Restriction of adenoviral replication to the transcriptional intersection of two different promoters for colorectal and pancreatic cancer treatment

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Abstract

In our current study, we developed oncolytic adenoviruses which preferentially lyse pancreatic and colon cancer cells by replacing viral E1 and/or E4 promoter with the tumor/tissue-specific promoters, cyclooxygenase-2 (COX-2), midkine (MK), or the cell cycle–dependent promoter, E2F1. We generated three sets of recombinant adenoviral vectors. In the first set, only the native E1A promoter was replaced by the COX-2, MK, or E2F1 promoter, respectively. In the second set, the viral E4 promoter was substituted by these heterologous promoters and the viral E1A promoter was substituted by the ubiquitously active cytomegalovirus-IE promoter. In the third set, we substituted the viral E1A and E4 promoters with the COX-2, MK, or E2F1 promoter, respectively. In our system, transcriptional targeting of solitary viral E1A resulted in 50% enhanced restricted vector replication when compared with an unrestricted replication-competent adenovirus. Furthermore, a targeted expression of the viral E1A gene products had a greater effect on restricted adenoviral replication than that of the E4 region. With our vectors, Ad.COX·MK and Ad.MK·COX, using two different heterologous promoters to control E1A and E4 expression, we showed enhanced viral replication specificity when compared with Ad.COX·COX or Ad.MK·MK, respectively. In a s.c. xenograft tumor model, there was no significant difference in the antineoplastic efficacy of the double heterologous promoter–controlled vectors when compared with our unrestricted replication-competent control adenovirus or vectors with only E1A transcriptionally driven by a heterologous promoter. [Mol Cancer Ther 2006;5(2):374–81]

Introduction

Oncolytic adenoviruses are potent chemosensitizers, particularly in cells with functional p53 (1), and have shown encouraging antineoplastic activity in clinical trials in combination with chemotherapy (2, 3). Furthermore, replication-competent adenoviral vectors promise to be more efficient gene delivery vehicles than their replication-deficient counterparts (4), and they are also associated with a higher risk for adverse effects, especially due to the lack of an established effective therapy for serious, disseminated adenovirus infection (5).

To improve the safety and therapeutic index of replication-competent adenoviral vectors, attempts have been made to restrict vector replication to tumors. Many levels in adenovirus replication may be regulated to generate conditionally replication-competent adenoviruses targeting tumor cells. Three strategies have been pursued. First, the complementary defects approach, in which cellular dysfunctions complement viral defects. However, this strategy requires a detailed understanding of the function of both the viral genes and the cellular pathways defective in cancer. The original virus of this type, *dl*1520, has a deletion of the *E1B 55K* gene (6). A related tumor-targeting strategy based on the complementation of viral E1A conserved region 2 deletion (E1Δ24), which is necessary for retinoblastoma protein binding, has recently been described (7).

Second, the transductional targeting approach is based on the modification of the fiber, which is critical for the first step of the adenoviral adsorption, in an attempt to redirect natural vector tropism through receptors expressed at high levels on tumor cells (8–11). However, because adenoviral transduction is a two-step process which also involves ubiquitously expressed integrins, actual re-targeting, without just broadening the tropism, is difficult to achieve.

Third, transcriptional targeting, which is less dependent on a complete understanding of virus biology, is based on the replacement of viral promoters by tumor/tissue-specific promoters which are critical for replication. Following adenovirus infection, E1A is the first viral gene product to be expressed, and is required for the efficient transcription of all other viral early gene regions (12). E1A also promotes S phase entry, partly through binding and inactivation of retinoblastoma protein and its related family members (13). Like the E1 gene products, the adenoviral E4 gene region is expressed early after infection and is an

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essential component of the viral life cycle. The adenoviral E1 and E4 gene products function in concert to create a cellular environment permissive for efficient expression and processing of viral gene products, and ultimately, a productive viral infection (14).

In this strategy, the adenovirus E1A gene has been placed, e.g., under the transcriptional control of the α-fetoprotein, MUC-1 (15, 16), prostate-specific antigen (17), and tumor-specific transcription factor promoters (18). However, this approach is hampered by the fact that small amounts of E1A gene products are sufficient to initiate adenoviral replication, resulting in an accumulation of E1A gene products, and thus, consequentially, in enhanced viral replication (19). Furthermore, the upstream adenoviral packaging sequence contains two enhancer elements (20) of the E1A promoter which are active in most cell types tested (21, 22) and cannot be removed without the abolition of adenoviral encapsidation (23). In addition, the region contains ''cryptic'' transcription initiation sites, which significantly contribute to the interference with adjacent promoters (24), resulting in the attenuation of their fidelity. In a previous study, we showed that the adenoviral E1A upstream regulatory region and viral gene products interact with some, but not all, heterologous promoters and that the basal promoter activity could often be reduced with an upstream polyadenylation sequence (25). Recent studies showed tumor-restricted adenoviral replication and lysis by replacing the viral E1A and E4 promoter with heterologous promoters (26–28).

