

A Novel A33 Promoter – Based Conditionally Replicative Adenovirus Suppresses Tumor Growth and Eradicates Hepatic Metastases in Human Colon Cancer Models

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Abstract **Purpose:** A33 antigen is a membrane-bound protein expressed in intestinal epithelium that is overexpressed in 95% of primary and metastatic colorectal carcinomas but is absent in most epithelial tissues and tumor types. We hypothesized that A33 promoter might be useful in the design of a conditionally replicative adenovirus for the treatment of colorectal cancer (CRC). **Experimental Design:** We cloned an A33 promoter fragment (A33Pr) that extends from -105 to +307 bp. Using luciferase activity as a reporter gene, we showed that A33Pr was active in CRC cell lines. We next constructed a conditionally replicative adenovirus named AV22EL where E1A was placed under the control of A33Pr. The tumor-specific oncolytic effect of AV22EL was investigated both *in vitro* and *in vivo*. **Results:** AV22EL induced specific *in vitro* lysis of human CRC cell lines that expressed A33 and have negligible lytic capacity on cells that lacked or had minimal A33 expression, including normal human colonic cells. *In vivo*, a marked reduction of tumor growth and increased long-term survival rates were observed in nude mice xenografted with s.c. CRC tumors. Combination with 5-fluorouracil induced an additive effect *in vitro* with no toxic effects *in vivo*. Remarkably, AV22EL completely eliminated established hepatic metastases in >90% of mice and restored hepatic function according to biochemical parameters. Its systemic administration induced E1A expression only in the hepatic metastasis but not in normal organs. **Conclusions:** These data show that AV22EL is a stringently regulated and potent oncolytic agent for the treatment of CRC.

Colorectal cancer (CRC) is the second most common cause of cancer mortality in Western countries and claimed >50,000 lives a year only in the United States.⁴ Close to 70% of patients that are affected by colorectal carcinoma undergo surgical resection and 30% to 40% of them develop a recurrent disease (1). The liver is the most common site of metastatic CRC and complete resection of hepatic metastases is the only curative option; however, surgery can be done only in 20% of patients

at the time of diagnosis and 5-year survival rates average 25% to 40% despite adjuvant chemotherapy (2). Among patients with metastatic colorectal carcinoma receiving as first-line chemotherapy 5-fluorouracil (5-FU) and new medications, such as irinotecan, Xeloda, oxaliplatin, Erbitux, and Avastin, the median time to progression is 8 to 9 months and their mean survival is 15 to 20.5 months (3–6). It seems therefore that current CRC treatments are rather ineffective on advanced disease, showing the need for more effective and specific therapeutics to significantly increase patients' survival.

Conditionally replicative oncolytic adenoviruses (CRAd) have shown promising applications in cancer gene therapy (7, 8). One strategy to achieve specific elimination of the tumor mass, avoiding negative undesired effects in contiguous normal tissue, is the use of tumor-selective transcriptional regulation to control the essential early E1 genes, which are required to transactivate the other adenoviral genes (9, 10). Several promoters have been used to drive adenoviral replication and oncolytic activity in colorectal carcinoma, including the carcinoembryonic antigen (CEA), the telomerase, and the cyclooxygenase-2 (COX-2) promoters, which might provide a broad spectrum of therapeutic efficacy. Despite the strong oncolytic efficacy of these viruses, there is still a concern about their stringent selectivity. CEA and COX-2 promoters were

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Received 5/5/08; revised 12/30/08; accepted 1/20/09; published OnlineFirst 3/31/09.

Grant support: Rene Baron Foundation, Friends of Leloir Foundation for the struggle against cancer, and Programa Bicentenario-Banco Mundial, Conicyt, Chile CTE-06.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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© 2009 American Association for Cancer Research.
doi:10.1158/1078-0432.CCR-08-1161

⁴ American Cancer Society. Cancer facts and figures 2008. Atlanta: American Cancer Society; 2008.

Translational Relevance

We provide here preclinical data about the therapeutic efficacy of a novel oncolytic adenoviral vector, AV22EL, for treatment of colorectal cancer (CRC). AV22EL replication was driven by a CRC-specific A33 antigen promoter. A33 is a membrane-bound protein located in the intestinal epithelium that is overexpressed in 95% of primary and metastatic CRC but is absent in most epithelial tissues and tumor types. A specific anti-A33 antibody is already in clinical use, suggesting its clinical relevance. AV22EL induced specifically the *in vitro* lysis of cells of CRC origin while showing almost no lytic activity on other cancer cell lines. AV22EL exhibited no cytotoxic effect on normal human colonic cells and hepatocytes, whereas these cells were eliminated by wild-type adenovirus. AV22EL markedly reduced the *in vivo* growth in nude mice of established CRC and showed an additive effect *in vitro* when combined with 5-fluorouracil with no evidence of further toxic effects *in vivo*. Its systemic administration induced the disappearance of hepatic metastatic nodules in >90% of mice and restored normal levels of hepatic enzymes following metastases disappearance, exhibiting no evidence of viral activity in normal organs, including normal intestine, as assessed by E1A and luciferase expression. Thus, AV22EL fulfills the criteria to become a useful and strongly specific tool for treatment of patients with CRC.

shown to be active in CEA-negative and COX-2-negative cells, respectively (11–13), the telomerase promoter could be active in CD34⁺ and other progenitor cells (14), whereas the use of an oncolytic virus based on the COX-2 promoter could be hampered by the rise of COX-2 expression in transient or chronic inflammation (15). An oncolytic virus was also designed based on a wnt-responding element because this pathway is strongly up-regulated in CRC; however, the wnt pathway is also active in normal stem cells in skin, the hematopoietic system, and in intestine, as well as in neurons in several brain regions (16), although adenovirus hardly infects keratinocytes and hematopoietic cells, diminishing the risks for undesirable collateral effects (17, 18). However, the possibility of generating an oncolytic virus based on stringent, cancer type-specific promoter might have the advantage of its strong attenuation in normal organs, increasing the effectiveness for systemic treatment of disseminated disease.

The A33 antigen is a cell surface glycoprotein present only in the small intestine and colonic epithelium with homology to proteins of the immunoglobulin superfamily, such as the coxsackievirus and adenovirus receptor (19). In human cancer, A33 was found in 95% of primary and metastatic colorectal carcinomas, 63% of diffuse gastric cancers, 83% of intestinal-type gastric cancers, 50% of pancreatic cancers, and ~50% of the diffuse-type and mucinous cancers; it was absent in epithelial cancers, sarcomas, neuroectodermal tumors, and lymphoid neoplasms (20, 21). A33 is the first example of a constitutively expressed, organ-specific epithelial membrane antigen present in patients with gastrointestinal cancer (20). A33 seems to be expressed by normal replicating colonocytes located at the base of the crypt that in principle should not be

accessible to an oncolytic virus (20). This restricted tissue localization and specific expression of A33 in gastrointestinal cancer led to the initiation of clinical trials in CRC aiming to target A33 by using specific antibody immunotherapy (22–26).

Initial data obtained from clinical studies using oncolytic viruses have been encouraging in terms of efficacy with minimal, if any, toxicity (27). However, their efficacy in the treatment of systemic metastasis remains a major challenge. We hypothesized that the A33 promoter (A33Pr) might be useful in the design of a CRAd. Thus, we constructed a new CRAd (AV22EL) in which E1A was placed under the control of A33Pr. We evaluated cell selectivity and cytotoxicity in a variety of normal and human cancer cell lines *in vitro* and determined the antitumor activity of the oncolytic vector on established s.c. tumors and liver metastasis following xenotransplantation of human CRC cells in nude mice. The whole data show that AV22EL is a potent oncolytic virus that can be combined with current chemotherapeutic agents.

Materials and Methods

Cell lines

The human CRC (LoVo, T84, and HT29), breast cancer (T47D), hepatocellular carcinoma (Hep-3B2), and fetal lung fibroblast (WI-38 and HFL-1) cell lines and cell lines derived from normal colon epithelia (FHC and CCD841) and human embryonic kidney cells (HEK 293) were obtained from the American Type Culture Collection. The human melanoma cell lines A375N and SB2 were kindly provided by Dr. Estela Medrano (Huffington Center on Aging, Houston, TX), and bovine aortic endothelial cells were kindly provided by Helene Sage (University of Washington, Seattle, WA). All the cell lines were grown in the recommended medium supplemented with 10% fetal bovine serum (Invitrogen Corp.), 2 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin and maintained in a 37°C atmosphere containing 5% CO₂.

