 42–48). More recently, loss of the mdm-2 and p53 regulator p14ARF (49–51) in tumor cells was reported to facilitate the replication of dl1520/ONYX 015 in a cell line (HCT-116) with wild-type p53 (52). However, it remains to be determined whether p53 and p14ARF can generally cooperate to antagonize virus growth. Thus, the role of p53 in adenovirus replication remains unclear, but the possible development of tumor-selective viruses for use in therapy implies the need for clarification.

To address this role of p53, several approaches can be taken. The most obvious approach is to compare the replication efficiency of wild-type adenovirus with a virus that lacks E1B-55 kDa and, therefore, cannot inactivate p53. However, such a mutant virus is also defective in all of the additional activities that involve E1B-55 kDa. It is known that E1B-55 kDa is necessary not only for p53 inactivation, but also for the modulation of mRNA export during adenovirus infection (53–59). In addition, there might be unknown activities of E1B-55 kDa that would also be missing in a virus that does not express the protein. As an alternative approach, it would be desirable to create a mutant of E1B-55 kDa that is not capable of binding p53 but potentially able to perform all of the other functions of E1B-55 kDa. This mutant could then be tested in a recombinant adenovirus to assess the role of p53 binding during virus replication. However, such a mutant has not been described thus far. Most mutations in E1B-55 kDa have led to pleiotropic effects on its functions (3, 5, 8, 60), possibly attributable to a shift in protein conformation. Furthermore, even if a functionally selective mutant E1B-55 kDa could be obtained, it could not be tested as to whether unknown functions of the protein would be affected in addition to the inhibition of p53 activity. Therefore, we took a third approach to assess the role of p53 in virus...
replication. We infected cells of various origin with wild-type adenovirus and simultaneously used an adenoviral vector to express a mutant version of p53, p53mt24-28, which is resistant to the inhibitory effect of E1B-55 kDa. p53mt24-28 remained stable and active in adenovirus-infected cells. However, even in the presence of high levels of active p53, adenovirus replication was not detectably affected.

MATERIALS AND METHODS

Cell Culture and Infection. All of the cells except HUVECs were maintained in DMEM (Life Technologies, Inc.) containing 10% FBS. HUVECs were cultivated in a special low-FBS-medium (Promocell). For infection of all cells treated with the indicated amount of virus in 500 μl of DMEM without serum. After 3 h of gentle rocking in an incubator, the cells were further incubated in 2 ml of DMEM/10% FBS. HUVECs were infected by adding the virus stock to the medium (2 ml) overlaying the cells, followed by rocking for 5 min. After incubation, virus was harvested by triple freeze-thawing of the infected cells in PBS. Virus titers were determined by infecting H1299 cells (adenoviruses Wt, d309) or 911 cells (adenovirus vectors) with serial dilutions of the harvest, followed by immunostaining of the viral E2A-72 kDa protein as described previously (59).

Recombinant Adenoviruses. The p53-coding region was excised from the plasmid pRcCMVp53 (4) and from the plasmid pRcCMV/p53mt24-28 (12) with XhoI and HindIII and ligated into pAdtrackCMV (61) with the same enzymes. Recombinant adenovirus was generated by homologous recombination to the plasmid pAdEasy1 (61) and transfection of 293 cells, as described (61). A virus expressing solely the GFP was generated in parallel. Viruses were amplified, and titers were determined as described (59).

Immunofluorescence. Cells were infected as described above, but all of the quantified were reduced by a factor of four, and the cells were seeded on plastic chamber slides (Nunc) suitable for microscopy. The cells were then fixed with paraformaldehyde (4% in PBS; 15 min), permeabilized with Triton X-100 (0.2% in PBS; 25 min), and incubated with antibody as described (62). The E2A-72 kDa DNA-binding protein was stained with a mouse monoclonal antibody (clone B6-6). The p53 protein was stained with a polyclonal rabbit antibody (FL-393; Santa Cruz Biotechnology) that has been raised against a full-length p53. The monoclonal antibody Ab-1 to p14ARF were from Calbiochem. The monoclonal antibody to lamin B was purchased from Zymed.