The goal of our current study was to examine whether adenoviral replication could be restricted more efficiently by targeting adenoviral E1 and E4 expression using two different tumor/tissue-specific promoters when compared with vectors with the same heterologous promoter driving E1 and E4. We also analyzed whether adenoviral replication and cell lysis could be restricted to the transcriptional intersection of these two promoters and compared whether tight transcriptional control of adenovirus E1A or E4 is more important for restricted viral replication. In addition, we examined the antineoplastic efficacy of the vectors in a s.c. colon cancer model in nude mice.

Materials and Methods

Cells and Cell Culture

The human tumor cell lines BxPc3 (CRL-1687), Panc1 (CRL-1469), CaCo2 (HTB-37), HT-29 (HTB-38), HeLa (CCL-2), and 293 cells (CRL 1573) were obtained from the American Type Culture Collection (Manassas, VA) and propagated in D-10 medium, consisting of high-glucose DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, 50 μg/mL gentamicin, and 2 mmol/L glutamine (Invitrogen/Life Technologies, Paisley, United Kingdom).

HaCaT cells were kindly provided by Dr. N. Fusenig (Division of Differentiation and Carcinogenesis *In vitro*, at the German Cancer Research Center) and maintained in D-10 medium. Primary human keratinocytes (HKC and HKC2) were kindly provided by Dr. K. Reimers (Klinik für Plastische, Hand- und Wiederherstellungschirurgie, MHH, Hannover, Germany) and propagated in PromoCell Keratinocyte Growth Medium 2 (Promocell GmbH, Heidelberg, Germany).

We established short-term cultures of pancreatic and colorectal cancer cells, designated PPC and PCC, from patients who underwent surgery for treatment of histologically confirmed pancreatic or colon cancers, respectively. Ethical clearance was obtained from the local ethics committee (reg. no. 2487). In addition, we were able to initiate cultures from normal colorectal mucosa. Cells were obtained from tissue by mechanical disruption and enzymatic digestion with DNase I (80 mg/mL), hyaluronidase (2,000 units/mL), and collagenase (40 mg/mL; Sigma-Aldrich, St. Louis, MO). Cells were maintained in DMEM/F12 (1:1) medium supplemented with 10% heat-inactivated fetal bovine serum, 50 μg/mL gentamicin, and 2 mmol/L glutamine. Cell lines were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Plasmids, DNA Transfections, and Luciferase Reporter Assays

Indicated fragments of the cyclooxygenase-2 (COX-2, −327/+59; ref. 29), midkine (MK −1009/+25; ref. 30), and E2F1 (−218/+66; ref. 31) promoter were PCR-amplified and cloned into the *Sac*I/*Xho*I restriction sites of the luciferase reporter vector pGL3-Basic (Promega, Madison, WI). Promoter studies were carried out using a dual luciferase reporter assay (Promega), allowing the analysis of test promoters via Firefly luciferase activity and normalization of transfection efficiency via *Renilla reniformis* luciferase driven by the constitutive cytomegalovirus (CMV)-IE promoter in a single well. For promoter analysis in subconfluent cell monolayers, cells were seeded 1 day prior to transfection at a density of 1×10^4 cells per well in 24-well tissue culture plates. Cell monolayers were cotransfected with 9.9 μg Firefly reporter vector DNA and 0.1 μg DNA of the *Renilla* luciferase expression plasmid vector pRL-CMV (Promega) using the calcium phosphate method (32). Forty-eight hours after transfection, cell lysates were analyzed using a microplate luminometer (Berthold ORION-96, Bad Wildbad, Germany), as described previously (25). Studies were carried out in four independent experiments using two preparations of plasmid DNA from the *Escherichia coli* strains, DH5α and Stbl2 (Invitrogen/Life Technologies).

Arrest and Analysis of Cell Cycle

Cells were arrested at the beginning of S phase by incubation with 2 mmol/L thymidine and 1 μg/mL aphidicolin (Sigma-Aldrich; ref. 33) 8 hours after transfection or at the time of adenovirus infection until analysis. Cell cycle distribution was determined by flow cytometric analysis (FACSCalibur flow cytometer; Becton Dickinson Immunocytometry Systems, Mansfield, MA), as described previously (25).

Recombinant Adenoviruses

Vectors were generated with a modified Ad-Easy system, provided by Prof. Bert Vogelstein (Johns Hopkins Kimmel

Cancer Center, Baltimore, MD; ref. 34), in which the E1 region had been restored. To generate adenoviral vectors with heterologous E1A promoters, a herpes simplex virus thymidine kinase (HSV-*tk*)—internal ribosome entry site—Ad5 E1 expression cassette driven by the human CMV-IE promoter (35) was cloned into pShuttle as a *Not*I/*Afl*II fragment. The CMV-IE promoter was replaced by the heterologous promoter with an upstream synthetic polyadenylation sequence (36). Recombinant vectors were generated as described previously (34). Adenoviral infection susceptibility of the cell lines was determined with adenoviral-green fluorescent protein, a previously described vector encoding green fluorescence protein driven by the CMV-IE promoter (37).

