ACTIVATION OF TGF-β/SMAD SIGNALING PATHWAY FOLLOWING POLYAMINE DEPLETION IN INTESTINAL EPITHELIAL CELLS

Lan Liu,1,2 Rachel Santora,1,2 Jaladanki N. Rao,1,2 Xin Guo,1,2 Tongtong Zou,1,2 Huifang M. Zhang,1,2 Douglas J. Turner,1,2 and Jian-Ying Wang1,2,3

Departments of 1Surgery and 3Pathology, University of Maryland School of Medicine and
2Baltimore Veterans Affairs Medical Center, Baltimore, Maryland 21201

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Submitted to: Am J Physiol-GI and Liver Physiology
Address Correspondence to:
Dr. Jian-Ying Wang
Department of Surgery
Baltimore Veterans Affairs Medical Center
10 North Greene Street
Baltimore, MD 21201

Phone: 410-605-7000 Ext. 5678
Fax: 410-605-7919
E-mail: jwang@smail.umaryland.edu
ABSTRACT

Smad proteins are transcription activators that are critical for transmitting transforming growth factor-β (TGF-β) superfamily signals from the cell-surface receptors to the nucleus. Our previous studies have shown that cellular polyamines are essential for normal intestinal mucosal growth and that a decreased level of polyamines inhibits intestinal epithelial cell proliferation, at least partially, by increasing expression of TGF-β/TGF-β receptors. The current study went further to determine the possibility that Smads are the downstream intracellular effectors of activated TGF-β/TGF-β receptor signaling following polyamine depletion. Studies were conducted in IEC-6 cells derived from rat small intestinal crypts. Depletion of cellular polyamines by α-difluoromethylornithine (DFMO) increased basal levels of Smad3 and Smad4 proteins, induced their nuclear translocation, and stimulated Smad sequence-specific DNA-binding activity. Polyamine depletion-induced Smads also were associated with a significant increase in transcription activation as measured by luciferase reporter gene activity of Smad-dependent promoters. Inhibition of Smads by a dominant negative mutant Smad4 in the DFMO-treated cells prevented the increased Smad transcription activation. Polyamine-deficient cells highly expressed TGF-β and were growth-arrested at the G₁ phase. Inhibition of TGF-β by treatment with either immunoneutralizing anti-TGF-β antibody or TGF-β antisense oligodeoxyribonucleotides not only blocked the induction of Smad activity, but also decreased the Smad-mediated transcriptional activation in polyamine-depleted cells. These findings suggest that Smads are involved in the downstream cellular processes mediated by cellular polyamines and that increased TGF-β/TGF-β receptor signaling following polyamine depletion activates Smads, thus resulting in the stimulation of Smad-target gene expression.
Key Words: growth arrest; TGF-β and TGF-β receptor; ornithine decarboxylase; α-difluoromethylornithine; crypts
INTRODUCTION

The Smad proteins are a family of transcriptional activators that are critical for transmitting the transforming growth factor-\(\beta\) (TGF-\(\beta\)) superfamily signals from the cell surface to the nucleus \((1,54)\). Based on distinct functions, Smads are grouped into three classes: the receptor-regulated Smads (R-Smads), Smad2 and Smad3; the common-Smad (co-Smad), Smad4; and the inhibitory Smads (I-Smads), Smad6 and Smad7 \((10,12,24,29)\). All TGF-\(\beta\) family members, including TGF-\(\beta\)s, activins and bone morphogenetic proteins, use type I and type II transmembrane serine/threonine kinase receptors in a variety of cell types \((1,10,12,29,32)\). Upon ligand binding, the activated type II kinase phosphorylates the type I receptor, which subsequently phosphorylates the R-Smads on a C-terminal SSXS motif. This induces dissociation of the R-Smad from the receptor, stimulates the assembly of a heteromeric complex between the phosphorylated R-Smads and Smad4, and results in the nuclear accumulation of this heteromeric Smad3/Smad4 complex \((4,10,29)\). In the nucleus, Smads bind to a specific DNA site \((\text{GTCTAGAC})\) and cooperate with various transcription factors in regulating target gene expression \((5,23,51)\).

The epithelium of the intestinal mucosa has the most rapid turnover rate of any tissue in the body and its integrity depends on the balance between cell proliferation, growth arrest, and apoptosis \((26,37)\). Intestinal epithelial cells continuously replicate in the proliferating zone within the crypts and differentiate as they migrate up the luminal surface of the colon and villous tips in the small intestine \((13,14)\). Mature differentiated cells at the luminal surface and villous tips are quickly lost through the process of apoptotic cell death and are replaced by new cells. This rapid dynamic turnover rate of intestinal epithelial cells is highly regulated and critically controlled by numerous factors including cellular polyamines \((27,30,48)\). The natural
polyamines spermidine and spermine and their precursor, putrescine, are ubiquitous organic cations of low molecular weight in eukaryotic cells and are intimately involved in distinct cellular functions (30,45). A series of observations from our previous studies (19,34,35,48,49) and others (27,30) have shown that normal intestinal mucosal growth depends on the supply of polyamines to the dividing cells within the crypts and that polyamines, either synthesized endogenously or supplied luminally, are absolutely required for epithelial cell division. Increasing cellular polyamines stimulates intestinal epithelial cell renewal, and decreasing cellular polyamines inhibits cell proliferation both in vivo and in vitro.

