

Recombinant Adeno-associated and Adenoviral Vectors for the Transduction of Pancreatic and Colon Carcinoma

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Abstract. Background: Recombinant adeno-associated viral vectors serotype 2 (rAAV2) can transduce several tissues with high efficiency. Materials and Methods: The transduction efficiency of rAAV2 in pancreatic and colon cancer was compared to that of recombinant adenovirus (rAd) *in vitro* and *in vivo* using green fluorescent protein (GFP). Results: With the exception of SU.86.86, the percentage of GFP-positive cells was below 10% at a multiplicity of infection (MOI) of 100 for rAAV2. At the same MOI, almost 100% of cells expressed GFP when rAd was used. However, the transduction efficiency for rAAV2 was comparable to that of rAd when co-infected with wt adenovirus, leading to a dramatic increase in the amount of double-stranded rAAV2 DNA. Similar results were obtained *in vivo*. While widespread GFP expression was readily detected in all xenografts injected with rAd, only one section of all tumors injected with rAAV2 contained GFP-positive cells. Conclusion: The results of this study indicate that rAAV2 might be useful for an *ex vivo* approach in cancer gene therapy, but it does not seem to be feasible for the *in vivo* treatment of malignant tumors.

The overall 5-year survival rate of pancreatic carcinoma remains below 3%, while the rate for colon carcinoma is approximately 30%. The development of new therapeutic options, including gene therapy approaches, is warranted.

In gene therapy, the choice of the vector is generally crucial. Two goals need to be achieved in order to minimize toxicity: efficient transduction of cells or tissue and high

selectivity. One way to achieve this is to deliver the therapeutic gene by directly injecting the vector into the tumor. The most commonly employed viral vector for intratumoral injection is the recombinant adenovirus (rAd). Adenoviral vectors have the advantage of efficiently transducing a wide variety of cells (1). The main drawback of this viral vector is its high immunogenicity (2).

The recombinant adeno-associated virus (rAAV) has emerged as a promising vector in gene therapy. Its advantages include efficient transduction of many different tissues *in vivo*, leading to stable, long-term expression of the transgene and no known pathogenicity in humans (3). Furthermore, no toxicity has yet been reported with the use of recombinant adeno-associated virus serotype 2 (rAAV2) vectors in animal models and clinical trials (4). To date, rAAV2 has not been widely used as a vector in cancer gene therapy. However, the transduction of a variety of human tumor cells, *e.g.*, small lung cell carcinoma, ovarian carcinoma, melanoma, cervical carcinoma and osteosarcoma, *in vitro* has been reported (5, 6). Other investigators demonstrated that rAAV2 transduces tumor cells *in vivo*, resulting in a therapeutic benefit (7).

The purpose of this study was to systematically evaluate the transduction efficiency of rAAV2 compared to that of rAd *in vitro* and *in vivo* in a panel of pancreatic and colon carcinoma cell lines. Two constitutively different active promoters were tested: the cytomegalovirus immediate early enhancer/promoter (CMV promoter) and the promoter of the elongation factor-1 α (EF-1 α) (8).

Materials and Methods

Cell lines. The human embryonic kidney cell line 293 and the human colon carcinoma cell line HT-29 were maintained in DME-media supplemented with 10% fetal calf serum (FCS) and antibiotics. The human pancreatic carcinoma cell lines SU.86.86 (ATCC, USA), BXPC3 (ATCC) and Hs766T (Dr. Schwarte-Waldhoff, University of Bochum, Germany) were maintained in RPMI 1640 supplemented with 10% fetal calf serum and

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antibiotics. The murine pancreatic carcinoma cell line Pan-02 was clonally established from a Pan-02 tumor (obtained from the Frederick Cancer Research Laboratories, USA) by selective trypsinization and was maintained in RPMI 1640.

In vivo tumor models. Pan-02 grows as a syngeneic pancreatic tumor in C57/Bl6 mice. This tumor was maintained by passaging tumor material from mouse to mouse. The human pancreatic carcinoma BXPC3 was maintained in the same fashion as a xenograft in nude mice. HT-29 and SU.86.86 xenograft tumors were generated by subcutaneous injection of 10^7 cells in a total volume of 200 μ l in a single flank of a nude mouse. The growth of SU.86.86 tumors was supported by the addition of an equal volume of Matrigel® (Becton-Dickinson, San Jose, CA, USA).