To generate adenoviral vectors with the E4 region under the control of heterologous promoters, the viral E4 promoter (Ad5 nucleotides 35528–35832, GenBank M73260) of the plasmid pAd-Easy-1 was replaced with heterologous promoters by homologous recombination with a plasmid carrying the promoter fragment and a removable kanamycin expression cassette. The adenoviral vector Ad.CMV·E4 was initially published as Ad.OW34 (35), and in this study, was renamed to unify adenoviral vector nomenclature. All adenoviral vectors had a deletion of the E3 region and were amplified in HeLa cells, purified by two rounds of CsCl density centrifugation and subsequent dialysis (38). Concentration and bioactivity of the adenovirus vectors were determined by measuring absorbency at 260 nm and 50% tissue culture infective doses ($TCID_{50}$) using 293 cells or the indicated cell line (39). The titer obtained by $TCID_{50}$ was 0.7 log higher than the titer by plaque assay. In all of the experiments, infections were normalized on the basis of plaque-forming unit titers.

Adenoviral Infectivity Assay

BxPc3, Panc1, CaCo2, HT-29, PPC, PCC, HKC, HKC2, normal colorectal mucosa, and 293 cells were seeded (1×10^6 cells per well) into six-well plates, and 12 hours later, incubated for 4 hours with adenoviral-green fluorescence protein at a multiplicity of infection of 5, 1, and 0.5 plaque-forming units per cell (determined on 293 cells). Forty-eight hours later, green fluorescent protein expression was analyzed by flow cytometry.

Cytotoxicity Assay

To determine viral cytopathic effect, 1×10^6 cells were seeded into six-well plates and infected with adenoviruses in 5 mL D-10 growth medium at a multiplicity of infection of 5 plaque-forming units/cell (determined for each cell line individually). Four hours later, cell monolayers were washed and incubated with fresh growth medium. Within a day, when complete lysis of monolayers infected with the control virus Ad.CMV·E4 occurred (approximately at 5 days postinfection), the remaining cells were washed, fixed with 2% paraformaldehyde, and subsequently stained with 1% crystal violet. Plates were photographed and the viral cytopathic effect was quantified with the software NIH ImageJ 1.33 (NIH, Bethesda, MD). Cell lysis was normalized to mock-infected cells.

Animal Studies

This study was approved by the local animal care and use committee. The antineoplastic efficacy of the adenoviral vectors was determined in a s.c. HT-29 tumor xenograft model in nude mice. For this, 5- to 7-week-old BALB/c *nu/nu* mice (Universitätsklinikum Essen, Germany) were injected with 1×10^7 HT-29 cells in 100 μL s.c. into the hind flanks. At least once a week, minimum and maximum perpendicular tumor axes were measured using vernier calipers, and tumor volume was calculated using the simplified formula of a rotational ellipse ($l \times w^2 \times 0.5$).

When the tumors reached a volume of ~180 mm^3, animals were randomly assigned to treatment groups and received two intratumoral injections of 5×10^9 virus particles diluted in a volume of 100 μL PBS on days 0 and 2. The untreated group received PBS. Excised tumors were dried overnight in a vacuum desiccator before measuring weight.

Statistical Analysis

The software SPSS 12 (SPSS Inc., Chicago, IL) was used for statistical analysis with the indicated test.

Results

Analysis of Promoter Activity

The specificity of the COX-2, MK, and E2F1 promoter fragments were examined with a dual luciferase reporter gene assay in subconfluent pancreatic (BxPc3 and Panc1) and colorectal (CaCo2 and HT-29) tumor cell lines. As a control, we used the nontumorigenic keratinocyte cell line HaCaT.

As shown in Fig. 1A, all promoters were active in subconfluent pancreatic and colorectal cells. The activity of the COX-2 promoter in BxPc3, Panc1, CaCo2, HT-29, and HaCaT cells was 4.5 ± 0.2%, 41.1 ± 2.9%, 16.0 ± 1.0%, 16.9 ± 3.7%, and 0.4 ± 0.2% when compared with the CMV-IE promoter, respectively. Similar results were obtained for the MK promoter: 5.1 ± 0.7%, 32.6 ± 1.4%, 6.3 ± 0.5%, 26.7 ± 1.4%, and 0.6 ± 0.1%, respectively. The activity of the E2F1 promoter was in subconfluent cells 6.8 ± 0.1%, 64.8 ± 6.0%, 26.1 ± 1.4%, 48.6 ± 3.1%, and 44.9 ± 6.4%, respectively. When the cells were mitotically arrested, the activity of the COX-2 and the MK promoter did not change significantly (P = not significant, Student's t test; Fig. 1B). However, the activity of the proliferation-associated E2F1 promoter was at least 23-fold reduced, when compared with the respective subconfluent cell line. Flow cytometric analysis of cell cycle distribution revealed that ~20% of the proliferating and ~4% of the mitotically arrested cells were in S phase (data not shown).

