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Enhanced Prostate Cancer Gene Transfer and Therapy Using a Novel Serotype Chimera Cancer Terminator Virus (Ad.5/3-CTV)

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Abstract

Few options are available for treating patients with advanced prostate cancer (PC). As PC is a slow growing disease and accessible by ultrasound, gene therapy could provide a viable option for this neoplasm. Conditionally replication-competent adenoviruses (CRCAs) represent potentially useful reagents for treating PC. We previously constructed a CRCA, cancer terminator virus (CTV), which showed efficacy both *in vitro* and *in vivo* for PC. The CTV was generated on a serotype 5-background (Ad.5-CTV) with infectivity depending on Coxsackie-Adenovirus Receptors (CARs). CARs are frequently reduced in many tumor types, including PCs thereby limiting effective Ad-mediated therapy. Using serotype chimerism, a novel CTV (Ad.5/3-CTV) was created by replacing the Ad.5 fiber knob with the Ad.3 fiber knob thereby facilitating infection in a CAR-independent manner. We evaluated Ad.5/3-CTV in comparison with Ad.5-CTV in low CAR human PC cells, demonstrating higher efficiency in inhibiting cell viability *in vitro*. Moreover, Ad.5/3-CTV potently suppressed *in vivo* tumor growth in a nude mouse xenograft

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model and in a spontaneously induced PC that develops in Hi-myc transgenic mice. Considering the significant responses in a Phase I clinical trial of a non-replicating Ad.5-*mda-7* in advanced cancers, Ad.5/3-CTV may exert improved therapeutic benefit in a clinical setting.

Prostate cancer (PC) is the most frequently diagnosed cancer and is the second leading cause of cancer death among men in the United States (Damber and Aus, 2008; Siegel et al., 2012). It is estimated that 238,590 new PC cases will be diagnosed in 2013 and 29,720 men will die of PC. Patients with localized disease may be treated with surgery or radiation, whereas the treatment options for patients with metastatic disease is purely palliative. Current therapies include hormonal therapy, radiotherapy, and cytotoxic chemotherapeutic agents (Sternberg, 2002; Siegel et al., 2012). Although existing approaches are beneficial in men with various stages of PC, the complications frequently associated with these conventional treatment options diminish positive clinical outcomes. Consequently, more efficient and innovative treatments are mandatory, and genetic therapies represent promising approaches for the treatment of this neoplasm.

Using subtraction hybridization combined with induction of cancer cell terminal differentiation, our laboratory cloned melanoma differentiation-associated gene-7/interleukin-24 (*mda-7/IL-24*) (Jiang et al., 1995, 1996), a novel member of the IL-10-related cytokine gene family (Sarkar et al., 2002a; Wolk et al., 2002; Sauane et al., 2003; Dash et al., 2010a). Subsequent studies documented that *mda-7/IL-24* displays almost ubiquitous anti-tumor properties in vitro and in vivo, leading to its rapid entry into the clinic, where its safety and clinical efficacy, when administered by adenovirus (Ad.*mda-7*; INGN 241), was observed in a phase I clinical trial in humans with advanced carcinomas and melanomas (Jiang et al., 1996; Fisher et al., 2003, 2007; Cunningham et al., 2005; Tong et al., 2005; Lebedeva et al., 2007a,c). *mda-7/IL-24* preferentially induces apoptosis in cancer cells while exerting no discernible toxic effects toward normal cells (Sarkar et al., 2002b; Sauane et al., 2008; Dash et al., 2010a, 2011a) and it also elicits potent “anti-tumor bystander activity” in distant cancer cells as a consequence of autocrine and paracrine secretion of MDA-7/IL-24 (Su et al., 2001a, 2005a; Fisher, 2005; Lebedeva et al., 2007a,b; Sauane et al., 2008; Dash et al., 2010b).

Since PC is generally a relatively slow-growing disease, it may require repeated gene therapy treatments, with single or multiple genes, over the lifespan of the patient. Conditionally replication-competent adenoviruses (CRCA) provide a potentially valuable reagent for gene therapy (Curiel and Fisher, 2012). Using subtraction hybridization we cloned a novel rodent gene, progression elevated gene-3 (*PEG-3*), in the context of tumor progression in transformed rat embryo cells (Su et al., 1997). *PEG-3*: (i) displays elevated expression as a function of oncogenic transformation (by diverse oncogenes) (Su et al., 1997, 2002); (ii) induces an aggressive cancer phenotype without promoting transformation when expressed in normal cells (Su et al., 1999, 2002); and (iii) the gene promoter (*PEG-Prom*) has been isolated and shown to display elevated expression in both rodent and human tumors (including PC), with negligible expression in normal cells (including human prostate epithelium) (Su et al., 2001b, 2005b; Sarkar et al., 2005a,b, 2006a,b, 2007b, 2008; Bhang et al., 2011; Das et al., 2012). Considering the cancer-specific expression aspects of the *PEG-*

Prom, we constructed a bipartite serotype 5 CRCA (called a cancer terminator virus, Ad.5-CTV) in which the expression of *E1A* and *E1B* genes of Ad, necessary for replication, is controlled by the *PEG*-Prom (Sarkar et al., 2005a, 2006, 2007b, 2008). This novel Ad.5-CTV also expressed *mda-7/IL-24* from the E3 region (Ad.PEG-E1A-*mda-7*). The ability of Ad.5-CTV to infect and express MDA-7/IL-24 in PC cells depends on the presence of Coxsackie-Adenovirus Receptors (CARs) on their surface. Ad.5-CTV is capable of efficiently infecting high CAR cells (such as DU-145) and expressing robust levels of *mda-7/IL-24*, whereas infection is restricted and expression of MDA-7/IL-24 is minimal in low CAR cells, such as PC-3 (Dash et al., 2010b, 2011b).

An approach to circumvent the low efficiency of Ad.5 infection of tumor cells involves “tropism modification” in which virus capsid proteins that normally associate with CAR are modified, permitting both CAR-dependent and CAR-independent infectivity of tumor cells. Studies using various tumor cell types have shown that inclusion of the infective type 3 Ad sequence within the Ad type 5 virus knob (Ad.5/3 recombinant virus) promotes viral infectivity in tumor cells displaying reduced or no CAR expression (Hamed et al., 2010; Dash et al., 2010b; Eulitt et al., 2011; Park et al., 2011; Azab et al., 2012). It is worth noting that Ad.5/3 also retains high infectivity in CAR-expressing tumor cells showing equal efficacy when compared with Ad.5, thereby providing an expanded range of utility for Ad.5/3, in both low and high CAR-expressing tumor cells.