Construction of plasmids. The recombinant AAV2 plasmid pTR-UF5-EF-1 α was derived from pTR-UF5 by exchanging the CMV promoter with a 1500-bp fragment of pHEF-B70 (L.G. Chang, University of Florida, USA) containing the promoter for the elongation factor-1 α including the first intron. The adenoviral shuttle plasmid pTR-UF7-EF1 was constructed accordingly, based on pTR-UF7. The green fluorescence protein (GFP) gene was used as a reporter gene.

Production of recombinant AAV2. RAAV2 vectors were produced by a two-plasmid transfection as described by Grimm *et al.*, and purified by the method of Zolotukhin *et al.* (9, 10). The titers for rAAV2, as determined by the infectious center assay on C12 cells (gift of P. Johnson) (11), were UF5 8×10^9 IU/ml and UF5-EF1 4×10^9 IU/ml.

Production of rAd. RAd was produced by using the Cre/lox recombination system (12) and was propagated in 293 cells. The titers for rAd, as determined by plaque assay, were UF7 4×10^{10} pfu/ml and UF7-EF1 1.8×10^{10} pfu/ml.

Infection of cell lines with rAAV2 and rAd in vitro. Cells ($0.5-1 \times 10^6$) were incubated in suspension with the viruses at the specified multiplicity of infection (MOI) in a total volume of 200 μ l of media, without fetal calf serum or antibiotics, at 37°C. After 3 h, 1000 μ l of complete media were added.

FACS analysis. The cells were assayed for GFP expression by FACS analysis 48 h after infection (Becton Dickinson Flow Cytometer, University of Florida Core Facility for Flow Cytometry, USA). In the case of the time-course experiments, the cells were fixed in 1% paraformaldehyde (Sigma, St. Louis, MO, USA) and stored at 4°C, protected from light. A cell was scored positive for green fluorescent protein (GFP) fluorescence when its fluorescence was higher than a certain threshold, set in the control sample and determined by the background fluorescence.

Transduction of tumors in vivo. Once the tumors that had been grown in nude and C57/Bl6 mice had reached a diameter of 5 mm, they were injected with 10^8 IU rAAV2 or 10^8 pfu rAd in a total volume of 25 μ l. The mice were sacrificed at the indicated time-points. The tumors were harvested and fixed in a 4% paraformaldehyde solution in phosphate-buffered saline (PBS), pH 7.4. After 48 h, they were equilibrated for 3 days in 30% sucrose in PBS, pH 7.4. The tumors were stored at -80°C until further analysis.

Fluorescent microscopy. The fixed tumors were cut on a cryostat (15-20 μ m thickness). In an attempt to at least semi-quantitatively assess the transduction efficiency of rAd, the tumors were completely sectioned and every third section was analyzed under the fluorescent microscope (Zeiss, Microscope Facility, Brain Institute, University of Florida, Gainesville, FL, USA). Due to the size of each tumor section, several overlapping digital pictures were taken (Spot32, Diagnostics Instrument Inc.) and later reassembled into a composite image using Adobe Photoshop 8.0.

Southern blot of Hirt-DNA. Cells (10^6) were infected at an MOI of 10, as described above. Hirt DNA was extracted as previously described (13). Ten mg of native DNA were separated on a 1% agarose gel. The DNA was transferred to a nylon membrane by a standard technique. The blot was hybridized with a randomly 32 P-labelled probe that was generated from the neomycin fragment of pTR-UF5 as a template.

Results

Infection of tumor cell lines with rAAV2 in vitro. The results for HT-29, a human colon carcinoma cell line, SU.86.86, BXPC3 and Hs766T, three human pancreatic carcinoma cell lines and Pan02, a murine pancreatic carcinoma cell line, are summarized in Figure 1 A and B. The only cell line that was efficiently transduced by rAAV2 was SU.86.86. At an MOI of 100, 79.9% and 85.2% of cells scored positive for GFP when infected with AAV-CMV and AAV-EF-1 α , respectively. At the same MOI, only 30.5% of Hs766T cells expressed GFP. All the other tumor cell lines tested displayed much lower transduction rates for both rAAV2 used. The percentage of GFP-positive cells ranged from 1.4% to 9.4% at the highest MOI.