Assessment of A33 mRNA expression

Total RNA was extracted using Tri Reagent (Sigma-Aldrich Co.). RNA (5 µg) was reverse transcribed with 200 units of SuperScript II Reverse Transcriptase (Invitrogen) using 500 ng of oligo(dT) primers. cDNAs were subjected to semiquantitative PCR. Each 25 µL reaction volume contained 1 unit Taq DNA polymerase (Invitrogen), 1× PCR reaction buffer [20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl], 1.5 mmol/L Mg₂Cl₂, 200 µmol/L of deoxynucleotide triphosphates, and 0.4 µmol/L of each specific primer. For the list of primers, see Supplementary Table S1. PCR conditions were set as follows: 90 s at 94°C and then 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. All the reactions were done in triplicates, and the results obtained for A33 were normalized to glyceraldehyde-3-phosphate dehydrogenase.

Construction of AV22EL and other recombinant adenoviruses

A 412-bp fragment of the human A33 promoter region (-105 to +307 bp relative to the transcription start site) was amplified from human lymphocyte genomic DNA using specific primers (Supplementary Table S1). The PCR product was cloned in pGEM-T easy vector (Promega Corp.) followed by subcloning in the *Bgl*III/*Xho*I sites of the promoterless firefly luciferase gene reporter vector pGL3-Basic (Promega) to obtain pGL3-A33.

We replaced the Rous sarcoma virus promoter of the shuttle vector pADPSY (28) with a multiple cloning site (*Spe*I, *Bcl*I, *Kpn*I, *Nhe*I, *Mlu*I, *Bgl*III, *Eco*RV, *Cl*aI, *Sna*BI, and *Sal*I) to create pAd-Xp. Next, a 234-bp fragment corresponding to the stop codon region of the growth hormone gene (29) was PCR cloned in the *Spe*I/*Kpn*I sites downstream of the inverted terminal repeat to create a new shuttle vector named pAd-I-Xp (see Supplementary Table S1 for the list of primers). A

+560/+1,632-bp fragment of E1A genomic sequence was PCR cloned from 293 cells, subcloned in TOPO-pCR4, and sequenced (see Supplementary Table S1). After subcloning in pcDNA3, HeLa cells were transfected to express E1A that was identified using a specific anti-E1A antibody (M73; Santa Cruz Biotechnology). A *Bgl*III/*Bam*HI E1A fragment was subcloned into the *Bgl*III site of pAd-I-Xp followed by cloning of A33Pr upstream of E1A in *Mlu*I/*Bgl*III sites to obtain the shuttle plasmid pAd-I-A33-E1A. The identity of the final construct was confirmed by sequencing. To construct AV22EL, the cotransfection method in 293 cells was used (30). Adenovirus amplification and purification were done as described (31). Physical particle concentration [viral particles (vp)/mL] was determined by A_{260} reading and biological particle concentration [determination of 50% tissue culture infective dose (TCID₅₀)] was determined by standard plaque assay on 293 cells (31).

Obtention of pAd-SV40-luc, pAd-CMV-*Renilla*, and pAd-AV-Luc vectors is described in Supplementary Data. Viral stocks were obtained after cotransfection in HEK 293 cells as above.

Luciferase assays

Luciferase assay using plasmids. Cells seeded in 24-well plates (4×10^4 per well) were cotransfected with 0.8 μ g of plasmid expressing luciferase as a reporter gene and 0.1 μ g of pRL-CMV (Promega) using Lipofectamine 2000 (Invitrogen). Cells were harvested 46 h later and the dual luciferase assay was done following the manufacturer's recommendations (Promega). Luciferase activity was measured using a GENios luminometer (TECAN). Data are expressed as fold induction over pGL3-Basic vector and obtained from at least three independent experiments.

In vitro luciferase assay using adenovirus. Cells seeded in 24-well plates (4×10^4 per well) were transduced with AV-Luc [multiplicity of infection (MOI), 100] and Ad-CMV *Renilla* (MOI, 10) in 200 μ L of DMEM/F12 containing 2% fetal bovine serum. Fresh medium containing serum (800 μ L) was added 2 h later followed by cell harvesting after 48 h. Quantification of luciferase activity followed the manufacturer's recommendations (Promega). Luciferase activity was measured using a GENios luminometer. The luciferase activity was normalized by Ad-CMV *Renilla* activity.

In vivo luciferase assay using adenovirus. LoVo cells (5×10^6) were injected s.c. and allowed to form tumors in the flank of 5- to 6-wk-old athymic N:NIH (S)-nu mice. When the average tumor volume reached 100 mm³, mice received 1×10^{10} vp/mouse of AV-Luc administered through the tail vein. Forty-eight hours later, luciferase activity in various tissues and in the liver metastatic nodules was determined. Quantification of luciferase activity followed the manufacturer's recommendations (Promega). Luciferase activity was measured using a GENios luminometer. The luciferase activity was normalized by milligram of protein Ad.

β -Galactosidase staining assay

Cell infectivity was assessed using an adenovirus expressing the bacterial β -galactosidase protein (Ad- β gal). Twenty-four hours after plating, cells were infected with Ad- β gal at different MOI in serum-free medium for 42 h, and the cells were incubated for an additional 72 h in DMEM/F12 containing 10% fetal bovine serum. Infected cells were washed with PBS, fixed, and evaluated for β -galactosidase activity by incubation in 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) staining solution (32).

Assessment of E1A levels

For determination of E1A mRNA levels, 1.5×10^5 cells per well were seeded in a six-well plate. The next day, cells were infected with the indicated viruses at 1,000 MOI or mock infected. After 24, 48, and 72 h, cells were lysed and total RNA was obtained. A semiquantitative PCR for E1A was done using the set of primers described in Supplementary Table S1.

Western blot

LoVo cells were transduced with AV22EL or Ad-WT (kindly provided by Dr. Andre Lieber, Seattle, WA) at various MOIs. After 72 h, total

protein extracts were prepared in lysis buffer [10 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100 (Sigma), 0.5% deoxycholic acid (Sigma), 0.1% SDS with a protease inhibitor cocktail]. Total protein extracts were separated in 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories). The membranes were probed with anti-E1A antibody (M73; Santa Cruz Biotech) and anti- β -actin antibody (A4700; Sigma) was used as a loading control. Enhanced chemiluminescence reagents were used to detect the signals following the manufacturer's instructions (Amersham).

Cytopathic effect

Cells were seeded in 24-well plates at a density of 1×10^4 per well. The next day, cells were infected with AV22EL or Ad-WT adenovirus at a MOI ranging from 1 to 1,000. The cytopathic effect was monitored by staining viable cells with crystal violet 10 d after infection (31). Density of gel bands was analyzed by using the ImageJ program developed by Wayne Rasband (NIH, Bethesda, MD).

T84-EGFP cells correspond to a single-cell clone obtained by limiting dilution after cell transfection with a plasmid expressing EGFP and selection in G418 (750 μ g/mL). WI-38 and T84 EGFP cells were seeded in 24-well plates at a density of 5×10^4 per well, each. The next day, cells were infected with AV22EL or Ad-WT at MOI of 500. Cells were photographed at day 7 after the initiation of the experiment under light and UV microscope. At the end of the experiments, cells were counted in a Neubauer chamber under light and UV microscope. The experiments were done thrice.

Hepatocyte isolation

Liver samples were collected from one patient undergoing partial hepatectomy. Sample collection was obtained under informed consent with the approval of the ethics committee of the Hospital University Austral. Hepatocyte isolation was done under sterile conditions using a modified two-step collagenase perfusion technique (33). Liver samples were homogenized with 0.1% collagenase for 30 min, twice. Cell suspension was centrifuged at $50 \times g$ for 5 min at 4°C. The cells were purified by density gradient centrifugation ($1,278 \times g$, 20 min, 4°C) using a 25% Percoll solution (density of 1.124 g/mL) in cold PBS. After isolation, hepatic cells were counted and viability was assessed by trypan blue exclusion test. Cells were seeded in collagen type I. At day 5, glycogen storage was detected by procedure periodic acid-Schiff.