We (35) have recently demonstrated that depletion of cellular polyamines induces the activation of TGF-β gene expression through posttranscriptional regulation and that increased gene product, TGF-β, plays a critical role in the inhibition of normal intestinal epithelial cell proliferation. Polyamine-deficient cells also highly express the TGF-β type I receptor, which associates with increased sensitivity to growth inhibition when these cells are exposed to exogenous TGF-β (39). The current study went further to determine the possibility that Smad proteins are the downstream intracellular effectors of activated TGF-β/TGF-β receptor signaling in polyamine-deficient cells. First, we examined whether polyamine depletion by inhibition of ornithine decarboxylase (ODC), the rate-limiting enzyme in the biosynthesis of polyamines, increased Smad activity in normal intestinal epithelial cells (IEC-6 line). Second, we examined whether this increased Smad activity following polyamine depletion specifically activated Smad-dependent promoters. Third, we determined whether the observed Smad activation resulted from increased expression of TGF-β in polyamine-deficient cells. Some of these data have been published in abstract form (25).
MATERIALS AND METHODS

Chemicals and supplies. Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Tissue culture media and dialyzed fetal bovine serum (dFBS) were obtained from Invitrogen (Carlsbad, CA), and biochemicals were from Sigma (St. Loius, MO). The double-stranded oligonucleotides used in electromobility shift assay and specific antibodies against Smad3 and Smad4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). a-difluoromethylornithine (DFMO) was purchased from Ilex Oncology Inc. (San Antonio, TX). The [α-32P]dCTP (3,000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL).

Reporter plasmids and recombinant adenovirus construction. Three Smad-dependent reporters used in this study were derived from the pGL3 promoter luciferase vector (Promega, Madison, WI). The pJun-Luc construct containing the −79 to +170 sequence of the human c-Jun promoter with three copies of connective Smad-binding element (SBE) is a gift from Dr. XF Wang (Duke University School of Medicine, Durham, NC) (51). The pPAI1-Luc construct contains the −800 to +22 sequence of the type-1 plasminogen activator inhibitor (PAI-1) gene promoter that has three copies of separated SBE (gift of Dr. ZJ Sun, Stanford University School of Medicine, Palo Alto, CA) (9,43). The reporter plasmid pSBE-Luc containing the synthetic sequence with four copies of connective SBE is a gift from Dr. J Duyster (Technical University of Munich, Munich, Germany) (44,54). A recombinant adenovirus expressing a dominant negative mutant Smad4 (AdMSmad4) was a generous gift from Dr. DM Simeone (University of Michigan, Ann Arbor, MI) (41). The dominant negative Smad4 construct was bluntly ligated into the EcoRV site of the shuttle vector (pAdTrack), which has an independent cytomegalovirus-driven green fluorescent protein (GFP) expression construct as a marker. Recombinant adenoviral plasmids were packaged into infectious adenoviral particles by
transfecting human embryonic kidney (HEK) 293 cells by using lipofectAMINE PLUS reagent. The adenoviral particles were propagated in HEK293 cells and purified on cesium chloride ultracentrifugation. Titers of the adenoviral stock were determined by standard plaque assay. Recombinant adenoviruses were screened for expression of the introduced genes by fluorescent microscopy. pAdeno-X, which was the recombinant replication-incompetent adenovirus carrying no cDNA insert (AdNull), was grown and purified as described above and served as a control adenovirus. Cells were infected by various concentrations of AdMSmad4 or AdNull and cell samples were collected for various measurements 72 h after the infection.

**Cell culture.** The IEC-6 cell line was purchased from ATCC at passage 13. The cell line was derived from normal rat intestinal crypt cells and was developed and characterized by Quaroni et al (38). Stock cells were maintained in T-150 flasks in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% heat-inactivated FBS, 10 μg/ml insulin, and 50 μg gentamicin sulfate/ml. Flasks were incubated at 37°C in a humidified atmosphere of 90% air-10% CO₂ and passages 15-20 were used in the experiments. There were no significant changes of biological function and characterization of IEC-6 cells at passages from 15 to 20 (19,34).

**Experimental design.** The first series of studies were to examine whether polyamine depletion by treatment with DFMO increased Smad activity in IEC-6 cells. The general protocol of the experiments and methods were similar to those described previously (35,39). Briefly, IEC-6 cells were plated at 6.25 × 10⁴ cells/cm² and grown in control medium (DMEM + 5% dialyzed FBS + 10 μg insulin and 50 μg gentamicin sulfate/ml) or the DMEM medium containing 5 mM DFMO or DFMO plus 5 mM spermidine for 4, 6, and 8 days. The Smad3 and Smad4 protein levels, their cellular distribution, and Smad sequence-specific DNA binding
activity were measured by Western blotting analysis, immunohistochemical staining, and electrophoretic mobility shift assays, respectively.

The second series of studies were to determine whether observed activation of Smads following polyamine depletion had the transcription activity in IEC-6 cells. Three widely accepted luciferase reporters of Smad-dependent promoters were used in this study and the activity of luciferase reporters was measured in cells grown in the DMEM medium containing DFMO alone or DFMO plus SPD for 6 days.

The third series studies were to define the relationship between increased expression of TGF-β and Smad activation in polyamine-deficient cells. Functions of TGF-β were examined by using immunoneutralizing anti-TGF-β antibody or antisense TGF-β oligodeoxynucleotides. Cells were initially treated with DFMO for 3 days, exposed to the media containing DFMO plus either anti-TGF-β antibody or antisense TGF-β oligodeoxynucleotides for 24 h, and then transfected with various luciferase reporter constructs. Levels of Smad3 and Smad4 proteins and their transcriptional activity were measured 48 h after transfection.

