Adenovirus-mediated Expression of both Antisense Ornithine Decarboxylase (ODC) and S-adenosylmethionine Decarboxylase (AdoMetDC) inhibits human esophageal squamous carcinoma cell growth

Research Article

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Abbreviations: bicinchoninic acid (BCA); coxsackie adenovirus receptor (CAR); cyclin-dependent kinases (cdks); cytomegalovirus (CMV); decarboxylated Sadenosylmethionine (dcSAM); Difluoromethylornithine (DFMO); dodecyl sulfate (SDS); Dulbecco’s modified Eagle’s medium (DMEM); green fluorescent protein (GFP); high-performance liquid chromatography (HPLC); methylglyoxalbis (guanylylhydrazone) (MGBG); monoclonal antibody (mAb); multiplicities of infection (MOIs); ornithine decarboxylase (ODC); reverse-transcription polymerase chain reaction (RT-PCR); S-adenosylmethionine decarboxylase (AdoMetDC)

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Summary
Polyamine biosynthesis is controlled primarily by ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC). Antisense ODC and AdoMetDC sequences were cloned into an adenoviral vector (Ad-ODC-AdoMetDCas). To evaluate the effect of recombinant adenovirus Ad-ODC-AdoMetDCas which can simultaneously express both antisense ODC and S-adenosylmethionine decarboxylase (AdoMetDC), the human esophageal squamous carcinoma cell line Eca109, was infected with Ad-ODC-AdoMetDCas as well as with control vector. Viable cell counting, determination of polyamine concentrations, cell cycle analysis, and Matrigel invasion assays were performed in order to assess the properties of tumor growth and invasiveness. Our study demonstrated that adenovirus-mediated ODC and AdoMetDC antisense expression inhibits tumor cell growth through a blockade of the polyamine synthesis pathway. This inhibitory effect cannot be reversed by the administration of putrescine. Tumor cells were arrested at the G₁ phase of the cell cycle after gene transfer and had reduced invasiveness. Our study suggests that as a new anticancer reagent, the recombinant adenovirus Ad-ODC-AdoMetDCas holds promising hope for the therapy of esophageal cancers.

I. Introduction
Polyamines are naturally occurring aliphatic polycations found in almost all living organisms. Polyamines include spermidine, spermine, and their diamine precursor, putrescine (Tian et al, 2006a). Polyamines have critical physiological functions in cell growth and differentiation. In mammalian cells, the intracellular polyamine biosynthetic pathway is primarily regulated by the action of two rate-limiting enzymes. Ornithine decarboxylase (ODC) is the first key enzyme required for polyamine synthesis, decarboxylating ornithine to produce putrescine (Tian et al, 2006b). The
second, rate-limiting enzyme is S-adenosylmethionine decarboxylase (AdoMetDC). It generates the aminopropyl donor, decarboxylated Sadenosylmethionine (dSAM), by decarboxylating adenosylmethionine. DcSAM donates its propylamine moiety for the formation of spermidine and spermine via catalysis by spermidine synthase and spermine synthase, respectively.

The association of increased polyamine synthesis with cell proliferation and cancer progression was first reported in the late 1960s. High polyamine levels and elevated polyamine synthesis activity were found in many tumors. Environmental and genetic risk factors for cancer, such as ultraviolet light (Ahmad et al., 2001) and various oncogenes (Holttta et al., 1988; Sistonen et al., 1989; Auvinen et al., 1992), have been reported to induce high ODC activity in normal tissues. Overexpression of ODC or AdoMetDC was also reported to cause malignant transformation of NIH3T3 cells (Auvinen et al., 1992; Paasinen-Sohns et al., 2000). Therefore, inhibition of ODC and/or AdoMetDC activity might induce a depletion of intracellular polyamines, providing an effective anticancer treatment strategy. Previous work has primarily focused on the development of polyamine synthesis inhibitors. Difluoromethylornithine (DFMO) irreversibly inactivates ODC activity and has been used in clinical chemoprevention trials for epithelial cancers, including colon, breast, cutaneous, and prostate malignancies (Meyskens and Gerner, 1999). AdoMetDC inhibitors, such as methylglyoxalbis (guanilylhydrazone) (MGBG), have also been shown to inhibit tumor growth (Warrell and Burchenal, 1983). SAM486A is a new AdoMetDC inhibitor that has been shown to possess anti-proliferative activity in both tissue culture cells and preclinical animal studies (Regenass et al., 1994).