Co-infection of tumor cell lines with rAAV2 and wild-type adenovirus (wtAd). The wild-type adenovirus (wtAd) is known to accelerate second-strand synthesis of AAV (14). In all human tumor cell lines, the transduction efficiency for rAAV2 was dramatically improved after co-infection with wtAd, as shown in Figure 1 C and D. The percentage of GFP-positive cells following infection with AAV-CMV and wtAd increased to 47.3-95.6% at an MOI of 100. A similar result was obtained for AAV-EF-1 α . The co-infection with wtAd not only resulted in a dramatic increase of the transduction rate, but also in a many-fold higher mean expression level of GFP (data not shown). The levels ranging from 8.4-146.6 in cells infected with rAAV2 alone increased to 17.8-1886.6 when cells were co-infected with wtAd. However, the murine cell line Pan02 could not be transduced efficiently after co-infection with rAAV2 and wtAd. The percentage of GFP-positive cells remained below 10%. The same observation was made in the murine sarcoma cell line KHT (data not shown).

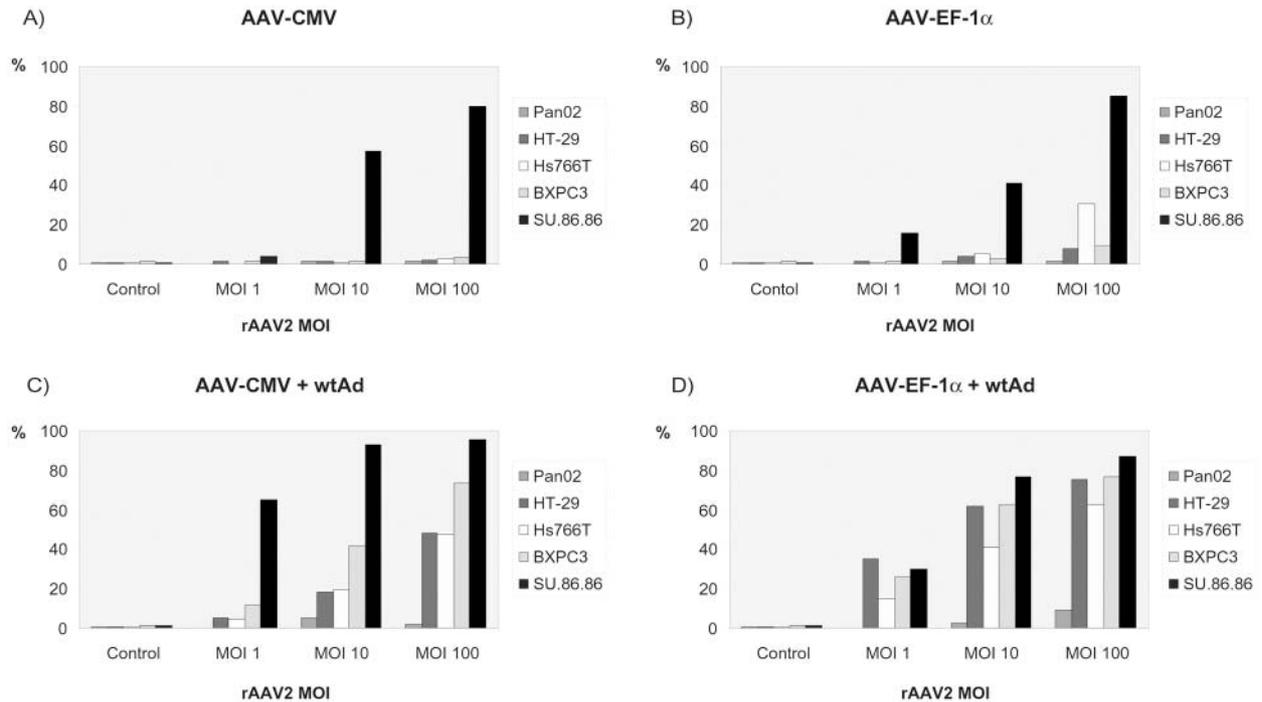


Figure 1. *In vitro* infection of tumor cell lines with recombinant adeno-associated virus serotype 2 (rAAV2) alone (AAV-CMV (A) and AAV-EF-1 α (B)) and together with wild-type adenovirus (C and D) (MOI of 10). Infection was carried out at the MOI's indicated, as described in Materials and Methods. The cells were analyzed for GFP expression (percentage) by FACScan.

