Amino Acids (2007) 33: 241–252
DOI 10.1007/s00726-007-0518-z
Printed in The Netherlands

Amino Acids

Polyamines and mRNA stability in regulation of intestinal mucosal growth

Review Article

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Received December 2, 2006
Accepted February 1, 2007
Published online April 4, 2007;

Summary. The mammalian intestinal epithelium is a rapidly self-renewing tissue in the body, and its homeostasis is preserved through strict regulation of epithelial cell proliferation, growth arrest, and apoptosis. Polyamines are necessary for normal intestinal mucosal growth and decreasing cellular polyamines inhibits cell proliferation and disrupts epithelial integrity. An increasing body of evidence indicates that polyamines regulate intestinal epithelial cell renewal by virtue of their ability to modulate expression of various genes and that growth inhibition following polyamine depletion results primarily from the activation of growth-inhibiting genes rather than a simple decrease in expression of growth-promoting genes. In this review article, we will focus on changes in expression of growth-inhibiting genes following polyamine depletion and further analyze in some detail the mechanisms through which mRNA stability is regulated by RNA-binding proteins.

Keywords: Intestinal epithelium – Cell proliferation and growth arrest – Posttranscriptional regulation – mRNA stability – RNA-binding proteins

Abbreviations: AMPK, AMP-activated protein kinase; AP-1, activator protein-1; co-Smad, common-Smad; CR, coding region; CRE, cAMP responsive element; C-siRNA, control siRNA; DENSPM, N^1,N^{11}-diethylnorspermdine; DFMO, D,L-α-difluoromethylornithine, IEC, intestinal epithelial cell; I-Smads, inhibitory Smads; NPM, necleophosmin; ODC, ornithine decarboxylase; Q-PCR, real-time quantitative PCR; R-Smads, receptor-regulated Smads; siHuR, siRNA targeting HuR mRNA; siRNA, small interfering RNA; TGF-β, transforming growth factor-β; TGFβRII, TGF-β type II receptor; TRE, TPA responsive element; 3′-UTRs, 3′-untranslated regions

Introduction

The epithelium of mammalian intestinal mucosa has the most rapid turnover rate of any tissue in the body, and maintenance of its integrity depends on a complex interplay between processes involved in cell proliferation, differentiation, migration, and apoptosis (Johnson, 1988; Jones and Gores, 1997; Loeffler et al., 1993). Under physiological conditions, undifferentiated epithelial cells continuously replicate in the proliferating zone within the crypts and differentiate as they migrate up the luminal surface of the colon and the villous tips in the small intestine (Potten, 1997; Potten and Loeffler, 1990). To maintain stable numbers of enterocytes, cell division must be dynamically counterbalanced by apoptosis, a fundamental biological process involving selective cell deletion to regulate tissue homeostasis (Loeffler et al., 1993; Potten and Loeffler, 1990; Potten et al., 1997). Apoptosis occurs in the crypt area, where it maintains the critical balance in cell number between newly divided and surviving cells, and at the luminal surface of the colon and villous tips in the small intestine, where differentiated cells are lost (Jones and Gores, 1997; Loeffler et al., 1993; Potten, 1997; Potten and Loeffler, 1990). This rapid dynamic turnover rate of the intestinal epithelium is highly regulated and critically controlled by numerous factors, including cellular polyamines (McCormack and Johnson, 1991; Wang and Johnson, 1991; Wang et al., 1991).

Over the past several years, our group and others have demonstrated that polyamines, either synthesized endogenously or supplied luminally, stimulate normal intestinal epithelial cell proliferation and enhance differentiation during early stage of mucosal development (Celano et al.,

1989; McCormack and Johnson, 1991; Wang and Johnson, 1991; Wang et al., 1991, 1993). In contrast, decreased levels of cellular polyamines inhibit mucosal growth and slow down its maturation (Kramer et al., 1999; Li et al., 1999, 2002; Patel and Wang, 1999; Patel et al., 1998; Rao et al., 2000). To define the exact role of polyamines in normal intestinal epithelial cell proliferation at the molecular level, an increasing body of evidence indicates that polyamines regulate cell proliferation by virtue of their ability to modulate expression of various growth-related genes. Polyamines positively regulate the transcription of growth-promoting genes such as *c-fos*, *c-jun*, and *c-myc* (Celano et al., 1989; Wang et al., 1993) and negatively affect expression of growth-inhibiting genes including *p53* (Li et al., 1999; Kramer et al., 1999), *junD* (Li et al., 2002; Patel and Wang, 1999), and *TGFβ*/*TGFβ* receptors (Patel et al., 1998; Rao et al., 2000) through modulations at the posttranscriptional level. In this article, we will overview changes in cellular signals that are activated following polyamine depletion, and then analyze in some detail the mechanisms by which polyamine depletion stabilizes mRNAs of growth-inhibiting genes, leading to growth inhibition of intestinal epithelial cell proliferation.

Polyamines are required for intestinal mucosal growth

The natural polyamines, spermidine, spermine and their precursor putrescine, are ubiquitous organic cations of low molecular weight found in all eukaryotic cells (Gerner and Meyskens, 2004; Tabor, 1984). Polyamines are implicated in a wide variety of biological functions and regulation of cellular polyamines has been recognized to be the central convergence point for the multiple signaling pathways driving different epithelial cell functions (McCormack and Johnson, 1991; Gerner and Meyskens, 2004; Tabor, 1984). Cellular polyamines are highly regulated by their biosynthesis, degradation, and transport. Polyamine synthesis depends on the activation or inhibition of ornithine decarboxylase (ODC), which catalyzes the first rate-limiting step in polyamine synthesis. ODC decarboxylates the amino acid ornithine to form putrescine; propylamine groups are then added to one or both amino groups of putrescine to form spermidine and spermine (Tabor, 1984). On the other hand, polyamines are degraded by diamine oxidase and spermidine/spermine-N-acetyltransferase (Gerner and Meyskens, 2004; Tabor, 1984).