Measurement of TGFβ content. The levels of TGFβ in culture supernants was measured with the use of the TGFβ1 ELISA system (Promaga, Madison, WI). After cells in 30-mm dishes were grown in the presence or absence of DFMO for various times, the monolayer of cells was washed once with D-PBS, and then 1 ml of fresh medium was added. The medium was collected following 12 h of further culture, and the content of TGFβ was measured according to the manufacture’s instructions. Cells were dissolved in 0.5 ml of 0.5 N NaOH at 37°C in humidified air for 90 min. The protein content of an aliquot of cell lysate was determined by the method described by Bradford (2). The levels of TGFβ content was normalized by protein and expressed as picograms per milliliter per milligram of protein.
**Western blot analysis.** Cell samples, placed in SDS sample buffer, were sonicated and then centrifuged (2,000 rpm) at 4°C for 30 min. The supernatant from cell samples was boiled for 10 min and then subjected to electrophoresis on 10% acrylamide gels according to Laemmli (16). After the transfer of protein onto nitrocellulose filters, the filters were incubated for 1 h in 5% non-fat dry milk in 1³ phosphate-buffered saline/Tween 20 (PBS-T: 15 mM NaH₂PO₄, 80 mM Na₂HPO₄, 1.5 M NaCl, pH 7.5, and 0.5% (vol/vol) Tween 20). Immunological evaluation was then performed for 1 h in PBS-T buffer containing 0.2 mg/ml the specific antibody against Smad3 or Smad4 protein. The filters were subsequently washed with 1³ PBS-T and incubated for 1 h with the second antibody conjugated to peroxidase. After extensive washing with 1³ PBS-T, the immunocomplexes on the filters were reacted for 1 min with Chemiluminescence Reagent (Du Pont NEN, NEL-100). Finally, the filters were placed in a plastic sheet protector and exposed to autoradiography film.

**Preparation of nuclear protein and electrophoretic mobility shift assays.** Nuclear proteins were prepared by the procedure described previously (20,53) and the protein contents in nuclear preparation were determined by the method described by Bradford (2). The double-stranded oligonucleotides used in these experiments were 5'-TCGAGAGCCAGACA-AAAAGCCAGACATTAGCCAGACAC-3', which contains the consensus Smad binding sites that are underlined. These oligonucleotides were radioactively end-labeled with [γ³²P] ATP and T4 polynucleotide kinase (Promega). For mobility shift assays, 0.035 pmol ³²P-labeled oligonucleotides (~30,000 cpm) and 10 μg nuclear protein were incubated in a total volume of 25 μl in the presence of 10 mM Tris-HCl (pH7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% of glycerol, and 1 μg poly (dl-dc). The binding reactions were allowed to proceed at room temperature for 20 min. Thereafter, 2 μl of bromophenol blue (0.1% in water) were added, and
protein-DNA complexes were resolved by electrophoresis on nondenaturing 5% of polyacrylamide gels and were visualized by autoradiography. The specificity of binding interactions was assessed by competition with an excess of unlabeled double-stranded oligonucleotide of identical sequence.

**Immunohistochemical staining.** The immunofluorescence procedure was carried out according to the method of Vielkind and Swierenga (47) with minor changes (7). Cells were incubated with the primary antibody against Smad3 or Smad4 at 4°C overnight and then incubated with secondary antibody conjugated with FITC for 2 h at room temperature. After the slides were rinsed three times, they were mounted and viewed through a Zeiss confocal microscope (model LSM410). Images were processed using Photoshop software (Adobe, San Jose, CA).

**Luciferase assays and transfection.** Transient transfections were performed using the LipofectAMINE kit and performed as recommended by the manufacturer (Invitrogen, Carlsbad, CA). The cells were collected at 48 h after the transfection and luciferase activities of reporters were assayed with a commercial kit (Promega). The luciferase activity from individual transfections was normalized by β-gal activity from cotransfected plasmid pCMV β-galactosidase. The experiments were done in triplicate and are reported as mean relative light units/β-galactosidase.

**Statistics.** All data are expressed as means ± SE from six dishes. Autoradiographic and immunohistochemical staining experiments were repeated three times. The significance of the difference between means was determined by analysis of variance. The level of significance was determined using the Duncan’s multiple range tests (8).
RESULTS

Effect of polyamine depletion on Smad3/Smad4 protein expression and their cellular distribution. Our previous studies demonstrated that exposure of IEC-6 cells to 5 mM DFMO for 4, 6, and 8 days almost completely depleted cellular polyamines and that decreased levels of polyamines resulted in a significant increase in expression of TGFβ and the TGFβ type I receptor (17,49). Because the activated TGFβ receptors propagate the signal by increasing Smad activity in a variety of cell types (1,54), the focus of the current study was to determine whether the altered TGFβ expression following polyamine depletion is associated with changes in Smad proteins in normal intestinal epithelial cells. Results presented in Figure 1A show that depletion of cellular polyamines by treatment with DFMO increased TGFβ content in culture medium, which was completely prevented by exogenous spermidine. Consistently, basal levels of Smad3 and Smad4 proteins also increased significantly in DFMO-treated cells. The induction of protein levels for Smad3 and Smad4 occurred at day 4 and remained elevated 6 and 8 days after exposure to DFMO (Fig. 1, B and C). The levels of Smad3 in cells exposed to DFMO were ~5.1, ~5.3, and ~5.5 times the normal values (without DFMO) at 4, 6, and 8 days after DFMO, respectively. Although basal expression of Smad4 protein was higher than that of Smad3 in IEC-6 cells, levels for the Smad4 also increased significantly and were more than twice the normal values 4, 6, and 8 days after DFMO treatment. Spermidine (5 mM) given together with DFMO completely prevented the increased levels of Smad3/Smad4 proteins. Putrescine (10 mM) had an effect equal to spermidine on Smad3/4 expression when it was added to cultures that contained DFMO (data not shown).