In vitro assays combining AV22EL with 5-FU

Cells seeded in 96-well plates (2×10^3 per well) were infected the next day with AV22EL or treated with 5-FU (Microsules Bernabo). In one experimental condition, cells were first treated with 5-FU for 24 h, the medium was then removed, and the cells were transduced with AV22EL. In another experimental condition, cells were first infected with AV22EL for 24 h and then the medium was replaced with fresh medium containing 5-FU. Five days after treatment, cell viability was established with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the absorbance was read at 570 nm using a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories). All assays were carried out in six different replicates.

Multicellular spheroids

The semisolid liquid overlay culture technique was used to produce multicellular spheroid cultures (34). Briefly, 1×10^4 cells were seeded onto a semisolid 1% (w/v) agarose per well layer in 96-well plates in 200 μ L of medium. Seventy-two-hour-old spheroids were infected at MOIs of 10, 100, and 500 for 7 d with the different adenoviruses. At the end of the experiment, spheroids were photographed and growth was quantified by regular measurement of cross-sectional area of individual spheroids.

Virus yield

Six-well plates were seeded with 1×10^5 cells per well 24 h before infection. Cells were infected at a MOI of 50 for 2 h in serum-free

medium. Then, the virus-containing media were removed, monolayers were washed twice with PBS, and 1 mL of complete medium was added to each well. At 72 h after infection, the cells were scraped into culture medium and lysed by three cycles of freezing and thawing. The supernatant was tested for the viral titer by an end point cytocidal assay as described by Li et al. (35).

***In vivo* studies on s.c. tumors and hepatic metastases**

Five- to 6-wk-old female and male athymic N:NIH (S)-nu mice (obtained from the animal facility of the Faculty of Veterinary, University of La Plata) were s.c. injected in one flank with 5×10^6 cells. When the average tumor volume reached 100 mm^3 , mice received 1×10^{10} vp/mouse of AV22EL or vehicle administered intratumorally on days 1, 4, and 7. For *in vivo* assays combining AV22EL with 5-FU, mice were injected with AV22EL followed by i.p. administration of 5-FU (30 mg/kg for 5 d) starting 1 d after the last AV22EL injection. Tumor volumes were estimated twice a week from caliper measurements [volume = $0.52 \times (\text{width})^2 \times \text{length}$]. *In vivo* experiments were done following institution guidelines approved by the NIH, and all animals under study received food and water *ad libitum*. For colorectal liver metastases studies, LoVo cells (1×10^6) were injected via the portal vein of female and male nude mice. At day 7 after cell inoculation, when multiple tiny spots of tumor metastases were confirmed in parallel experimental groups, mice received tail vein injections of AV22EL, Ad- β gal, or vehicle. Administration was repeated 48 and 72 h later. Animals were bled and serum was used to determine albumin, aspartate aminotransferase, and alkaline phosphatase levels.

For assessment of E1A expression *in vivo*, mice, harboring or not established liver metastases, were injected once via the tail vein with AV22EL or PBS. Three days later, mice were sacrificed and organs were removed for obtention of total RNA.

For *in vivo* detection of viral replication, hepatic metastases were fixed in 4% paraformaldehyde for 1 h, cryopreserved overnight in 30% sucrose, embedded in tissue OCT, and stored at -20°C . Cryostat sections (9 μm) were mounted on gelatin-coated slides and incubated with goat anti-adenoviral hexon protein antibody AB1056 (Chemicon International). Biotinylated secondary antibodies were used in conjunction with the Vectastain avidin-biotin complex method kit (Vector Laboratories), and finally, the reaction was visualized by using 3,3'-diaminobenzidine chromogen (Dako). Slides were counterstained with hematoxylin and photographed on an Olympus BX60 microscope. The liver metastasis was sectioned in three pieces, each $\sim 0.5 \text{ mm}$ in width. Histologic analysis was done on one 5- μm tissue section of each piece.

Assessment of intratumor viral replication

LoVo cells (5×10^6) were injected s.c. and allowed to form tumors in the flank of 5- to 6-wk-old athymic N:NIH (S)-nu mice. When the average tumor volume reached 100 mm^3 , mice received 1×10^{10} vp/mouse of AV-Luc or AV22EL administered intratumorally on day 1. Seven days after injection, total DNA was obtained from tumors (Illustra tissue & cells genomicPrep Mini Spin, GE Healthcare) and E4 gene was measured by quantitative real-time PCR (36, 37). Genomic DNA was subjected to real-time PCR in an iCycler iQ System (Bio-Rad Laboratories). Each 25 μL reaction volume contained 1 unit Platinum Taq DNA polymerase (Invitrogen), $1 \times$ PCR reaction buffer [20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl], 1.5 mmol/L Mg_2Cl_2 , 2.5 g bovine serum albumin, 0.01% glycerol, 0.4 $\mu\text{mol/L}$ of each specific primer targeting the E4 region (Ad5, nucleotides 33806-34074; Supplementary Table S1), 200 $\mu\text{mol/L}$ of deoxynucleotide triphosphates, and $0.3 \times$ SYBR Green Solution. PCR conditions were set as follows: 150 s at 94°C and then 39 cycles of 45 s at 94°C , 30 s at 60°C , and 30 s at 72°C . All the reactions were done in triplicate. Analysis of data was carried out using the iCycler software (Bio-Rad Laboratories) by comparing test sample with a standard. Standard curves were generated by serial dilutions of 10^{10} copies of adenoviral DNA in a solution of control cellular genomic DNA.

Statistical analysis

Statistical analyses of luciferase studies, spheroids, and *in vivo* experiments were determined by ANOVA followed by Tukey's test. Statistical analyses of 5-FU curves and viral replication *in vivo* were determined by ANOVA (two-way ANOVA) followed by Bonferroni's test. A *P* value of <0.05 was considered significant. Statistical analyses of serum liver chemistries were done by the Dunnett's multiple comparison test. Survival was determined by the method of Kaplan-Meier. Log-rank test was used to compare differences in survival. Data analysis was done with the GraphPad Prism (GraphPad Software, Inc.).

Results

Analysis of A33 mRNA expression and A33 promoter activity in different human cell lines. To establish whether A33 gene promoter could be useful for the construction of a CRAd, we first evaluated A33 mRNA expression levels in different malignant and normal human cells by semiquantitative PCR. Only colorectal carcinoma cell lines LoVo, T84, and HT29 showed A33 mRNA expression, whereas normal colonic cells CCD841 showed approximately one tenth the levels of A33 mRNA observed in LoVo cells (Fig. 1A). A33 mRNA was not expressed in additional malignant cells, normal fibroblasts, and endothelial cells (Fig. 1A). A33Pr, cloned in the promoterless plasmid pGL3 basic, was essentially active only in human CRC cells LoVo, T84, and HT29, whereas human mammary and melanoma cells showed strongly reduced activity (Supplementary Fig. S1A). In coincidence, an adenoviral vector expressing the luciferase gene driven by A33Pr (AV-Luc) was highly active only in CRC cells; among A33-nonexpressing cells, AV22EL showed a slight activity in SB2 melanoma cells (Fig. 1B and see below).

We next constructed a CRAd (AV22EL) where E1A was placed under the control of A33Pr (Supplementary Fig. S1B). AV22EL genome lacks the adenoviral E3 region and contains the I-A33Pr-E1A cassette introduced in the E1 region of the viral backbone. E1A expression driven by A33Pr was confirmed by Western analysis after transduction of LoVo cells at different MOIs of AV22EL (Fig. 1C).

Selective replication and lytic activity of AV22EL in CRC cells. AV22EL showed marked *in vitro* cytocidal effect against LoVo and T84 human CRC cells expressing the highest A33 mRNA levels (Fig. 2A and B). AV22EL was at least 2 orders of magnitude less effective on HT29 human colorectal carcinoma cells that expressed the lowest A33 mRNA level among the CRC cells (Fig. 2A and B). Interestingly, the presence of the E3 region was slightly detrimental for the *in vitro* lytic activity of the A33Pr-based CRAd (Supplementary Fig. S2A). AV22EL was only effective at 1,000 MOI on human Hep-3B hepatoma and SB2 melanoma cells, whereas it had essentially no cytocidal effect on human A375 melanoma cells, T47D breast cancer cells, WI-38 and HFL-1 fibroblasts, and bovine aortic endothelial cells (Fig. 2A and B). Ad-WT was active in CRC cells at a lower MOI than AV22EL (Fig. 2A and B; Supplementary Fig. S2B); however, its lytic activity was independent of the A33 levels expressed by the different cell types (Fig. 2A and B).