Control of mammalian intestinal mucosal growth is a complex process that is regulated at different levels by numerous factors. Maintenance of mucosal integrity requires epithelial cell decisions that regulate signaling networks controlling expression of various genes (Johnson, 1988; McCormack and Johnson, 1991). Our previous studies (Liu et al., 2005, 2006; McCormack and Johnson, 1991; Wang and Johnson, 1991, 1994; Wang et al., 1991) have demonstrated that polyamines are required for the stimulation of mucosal growth, in part, through their ability to regulate expression of nuclear transcription factors including c-Fos, c-Jun, and c-Myc proteins. In a gastric mucosal stress ulcer model, the increase in cell renewal during mucosal repair is associated with increases in both tissue polyamines and expression of *c-fos*, *c-jun*, and *c-myc* genes (Wang and Johnson, 1994). Depletion of cellular polyamines by α-difluoromethylornithine (DFMO), a specific inhibitor of ODC, decreases expression of these genes, inhibits cell division, and delays mucosal healing. Similarly, in cultured intestinal epithelial cells, increased polyamines induce expression of *c-fos*, *c-myc* and *c-jun* and promote cell proliferation (Liu et al., 2005; McCormack and Johnson, 1991). Polyamines also modulate the activity of Akt kinase (Zhang et al., 2004) and NF-κB signaling (Li et al., 2001), contributing to the control of epithelial cell apoptosis under physiological conditions.

Polyamine depletion inhibits mucosal growth by stabilizing specific mRNAs

Negative growth control, including growth arrest and apoptosis, must be understood to comprehend how appropriate cell numbers are maintained in normal intestinal mucosa. Alterations in any part of the equation contribute to mucosal atrophy or loss of epithelial integrity, and this has attracted considerable interest recently. Our previous studies have demonstrated that inhibition of intestinal mucosal growth following polyamine depletion is not due to a simple decrease in expression of growth-promoting genes, because polyamine-deficient cells continuously maintain high basal levels of c-Myc and c-Jun (Liu et al., 2005; McCormack and Johnson, 1991; Patel and Wang, 1997). In the other words, inhibition of intestinal mucosal growth following polyamine depletion is an active process and results primarily from the activation of expression of genes that are involved in growth arrest and apoptosis. Our previous studies and others have demonstrated that several growth-inhibiting genes including *p53*, *junD*, and *TGF-β* are activated following polyamine depletion and that polyamines modulate expression of these genes at the level of posttranscription rather than transcription in intestinal epithelial cells.

Polyamine depletion stabilizes p53

The p53 gene encodes for a nuclear phosphoprotein, which was originally discovered as a cellular protein bound to the SV40 T antigen in transformed cells. p53 is present in low concentrations in normal cells and has a half-life of about 6–20 min (Appella and Anderson, 2001; Slee et al., 2004). Expression of the p53 gene is highly regulated by the cell according to its state of growth. Steady-state levels of p53 mRNA are significantly increased in certain growth conditions and in cells transformed by a variety of means including viral infection, chemical treatment and transfection of oncogenes (Fang et al., 2000; Kurki et al., 2004). Inactivation of the p53 gene occurs in over half of all human tumors, implying that expression of the p53 gene has an important physiological role in the control of the cell cycle and that loss of this gene represents a fundamental step in the pathogenesis of cancer (Slee et al., 2004; Appella and Anderson, 2001). This action of p53 has been ascribed to its ability to induce expression of a cellular gene WAF1/CIP1 that encodes a 21 kDa inhibitor of G1 cyclin-dependent kinases (Appella and Anderson, 2001; Fang et al., 2000). In addition, there is increasing evidence that the p53 protein also induces apoptosis (Slee et al., 2004). Activation of the p53 gene expression induces growth arrest, apoptosis or both.

The first evidence showing the role of the p53 gene expression in growth inhibition following polyamine depletion is from our observations (Li et al., 1999, 2001) and others (Kramer et al., 1999, 2001; Ray et al., 1999). Using cultured IEC-6 cells that are derived from normal rat intestinal crypts, we have made the unique observation that inhibition of polyamine synthesis with DFMO significantly increases p53 gene expression, which is associated with an increase in G_1 phase growth arrest but not apoptosis (Li et al., 1999). Increases in p53 following polyamine depletion are associated with increases in other cell cycle inhibitors, including $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ (Ray et al., 1999). In control cells, steady-state levels of p53 mRNA were present at 4 days and then almost completely disappeared at 6 days, with minimal expression at 12 days after plating. Depletion of cellular polyamines increased expression of the p53 gene. This increase in mRNA levels for the p53 gene was noted at 4 days and remained elevated at 12 days after exposure to DFMO. Maximum increases in p53 mRNA levels occurred between 6 and 12 days after addition of DFMO and were more than 10 times control values. The increased levels of p53 mRNA in polyamine-deficient cells were paralleled by increases in p53 protein. Spermidine given together with DFMO completely prevented the increased expression of the p53 gene. The concentrations of p53 mRNA and protein in cells treated with DFMO plus spermidine were indistinguishable from those in cells grown in control cultures. The effect of polyamines on p53 gene expression is specific, since polyamine depletion did not induce expression of the *Rb* gene in IEC-6 cells. These results suggest that p53 is involved in the regulation of intestinal mucosal growth by cellular polyamines.

Consistent with our findings, Kramer et al. (1999) and Ray et al. (1999) have reported that exposure of human melanoma cells (MALME-3 M cell) to a polyamine analogue, N^1,N^{11}-diethylnorspermdine (DENSPM), not only decreases cellular polyamines but also increases p53 and p21 gene expression as well. DENSPM is known to deplete polyamine pools by inhibiting biosynthetic enzymes and potently inducing the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase. Treatment with DENSPM increases wild-type p53 ($\sim$10-fold at maximum), which is concomitant with an increase in p21 in MALME-3 M cells. Another cyclin-dependent kinase inhibitor, p27, and cyclin D1 increase slightly, whereas proliferating cell nuclear antigen and p130 remain unchanged. Induction of p21 protein is paralleled by an increase in its mRNA, but induction of p53 protein is not, suggesting that cellular polyamines dictate transcriptional activation of the p21 gene and posttranscriptional regulation of the p53 gene. Inconsistent with observations in IEC-6 cells, polyamine depletion by DENSPM in MALME cells also causes an increase in hypophosphorylated Rb protein.