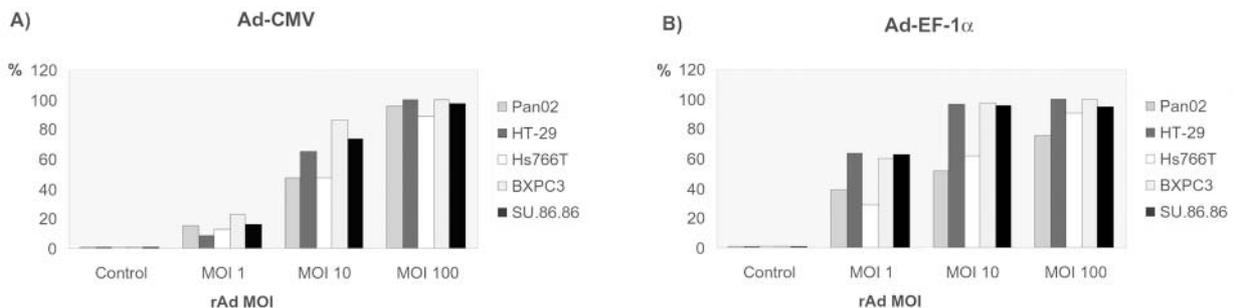


Figure 2. *In vitro* infection of tumor cell lines with recombinant adenovirus (Ad-CMV (A) and Ad-EF-1 α (B)). Infection was carried out at the MOI's indicated, as described in Materials and Methods. The cells were analyzed for GFP expression (percentage) by FACScan.

Infection of tumor cell lines with rAd in vitro. All 5 cell lines were readily transduced with rAd and expressed GFP at high levels, as shown in Figure 2. The percentage of GFP-positive cells increased with higher MOI. Infection with Ad-CMV at an MOI of 1 resulted in 8.4% (HT-29) to 22.73% (BXPC3) GFP-positive cells. This fraction increased to 88.4% (Hs766T) and to 99.8% (BXPC3) at an MOI of 100. Similar results were obtained for Ad-EF-1 α . At an MOI of 100, all cell lines, except for the murine pancreatic carcinoma Pan02, were greater than 90% GFP-positive.

Time-course of the rAAV2 infection in vitro. In SU.86.86, the percentage of GFP-expressing cells increased from 2.1% at 12 h to a maximum of 35.5% at 120 h post-infection. In the positive control, which was co-infected with wtAd, 62.0% of cells expressed GFP at 24 h. In the HT-29 cells, the portion of GFP-positive cells increased from 0.3% at 12 h to a maximum of 10.6% at 120 h. When co-infected with wtAd, 48.3% of the cells expressed GFP at 24 h post-infection.

Hirt-DNA from infected HT-29 and SU.86.86 cells was analyzed by Southern blot (Figure 3). In the SU.86.86 cells,