In the present study, we constructed and evaluated the in vitro and in vivo efficacy in low and high CAR PCs of a novel tropism-modified CTV in which the virus capsid proteins that normally associate with CAR were modified, Ad.5/3-CTV, permitting CAR-independent infectivity of tumor cells. In low CAR PC-3 cells, Ad.5/3-CTV is more efficient than Ad.5-CTV in infecting tumor cells, delivering a transgene (*mda-7/IL-24*), expressing MDA-7/IL-24 protein and inducing cancer-specific apoptosis. In an in vivo context, Ad.5/3-CTV is superior to the Ad.5-CTV in inhibiting in vivo tumor growth and exerting an anti-tumor “bystander” effect in nude mouse human PC xenografts and Ad.5/3-CTV potently suppresses PC development in an immunocompetent Hi-Myc transgenic mouse model of PC.

Materials and Methods

Cell lines, culture conditions, and viability assays

DU-145 and PC-3 PC cells were obtained from the American Type Culture Collection and cultured as described (Lebedeva et al., 2003). Construction and characterization of PC-3 over-expressing *Bcl-2*, PC-3-*Bcl-2*, and control clones containing the neomycin vector, PC-3-Neo, were described previously (Lebedeva et al., 2003). Cell viability was determined by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Lebedeva et al., 2003). Cell cultures were routinely tested for mycoplasma using a kit from Sigma (MP-0025) and only mycoplasma free cells were used for these studies.

Construction of Ad.5/3-CTV

The genome of Ad.5/3-PEG-E1-*mda-7* was generated in three consecutive steps (Fig. 1). (1) Homologous recombination of pAd.5/3 genomic plasmid with pShuttleE3 plasmid containing the *mda-7/IL-24* expression cassette and kanamycin selection results in the pAd.5/3-E3-*mda-7* genome. (2) pAd.5/3-E3-*mda-7* was cut with *SwaI* to excise the kanamycin resistance gene. (3) The resultant pAd.5/3-E3-*mda-7* plasmid was recombined with pShuttleE1 plasmid containing E1A and E1B genes under control of the *PEG-3* promoter resulting in Ad.5/3-PEG-E1-*mda-7* (Ad.5/3-CTV) genomic plasmid. This plasmid was digested with *PacI* to release viral ITRs and transfected in A549 cells to rescue the CRCA, Ad.5/3-CTV.

Preparation of whole-cell lysates and Western blot analyses

Preparation of whole-cell lysates and Western blot analyses were performed as previously described (Sarkar et al., 2005b). The primary antibodies used were anti-MDA-7/IL-24 (Gen Hunter Corporation, Nashville, TN), anti-EF1 α (1:1,000; mouse mono-clonal; Millipore, Lake Placid, NY), anti-Mcl-1 (1:500; mouse monoclonal; Santa Cruz, Santa Cruz, CA), anti-BiP/GRP78 (1:500; rabbit monoclonal; Santa Cruz, CA), anti-GRP94 (1:1,000; rabbit monoclonal; Sigma, St Louis, MO), and anti-PARP (1:1,000; rabbit monoclonal; Cell Signaling, Danvers, MA).

Human PC xenografts in athymic nude mice

PC-3-Bcl-2 cells (2×10^6) were injected s.c. in 100 μ l of 1:1 PBS and Matrigel in the left and right flanks of male athymic nude mice (NCRnu/nu, 6–8 weeks old, ~20 g body weight) (Sarkar et al., 2005a). After establishing palpable tumors of ~100 mm³, requiring ~7–10 days, intratumoral injections of different Ads were given only to the tumors on the left flank at a dose of 1×10^{10} viral particles in 100 μ l. The injections were given twice a week for four weeks. A minimum of five animals was used per experimental point. Tumor volume was calculated using the formula: $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$. At the end of the experiment, the animals were sacrificed, and the tumors were removed and weighed.

Hi-Myc mice and animal husbandry protocols

The VCU Institutional Animal Care and Use Committee approved the experimental protocol used in this study and the animals were cared for in accordance with institutional guidelines. This study used Hi-Myc transgenic mice in which prostate-specific expression of human c-*Myc* is driven by the rat probasin promoter with two androgen response elements (ARR2/probasin promoter) (Ellwood-Yen et al., 2003). Mice were obtained from the Mouse Repository of the National Cancer Institute Mouse Models of Human Cancer Consortium at NCI Frederick, MD, USA. Mouse-tail DNA was isolated using the DNeasy Blood & Tissue Kit from QIAGEN (Valencia, CA) and subjected to a PCR-based screening assay for genotyping. For genotyping Hi-Myc mice, the upstream primer (located within the ARR2-PB promoter), 5'-AAACATGATGACTACCAAGCTTGGC-3' and the downstream primer (within the MYC cDNA sequence) 5'-ATGATAGCATCTTGTTCTTAGTCTTTTTCTTAATAGGG-3' were used to generate a PCR product of 177 base pairs.

Preparation of microbubbles (MBs), ultrasound (US) platform, ultrasound-targeted microbubble destruction (UTMD), and BI-97C1 (Sabutoclax)

Preparation of MBs followed by UTMD for delivery of *mda-7/IL-24* expressing Ads has been described previously (Dash et al., 2011a). Targeson (Targeson) custom synthesis US contrast agent (perfluorocarbon MBs, encapsulated by a lipid coat and poly (ethyleneglycol) stabilizer) were obtained. MBs were reconstituted in the presence or absence of 1 ml of 1×10^{11} viral particles of indicated Ads and unenclosed surface-associated Ads were treated with complement as previously described (Cianfriglia et al., 1999; Howard et al., 2006). For in vivo experiments, US exposure was achieved with a Micro-Maxx SonoSite (SonoSite, Oceanside, CA) US machine equipped with the transducer L25 set at 0.7 Mechanical Index, 1.8 MPa for 10 min. Mice were sedated in an IMPAQ6 anesthesia apparatus (VetEquip, Pleasanton, CA) that was saturated with 3–5% isoflurane and 10–15% oxygen with the aid of a precision vaporizer to deliver the appropriate amount of anesthetic and to induce anesthesia. For MB/Ad injection a 27-gauge needle with a heparin lock was placed within a lateral tail vein for administration of contrast material. The mice received injections of 100 μ l of MBs with Ads through the tail vein eight times in the span of 4 weeks. Ultrasound (sonoporation) was performed with a SonoSite scanner (SonoSite) equipped with the transducer L25 set at 0.7 Mechanical Index, 1.8 MPa for 10 min in the ventral side of mice in the prostatic area. BI-97C1 (Sabutoclax) was administered intraperitoneally at a dose level of 3 mg/kg three times a week for the duration of the study (total 12 injections). Compounds dissolved in 500 μ l of solvent (ethanol/Cremophor EL/saline = 10:10:80) were injected intraperitoneally. At the end of the experiment, the Hi-*Myc* mice were sacrificed and the prostate was dissected. The harvested prostate was preserved in neutral buffered formalin at 4°C before embedding in paraffin for immunohistochemical analysis.