Differential Adenoviral Infection Susceptibility

Because every cell line expresses different amounts of coxsackie adenovirus receptor, which determines adenoviral infectivity, we analyzed adenovirus transduction efficiency of BxPc3, Panc1, CaCo2, HT-29, PPC, PCC, HKC, HKC2, normal colorectal mucosa, and 293 cells. Infection of these cells with adenoviral-green fluorescence protein at a multiplicity of infection of 0.5 plaque-forming units/cell (determined on 293 cells) resulted in 41.7%,

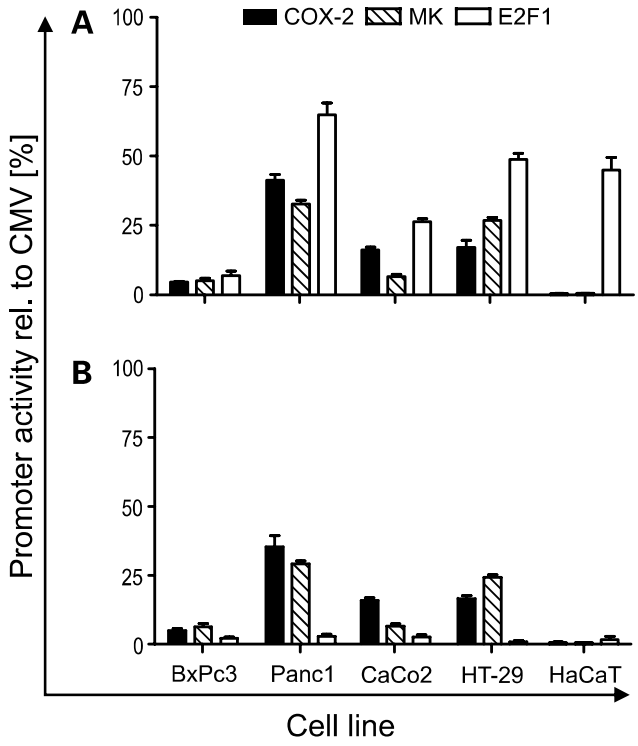


Figure 1. Luciferase reporter assays. Specificity of the COX-2, MK, and E2F1 promoter was analyzed using a dual luciferase reporter assay. Promoter activity was measured in subconfluent (**A**) or mitotically arrested BxPc3, Panc1, CaCo2, HT-29, and HaCaT cells (**B**) and compared with the CMV-IE promoter. *Columns,* means from four independent experiments; *bars,* ± SD.

46.8%, 44.2%, 43.4%, 11.8%, 16.1%, 9.8%, 10.2%, 9.4%, and 47.7%, respectively. Based on these results, for all subsequent experiments, we established individual infection conditions for each cell line to compensate for differences in adenoviral transduction efficiency.

As shown in Fig. 2, we generated 13 replication-competent adenoviral vectors, which can be grouped into three sets. The viral E1 and/or E4 promoter was replaced by the COX-2, MK, or E2F1 promoter, respectively.

Cytopathic Effect of Adenoviruses with E1A and/or E4 Driven by a Tumor/Tissue-Specific Promoter

BxPc3, Panc1, CaCo2, and HT-29 cells were used to evaluate the cytopathic effect of these replication-competent adenoviruses. As a control for normal cells, we used primary human keratinocytes and for unrestricted adenoviral replication, similar to that of wild-type Ad5, Ad.CMV·E4. When compared with mock-infected cell monolayers, infection of the tumor cell lines with Ad.CMV·E4, Ad.COX·E4, Ad.MK·E4, Ad.CMV·COX, or Ad.CMV·MK resulted in 100%, ≥90.3%, ≥88.7%, ≥94.0%, and ≥90.7% lysis of the cell monolayers, respectively (Fig. 3A-C). Infection of the primary keratinocytes resulted in 100%, ≥65.6%, ≥67.4%, ≥92.2% and ≥71.8% lysis, respectively.

As shown in Fig. 3D, infection of the tumor cell lines with the dual heterologous promoter controlled adenoviral vectors, Ad.COX·COX, Ad.MK·MK, Ad.COX·MK, or Ad.MK·COX resulted in 74.2% to 93.3% cell lysis. Infection of the primary keratinocytes with Ad.COX·COX or Ad.MK·MK resulted in ~15% cell lysis when compared with uninfected cells. However, infection with Ad.COX·MK or Ad.MK·COX resulted only in ~4% cell lysis.

Cytopathic Effect of Adenoviruses with E1A and/or E4 Driven by the E2F1 Promoter

Next, we determined whether E2F1 allows cell cycle–dependent replication of adenoviral vectors. Furthermore, we analyzed whether it is more important to have a tight expression control of the adenoviral E1A or E4 gene products. For this, we infected the same panel of cell lines, arrested and proliferating, with the adenoviral vectors Ad.CMV·E4, Ad.CMV·E2F, Ad.E2F·E4, Ad.E2F·E2F, Ad.E2F·COX, Ad.E2F·MK, Ad.COX·E2F, or Ad.MK·E2F.

As shown in Fig. 4A, infection of cells with Ad.CMV·E4, or Ad.CMV·E2F resulted, irrespective of their cell cycle status, in 100% and ~80% cell lysis, respectively. Infection of proliferating and arrested cells with Ad.E2F·E4 resulted in ~98% and ~24% lysis of the cell monolayers, respectively.

