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Adenovirus-mediated Expression of Both Antisense Ornithine Decarboxylase and S-adenosylmethionine Decarboxylase Induces G_1 Arrest in HT-29 Cells

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To evaluated the effect of recombinant adenovirus Ad-ODC-AdoMetDCas which can simultaneously express both antisense ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) on cell cycle distribution in colorectal cancer cell and investigated underlying regulatory responses, human colorectal cancer cells HT-29 were cultured in RPMI 1640 medium and infected with Ad-ODC-AdoMetDCas. Cell cycle progression was detected by flow cytometry analysis. The expression levels of cell cycle regulated proteins were measured by Western blot analysis. The mRNA level of cyclin D1 was measured by RT-PCR. And a luciferase reporter plasmid of cyclin D1 promoter was constructed to observe the effect of Ad-ODC-AdoMetDCas on cyclin D1 promoter activity. The results showed that recombinant adenovirus Ad-ODC-AdoMetDCas significantly induced G_1 arrest, decreased levels of cyclin D1 protein and mRNA and suppressed the promoter activity. Ad-ODC-AdoMetDCas also inhibited nuclear translocation of β-catenin. In conclusion, downregulation of ODC and AdoMetDC mediated by Ad-ODC-AdoMetDCas transfection induces G_1 arrest in HT-29 cells and the arrest was associated with suppression of cyclin D1 expression and inhibition of β-catenin nuclear translocation. As a new anticancer reagent, the recombinant adenovirus Ad-ODC-AdoMetDCas holds promising hope for the therapy of colorectal cancers.

Keywords: Colorectal cancer, Cyclin D1, Ornithine decarboxylase, Polyamine, S-adenosylmethionine decarboxylase

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Introduction

The polyamines putrescine, spermidine and spermine are organic cations shown to participate in a bewildering number of cellular reactions (Janne *et al.*, 2004). Their positive charges enable polyamines to interact electrostatically with polyanionic macromolecules within the cell, such as DNA and RNA (Roberts *et al.*, 1992; Basu *et al.*, 1993). There are two critical enzymes controlling the polyamine metabolic pathway in eukaryotic cells. The first one is ornithine decarboxylase (ODC), which catalyzes the decarboxylation of ornithine to produce putrescine and has been the major target for anticancer investigation (Thomas *et al.*, 2003). The second rate-limiting enzyme is S-adenosylmethionine decarboxylase (AdoMetDC), which catalyzes the formation of decarboxylated S-adenosylmethionine (dcSAM). DcSAM donates its propylamine moiety for the biosynthesis of the spermidine and spermine by spermidine synthase and spermine synthase, respectively. AdoMetDC has been implicated to play a predominant role in tumor growth by promoting the formation of the more distal polyamines spermidine and spermine (Manni A *et al.*, 1995). Polyamine content is high in rapidly proliferating normal and neoplastic cells (Seile *et al.*, 1998; Faaland *et al.*, 2000). Polyamine and both rate-limiting enzymes play important roles in cell growth and cell cycle progression of normal and malignant cells (Gerner *et al.*, 2004).

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) (Wallace *et al.*, 2003a). 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 (Fredlund *et al.*, 1995). Both polyamines and cyclin/cdks show phased changes through the cell cycle, however, the interaction between these two sets of regulatory molecules remains to be defined.

In our previous study, we have constructed a replication-deficient recombinant adenovirus (Ad-ODC-AdoMetDCas) to transfer antisense ODC and AdoMetDC gene to cells. And we found that the downregulation of ODC and AdoMetDC induced by Ad-ODC-AdoMetDCas could lead to significant suppression of colorectal cancer cell growth (Zhang *et al*., 2006). In the present study we had evaluated the effect of Ad-ODC-AdoMetDCas on cell cycle distribution and investigated underlying regulatory responses. Our findings indicated a close association between G_1 arrest induced by Ad-ODC-AdoMetDCas and the suppression of cycling D1. We presumed that inhibition of cyclin D1 induced by Ad-ODC-AdoMetDCas is through β-catenin which is the key component in Wnt/β-catenin pathway.

