Original Article

Prostate Specific Antigen Promoter-Driven Adenovirus-Mediated Expression of Both ODC and AdoMetDC Antisenses Inhibit Prostate Cancer Growth

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ABSTRACT

Objective: To generate recombinant adenovirus that could simultaneously express ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) antisenses specifically in prostate cancer cells, and evaluate its inhibitory effect on prostate cancer *in vivo*.

Methods: Fragments of ODC and AdoMetDC genes were generated by PCR, cloned into the pPGL-PSES, and then recombined with pAdEasy-1 vectors in AdEasy-1 cells. Ad-PSES-ODC-AdoMetDCas virus was produced in HEK293 cells. Following transfection with Ad-PSES-ODC-AdoMetDCas, the levels of ODC or AdoMetDC were determined by RT-PCR and western blot assays. The effect of Ad-PSES-ODC-AdoMetDCas treatment on tumor formation and growth was evaluated in xenograft models of prostate cancers *in vivo*.

Results: The plasmid pAdEasy-PSES-ODC-AdoMetDCas was successfully constructed and the recombinant Ad-PSES-ODC-AdoMetDCas adenovirus was produced. Transfection with Ad-PSES-ODC-AdoMetDCas adenovirus significantly inhibited the expression of ODC and AdoMetDC genes specifically in prostate DU145 cells, but not H1299, HT29 and HepG2 cancer cells, and disrupted the ability of DU145 cells to form solid prostate cancer *in vivo*. Intratumoral treatment with Ad-PSES-ODC-AdoMetDCas adenovirus significantly inhibited the growth of engrafted prostate tumors *in vivo*.

Conclusion: The recombinant Ad- PSES-ODC-AdoMetDCas adenovirus specifically reduces the expression of both ODC and AdoMetDC genes in prostate cells and may be used for treatment of prostate cancers at the clinic.

Key words: Prostate androgen independent promoter; Ornithine decarboxylase; S-adenosylmethionine decarboxylase; Adenovirus; Prostate cancer

INTRODUCTION

Prostate cancer is the most commonly diagnosed non-cutaneous neoplasm in men and the second leading cause of cancer mortality among American men[1]. Prostate specific antigen (PSA) is selectively expressed by benign, hyperplastic, and malignant prostate epithelium[2–4] and significantly increased levels of PSA in patient's sera is a valuable indicative of prostate diseases[5]. The incidence of prostate cancers is rapidly rising in developed countries due to increased sensitivity of PSA detection[4, 6]. The unique property of PSA expression allows to target therapeutic gene expression selectively in prostate tissues for the treatment of prostate cancers[7]. Indeed, previous studies have shown that the immediate 5' region of

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the PSA promoter was sufficient in driving the expression of interesting genes specifically in prostate tissue[8].

Polyamines, which belong to aliphatic compounds, exit in almost all living species and physiologically regulate the growth and differentiation of normal cells[9–11]. In mammalian cells, the intracellular polyamine biosynthesis is mainly regulated by two rate-limiting enzymes[12] ornithine decarboxylase (ODC) and S-adenosyl-methionine decarboxylase (AdoMetDC), which have been implicated in tumor growth by promoting the formation of more distal polyamines spermidine and spermine[13]. High levels of polyamine and elevated levels of polyamine synthetic enzyme activity have been detected in many kinds of cancers, including prostate cancer[14–17]. Indeed, inactivation of polyamine catabolism in Du145 prostate carcinoma cells attenuated their proliferation[18]. Therefore, down-regulation of ODC and AdoMetDC expression and depletion of polyamine content in prostate cancer by novel protocols of gene therapy may be a promising approach to treat prostate cancer[19].

In this study, we generated novel recombinant adenovirus using the PSA promoter controlled expression of two antisense RNAs for the ODC and AdoMetDC mRNAs, allowing disrupting the expression of ODC and AdoMetDC specifically in prostate epithelial cells. Its therapeutic efficacy in inhibiting the growth of prostate cancers was evaluated *in vivo*.