Infection of the tumor cell lines with the vectors Ad.E2F·E2F, Ad.E2F·COX, or Ad.E2F·MK resulted in ~90% cell lysis, when the cells were proliferating at the time point of infection. Infection of proliferating human keratinocytes with Ad.E2F·COX or Ad.E2F·MK resulted in ~34% cell lysis. However, under the same conditions, infection of HKC with the vector Ad.E2F·E2F resulted in 83.9 ± 5.9% cell lysis. In contrast, when the tested panel of cells was chemically arrested, virtually no cell lysis with these adenoviral vectors was observed (Fig. 4B).

In contrast, infection of the cell lines with adenoviral vectors in which the E2F1 promoter was placed upstream of the adenoviral E4 gene products and E1A was driven by the COX-2 or MK promoter, no significant difference in cell lysis was observed between proliferating and arrested cells (P = not significant, Student's t test; Fig. 4C).

Cytopathic Effect of Adenoviruses in Primary Cells

We verified whether the cytopathic effect of the replication-competent adenoviral vectors obtained with commercially available permanent tumor cell lines could also be obtained in short-term primary pancreatic and colon cancer cells. As a control, we used cultures of human primary keratinocytes and normal colon mucosa cells. As shown in Fig. 5, infection of the primary tumor cells with Ad.CMV·E4, Ad.E2F·E2F, Ad.COX·MK, Ad.E2F·E4, or Ad.COX·E4 resulted in complete lysis of the cell monolayers. Infection of the nonneoplastic cells with Ad.CMV·E4 produced complete cell lysis. In contrast, monolayers infected with Ad.E2F·E4, Ad.COX·E4, or Ad.E2F·E2F showed only significant cytopathic effects. Infection with Ad.COX·MK produced no visible cytopathic effect in the tested nonneoplastic cells.

Animal Studies

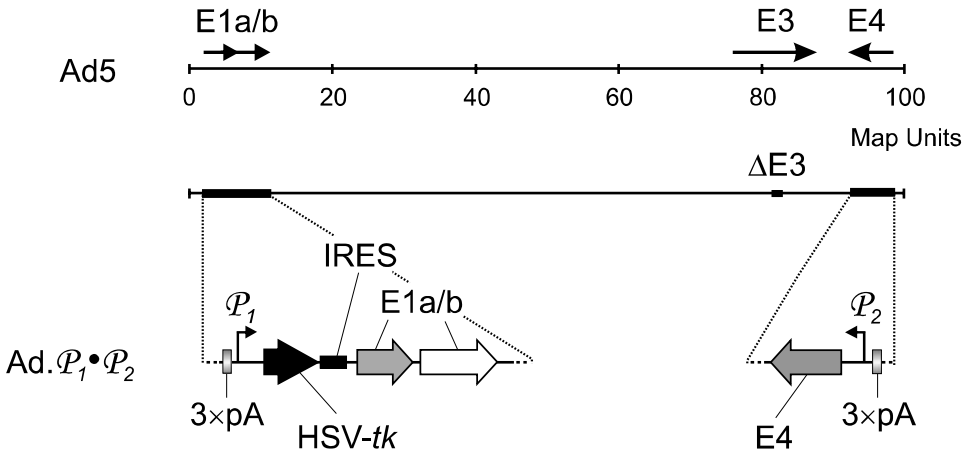
We tested the vectors Ad.CMV·E4, Ad.COX·MK, Ad.E2F·E2F, Ad.E2F·E4, and Ad.COX·E4 for their antineoplastic efficacy in an HT-29 s.c. colon carcinoma model in nude mice. As shown in Fig. 6A, the mean tumor volume of

untreated animals was 1,401.9 ± 233.3 mm^3, and the mean tumor volume of the treated animals was 329.5 ± 60.7 mm^3. On day 30, the mean dry tumor weight of untreated animals was 517.0 ± 51.8 mg, and that of adenoviral-treated animals was 159.1 ± 16.8 mg (Fig. 6B). Using the ANOVA Tukey's multiple comparison test, the tumor weight of the treated animals was significantly lower than that of the control group ($P \leq 0.001$). Furthermore, there was no significant difference among the treatment groups (P = not significant).

Discussion

Previously, several groups reported restricted adenoviral replication just by transcriptional targeting of viral E1A by a tumor/tissue-specific promoter (15–18). Somewhat improved targeting was shown by controlling E1A and E1B using a tumor/tissue-specific promoter or enhancer, respectively (40). Recently, several groups have reported the development of restricted replication-competent adenoviral vectors based on targeted expression of adenoviral E1A and E4 using the same heterologous promoter (26–28). Banerjee et al. compared adenoviral E1Δ24-deletion mutants with tyrosinase enhancer/promoter (hTyr2E/P) driving the expression of viral E1A, E4, or both. The authors showed enhanced selectivity of viral replication for melanoma cells of viruses with E1A and E4 driven by the hTyr2E/P when compared with viruses with E1A driven by the hTyr2E/P, and furthermore, that solitary driving of E4 by the hTyr2E/P does not sufficiently restrict adenoviral replication to melanoma cells.