To extend the finding of increased Smad expression following polyamine depletion, we further explored the cellular distribution of Smad3/Smad4 proteins in the cells grown in the
presence or absence of DFMO for 6 days with the use of immunohistochemical staining technique. In control cells, the immunostainings for Smad3 were just visible (Fig. 2A, left), but the apparent immunoreactivities for Smad4 were observed in a loosely defined perinuclear area (Fig. 2A, right). Consistent with our data from Western blot analysis, these immunostainings for Smad3/Smad4 increased markedly after depletion of cellular polyamines by DFMO (Fig. 2B). Increased Smad3/Smad4 proteins in DFMO-treated cells were present just inside a defined nuclear area. In the presence of DFMO, spermidine prevented the increased immunostaining levels for Smad3/Smad4 and blocked their translocation to the nucleus (Fig. 2C). The cellular distribution of Smad3/Smad4 in cells treated with DFMO plus spermidine was indistinguishable from that in control cells (Fig. 2, A vs. C).

Changes in Smad sequence-specific DNA binding activity in polyamine-deficient cells. Consistently, increased levels of Smad3/Smad4 proteins in cells exposed to DFMO were paralleled by a marked increase in Smad-binding activity as measured by electrophoretic mobility shift assays (Fig. 3A). The Smad-binding activities were increased by approximately six-folds 4, 6, and 8 days after DFMO treatment. Spermidine given together with DFMO completely prevented the increase in Smad-binding activity. To evaluate the specificity of the binding reaction in Fig. 3A, competitive inhibition experiments were carried out in control cells and cells exposed to DFMO for 6 days. The Smad-binding activity in both control (Fig. 3Bα) and DFMO-treated cells (Fig. 3Bβ) was dose-dependently inhibited when various concentrations of the unlabeled Smad oligonucleotide were added to the binding reaction mixture. We also examined the effect of the unlabeled oligonucleotide containing a mutated Smad binding site on Smad binding activity and demonstrated that the Smad-mutated oligonucleotide did not inhibit Smad-binding activity in IEC-6 cells (data not shown). Results presented in Fig. 3Bγ further
show that preincubation of nuclear extracts with the specific antibody against Smad3 or Smad4 significantly inhibited the formation of Smad-binding complex. In contrast, preincubation with IgG had no inhibitory effect on Smad-binding activity. These results indicate that polyamine depletion activates Smad signaling in normal intestinal epithelial cells.

Effect of polyamine depletion on Smad-dependent transcriptional activity. To determine the possible transcription activation of induced Smad activity following polyamine depletion, the luciferase reporter activities of three Smad-dependent promoter constructs were measured in control cells and cells treated with DFMO alone or DFMO plus spermidine for 6 days. Results presented in Fig. 4 show that polyamine depletion-induced Smads were associated with significant increases in luciferase reporter activities of Smad-dependent promoters. Levels of pJun-Luc, pPAI1-Luc, and pSBE-Luc luciferase reporter activity in cells exposed to DFMO for 6 day were ~7.8, ~3.1, and ~7.2 times the control values, respectively. On the other hand, spermidine at the concentration of 5 mM given together with DFMO completely prevented increases in luciferase reporter activity of Smad-dependent promoters.

To further define the relationship between induced Smads and the transcription activation following polyamine depletion, we examined the effect of inhibition of wild-type Smad4 by ectopic expression of a dominant negative mutant Smad4 (MSmad4) on transcriptional activation of Smad-dependent promoters in IEC-6 cells. Our previous studies have demonstrated that adenoviral vectors infect intestinal epithelial cells nearly 100% efficiency within 48 h (40). In fact, more than 95% of IEC-6 cells were positive when they were infected with the adenoviral vector encoding green fluorescent protein (GFP) served as the marker for 24 h (data not shown). Results presented in Fig. 5 show that infection of polyamine-deficient cells with the recombinant adenoviral vector encoding MSmad4 cDNA (AdMSmad4) decreased luciferase reporter activity
of Smad-dependent promoters. Levels of pJun-Luc and pSBE4-Luc luciferase reporter activity were decreased by ~40% and ~55% when the adenovirus at the concentration of 20 plaque-forming units per cell was used, respectively. An adenovirus that lacked exogenous MSmad4 cDNA was used as the negative control and did not alter levels of Smad-dependent transcription activation in polyamine-deficient cells. These results clearly indicate that increased Smads have the transcription activation following polyamine depletion.

*Effect of polyamine depletion on TGF-β expression and cell proliferation.* Depletion of cellular polyamines by DFMO also significantly induced TGF-β mRNA expression and increased TGF-β content. The levels of TGF-β in the cells treated with DFMO for 6 days were ~two times the normal values (data not shown). Increased expression of TGF-β in the polyamine-deficient cells was associated with an inhibition of cell growth, in which cells were arrested in G1 phase. Cell numbers were decreased by ~50% in DFMO-treated cells and population of G1 cells increased from ~39% in control cells to ~70% in cells exposed to DFMO for 6 days. TGF-β expression and cell growth were returned to normal when DFMO was given together with DFMO (data not shown). Similar results have been published previously (35).

*Effects of inhibition of TGF-β on Smad activation in polyamine-deficient cells.* To elucidate whether the increased Smad activity following polyamine depletion results from an increased expression of TGF-β, the following three logically linked studies were carried out. First, we examined the effect of exposure of normal IEC-6 cells (without DFMO) to exogenous TGF-β on Smad activity. Figure 6A clearly shows that treatment with TGF-β at the concentration of 5 ng/ml increased expression of Smad3 protein but had no effect on Smad4. Significant increases in Smad3 protein occurred at 1.5 h and peaked between 3 and 6 h after treatment with TGF-β. Maximum increases in Smad3 protein were >5 times the pretreatment
control level. Furthermore, increase in Smad3 protein was paralleled by that of Smad-binding activity (Fig. 6B). Levels of Smad-binding activity were ~2.3, ~7.2, and ~6.5 times the control levels at 1.5, 3, and 6 h after treatment with TGF-β, respectively.

