A Novel Targeting Modality to Enhance Adenoviral Replication by Vitamin D₃ in Androgen-independent Human Prostate Cancer Cells and Tumors

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ABSTRACT

We report the development of a novel replication-competent adenoviral vector, Ad-hOC-E1, containing a single bidirectional human osteocalcin (hOC) promoter to drive both the early viral E1A and E1B gene. This vector selectively replicated in OC-expressing but not non-OC-expressing cells, with viral replication enhanced at least 10-fold on vitamin D₃ exposure. Both the artificial TATA-box and hOC promoter element in this bidirectional promoter construct were controlled by a common OC regulatory element which selectively activated OC expression in cells. The expression of E1A and E1B gene by Ad-hOC-E1 can be markedly induced by vitamin D₃. Unlike Ad-sPSA-E1, an adenoviral vector with viral replication controlled by a strong super prostate-specific antigen (sPSA) promoter which only replicates in PSA-expressing cells with androgen receptor (AR), Ad-hOC-E1 retarded the growth of both androgen-dependent and androgen-independent prostate cancer cells irrespective of their basal level of AR and PSA expression. A single i.v. administration of 2 × 10⁹ plaque-forming units of Ad-hOC-E1 inhibited the growth of previously established s.c. DU145 tumors (an AR- and PSA-negative cell line). Viral replication is highly enhanced by i.p. administration of vitamin D₃. Ultimately, enhancing Ad-hOC-E1 viral replication by vitamin D₃ may be used clinically to treat localized and osseous metastatic prostate cancer in men.

INTRODUCTION

One of the biggest challenges facing gene therapy is the development of site-specific vectors for therapeutic genes. Two main approaches using Ad¹-mediated cancer gene therapy have been attempted. The first is targeting tumor cells with genetically modified Ad through either direct modification of viral fiber protein (1–4) or through conjugation with bispecific single-chain antibodies (5, 6) that interact with specific cell surface receptors. The second approach is selective targeting with tumor- and tissue-specific promoters driving the expression of therapeutic genes or viral replication in a tumor cell-specific manner (7–9). In replication-competent Ad vectors, the tissue or tumor-specific promoter-enhancer has exclusively been inserted proximal to the E1A promoter-enhancer region (10–12) with the rationale that expression of E1A and, therefore, the whole Ad transcription program will depend on these tissue- or tumor-specific promoters. However, leakage of foreign promoters in E1A control, yielding low levels of E1A, may result in loss of specificity (13). To control the viral replication more stringently, separate promoter control of both the E1A and E1B genes significantly improves specificity (14, 15). However, with promoter interference and homologous recombination between closely juxtaposed promoters within the Ad vectors, modulation or loss of promoter activity and tissue specificity may result (16). Therefore, we devised an alternate strategy to retain a high level of specificity for target cells by controlling expression of E1A and E1B genes with a single bidirectional promoter fused with a reversed artificial TATA-box upstream of the enhancer/promoter region.

In transgenic mice carrying the hOC promoter-chloramphenicol acetyltransferase fusion gene, OC expression was restricted to bone-associated tissues and the brain (17). Our laboratory also demonstrated that OC expression is detectable in both primary and bone metastatic prostate tumor specimens (18). We previously showed that mouse OC-mediated hsv-TK (OC-TK), plus the prodrug ganciclovir (GCV) or acyclovir (ACV), efficiently blocks the growth of localized prostate tumors and their skeletal xenografts (19). We recently showed that a single i.v. administered dose of Ad-OC-E1a markedly inhibited previously established prostate tumor grown in the skeleton (18). A Phase I OC dose escalation trial has demonstrated the safety of intratumoral delivery of Ad-OC-TK followed by an oral ACV analogue, valacyclovir (20). To improve on mouse OC promoter, we developed a human version, hOC, which contains a VDRE. Its activity can be induced by vitamin D (21). We now demonstrate that a hOC-E1 bidirectional E1A/E1B expression cassette (Ad-hOC-E1) can be effectively up-regulated by vitamin D₃. Concomitant Ad-hOC-E1 and vitamin D₃ treatment showed a highly specific and effective kill of AI and metastatic prostate cancer cells in vitro and markedly reduced the growth of both AR- and PSA-negative DU145 tumor xenografts in nude mice by a single systemic administration of Ad-hOC-E1.