In an effort to develop oncolytic adenoviral vectors restricted to colon and pancreatic cancers, we explored three sets of vectors in this study. In the first set, only the native E1A promoter was replaced by the COX-2, MK, or



	Virus	E1A Promoter ($\mathcal{P}_1$)	Transgene	E3 Region	E4 Promoter ($\mathcal{P}_2$)
control	Ad.CMV•E4	CMV-IE	HSV-*tk*	Δ	
Set 1	Ad.COX•E4	COX-2	HSV-*tk*	Δ	
	Ad.MK•E4	MK	HSV-*tk*	Δ	
	Ad.E2F•E4	E2F1	HSV-*tk*	Δ	
Set 2	Ad.CMV•COX	CMV-IE	HSV-*tk*	Δ	COX-2
	Ad.CMV•MK	CMV-IE	HSV-*tk*	Δ	MK
	Ad.CMV•E2F	CMV-IE	HSV-*tk*	Δ	E2F1
Set 3	Ad.COX•COX	COX-2	HSV-*tk*	Δ	COX-2
	Ad.COX•MK	COX-2	HSV-*tk*	Δ	MK
	Ad.COX•E2F	COX-2	HSV-*tk*	Δ	E2F1
	Ad.MK•COX	MK	HSV-*tk*	Δ	COX-2
	Ad.MK•MK	MK	HSV-*tk*	Δ	MK
	Ad.MK•E2F	MK	HSV-*tk*	Δ	E2F1
	Ad.E2F•COX	E2F1	HSV-*tk*	Δ	COX-2

Figure 2. Genomic organization of replication-competent adenoviral vectors. In all vectors, an expression cassette driven by the promoter $\mathcal{P}_1$ (COX-2, MK, or E2F1) with an upstream triple polyadenylation sequence was inserted into the E1 region of an E3-deleted adenovirus vector. In this expression cassette, the Ad5 E1 expression is transcriptionally linked by an internal ribosome entry site sequence to the herpes simplex virus thymidine kinase. The viral E4 promoter of double heterologous promoter–controlled vectors was replaced by the promoter $\mathcal{P}_2$ (COX-2, MK, or E2F1).

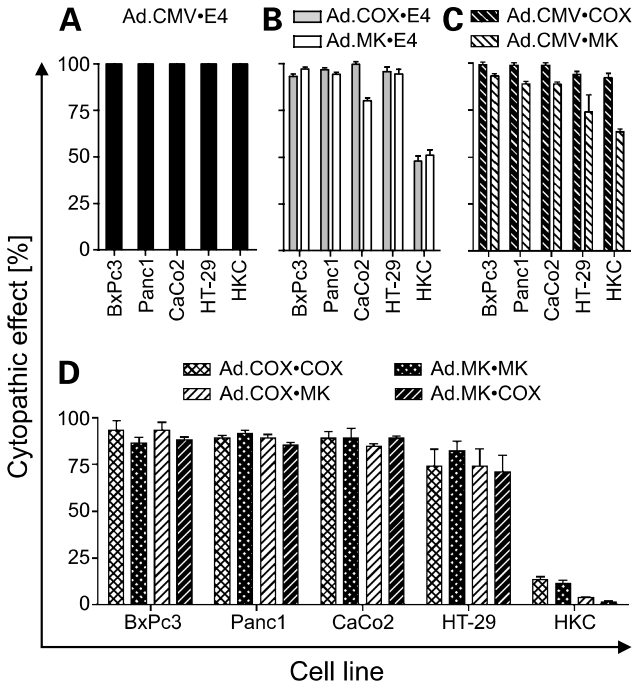


Figure 3. Cytopathic effect assay. Indicated cell lines were infected with adenoviral vectors carrying the viral E4 and/or E1 region under the transcriptional control of a heterologous promoter with an equivalent of 5 plaque-forming units/cell, determined by $TCID_{50}$ for each cell line individually. Infection of cells with the unrestricted replication-competent Ad.CMV·E4 served as control. **A,** the cytopathic effect was quantified within a day using an image analysis software and normalized to mock-infected cells when Ad.CMV·E4-infected cells were completely lysed. **B,** cytopathic effect of cells infected with vectors encoding E1 under the control of the COX-2 or MK promoter. **C,** cell killing of vectors in which the native E4 promoter has been replaced by the COX-2 or MK promoter. **D,** cytopathic effect of the E1 and E4 regions controlled by COX-2 or MK promoter. The results obtained with HKC were similar to those obtained with the nontumorigenic keratinocyte cell line HaCaT (data not shown). *Columns,* means from four independent experiments; *bars,* ± SD.

E2F1 promoter. In the second set, the viral E4 promoter was substituted with these heterologous promoters and the viral E1A promoter was replaced by the ubiquitously active CMV-IE promoter (41). In the third set, we substituted the viral E1 and E4 promoter by the COX-2, MK, or E2F1 promoter. In a previous study, we showed that the COX-2 and MK promoters were neither significantly affected by the Ad5 E1 enhancer nor by adenoviral gene products (25).