Immunoblots. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose, followed by incubation with antibodies in PBS containing 5% milk powder and 0.05% Tween 20 and chemiluminescent detection (Pierce) of a peroxidase-coupled secondary antibody against mouse enzymes. Recombinant adenovirus was generated by homologous recombination to the plasmid pAdEasy1 (61) and transfection of 293 cells, as described (61). A virus expressing solely the GFP was generated in parallel. Viruses were amplified, and titers were determined as described (59).

RESULTS

A Chimera of p53 and p73, p53mt24-28, Induces p53-responsive Genes but Is Resistant to Destabilization during Adenovirus Infection. We previously reported that p53 can be made resistant to the destabilization by adenovirus oncoproteins without compromising its transcriptional activity (12). This is achieved by replacing the E1B-55 kDa-interacting residues 24–28 of p53 with the homologous sequence from p73, which itself is not subject to degradation in adenovirus-infected cells. To express such a nondegradable p53 variant concomitantly in adenovirus-infected cells, a first generation adenovirus vector was used. Recombinant viruses were generated that have the E1 region deleted and replaced by expression cassettes, either for the GFP alone (AdGFP) or, in addition, for wild-type p53 (Adp53) or the p53-mutant p53mt24-28 (Adp53mt24-28).

Upon infection of H1299 cells (a p53−/− cell line derived from a human adenocarcinoma of the lung) and HUVECs, the viruses were first tested for p53 expression using immunofluorescence (Fig. 1) and immunoblot (Fig. 2A). Furthermore, p53 activity was assessed by

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4 The abbreviations used are: HUVEC, human umbilical vein endothelial cell; FBS, fetal bovine serum; GFP, green fluorescent protein; MOI, multiplicity of infection.

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determining the levels of p21/cip1/waf1, the product of a well-characterized p53-responsive gene (63), by Western blot analysis (Fig. 2C). It was found that Adp53 and Adp53mt24-28, but not AdGFP, express readily detectable amounts of p53 (Figs. 1 and 2A). p53 was detected in more than 95% of the cells. Also, transduction of H1299 cells with Adp53 and Adp53mt24-28 strongly increased p21 expression (Fig. 2C). In another set of experiments, p53 levels and activity were determined with the same assays, but in addition to the recombinant viruses, the cells were simultaneously infected with wild-type adenovirus WtD (B, D, and F). Each virus was used at a MOI of 10. Twenty-four h after transduction, the cells were harvested, and the proteins p53 (A and B), p21 (C and D), and p27 (E and F; loading control) were detected by immunoblot analysis.

Fig. 2. p53 levels and activity upon adenovirus infection. H1299 cells were mock-infected (Lane 1) or transduced with recombinant adenoviruses (Lanes 2–4) to express GFP (Lane 2), p53 (Lane 3), or p53mt24-28 (Lane 4). Where indicated, the cells were simultaneously infected with wild-type adenovirus WtD (B, D, and F). Each virus was used at a MOI of 10. Twenty-four h after transduction, the cells were harvested, and the proteins p53 (A and B), p21 (C and D), and p27 (E and F; loading control) were detected by immunoblot analysis.

doing p53, but p21 levels were high in the presence of p53mt24-28 (Fig. 2D). Thus, the mutation of residues 24–28 renders p53 stable and functional throughout an adenovirus infection.