AV22EL specificity was further confirmed when T84 CRC cells expressing EGFP were mixed *in vitro* with WI-38 fibroblasts. Under these conditions, only T84-EGFP cells were lysed by AV22EL, whereas neighbor WI-38 fibroblasts were not affected (Supplementary Fig. S2C and D). On the other hand,

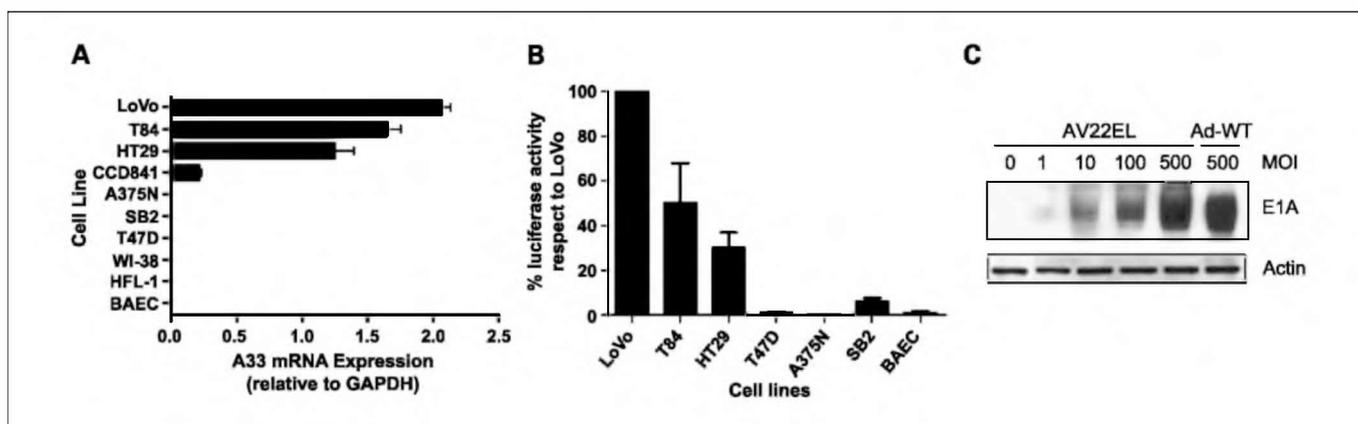


Fig. 1. Characterization of A33 promoter activity in different cell types. **A.** A33 mRNA levels in different cell types. A33 mRNA levels were assessed as described in Materials and Methods. All the experiments were done in triplicate, and the data were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels. Columns, mean; bars, SD. **B.** activity of the A33 promoter. Cells were transduced with 5×10^6 vp/mL of AV-Luc and Ad-CMV *Renilla* as described in Materials and Methods. Cell extracts were assayed 2 d later for firefly and *Renilla* luciferase. Columns, mean of three independent experiments; bars, SD. **C.** Western blot analysis showing E1A expression in LoVo cells infected with AV22EL.

Ad-WT efficiently lysed both cell types (Supplementary Fig. S2D).

As an alternative way to establish the selectivity of AV22EL, cells were infected with AV22EL or Ad-WT and the viral yield was measured by plaque assay at 72 hours after infection. AV22EL replicated very efficiently in LoVo and T84 colon cancer cells, although at a lower level than Ad-WT (Fig. 2C). On the other hand, AV22EL replicated inefficiently in A33-nonexpressing melanoma and breast cancer cell lines that produced 10^5 - to 10^6 -fold less progeny than A33-positive cells. The virus did not replicate at all in normal colon cells, fibroblasts, and endothelial cells (Fig. 2C). Interestingly, HT29 colon cancer cells that express A33 showed viral yield levels similar to those observed in SB2 melanoma cells that did not express A33 (Fig. 2C). Despite that, AV22EL showed 10-fold higher lytic capacity on HT29 cells compared with SB2. By using a replication-defective adenovirus expressing the β -galactosidase gene followed by X-Gal staining, we observed that at the largest MOI of 1,000, only 40% of HT29 cells were transduced compared with $\sim 100\%$ of SB2 melanoma cells (Supplementary Fig. S2E). On the other hand, HT29 CRC cells infected with 1,000 MOI of AV22EL exhibited clearly detectable levels of E1A, whereas only faint levels of E1A were seen in SB2 melanoma cells under the same conditions (Supplementary Fig. S2F). Ad-WT infection led to similar E1A expression levels in both HT29 and SB2 cells (Supplementary Fig. S2F). These data show that AV22EL replicates in HT29 cells at a better extent compared with SB2 melanoma cells, although, based on E1A expression, it seems that SB2 melanoma cells can support a certain level of AV22EL replication.

Complete attenuation of AV22EL lytic capacity on normal human colonic cells and hepatocytes. To further establish that AV22EL activity was attenuated in normal cells, we assessed its cytotoxic effect on CCD841 and FHC cells derived from normal colon epithelia. Ad-WT efficiently lysed both LoVo colorectal carcinoma cells and normal colonic epithelial cells at a MOI of 100 (Fig. 3A). However, AV22EL exerted a cytotoxic effect on LoVo cells but had no lytic effect on CCD841 and FHC cells, suggesting that AV22EL is strongly attenuated in normal colonic cells (Fig. 3A). Of relevance was also the evidence that

AV22EL had no lytic activity on normal human hepatocytes at the largest MOI of 1,000, whereas Ad-WT exhibited a strong lytic effect (Fig. 3B). The inset also shows that Ad-WT, but not AV22EL, could replicate in normal hepatocytes (Fig. 3B).

AV22EL lytic capacity on cells growing in multicellular spheroids. Multicellular spheroids represent a tridimensional *in vitro* model of an avascular growing tumor. Three-day-old spheroids were infected *in situ* with AV22EL or Ad-WT at different MOIs and followed for additional 7 days. At the end of the experiment, spheroids were photographed and sized. Zero MOI corresponds to the average volume of noninfected spheroids at the end of the experiment (Fig. 4; Supplementary Table S2). Spheroids contained in average 5,000 cells at the beginning of the experiment, whereas noninfected spheroids grew up to an average of 30,000 cells/each after 7 days (Supplementary Fig. S3A). In the photography, A375N spheroid volume seems larger due to the fact that A375N cell size is larger than the others (data not shown). Ad-WT inhibited $\sim 50\%$ the growth of spheroids made of A375N and SB2 melanoma cells, whereas AV22EL had essentially no inhibitory effect (Fig. 4A; Supplementary Table S2). On the other hand, AV22EL was as effective as Ad-WT on the growth inhibition of spheroids made of LoVo cells (Fig. 4A; Supplementary Table S2). These results confirmed the selective oncolytic capacity of AV22EL on CRC cells.

In vivo antitumor effect of AV22EL on established tumors. Nude mice were initially xenotransplanted in the flank with tumorigenic inocula of LoVo or SB2 cells. When the average tumor volume reached 100 mm^3 , mice received 1×10^{10} vp/mouse of AV22EL or vehicle administered via intratumor injection at days 1, 4, and 7. AV22EL was effective in inhibiting tumor growth in 100% of mice harboring established LoVo tumors (Fig. 5A, left), including complete tumor regression in two mice that lasted for 200 days (data not shown). However, AV22EL did not affect SB2 melanoma cell growth despite an initial inhibitory effect (Fig. 5A, left) that is probably in accordance with the faint E1A levels reflecting viral replication that were observed after SB2 infection with AV22EL (Supplementary Fig. S2F). No evidence of tumor growth inhibition was observed in mice harboring