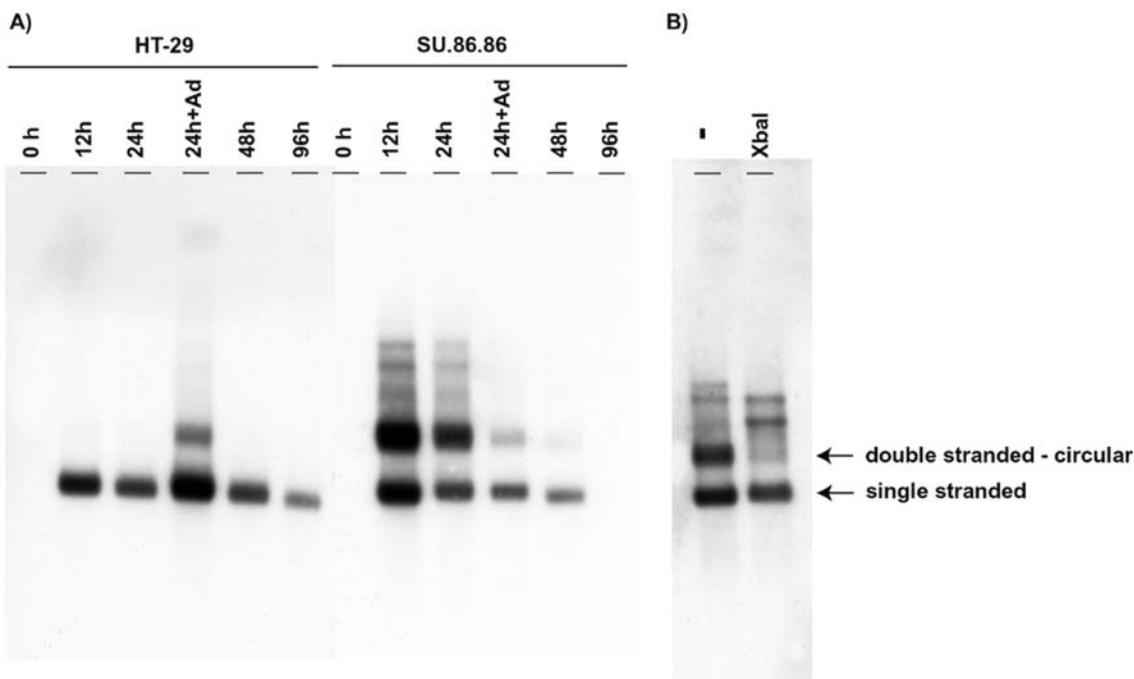


Figure 3. A) Southern blot analysis of Hirt-DNA from HT-29 and SU.86.86 cells infected with AAV-CMV at a MOI of 10. The cells were harvested at the time-points indicated. Hirt-DNA was extracted and separated on an agarose gel. The membrane was hybridized with a probe against the neomycin-resistant gene. B) Digestion of Hirt-DNA with *Xba*I, a single cutter in pTR-UF5.

an apparently circular species of the AAV-CMV genome was present, in addition to the single-stranded input DNA from the first time-point onwards. In the HT-29 cells, this circular species could only be detected in the sample, which was co-infected with wtAd. This quicker conversion of the single-stranded input DNA into the intermediate circular form might, at least in part, account for the differences in the transduction efficiency between those two cell lines.

Transduction of tumors in vivo with rAAV2. Subcutaneously-grown xenografts were injected with 108 IU rAAV2. The tumors were harvested at approximately week 1 and week 2 post-injection, depending on the dynamics of the tumor growth. In addition, the slowest growing tumors, SU.86.86 and BXPC3, were carried on to days 24 and 42 post-injection, respectively. GFP expression could not be detected in any of the sections of any of the tumors injected with rAAV2, except an SU.86.86 tumor injected with AAV-EF-1 α harvested on day 7 post-injection. In both adjacent sections, GFP expression had already faded to levels below the limit of detection.

Transduction of tumors in vivo with rAd. In sharp contrast to rAAV2, all the tumors injected with rAd showed GFP expression. A representative section for each tumor is shown in Figure 4. Six sections of a BXPC3 tumor injected

with Ad-EF-1 α after 1 week are provided in Figure 5. The 15x6x4 mm³ tumor is completely sectioned into a total of 321, 25- μ m sections. A semi-quantitative analysis of all tumors injected with rAd is summarized in Table I. The maximum GFP expression in all 4 tumors examined was seen at week 1, with decreasing levels thereafter. The level of GFP expression per cell, as judged by the intensity under the fluorescent microscope, was lower at late time-points. There were hardly any GFP-positive cells in the SU.86.86 tumor 24 days post-injection.

Discussion

In the present study, the transduction efficiency of rAAV2 vectors was tested *in vitro* and *in vivo* using GFP as a transgene in a panel of human and murine tumor cell lines. rAd was used in comparison. rAAV2 was much less effective compared to rAd, with respect to transduction efficacy as well as to the intensity of GFP expression. However, we were able to dramatically increase the transduction rate for rAAV2 by co-infection with wtAd. This indicates that rAAV2 successfully enters the cell, but is apparently lacking essential steps for transcription and translation of the transgene. Similar results were obtained in ovarian cancer cells and hepato-cellular carcinoma (15-17). rAAV2 is known to have a delayed expression of the