Second, we examined whether dysfunction of TGF-β by using immunoneutralizing anti-TGF-β antibody prevented the induced Smads in polyamine-deficient cells. Cells were grown in the presence of DFMO for 3 days, exposed to the medium containing DFMO and the antibody against TGF-β at different concentrations for 24 h, and then transfected using the pPAI1-luc or pSBE4-Luc luciferase reporter construct. Levels of Smad3/Smad4 proteins and their transcription activity were measured 48 h after the transfection in the presence of DFMO. Administration of the anti-TGF-β antibody not only significantly prevented the increase in Smad3 protein (Fig. 7A and B) but also inhibited Smad-dependent transcription activity (Fig. 7C), although it had no effect on induced levels of Smad4 in DFMO-treated cells. In the cells treated with the antibody, levels of Smad3 protein were decreased by ~45% at 0.5 μg/ml and ~62% at 2 μg/ml, respectively. Consistently, luciferase activities of the pPAI1-luc reporter construct were decreased by ~55% at 0.5 μg/ml and ~65% at 2 μg/ml the antibody (Fig. 7Ca), and levels of the pSBE4-luc luciferase activity were decreased by ~33% at 0.5 μg/ml and ~45% at 2 μg/ml (Fig. 7Cb) in the DFMO-treated cells. The heat-inactivated anti-TGF-β antibody served as control in this experiments and showed no additional effect on Smad activity.

Third, we determined the effect of inhibition of TGF-β expression by using TGF-β antisense oligomers on Smads in polyamine-deficient cells. The protocol of this study was similar to the previous study involving the anti-TGF-β antibody. After polyamine-deficient cells were treated with TGF-β antisense or sense oligomers for 24 h, they were transfected with Smad-dependent luciferase reporter constructs. Levels of Smad3/Smad4 proteins and the luciferase
reporter activity were examined 48 h after the transfection. As shown in Figure 8A and B, treatment with TGF-β antisense oligomers decreased levels of Smad3 protein but had no effect on Smad4 in polyamine-deficient cells. When the DFMO-treated cells were exposed to 4 or 6 nm TGF-β antisense oligomers for 72 h, the Smad3 protein levels were decreased by >80%. Treatment with TGF-β sense oligomers at the same concentrations had no effect on expression of both Smad3 and Smad4. Inhibition of Smad3 protein levels by TGF-β antisense oligomers also suppressed transcription activation of Smad-dependent promoters in polyamine-deficient cells (Fig. 8C). The luciferase activities of pJun-Luc and pPAI1-luc reporter constructs were decreased by ~85% after exposure to 4 nm TGF-β antisense oligomers. In addition, there was no apparent loss of cell viability in cells treated with DFMO alone or DFMO-treated cells exposed to TGFβ antisense or sense oligomers (data not shown). Taken together, these findings strongly suggest that induced Smad activation following polyamine depletion is due to increased TGF-β in normal intestinal epithelial cells.

**DISCUSSION**

As discussed in the introduction, normal intestinal mucosal growth depends on an adequate supply of polyamines in the dividing cells within the crypts. Although few specific functions of polyamines at cellular and molecular levels are defined, there is little doubt that polyamines regulate intestinal epithelial cell proliferation by virtue of their ability to modulate expression of various growth-related genes (3,19,20,34,49). Increasing evidence indicates that polyamines positively regulate the transcription of growth-promoting genes such as c-Fos and c-Myc (3,34,50) and negatively control expression of growth-inhibiting genes including TGF-βTGF-β receptor (35,39), p53 (19,21), and JunD (22,36) at the posttranscriptional level in
normal intestinal epithelial cells. It has been shown that increased polyamines not only stimulate expression of c-Fos and c-Myc genes by enhancing their mRNA synthesis, but also inhibit expression of TGF-β TGF-β receptor, p53, and JunD genes by increasing their mRNA degradation, leading to stimulation of intestinal epithelial cell proliferation. In contrast, decreased levels of polyamines lead to reduction in levels of growth-promoting proteins through inhibition of their gene transcription and increase levels of growth-inhibiting factors through stabilization of their mRNAs, which are associated with an increase in G1 phase growth arrest. However, the exact signaling pathways by which products of these growth-related genes mediate intestinal epithelial cell proliferation after increased or decreased cellular polyamines remain to be elucidated.

The TGF-β family consists of a group of closely related genes that are widely distributed in a variety of human and animal tissues including the gastrointestinal mucosa (5,6,10,29). TGF-β is an important physiological regulator for normal intestinal mucosal growth, and administration of TGF-β inhibits proliferative activity and promotes the development of differentiated function in intestinal epithelial cells (12,28,32,54). We have recently demonstrated that polyamine depletion by DFMO dramatically stabilizes TGF-β mRNA and inhibits the mRNA degradation, leading to an increase in TGF-β expression (35). Addition of immunoneutralizing anti-TGF-β antibody to the culture medium significantly prevents growth inhibition in DFMO-treated cells. Polyamine depletion also increases expression of the TGF-β type-I receptor in IEC-6 cells (39). Inhibition of TGF-β type-I receptor expression decreases TGF-β-mediated growth inhibition in normal cells and prevents the increased sensitivity to exogenous TGF-β in polyamine-deficient cells. These findings strongly suggest that increased
TGF-b/TGF-b receptor expression is one mechanism by which decreased levels of polyamines inhibit cell renewal in the intestinal mucosa.