MATERIALS AND METHODS

Cells and Reagents

HT-29, H1299, HepG2 and Du145 cells were stored in our institute. HEK 293 packaging cells were purchased from Shanghai Institute of Biochemistry and Cell biology, Chinese Academy of Sciences (Shanghai, China).

The shuttle vectors of TA-ODC and TA-AdoMetDC were constructed in our laboratory. PGL3-PSES vector was a gift from Prof. Chinghai Kao in Indiana University (Indianapolis, IN). Escherichia Coli pAdTrack and pAdeasy-1 cells were stored in our laboratory.

Plasmids Constructions: Construction of pAdeasy-PSES-ODC-AdoMetDC

Plasmids TA-ODC and TA-AdoMetDC were used as the templates for the amplification of the ODC (140 bp) and AdoMetDC (220 bp) gene fragments by polymerase chain reaction (PCR). The target fragments were purified after agarose-electrophoresis and inserted into the pMD19-T simple vector, achieving the plasmid, designated as TA-ODC-AdoMetDC. Subsequently, the designed 360 bp fragment was further cloned into the Kpn I and EcoR V sites of the pAdTrack null vector, which generated the recombinant plasmid pAdTrack-ODC-AdoMetDC. Next, the Hind III DNA fragment of pAdTrack-ODC-AdoMetDC was further inserted into the corresponding site of the pPGL-PSES (bearing prostate androgen independent promoter) vector and formed pAdTrack-PSES-ODC-AdoMetDC. Finally, pAd-Track-PSES-ODC-AdoMetDC was linearized by digestion with Pem I, purified, and then transformed (1 μl linearized plasmid DNA) into highly competent cells, AdEasy-1, for generating recombinant plasmid pAdeasy-PSES-ODC-AdoMetDC. Its authenticity was confirmed by DNA sequencing.

Viral Preparation and Analysis

The recombinant plasmid pAdeasy-PSES-ODC-AdoMetDC was linearized with Pac I, and transformed into 293 packing cells for the generation of recombinant adenovirus, Ad-PESE-ODC-AdoMetDCas, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Sixteen hours post transfection, the cells were harvested and lyzed by freezing and thawing repeatedly, followed by centrifuging at 10,000 rpm for 10 min for collecting the viral supernatant. The recombinant viral particles were purified by ultracentrifugation in cesium chloride step gradients. The genes contained in the virus were analyzed by RT-PCR. The titers of purified adenoviruses were measured by green fluorescent protein (GFP) expression.

Evaluation of Gene Transduction Efficiency *In Vitro*

HT-29, H1299, HepG2 or Du145 cells at 7×10^4/well were infected with Ad-PESE-ODC-AdoMetDCas at multiplicities of infection (MOI) of 30, 50, 70, 90, or 110, respectively, for 24 h. The GFP-positive cells were quantified under a fluorescence microscope.

PT-PCR Analysis of ODC and AdoMetDC mRNA Levels

Following infection with the Ad-PESE-ODC-

AdoMetDCas adenoviruses (90 MOI) for 72 h, HT-29, H1299, HepG2 and Du145 cells were harvested and their total RNA was extracted using Trizol reagent (GIBCO), according to the manufacturer's instructions. Total RNA (5 μg) from each sample was reversely transcripted into cDNA using the cDNA synthesis kit. The levels of ODC and AdoMetDCas mRNA transcripts were analyzed by PCR using the specific primers. Their sequences were 5'-TGTGAATGATGGCGTCTATGG A-3' (sense) and 5'-AGGCTGCTCTGTGGC GTTT-3' (antisense) for ODC; and 5'-AGAGAGTCGGG-TAATCAGTCAGC-3' (sense) and 5'-GAACA-TAGCACTCTGGCAATCAA-3' (antisense) for AdoMeODC. A total of 20 μl PCR reaction was first denatured at 94°C for 5 min, and then subjected to 30 cycles of 94°C 30 s, 60°C 30 s, and 72°C 40 s, followed by extending at 72°C for 10 min. An aliquot of each product was analyzed by agarose- electrophoresis. The images were obtained after being stained with ethidium bromide.