Esophageal cancer is one of the most lethal cancers known to mainland in China because of the high incidence and high mortality. Metastatic esophageal cancer is essentially resistant to systemic cytotoxic chemotherapy, while external beam and radioisotope radiotherapy offers only symptom palliation. The development of novel therapies, such as gene therapy, is of high priority.

In the present study, we constructed a replication-deficient recombinant adenovirus containing antisense sequences of both ODC and AdoMetDC (Ad-ODC-AdoMetDCas) to downregulate their gene expression levels simultaneously. Our data show that downregulation of these two key enzymes by Ad-ODC-AdoMetDCas significantly inhibited esophageal cancer cell growth and tumor invasiveness in vitro. The tumor cells were arrested in the G1 phase of the cell cycle. Polyamine levels were significantly decreased in Ad-ODC-AdoMetDCas-treated cells compared with controls.

II. Materials and methods
A. Cell culture and reagents
Human esophageal cancer Eca109 cell line obtained from the Chinese Academy of Sciences, were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin. HEK293 cells (transformed human embryonic kidney cells), also purchased from the Chinese Academy of Sciences, were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen USA) containing 10% fetal bovine serum. All cells were cultured in a 5% CO2 incubator at 37°C. The polyamine standards (putrescine, spermidine, and spermine) and dyesulf chloride for high-performance liquid chromatography (HPLC) were purchased from Sigma (St. Louis, MO, USA). An anti-ODC mouse monoclonal antibody (mAb) and an anti-AdoMetDC mouse polyclonal antibody were prepared in our laboratory. An anti-p21 (sc-6246) mouse mAb and an antiactin (sc-1616) goat polyclonal antibody were purchased from Santa Cruz Biotechnology. Matrigel and Transwell plates were obtained from BD Biosciences (Bedford, MA, USA) and Costar (Cambridge, MA, USA), respectively.

B. Construction of Ad-ODC-AdoMetDCas
The construction of the adenoviral vector, rAd-ODC/EX3as, containing antisense ODC sequence with both a cytomegalovirus (CMV) promoter and a green fluorescent protein (GFP) gene, was reported previously (Zhang et al., 2005). To construct an adenoviral vector harboring additional antisense AdoMetDC sequence, a 205-bp cDNA fragment of the 5′ end of the AdoMetDC gene was amplified by reverse-transcription polymerase chain reaction (RT-PCR) using specific primers and was subcloned downstream of the ODC gene in the pAd-ODCas vector in the reverse direction. The forward primer was 5′GTTCTAGATTTGCTAGTCCTACAGGGTATG′3′ and the reverse primer was 5′GGCTCGAGTAAGCTTCCTGCTTGTCAGT′3′. The sequence of the resulting clone, pAd-ODC-AdoMetDCas, was confirmed by sequencing and was then linearized by digestion with Pme I and transformed into Adeasier-1 cells containing the 33-kb pAdeasy-1 vector to generate recombinant clones as previously described (He et al., 1998). The recombinant adenovirus genome was digested with Pac I and transfected into HEK293 cells with Lipofectamine2000 (Invitrogen USA) for the isolation of recombinant adenovirus. Recombinant viral plaques were identified and amplified by PCR in order to verify ligation success. The recombinant virus particles were purified by CsCl ultracentrifugation (Prevec et al., 1991) and a standard plaque assay was performed to measure the titer of the purified viral stock. The control virus, Ad-GFP, contained no gene insertion in the multiple cloning site.