The data from the current studies provide new evidence that Smad proteins are involved in the process mediating functions of cellular polyamines in intestinal epithelial cells. Exposure of IEC-6 cells to DFMO for 4, 6, and 8 days significantly increased levels of both Smad3 and Smad4 proteins and induced their nuclear translocation. Smad3 is shown to be highly expressed in intestinal epithelial cells and activation of this R-Smad is ligand-specific (10,17,29,31). It is not surprising that polyamine depletion by DFMO increases Smad3 protein and enhances its nuclear translocation, because treatment with DFMO is known to stimulate expression of TGF-b/TGF-b receptor in IEC-6 cells (35,39). It is likely that activated Smad3 results from the increased expression of TGF-b/TGF-b receptors in polyamine-deficient cells. This contention is strongly supported by data presented in Figures 7 and 8 that show that inhibition of TGF-b by treatment with either immunoneutralizing anti-TGF-b antibody or TGF-b antisense oligomers prevented the increased Smad activation in the absence of cellular polyamines.

The current studies also demonstrated that polyamine depletion induced Smad4 nuclear translocation in IEC-6 cells. Smad4 functions as a common mediator for all R-Smads and forms heteromeric complexes with Smad3 after ligand activation (12,31,54). The observed change in Smad4 in the current studies seems to be a secondary response to the activation of Smad3 in DFMO-treated cells. In support of this possibility, treatment with exogenous TGF-b did not alter levels of Smad4 protein in normal IEC-6 cells (without DFMO), although it significantly increased Smad3 expression (Fig. 6). Furthermore, exposure to immunoneutralizing anti-TGF-b antibody or TGF-b antisense oligomers did not prevent the increased levels of Smad4 protein in polyamine-deficient cells. The other possibility also exists, that polyamine depletion induces
Smad4 expression through a mechanism independent from the activated Smad3 in intestinal epithelial cells. In addition, this increased Smad expression and nuclear translocation in DFMO-treated cells are specifically related to polyamine depletion rather than to a nonspecific effect of DFMO, because the stimulatory effect of this compound on Smads was completely prevented by the addition of exogenous spermidine.

Results presented here further showed that polyamine depletion-induced Smad activity was associated with a significant increase in transcription activation of Smad-driven promoters in IEC-6 cells. Using EMSA method and luciferase reporter assays, we demonstrated that polyamine depletion significantly increased Smad sequence-specific DNA binding and induced luciferase reporter activity of Smad-dependent promoters. Our results are consistent with others that indicated that induced nuclear Smad proteins mediate transcription of Smad-target genes in a variety of cell types (10,29,52). The current studies also show that increased transcriptional activation following polyamine depletion is primarily due to the function of Smad3/Smad4 heteromeric complexes because ectopic expression of a dominant negative mutant Smad4 prevented the increased Smad transcription activation in DFMO-treated cells. Recently, Smad DNA binding sites have been identified in various gene promoters including PAI-1, JunB, c-Jun, and p21 by different approaches (15,23,33,44,55). In general, these sequences reported by different groups are essentially identical; regardless of whether one defines a Smad-binding site as the palindrome AGACGTCT, as the CAGA box, or as repeats of GACA, all identified sites contain the Smad box, 5’-GTCT-3’, or its reverse complement, 5’-AGAC-3’. Our results clearly indicate that induced Smad3/Smad4 heteromeric complexes in polyamine-deficient cells are able to bind to this specific DNA site, suggesting that Smads mediate transcriptional activation, probably through a direct interaction with SBE sites in target promoters.
The exact mechanism by which activated Smads induced by TGF-β following polyamine depletion mediate target gene transcription is obscure. Although initial studies show that Smads can bind directly to DNA and activate transcription of target genes, increasing evidence reveals that Smads primarily function as co-modulators of transcriptional activity and that interaction between Smads and their DNA-binding partners are essential for Smads to function in regulating specific transcriptional response (1,18,42). Recently, Smad3/AP-1 interactions are known to control transcriptional responses to TGF-β in a promoter-specific manner (11,23,46,51,55). We have recently demonstrated that polyamine depletion significantly increases JunD/AP-1 activity in IEC-6 cells and that increased JunD/AP-1 plays an important role in growth inhibition of polyamine-deficient cells (22,36). It is not clear at present whether Smads interact with JunD/AP-1 and if observed Smad/JunD complexes cooperatively regulate transcription of specific target genes and are involved in the G1 phase growth arrest following polyamine depletion. Further studies are needed to define the exact relationship between Smads and JunD/AP-1 in polyamine-deficient IEC-6 cells.

Combining the current findings with our previous studies (35,39), we propose a model delineating the role of TGF-β/Smad signaling pathway in the inhibition of intestinal epithelial cell proliferation following polyamine depletion (Fig. 9). In this model, polyamines negatively regulate posttranscription of the TGF-β gene, while Smads are the downstream intracellular effectors of activated TGF-β receptors. Decreased cellular polyamines stabilize TGF-β mRNAs, increase TGF-β synthesis, and cause the activation of TGF-β receptors. The resultant activation of TGF-β receptors induces Smad3/Smad4 levels and stimulates Smad nuclear translocation, resulting in the accumulation of Smad3/Smad4 complexes. In the nucleus, Smads bind to the specific DNA site and cooperate with their DNA-binding partners such as JunD/AP-1 to activate
or repress transcription of specific target genes, thus leading to the inhibition of intestinal epithelial cell proliferation. In contrast, increased polyamines down-regulate TGF-b/Smad signaling and enhance intestinal epithelial cell proliferation. These findings suggest that Smads are involved in the negative control of intestinal mucosal growth and that polyamines stimulate intestinal epithelial cell renewal \textit{in vivo} at least partially through inhibition of TGF-b/Smad signaling pathway under physiological conditions.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig. 1. Changes in expression of TGFβ and Smad3/Smad4 proteins in control IEC-6 cells and cells treated with either α-difluoromethylornithine (DFMO) alone or DFMO plus spermidine (SPD). **A**: levels of TGFβ. Cells were grown in DMEM containing 5% dialyzed FBS in the presence or absence of DFMO (5 mM) or DFMO plus SPD (5 μM) for 4, 6, and 8 days, monolayer of cells was washed once with D-PBS, and then 1 ml of fresh medium was added. The medium was collected following 12 h of further culture, and the content of TGFβ was measured. **B**: representative autoradiograms of Western blots from cells described in **A**. Whole cell lysates were harvested, applied to each lane (30 μg) equally, and subjected to electrophoresis on 10% acrylamide gel. Levels of Smad3 (≈ 55kDa) and Smad4 (≈ 60 kDa) were identified by probing nitrocellulose with the specific antibodies. After the blot was stripped, actin (≈ 42 kDa) immunoblotting was performed as an internal control for equal loading. **C**: quantitative analysis of Western blots by densitometry from cells described in **B**: *a*, Smad3; and *b*, Smad4. Values are means ± SE of data from 3 separate experiments; relative levels of Smad3 and Smad4 were corrected for loading as measured by densitometry of actin. *P < 0.05 compared with control and DFMO plus SPD.*