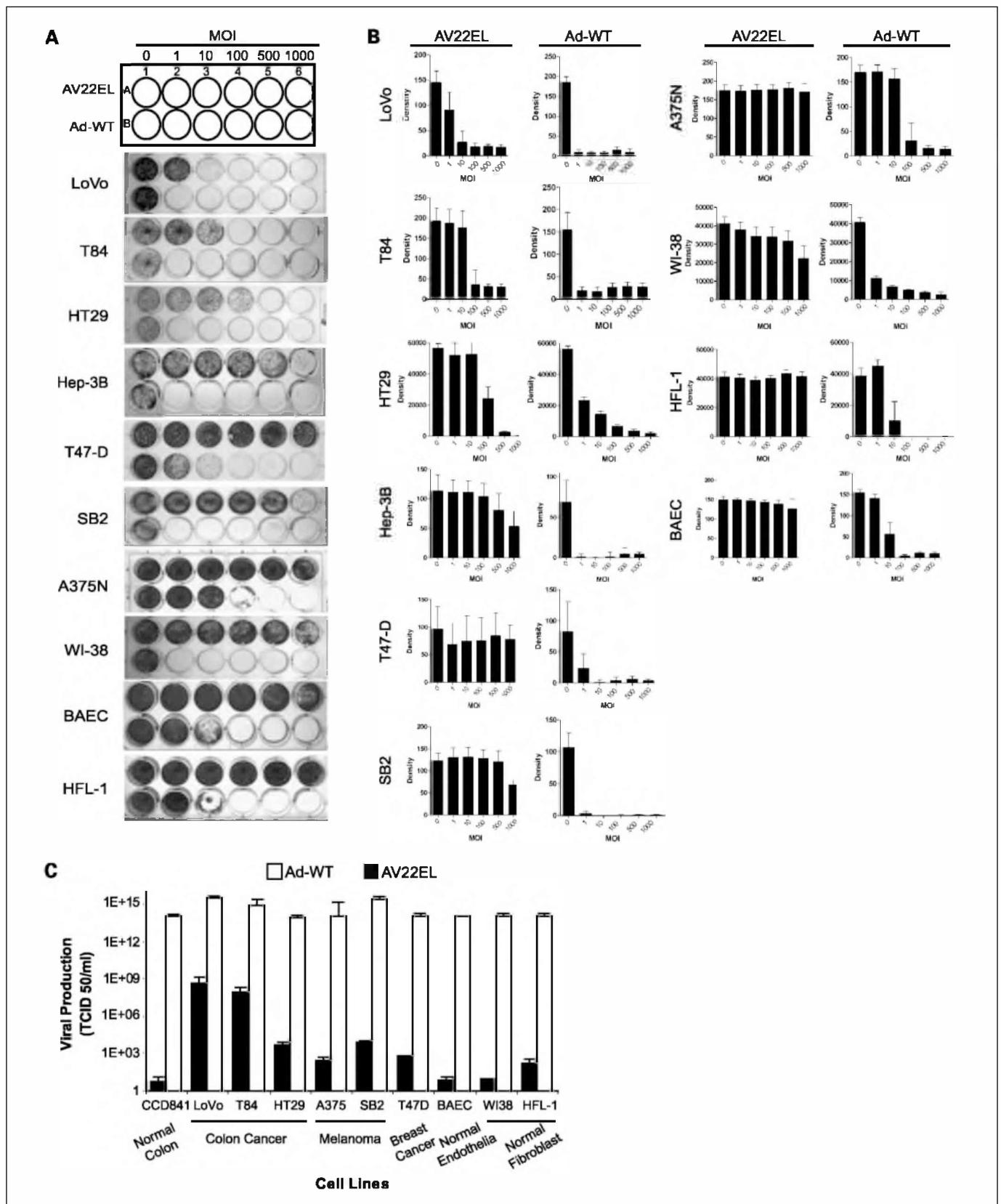


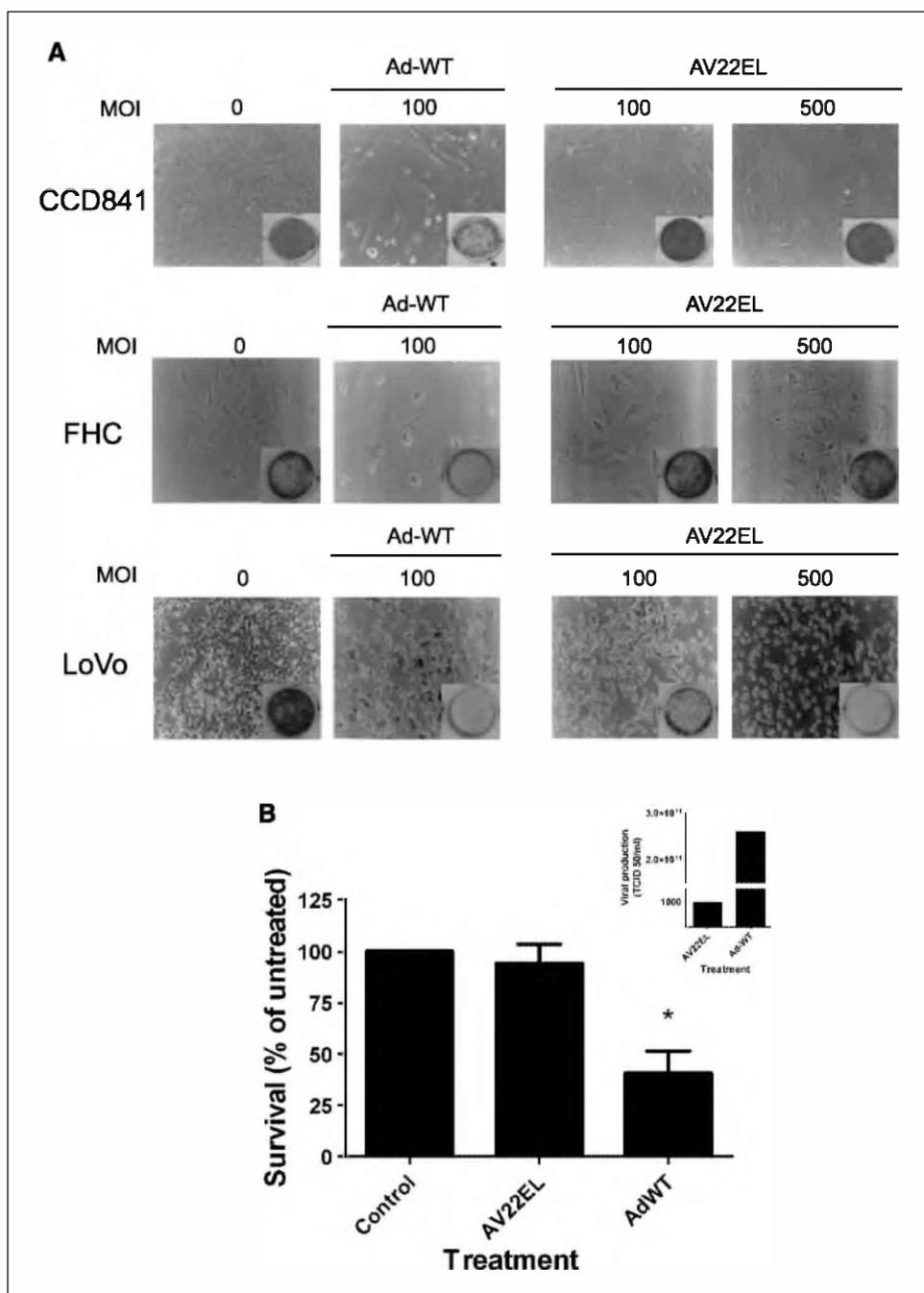
Fig. 2. AV22EL cytotoxic effect and replication capacity in different cell types. *A*, cytotoxic effect of AV22EL on different cell types. AV22EL exerted a cytotoxic effect only on LoVo, T84, and HT29 colon cancer cells, whereas Ad-WT showed cytotoxic effect on every cell type. Cells were cultured for 10 d before staining with crystal violet. *B*, quantification of crystal violet levels was assessed as described in Materials and Methods. *C*, virus yield study of AV22EL on different normal and tumor cell lines. Columns, mean; bars, SD.

LoVo tumors treated with PBS. Kaplan-Meier curve showed that AV22EL-treated mice survived significantly longer than control mice ($P < 0.01$; Fig. 5A, right). There was no sign of virus-related toxicity or weight loss associated with the treatment (data not shown). To confirm that AV22EL replicated *in situ*, we injected intratumorally mice harboring colon cancer tumors of 100 mm³ average volumes. Seven days later, 1.2×10^{10} viral copies per tumor were recovered after AV22EL administration compared with 1.7×10^8 copies per tumor obtained after administration of a nonreplicating adenovirus expressing luciferase (AV-Luc; Fig. 5A, right).