With the first set of our adenoviral vectors, we were able to confirm that transcriptional targeting of solitary E1A results in a semirestricted adenoviral replication. Despite the fact that the COX-2 and MK promoter showed virtually no activity in luciferase reporter assays in primary human keratinocytes, infection of these cells with Ad.COX·E4 or Ad.MK·E4 resulted in ~50% cell lysis. However, infection of these cells with our E3-deleted unrestricted replication-competent control vector, Ad.CMV·E4, resulted in complete lysis. Qualitatively, these results were confirmed by the infection of proliferating and arrested cells with Ad.E2F·E4. Although the E2F1 promoter had virtually no activity in the tested blocked cell lines, the Ad.E2F·E4 vector still caused ~25% cell lysis. Under the same experimental conditions, infection with the Ad.CMV·E4 vector resulted in complete cell lysis.

With the second set of adenoviral vectors, we showed that transcriptional targeting of solitary E4 with the COX-2 promoter resulted in ≤30% reduced cells lysis when compared with the control vector Ad.CMV·E4. However, driving E4 by the MK promoter did not restrict adenoviral replication significantly. In addition, infection of proliferating cells with Ad.CMV·E2F resulted in a similar cytopathic effect as in mitotically arrested cells. These data indicate that exclusive transcriptional targeting of E4 could improve adenoviral replication specificity. However, not all promoters are suitable for specific expression of adenoviral E4, confirming results by Banerjee et al. (27). Furthermore, a tight transcriptional control of viral E1 gene products seems to have a greater effect on restricted adenoviral replication than that of the E4 region.

With the third set of our adenoviral vectors using the same heterologous promoter driving adenoviral E1 and E4, the adenoviral replication selectivity was improved

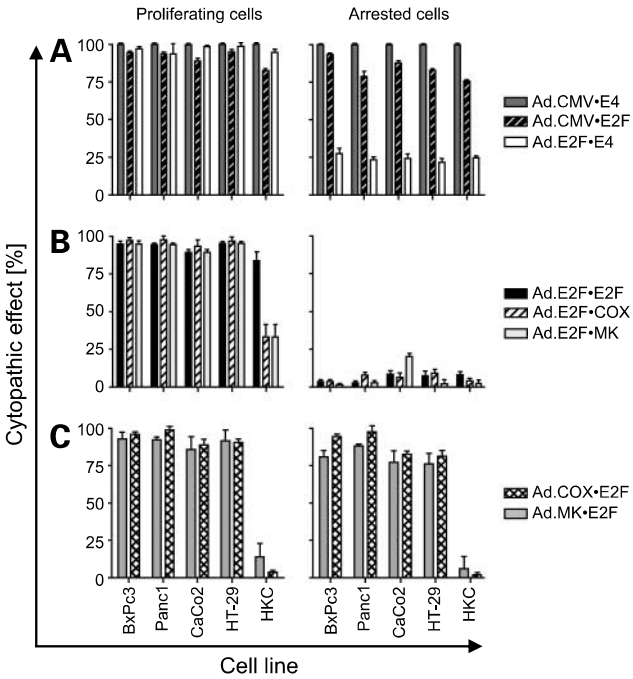


Figure 4. Adenoviral cytopathic effect assay in arrested and proliferating cells. **A,** under the same experimental conditions as described in Fig. 3, cell monolayers were infected with adenoviruses encoding E1 or E4 driven by the E2F1 promoter. **B,** the cytopathic effect of adenoviruses with the viral E1A region under the control of the E2F1 promoter and E4 driven by the COX-2, MK, or E2F1 promoter, respectively. **C,** the cytopathic effect of adenoviruses with an E1A region controlled by the COX-2 or MK promoter and the E4 region by the E2F1 promoter, respectively. The results obtained with HKC were similar to those obtained with HaCaT (data not shown). The susceptibility of the cell lines to adenovirus was normalized. *Columns,* means from four independent experiments; *bars,* ± SD.

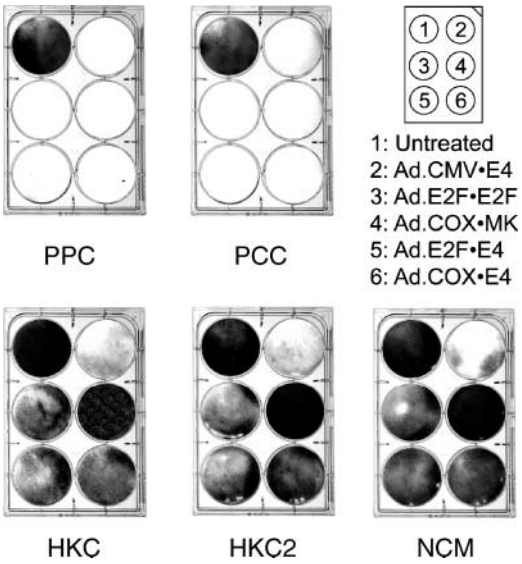


Figure 5. The cytopathic effect of replication-competent adenoviral vectors. Under the same experimental conditions as described in Fig. 3, monolayers of primary pancreatic carcinoma (*PPC*), primary colorectal carcinoma (*PCC*), primary human keratinocytes (*HKC* and *HKC2*), and normal colorectal mucosa cells (*NCM*) were left untreated or infected with adenoviral vectors carrying the adenoviral E1A region under the transcriptional control of the COX-2, E2F1, or CMV-IE promoter and the E4 region driven by the native E4, MK, or E2F1 promoter, respectively. Within a day, when complete cell lysis of the monolayers infected with Ad.CMV·E4 occurred (5 d postinfection), all cell monolayers were washed and stained. One representative experiment out of three.