Fig. 2. Cellular distribution of Smad3 (*left*) and Smad4 (*right*) proteins in IEC-6 cells described in **Fig. 1**. The IEC-6 cells were plated in a 4-well chamber slide and grown in DMEM containing 5% dialyzed FBS in the presence or absence of DFMO or DFMO plus SPD for 6 days, and then fixed for immunostaining. Cells were permeabilized and incubated with the specific antibody against Smad3 or Smad4 protein, and then with anti-IgG
conjugated with FITC. Slides were viewed through a Zeiss confocal microscope, and all photos were taken at identical magnification, exposure, and shutter speeds. 

A: control. 

B: cells treated with DFMO alone for 6 days. 

C: cells treated with DFMO plus SPD for 6 days. Original magnification: x1000. Three experiments were performed that showed similar results.

Fig. 3. Changes in sequence-specific Smad DNA binding activity in IEC-6 cells described in Fig. 1. 

A: Smad DNA binding activity after different treatments. a, representative autoradiograms of electrophoretic mobility shift assay (EMSA) for Smad binding activity. Nuclear extracts were prepared from cells grown in the presence or absence of 5 mM DFMO with or without 5 mM SPD for 4, 6, and 8 days. EMSA was performed using 10 μg of nuclear protein and 0.035 pmol of 32P-end-labeled oligonucleotides containing a consensus Smad binding site. Positions of the specifically bound DNA-protein complex and freely migrating probes are indicated. 

b, quantitative analysis of EMSA by densitometry from cells described in Aa. Values are means ± SE of data from 3 separate experiments. *P< 0.05 compared with control groups. 

B: effects of unlabeled Smad oligonucleotide or the antibody against Smad3 or Smad4 added to the binding reaction mixture on Smad binding activity. a, representative autoradiograms from control cells (Control); b, representative autoradiograms from cells treated with DFMO for 6 days (DFMO); and c, effects of addition of the antibody against Smad3 or Smad4 on the Smad binding activity. Nuclear extracts (10 μg) isolated from cells exposed to DFMO for 6 days were initially incubated with 2 μg of the indicated antibody for 40 min and then
subjected to EMSA as described in A. IgG: lane with nuclear extracts and IgG. Three experiments were performed that showed consistent results.

**Fig. 4.** Transcription activation of Smad-target genes in IEC-6 cells described in **Fig. 1.** A: luciferase (Luc) reporter activity of the –79 to +170 region of the human c-Jun promoter (pJun-Luc). a, schematic structure of pJun-Luc reporter. The 3’ boundary is 170 base pairs upstream of the c-Jun transcription initiation site, fused to the Luc reporter gene. SBEs indicate the relative locations of the 3 Smad DNA-binding sites. b, luciferase activity. Cells were grown in the media containing DFMO alone or DFMO plus SPD for 4 days, and then transfected using the pJun-Luc reporter by lipofectamine technique. After 48-h of incubation in the presence of DFMO, transfected cells were harvested and assayed for luciferase activity. Luciferase activity of the pJun-Luc reporter was normalized by β-galactosidase activity from co-transfection of plasmid pCMV β-galactosidase. Values are means ± SE of data from 6 dishes. *P < 0.05 compared with control groups. B: luciferase reporter activity of the type-1 plasminogen activator inhibitor gene promoter (pPAI1-Luc): a, schematic structure of pPAI1-Luc reporter and b, luciferase activity. Values are means ± SE of data from 6 dishes. *P < 0.05 compared with control groups. C: luciferase reporter activity of the construct containing four copies of the synthetic SBE (pSBE4-luc): a, schematic structure of pSBE4-Luc reporter and b, luciferase activity. Values are means ± SE of data from 6 dishes. *P < 0.05 compared with control groups.
Fig. 5. Effect of ectopic expression of the dominant negative mutant Smad4 (MSmad4) on Smad transcription activation in polyamine-deficient IEC-6 cells.  

**A**: luciferase activity of the pJun-Luc reporter.  **B**: luciferase activity of the pSBE4-Luc reporter.  

Cells were grown in the medium containing 5 mM DFMO for 3 days, and then infected with the recombinant adenoviral vector encoding MSmad4 cDNA (AdMSmad4) or an adenovirus lacking DNMSmad4 (Adnull) at a multiplicity of infection of 20 plaque-forming units per cells (pfu/cell) for 24 h. These infected cells were then transfected with the pJun-Luc reporter or pSBE4-Luc construct by lipofectamine technique, and luciferase activity was measured after 48-h incubation in the presence of DFMO. Values are means ± SE of data from 6 dishes. *, + P < 0.05 compared with control cells and DFMO-treated cells infected with Adnull, respectively.

Fig. 6. Effect of treatment with exogenous TGF-β on expression of Smad proteins and sequence-specific Smad DNA binding activity in normal IEC-6 cells (without DFMO).  