Immunohistochemical staining

For immunohistochemical (IHC) analysis, formalin-fixed and paraffin-embedded specimens were sectioned 3–4- μ m thick. Sections were deparaffinized, re-hydrated and then quenched in 3% H₂O₂ for 20 min. Sections were washed with PBS and blocked in PBS containing 1% BSA for 20 min at 37°C. Monoclonal anti-MDA-7/IL-24 (1:200) was incubated for 3 h at room temperature and then washed three times in PBS. Sections were incubated with an avidin–biotin–peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) and then washed two times in PBS. The immunoreactivity was determined using diaminobenzidine (DAB) as the final chromogen. Finally, sections were counterstained with Meyer's Hematoxylin, dehydrated through a sequence of increasing concentrations of alcohol, cleared in xylene and mounted with epoxydic medium. Sections were also processed for hematoxylin and eosin (H&E) staining.

Determination of apoptotic cells by TUNEL assay

For TUNEL assays, we used the DeadEnd Colorimetric TUNEL Assay kit (Promega, Madison, WI) performed according to the manufacturer's instructions. Briefly, paraffin-embedded slides were deparaffinized and rehydrated. Pre-equilibrated slides were labeled with a labeling DNA-strand break solution containing a biotinylated nucleotide mix (60 min at 37°C). After several washes in two times SSC and PBS, slides were blocked with

hydrogen peroxide (3–5 min at room temperature). After several washes in PBS, the slides were mounted with mounting solution with DAPI. Apoptotic cells on the slides were observed under an Olympus epifluorescence microscope (X10 magnification; Olympus, Center Valley, PA) in randomly chosen fields. For detection of apoptosis in a time-dependent manner in vitro, PC-3 cells were grown in microscopic slide culture chambers (BD Bioscience, San Jose, CA) and cells were treated with Ad.5/3-CTV and BI-97C1 (Sabutoclax, Sanford Burnham Medical Research Institute, La Jolla, CA) after which the cells were fixed with 4% formaldehyde at the indicated time and TUNEL assays were performed as per the manufacturer's instruction using an Olympus epifluorescence microscope (X10 magnification; Olympus, Center Valley, PA) (Dash et al., 2011a).

Statistical analysis

Statistical analysis was done using Student's *t*-test, followed by Fisher's protected least significant difference analysis. $P < 0.05$ was considered significant.

Results

Ad.5/3-CTV displays enhanced *mda-7/IL-24* expression and inhibition of cell viability in low CAR PC cells

The scheme for constructing Ad.5/3-CTV, in which viral replication is controlled by the PEG-Prom and which also expresses *mda-7/IL-24* in an Ad.5/3 background, is shown in Figure 1 and described in detail in Materials and Methods Section. As controls we used Ad.5-vec (replication-incompetent empty Ad.5), Ad.5/3-vec (replication-incompetent empty Ad.5/3), Ad.5-PEG-E1A, in which viral replication is controlled by the PEG-Prom in an Ad.5 background, and Ad.5/3-PEG-E1A, in which viral replication is controlled by the PEG-Prom in an Ad.5/3 background. We compared MDA-7/IL-24 expression upon infection of Ad.5/3-CTV and Ad.5-CTV in PC cells that contain low or high CAR on their surface. For this purpose, we used PC-3, which have a reduced level of CAR (D value 0.32) in comparison with DU-145, which express a high level of CAR (D value 0.92) (Lebedeva et al., 2003; Dash et al., 2010b). In PC-3, MDA-7/IL-24 expression was significantly higher upon infection with Ad.5/3-CTV, as compared to Ad.5-CTV, whereas infection with both Ad.5/3-CTV and Ad.5-CTV in DU-145 resulted in comparable expression of MDA-7/IL-24 protein (Fig. 2A and B). Infection with control Ad vectors did not result in MDA-7/IL-24 expression. These findings indicate that infection with Ad.5/3-CTV promotes enhanced transgene delivery in low CAR containing PC cells compared to Ad.5-CTV, and both CTVs are comparable in transgene delivery in high CAR PC cells. An analogous finding was evident when analyzing Ad replication by monitoring E1A protein levels in PC-3 and DU-145 cells (Fig. 2A and B). In DU-145 cells, a similar pattern of virus replication was apparent following infection with Ad.5 or Ad.5/3 background viruses, while in PC-3 cells replication of Ad.5/3 was significantly elevated in comparison with infection with Ad.5 (Fig. 2A and B).

The efficacy of Ad.5/3-CTV and Ad.5-CTV in reducing cell proliferation of PC cells was evaluated in vitro by MTT assays. Ad.5/3-CTV infection resulted in enhanced reduction in the viability of PC-3 cells as compared to Ad.5-CTV infection at m.o. i.'s of 500 and 1,000

VP/cell on day 3-post and day 6-post infection (Fig. 2C). In DU-145 cells both Ad.5/3-CTV and Ad.5-CTV showed parallel efficiencies in reducing growth when assayed using equivalent viral titers and evaluated at parallel time points (Fig. 2D). It should be noted that Ad.5-PEG-EIA and Ad.5/3-PEG-EIA was as effective as Ad.5-CTV and Ad.5/3-CTV in reducing cell viability in both cell lines indicating that the profound effect of Ad replication in inhibiting cell viability might mask the in vitro growth inhibitory effects of *mda-7/IL-24*. In these contexts, in vivo evaluation of CTV is mandatory to confirm the “anti-tumor bystander” effect exerted by MDA-7/IL-24.