Combination of AV22EL with 5-FU. Because s.c. tumors were not completely eliminated by AV22EL treatment, we assessed

whether the combination of AV22EL with the commonly used chemotherapeutic agent 5-FU will improve therapeutic efficacy. After initial attempts to combine AV22EL and 5-FU in different ways, we observed that preincubation of cells with AV22EL followed by washing and addition of 5 $\mu\text{g}/\text{mL}$ 5-FU for 4 additional days enhanced the lytic effect of 5-FU both on LoVo and HT29 cells, although in the latter the effect was less pronounced (Fig. 5B, a). T84 cells were highly sensitive to 5-FU alone (Fig. 5B, a). When 5-FU concentration was lowered to 0.1 $\mu\text{g}/\text{mL}$, we observed that 10 MOI of AV22EL significantly enhanced the lytic effect of 5-FU on the three colon cancer cell lines compared with 5-FU alone (Fig. 5B, b). AV22EL was completely attenuated in normal cells because it was unable to

Fig. 3. Lack of AV22EL cytotoxic effect on normal cells. **A**, lack of cytotoxic effect of AV22EL on normal colonic cells. AV22EL exerted a cytotoxic effect only on LoVo CRC cells but had no effect on CCD841 and FHC normal colonic cells grown as monolayers. Ad-WT lysed both normal and tumor cells. Cells were photographed under phase-contrast light microscope 7 d after the initiation of the experiment. Insets, crystal violet staining. **B**, lack of cytotoxic effect of AV22EL on human normal hepatocytes. Cells obtained as described in Materials and Methods were infected with AV22EL or Ad-WT and survival was established with the MTT assay at the end of the experiments. Columns, mean of two independent experiments with six replicates; bars, SD. *, statistically significant differences compared with the control ($P < 0.05$). Inset, viral yield after infection of the hepatocytes with the different viruses.



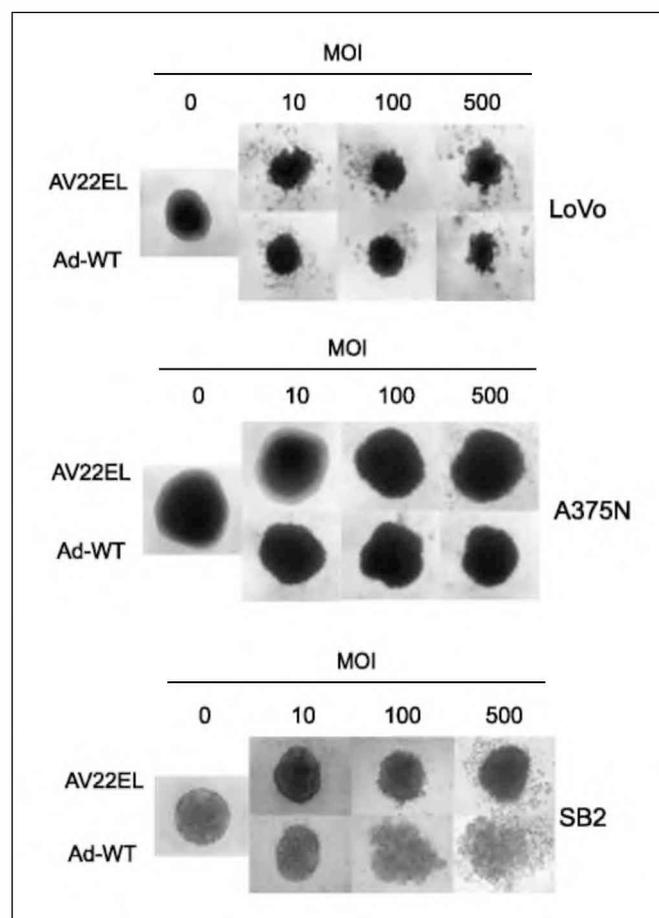


Fig. 4. AV22EL effects on spheroid growth. Spheroids prepared with A375N and SB2 human melanoma cells and LoVo CRC cells were transduced with increasing MOI of AV22EL or Ad-WT. Photographs of the spheroids were taken under phase-contrast light microscopy.

enhance the growth inhibition induced by 5-FU on normal colon cells and fibroblasts (Fig. 5B, c).

We next evaluated if this improved inhibitory effect *in vitro* could be extrapolated *in vivo*. Mice harboring s.c. LoVo tumors were treated with PBS, 5-FU alone, AV22EL alone, or the combination of AV22EL followed by 5-FU. 5-FU alone did not significantly inhibit tumor growth (Fig. 5C), whereas mice treated with AV22EL induced a strong delay in tumor growth in 100% of mice (Fig. 5C). Adding 5-FU in combination with the CRAd did not significantly improve the tumor growth-inhibitory effect observed with AV22EL alone (Fig. 5C). We observed no evidence of toxicity or weight loss associated with

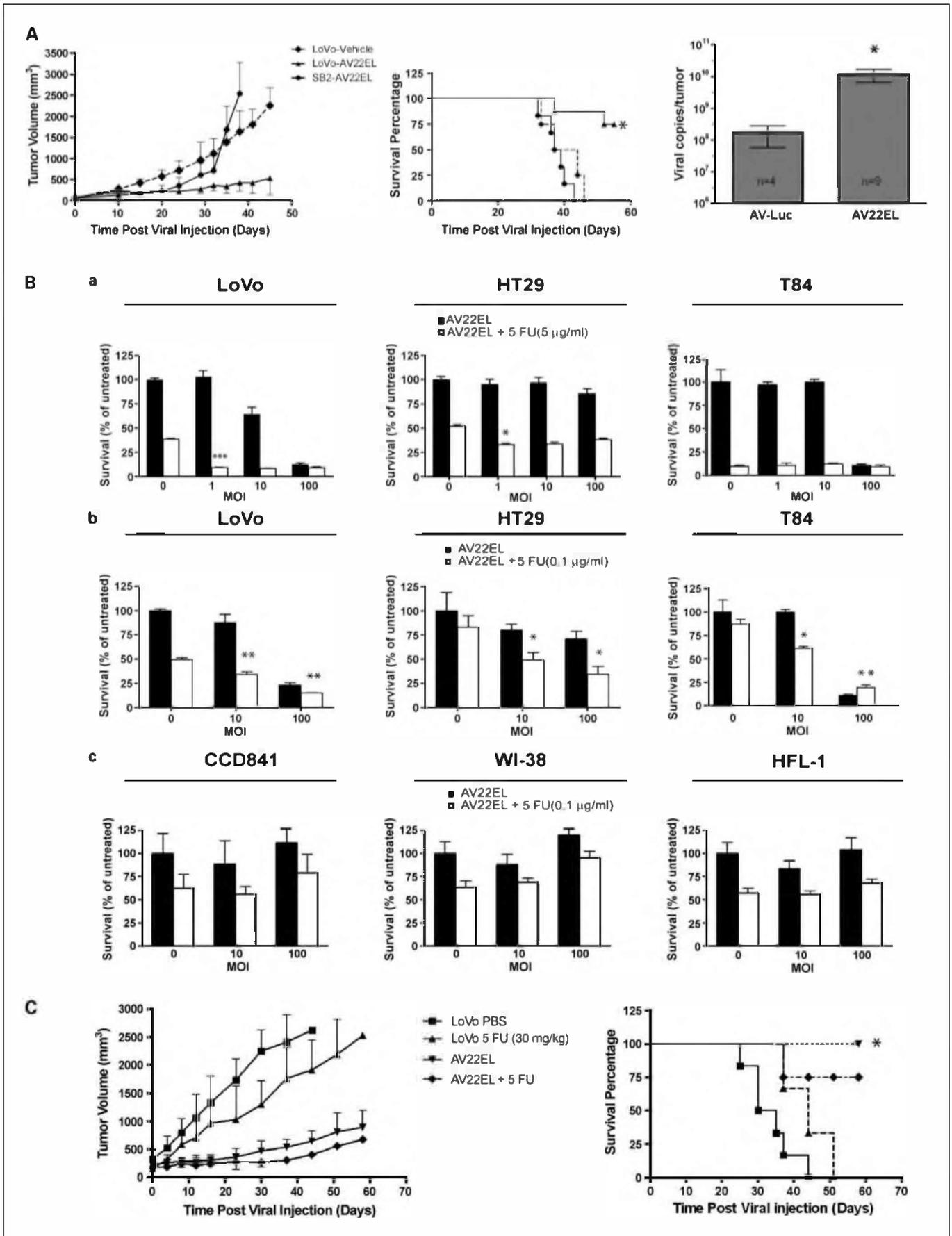
the treatments that included the CRAd alone or combined with 5-FU (data not shown).

Liver metastases elimination following systemic AV22EL delivery. Eradication of hepatic metastases is a key factor for improved CRC prognosis (1). Therefore, we used an efficient model for metastases development in the liver by injecting nude mice in their portal vein with CRC cells. After we have confirmed that hepatic metastases developed, we assessed whether systemic administration of AV22EL might suppress growth of established tumor nodules.