MATERIALS AND METHODS

RT-PCR Analysis. Cells were treated with 5 nM vitamin D₃ analogue (Ro 25-9022; Roche, Nutley, NJ) or ethanol as the control group for 48 h. RNA was extracted using RNAzolB (Teltest, Friendswood, TX) and RT-PCR was performed according to the manufacturer’s protocol with Moloney Murine Leukemia Virus reverse transcriptase (Life Technologies Inc., Rockville, MD). The primer sequences for hOC are 5'-ACACTCTCGCCCTATTG-3' (forward) and 5'-GATGTGGTCAGCCAACTC-3' (reverse); for PSA 5'-GTAGACTCCAGCCACGAC-3' (forward) and 5'-CACAGACACCCCATCATA-3' (reverse); for VDR 5'-AGGAAATCTTTATGAC-3' (forward) and 5'-CAGAGCATGCATGCAC-3' (reverse); for AR 5'-CCCTAGTTGATCCCTACT-3' (forward) and 5'-GCACAGCTTTGATCCAAC-3' (reverse). For GAPDH the primer sequences are 5'-ACACAGCTCAGCACATTACA-3' (forward) and 5'-TCCACACTTGCTGCT-3' (reverse).

Plasmid and Virus Construction. A 3.9-kb hOC promoter was cloned from genomic DNA of DU145, using Genome Walker kits (Clontech, Palo Alto, CA). The 2.3-kb E1B gene was amplified from the Ad5 genome and fused with the E1A region. The hOC promoter was amplified from the human osteocalcin gene by PCR, using genomic DNA of DU145 as template. The amplification products were subcloned into the pBluescriptII vector. The bidirectional promoters were amplified from the plasmids using primers with unique restriction sites and subcloned into the pBluescriptII vector. The plasmids were linearized with EcoRI and transcribed in vitro with SP6 RNA polymerase. The transcription products were transfected into 293 cells using calcium phosphate precipitation. The high-titer recombinant adenovirus was generated using the packaging cell line 293Ad. The virus titer was determined by plaque assay.

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3 The abbreviations used are: Ad, adenoviral vector; hOC, human osteocalcin; PSA, prostate-specific antigen; sPSA, super PSA; VDR, vitamin D receptor; AR, androgen receptor; VDR, vitamin D receptor; AD, androgen dependent; AI, androgen independent; MIQ, multiplicity/multiplicity of infection; FACs, fluorescence-activated cell sorting/sorter; p.i., postinfection; pfu, plaque-forming unit(s); RT-PCR, reverse transcription-PCR; CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAR, coxackie and adenovirus receptor.
and GAPDH expression by RT-PCR was used as an internal standard of RNA loading in each sample.

Northern Blot Analysis. Cells were infected with 10 MOI of Ad-hOC-E1 for 2 h and then cultured in 5 nM vitamin D_{3} analogue or ethanol for 48 h. Cells were lysed in triple-detergent lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% NaN_{3}, 1% SDS, 1% NP40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and 1 X protease inhibitor cocktail (Roche, Nutley, NJ)]. Fifty μg of protein were used for immunoblotting using the NOVEX (Invitrogen, Carlsbad, CA) system. Membrane was probed with a 1:200 dilution of adenovirus-2 E1A antibody (13 S-5), followed by a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody against rabbit IgG. Both antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). ECL plus system (Amersham Pharmacia Biotech) was used to detect the signal. Cell lysate from 293 cells was used as quantitative reference in each blot and the intensity of bands was measured by Quantity one-4.1.1 Gel Doc gel documentation software (Bio-Rad).