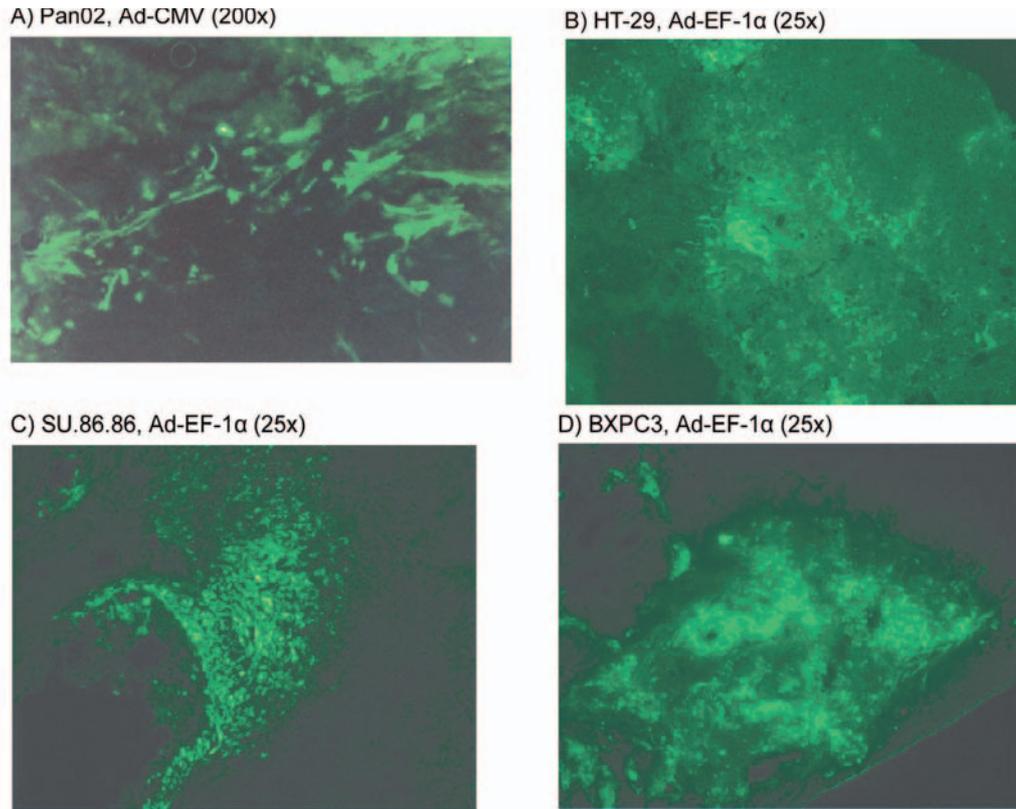


Figure 4. Transduction of tumors (Pan02 (A), HT-29 (B), SU.86.86 (C) and BXP3 (D)) in vivo with recombinant adenovirus (rAd). The tumors were harvested 1 week after injection. A representative section of each tumor is shown.