Materials and Methods

Cells and reagents. Human colorectal cancer cell line HT-29 was obtained from Chinese Academy of Sciences. HT-29 cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated bovine serum and the antibiotics penicillin G potassium and streptomycin sulfate at 37ºC in a humidified 5% CO_2-containing incubator. Luciferase reporter plasmids pGL3-basic and pRL-TK were kindly supplied by Dr Yaoqin Gong (Institute of Medical Genetics, School of Medicine, Shandong University). Daul-Luciferase reporter assay kit were purchased from Promega. Mouse anti-β-actin monoclonal antibody were purchased from Santa Cruz; rabbit anti-human polyclonal antibody against cyclin D1, CDK4 and β-catenin protein were purchased from Neomarkers.

Flow cytometry analysis. The HT-29 cells were plated in 6-well plates at density of 3×10^5 cells/well and treated with 50 MOI Ad-GFP, Ad-ODC-AdoMetDCas, or PBS as a mock control. After the treatment for 72 h, cells were harvested via trypsinization, washed once with cold PBS, fixed with 70% ice-cold enthanol and stored at 4ºC. Cells were then washed with PBS and treated with RNase, and the DNA was stained with propidium iodide. The cell cycle phase distribution was analyzed with CXP Cytometer 1.0 (Beckman Coulter Cytomics" 500).

Western blot analysis. Proteins were extracted three days after treated with 50 MOI Ad-GFP, Ad-ODC-AdoMetDCas or PBS as a mock control. Whole cell lysates were prepared in extraction buffer containing 50 mM Tris (pH8.0), NP-40 (1%), aprotinin (1 μg/mL), sodium dodecylsulfate (0.1%), sodium azide (0.02%), NaCl (150 mM), phenylmethylsufonyl fluoride (100 μg/mL). The cytoplastic and nuclear proteins were prepared as described previously (Jiang *et al*., 2005). Sample protein concentrations were determined by Bicinchoninic Acid (BCA) protein assay. Proteins were subjected to 5% stacking and 12% resolving SDS-PAGE gels and transferred onto the nitrocellulose membranes (Millipore Bedford). After incubation with primary antibody followed by secondary antibody conjugated to horseradish peroxidase, the membranes were reacted for 5 min with SuperSingal west pico chemiluminescent substrate (Pierce) and exposed to X-rays films (Kodak). X-rays were analyzed using light-density analysis software (AlphaImager).

RT-PCR analysis of cyclin D1 mRNA expression. Total RNA was extracted from untreated and treated cells using Trizol reagent and 5 μg of RNA of each sample was used for subsequent reverse transcription according to the manufacturer's protocols (Invitrogen). Polymerase chain reaction (PCR) analysis for cyclin D1 and internal housekeeping gene β-actin were performed by specific pairs of primers, cyclin D1 forward 5' CCAGAGGCGGAGGAGA ACAA 3', cyclin D1 reverse 5' GAGGCGGTAGTAGGACAGGA 3', β-actin forward 5' CCACTGGCATCGTGATGGAC 3', β-actin reverse 5' ACGGATGTCCACGTCACACT 3'. PCR reactions (35 cycles) were 94ºC for 40 s, 59ºC for 40 s, 72ºC for 1 min with initial inactivation of enzyme at 94ºC for 5 min and another 7 min for elongation at 72ºC after the cycles were finished. The PCR products were analyzed by electrophoresis on a 1% agarose gel.

Construction of luciferase reporter plasmid pGL3-cyclin D1. Total DNA was extracted from human blood sample. Then, we used the DNA as the templates to perform PCR for the amplification of cyclin D1 promoter region from −382 to +104, which harbored TCF-4 response elements from −81 to −73 (Shtutman *et al*., 1999). The upstream primer is 5' TTGAGCTC CGGCCTTCCTAGTTGTC 3' and the downstream primer is 5' TCAAGCTTCGCTCGGCTCTCGCTTC 3' (enzyme recognition sites are underlined). PCR reactions (35 cycles) were 94ºC for 40 s, 59ºC for 40 s, 72ºC for 1 min 30 s with initial inactivation of enzyme at 94ºC for 5 min and another 7 min for elongation at 72ºC after the cycles were finished. The amplified products were purified by Qiagen gel extraction kit (Qiagen) and then ligated into the TA clone vector (pMD18-T). Both the recombinant TA vector pMD18-cyclin D1 and pGL3-basic null vector were digested with *Sac*I and *Hind*III. The desired fragments of 480 bp and 4.8kb were purified separately. The two purified DNA fragments were ligated with T_4 ligase at 16ºC overnight and then transformed into competent cells DH5α. The positive clones (pGL3-cyclin D1) and their insert directions were confirmed by digestion and sequencing.