It has been shown that p53 is an ephemeral protein and its half-life is approximately 25–40 min in variety of cell types (Appella and Anderson, 2001). In addition to transcriptional regulation, expression of the p53 gene is primarily regulated at the posttranscriptional level. Polyamine depletion increases the stability of p53 mRNA as measured by the mRNA half-life, but has no effect on p53 gene transcription in IEC-6 cells (Li et al., 2001). p53 mRNA levels in control cells declined rapidly after inhibition of gene transcription by addition of actinomycin D. The half-life of p53 mRNA in control cells was $\sim$45 min. However, the stability of p53 mRNA was dramatically increased by polyamine depletion with a half-life of $>$18 h. Increased half-life of p53 mRNA was prevented when spermidine was given together with DFMO. The half-life of p53 mRNA in cells treated with DFMO plus spermidine was $\sim$48 min, similar to that of controls (without DFMO). In contrast, inhibition of polyamine synthesis did not increase p53 gene transcription as measured by using nuclear run-on transcription assays. There were no

significant differences in the rate of p53 gene transcription between control cells and cells exposed to DFMO in the presence or absence of spermidine for 6 days. These findings clearly indicate that polyamines regulate the p53 gene expression posttranscriptionally in intestinal epithelial cells and that depletion of cellular polyamines induces p53 mRNA levels primarily through the increase in its stability.

Our studies have also shown that polyamine depletion stabilizes p53 protein through the interaction with nucleophosmin (NPM) (Zou et al., 2005). The levels of p53 protein in control cells declined rapidly after inhibition of protein synthesis by administration of cycloheximide. The half-life of p53 protein in control IEC-6 cells was ~15 min and increased to ~38 min in cells exposed to DFMO for 6 days. When DFMO was given together with spermidine, p53 protein levels decreased at the rate similar to that observed in controls, with a half-life of ~18 min. These data indicate that cellular polyamines are essential for the degradation of p53 protein and that induced accumulation of p53 protein in polyamine-deficient cells results, at least partially, from its protein stabilization.

NPM is a multifunctional protein that was originally identified as a nucleolar protein involved in ribosome biogenesis and has been recently shown to regulate p53 activity (Chan et al., 1989; Schmidt-Zachmann et al., 1987). We have recently demonstrated that polyamines modulate NPM activity in IEC-6 cells (Zou et al., 2005). Depletion of cellular polyamines by DFMO stimulates expression of the NPM gene and induces nuclear translocation of NPM protein. Polyamine depletion stimulates NPM expression primarily by increasing both NPM gene transcription and its mRNA stability, and induced NPM nuclear translocation through the activation of phosphorylation of mitogen-activated protein-kinase kinase. Increased NPM physically interacts with p53 and forms a NPM/p53 complex in polyamine-deficient cells. Inhibition of NPM expression by small interfering RNA (siRNA) targeting of a specific site on the NPM mRNA not only destabilizes p53 as indicated by a decrease in its protein half-life but also prevents the increased p53-dependent transactivation as indicated by a decrease in p21-promoter activity.

Polyamines modulate junD mRNA stability

JunD is a member of the *jun* family proto-oncogenes which are primary components of the activator protein-1 (AP-1) transcription factors (Hirai et al., 1989; Ryder et al., 1989). The Jun proteins (c-Jun, JunB, and JunD) are basic-leucine transcription factors that can form either AP-1 homodimers (Jun/Jun) or AP-1 heterodimers with members of related Fos family (c-Fos, FosB, Fra-1 and Fra-2) or the ATF family (ATF2, ATF3, and ATF4) (Ryder et al., 1989). Jun/Jun and Jun/Fos dimers bind to the TPA responsive element (TRE) TGACTCA present in many gene promoters, whereas Jun/ATF dimers bind preferentially to the cAMP responsive element (CRE) TGAC GTCA (Angel et al., 1988). The three Jun proteins are similar in their DNA-binding affinity which is determined by the C-terminal leucine zipper dimerization domain (Nakabeppu et al., 1988). The N-terminal transactivation domain is less conserved and may account for the different transactivation characteristics of the Jun proteins. There is increasing evidence that individual AP-1 dimers play distinct functions in different cellular contexts, including cell proliferation, growth arrest, differentiation, and apoptosis (Angel et al., 1988; Hirai et al., 1989; Nakabeppu et al., 1988; Ryder et al., 1989). For example, *c-jun* and *junB* function as immediate early response genes and activation of these two genes enhances the transition from a quiescent state to proliferating state, indicating that c-Jun and JunB are positive AP-1 factors for cell proliferation. In contrast, the activation of *junD* gene expression slows cell proliferation in some cell types and increases the percentage of population of cells arrested in the G_0/G_1 phase of the cell cycle (Hirai et al., 1989; Pfarr et al., 1994), suggesting that JunD is a negative AP-1 factor and down-regulates the G_1 to S phase transition.

We have demonstrated that depletion of cellular polyamines is associated with an increase in JunD/AP-1 activity in intestinal epithelial cells (Patel and Wang, 1999). Exposure of IEC-6 cells or Caco-2 (a human colon carcinoma cell line) to DFMO for 4 and 6 days increases AP-1 binding activity as measured by electrophoretic mobility shift assays, which is prevented by exogenous spermidine given together with DFMO. The anti-JunD antibody, when added to the binding reaction mixture, dramatically supershifts the AP-1 complexes present in IEC-6 cells exposed to DFMO for 4 and 6 days. The AP-1 activity attributed to JunD in the DFMO-treated cells is approximately one third of the total AP-1 binding activity on *day 4*, and about half on *day 6*, respectively. In control cells and cells exposed to DFMO and spermidine, the AP-1 binding activity is slightly supershifted by the anti-JunD antibody. On the other hand, addition of antibodies against c-Jun and JunB to the binding reaction mixture has no effect on the AP-1 binding activity in all 3 treatment groups. Although the anti-Fos antibody also

partially supershifts the AP-1 complexes, there are no significant differences in the AP-1 activity attributed to Fos between control cells and polyamine-deficient cells. The increased AP-1 binding activities in polyamine-deficient cells are not supershifted by the anti-Myc antibody. These results indicate that the increase in AP-1 activity in polyamine-deficient cells is primarily contributed by an increase in JunD/AP-1 while c-Jun/AP-1 and JunB/AP-1 activity remains essentially decreased or unchanged.