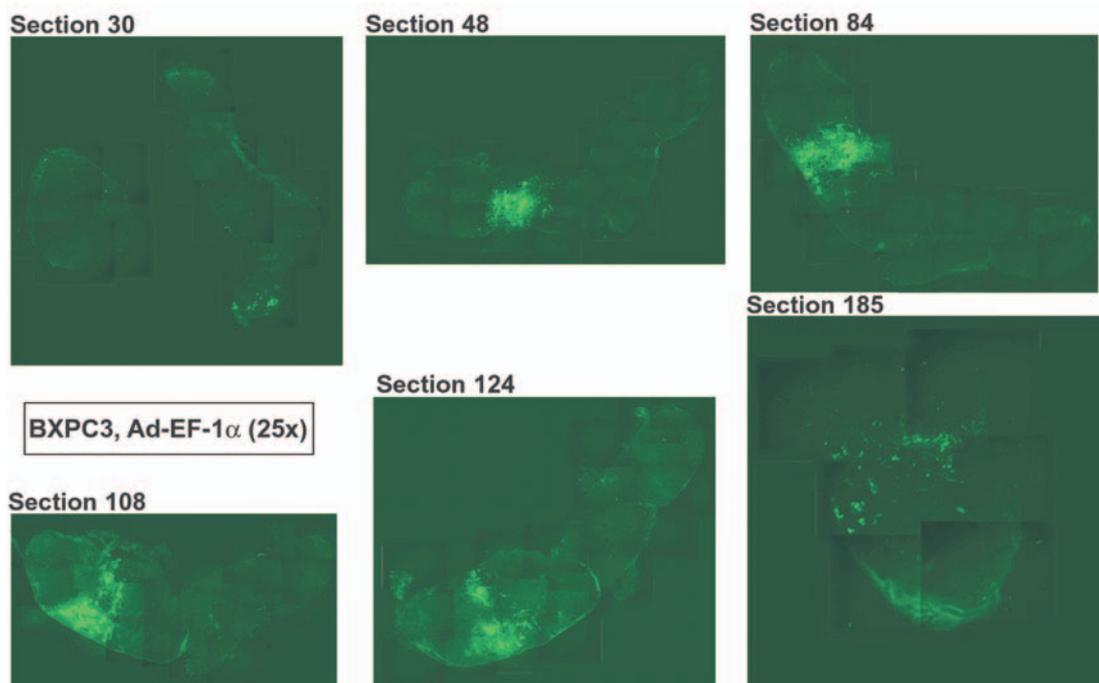


Figure 5. Extent of transduction of a whole tumor by recombinant adenovirus (rAd) (Ad-EF-1 α) in vivo. A representative BXP3 tumor injected with Ad-EF-1 α and harvested after 1 week is shown. Due to the size of the tumors, each section is a composite digital image of the entire tumor. The 15x6x4 mm³ tumor was completely sectioned into 321, 25- μ m sections.

Table I. Semi-quantitative analysis of the transduction of tumor tissue by recombinant adeno-associated virus serotype 2 (AAV-CMV; AAV-EF-1 α) compared to that of recombinant adenovirus (Ad-CMV; Ad-EF-1 α). Four different tumors were transduced (Pan02 (A), HT-29 (B), SU.86.86 (C) and BXPC3 (D)).

A) Pan-02			B) HT-29				
	Week 1	Week 2		Week 1	Week 2		
Ad-CMV	++++	++	Ad-CMV	+++	+		
AAV-CMV	-	-	AAV-CMV	-	-		
Ad-EF-1 α	+++	+	Ad-EF-1 α	++++	++		
AAV-EF-1 α	-	-	AAV-EF-1 α	-	-		
C) SU.86.86			D) BXPC3				
	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	
Ad-CMV	+++	+	-	Ad-CMV	+++	+	n.d.
AAV-CMV	-	-	-	AAV-CMV	-	-	n.d.
Ad-EF-1 α	++++	++	+	Ad-EF-1 α	++++	++	n.d.
AAV-EF-1 α	+/-	-	-	AAV-EF-1 α	-	-	-

transgene in the absence of adenoviral helper functions (18, 19). Typically, there is a lag of 10-20 days between infection and the expression of the transgene. This has, in part, been contributed to slow second-strand synthesis in the absence of helper virus function (20). In addition, the phosphorylation status of the FK506-binding protein plays a role in transgene expression by rAAV2 (21). Although we were able to greatly improve the transduction efficiency by co-infection with adenovirus in all the human tumor cell lines, this was not the case in the murine pancreatic carcinoma cell line Pan02. Apparently, this murine tumor cell line lacks surface receptors or other functions, which are essential for entry into the cell. Similar observations were made by Maas *et al.* (5).

As stated above, the transgene expression in tissues transduced with rAAV2 *in vivo* was delayed and did not reach a plateau until 4-6 weeks post-injection. The majority of these studies were performed in quiescent tissues, such as muscle, liver and neuronal cells. Some studies have suggested that cells in S-phase are transduced more efficiently (22, 23). Therefore, transgene expression by rAAV2 may be facilitated in a tumor *in vivo* since these cells are actively dividing. Furthermore, this experimental design would allow rAAV2 sufficient time for transgene expression compared to the *in vitro* situation. However, in our studies, after intratumoral injection of rAAV2 and rAd, a picture very similar to the *in vitro* results emerged. rAd was able to efficiently transduce tumor cells *in vivo*, as reported in other investigations (24). rAAV2, however, performed very poorly when directly injected into the tumor. In only one section of all the tumors injected was GFP expression detected in a few cells. This is contradictory to the *in vitro* data, where

transduction to some extent, at least entry into the cell, was demonstrated. A possible reason for the discrepancy could be the dilution of the vector by the fast tumor growth. Another limiting factor might be the penetration of the tumor. Heparin sulfate is a widespread surface antigen and, since AAV2 binds to this receptor, penetration of the tumor would be hampered (25).

We conclude that rAAV2 serotype 2 failed to transduce tumor cells *in vivo* in the tested human pancreatic and colon carcinoma cell lines after direct intratumoral injection. The *in vitro* results, however, indicated that rAAV2 might be useful for infecting tumor cells *ex vivo*. We have demonstrated that rAAV2 is particularly useful for the generation of stable cell lines, since rAAV2 leads to stable expression of the desired transgene (26). Other useful observations for rAAV in cancer gene therapy have been the stable expression of anti-angiogenic factors after transduction of muscle or liver (27, 28).

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