C. Analysis of gene transduction efficiency in vitro
The efficiency of adenovirus-mediated gene transfer was assessed by detection of GFP. Eca109 cells (3×10^5 cells/well) seeded in 6-well plates were infected with Ad-GFP at different multiplicities of infection (MOIs) of 5, 10, 20, 50 and 100. GFP expression was analyzed at 48 h after the infection using a flow cytometer (Beckman Coulter, Miami, FL, USA).

D. Western blot analysis
After the Eca109 cells had been treated with phosphate-buffered saline (PBS), Ad-GFP, Ad-ODCas, and Ad-ODC-AdoMetDCas for 72 h, total cell lysates were prepared in extraction buffer containing 50 mM Tris (pH8.0), 1% NP-40, 1 µg/ml aprotinin, 0.1% sodium dodecyl sulfate (SDS), 0.02% sodium azide, 150mM NaCl, and 100 µg/ml phenylmethylsulfonyl fluoride. Sample protein concentrations were quantified using the bicinchoninic acid (BCA) protein assay. After electrophoresis samples were transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). After an incubation with appropriate antibodies in PBS containing 5% nonfat dry milk and 0.02% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, developed using the Western blotting luminol reagent
(Santa Cruz Biotechnology, USA), and exposed to X-ray film (Kodak, Shantou, China).

E. Measurement of polyamine content

Polyamine content was measured as previously described (Aboul-Enein and al-Duraihi, 1998). After an incubation with PBS, Ad-GFP, Ad-ODCas, and Ad-ODC-AdoMetDCas for 3 days, Eca109 cells were harvested by scraping and permeabilized with 5% trichloroacetic acid. The polyamines in the supernatant were separated and quantified on an ionpaired, reversed-phase HPLC system. Protein content was subsequently measured in the precipitate.

F. Measurement of cell growth

Viable cell counts were used to evaluate the effects of recombinant adenovirus on cell proliferation. Eca109 cells were plated in 6-well tissue culture plates at a density of 5x10⁶ cells/well. After 24 h, tumor cells were treated with Ad-GFP, Ad-ODCas, and Ad-ODC-AdoMetDCas at an MOI of 50 or with PBS as a control. Cells in each treatment group were plated in triplicate and cultured for 6 days. Cells were then treated with trypsin and harvested every 24 h and subsequently stained with 0.4% trypan blue (Gibco, USA) for the identification of dead cells. Viable cells were then counted using a hemocytometer.

G. Cell cycle analysis

Eca109 cells were seeded at a density of 3x10⁵ cells/well in 6-well plates and treated with Ad-GFP, Ad-ODCas, or Ad-ODC-AdoMetDCas at an MOI of 50 or treated with PBS as a control. Three days following treatment, cells were harvested as described above, washed once with cold PBS, and treated with 70% ethanol. Cells were then washed with ice-cold PBS and treated with RNase. DNA was subsequently stained with propidium iodide. Cell cycle phases were analyzed using FACSscan (Becton Dickinson).

H. Matrigel invasion assay

Eca109 cells were infected with Ad-GFP, Ad-ODCas, or Ad-ODC-AdoMetDCas at an MOI of 50 for 2 days. Invasiveness was measured by counting cells that had traveled through Matrigel-coated Transwell inserts. Transwell inserts (6.5 mm) with a 8.0-μm pore size were coated with 30 μl of Matrigel and dried for 2 h at room temperature. Cells were harvested as described above. A 100-μl cell suspension containing 5x10⁵ cells was added to wells in triplicate. After 24 h of incubation, nonmigrated cells were scraped from the upper side of the membrane with cotton swabs. Cells that passed through the filter into the bottom side of the membrane were fixed and stained with hematoxylin. Five representative fields in each well were quantified to determine the number of invasive cells under a light microscope at 200 x magnification.

I. Statistical analysis

Data are reported as the mean ± standard deviation (SD). Statistical differences between control and treated cells were evaluated using Student’s t-test. A value of P < 0.05 was considered significant.