We have further demonstrated that the increased JunD/AP-1 activity following polyamine depletion is primarily due to the activation of *junD* gene expression and that polyamine depletion fails to induce *junD* gene transcription but stabilizes JunD mRNA. There are significant increases in JunD mRNA and protein in polyamine-deficient IEC-6 cells (Li et al., 2002), although expression of the *c-fos, c-jun*, and *junB* genes are decreased (Wang et al., 1993). Increased JunD mRNA levels are ~6.6 times the control levels on *day 4* and ~9 times on *day 6* after treatment with DFMO. Increased levels of JunD mRNA are paralleled by a significant increase in JunD protein, which is clearly located in the nucleus. These increases in both JunD mRNA and protein in DFMO-treated cells are prevented by addition of exogenous spermidine.

To test the possibility that the increase in JunD mRNA level in polyamine-deficient cells results from an increase in the mRNA synthesis, we have examined changes in the rate of *junD* gene transcription by using nuclear run-on transcription assay (Li et al., 2002). Our results showed that inhibition of polyamine synthesis by DFMO did not increase *junD* gene transcription in IEC-6 cells. There were no significant differences in the rate of *junD* gene transcription between control cells and cells exposed to DFMO in the presence or absence of spermidine for 4 and 6 days. We also examined rapid effect of addition of exogenous spermidine on *junD* gene transcription in control cells and demonstrated that exposure of normal IEC-6 cells (without DFMO) to 5 μM spermidine for 2 and 4 h did not alter the rate of *junD* gene transcription. On the other hand, rates of *c-myc* and *c-jun* gene transcription were significantly decreased following polyamine depletion (Liu et al., 2006; Patel and Wang, 1997; Wang et al.,

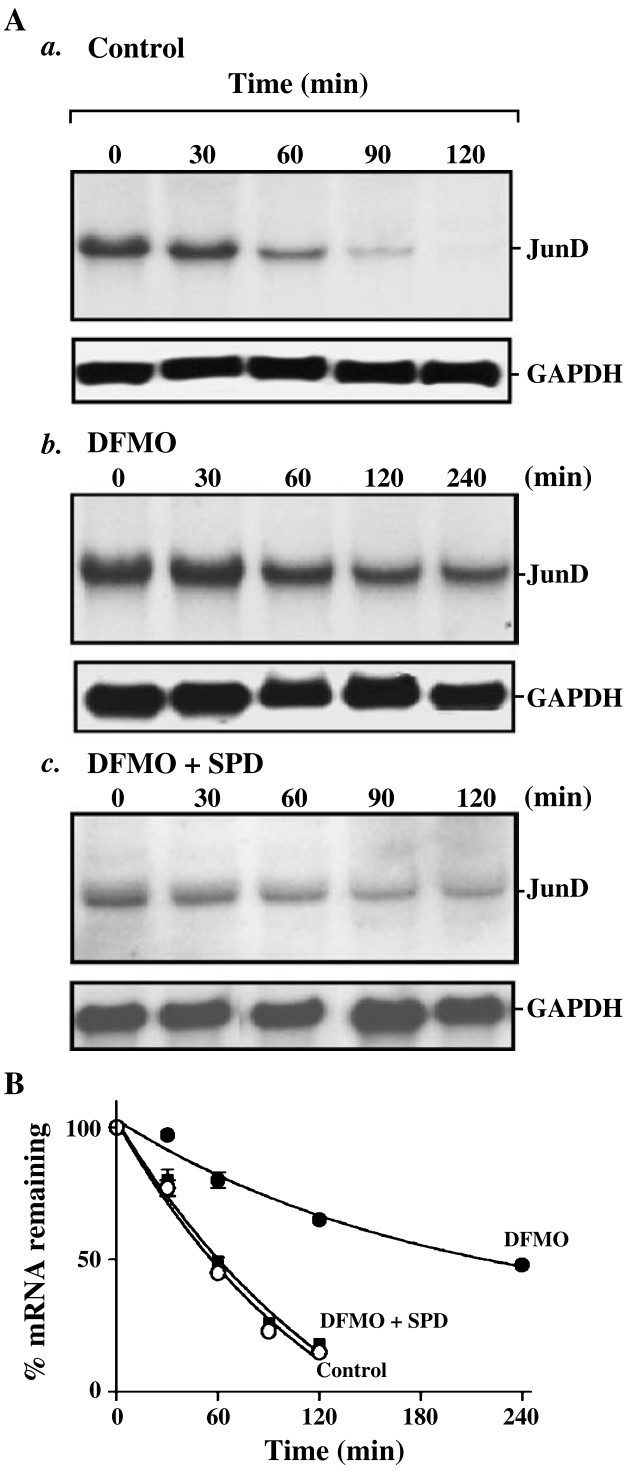

Fig. 1. Cytoplasmic half-life studies of JunD mRNA from IEC-6 cells in the presence or absence of cellular polyamines. **A** Representative autoradiograms of Northern blots. Cells were untreated (*a*), or treated with 5 mM α-difluoromethylornithine (DFMO) alone (*b*), or DFMO plus spermidine (SPD) (*c*) for 6 days and then incubated with 5 μg actinomycin D/ml for indicated times. Total cellular RNA was isolated and JunD mRNA levels were assayed by Northern blotting analysis using a JunD cDNA probe. Loading of RNA was monitored by hybridization to labeled GAPDH probe. **B** Percent of JunD mRNA remaining in IEC-6 cells described in **A**. Values are means ± SE of data from 3 separate experiments, and relative levels of JunD mRNA were corrected for RNA loading as measured by densitometry of GAPDH

1993). These results clearly indicate that cellular polyamines play only a minor role in the regulation of *junD* gene transcription and that the increase in steady-state levels of JunD mRNA after polyamine depletion is related to a mechanism other than the stimulation of *junD* gene transcription in intestinal epithelial cells.

To determine the involvement of posttranscriptional regulation in this process, JunD mRNA stability was examined by measurement of the mRNA half-life (Li et al., 2002). As shown in Fig. 1, depletion of cellular polyamines increased the stability of JunD mRNA in IEC-6 cells. In control cells, the half-life of JunD mRNA was ~50 min. However, the stability of JunD mRNA was increased by polyamine depletion with a half-life of >4 h. Spermidine, when given together with DFMO, almost completely prevented the increased half-life of JunD mRNA in polyamine-deficient cells. The half-life of JunD mRNA in cells exposed to DFMO plus spermidine was ~60 min, similar to that of controls (without DFMO). These findings indicate that polyamines regulate the *junD* expression posttranscriptionally and that depletion of cellular polyamines induces JunD mRNA levels primarily by increasing its stability. However, the exact mechanisms by which polyamine depletion stabilizes JunD mRNA remain to be demonstrated.

