Supplementary Methods

Cell culture

A549, HT29, Caco-2, MC38-cea-2 (1), 293 and LS174T were grown in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Cellegro, Washington, DC) with penicillin-streptomycin (Invitrogen, Carlsbad, CA), 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA) and 1.0 mM sodium pyruvate (Invitrogen, Carlsbad, CA). H2122 was grown in RPMI1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum with penicillin-streptomycin. sCAR-MFE was prepared as described previously (1, 2).

Stable transfection

To establish stable cell lines that over express *Renilla* luciferase (rLuc), LS174T cells were transfected with a pcDNA3.1 expression vector in which the *Renilla* luciferase coding unit (rLuc) is driven by an SV40 promoter, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Neomycin-resistant cells were selected in medium containing G418 (1 mg/ml). Extracts of G418 resistant cells were screened for rLuc expression with the Luciferase assay system (Promega, Madison, WI).

Immunoblot Analysis

Cells were washed twice with phosphate-buffered saline (PBS) and incubated on ice for 10 min in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% NP-40, 1% triton, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and protease inhibitors (Complete Tablet; Roche, Indianapolis, IN). After 10,000 x *g* centrifugation for 10 minutes, supernatant protein concentrations were measured with the Bio-Rad Protein Assay (Bio-Rad). The protein extracts were denatured in denaturing buffer. Equal amounts of protein (100 µg cell lysate) were loaded on SDS-polyacrylamide gels (8% for CEA and 12% for all others) for electrophoresis. Proteins were subsequently transferred to

nitrocellulose membranes. The membranes were probed with anti-CEA antibody, cT84.66 (1:10,000 dilution; (3)), anti-human COX-2 monoclonal antibody (1:300 dilution; Cayman Chemical), anti-CAR antibody (RmcB), anti-GAPDH antibody (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-14-3-3θ antibody (1:3,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C, followed by incubation at room temperature for 60 min with horseradish peroxidase (HRP)-conjugated secondary antibody. Immunoreactivity was determined by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ).

Analysis of *COX-2* promoter activity in cell culture

Tumor cell lines were infected with Ad.cox2fLuc or Ad.CMVfLuc (MOI=100). The adenovirus were diluted to 150 µl with medium containing 2% FBS, then added to cell monolayers in 24-well plates (2×10^5 cells/well). Virus-treated cells were incubated at 37 °C for 90 minutes. 1 mL medium containing 10% FBS was added after the 90 minutes incubation. After a 40-hour incubation at 37 °C in medium containing 10% FBS, cells were lysed and luciferase activity was measured. The relative activities of the Cox-2 promoters are presented as Relative Luciferase Units (RLU) of the Ad.cox2fLuc infected cells divided by the RLU of Ad.CMVfLuc infected cells.

CEA competition of virus infection

Ad.CMVfLuc (3×10^8 vp) was mixed with 63 ng of sCARfMFE or with 63 ng of sCAR6hMFE in 10 µl and incubated for 60 minutes at room temperature. The sCAR-MFE:adenovirus complexes were diluted to 200 µl with medium containing 2% FBS and increasing amounts of sCEA (0, 50, 150, 500, 1500, 3000 and 5000 ng sCEA; soluble CEA, Protein Sciences Corporation, Meriden, CT), then added to MC38-cea-2 cell monolayers in 24-well plates (2×10^5 cells/well). Virus-treated cells were incubated at 37 °C for 90 minutes. Medium was aspirated and cells were washed with PBS. After a 40-

hour incubation at 37 °C in medium containing 10% FBS, cells were lysed and luciferase activity was measured.