Ad.5/3-CTV, but not Ad.5-CTV, induces ER stress and apoptosis, and overcomes therapy resistance in PC-3-*Bcl-2* tumor cells

We next analyzed the expression of *mda-7/IL-24*-downstream genes and signals that confer its tumor suppressor properties upon infection with Ad.5/3-CTV and Ad.5-CTV in low CAR PC-3 cells. Ad.5/3-CTV induces an ER stress response (unfolded-protein response) and we therefore determined the expression levels of ER-stress markers. In PC-3-Neo cells, the levels of BiP/GRP78 and GRP94 were significantly higher upon infection with Ad.5/3-CTV as compared with Ad.5-CTV. Ad.5/3-CTV also efficiently induced apoptosis as evidenced by increased cleavage of PARP (Fig. 3A). It should be noted, that infection with the conditionally replication competent Ad.5/3, Ad.5/3-PEG-EIA, also induced a stress response, as did Ad.5/3-CTV, as indicated by enhanced BiP/GRP78, GRP94 and PARP cleavage (Fig. 3A). However, this effect was not evident with Ad.5-PEG-EIA or Ad.5-CTV infection.

The *Bcl-2* gene family plays a central role in PC and over expression of *Bcl-2* gene family members confers resistance to specific cancer therapeutics (Lebedeva et al., 2003). In this context, we evaluated the efficacy of Ad.5/3-CTV versus Ad.5-CTV in PC-3-*Bcl-2* cells (PC-3 cells that stably over express *Bcl-2*), which are resistant to *mda-7/IL-24*-mediated killing (Lebedeva et al., 2003). As control, we used PC-3-Neo cells that are stably transformed with the same vector expressing only the neomycin-resistance gene and not the *Bcl-2* gene. In MTT assays, Ad.5-PEG-EIA and Ad.5-CTV were less effective in inhibiting cell proliferation (viability) of PC-3-*Bcl-2*, whereas PC3-Neo cells were sensitive to these viruses (Fig. 3B and C). Accordingly, the effect of Ad.5-CTV was more robust in suppressing growth of PC-3-Neo as compared to PC-3-*Bcl-2* cells, whereas the Ad.5/3-CTV displayed equivalent vigorous in vitro anti-proliferative activity in both cell types (Fig. 3B and C). These findings support the enhanced potential therapeutic application of Ad.5/3-CTV versus Ad.5-CTV in PC patients frequently showing *Bcl-2* over expression and down-regulation of CAR.

Ad.5/3-CTV eradicates primary and inhibits distant PC-3-*Bcl-2* xenografts in nude mice

Experiments were performed to determine if the enhanced in vitro activity of Ad.5/3-CTV compared to Ad.5-CTV in low CAR PC-3-*Bcl-2* cells translates into enhanced in vivo activity. PC-3-*Bcl-2* tumor cells were inoculated in both the right and left flanks of athymic nude mice. After ~7–10 days palpable tumor xenografts of ~100 mm³ developed and the mice received eight intratumoral injections only in the left flank tumors over a 4-week period with 1×10^{10} viral particles per 100 μ l. The Ads used for this study included Ad.5-

vec, Ad.5/3-vec, Ad.5-PEG-E1A, Ad.5/3-PEG-E1A, Ad.5-CTV, and Ad.5/3-CTV. No injections were administered to right flank tumors. PC-3-Bcl-2 formed large, aggressive and actively proliferating tumors on both flanks that were not affected by treatment with Ad.5-vec, Ad.5/3-vec, Ad.5-PEG-E1A, or Ad.5-CTV. Although Ad.5/3-PEG-E1A inhibited the growth of tumors on the left flank, it had no effect on the distant tumors on the right flank (Fig. 4A–D). In contrast, Ad.5/3-CTV dramatically inhibited tumor growth on the injected left flank and markedly inhibited tumor growth on the right flank, exceeding the therapeutic effect of any other viral treatment. These results provide definitive evidence for enhanced therapeutic efficacy of Ad.5/3-CTV as compared to Ad.5-CTV in prostate tumor cells with reduced CAR, and highlight the potent “bystander anti-cancer” activity of *mda-7/IL-24* resulting in growth inhibition of right-side, non-injected tumors. The effectiveness of Ad.5/3-CTV and Ad.5-CTV in transducing MDA-7/IL-24 in vivo was confirmed by Western blotting using total protein extracts from the harvested tumors and probing with MDA-7/IL-24 antibody. Ad.5/3-CTV generated more MDA-7/IL-24 protein in both flanks, validating the previously reported “bystander anti-tumor” effect of MDA-7/IL-24 (Su et al., 2001a, 2005a; Chada et al., 2004; Lebedeva et al., 2007a; Sarkar et al., 2007b). In contrast, only weak MDA-7/IL-24 protein expression was evident in Ad.5-CTV injected PC-3-Bcl-2 tumor (left flank) and no protein expression was evident on the right flank tumor (Fig. 4E). These findings demonstrate that in low CAR PC cells, Ad.5/3-CTV can generate robust expression of MDA-7/IL-24 protein that is sufficient to inhibit tumor cell proliferation, and exert “bystander anti-tumor” activity mediated by MDA-7/IL-24 in distant tumors.

Combination treatment of Ad.5/3-CTV and BI-97C1 (Sabutoclax) potentiates inhibition of prostate tumor growth in vivo in immune competent animals

Because PC is a relatively slow-growing disease, repeated systemic gene therapy applications in combination with anti-tumor chemotherapeutic agents over the life span of the patient may be necessary to provide enduring clinical responses (Di Lorenzo and De Placido, 2006; Damber and Aus, 2008). Previous studies demonstrated that BI-97C1 (Sabutoclax), which is a pure optical derivative of Apogossypol (Wei et al., 2010), has significant activity as a single agent against PC cells in vitro and in vivo in nude mouse xenograft studies. Apogossypol derivatives antagonize the anti-apoptotic Bcl-2 family members including Bcl-2 and myeloid cell leukemia-1 (Mcl-1) (Wei et al., 2009). MDA-7/IL-24 induces cancer-specific apoptosis through the translational inhibition of Mcl-1 (Dash et al., 2010c). BI-97C1 (Sabutoclax) sensitizes PC cells to *mda-7/IL-24*-mediated toxicity in vitro and in vivo (Dash et al., 2011a).