III. Results

A. Ad-ODC-AdoMetDCas inhibits ODC and AdoMetDC gene expression in cancer cells in vitro

Adenovirus infects host cells through the coxsackie and adenovirus receptor (CAR) (Bao et al, 2005). As the CAR status in cancer cells is largely unknown, we first evaluated adenoviral gene transfer efficiency in tumor cells using Ad-GFP. Eca109 tumor cells were infected with AdGFP at MOIs of 5, 10, 20, 50 and 100 for 48 h. We demonstrated that 73.6 ± 2.3% of A-549 cells were positive for GFP at an MOI of 50; this MOI was used for further study. To study the inhibitory effects of adenooviral vector-gene transfer on both ODC and Ad-ODCas gene expression, Eca109 cells were infected with Ad-GFP, Ad-ODCas, and Ad-ODC-AdoMetDCas at an MOI of 50 for 72 h. Protein extracted from both adenoviral vector-treated and control conditions were probed with antibodies against ODC and AdoMetDC. Figure 1 shows that Ad-ODC-AdoMetDCas induced a greater than 50% reduction of both ODC and AdoMetDC protein in Eca109 cells compared with Ad-GFP-infected or uninfected cells. Similarly, Ad-ODC-AdoMetDCas induced a greater than 50% reduction of both ODC and AdoMetDC protein in Eca109 cells compared with control conditions. Not surprisingly, ODC protein levels dropped more than 50% in Eca109 cells after Ad-ODCas treatment compared with Ad-GFP-infected or uninfected cells. However, there was no appreciable change in AdoMetDC protein levels in Ad-ODCas-treated cells compared with control cells.

B. Ad-ODC-AdoMetDCas gene transfer decreases polyamine content in cancer cells

After demonstrating that Ad-ODC-AdoMetDCas depressed ODC and AdoMetDC protein expression levels in Eca109 cells, we next evaluated whether the polyamine content could be decreased accordingly by adenoviral gene transfer into these tumor cells. Polyamines in adenovirus-infected or uninfected cancer cells were separated by ion-paired, reversed-phase HPLC. As shown in Table 1, both Ad-ODCas and Ad-ODC-AdoMetDCas decreased the polyamine content of Eca109 cells, correlating with the downregulation of polyamine biosynthesis. Table 1 also shows that incubation with Ad-ODCas alone caused a drop in putrescine content in Eca109 cells. Spermidine concentrations decreased, while spermine levels remained low too. In cells treated with Ad-ODC-AdoMetDCas, all three polyamines were reduced to very low levels. After a comparison of Ad-ODC-AdoMetDCas- and Ad-ODCas-infected cells, both spermidine and spermine were significantly reduced (P<0.05).

C. Ad-ODC-AdoMetDCas inhibits cancer cell proliferation

After confirming the suppression of ODC and AdoMetDC gene expression and polyamine reduction by adenoviral gene transfer, we then asked whether these inhibitory effects could be translated into inhibition of cell growth. We used viable cell counts to determine rates of tumor cell proliferation. The results in Figure 2 demonstrate significant inhibition of cell proliferation in cancer cell lines treated with either Ad-ODCas or Ad-ODC-AdoMetDCas (P < 0.05) compared with control cells treated with either Ad-GFP or PBS. This inhibition of cell growth was maintained for 7 days (data not shown). Significant differences in the inhibitory effects existed
between Ad-ODCas- and Ad-ODC-AdoMetDCas-mediated transduction (P < 0.05). When compared with Ad-ODCas, Ad-ODC-AdoMetDCas was shown to be more effective in inhibiting proliferation of Eca109 cell.

Figure 1. Western blot analysis of ODC and AdoMetDC gene expression in Eca109 cells. Total protein was extracted 3 days after infection with Ad-GFP, Ad-ODCas, or Ad-ODC-AdoMetDCas at an MOI of 50. Each lane was loaded with 50 μg protein and electro-transferred onto a nitocellulose membrane. The blot was probed with either an ODC monoclonal antibody or an AdoMetDC polyclonal antibody.