p53 and p53mt24-28 Do Not Dectectably Interfere with Adenovirus Replication. Despite the presence of E1B-55 kDa-resistant p53, staining with an antibody against the viral E2A-72 kDa gene product revealed that the viral replication centers were formed (Fig. 3, A, part c, and B, part c), and at least in HUVECs, p53 even seemed to accumulate in these centers (Fig. 3B, part d, and Ref. 64). The presence of replication centers despite high levels of active p53 suggested that p53 does not block virus replication. To further investigate this, we quantitated adenovirus replication in the presence or absence of active p53 in H1299 cells and HUVECs. These cells were simultaneously infected with two different viruses: wild-type adenovirus, providing the factors necessary for virus replication, and a first generation adenovirus vector expressing GFP, p53, or p53mt24-28. After 48 h, the cells were harvested, and virus was released by freeze-thawing. The resulting virus titer was then determined by infection of H1299 cells with serial dilutions of the harvest, followed by immunostaining of the viral E2A-72 kDa protein. As shown in Fig. 4, the expression of p53 or p53mt24-28 did not reduce virus yield in either cell type. No significant effect of p53 expression on virus yield was observed either when replacing wild-type adenovirus with the virus dl1520/ONYX 015 in this experimental setup or when transduction with the p53 expression vectors preceded infection with wild-type adenovirus by 12 h (data not shown). In another experiment, HUVECs determining the levels of p21/cip1/waf1, the product of a well-characterized p53-responsive gene (63), by Western blot analysis (Fig. 2C). It was found that Adp53 and Adp53mt24-28, but not AdGFP, express readily detectable amounts of p53 (Figs. 1 and 2A). p53 was detected in more than 95% of the cells. Also, transduction of H1299 cells with Adp53 and Adp53mt24-28 strongly increased p21 expression (Fig. 2C). In another set of experiments, p53 levels and activity were determined with the same assays, but in addition to the recombinant viruses, the cells were infected with a wild-type adenovirus termed WtD (29). Under these conditions, wild-type p53 levels were strongly reduced, whereas the levels of p53mt24-28 remained unaffected (Fig. 2B and Fig. 3). Again, the activity of wild-type p53 was determined by measuring the levels of p21/cip1/waf1. In adenovirus-infected cells, p21 expression was induced less efficiently by wild-type p53, but p21 levels were high in the presence of p53mt24-28 (Fig. 2D). Thus, the mutation of residues 24–28 renders p53 stable and functional throughout an adenovirus infection.

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were growth arrested by a 4-day period of confluence before infection. Again, expression of p53 or p53mt24-28 did not impair viral replication in this experimental setup, and essentially the same results as with growing HUVECs (Fig. 4) were obtained (data not shown). Thus, p53 activity has no detectable effect on virus yield in H1299 cells and HUVECs.

p53 and p53mt24-28 Do Not Impair the Replication and Release of Recombinant Adenovirus in an E1-transformed Cell Line. In the system described above, cells were coinfected with two different viruses. To avoid any influence that the use of two different viruses might have on replication, we also used a system in which adenovirus recombinants lacking the E1 region could replicate efficiently. To validate this system, the three recombinant viruses AdGFP, Adp53, and Adp53mt24-28 were used to infect 911 cells, a cell line derived from human embryonic retinoblasts that stably expresses the adenovirus E1 region, including E1B-55 kDa (65). p53 and p21 levels were determined in the infected cells by immunoblot analysis. Mock-infected cells contained detectable levels of endogenous p53, in accordance with the stabilizing effect of E1B-55 kDa alone on p53 (66). In contrast, p53 was not detectable after infection with AdGFP (Fig. 5A), presumably because of its degradation that is mediated together by the proteins E1B-55 kDa (expressed by the 911 cells) and E4orf6 (expressed by the recombinant virus; Refs. 9–12).

Upon infection with Adp53 and even more strongly with Adp53mt24-28, p53 was detectable, suggesting that p53 is overexpressed from Adp53 but destabilized by E1B-55 kDa and E4orf6, whereas p53mt24-28 is overexpressed and stable. AdGFP suppressed p21 expression (Fig. 5B; compare Lanes 1 and 2), in accordance with the elimination of endogenous p53 (Fig. 5A, Lanes 1 and 2). In contrast, upon infection with Adp53, p53 activity was sufficient to allow p21 expression. As expected, p21 induction by Adp53mt24-28 was even stronger (Fig. 5B). Thus, p53mt24-28 is active and stable after infection of 911 cells with Adp53mt24-28.

Next, we tested the replication of adenovirus vectors that express p53 or p53mt24-28 in cells that complement the viral E1 functions. In parallel assays, 911 cells were infected with AdGFP, Adp53, or Adp53mt24-28. Twenty-four, 48, and 72 h after infection, the cells were harvested and lysed by triple freeze-thawing. Virus yield was determined by infection of a fresh monolayer of 911 cells and immunostaining of infected cells using an antibody against the viral E2A protein. No significant difference in virus replication was found between any of the three viruses (Fig. 6), despite the stark differences in p53 levels and activity that were found under identical conditions (Fig. 5). Thus, active p53 does not detectably affect adenovirus replication in 911 cells.