Experiments were performed to determine if Ad.5/3-CTV in combination with BI-97C1 could also inhibit prostate tumor growth in vivo. For this analysis we used an immunocompetent transgenic mouse model of PC (the Hi-*Myc* mouse) that spontaneously develops PC. In Hi-*Myc* mice, prostate-specific *c-Myc* gene expression is controlled through the rat probasin promoter with two androgen response elements (ARR2/probasin promoter). Hi-*Myc* mice develop prostatic intraepithelial neoplasia (mPIN) as early as 2–4 weeks of age and invasive adenocarcinoma of the prostate at 6 months (Ellwood-Yen et al., 2003). Treatment was initiated at 22 weeks of age. The ability to deliver adenoviruses systemically is limited by sequestering of the virus in the liver and clearance of the virus by the immune

system (Muruve, 2004; Koizumi et al., 2007; Schenk et al., 2010). To overcome these formidable problems we employed a microbubble-targeted ultrasound destruction (UTMD) approach (Lu et al., 2003; Howard et al., 2006; Greco et al., 2010; Das et al., 2012) in which MBs incorporating adenoviruses are targeted to release therapeutic viruses at the tumor site using US. Using US to sonicate the MBs creates transient non-lethal perforations in cells and other membranes. In this way, systemic and targeted delivery of *mda-7/IL-24* to the prostate of Hi-*Myc* mice was performed by tail vein injection of MBs incorporating Ad.5/3-vec, Ad.5/3-PEG-*EIA*, or Ad.5/3-*CTV* followed by sonoporation in the prostatic area (Dash et al., 2011a). A total of eight tail vein injections of each adenovirus were administered over a 4-week period. BI-97C1 was administered intraperitoneally (i.p.) in each group at 3 mg/kg three times a week throughout the study. The prostates of Hi-myc mice were sectioned and stained for MDA-7/IL-24 and Ki-67, proliferation marker, and apoptosis induction was analyzed by TUNEL assay. MDA-7/IL-24 expression was accompanied by increased TUNEL positive cells and decreased Ki-67 positive cells in the Ad.5/3-*CTV* and BI-97C1-treated group compared to the control groups (Fig. 5). Although, Ad.5/3-*CTV* alone induced significant apoptosis it was markedly augmented when used in combination with BI-97C1.

Discussion

The progression of PC is often slow and different therapeutics may be required during various stages of disease progression and at multiple times during the lifespan of the patient. In the context of gene therapy, it may be necessary to employ different genes, used alone or in combination, and systemic viral delivery approaches over extended time frames (Damber and Aus, 2008; Dash et al., 2011b). PC clinical trials have been performed with conditionally replicating adenoviruses (DeWeese et al., 2001; Small et al., 2006; Fukuhara et al., 2010), resulting in partial clinical responses. CG706 (created by inserting the PSE, a minimal promoter construct of human PSA gene to drive the *EIA*) and CG7870 (rat probasin promoter-driven *EIA* gene and the PSE-driven *EIB* gene) were tested in respective phase I trials, in which patients with local recurrence received an intraprostatic injection of the viruses following primary radiotherapy. Liver function toxicities with CV706 administration were not observed (DeWeese et al., 2001). The responses of patients were dose-dependent and there was a reduction of 50% of PSA. With CG7870 a mild liver inflammation was observed, but approximately 25–49% reduction in PSA was observed (Small et al., 2006). While this conditional replicating viral approach has potential it needs further validation as a frontline therapy for patients with PC, including monitoring changes in PSA and evaluating disease progression over time.

To enhance therapeutic efficacy of Ad gene therapy for PC, we have presently used a number of strategies. We earlier constructed a bipartite Ad.5 where viral replication is controlled by the minimal active region of the promoter of the *PEG-3* gene (Su et al., 1997, 2005b), restricting viral replication to cancer cells with limited activity in normal cells, and *mda-7/IL-24* is driven by a CMV promoter from the E3 region of Ad.5 (Sarkar et al., 2005a, 2006, 2007b, 2008). These viruses, termed *CTVs* (reviewed in Das et al., 2012), have displayed profound activity in athymic nude mouse human xenograft models, including breast carcinomas, PC (including therapy-resistant PCs over expressing *Bcl-2* or *Bcl-X_L*) and metastatic melanoma (Sarkar et al., 2005a, 2006, 2007b, 2008; Greco et al., 2010).

To enhance viral entry into CAR deficient cancer cells, we engineered chimeric adenoviruses containing the Ad.3 sequence within the Ad.5 virus knob (Ad.5/3) (Dash et al., 2010b; Azab et al., 2012), which redirects binding of the vector to the Ad.3 receptor, desmoglein 2 (Wang et al., 2011). In this study, we document that Ad.5/3-CTV eradicates, thereby further verifying the anti-cancer potency of this therapeutic virus not only in primary-injected tumors but also distant non-injected tumors derived from a resistant PC cell line in a nude mouse xenograft model. As discussed, Ad.5/3-CTV is capable of infecting cancer cells regardless of their cell surface CAR status, which makes it potentially more efficacious than Ad.5-CTV that fails to efficiently infect and deliver the therapeutic genes in low CAR PC cells. The Ad.5/3 chimeras are directed to CD46 and CD80/CD86 receptors that are highly expressed on tumor cells significantly improving anti-tumor efficacy (Sirena et al., 2004; Ulasov et al., 2007). Tropism modified Ad.5/3 conditional replication has also been used to express GMCSF and this approach was also found to be safe and efficacious in treating cancer patients (Koski et al., 2010). Additionally, it has been reported that Ad.5/3 serotype chimerism could also be a viable strategy for circumventing the generation of neutralizing antibodies in patients receiving multiple rounds of conditionally replicating viruses (Raki et al., 2011).

Systematic delivery of Ads is challenging because of sequestration of viruses in the liver restricting efficient delivery to disseminated tumors (Koizumi et al., 2007) and neutralization of viruses by the immune system (Curiel and Fisher, 2012). To prevent trapping of CTV in the liver and elimination of viruses by the immune system we have developed an innovative approach that involves the use of perfluorocarbon MBs and US (Greco et al., 2010; Dash et al., 2011a). This approach is called ultrasound-targeted microbubble destruction (UTMD). We have applied the UTMD approach using a tropism-modified Ad.5/3-CTV in Hi-*Myc* transgenic mice, which develop PC (Ellwood-Yen et al., 2003; Dash et al., 2011a). Ad.5/3-CTV in complement-treated MBs were administered systemically through the tail vein of mice and released in the prostate area through US in this syngeneic immunocompetent PC mouse model (Hi-*Myc* mouse) using UTMD (Dash et al., 2011a). Hi-*Myc* mice develop, with high penetrance, prostatic intraepithelial neoplasia (PIN) that advances over time to invasive adenocarcinomas in all lobes of the prostate gland (Ellwood-Yen et al., 2003). In the present study, we show the combinatorial anti-cancer effect of Ad.5/3-CTV and the novel Mcl-1 antagonist, BI-97C1 (Sabutoclax), which significantly inhibits PC in Hi-*Myc* transgenic mice. For combination studies we chose an Mcl-1 antagonist based on our previous observations where we demonstrated that suppression of the pro-survival Bcl-2 family member, Mcl-1, is required for *mda-7/IL-24*-mediated apoptosis of prostate carcinomas (Dash et al., 2010c, 2011a). Here, we demonstrate that pharmacological inhibition of Mcl-1 expression with the novel Apogossypol derivative BI-97C1 is sufficient to sensitize prostate tumors to *mda-7/IL-24*-induced (Ad.5/3-CTV) apoptosis.