Transient transfection and luciferase activity assay. The HT-29 cells were plated in 24-well plates at density of 1×10^5 cells/well. pGL3-basic and pGL3-cyclin D1 were transfected into cells separately. And pRL-TK control vector was co-transfected for normalization of transfection efficiency. Transfection was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacture's instructions, and cells were harvested 72 h post-transfection for promoter activities assay.

The same as described above, pGL3-cyclin D1 and pRL-TK were co-transfected into HT-29 cells in 24-well plates (1×10^5 cells/well). After 24 h, cells were then respectively treated with 50 MOI Ad-GFP, Ad-ODC-AdoMetDCas or PBS as a mock control. After the treatment for 72 h, cells were harvested for promoter activities assay. Luciferase activity assays were performed using the Daul-Luciferase reporter assay system according to the manufacturer's protocol.

Statistical analysis. Data in this paper are presented as mean ± SD from three separated experiments. Student's *t*-test was used to compare the data, and $p < 0.05$ was taken as the level of significance. All results were analyzed by statistical software SPSS 10.0.

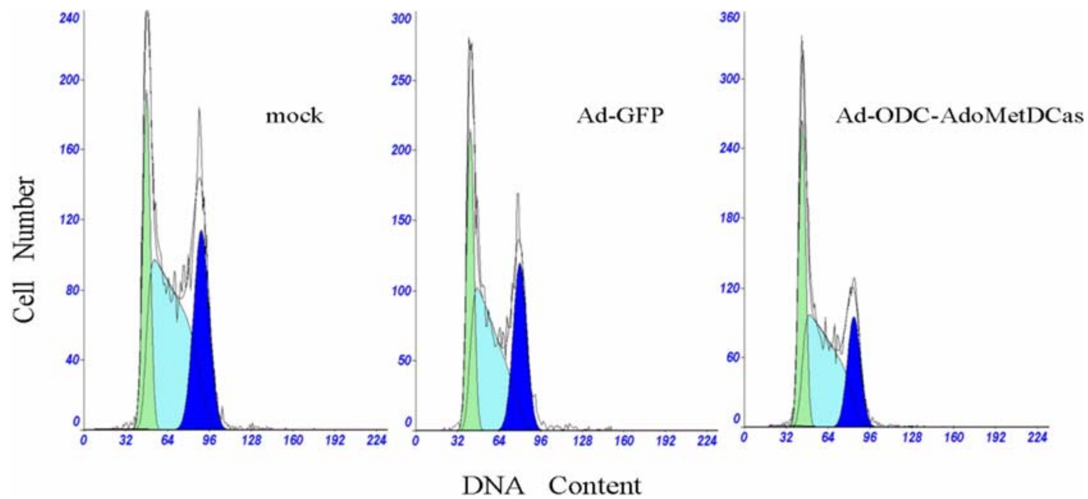

Fig. 1. Flow cytometer analysis of HT-29 cells (G_1 phase in ■, S phase in ■, G_2 phase in ■). Cells were treated with 50 MOI Ad-GFP, Ad-ODC-AdoMetDCas or PBS as a control for three days and then collected and dyed by propidium iodide for cell cycle analysis. The data are representative of three separate experiments.

Results

Ad-ODC-AdoMetDCas induced G_1 arrest of HT-29. As shown in Fig. 1, treatment with Ad-ODC-AdoMetDCas induced a progressive accumulation in G_1 phase cells, a profile characteristic of G_1 arrest ($p < 0.05$). Results in Table 1 showed that Ad-ODC-AdoMetDCas treatment induced 59.3 ± 3.9% of cells to arrest in G_1 phase, compared with 37.5 ± 2.1% in PBS and 41.8 ± 3.5% in Ad-GFP-treated conditions. There was no significant difference in Ad-GFP-treated and uninfected cells ($p > 0.05$).