**A**: representative autoradiograms of Western blots. Cells were grown in the DMEM containing 5% dialyzed FBS for 4 days and then TGF-β at the concentration of 5 ng/ml was given. Levels of Smad3 and Smad4 proteins were measured at various times after treatment with TGF-β. Three experiments were performed that showed consistent results.  

**B**: sequence-specific Smad DNA binding activity.  

**a**, representative autoradiograms of EMSA for Smad binding activity from cells described in **A**. Positions of the specifically bound DNA-protein complex and freely migrating probes are indicated.  

**b**, quantitative analysis of EMSA by densitometry from cells described in **Ba**. Values are means ± SE of data from 3 separate experiments. * P < 0.05 compared with controls (0 h).
Fig. 7. Effect of immunoneutralizing anti-TGF-β antibody added to cultures containing DFMO on Smad protein expression and the transcriptional activity in IEC-6 cells. A: expression of Smad3 and Smad4 proteins in cells exposed to DFMO and anti-TGF-β antibody. Cells were pretreated with DFMO for 3 days, and then incubated with the antibody against TGF-β at different concentrations for additional 72 h in the presence of DFMO. Whole cell lysates were harvested and levels of Smad3 and Smad4 proteins were assayed by Western blot analysis. Loading of proteins was monitored by actin immunoblotting. B: quantitative analysis of Western blots by densitometry from cells described in A: a, Smad3; and b, Smad4. Values are means ± SE of data from 3 separate experiments. * P < 0.05 compared with cells exposed to DFMO alone. C: changes in transcriptional activity in cells described in A: a, luciferase activity of pPAI1-Luc reporter; and b, luciferase activity of pSBE4-Luc reporter. After cells were grown in the presence of DFMO for 3 days, they were incubated with the media containing DFMO plus the anti-TGF-β antibody at various concentrations for 24 h and then transfected using either the pPAI1-Luc or pSBE4-Luc reporter construct by lipofectamine technique. The activity of pPAI1-Luc and pSBE4-Luc luciferase reporter was measured 48 h after transfection. Values are means ± SE of data from 6 dishes. * P < 0.05 compared with cells exposed to DFMO alone.

Fig. 8. Effect of inhibition of TGF-β expression by using antisense TGF-β oligodeoxyribonucleotides on Smad protein expression and the transcriptional activity in polyamine-deficient IEC-6 cells. A: Smad3 and Smad4 protein levels. a: representative
autoradiograms of Western blots. Cells were initially treated with 5 mM DFMO for 3 days and then exposed to different concentrations of TGF-b antisense oligomers (ASO) or sense oligomers (SO) in the presence of DFMO. Smad3 and Smad4 protein levels were measured 72 h after treatment with TGF-b antisense or sense oligomers by Western immunoblotting analysis. Equal loading of proteins was monitored by actin immunoblotting. B: quantitative analysis of Western blots by densitometry from cells described in A: a, Smad3; and b, Smad4. Values are means ± SE of data from 3 separate experiments. * P < 0.05 compared with cells treated with TGF-b sense oligomers. C: changes in transcriptional activity in cells described in A: a, luciferase activity of pJun-Luc reporter; and b, luciferase activity of pPAI1-Luc reporter. After cells are grown in the presence of DFMO 3 day, they were exposed to TGF-b antisense or sense oligomers (4 mM) for 24 h, and transfected using the pJun-Luc or pPAI1-luc reporter. The activity of luciferase reporters was measured 48 h after the transfection. Values are means ± SE of data from 6 dishes. * P < 0.05 compared with cells treated with sense oligomers.

Fig. 9. Schematic diagram depicting the proposed role of TGF-b/Smad signaling pathway in the inhibition of normal intestinal epithelial cell proliferation following polyamine depletion. In this model, polyamines are the negative regulators for expression of the TGF-b gene, while Smad proteins are the downstream intracellular effectors of activated TGF-b receptors. Decreased cellular polyamines, by either inhibition of their synthesis, stimulation of the catabolism, or suppression of polyamine uptake, increase expression of TGF-b through stabilization of TGF-b mRNA, enhance the release of TGF-b, and subsequently phosphorylates TGF-b type II receptor (R-II). The phosphorylated R-II
activates TGF-β type I receptor (R-I), induces the formation of Smad3/Smad4 heteromeric complexes, and stimulates their nuclear translocation. The activated Smads in the nucleus bind to the specific DNA site and cooperate with Smad DNA-binding partners (DBP) such as some AP-1 proteins to activate or repress transcription of specific target genes, thus leading to the inhibition of normal intestinal epithelial cell proliferation following polyamine depletion.
Fig. 1

A.

Levels of TGFβ (pg/ml)

B.

kDa

-Smad3

-Smad4

-Actin

C.

a. Smad3

Relative levels

b. Smad4

Relative levels
A. Control

B. DFMO

C. DFMO+SPD

Smad3

Smad4

Fig. 2
A

a. DNA Binding

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b. Relative Levels

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B

a. Control

Unlabeled Oligos

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b. DFMO

-Smad

c. Anti-Smad Antibody

IgG Anti-Smad3 AB Anti-Smad4 AB

-Smad

Fig. 3
Luciferase activity

**A**

a. pJun-Luc

-79  +62  +170
SBEs  Luc

b. Reporter Activity

![Diagram of Luciferase activity for pJun-Luc](image)

**B**

a. pPAI1-Luc

-300  -730  -580  -280  +22
SBE  SBE  SBE  Luc

b. Reporter Activity

![Diagram of Luciferase activity for pPAI1-Luc](image)

**C**

a. pSBE4-Luc

SBEs  Luc

b. Reporter Activity

![Diagram of Luciferase activity for pSBE4-Luc](image)

Fig. 4
Fig. 5
Fig. 6
Fig. 7

A

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B

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b. Smad4

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C

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Fig. 8