In summary, if specific technical impediments can be overcome, gene therapy for PC holds substantial promise in treating this disease (Curiel and Fisher, 2012; Das et al., 2012). (1) The prostate gland is dispensable and not vital for survival, and it is accessible by US. (2) Ad.5/3-CTV can be either directly injected into the primary tumor or delivered through

UTMD with complement-treated MBs incorporating Ads thereby resulting in cancer cell lysis and expression of MDA-7/IL-24. (3) The replication of this virus and the expression of the therapeutic genes can be directly targeted in cancer cells without non-specific expression in normal cells by using the cancer-specific *PEG-3* promoter. (4) Disease progression can be monitored (Cookson, 2001; Gopalkrishnan et al., 2001). In these contexts, the use of Ad.5/3-*CTV* to administer the therapeutic and cancer-specific cytotoxic MDA-7/IL-24 protein to selectively induce cytolysis and apoptosis in prostate tumor cells may represent a potentially viable treatment option (Anderson, 1998; Sarkar et al., 2007a; Das et al., 2012).

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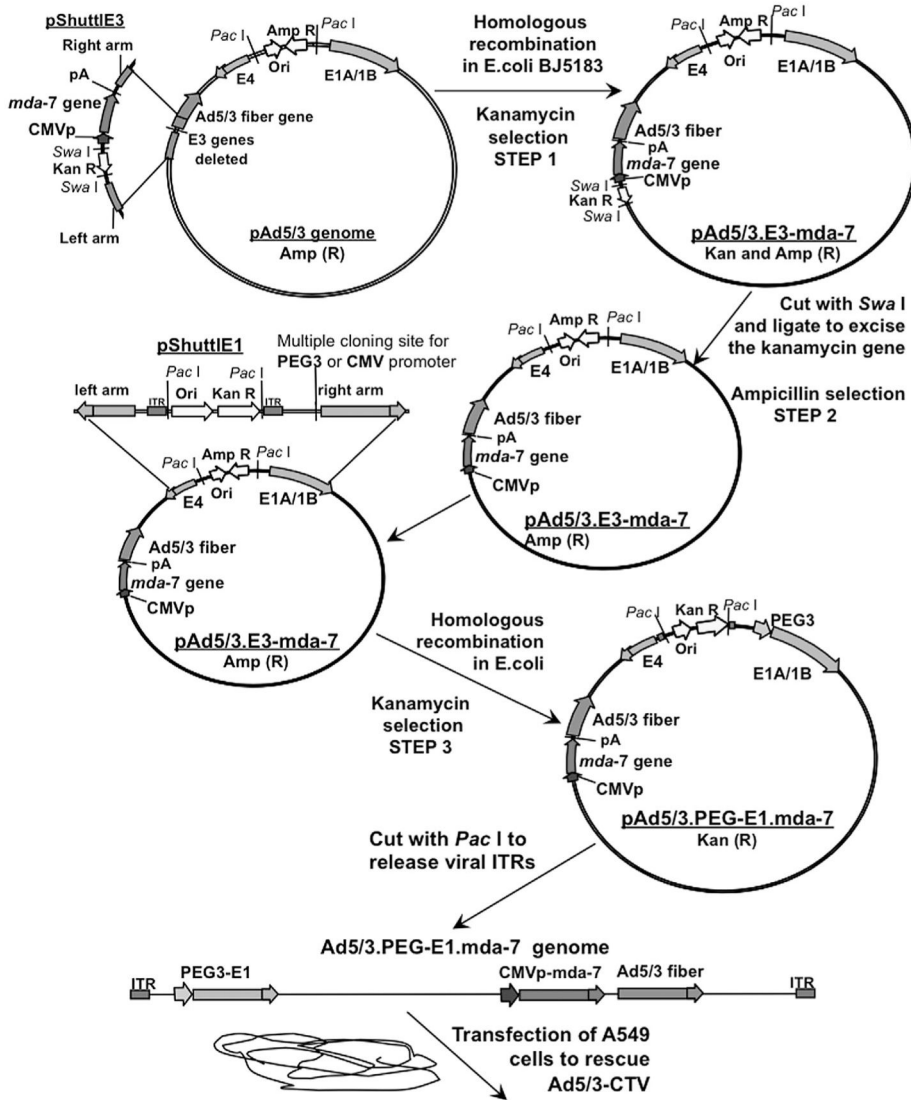


Fig. 1. Generation of a tropism-modified cancer terminator virus (Ad.5/3-CTV). Schematic representation outlining the construction of a tropism-modified cancer terminator virus for delivery of *mda-7*/IL-24. The detailed procedures are described in Materials and Methods Section.