Ad-ODC-AdoMetDCas inhibited expression of cyclin D1. We further analyzed whether the main cell cycle regulatory proteins of G_1 phase were altered after adenoviral gene transfer and whether they correlated with cell cycle arrest. Expression of cyclin D1 and CDK4 in HT-29 cells were detected by Western blot analysis. The results showed that cyclin D1 protein decreased more than 60% in Ad-ODC-AdoMetDCas-treated cells compared with Ad-GFP or PBS-treated cells, but there were no obvious changes in the CDK4 expression in three groups (Fig. 2A). We also analyzed the mRNA level of cyclin D1 by RT-PCR, which showed that the fragment of cyclin D1 (183 bp) decreased significantly in Ad-ODC-AdoMetDCas-treated cells compared with the other groups (Fig. 2B). Our data indicate that downexpression of cyclin D1 correlates with cell cycle arrest in G_1.

Table 1. G_0-G_1 cell cycle phase distribution of HT-29 cells

Treatment	G_0-G_1 (%) ($\overline{X} \pm s$)
+mock	22 ± 2.1
+Ad-GFP	25 ± 3.5
+Ad-ODC-AdoMetDCas	41 ± 3.9*

*$p < 0.05$ versus Ad-GFP- and PBS-treated cells.

Identification of luciferase reporter plasmid pGL3-cyclin D1. After demonstrating Ad-ODC-AdoMetDCas suppressed cyclin D1 protein and mRNA expression levels in HT-29 cells, we next evaluated the effect of Ad-ODC-AdoMetDCas on cyclin D1 promoter. The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway (Shtutman *et al*., 1999) and the pathway is active in HT-29 cells, so we constructed luciferase reporter plasmid of cyclin D1 promoter which harbored TCF/LEF response elements but not full-length promoter region. The cyclin D1 promoter region harbored TCF/LEF response elements was amplified by PCR, the PCR product was performed on agarose gel electrophoresis. Approximately 486 bp band was obtained which was consistent with the size of the promoter region from −382 to +104 (Fig. 3A, lane 1). A fragment with equal size to the promoter region was also produced after the digestion of constructed pGL3-cyclin D1 plasmid by *Sac*I and *Hind*III (Fig. 3A, lane 2), which suggested the PCR product was successfully inserted into pGL3-basic null vector. The sequencing results further confirmed the exactness of the fragment's sequence and direction (data not shown).

Luciferase activity assay. After cyclin D1 promoter luciferase reporter plasmid was constructed successfully, the plasmid was transfected into HT-29 cells for luciferase activity assay. M1/M2 represents luciferase relative activity. The results showed that the 486bp-fragment presented a promoter activity (2.6 ± 0.2) which was 50 fold stronger than that of pGL3-basic (0.05 ± 0.001).

We next examined whether pGL3-cyclin D1 could be regulated by Ad-ODC-AdoMetDCas. Luciferase activity assay showed that cyclin D1 promoter activity decreased significantly in Ad-ODC-AdoMetDCas-treated cells compared with Ad-GFP or PBS-treated cells (Fig. 3B). As shown in Table 2, cyclin D1 promoter activity was 1.4 ± 0.22 in Ad-ODC-