Polyamine depletion stabilizes TGFβ mRNA leading to activation of smad signaling

The transforming growth factor-β (TGF-β) family is a group of multifunctional peptides involved in regulation of epithelial cell growth and phenotype (Barnard et al., 1990, 1993). There are three distinct but highly related mammalian isoforms of TGFβs, named β1, -β2, and -β3. TGF-βs exert their multiple actions through heteromeric complexes of two types (type I and II) of transmembrane receptors with a serine/threonine kinase domain in their cytoplasmic region (Franzen et al., 1993). The receptors for TGF-βs are found on nearly all cell types, but the nature of the biological response to TGF-βs varies with cell type. Exposure of epithelial cells to TGF-βs leads to inhibition of growth, induction of extracellular matrix protein formation, modulation of proteolysis, and stimulation of cell migration (Barnard et al., 1990, 1993). To initiate the signaling of these responses, TGF-β binds directly to the TGF-β type II receptor (TGFβRII) that is a constitutive active kinase, after which the TGF-β type I receptor (TGFβRI) is recruited into the complex (Franzen et al., 1993). The TGFβRII in the complex phosphorylates the GS domain of TGFβRI and then leads to propagation of further downstream signals. Mutational analyses altering serine and threonine residues in the TGFβRI GS domain have indicated that the phosphorylation by TGFβRII is indispensable for TGF-β signaling, although its signaling activity does not appear to depend on the phosphorylation of any particular serine or threonine residue in the TTSGSGSG sequence of the GS domain (Barnard et al., 1990, 1993; Franzen et al., 1993).

We have reported that polyamine depletion activates expression of the TGF-β gene through stabilization of TGF-β mRNA but not its gene transcription (Patel et al., 1998; Rao et al., 2000). Depletion of cellular polyamines increases the mRNA levels of TGF-β, which is paralleled by an increase in TGF-β content. To determine the mechanisms by which polyamine depletion induces the TGF-β gene expression, our results show that depletion of cellular polyamines has no effect on the rate of TGF-β gene transcription, but significantly increases half-life of mRNA for TGF-β. In control cells, the half-life of TGF-β mRNA is ~65 min, while the stability of TGF-β mRNA in polyamine-deficient cells is dramatically increased, with a half-life of >16 h. This increased stability of TGF-β mRNA in DFMO-treated cells is prevented when spermidine is given together with DFMO. These results clearly indicate that polyamines affect the TGF-β gene posttranscriptionally rather than transcriptionally, and that polyamine depletion induces the activation of TGF-β gene by increasing TGF-β mRNA stability.

Our results also indicate that increased stabilization of TGF-β leads to the activation of Smad signaling pathway following polyamine depletion. The Smad proteins are a family of transcriptional activators that are critical for transmitting the TGF-β superfamily signals from the cell surface to the nucleus (Attisano and Wrana, 2000; Zawel et al., 1998). 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. All TGF-β family members, including TGF-βs, activins and bone morphogenetic proteins, use TGF-βRI and TGF-βRII receptors in a variety of cell types (Itoh et al., 2000). Upon ligand binding, the activated TGF-βRII kinase phosphorylates the TGF-βRI 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 (Attisano and Wrana, 2000; Zawel et al., 1998; Itoh et al., 2000). In the nucleus, Smads bind

to a specific DNA site (GTCTAGAC) and cooperate with various transcription factors in regulating target gene expression (Attisano and Wrana, 2000).

We have found that exposure of IEC-6 cells to DFMO for 4, 6, and 8 days increased levels of both Smad3 and Smad4 proteins and induced their nuclear translocation (Liu et al., 2003). Smad3 is shown to be highly expressed in intestinal epithelial cells and activation of this R-Smad is ligand-specific. It is not surprising that polyamine depletion increases Smad3 protein and enhances its nuclear translocation, because decreased levels of cellular polyamines are known to stimulate expression of TGF-β/TGF-β receptor in IEC-6 cells (Patel et al., 1998; Rao et al., 2000). Activated Smad3 results primarily from the increase in expression of TGF-β in polyamine-deficient cells, since inhibition of TGF-β by treatment with either immunoneutralizing anti-TGF-β antibody or TGF-β antisense oligomers prevents the increased Smad activation in the absence of cellular polyamines.

Polyamine depletion also induces Smad4 nuclear translocation in IEC-6 cells (Liu et al., 2003). Smad4 functions as a common mediator for all R-Smads and forms heteromeric complexes with Smad3 after ligand activation (Zawel et al., 1998; Itoh et al., 2000). The observed change in Smad4 in polyamine-deficient cells, however, seems to be a secondary response to the activation of Smad3. In support of this possibility, treatment with exogenous TGF-β did not alter levels of Smad4 protein in normal IEC-6 cells (without DFMO), although it significantly increased Smad3 expression. Furthermore, exposure to immunoneutralizing anti-TGF-β antibody or TGF-β 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 addition, this increased Smad expression and nuclear translocation in the 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.

Furthermore, polyamine depletion-induced Smad activity is associated with a significant increase in transcription activation of Smad-driven promoters. Using electrophoretic mobility shift method and luciferase reporter assays, we have demonstrated that polyamine depletion increases Smad sequence-specific DNA binding and induces luciferase reporter activity of Smad-dependent promoters (Liu et al., 2003). Our 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 polyamine-deficient cells. These findings clearly indicate that TGF-β stabilization following polyamine depletion induces the formation of Smad3/Smad4 heteromeric complexes and activates transcription of Smad target genes, leading to the inhibition of intestinal epithelial cell proliferation.