In the previous experiments, viruses were harvested by freeze-thawing. We also determined whether p53 might affect the release of virus at the end of a lytic cycle, as has been suggested previously (42, 67). To address this, 911 cells were infected with adenovirus vectors expressing GFP alone, p53, or p53mt24-28. After 48–120 h postinfection, a small fraction of the media overlaying the cells was removed, and the amount of released virus particles was determined by titration. As shown in Fig. 7, p53 or p53mt24-28 did not negatively regulate the release of virus. Apparently, p53 activity does not inhibit the late stages of the viral life cycle.

H1299 Cells and HUVECs Express p14ARF. It was reported recently (52) that dl1520/ONYX 015 grows poorly in cells that express both wild-type p53 and p14ARF, possibly because the com-

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**Fig. 4. Replication of wild-type adenovirus in the presence of p53.** H1299 cells or HUVECs were infected with wild-type adenovirus WtD and, where indicated, simultaneously with adenovirus vectors expressing GFP, p53, or p53mt24-28. For H1299 cells, the MOI was 10 for each virus. In the case of HUVECs, this number was 50 for the expression vectors and 25 for wild-type adenovirus. Forty-eight h after infection, the cells were harvested, and virus was released by repeated freeze-thawing. The virus yield was determined by infecting H1299 cells, followed by immunostaining against the viral E2A protein, and divided by the number of infected cells. The average result of at least three independent experiments is represented by the columns, along with the SE.

**Fig. 5. p53 levels and activity upon infection of E1-transformed cells with adenovirus vectors.** 911 cells (human cells that express the adenoviral E1 genes) were infected with adenovirus vectors as indicated (MOI = 5). Twenty-four h after infection, the cells were harvested, and the proteins p53 (A), p21 (B), and p27 (C) were detected by immunoblot analysis.

**Fig. 6. Replication of adenovirus vectors expressing p53 in E1-transformed cells.** 911 cells were infected with adenovirus vectors as indicated (MOI = 5). At the indicated time points after infection, the cells were harvested, and virus yield was determined by infecting a new monolayer of 911 cells as described in the legend to Fig. 4. The results are shown on a logarithmic scale.
bined activity of both tumor suppressors may inhibit adenovirus growth. Therefore, we assessed the amount of p14ARF in the cells under study. Because the adenovirus E1A protein was shown to induce p14ARF expression (68), p14ARF levels were also determined in adenovirus-infected cells. H1299 cells, HCT116 cells [found previously (52) to express no detectable p14ARF and, therefore, serving as a negative control], 911 cells, and HUVECs were either mock-infected or infected with wild-type adenovirus for 24 h before harvest. A monoclonal antibody against p14ARF was used to detect the protein in whole cell lysates by Western Blot analysis (Fig. 8). It was found that H1299 cells express high levels of p14ARF, and the protein was also detected in 911 cells. p14ARF levels in HUVECs were insufficient for detection but could be raised to detectability by adenovirus infection. Hence, p53 does not inhibit adenovirus replication in the cells studied here, although p14ARF is expressed.

DISCUSSION

Our results show that the elimination of p53 is not required for efficient replication and release of adenovirus type 5. The observations were made in primary cells or in cell lines that are not compromised for the expression of p14ARF. These results challenge the concept of using adenoviruses deficient in antagonizing p53 to treat cancer patients, raising the need to develop alternative strategies to construct tumor-selective viruses.

Previously (28), it has been reported that the replication of the virus dl1520/ONYX 015 lacking E1B-55 kDa is suppressed in cells containing wild-type p53, at least when p14ARF is expressed in the same cells (52). The proposed correlation between p53 status and replication of this virus was not supported in many subsequent reports (34, 42–48). Some of the cells that replicate the virus efficiently despite the presence of wild-type p53 may indeed lack functional p14ARF, thereby leaving open the possibility that p53 might act as an antagonist to virus replication when all of the p53 regulation pathways are intact. However, the results presented in this study were obtained with primary cells (HUVECs) or with cell lines that express p14ARF (H1299, 911). Unlike transformed cells, HUVECs were not selected for alterations in growth regulation, and they have intact pathways leading to p53-mediated transcription (69). Nonetheless, p53 activity did not affect virus replication in these cells. From this, we conclude that p53 activity does not prevent adenovirus replication.