Southern Blot Analysis. Cells were infected with 10 MOI of Ad-hOC-E1 or Ad-CMV-pA. After 2 h of absorption, cells were washed with PBS twice and then cultured in fresh medium containing 5 nM vitamin D_{3} analogue or ethanol for 48 h. Cells were lysed in triple-detergent lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% NaN_{3}, 1% SDS, 1% NP40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and 1 X protease inhibitor cocktail (Roche, Nutley, NJ)]. Fifty μg of protein were used for immunoblotting using the NOVEX (Invitrogen, Carlsbad, CA) system. Membrane was probed with a 1:200 dilution of adenovirus-2 E1A antibody (13 S-5), followed by a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody against rabbit IgG. Both antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). ECL plus system (Amersham Pharmacia Biotech) was used to detect the signal. Cell lysate from 293 cells was used as quantitative reference in each blot and the intensity of bands was measured by Quantity one-4.1.1 Gel Doc gel documentation software (Bio-Rad).

Indirect Immunofluorescence Analysis of CAR Antigen. One × 10^{6} cells of each cell line were washed extensively with cold PBS, incubated with 1 μg/ml mouse anti-CAR antibody (RmcB, provided by Dr. Jer-Tsong Hsieh, University of Texas Southwestern, Dallas, TX) or a 1:50 diluted isotope control antibody in 100 μl of FACS buffer (0.1% BSA and 1% sodium azide in PBS) at 4°C for 1 h. After incubation, cells were washed with cold FACS buffer twice. Cells were incubated with FITC-conjugated goat antimouse IgG (Jackson ImmunoResearch Laboratories Inc.) in a 1:50 dilution at 4°C for 1 h and then analyzed by flow cytometry (FACS Calibur,
ENHANCED ADENOVIRUS REPLICATION BY VITAMIN D₃

Vitamin D₃ Up-Regulates hOC mRNA Expression in AI Prostate Cancer Cell Lines. RT-PCR was performed to compare the expression profiles of OC, PSA, and AR mRNA among several human cancer cell lines. Fig. 1 shows the basal level of OC mRNA expression, based on RT-PCR, can be detected clearly in human osteosarcoma MG-63, and AI metastatic prostate cancer cell lines, PC3, PC3M, and DU145. A very low level of OC transcripts was detected in LNCaP, an AD line, and its derivative AI and metastatic subline C4-2. PSA mRNA can only be detected in AR-expressing prostate cancer cells, such as LNCaP and C4-2, but not in the AR-expressing osteoblastic cell line MG-63 or other AR-negative but OC-expressing prostate cancer cell lines. In addition, OC mRNA expression can be up-regulated markedly by a vitamin D₃ analogue in all metastatic prostate cancer cell lines including C4-2 cells, which only express a trace basal level of hOC. In renal cancer RCC 52 cells, the basal level of OC mRNA was undetectable. However, vitamin D₃ can also activate OC promoter by an unknown mechanism (Fig. 1). Cell lines in which OC can be induced by vitamin D₃, all express VDR, which suggests that the VDR complex with VDRE in the proximal region of hOC promoter must be responsible for up-regulating OC transcriptivity in prostate and bone cancer cell lines. We ob-

Fig. 3. Basal and vitamin D₃-induced E1A and E1B mRNA transcription by Ad-hOC-E1. AI human prostate cancer cell lines C4-2, PC3, and DU145, and human renal cell carcinoma cell line RCC52, infected with 10 pfu/cell Ad-hOC-E1 or Ad-CMV-pA, were cultured in the presence or absence of 5 nM vitamin D₃. RNA was extracted at 48 h.p.i. E1A and E1B mRNA were detected by Northern blot and probed with 32P-labeled E1A and E1B DNA probes, respectively. 28S RNase was used as an internal control of RNA loading of each sample.

<image>

Fig. 4. Basal and vitamin D₃-induced E1A protein expression by Ad-hOC-E1. AI human prostate cancer cell lines C4-2, PC3, and DU145, and human renal cell carcinoma cell line RCC52, infected with 10 pfu/cell of Ad-hOC-E1 or Ad-CMV-pA, were cultured in the presence or absence of 5 nM vitamin D₃. Proteins were prepared 48 h.p.i. E1A protein was detected by Western blot and probed with a polyclonal antibody to Ad2 E1A protein. Cell lysate from 293 cells was used as a positive control of E1 protein expression. KDs, molecular weight (Mᵣ) in thousands.
served that vitamin D$_3$ also slightly enhanced PSA mRNA expression in LNCaP and C4-2 cells by an unknown mechanism (31, 32). These results show that the hOC promoter has a broader spectrum of activity than the PSA promoter in AD and AI prostate cancers, and could be highly inducible by vitamin D$_3$. The results suggest that therapeutic genes driven by the hOC promoter can target primary and metastatic prostate cancers.