Polyamines modulate mRNA stability through RNA-binding protein HuR

Although gene expression is crucially modulated at the transcriptional level, the essential contribution of post-transcriptional events, such as mRNA processing, transport, turnover, and translation, is becoming increasingly recognized. In particular, regulation of mRNA stability is shown to play a critical role in activation of specific gene expression during the cellular response to mitogens, immunological triggers, stressful stimuli, and differentiation agents (Bashirullah et al., 2001; Fan et al., 2002). The mRNA turnover is primarily controlled through the association of RNA-binding proteins that bind to specific RNA sequences and either increase or decrease transcript half-life, and thus alters the profiles of expressed gene products (Gorospe, 2003; Brennan and Steitz, 2001). The best characterized *cis*-acting elements of mRNA turnover are U-rich and AU-rich sequences that are usually located in the 3′-untranslated regions (3′-UTR) of many labile mRNAs, such as those of certain protooncogenes, cytokines, and cell cycle regulatory proteins (Brennan and Steitz, 2001). HuR is a pivotal posttranscriptional regulator of gene expression and binds with great affinity and specificity to U-rich and AU-rich elements in a variety of mRNAs which typically present one or several hits of a recently identified RNA motif (de Silanes et al., 2004). Upon binding to a target mRNA, HuR has been shown to stabilize the mRNA, alter its translation, or carry out both functions (Brennan and Steitz, 2001; de Silanes et al., 2004). HuR is a ubiquitously expressed member of the Hu/ELAV (embryonic lethal abnormal vision in *D. melanogaster*) family of RNA-binding proteins that also comprises the primarily neuronal members HuB, HuC, and HuD (Brennan and Steitz, 2001; Gorospe, 2003). HuR is predominantly nuclear in unstimulated cells, but it rapidly translocates to the cytoplasm in response to various stimuli (Heinonen et al., 2005; Mazan-Mamczarz et al., 2003; Seko et al., 2004). Although the precise processes

regulating HuR function remain to be fully understood, it is clear that its subcellular localization is intimately linked to its effects upon target transcripts.

Depletion of cellular polyamines increases cytoplasmic levels of HuR

We (Zou et al., 2006) have recently reported that polyamine depletion enhanced the cytoplasmic accumulation of HuR, although it had no effect on the levels of total cellular HuR (Fig. 2). The induction of cytoplasmic HuR occurred as early as day 4 after exposure to DFMO and remained elevated on day 6, associated with a significant decrease in nuclear HuR. In keeping with the fact that the vast majority of HuR is nuclear at all times, there was only a slight reduction of nuclear HuR on day 4 after DFMO treatment, although the decrease was more noticeable by day 6. Supplementation with the polyamine putrescine reversed the effects of DFMO, both preventing the accumulation of cytoplasmic HuR and the reduction in nuclear HuR. These results indicate that decreased levels of cellular polyamines increase HuR translocation to the cytoplasm in intestinal epithelial cells.

In contrast, increased cellular polyamines by ectopic expression of the *ODC* gene inhibit HuR cytoplasmic translocation. We (Liu et al., 2006) have established two clones of stable ODC-expressing IEC (ODC-IEC) cells which exhibited dramatic high levels of ODC protein (>50-folds) and cellular polyamines putrescine (12-fold), spermidine (2-fold), and spermine (~25%). Interestingly, increased polyamines by ODC overexpression caused a decrease (by ~50%) in the levels of cytoplasmic HuR and a corresponding increase in its nuclear abundance (by 2-fold); no net changes in whole-cell HuR levels were seen in ODC-IEC cells compared with control cells (Zou et al., 2006). The effect of ODC overexpression on the subcellular distribution of HuR was not simply due to clonal variation, since two stable cell clones exhibited identical results. These results suggest that increases in cellular polyamines promote the nuclear accumulation of HuR and reduce its cytoplasmic levels in intestinal epithelial cells. How polyamines influence HuR abundance in the cytoplasm remains an open question, but we have preliminary evidence which supports the involvement of the AMP-activated protein kinase (AMPK), a kinase previously shown to inhibit the cytoplasmic export of HuR (Wang et al., 2002). Interventions to decrease cellular polyamines significantly reduced AMPK enzyme activity, while ectopic constitutive activation of AMPK prevented the elevation in cytoplasmic HuR levels in polyamine-deficient cells. The precise mechanisms by which polyamines modulate AMPK function and HuR distribution are the focus of our ongoing studies.