Immunohistochemistry for Ki67

Livers were immersed in 10% formalin at room temperature overnight, then incubated in 50% ethanol overnight prior to preparing paraffin sections. The paraffin section slides received two five minute room temperature xylene immersions at room temperature, washed with 100 % ethanol (2 x, 5 min), then rehydrated by sequentially immersing the slides through graded ethanol washes (95%, 85%, 70% and 50%; 3 min each at room temperature). The sections were then immersed in 0.5% v/v hydrogen peroxide/methanol for 10 min, washed in distilled water and immersed in citrate buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0) in Coplin jars at 95-100 °C for 20 min. The whole Coplin jar was placed at room temperature to allow the slides cool for 20 min. The slides were washed with 1x TBS (0.05 M Tris Base, 0.9% NaCl, pH 7.6). For serum blocking, the sections were incubated with normal goat serum blocking solution (2% goat serum, 1% BSA, 0.1% Triton X-100, 0.05% Tween 20, 0.01 M PBS, pH 7.2) for 30 min at room temperature. For primary antibody incubation, the sections were incubated with rabbit anti-human Ki-67 (1:1000 dilution; Novocastra, # NCL-Ki67p, Norwell, MA) in 0.01 M PBS pH 7.2 for 1 h at room temperature. Slides were washed twice (5 min) with 1x TBS, then incubated with biotinylated goat anti-rabbit IgG (1:500 dilution; Vector Laboratories) for 30 min at room temperature. After two 5 min washes with 1x TBS the section were incubated with HRP-Streptavidin (1:500 dilution; Vector Laboratories, Burlingame, CA) in 0.01 M PBS pH 7.2 for 30 min at room temperature. Following two additional 5 min washers with 1x TBS, the sections were incubated with DAB peroxidase substrate (0.05% DAB, 0.015% H_2O_2 in 0.01 M PBS pH 7.2) until a light brown background appeared (5~10 min). The slides were rinsed in running tap water for 5 min, then dehydrated through 95% ethanol for 2 min and 100% ethanol for 5 min. This

procedure is modified from the protocol described by Novocastra for Ki67 staining. Slides were mounted with Permount Mounting Medium (Fisher Scientific, Pittsburgh, PA) and the images were taken with the ScanScop XT (Aperio, Vista, CA) microscope.

Supplementary Figure Legends

Supplementary Figure 1. COX-2 and CEA expression in human lung and colon cancer cell lines. COX-2 and CEA protein expression were analyzed by Western Blotting in the cell lines shown. 14-3-3 was used as a loading control..

Supplementary Figure 2. Structure of the Ad.cox2NTP imaging/therapeutic virus, and *COX-2* promoter-restricted transgene expression by Ad.cox2fLuc and Ad.cox2NTP in liver and in LS174T hepatic tumors. (A) Ad.cox2NTP genomic structure. The human hcox-2L promoter encompasses nucleotides -1432 to +59. "I" is a truncated intron, "ttk" is the truncated thymidine kinase coding region; "tsfLuc" is a thermal stable firefly luciferase coding region and "egfp" is enhanced green fluorescent protein. "2A" is the self-cleaving peptide of the foot-and-mouse disease virus. (B) Tumor-free mice and mice bearing LS174T(rLuc) hepatic tumors were injected intravenously with Ad.CMVfLuc, Ad.cox2fLuc or Ad.cox2NTP (1×10^9 ifu/mouse) 14 days after tumor transplantation. LS174T(rLuc) tumor burden was monitored by coelenterazine-dependent rLuc bioluminescence in living animals (a-f) at 18 days after hepatic tumor cell implantation into nude mice. Adenovirus-directed transgene expression was monitored by luciferin-dependent fLuc bioluminescence five days after virus administration (g-l). Following FL imaging, the mice were sacrificed and the tissues were removed and imaged for tumor-derived rLuc activity (s-x) and for Ad-directed fLuc transgene expression (m-r). (H: heart, I: intestine, K: kidney, L: liver, Ln: lung, S: spleen)

Supplementary Figure 3. Effect of volume dilution on the targeting efficacy of adenovirus monomeric sCARhMFE and trimeric sCARfMFE complexes. Ad.CMVfLuc (3×10^8 vp/well) was pre-incubated with increasing amounts of sCARhMFE

or sCARfMFE in 0.5 µl. MC38-cea-2 cells were then infected with the viral preparations in two different volumes, 0.2 ml/well (left) or 1.0 ml/well (right). After 40 hours, cell extracts were prepared and assayed for luciferase activity and protein concentration. Data are averages ± SEM of three cultures at each time point, and are plotted relative to the luciferase activity observed in extracts from cells infected with [Ad.CMVfLuc][trimeric sCARfMFE (100ng)] in 0.2 ml (left) and in 1.0 ml (right). Data are averages ± SEM (n = 3; **p < 0.01) compared to cells infected with [Ad.CMVfLuc][sCARhMFE]) at the same adapter concentration).

Supplementary Fig. 4. Soluble CEA blocks monomeric sCARhMFE retargeting of Ad to CEA-positive cells more effectively than it blocks trimeric sCARfMFE Ad retargeting. MC38-cea-2 cells were infected with Ad.CMVfLuc (3×10^8 vp/well), pre-incubated with sCARhMFE (63 ng/well) or sCARfMFE (63 ng/well), in medium containing increasing amounts of sCEA. Cell extracts were prepared and assayed for luciferase activity and protein concentration. The Y axis indicates the percent of luciferase activity relative to the activity of cells infected in the absence of any sCEA competitor. Data are averages ± SEM (n = 3; **p < 0.01).

References

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