E1A and E1B genes Are Expressed in OC-expressing Cells by Ad-hOC-E1. To control both E1A and E1B genes with a single promoter, we generated bidirectional hOC and strong sPSA promoters by inserting an artificial TATA box lined in the opposite direction to hOC or sPSA enhancer/promoter. The E1A CDNA was cloned downstream of an artificial TATA box promoter, and E1B cDNA was cloned downstream of the hOC or sPSA enhancer/promoter region. Replication-competent Ad-hOC-E1 and Ad-sPSA-E1 vectors were constructed by inserting these bidirectional E1A/E1B expression cassettes at the deleted E1 region of the sPSA-E1 vectors. In parallel, replication-defective Ad-hOC-E1 DNA was detected only as trace in C4-2, PC3, and DU145, and a non-OC-expressing cell line, RCC52. After vitamin D$_3$ induction, however, these transcripts were enhanced 10- to 50-fold above the basal level of Ad-hOC-E1 DNA replication. The induction of viral replication in C4-2, PC3, and DU145 correlated with hOC mRNA expression (Fig. 1). In RCC52

![Image](image_url)

**Fig. 5.** Basal and vitamin D$_3$-induced Ad-hOC-E1 replication. A, cells infected with 10 pfu/cell Ad-hOC-E1 were cultured in the presence or absence of 5 nm vitamin D$_3$. Viral DNA was isolated at the indicated hours p.i. DNA from an equal number of cells was digested with HinIII and subjected to Southern blot analysis. The HinIII-digested DNA fragments of Ad-w.t. were labeled with $[^{32}P]CTP$ and used as a probe to detect all viral HinIII fragments. B, a serial diluted Ad-hOC-E1 DNA was digested with HinIII and used as a positive control of Southern blot.

![Image](image_url)

**Fig. 6.** Immunofluorescence FACS analysis of CAR expression. The indicated cells were plated and infected as described in "Materials and Methods." The titer of culture supernatants was determined by plaque assay. Data represent the mean of three experiments. Titers were normalized to $1 \times 10^8$ pfu/ml in 293 cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ad-CMV-pA</th>
<th>Ad-hOC-E1</th>
<th>Ad-hOC-E1 $+$ vitamin D$_3$</th>
<th>Ad-w.t. (d5809)</th>
</tr>
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<tbody>
<tr>
<td>C4-2</td>
<td>$1 \times 10^8$</td>
<td>$1 \times 10^8$</td>
<td>$1 \times 10^8$</td>
<td>$1 \times 10^8$</td>
</tr>
<tr>
<td>PC3</td>
<td>$3 \times 10^3$</td>
<td>$1 \times 10^7$</td>
<td>$1 \times 10^7$</td>
<td>$6 \times 10^7$</td>
</tr>
<tr>
<td>DU145</td>
<td>$3 \times 10^3$</td>
<td>$1 \times 10^7$</td>
<td>$5 \times 10^7$</td>
<td>$1 \times 10^7$</td>
</tr>
<tr>
<td>RCC52</td>
<td>$3 \times 10^3$</td>
<td>$6 \times 10^7$</td>
<td>$4 \times 10^7$</td>
<td></td>
</tr>
</tbody>
</table>

*nd, not detectable.*
cells, Ad-hOC DNA was strongly detected at 48 h p.i. with vitamin D₃ induction but was barely detectable without induction. This result is consistent with the expression of hOC mRNA (Fig. 1). Because RCC52 cells expressed a high level of CAR on the cell surface (Fig. 6), obviously the failed Ad-hOC-E1 replication in non-hOC-expressing cells is attributable to the stringent specificity of hOC promoter and is not related to the efficiency of viral entry.