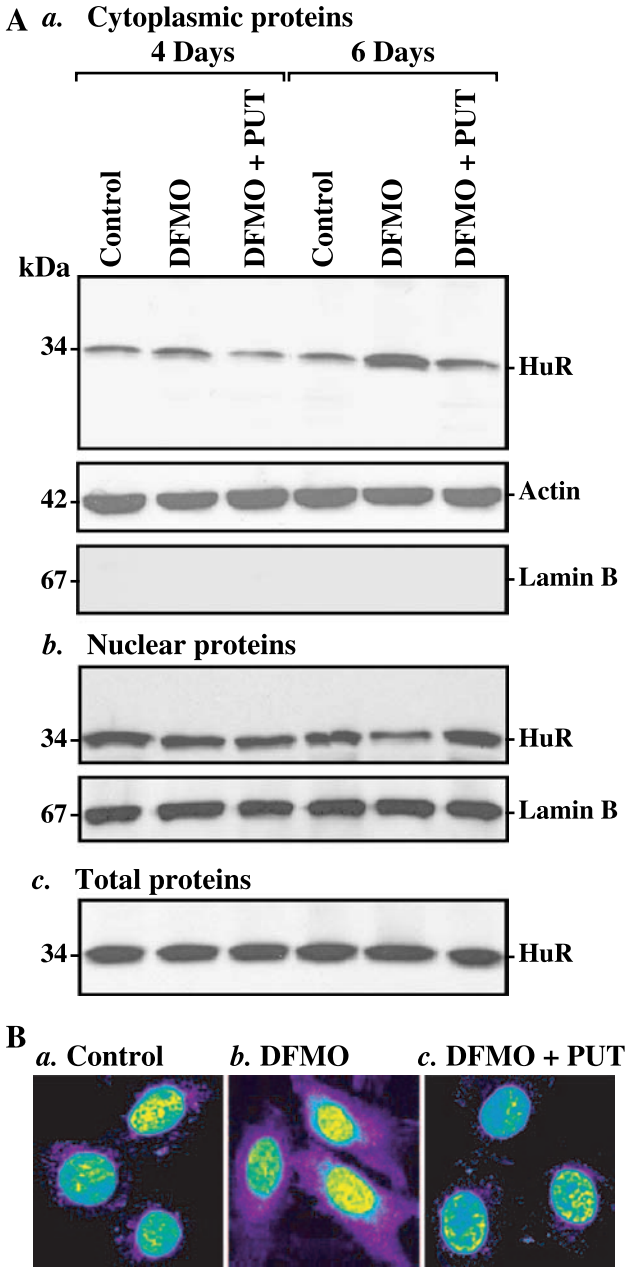

Fig. 2. Changes in levels and cellular distribution of HuR in control IEC-6 cells and cells treated with either DFMO alone or DFMO plus putrescine (PUT) for 4 and 6 days. **A** Representative Western blots: *a* cytoplasmic proteins; *b* nuclear proteins; *c* total proteins. After detecting HuR (~34 kDa), blots were reprobed to detect β-actin (~42 kDa) in cytoplasmic and whole-cell lysates or lamin B in nuclear lysates to control for the quality of the fractionation procedure and the even loading of samples. **B** Cellular distribution of HuR in cells described in **A**: *a* control; *b* DFMO treatment for 6 days; *c* DFMO + PUT treatment for 6 days. Purple: HuR; yellow: nuclei. Original magnification, ×1000. Three experiments were performed that showed similar results

Cytoplasmic HuR directly binds to p53 and NPM 3′-UTRs

Based on the reported association of HuR with the p53 mRNA (Mazan-Mamczarz et al., 2003) and its predicted affinity for the NPM mRNA (de Silanes et al., 2004) at the U-/AU-rich 3′-UTRs of these transcripts, we hypothesized that the p53 and NPM 3′-UTRs were bound to HuR in IEC cells and further postulated that this association would increase in the cytoplasm following polyamine depletion. To test these possibilities, we performed two experiments. First, we used biotinylated transcripts spanning the NPM or p53 3′-UTRs in RNA pull down assays using streptavidin-coated beads and lysates prepared from either untreated or polyamine-deficient cells. Our results showed that the NPM 3′-UTR transcript readily associated with cytoplasmic HuR, as detected by Western blot analysis of the pull down products. The intensity of the binding increased significantly when using lysates prepared from cells that were rendered polyamine-deficient by treatment with DFMO for 4 or 6 days (by 1.8- and 3.3-fold, respectively), but was reduced when using lysates from cells treated with exogenous putrescine. On the other hand, transcripts corresponding to the coding region (CR) of NPM showed undetectable binding to HuR present in cytoplasmic lysates, regardless of the presence or absence of cellular polyamines. Similarly, HuR binding to the p53 3′-UTR (but not the CR) also increased by 1.4-fold and 2.9-fold when using lysates prepared from cells that had been depleted of polyamines for 4 and 6 days, respectively. Again, administration of putrescine together with DFMO completely prevented the increase in HuR binding to the p53 3′-UTR.

Second, we examined the *in vivo* association of endogenous NPM or p53 mRNAs with HuR in intestinal epithelial cells following polyamine depletion through immunoprecipitation of HuR under conditions that preserved its association with target mRNAs (Zou et al., 2006). Our results demonstrated that the products immunoprecipitated with the anti-HuR antibody contained endogenous NPM and p53 mRNAs, as measured by RT-PCR analysis and real-time quantitative PCR (Q-PCR) analysis. The association of endogenous NPM and p53 mRNAs with endogenous HuR increased in cells following DFMO-triggered depletion of cellular polyamines. Significantly, NPM and p53 mRNAs were undetectable in materials immunoprecipitated by using nonspecific IgG1. Together, these findings support the notion that cytoplasmic HuR in intestinal epithelial cells specifically binds to the 3′-UTRs of NPM and p53 mRNAs and that binding increases following polyamine depletion.

HuR silencing abolishes the elevations in the stability of p53 and NPM mRNAs

We designed the siRNA molecule targeting the HuR mRNA (siHuR) in order to reduce HuR levels and thus directly examine its putative role in the previously reported changes in p53 and NPM mRNA stability following polyamine depletion (Li et al., 2002; Zou et al., 2005). Transfection with siHuR potently silenced HuR expres-

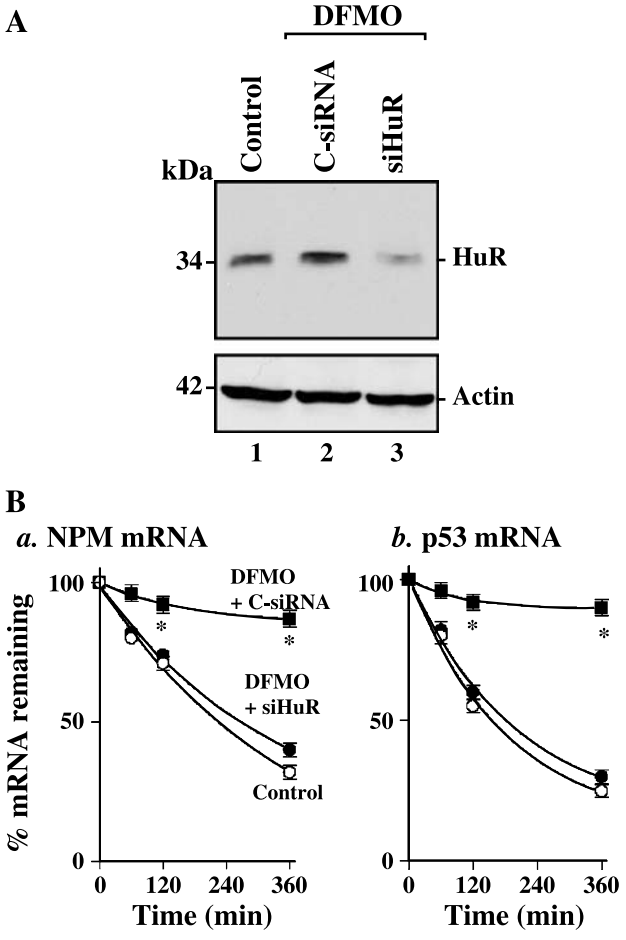

Fig. 3. Effect of HuR silencing on the expression and stability of NPM and p53 mRNAs. **A** Representative HuR immunoblots. After DFMO treatment for 4 days, cells were transfected with either control siRNA (C-siRNA) or siHuR, and whole cell lysates were harvested 48 h thereafter in the presence of DFMO. The levels of HuR protein were measured by Western blot analysis. **B** Half-life of the NPM mRNA (*a*) and p53 mRNA (*b*), assessed by using actinomycin D. Total cellular RNA was isolated at the times shown, and the remaining levels of NPM, p53, and β-actin mRNAs were measured by Q-PCR analysis. Values are the means ± SE from triplicate samples. $^{*}P < 0.05$ when comparing control and DFMO-treated cells transfected with siHuR