Western Blot Analysis

HT-29, H1299, HepG2 or Du145 cells were infected with, or without, the Ad-PESE-ODC-AdoMetDCas or Ad-GFP (MOI=90) recombinant adenoviruses, respectively, for 72 h. The cells were harvested and lysed. Equal amount of total proteins from each lysate was separated by SDS-PAGE and electronically transferred to PVDF membranes (Roche Applied Science, Indianapolis, IN). The membranes were probed with anti-ODC (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-MetDCas (Boster, Wuhan, China) and after washing, the bound primary antibodies were visualized by horseradish peroxidase-conjugated secondary antibodies and ECL Western Blotting Detection System (Pierce Inc, Rockford, IL). The levels of β-actin were probed with specific antibody and used as controls.

Prostate Tumor Models and Treatment

Male BALB/c nude mice at 4–6 w of age and weighing at 18–22 g were obtained from Experimental Animals Center, Chinese Academy of Medical Sciences (Beijing, China). All animal experiments were conducted in accordance with the principles and procedures of laboratory animals, and approved by the Animal Protective Committee of our campus. To establish prostate cancer models, individual mouse was subcutaneously injected with 5×10^6 Ad-PESE-AdoMetDCas or control Ad-GFP-infected Du145 cells into the right flank. Additional control group of mice received equal number of unmanipulated Du145 cells. Their tumor formation was blindly evaluated by measuring the tumor size twice per week for 40 d.

To evaluate whether intratumoral injection with the Ad-PESE-AdoMetDCas recombinant adenoviruses could inhibit the progression of established prostate cancer in vivo, unmanipulated Du145 cells (5×10^6 cells/mouse) were injected subcutaneously into mice to establish prostate cancer model. One week later, when the diameter of the tumor reached 5–7 mm the mice were randomly divided into three groups (n=6). Groups of mice were treated intratumorally with control Ad-GFP or recombinant Ad-PESE- ODC-AdoMetDCas adenoviruses (5×10^8 pfu/ mouse/ daily) or none for 3 consecutive days. The growths of prostate cancer were measured as described above every other day for 30 d.

Statistical Analysis

Tumor volume (mm^3) was determined by measuring the two largest diameters every three days using microcaliper and calculated as follows: V=(length × width 2) × 0.5236. The average tumor volume in each group was calculated and tumor growth curve was plotted versus observation time.

Data were presented as $\bar{x}\pm s$. One way ANOVA analysis was applied to compare tumor volumes among different groups to assess the therapeutic effect. Analysis was performed using the statistical software SPSS13.0 according to the instructions of the software. A value of $P<0.05$ was considered to be of statistical significance.

RESULTS

Generation of Ad-PESE-AdoMetDCas Recombinant Adenoviruses

To generate Ad-PESE-AdoMetDCas recombinant adenovirus, the gene fragments for ODC and AdoMetDC were amplified by PCR and cloned into the pAdTrack null vector, generating the recombinant plasmid pAdTrack-ODC- AdoMetDC. Following transformed into E. coli DH5α cells, three recombinants were randomly selected and digested with Kpn I/EcoR V. As shown in Figure 1, a clear band of about 360 bp displayed, indicating the ODC and AdoMetDC gene fragments, as designed, were fused. Next, the Hind III DNA fragment of the pAdTrack-ODC-AdoMetDC was