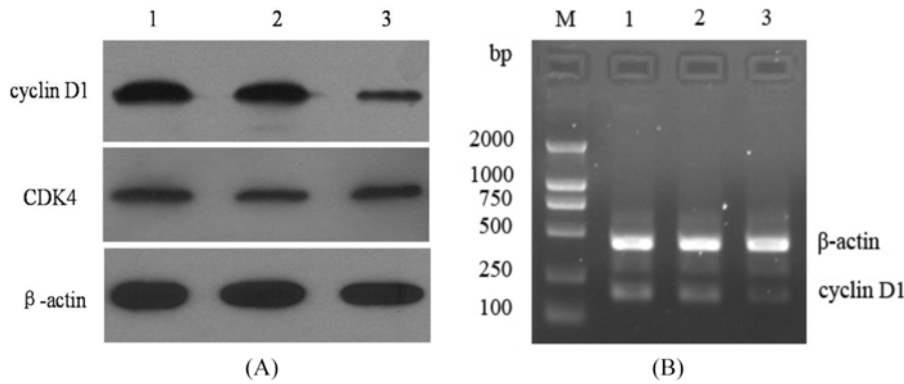

Fig. 2. Downregulation of cyclin D1 expression in HT-29 cells treated with Ad-ODC-AdoMetDCas. A, western blot analysis. Cyclin D1 protein in Ad-ODC-AdoMetDCas-treated cells (lane3) was significantly downregulated compared to mock-treated cells (lane1) and Ad-GFP-treated cells (lane 2). CDK4 protein was not affected by Ad-ODC-AdoMetDCas. B, RT-PCR analysis. Cyclin D1 mRNA (183bp) in Ad-ODC-AdoMetDCas-treated cells (lane3) was significantly downregulated compared to mock-treated cells (lane1) and Ad-GFP-treated cells (lane 2). Lane M was DL-2000 marker. The β-actin RT-PCR was set as the control.

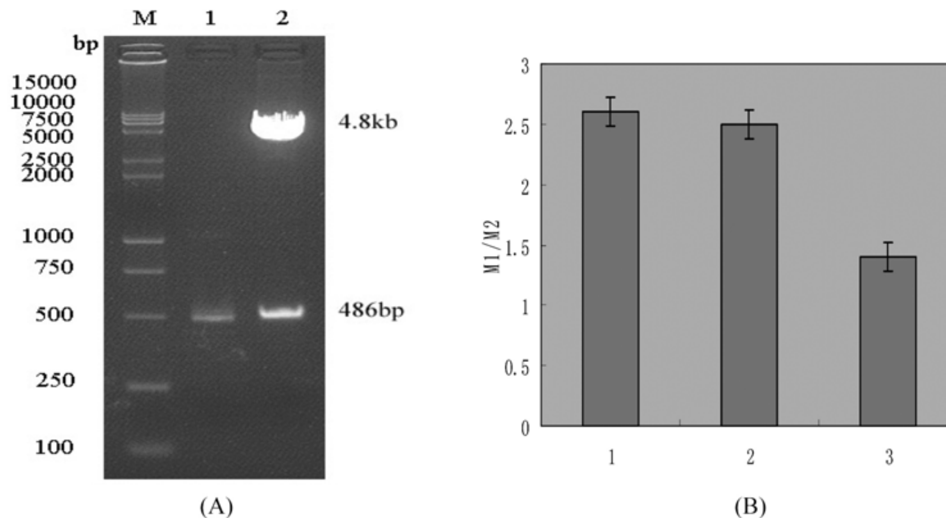

Fig. 3. Construction and activity assay of Cyclin D1 promoter luciferase reporter plasmid. A, product from PCR and restriction digestion of pGL3-cyclin D1, Lane M: DL-2000+15000 marker; lane 1: cyclin D1 PCR product (486bp); lane 2: pGL3-cyclin D1 digested by *Sac*I and *Hind*III (486bp and 4.8kb); B, luciferase activity assay of cyclin D1 promoter. The activity of cyclin D1 promoter was significantly downregulated in Ad-ODC-AdoMetDCas-treated cells (lane3), but in mock-treated cells (lane1) and Ad-GFP-treated cells (lane 2) didn't show significant difference.

AdoMetDCas-treated cells compared with 2.5 ± 0.25 in Ad-GFP-treated and 2.6 ± 0.18 in PBS-treated cells ($p < 0.05$). Our data indicate that Ad-ODC-AdoMetDCas inhibits cyclin D1 expression by suppressing promoter activity.

Table 2. Cyclin D1 promoter activity in HT-29 cells

Treatment	M1/M2 ($\overline{X}$ ± s)
+mock	2.6 ± 0.18
+Ad-GFP	2.5 ± 0.25
+Ad-ODC-AdoMetDCas	1.4 ± 0.22*

*$p < 0.05$ versus Ad-GFP- and PBS-treated cells.