sion in polyamine-deficient cells (Fig. 3), while transfection with control siRNA (C-siRNA) showed no inhibitory effect on HuR expression. Consistently, the increase in stability of NPM and p53 mRNAs in polyamine-deficient cells was abolished by silencing HuR (Fig. 3), as the half-lives of NPM and p53 mRNAs in DFMO-treated siRNA-transfected cells were again similar to those of control cells (without DFMO). Furthermore, in HuR-silenced populations, the increased expression of NPM and p53 proteins following polyamine depletion was also prevented. These findings strongly suggest that HuR critically contributes to increasing the stability of NPM and p53 mRNAs in polyamine-depleted cells, in turn elevating NPM and p53 expression and consequently inhibiting intestinal epithelial cell proliferation.

Conclusions

The experimental data summarized in this article provide evidence supporting the hypothesis that polyamine depletion causes inhibition of cell proliferation primarily through the activation of specific cellular signaling pathways, especially increased stability of mRNAs including p53, NPM, JunD, and TGF-β. Based on our previous studies (Li et al., 1999; Patel and Wang, 1999; Patel et al., 1998, Wang and Johnson, 1991; Wang et al., 1991; Zhang et al., 2006; Zou et al., 2006) and others (Bhattacharya et al., 2005; Kramer et al., 2001; Ray et al., 1999), we propose a model delineating the regulation of expression of these growth-inhibiting genes by cellular polyamines and the involvement of these proteins in the process of growth inhibition of small intestinal mucosa following polyamine depletion (Fig. 4). In this model, polyamines negatively regulate posttranscription of growth-inhibiting genes, and the activation of these genes plays a critical role in the negative control of intestinal mucosal growth. Depletion of cellular polyamines stabilizes mRNAs of these growth-inhibiting genes through RNA-binding protein HuR and increases levels of their mRNAs, thus enhancing their protein expression. The resultant accumulation of growth-inhibiting proteins following polyamine depletion activates the transcription of cell cycle arrest genes such as p21, which then blocks the G_1-S phase transition, leading to the inhibition of intestinal epithelial

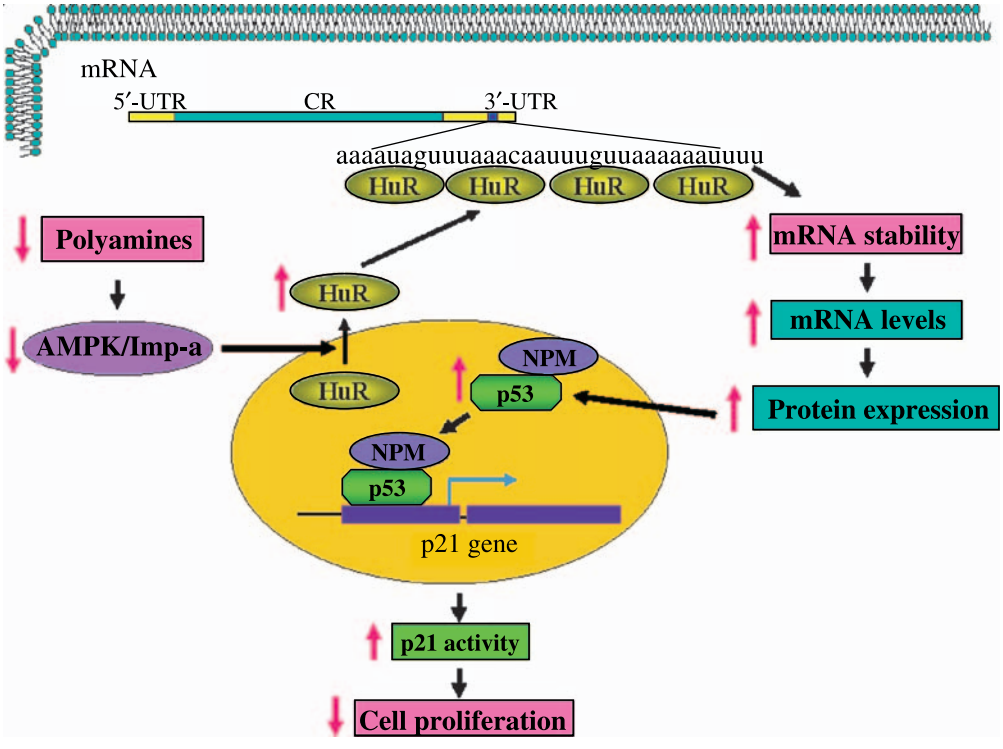

Fig. 4. Schematic diagram depicting the regulation of growth-inhibiting gene expression by cellular polyamines and the proposed role of these proteins in growth inhibition of intestinal epithelial cell proliferation following polyamine depletion. Decreased levels of polyamines stabilize mRNAs such as p53 and NPM by increasing cytoplasmic levels of RNA-binding protein HuR, resulting in the nuclear accumulation of growth-inhibiting proteins through the induction of their newly synthesis. The resultant increases in growth-inhibiting proteins such as p53 and NPM initiate and/or enhance transcription of cell cycle arrest genes such as p21, inhibits cell proliferation, and suppresses mucosal growth of the small intestine. *UTR* Untranslated region; *CR* coding region; *AMPK* AMP-activated protein kinase; *NPM* nucleophosmin; *Imp-α* importin-α

cell proliferation and mucosal growth. However, there are still many critical issues that remain to be addressed regarding this model. For example, studies to define the molecular process responsible for regulation of RNA-binding proteins by polyamines and how polyamine depletion-induced growth-inhibiting proteins interact with their downstream target signals are needed and will lead to a better understanding of the biological functions of cellular polyamines and the mechanism of polyamine depletion-induced growth arrest under physiological and various pathological conditions.

Acknowledgements

This work was supported by a Merit Review Grant from the Department of Veterans Affairs and by NIH Grants DK-57819, DK-61972, and DK-68491. J-Y. Wang is a Research Career Scientist, Medical Research Service, US Department of Veterans Affairs.

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