Mice were treated with AV22EL, Ad- β gal, or PBS at days 7, 10, and 14 after the initial cell injection. Seven days after the last viral administration, mice were sacrificed. Almost all the mice injected with Ad- β gal or PBS developed multiple tumor nodules throughout the liver by day 21 after cell injection (Fig. 6A). Mice treated with PBS or Ad- β gal showed metastatic nodules that displayed areas with glandular-like arrangement of tumor cells (Fig. 6A, left). Gross observation and histopathologic analysis of autopsies confirmed that 10 of 11 mice injected with AV22EL were free of metastatic nodules (Fig. 6A, right), whereas 1 animal showed strongly reduced metastatic areas (data not shown). None of the mice injected with PBS or Ad- β gal showed evidence of therapeutic benefit (Fig. 6A, right). In addition, abundant expression of the adenovirus hexon protein was detected in tumor metastasis, but not in the normal liver, 72 hours after AV22EL administration (Fig. 6B). Moreover, systemic administration of AV22EL led to E1A mRNA expression only in liver metastatic nodules, whereas other organs, such as spleen, kidney, intestine, lung, and nonmetastatic liver, exhibited no E1A expression (Fig. 6C, left). Similarly, systemic administration of AV-Luc, where luciferase activity was driven by the A33 promoter, showed activity exclusively in liver metastatic nodules (Fig. 6C, right).

To establish whether administration of AV22EL might affect liver function, we also assessed liver enzymes as a surrogate readout of liver toxicity. Growth of metastatic nodules in mice treated with PBS or control Ad- β gal adenovirus decreased albumin levels while inducing an increase in aspartate aminotransferase and alkaline phosphatase levels (Fig. 6D). Interestingly, albumin levels seemed normal in mice treated with AV22EL, whereas the differences in albumin levels remained statistically significant between the "no tumor" group compared with the Ad- β gal or PBS groups of mice (Fig. 6D). Aspartate aminotransferase levels also seemed normal in the AV22EL-treated group, whereas the Ad- β gal group also exhibited diminished levels compared with the PBS-treated group (Fig. 6D). Alkaline phosphatase differences were not statistically significant between the different groups, although alkaline phosphatase levels in the AV22EL group were similar

Fig. 5. *In vitro* and *in vivo* efficacy of AV22EL in combination or not with 5-FU. **A**, *in vitro* efficacy of AV22EL on s.c. established LoVo tumors. Left, mice carrying established LoVo ($n = 12$) or SB2 tumors ($n = 6$) were treated with AV22EL, whereas control mice ($n = 8$) received vehicle alone. AV22EL exerted a significant antitumor effect ($P < 0.005$ at day 35). Middle, Kaplan-Meier curve showing increased survival in mice induced by AV22EL treatment compared with the other groups. *, $P < 0.01$, when the AV22EL-treated group was compared with the vehicle-treated group. Right, intratumor replication of AV22EL compared with Ad-Luc. Adenovirus was injected intratumorally into LoVo tumors on day 0 at 10^{10} VP/tumor. Viral genome content was measured by quantitative real-time PCR. *, $P < 0.05$. **B**, a to c, *in vitro* cytotoxic effect of AV22EL combined or not with 5-FU. Cells were incubated with two different concentrations of 5-FU or with AV22EL alone or in sequential combination. In the latter case, cells were transduced with AV22EL at the indicated MOIs for 24 h and washed and then 5-FU was added for 4 additional days. Columns, mean; bars, SD. The asterisks indicate statistically significant differences compared with cells treated with 5-FU alone: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **C**, *in vivo* efficacy of AV22EL combined or not with 5-FU on s.c. established LoVo tumors. Left, mice carrying established LoVo tumors were treated with AV22EL alone ($n = 6$), 5-FU alone ($n = 4$), and AV22EL/5-FU in combination ($n = 6$). Control mice ($n = 4$) were treated with vehicle alone. AV22EL combined or not with 5-FU showed a statistically significant antitumor effect ($P < 0.005$ at day 45). Right, Kaplan-Meier survival curve showing the increased survival induced by AV22EL treatment combined or not with 5-FU compared with the vehicle-treated group. *, $P < 0.01$.



to those observed in the no tumor group (Fig. 6D). These data suggest that AV22EL not only was therapeutically efficient to inhibit metastatic growth but also was able to restore the normal function of the liver.

Discussion

Here, we show that a conditionally replicative oncolytic adenovirus based on the A33 promoter was able to inhibit the *in vivo* growth in nude mice of established colorectal carcinoma and eliminated established hepatic metastases. AV22EL was highly selective because it was completely ineffective on cells that did not express A33.

Most of the oncolytic viruses are developed based on promoters that can be active in more than a single cancer type to allow for a wider use without the therapeutic limitation of using the oncolytic virus in only one cancer type. This is the case for the CEA, COX-2, T-cell factor, and telomerase promoters that have been mentioned in Introduction. An additional CRAd based on a double heterologous promoter Ki67 and COX-2 showed significant oncolytic activity on s.c. established colorectal tumors, although its effectiveness on established hepatic metastases was not assessed (38). More recently, a CRAd expressing E1A driven by the nonspecific Rous sarcoma virus promoter and the proapoptotic protein tumor necrosis factor-related apoptosis-inducing ligand was effective in inducing the elimination of established metastases obtained from colon carcinoma cells (39). The concern is that these promoters are active in different types of normal cells or under mild pathologic conditions, such as inflammation, limiting their systemic use due to their potential toxicity to normal organs.

Instead, oncolytic viruses such as AV22EL that are based on stringent, cancer type-specific promoters have the potential advantage of their strong attenuation in normal organs, increasing their effectiveness for systemic treatment of disseminated disease. AV22EL has been cytotoxic *in vitro* on colorectal carcinoma cells expressing A33 antigen growing in monolayers and as spheroids, indicating that AV22EL was able to penetrate the cell-cell adhesive interactions established in spheroid systems. On the other hand, AV22EL was rather ineffective on cells that did not express A33, such as melanoma and normal colorectal cells, which expressed very low levels of A33. Although this novel CRAd exhibited a slight lytic activity on hepatoma cells and certain human melanoma cells at the highest MOI, normal hepatocytes were completely refractory to AV22EL while they were lysed by the wild-type (WT) adenovirus.

The present data indicate that AV22EL exhibited several orders lower viral yield than WT adenovirus. In preliminary studies, we compared AV22EL with an adenoviral construct prepared with the JM17 backbone that includes the E3 region (40). Side by side comparison of the two CRAds showed that the presence of the entire E3 region was detrimental to CRAd activity, leading us to pursue the experiments with the CRAd lacking the E3 region. The detrimental effect of the entire E3 region was consistent with previous data showing that the E3 products 14.5K and 10.4K down-regulated E1A at the translational level (41). However, more recent evidence indicates that restoration of the E3 adenoviral death protein or even the entire E3 coding region might enhance CRAd activity (42–44), strongly suggesting that AV22EL lytic activity could be greatly

improved by adding the adenoviral death protein coding region without losing tumor specificity.

To assess the potential capacity of AV22EL as a therapeutic agent in the clinics, we studied its combination with 5-FU that remains the mainstay of treatment for metastatic CRC. We did different combinations *in vitro* to establish (a) whether both compounds can result in additive or synergistic therapeutic effect, (b) if this effect might depend on the administration timing of both compounds, and (c) if they might render resistant cells sensitive to their combination. We observed that the combination of AV22EL and 5-FU was strongly effective *in vitro* even at concentrations where they were less effective as single agents. This improved *in vitro* inhibitory effect was observed in three different colon cancer cell types that differed in their A33 expression levels, mainly when the virus was administered before the chemotherapeutic agent, indicating that the virus was permissive for 5-FU effect. Despite the fact that we were unable to see enhanced therapeutic effect *in vivo* of their combination, the whole studies suggest that AV22EL can potentially induce an additive effect in combination with 5-FU against both p53 mutant (HT29 and T84) and WT (LoVo) colon carcinoma cells. This is of high importance as the cell cycle response to 5-FU treatment in colorectal carcinoma cells has been reported to be influenced by p53 status (45). It is expected that the therapeutic efficacy of AV22EL can be further improved by assessing different combination protocols with 5-FU or additional novel chemotherapeutic agents.