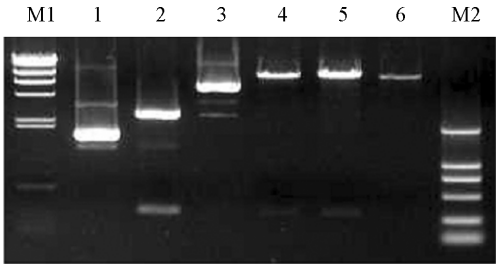

Figure 1. Restriction endonuclease digestion of the shuttle vector pAdTrack-ODC-AdoMetDC. M1 and M2: DNA molecular weight markers; Lane 1 and 2: TA-ODC-AdoMetDC and its digested products with both Kpn I and EcoRV; Lane3: pAdTrack-PSES-ODC-AdoMetDC-1; Lane4: pAdTrack-PSES-ODC-AdoMetDC-1-KpnI/EcoRV; Lane5: pAdTrack-PSES-ODC-AdoMetDC-2-KpnI/EcoRV; Lane 6: vector DNA pAdTrack. Data are representative of two separated experiments.

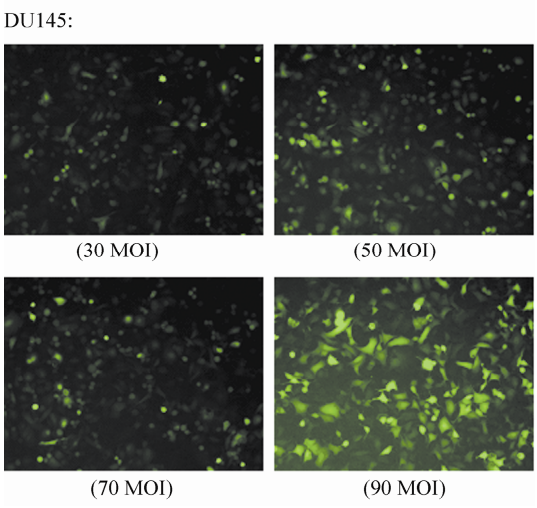

Figure 2. Analysis of GFP expression. Du145, H1299 and HT29 cells were transfected with Ad-PESE-ODC-AdoMetDCas at MOI of 30, 50, 70, 90 or 110, respectively. The expression of GFP was observed longitudinally under a fluorescence microscope. Data are representative of two independent experiments from Du145 cells.

further inserted into the corresponding site of the PGL-PSES (bearing prostate androgen independent promoter) vector and formed the construct pAdTrack-PSES-ODC- AdoMetDC. Subsequently, pAdTrack-PSES- ODC- AdoMetDC was linearized and transformed into AdEasy-1 cells to generate recombinant plasmid pAdeasy-PSES-ODC-AdoMetDC. Its authenticity was confirmed by DNA sequencing.

To produce the viruses, the pAdeasy-PSES-ODC-AdeMetDC plasmid was linearized and transfected into 293 cells, followed by purification of viral particles. Approximately, about 3×10^8 pfu recombinant Ad-PESE-ODC-AdoMetDCas adeno-viruses were yielded in 1 ml of cell cultures medium.

Characterization of the Ad-PESE-ODC-AdoMetDC as Adenoviruses

To determine the infection efficacy of the Ad-PESE-ODC-AdoMetDCas recombinant adeno-virus, Du145, H1299 and HT29 cells were infected with Ad-PESE-ODC-AdoMetDCas at MOI of 30, 50, 70, 90 or 110, respectively. The expression of florescent GFP was observed under a florescent microscope (Figure 2). Adenoviral infection of Du145, H1299 and HT29 cells at a MOI of 90 induced strong expression of GFP in all cells. Infection of Du145 cells with different doses of Ad-PESE-ODC-AdoMetDCas adenovirus caused dose-dependent GFP expression, indicating the efficient infection with the generated virus in the cells.