To further assess whether the amplified viral DNA can be

Fig. 7. Vitamin D₃-induced cytotoxicity by replication-competent Ad vectors. OC-positive C4-2, PC-3, and DU145 cells were infected with (A) Ad-hOC-E1, (B) Ad-hOC-E1, or Ad-sPSA-E1 at the indicated dose, and OC-negative RCC52 cells were infected with (C) indicated Ad vectors. Cells were subsequently cultured in the presence (A, B) or absence (A, C) of 5 μM vitamin D₃, and the cytotoxicity assay was performed using crystal violet staining at the indicated day after infection. The relative cell number was assessed by absorbance at 590 nm after staining; versus mock-infected group: *, P < 0.05; †, P < 0.005.
packed to form the infectious particle, we harvested culture me-

dium and performed a plaque assay. The differential titer of Ad

evectors in various human cell lines is shown in Table 1 and
demonstrates that Ad-hOC-E1 grew well in OC-expressing pros-
tate cancer cell lines, such as C4-2, PC3, and DU145, and that
vitamin D3 can induce a 5- to 25-fold increase in viral replication.
This induced viral titer is equal to Ad-w.t. in PC3. However,
Ad-OC-E1 cannot grow in non-OC-expressing RCC52 cells. The
titer of Ad-hOC-E1 in these cells is as low as that of Ad-CMV-pA,
a replication-defective Ad vector.

Replication-competent Ad Vectors Induce AI Prostate Cancer Cell Death. To test whether replication-competent Ad vectors can
grow and lyse prostate cancer cells, an in vitro cytotoxicity assay
comparing Ad-hOC-E1 and Ad-sPSA-E1 was performed. As con-
trols, Ad-w.t. (positive control) and Ad-CMV-pA (negative con-
tral) either effectively lysed or were completely ineffective in all
of the tested cell lines (data not shown). Fig. 7A shows that in
response to Ad-hOC-E1, marked cell lysis was observed in C4-2
cells (AR- and PSA-positive) at a dose level of 1 MOI (P < 0.05
versus mock-infected group) at 7 days posttreatment and day 5 by
vitamin D3 induction. The 10-fold-enhanced cell kill of Ad-
hOC-E1 by vitamin D3 also occurred in PC3 and DU145 cells (Fig.
7A), whereas there was no vitamin D-induced kinetic change of
cytotoxicity observed in Ad-sPSA-E1, Ad-CMV-pA, and Ad-w.t.-
treated groups in any of the tested cell lines (data not shown). With
vitamin D3, treatment (Fig. 7B), in C4-2 cells, Ad-sPSA-E1 showed
higher cell killing activity than did Ad-hOC-E1. These data cor-
relate with the endogenous PSA and OC promoter activity shown
in Fig. 1. In contrast, however, when Ad-hOC-E1 or Ad-sPSA-E1
was added to PC-3 cells (AR- and PSA-negative), a differential
inhibition of cell growth by Ad-hOC-E1 but not by Ad-sPSA-E1
was observed. Because of the lower level of CAR associated with
PC-3 cells (Fig. 6), a higher dose of Ad vector (e.g., 1–5 MOI)
was necessary to lyse these cells in vitro. This is supported by DU145
experimental data (AR- and PSA-negative cells), which have a
higher level of CAR (Fig. 6) and were inhibited by Ad-w.t. and
Ad-hOC-E1 at a dose of 0.1–1.0 MOI 5–7 days after viral expo-
sure. Ad-sPSA-E1 and Ad-CMV-pA were ineffective against the
growth of DU145 cells in vitro. As expected, the growth of RCC52
cells (completely deficient in OC expression) was sensitive only to
Ad-w.t. and completely insensitive to growth inhibition by Ad-
hOC-E1, Ad-sPSA-E1, or Ad-CMV-pA (Fig. 7C).
Ad-hOC-E1 Combined with Vitamin D3 Is Highly Effective against the Growth of DU145 Tumors in Vivo. To determine the therapeutic efficacy of Ad-hOC-E1 in AI prostate cancer in vivo, we evaluated the therapeutic effect of Ad-hOC-E1 in a DU145 xenograft model in nude mice. DU145 xenograft was shown to be a very aggressive tumor that grew to 40-fold of its initial volume at 5 weeks (Fig. 8A). A single tail vein injection of replication-defective Ad-CMV-pA barely inhibited tumor growth, but the identical protocol of Ad-hOC-E1 administration suppressed tumor growth significantly ($P < 0.05$). Similarly, vitamin D3 administration alone also inhibited DU145 tumor growth ($P < 0.05$) in vivo. The growth of DU145 tumors was markedly repressed when animals were treated with Ad-hOC-E1 plus vitamin D3 ($P < 0.005$). In controls, Ad-CMV-pA plus vitamin D3 did not further enhance tumor volume reduction when compared with vitamin D treatment alone. These results demonstrated that Ad-hOC-E1 and vitamin D3 combination therapy achieved additive antitumor efficacy ($P < 0.05$ versus Ad-hOC-E1 alone or vitamin D3 alone).