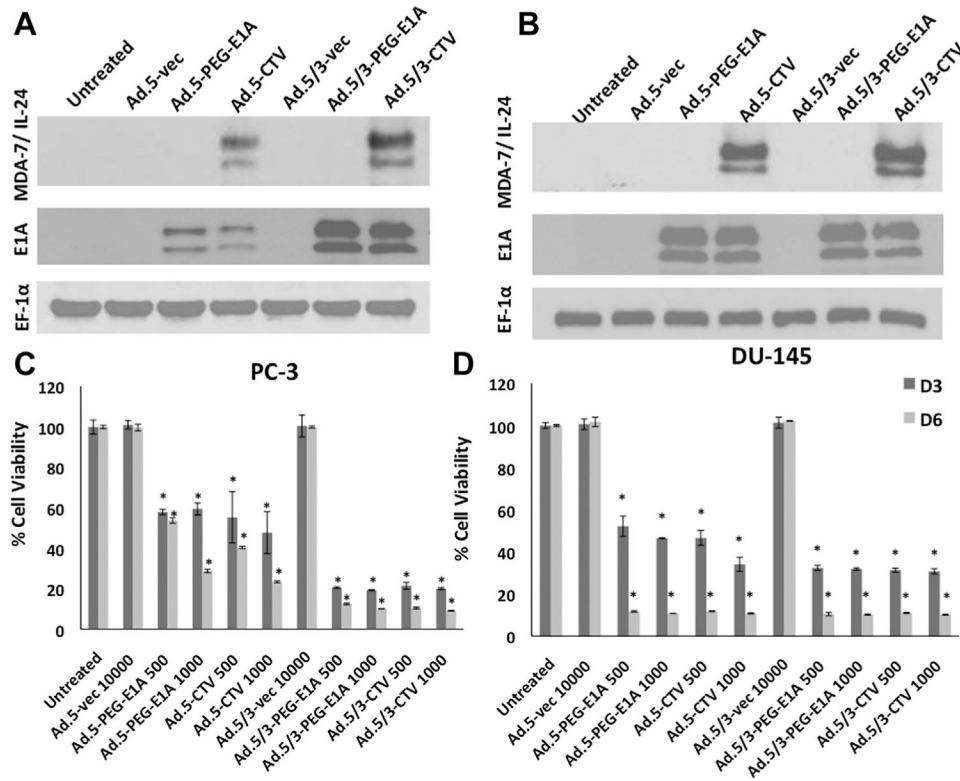


Fig. 2. Ad.5/3-CTV enhances *mda-7/IL-24* expression and inhibition of cell viability in low CAR prostate cancer cells. A and B: DU-145 and PC-3 cells were infected with the indicated vp/cell of Ad.5-vec, Ad.5-PEG-E1A, Ad.5-CTV, Ad.5/3-vec, Ad.5/3-PEG-E1A, and Ad.5/3-CTV for 48 h and total proteins were isolated. The expression of MDA-7/IL-24 (23.8 kDa protein, with 35–40 kDa glycosylated species detected on the gel), E1A and EF-1α (as a loading control) proteins were analyzed by Western blot analyses. C and D: Cell viability using the MTT assay was quantified after 3 and 6 days with the indicated doses of vp/cell of Ad.5-CTV, Ad.5/3-CTV and their respective controls. Results are the mean \pm SD (n = 3) * P < 0.05.

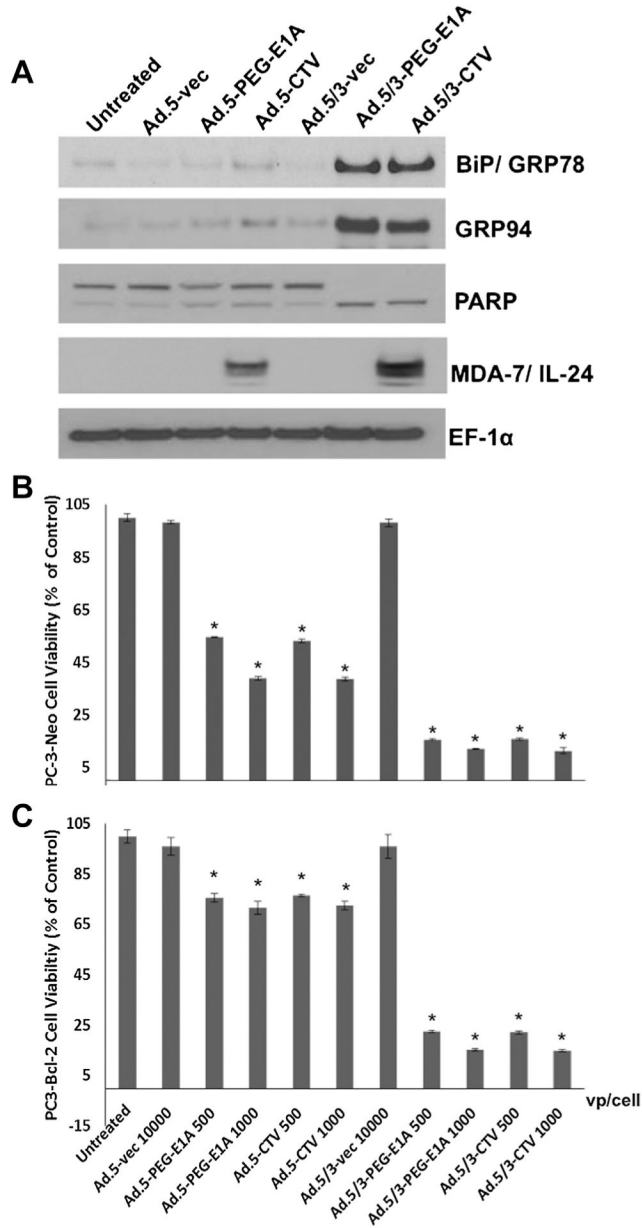


Fig. 3. Ad.5/3-CTV, but not Ad.5-CTV, induce ER stress and apoptosis, and overcome therapy resistance in PC-3-Bcl-2 tumor cells. A: Changes in BiP/GRP78, GRP94, and activation of PARP were detected by Western blot analysis after 2 days of treatment of PC-3 cells with the indicated Ads. B + C: Cell viability using the MTT assay was quantified after 6 days with the indicated doses of vp/cell of Ad.5-CTV, Ad.5/3-CTV, and their respective controls. Results are the mean \pm SD (n = 3). * $P < 0.05$ with the Ad.5-vec 10,000 vp/cell-infected group.