Liver metastases remain a significant clinical problem in CRC with limited therapeutic options. Few oncolytic vectors were systemically administered to target hepatic metastasis derived from CRC. Systemic administration of AV22EL induced the disappearance of metastatic nodules in >90% of mice and seemed to restore normal levels of hepatic enzymes. Moreover, human hepatocytes were completely refractory to AV22EL and autopsy done on mice that received AV22EL systemically confirmed the absence of hepatic toxicity. Because murine cells are not permissive for adenoviral replication, it was of relevance in terms of viral toxicity the information obtained from the use of Onyx-15 in the clinics, a type 5 oncolytic adenovirus that is attenuated due to the deletion in *E1b* 55K gene (46). Recent data show that its administration through the hepatic artery to patients with metastatic CRC involving the liver exerted only minimal toxicity (47). Because fresh human CRC samples exhibited high expression levels of coxsackie-adenoviral receptor, the natural receptor of adenovirus type 5 (48), it can be proposed that oncolytic viruses based on adenovirus type 5 with strong and selective lytic capacity, such as AV22EL, which is highly attenuated in hepatic cells, might represent an alternative to existing oncolytic viruses for systemic administration.

The human A33 antigen is a M_r 43,000 cell membrane glycoprotein that has become a target for antibody immunotherapy due to its restricted localization in colon cancer as well as in metastatic lesions of the same origin (24). The humanized antibody (huA33) has led to the initiation of a phase I clinical trials with encouraging results (26). The iodinated antibody has shown selective localization and deep penetration even in large necrotic tumors, suggesting that huA33 might become a therapeutic tool for cancer immunotherapy (24). However, in two clinical studies, patients developed anti-huA33 antibodies, limiting its potential therapeutic effect and the full manifestation of its potential clinical toxicities (49, 50),

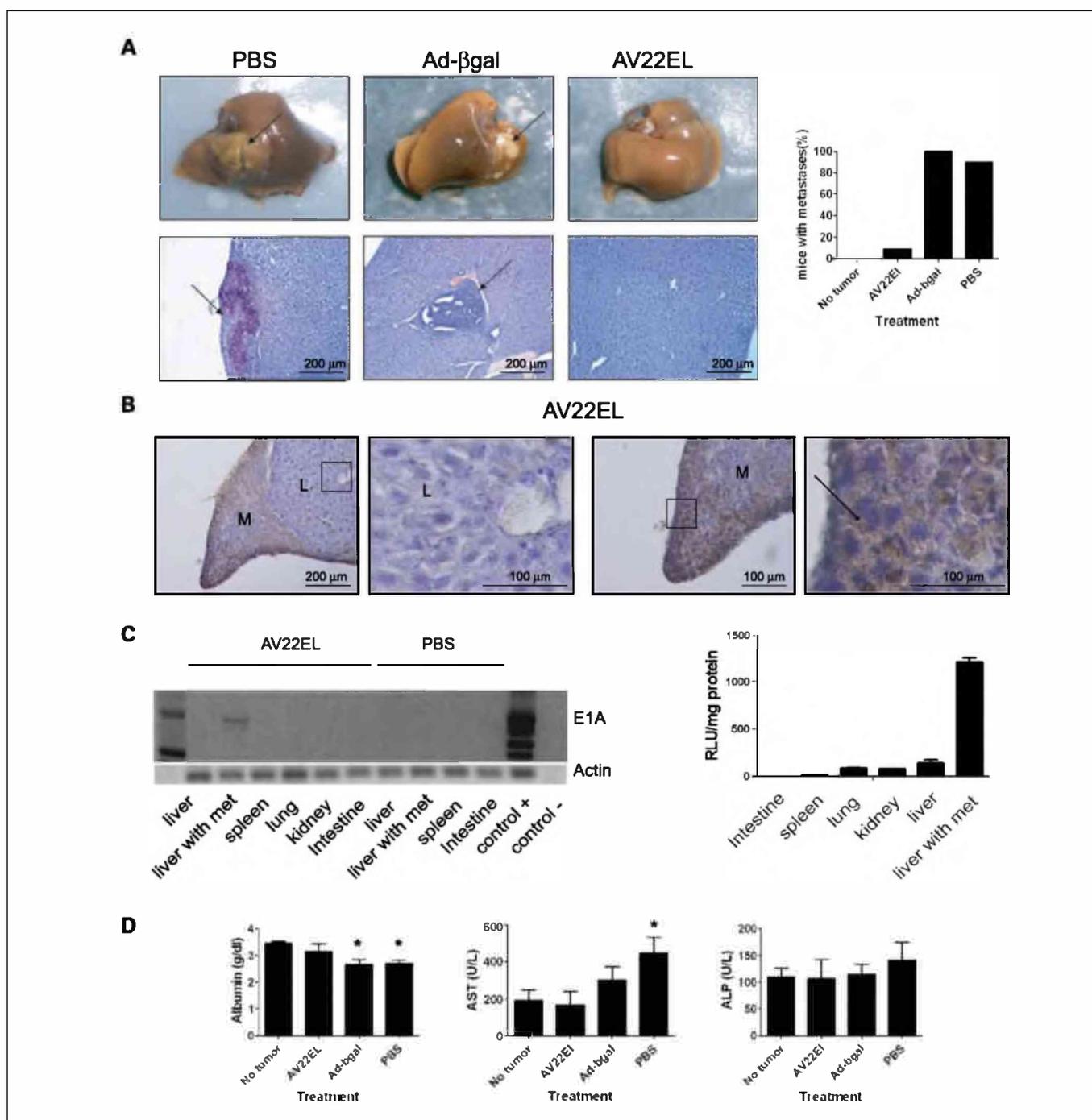


Fig. 6. *In vivo* efficacy of AV22EL on established hepatic metastases. *A*, representative photographs of mice carrying LoVc tumors treated or not with AV22EL. Arrow, light macroscopic photographs showing the presence of metastatic nodules in PBS-treated and Ad-βgal – treated mice. Top, AV22EL-treated mice show the liver clear of metastatic nodules. H&E staining of human liver sections. Bottom, arrows, LoVc tumors display areas with glandular-like arrangement of tumor cells. Magnification, ×200. Right, comparison of metastases number. Number of metastases were determined at the end of the experiments 14 d after administration of the first viral dose. *B*, immunohistochemical detection of hexon protein in metastatic areas (M) located in the liver (L). Arrows, positive staining in metastatic cells and negative staining in hepatocytes. The areas in the box are further examined at higher magnification. Magnifications, ×200 and ×400. *C*, E1A mRNA expression in different organs following AV22EL administration. PCR analysis was done on total RNAs obtained from different organs 72 h after injection of AV22EL or PBS via tail vein. Right, tissue distribution of AV-Luc. Six mice were administered i.v. 10^{10} vp of AV-Luc and 10^6 vp of Ad-CMV *Renilla*. Forty-eight hours later, luciferase activity in various tissues and liver metastatic nodules was determined. Results are expressed as mean of luciferase activity in light units per milligram of protein. Bars, SD. *D*, comparison of serum liver chemistries: hepatic panel function was determined at the end of the experiments 14 d after administration of the first viral dose. Serum enzyme levels are reported in units/mL. Albumin level is reported in g/dL. Columns, mean; bars, SD. *, statistically significant differences to no tumor mice ($P < 0.05$).

suggesting that a less immunogenic compound might be necessary.

Here, we have shown the therapeutic efficacy of a novel CRAD whose replication is driven by the A33 promoter,

providing an additional strategy that makes use of the tissue-specific expression of this protein. Despite the fact that AV22EL exhibited lower replication capacity than WT adenovirus *in vitro*, it showed intratumor replicating capacity similar to

that attained in breast cancer for a CRAd that targets the G₁-S checkpoint (Ad-dl922-947) and higher than Ad-Onyx-017 that targets p53 (36). Thus, AV22EL was highly effective on primary tumors and hepatic metastasis of CRC with no cytotoxic effect on normal colon derived cells, could be combined with current chemotherapeutic treatments, restored the normal hepatic function after the elimination of hepatic metastasis, and after systemic administration was strictly active in hepatic metastases, avoiding normal organs. Given the evidence of the mild adverse effects of oncolytic adenovirus therapeutics, we believe

that AV22EL might become a useful tool for treatment of patients with CRC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Florencia Straminsky and Fabic Fraga for their technical support.

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Clinical Cancer Research

A Novel A33 Promoter–Based Conditionally Replicative Adenovirus Suppresses Tumor Growth and Eradicates Hepatic Metastases in Human Colon Cancer Models

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