To assess viral distribution after a single i.v. Ad-hOC-E1 administration, we detected by PCR analysis the Ad viral DNA sequences in the prostate, liver, lung, brain, and tumor tissues. Liver and lung were the major organs trafficking viruses. Only a few viruses were found at the s.c. tumor site at week 1 (Fig. 8B). Viral DNA accumulated significantly thereafter and markedly increased in weeks 3 and 5. Vitamin D3 administration enhanced viral replication/accumulation consistently in tumor tissues, but not liver, during the entire course of the treatment period (week 1 to 5, see Fig. 8C). Toxicology studies with Ad vectors were hampered because human adenoviruses replicate only in human cells. Immunohistochemistry data are consistent with the characteristics of Ad type 5 virus in which the E1A viral protein was expressed only in human tumor tissue but not in mouse liver (Fig. 8D, E1A), although a steady accumulation of Ad-DNA was observed in mouse liver over 5 weeks (Fig. 8C). Tumor xenografts maintained in mice treated with Ad-hOC-E1 plus vitamin D3 together underwent a strong necrotic reaction within the tumor region without affecting the normal hepatocellular architecture (Fig. 8D, H&E). These results provide preclinical evidence of the specificity, efficacy, and safety of Ad-hOC-E1 and vitamin D3 for prostate cancer gene therapy.

DISCUSSION

The progression of prostate cancer to the AI bone metastatic state is lethal. Patients with hormone-refractory bone-metastatic prostate cancer survive 9 months or less (34). There is no effective therapy broadly targeting tumor cells with variable levels of AR and PSA. Our objective is an inducible gene therapy approach using replication-competent Ad vectors targeting the growth of human prostate cancer cells whether they express AR and PSA or not. Ad-hOC-E1 has advantages over Ad-sPSA-E1: (a) unlike As-sPSA-E1, Ad-hOC-E1 has a broad spectrum of cytotoxicity against the growth of human prostate cancer tumor cells in vitro and tumor xenografts in vivo irrespective of their basal AR and PSA status; and (b) the replication of Ad-hOC-E1 but not Ad-sPSA-E1 can be induced by vitamin D, with enhanced cytotoxicity against tumor cells in vitro and tumor xenografts in vivo.