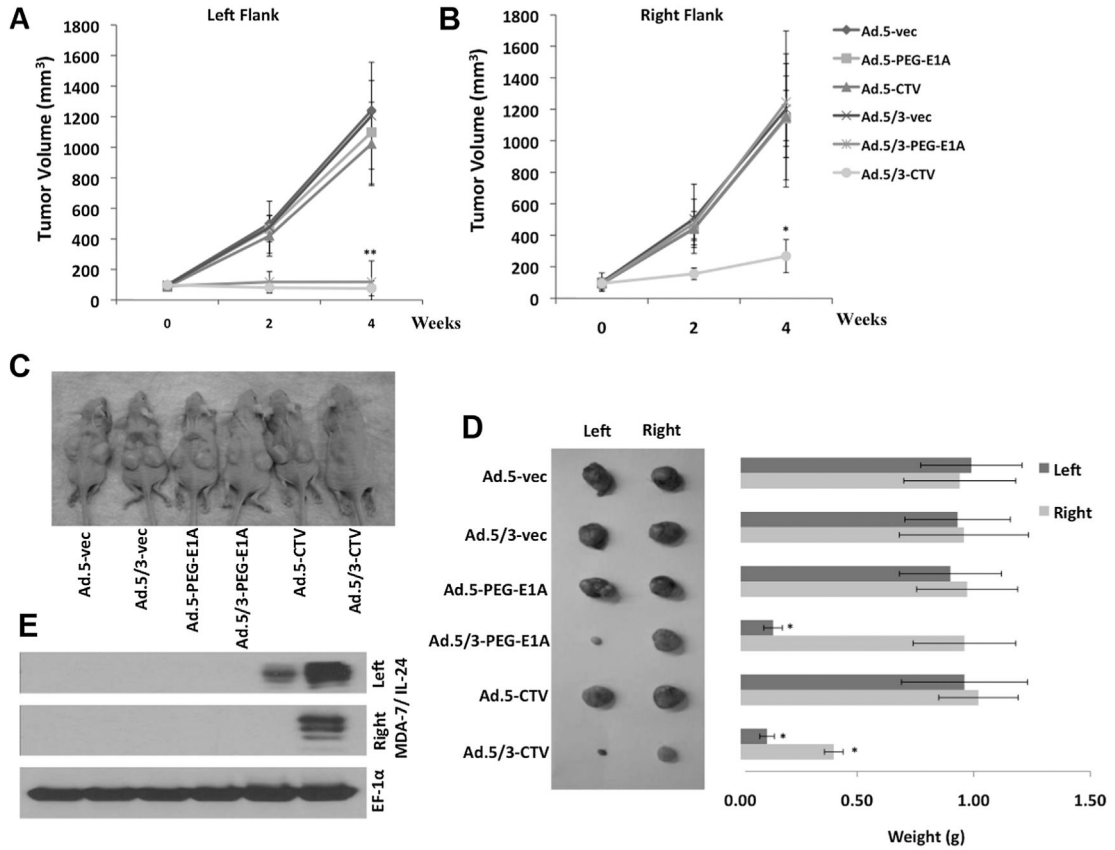


Fig. 4. Ad.5/3-CTV eradicates primary and inhibits distant PC-3-Bcl-2 xenografts in nude mice. Tumor xenografts with PC-3-Bcl-2 cells were established in athymic nude mice in both right and left flanks; and only tumors on the left side were injected with the indicated Ads over a 4-week period (total of nine injections). Measurements of PC-3-Bcl-2 xenograft tumor volumes on (A) left and (B) right flanks; points, average (with a minimum of five mice in each group); bars \pm SD. Inset contains a photograph of the animals of each representative group. C: Photograph of the PC-3-Bcl-2 xenograft tumor at the end of the study. D: Measurement of tumor weight at the end of the study; columns, mean (with at least five mice in each group); bars \pm SD. E: Western blot analysis of protein extracts from representative PC-3-Bcl-2 tumor samples treated with Ad.5-vec, Ad.5/3-vec, Ad.5-PEG-E1A, Ad.5/3-PEG-E1A, Ad.5-CTV, Ad.5/3-CTV. The immunoblot was reacted with anti-MDA-7/IL-24.

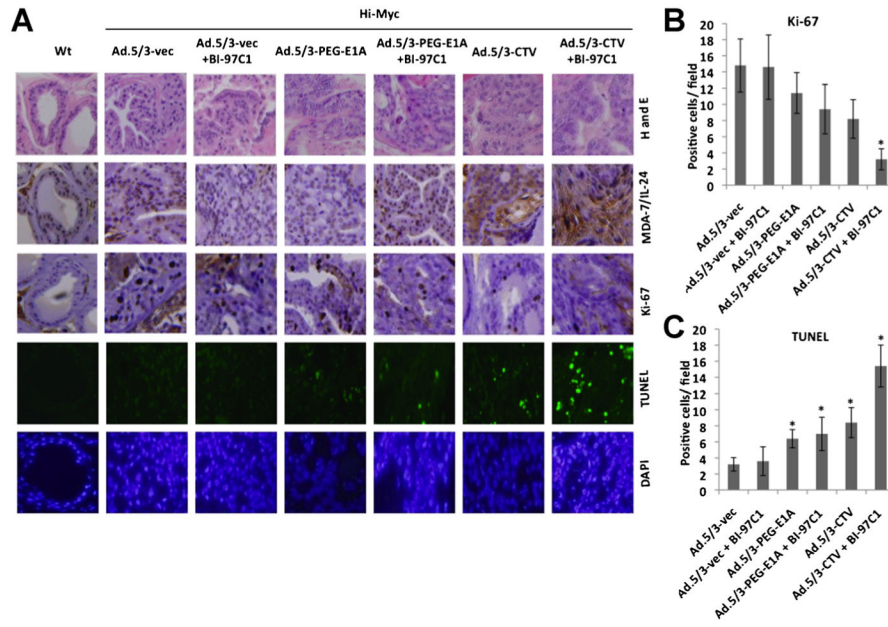


Fig. 5. Combination treatment of Ad.5/3-CTV and BI-97C1 (Sabutoclax) potentiates inhibition of prostate tumor growth in vivo in immune competent animals. A: The prostatic region of 22-week-old male Hi-*Myc* mice were sonoporated for 10 min following tail-vein injection of the indicated complement-treated MB/Ad complexes and treated as described in the Materials and Methods Section for 4 weeks (total eight injections of indicated viruses). BI-97C1 (Sabutoclax) was administered intraperitoneally (i.p.) in each group at 3 mg/kg three times a week throughout the study. At the end of the experiment, the mice were sacrificed and the prostates were collected. The paraffin-embedded sections were obtained from the prostate and immunohistochemistry was performed to measure systemic transgene delivery by staining with anti-MDA-7/IL-24, TUNEL assay and Ki-67 staining detected apoptosis and cell proliferation in the prostate sections. Nuclei were visualized with DAPI. Wild type mice of the same strain that do not develop prostate cancer served as a control for these experiments. (B) Quantification of microvessel density in prostate section per field followed by Ki-67 staining and (C) Quantification of TUNEL positive signals in the prostate section (**P* < 0.05 between the indicated groups). Data represent mean ± SD (n = 3).