Specific Suppression of ODC and AdoMetDC Expression in Prostate Carcinoma Cells

Next, we examined the efficacy of antisense-mediated modulation of ODC and AdoMetDC mRNA transcription in cancer cells. H1299, HT29, HepG2 or Du145 cells were infected with, or without, the Ad-PESE-ODC-AdoMetDCas or control Ad-GFP for 72 hours. The levels of ODC and AdoMetDC mRNA transcripts were semi-quantitatively analyzed by RT-PCR assay (Fig. 3A). Similar levels of ODC and AdoMetDC mRNA transcripts were observed in H1299, HT29 or HepG2 cells that had been infected with, or without, the Ad-PESE-ODC-AdoMetDCas or control Ad-GFP adenoviruses. In contrast, the levels of ODC and AdoMetDC mRNA transcripts in Du145 prostate cancer cells that had been infected with the Ad-PESE-ODC-AdoMetDCas were significantly reduced, as compared with the control groups (Figure 3A Lane 3). Similarly, the levels of ODC and AdoMetDCas proteins in Du145 cells, but not other cells tested, that had been infected with the Ad-PESE-ODC-AdoMetDCas were significantly lower than that in controls, determined by Western blot analysis (Figure 3B). The relative levels of ODC and AdoMetDC expression in Du145 cells infected with Ad-PESE-ODC-AdoMetDCas were reduced by 55% and 41%, respectively, as compared with that

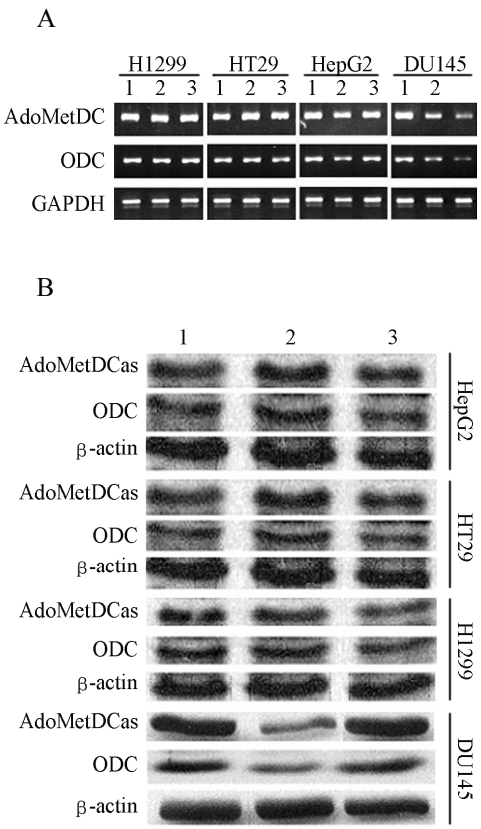

Figure 3. Analysis of ODC and AdoMetDC expression. Du145, HepG2, HT-29 or H1299 cells were transfected with, or without, the Ad-PESE-ODC-AdoMetDCas or control adenoviruses at a MOI of 90. Three days post transfection, the cells were harvested and the levels of ODC and AdoMetDC expression were characterized by RT-PCR. A: Western blot assays; B: Column 1, 2 or 3 represented the unmanipulated cells, the cells treated with control adenovirus or recombinant Ad-PESE-ODC-AdoMetDCas adenovirus, respectively. Data are representatives of three independent experiments.

in the cells treated with Ad-GFP. However, similar treatment of HepG2, H1299 or HT29 cells with Ad-PESE-ODC-AdoMetDCas did not significantly change the levels of ODC and AdoMetDC expression. These data clearly demonstrated that the expression of both ODC and AdoMetDC antisenses mediated by Ad-PESE-ODC-AdoMetDCas adenoviruses suppressed the expression of the targeted genes, specifically in prostate tumor cells.

Antitumor Activity of Recombinant Adenovirus Ad-PESE-ODC-AdoMetDCas *In Vivo*

Given that the levels of ODC and AdoMetDC expression are crucial for the development of prostate cancers, we further evaluated whether treatment of DU145 cells in vitro with the Ad-PESE-ODC-AdoMetDCas adenovirus or intratumoral treatment with the Ad-PESE-ODC-AdoMetDCas adenovirus *in vivo* could modulate the growth of engrafted prostate cancer *in vivo*.