Ad-ODC-AdoMetDCas inhibited the translocation of β-catenin. After confirming that Ad-ODC-AdoMetDCas inhibited cyclin D1 promoter activity which harbored TCF/LEF response elements, we then analyzed the effect of Ad-ODC-AdoMetDCas on the level of β-catenin which is the important upstream regulator of cyclin D1 in Wnt/β-catenin pathway. The results showed that there were no obvious changes of total β-catenin in three groups cells but the nuclear level of β-catenin decreased and the cytoplastic level increased in Ad-ODC-AdoMetDCas-treated cells compared with Ad-GFP-treated or PBS-treated cells (Fig. 4). The data suggest that Ad-ODC-AdoMetDCas has no effect on total β-catenin level but inhibits the translocation from cytoplasm to nucleus of β-

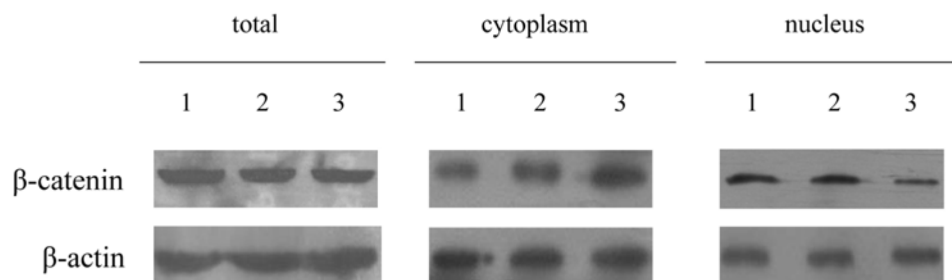

Fig. 4. Translocation inhibition of β-catenin in HT-29 cells treated with Ad-ODC-AdoMetDCas. The total, cytoplastic and nuclear proteins were estimated by western blot analysis respectively. No changes were found of total β-catenin in three groups. But the cytoplastic level of β-catenin increased and the nuclear level decreased in Ad-ODC-AdoMetDCas-treated cells (lane 3) compared to mock-treated cells (lane1) and Ad-GFP-treated cells (lane 2). An immunoblot with antibody against β-actin was set as a control.

catenin. The nuclear translocation was suppressed more than 50%. The reduction of nuclear β-catenin accounts for suppression of cyclin D1 promoter activity.

Discussion

Polyamines are essential for normal cell growth, and aberrant polyamine metabolism is known to play an important role in the development of tumors (Hillary *et al*., 2003). In colorectal cancer, the activities of ODC and AdoMetDCas and the level of polyamine content are increased by 3- to 4-fold over those found in the equivalent normal tissue (Milovica *et al*., 2001). A growing body of literature suggests that inhibition of the polyamine biosynthetic enzymes leads to cessation of cell growth (Yerlikaya, 2004). α-Difluoromethylornithine (DFMO) acting as a suicide inhibitor ODC was the first effective, rationally designed antiproliferative drug aimed at depleting polyamine from cells (Wallace *et al*., 2003b). Similarly, SAM486A as inhibitor of AdoMetDC has been applied to study the suppression effect on cancer cell growth (Paridaens *et al*., 2000; Hu *et al*., 2004). We constructed adenovirus vectors to transfer antisense ODC and AdoMetDCas gene to colorectal cancer cells which could inhibit both ODC and AdoMetDCas. Our previous study showed that Ad-ODC-AdoMetDCas significantly reduced both ODC and AdoMetDCas protein levels in HT-29 cells and suppressed the cell growth compared with vector infection.