Table 1. Effects of Ad-ODCas and Ad-ODC-AdoMetDCas on polyamine content (mmol/mg protein) in Eca109 cells. Cells were seeded at a density of 1 × 10⁶ cells/cm² and infected at an MOI of 50 with Ad-GFP, Ad-ODCas, or Ad-ODC-AdoMetDCas. After 3 days of infection, cells were collected and prepared for HPLC analysis. Results are presented as the mean ± SD of three separate experiments. *P < 0.05 vs. Ad-GFP or uninfected cells

<table>
<thead>
<tr>
<th>Cell lines and Treatment</th>
<th>Polyamine pools (pmol/mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>Put</td>
</tr>
<tr>
<td>Eca109</td>
<td>590</td>
</tr>
<tr>
<td>+ Ad-GFP</td>
<td>525</td>
</tr>
<tr>
<td>+ Ad-ODCas</td>
<td>254*</td>
</tr>
<tr>
<td>+ Ad-ODC-AdoMetDCas</td>
<td>76*</td>
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Figure 2. Effects of Ad-ODCas and Ad-ODC-AdoMetDCas on proliferation of Eca109 cell. Cells were seeded at 5 × 10⁴ cells/well and allowed to attach for 24 h. Viable cells were counted daily by trypan blue exclusion on days 0–5 after infection with Ad-GFP, Ad-ODCas and Ad-ODC-AdoMetDCas at an MOI of 50 and compared with uninfected cells.

- PBS; ●: Ad-GFP; ▲: Ad-ODCas; ▼: Ad-ODC-AdoMetDCas
D. Ad-ODC-AdoMetDCas arrests cancer cell cycles in G₁ phase

After we had established that Ad-ODC-AdoMetDCas inhibited tumor cell proliferation, we further analyzed cell cycle profiles of gene-transferred tumor cells. Eca109 cells were treated with PBS, Ad-GFP, Ad-ODCas, or Ad-ODC-AdoMetDCas at an MOI of 50 for 72 h (Figure 3). Cells were then harvested by treatment with trypsin. Propidium iodide staining was used to detect changes in DNA concentrations in different phases of the cell cycle. Results displayed in Table 2 show that Ad-ODC-AdoMetDCas and Ad-ODCas cause more Eca109 cells to arrest compared with controls (P < 0.05). Eca109 cells were arrested in G₀-G₁ phase (66±3.2% in Ad-ODC-AdoMetDCas- and 56±2.3% in Ad-ODCas-treated conditions) compared with 45 ± 2.5% in PBS and 49 ± 3.2% in Ad-GFP- treated conditions. Statistical analysis also revealed a significant difference between Ad-ODC-AdoMetDCas and Ad-ODCas-treated Eca109 cells (P < 0.05) and a greater number of Eca109 cells were arrested by Ad-ODC-AdoMetDCas.

The cell cycle regulatory protein, p21\textsuperscript{WAF1/CIP1/SDi1} (p21), is known to regulate the G₁-S transition (Kamb, 1995). We further analyzed whether p21 gene expression was altered after adenoviral gene transfer and whether it correlated with cell cycle arrest. Expression of p21 in Eca109 cell was detected by Western blot analysis. After 3 days of incubation, p21 was found increased up to 3-fold in Ad-ODC-AdoMetDCas treated cells (Figure 4). Our data indicate that Ad-ODCAdoMetDCas treatment arrests tumor cells in G₀-G₁ phase. This cell cycle arrest correlates with an increased level of p21 expression.

E. Ad-ODC-AdoMetDCas impairs tumor invasiveness in vitro

The Matrigel assay is a widely used protocol to evaluate tumor invasiveness in vitro. We therefore performed the Matrigel assay to evaluate whether either Ad-ODCas or Ad-ODC-AdoMetDCas could decrease tumor invasiveness in addition to their anti-proliferative effects reported above. Eca109 cells (5x10⁴ cells per insert) were allowed to invade the Matrigel-coated membrane. The numbers of invading cells were represented as the average of five randomly selected microscopic fields on the underside of the membrane (Figure 5A). As shown in Figure 5B, only 9 ± 3 cells in the Ad-ODCAndoMetDCas condition and 20 ± 5 cells in the Ad-ODCas condition passed through the membrane. In comparison, 51 ± 7 cells in the PBS condition and 48 ± 8 cells in the Ad-GFP condition passed through the filter (P <0.01). In addition, only 30% of Ad-ODC-AdoMetDCas-infected tumor cells successfully passed through the membrane. These results clearly demonstrate that Ad-ODC-AdoMetDCas significantly decreased tumor invasiveness in vitro.
Table 2. \(G_0 - G_1\) cell cycle phase distribution of Eca109 cells.