Du145 cells were infected with, or without, the Ad-PESE-ODC-AdoMetDCas or control Ad-GFP adenoviruses at a MOI of 90 for 24 h and implanted into nude mice. The formation and growth of prostate cancers in the recipients were monitored longitudinally (Figure 4). Mice implanted with Ad-GFP-infected DU145 cells developed a single big cancer, similar to the mice received unmanipulated prostate cells. However, the total volume of tumor in the mice received Du145 cells infected with Ad-GFP was significantly smaller than that in the mice received unmanipulated prostate cells. The progressive rates of tumors grown in the mice received unmanipulated DU145 cells or Ad-GFP infected DU145 cells were 26.2 mm^3/day or 14.7 mm^3/day, respectively. These suggested that expression of GFP or adenoviral infection inhibited the growth of engrafted prostate cancers in vivo. Importantly, the mice received Ad-PESE-ODC-AdoMetDCas-infected DU145 cells did not develop visible cancer throughout the experimental period. Therefore, induction of both ODC and AdoMetDC antisense expression in prostate cancer cells suppressed the ability of prostate cancer cells to form solid tumors *in vivo*.

We further assessed the effect of intratumoral injection with Ad-PESE-ODC-AdoMetDCas adenovirus after the establishment of solid tumor *in vivo*. Nude mice were implanted with Du145 cells and monitored the formation of prostate tumors. About 7 d post implantation when the tumors reached 5–7 mm in diameter, these mice were randomly divided and injected intratumorally with Ad-PESE-ODC-AdoMetDCas adenovirus, Ad-GFP (3×10^8 PFU/mouse) adenovirus or PBS. The growth of prostate cancers was measured and showed in Figure 4B. The solid tumors in the mice injected with PBS grew rapidly while the growth in the mice received Ad-GFP was slightly slow. One month post treatment, the average volumes of tumors in the mice treated with PBS and control adenovirus were 1059 mm^3 and 759 mm^3, respectively. Significantly, the average volume of tumors in the mice exposed to adenoviral Ad-PESE-ODC-AdoMetDCas was reduced to 343 mm^3. Furthermore, the tumor growth rates of individual groups were 33.5 mm^3/day (PBS), 23.1 mm^3/day (Ad-GFP) or 8.8 mm^3/day (Ad-PESE-