by ~60% when compared with the respective solitary E1 controlled vector, confirming previous observations (27, 28). In the vectors Ad.COX·MK, Ad.MK·COX, using two different promoters to drive viral E1 and E4, replication selectivity was enhanced by ~85% when compared with the respective solitary E1 controlled vector. Using the Student-Newman-Keuls test, this difference in restricted replication was significant when compared with Ad.COX·COX or Ad.MK·MK in the tested primary keratinocytes ($P \leq 0.001$). One possible explanation for this observation might be the reduced transcriptional interference between two different promoters using distinct sets of transcription factors which, in our setting, enhanced the overall promoter fidelity and thus targeting of adenoviral replication (42).

Because permanent cell lines are often in continuous culture for several years and, thus, are likely to have genetic aberrations, we established short-term cultures of primary pancreatic and colon cancer cells. As a control, we used cultures of human primary keratinocytes and normal colon mucosa cells. The cytopathic effect of the Ad.COX·MK did not differ significantly when compared with the tested permanent cell lines. As expected, in the proliferating cells, the cytopathic effect of Ad.E2F·E2F was similar to that of Ad.CMV·E4.

In a s.c. HT-29 colon cancer model in nude mice, we evaluated the intrinsic oncolytic effects of Ad.COX·E4, Ad.COX·MK, Ad.E2F·E4, and Ad.E2F·E2F. We compared the antineoplastic effect of these vectors with the control Ad.CMV·E4. This vector did not show any cell type specificity. However, because this vector has a deletion in the viral E3 region, like the other vectors, the replication kinetic of this vector is slower than that of wild-type Ad5 (35).

The key findings were: first, animals receiving intratumoral injections of the oncolytic vectors had a significant antitumor response when compared with untreated control animals, confirming that the replication-competent adenovirus has direct oncolytic activity (6, 43). Second, because there was no significant difference in the antineoplastic efficacy of the vectors, this indicates that neither controlling viral E1A nor E1A and E4 expression by tumor/tissue-specific promoter significantly influences the treatment efficiency of these vectors. Third, the oncolytic effect of these vectors is largely independent of the strength of the heterologous promoters, confirming previous results (19).

Interestingly, the vectors Ad.E2F·E4 and Ad.E2F·E2F had the same treatment efficacy as the other vectors, although at any given time, only a fraction of tumor cells are in S phase. However, infection of the cells with adenovirus expressing wild-type E1A urges cells into S phase and thus allows replication of these vectors.

All vectors in this study encoded the HSV-*tk* gene, which allows efficient stopping of viral replication as a failsafe mechanism with ganciclovir because there are currently no antiadenoviral agents approved for clinical use (44).

Our data shows that transcriptional targeting of viral E1A and E4 with different promoters enhances the restriction of viral replication when compared with vectors using the same promoter. Furthermore, transcriptional targeting of viral E1A and E4 did not significantly influence the antineoplastic effect of the vectors in our tumor model.

The use of two different heterologous promoters controlling E1A and E4 gene products is a promising step towards a tight tumor/tissue-specific adenoviral replication, which might be further enhanced by using the E1Δ24 mutant, which cannot release free E2F by interacting with retinoblastoma protein (45), instead of

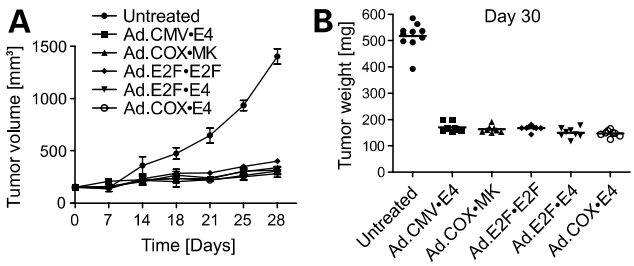


Figure 6. Antineoplastic efficacy of adenoviral vectors. HT-29 xenografts were grown s.c. to volumes of ~180 mm^3. Groups of mice ($n = 8$) were treated by intratumoral injection of 5×10^9 virus particles in 100 μL PBS on days 0 and 2. **A**, tumors were measured at the indicated time points and calculated volumes (mean ± SD) are presented as growth curves. **B**, dry tumor weight 30 d after vector administration. *Points,* individual tumors; *horizontal lines,* median values.

wild-type E1A. However, the lack of well-characterized tumor/tissue-specific promoters and interference with adenoviral promoters or enhancers limits the systematic application of this approach.

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