There are several examples of small DNA tumor viruses that do not actually require the inactivation of p53 to replicate efficiently. Examples include some papillomaviruses, such as human papillomavirus type 6, 11, etc., that belong to the class of weakly oncogenic papilloma viruses. Unlike highly oncogenic papillomaviruses (e.g., types 16, 18, etc.), human papillomaviruses of the types 6 and 11 express E6 proteins that do not detectably interfere with p53 activity, but yet these viruses were able to survive during evolution. Similarly, p53 did not seem to impair the replication of SV40 in vivo (70). More recently (71), it was found that some ovine adenoviruses lack detectable transforming activity but nonetheless replicate efficiently.

On the other hand, many DNA tumor viruses did evolve specific and efficient mechanisms to inactivate p53. If p53 does not detectably interfere with virus replication in cell culture, why were such mechanisms evolved and maintained? Possibly, the virus antagonizes the p53 pathway by several redundant mechanisms that may be used to different extents in different cell types, e.g., viral proteins not only inhibit p53 activity, they also antagonize p53-downstream targets. The p53-induced bax gene product is antagonized by adenovirus E1B-19 kDa (72, 73), and this appears to increase virus yield at least in some cell types. Furthermore, p53 induces p21, and the p21 protein inhibits the phosphorylation of the retinoblastoma protein pRb, but the viral E1A proteins bind and inactivate pRb (for review, see Ref. 74). Such redundancy may account for the failure of p53 activity to block virus replication. It remains to be determined whether viral proteins antagonize even more products of the large number of p53-induced genes (75, 76). Abolishing the inactivation of p53 and the downstream targets of p53 by mutations of adenovirus genes may ultimately lead to the successful construction of viruses that are blocked by p53 and that, therefore, replicate selectively in tumor cells.

Different approaches to the construction of tumor-specific adenoviruses also seem promising (77). These include the insertion of a tumor-specific promoter upstream of the essential E1A gene (78), the modification of the E1A proteins (79), e.g., to abolish binding to pRb (80), the overexpression of the adenovirus death protein encoded by the E3 region (80), overexpression of E4orf4 (81), and the modification of receptor-binding proteins on the viral surface (82). These strategies or combinations of them may increase antitumoral effectiveness and/or specificity of adenovirus constructs as anticancer agents.

The adenovirus mutant dl1520/ONYX 015 is currently being used as a therapeutic tool against cancer, and the preclinical results (30–34, 37, 38, 82) as well as the clinical results (27, 35, 38–40) reported thus
far seem very encouraging. Selective replication in and destruction of tumor cells was observed, raising hopes for broader application. Our results, as well as the previous reports (34, 42–48) about the replication behavior of dl1520/ONYX 015, do not necessarily contradict the clinical data. However, they do put into question the initial concept that was proposed for the cell-specific replication of dl1520/ONYX 015. In the initial report (28) describing the potential use of dl1520/ONYX 015 as a therapeutic tool, it was concluded that dl1520/ONYX 015 replicates selectively in those cells that do not express functional p53, whereas in normal cells, p53 should prohibit efficient virus replication. Our results do not support this concept. However, it remains possible that adenoviruses in general, including wild-type adenovirus type 5, have a preference for many tumor cells, as compared with normal tissue. In agreement with this, our unpublished observations show that the infectivity of adenovirus type 5 is 10–100-fold lower in primary cells (HUVECs, human fibroblasts, and normal human bronchial epithelia) than in most tumor-derived cell lines tested (e.g., H1299, HeLa, and A549). Such a “natural” selectivity of adenovirus for tumor tissue has already been suggested in a report from the year 1956 (83) about the use of wild-type adenovirus to treat cervical carcinoma. The authors treated patients with advanced cervical carcinoma by injection of wild-type adenovirus directly into the tumor or into blood vessels supplying the tumor. They observed damage to the tumor cells, resulting in tumor regression in some cases. Importantly, virus was reisolated from the tumor material but not from the surrounding tissue after treatment. Despite the limited tools available at that time, it seems that the virus had replicated in the tumors at least with some selectivity. On the basis of these considerations, it is possible that dl1520/ONYX 015 merely represents an attenuated adenovirus with no particularly enhanced specificity for tumor cells. On the other hand, the clinical success that was achieved even with this construct raises hopes that adenoviruses can ultimately be developed into specific and efficient therapeutic tools.

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