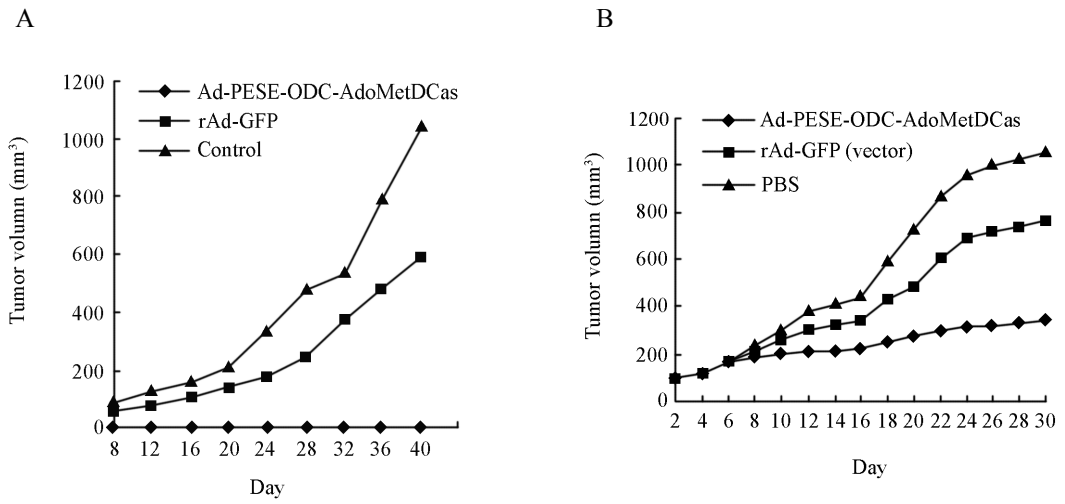

Figure 4. Anti-tumor activities of recombinant adenovirus Ad-PESE-ODC-AdoMetDCas *in vivo*. A: The effect of Ad-PESE-ODC-AdoMetDCas transfection on tumor formation. Du145 cells were transfected with, or without, Ad-PESE-ODC-AdoMetDCas or control Ad-GFP at a MOI of 90. Sixteen hours post transfection, the cells were harvested and injected into nude mice, followed by monitoring the formation and growth of solid prostate cancer (n=8 per group); B: The inhibitory effect of Ad-PESE-ODC-AdoMetDCas on tumor growth. Nude mice were implanted with Du145 cells and 7 d after implantation when the developed prostate tumors reached 5–7 mm in diameter, the mice were randomly divided. They were intratumorally injected with, or without, Ad-PESE-ODC-AdoMetDCas or control Ad-GFP (5×10^8 pfu/mouse/daily) adenoviruses for three consecutive days (n=6 per group). The growth of implanted tumors was monitored longitudinally.

ODC-AdoMetDCas), respectively.

Collectively, these data demonstrated that adenovirus-mediated specific suppression of both ODC and AdoMetDC expression in prostate cancers dramatically inhibited the formation and growth of prostate cancers, even after establishment of solid tumor *in vivo*.

DISCUSSION

Adenovirus-based gene therapy is an attractive approach to inhibit the growth of prostate cancers[20, 21]. ODC and AdoMetDC are crucial for the polyamine biosynthesis, associated with the growth of tumors in human[22]. Theoretically, aberrations of polyamine biosynthesis in tumor tissues may be an attractive target for anticancer drug therapy[20, 23]. Indeed, inhibitors for ODC and AdoMetDC enzymes, such as α-difluoromethylornithine, MGBG and SAM486A, have been found to effectively suppress the growth of tumor cells[24]. However, their antitumor effects are limited in clinic due to potential toxicity[25, 26].

In the current study, we successfully generated recombinant adenovirus, which can express both ODC and AdoMetDC antisenses specifically in prostate cells. Infection of prostate cancer cells, Du145, but not other human cancer cell lines tested, significantly down-regulated the expression of ODC and AdoMetDC genes, demonstrated by RT-PCR and Western blot assays. These data, as expected, demonstrate that the transcripts of ODC and AdoMetDC antisenses can effectively suppress the expressions of ODC and AdoMetDC genes specifically in prostate cells. Furthermore, infection of human prostate cancer cells with Ad-PESE-ODC-AdoMetDCas adenovirus abrogated the ability of the prostate cancer cells to form solid tumors in immunocompromised nude mice. Importantly, intratumoral injection of Ad-PESE-ODC-AdoMetDCas adenovirus inhibited the growth of implanted human prostate cancers *in vivo*. These data are extremely significant as it suggests that Ad-PESE -ODC-AdoMetDCas-based gene therapy may be used for treatment of small prostate cancers or after surgical resection of partial tumor tissues.

In summary, our data provide evidence that adenovirus-mediated expression of both ODC and AdoMetDC-specific antisenses effectively inhibits the growth of human prostate cancers *in vivo*. Conceivably, the PSA promoter driven Ad-PESE-ODC-AdoMetDCas-based therapy targeting specifically to prostate cells should have little toxic and adverse effect in prostate patients. Our results suggest that synergistic inhibition of ODC and AdoMetDC expression, through a gene therapy

approach, may represent a promising therapy for prostate caners.

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