Flow cytometry analysis showed that Ad-ODC-AdoMetDCas affected progression of HT-29 cells through the G_1/S check point. In fact, ODC plays an important role in the G_1/S transition in the cell cycle (Mayeur *et al*., 2005). Ordesson S.M. found that the increase in ODC activity in conjunction with the G_1/S transition took place and the doubling of the AdoMetDC mRNA content took place when there was a doubling of the AdoMetDC activity at the G_1/S transition (Ordesson, 2003). To study the mechanism arresting cell cycle in G_1 phase by Ad-ODC-AdoMetDCas, we detected the cyclin D1 and CDK4 levels in HT-29 cells. Cyclin D1 accumulates in the cell nucleus during the G_1 phase of the cell cycle and is rate limiting and essential for the progression through the G_1 phase (Li *et al*., 2003). Cyclin D1 mainly binds to and activates the cyclin-dependent kinases CDK4 to drive cell through G_1/S transition. Western blotting analysis demonstrated that Ad-ODC-AdoMetDCas infection significantly reduced cyclin D1 protein level but did not decrease CDK4 level. We also detected the mRNA level of cyclin D1 by RT-PCR and found that the mRNA level was reduced by Ad-ODC-AdoMetDCas. Previous studies have shown the link between polyamine levels and cyclin D1 examined by using a bis (benzyl) spermine analogue, $C_6H_5CH_2NH(CH_2)_3NH(CH_2)_7NH(CH_2)_3NHCH_2C_6H_5$ (Thomas *et al*., 1997). The polyamine analogue induced a decrease in polyamine levels associated with a decrease in cyclin D1 levels and cell cycle arrest in the G_1 phase. In consistency with the preceding finding, Ad-ODC-AdoMetDCas downregulated the expression of cyclin D1 which was involved in G_1 arrest of HT-29 cells.

Induction of cyclin D1 is growth factor-dependent and tightly regulated, either at the level of transcriptional activation, protein expression or cellular localization. Several transcription factors such as STAT proteins, NF-kB, Egr-1, Ets, CREB and nuclear receptors have been found to transactivate the cyclin D1 promoter (Olivier, 2002). In colorectal carcinomas, cyclin D1 protein overexpression is dependent on β-catenin protein dysregulation (Wong *et al*., 2002). Cyclin D1 transcription is activated by β-catenin, the main protein overexpressed in colorectal cancer following the loss of the tumor-suppressor gene adenomatous polyposis coli (APC). β-catenin activates transcription from the cyclin D1 promoter, probably through activation of TCF/LEF-binding sites (Tetsu *et al*., 1999). Through harnessing luciferase reporter plasmid pGL3-cyclin D1 successfully constructed by us, which harbored TCF/LEF response elements, the present study showed that Ad-ODC-AdoMetDCas reduced cyclin D1 promoter activity, so we presumed that Ad-ODC-AdoMetDCas downregulated cyclin D1 transcription through inhibited the activity of cyclin D1 promoter activated by β-catenin.

β-catenin-mediated signaling depends on its accumulation and subsequent translocation into the nucleus. Then, β-catenin combines with members of the TCF/LEF family to form

complexes that can promote the expression of several genes, such as c-myc and cyclin D1 (Morin, 1999). HT-29 cells lack wild type APC, which contain two truncated forms of APC. Loss of APC function results in elevated Wnt/β-catenin signaling and switched expression of β-catenin from intercellular junctions to the nucleus and cytoplasm, and translocation of the protein into the nucleus activates the transcription of candidate target genes (Aust *et al*., 2002). Our results showed that Ad-ODC-AdoMetDCas treatment inhibited the translocation of β-catenin from cytoplasm to nucleus. The inhibition of nuclear translocation could account for transcription inhibition of cyclin D1 which is the downstream target gene of β-catenin pathway. In normal intestinal epithelial cell (IEC-6), polyamine depletion induced by DFMO is associated with the decrease of E-cadherin and β-catenin protein levels (Wang, 2005). And further studies show depletion of cellular polyamines reduces intracellular Ca^{2+} concentration and decreases tyrosine phosphorylation of β-catenin, which accompany a decrease in epithelial cell migration (Guo *et al*., 2002). The underlying mechanisms that Ad-ODC-AdoMetDCas affects β-catenin protein remain to be studied further.

In summary, our data provided evidences that G_1 arrest in the HT-29 cells induced by Ad-ODC-AdoMetDCas were associated with suppression of cyclin D1 expression and downregulation of cyclin D1 promoter activity through Wnt/β-catenin pathway. Dowmregulation of ODC and AdoMetDC mediated by Ad-ODC-AdoMetDCas may be a potential therapeutic approach for colorectal cancers.

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