<table>
<thead>
<tr>
<th>Cell lines and treatment</th>
<th>Percent of total cells (G_0 - G_1) ((\bar{X} \pm S))</th>
</tr>
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<tbody>
<tr>
<td>Eca109 cell PBS)</td>
<td>45(\pm 2.5)</td>
</tr>
<tr>
<td>+Ad-GFP</td>
<td>49(\pm 3.2)</td>
</tr>
<tr>
<td>+Ad-ODC/Ex3as</td>
<td>56(\pm 2.3^*)</td>
</tr>
<tr>
<td>+Ad-ODC-AdoMetDCas</td>
<td>66(\pm 3.2^*)</td>
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\(^*\) P <0.05, Vs Ad-GFP- and PBS-treated cells.

Figure 4. Western blot analysis of p21 expression levels in Eca109 cell. Total protein was extracted 3 days after infection at an MOI of 50. Each lane was loaded with 80 \(\mu\)g of protein and probed with a p21 monoclonal antibody.

Figure 5. A. Ad-ODC-AdoMetDCas inhibited Eca109 cell invasion. Eca109 cells were treated with recombinant adenovirus at an MOI of 50 for 72 h and then allowed to invade transwell inserts (8-\(\mu\)m pores) coated with Matrigel for 24 h. The cells that invaded through the inserts were stained, counted, and photographed under light microscopy at 200\(\times\) magnification.

B. The numbers of cells that invaded through the Matrigel-coated inserts. The data are presented as the mean \(\pm\) SD for three separate experiments from each group.
IV. Discussion

It has been known for many years that normal cell growth is regulated in a cyclical manner by the increase and decrease of cyclins and cyclin-dependent kinases (cdks). Furthermore, there are also changes in polyamine, ODC and AdoMetDC concentrations during the cell cycle. Both ODC and AdoMetDC mRNA levels and polyamine concentration are doubled during the cell cycle. Elevated levels of ODC and AdoMetDC activity were found in various cancers (Cohen, 1998), such as prostate, breast, and colorectal cancer, and are related to cancer recurrence (Pegg and McCann, 1982; Gutman et al, 1995). Our recent work has proven that inhibition of ODC activity by recombinant antisense ODC adenovirus has had antitumor effects on human lung cancer (Tian et al, 2006a,b). This adenovirus, however, did not inhibit AdoMetDC, a critical enzyme that is normally elevated in tumor cells. We speculate that double inhibition of ODC and AdoMetDC might be a more effective way to suppress tumor growth. Our in vitro study demonstrated more robust antitumor effects by dual inhibition of both ODC and AdoMetDC activities compared to inhibition of ODC activity alone. Double inhibition by Ad-ODC-AdoMetDCs infection significantly reduced ODC and AdoMetDC protein levels more than 50% Eca109 cells compared to controls. A substantial decrease in ODC and AdoMetDC expression levels also causes a reduction of polyamine biosynthesis. Ad-ODC-AdoMetDCs infection depresses three types of polyamines. In contrast, only putrescine and spermidine were shown to be decreased after Ad-ODCs infection. Ad-ODCs treatment of tumor cells did not eliciting a statistical difference in spermine content compared with control treatment. We speculate that the inability of Ad-ODCs to block AdoMetDC activity might be responsible for this observation, consistent with results reported by other researchers who demonstrated that the ODC inhibitor, DFMO, had no effect on spermine levels in tumor cells. Spermine, however, plays an equally important role in carcinogenesis as do the other polyamines. Furthermore, high levels of spermine also contribute to cellular resistance to apoptotic cell death (Hashimoto et al, 1999). The inability of Ad-ODCs to decrease intracellular spermine levels therefore represents an inherent drawback in its potential antitumor effects.