One approach to creating conditionally replication-competent Ad vectors is to use tissue-specific promoters such as PSA to regulate the early viral gene, E1. CN706 has been engineered to include other tissue-specific promoters in tandem, such as human glandular kallikrein (hK2) or probasin promoter to selectively control transcription and translation of early viral $E1A$ and $E1B$ genes (14, 15) with efficient oncolytic action in cells that only express PSA. We have designed a novel Ad-vector, Ad-hOC-E1 with a single bidirectional hOC promoter to drive the expression of both $E1A$ and $E1B$ genes. Because of the VDRE in the hOC promoter, viral replication can be promoted 10-fold or more with vitamin D exposure. This new version of Ad-hOC-E1 was highly efficient in destroying human prostate tumor cells irrespective of their basal AR and PSA status. We have detailed the ability of hOC promoter to drive the expression of both $E1A$ and $E1B$ in a bidirectional manner and the ability of this virus to inhibit the growth of human prostate cancer cell lines in vitro and tumor xenografts in vivo. There are potential advantages of using a single bidirectional promoter to drive the adenoviral replication or adenovirus-directed toxic gene expression. We have observed that two copies of the same promoter that drives both $E1A$ and $E1B$ genes resulted in the deletion of the E1A sequence during viral replication (data not shown). Juxtaposing promoters with a homologous region could result in homologous recombination and deletion of transgenes important for viral replication. Conversely, juxtaposing promoters with heterologous sequences could prevent homologous recombination and result in promoter competition and the squelching of transcription factors. A single promoter to drive both $E1A$ and $E1B$ genes could avoid the homologous recombination, promoter competition, and squelching of transcription factors during gene transcription. In the present study, we demonstrated that a single hOC promoter, driving bidirectional $E1A$ and $E1B$ genes, replicated efficiently in AI prostate cancer cell lines (C4-2, PC-3, and DU145). The selectivity of this bidirectional hOC promoter and its inducibility by vitamin D was demonstrated in RCC52 and prostate cancer cell lines. The inducibility of viral replication by vitamin D3 enhances cytotoxicity and efficient viral replication during the early onset of virus accumulation at the site of tumor implantation. Vitamin D-induced viral replication can also be observed in cells that have undetectable basal OC promoter activity (such as RCC52 cell lines).

Ad-hOC-E1 plus vitamin D3 may be useful for the treatment of human renal cancers as long as these cancer cell lines contain functional VDR. Because of the presence of VDR in liver cells, potential hepatotoxicity is a concern, although other transcription factors may be required for OC promoter activation. Recently, Yeung et al. found that three groups of transcription factors, Runx2, JunD/Fra-2, and Sp1, were responsible for the high hOC promoter activity in AI prostate cancer cells by binding to the OSE2, AP-1/VDRE, and OSE1 elements, respectively (35). The specific requirement of general transcription factors and specific interaction among these transcription factors and their binding to the OC promoter could result in differential gene transcription (36–38). The involvement of vitamin D in triggering the formation of the VDR-retinoid X receptor complexes could trigger a threshold transcriptional factor that preferentially activates the hOC promoter, raising the possibility that Ad-hOC-E1, combined with vitamin D3, may be a useful regimen for the treatment of not only prostate cancer but also other cancers, including renal cancer.

Vitamin D has been found to affect the growth of prostate cancer in preclinical experiments (39), and there are indications that it may be useful for both prevention and treatment of prostate cancer (40–42). However, vitamin D-mediated antiproliferation of AI prostate cancer cells is still controversial (43). In this study, VDR was prevalently expressed in PC3 cells, followed by DU145 and C4-2 cells (Fig. 1). On vitamin D exposure, growth inhibition was most pronounced in C4-2 but was not observed in PC3 and DU145.
cells (data not shown). In contrast to the cells grown in culture, the growth of DU145 tumor in nude mice was markedly inhibited by vitamin D₃ alone without additional Ad vector (Fig. 7A). The precise antiproliferation mechanism(s) of vitamin D₃ against DU145 tumor growth as xenograft but not in cell culture is unclear. Possibly, vitamin D is a potent antangiogenic agent (44) that inhibits neovascularization during tumor development.

The major side effect of high-dose vitamin D administration is hypercalcemia, which could jeopardize its clinical utility. Although vitamin D₃ analogue, Ro 25-9022, used in the present study, has not been tested at the maximum tolerated dose, when mice treated with 4 ng of Ro 25-9022 twice a week for 3 weeks were compared with vehicle-treated mice, they showed only a mild side effect, a 10% body weight reduction for a period of 3 weeks, and they recovered after the vitamin D₃ treatment was stopped (43). The next goal of our study is to establish the optimal vitamin D₃ treatment protocol to minimize its side effects. A dual modality strategy combining Ad-E1 and vitamin D₃ may be translated rapidly into the clinic for the treatment of men with hormone-refractory metastatic prostate cancer.

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