P53, also known as tumor protein 53 (TP53), is a transcription factor that regulates the cell cycle and hence functions as a tumor suppressor. It is important in multicellular organisms as it helps to suppress cancer. p53 has been described as "the guardian of the genome", "the guardian angel gene", or the "master watchman", referring to its role in conserving stability by preventing genome mutation. It has also been found to play an important role in sun tanning. The alteration of gene p53 is a key event in esophagus cancer and if there is a relationship between ODC and AdoMetDC on this issue, we will study it in the future.

To further understand the underlying mechanism of tumor cell growth inhibition, cell cycle and cell-cycle-related proteins were examined. Previous studies have shown that DFMO arrests a broad spectrum of tumor cell types, such as IEC-6, Hep-2, MKN45, and HL-60, in G1 phase (Wallace et al, 2003). Our recent work also demonstrated that treatment of Eca109 cells with Ad-ODCas causes lung cell cycle arrest in G1 phase (Tian et al, 2006a). In agreement with these findings, we demonstrated that both Ad-ODC-AdoMetDCas and Ad-ODCas decreased the rate of DNA synthesis of cancer cells and blocked cell cycle at the G1/S boundary. This result also suggests that synergistic inhibition of ODC and AdoMetDC activities may be more effective in inducing cell cycle arrest and halting cell growth than a single blockade of ODC activity. These data are in agreement with a report that treatment of MALME-3M cells with either the ODC inhibitor, DFMO, or the AdoMetDC inhibitor, MDL-73811, slows cell growth but fails to induce cell cycle arrest, and treatment with a combination of both inhibitors halts cell growth and causes a significant G1 arrest (Kramer et al, 2001).

We also assessed the effects of the two antisense constructs in the context of tumor invasiveness. Both Ad-ODC-AdoMetDCas and Ad-ODCas reduced the invasiveness of Eca109 cells compared with vector controls. Furthermore, the data also showed that Ad-ODC-AdoMetDCas was superior in inhibiting cancer cell invasion compared with Ad-ODCas infection. Overexpression of ODC has been suggested to confer an invasive phenotype on cells. Kubota and colleagues reported in 1997 that overexpression of ODC in mouse 10T1/2 fibroblasts induced not only cell transformation and anchorage-independent growth in soft agar, but also invasiveness through a Matrigel-coated filter. Similar work had been done by this same group (Kubota et al, 1995) that compared the invasiveness of mouse mammary carcinoma FM3A and EXOD cell lines that overexpress ODC and found that EXOD cells showed more than a 5.6-fold increase in invasiveness compared with FM3A cells by Matrigel assay. Inhibition of ODC by DFMO reduced invasiveness in breast cancer cells significantly (Manni et al, 2002). Our previous work in which ODC levels were reduced using the adenovirus-delivered antisense ODC found that lower ODC levels also inhibited tumor invasion in lung cancer (Tian et al, 2006a). ODC, however, is not the sole enzyme responsible for olyamine biosynthesis or tumor invasion. AdoMetDC was also proven to strongly correlate with progression of tumor invasiveness. Overexpression of AdoMetDC alone has been reported to be sufficient to transform NIH 3T3 cells and induce highly invasive tumors in nude mice (Manni et al, 1995). High expression levels of AdoMetDC may compensate for and strengthen the activity of ODC through different molecular pathways (Ravanko et al, 2004). Therefore, we simultaneously targeted both these critical enzymes and obtained superior inhibition of cancer invasion.

In summary, we provide evidence that polyamine reduction by antisense techniques that targeted ODC and AdoMetDCas suppresses cancer cell growth and invasiveness in vitro. Synergistic inhibition of both ODC and AdoMetDC activities by gene therapy approaches therefore might represent a novel treatment option for esophageal cancer.
References


