miR-503 represses CUG-binding protein 1 translation by recruiting CUGBP1 mRNA to processing bodies

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ABSTRACT **microRNAs (miRNAs) and RNA-binding proteins (RBPs) jointly regulate gene expression at the posttranscriptional level and are involved in many aspects of cellular functions. The RBP CUG-binding protein 1 (CUGBP1) destabilizes and represses the translation of several target mRNAs, but the exact mechanism that regulates CUGBP1 abundance remains elusive. In this paper, we show that miR-503, computationally predicted to associate with three sites of the CUGBP1 mRNA, represses CUGBP1 expression. Overexpression of an miR-503 precursor (pre-miR-503) reduced the de novo synthesis of CUGBP1 protein, whereas inhibiting miR-503 by using an antisense RNA (antagomir) enhanced CUGBP1 biosynthesis and elevated its abundance; neither intervention changed total CUGBP1 mRNA levels. Studies using heterologous reporter constructs revealed a greater repressive effect of miR-503 through the CUGBP1 coding region sites than through the single CUGBP1 3′-untranslated region target site. CUGBP1 mRNA levels in processing bodies (P-bodies) increased in cells transfected with pre-miR-503, while silencing P-body resident proteins Ago2, RCK, or LSm4 decreased miR-503–mediated repression of CUGBP1 expression. Decreasing the levels of cellular polyamines reduced endogenous miR-503 levels and promoted CUGBP1 expression, an effect that was prevented by ectopic miR-503 overexpression. Repression of CUGBP1 by miR-503 in turn altered the expression of CUGBP1 target mRNAs and thus increased the sensitivity of intestinal epithelial cells to apoptosis. These findings identify miR-503 as both a novel regulator of CUGBP1 expression and a modulator of intestinal epithelial homoeostasis.**

Monitoring Editor
William P. Tansey
Vanderbilt University

Received: May 27, 2011
Revised: Sep 29, 2011
Accepted: Oct 31, 2011

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E11-05-0456) on November 9, 2011.

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Abbreviations used: Ago, Argonaute; ARE, AU-rich element; CAT-1, cationic amino acid transporter-1; CDK, cyclin-dependent kinase; CHX, cycloheximide; C-oligo, control oligo; CR, coding region; CUGBP1, CUG-binding protein 1; DFMO, α-difluoromethylornithine; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; HPLC, high-performance liquid chromatography; IAP, inhibitor of apoptosis; IEC, intestinal epithelial cell; IP, immunoprecipitation; miRNA, microRNA; NLS, nuclear localization signal; ODC, ornithine decarboxylase; P-bodies, processing bodies; pre-miR-503, miR-503 precursor; Q-PCR, quantitative PCR; RBP, RNA-binding proteins; RISC, RNA-induced silencing complex; siCUGBP1, siRNA targeting CUGBP1; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; UTR, untranslated region; YFP, yellow fluorescent protein.

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INTRODUCTION

The regulation of mRNA stability and translation critically influences gene expression, particularly in the mammalian intestinal mucosa, which has the most rapid turnover rate of any tissue in the body under physiological conditions (Rao and Wang, 2011). The mRNAs are targeted for rapid degradation and/or translational repression through a process involving the interaction of specific mRNA sequences (*cis*-elements) with specific *trans*-acting factors, such as RNA-binding proteins (RBPs) and microRNAs (miRNAs; Keene, 2007; Shen and Pili, 2008; Bartel, 2009; Krol *et al*., 2010). The AU-rich elements and GU-rich elements are the best-characterized *cis*-acting sequences found in the 3′-untranslated region (UTR) from a variety of labile mRNAs. RBPs usually function as regulators that coordinately regulate multiple transcripts and create regulatory networks or "regulons" that are defined by RBPs and their target transcripts. Although many RBPs have housekeeping functions and

interact with many cellular transcripts, several RBPs have emerged that associate with specific subsets of mRNAs and play important roles in controlling gene expression patterns in response to stressful environmental conditions (Kim *et al.*, 2011; Masuda *et al.*, 2011). The CUG-binding protein 1 (CUGBP1) is among the most prominent sequence-specific translation and turnover regulatory RBPs, and it influences many aspects of the cellular response to stress agents, proliferative and apoptotic signals, and developmental cues (Wang *et al.*, 2007; Vlasova *et al.*, 2008; Zhang *et al.*, 2008; Sen *et al.*, 2009; Xiao *et al.*, 2011).

CUGBP1 is a member of the CUGBP and embryonic lethal abnormal vision-like factor (CELF) family of RBPs that are evolutionarily conserved and play essential roles in posttranscriptional gene regulation. CUGBP1 contains three RNA recognition motifs through which it binds to specific mRNAs that often contain GU-rich elements in their 5′-UTRs, 3′-UTRs, or coding regions (CRs; Vlasova and Bohjanen, 2008). Human CUGBP1 and its homologues in chickens, zebra fish, frogs, flies, and worms have long been known to regulate gene expression at posttranscriptional levels and to control important developmental processes and cellular functions (Paillard *et al.*, 1998; Shen and Pili, 2008; Vlasova and Bohjanen, 2008). Knockout of the CUGBP1 homologue ETR1 in *Caenorhabditis elegans* is lethal and impairs muscle development (Milne and Hodgkin, 1999); CUGBP1 knockout in mice is also lethal in most cases, but the few mice that are born display severe fertility defects (Kress *et al.*, 2007), suggesting that the CUGBP1 regulon coordinates gene expression at multiple posttranscriptional levels. In addition to its well-defined role as a regulator of splicing, the interaction of CUGBP1 with its target mRNAs also enhances mRNA decay and represses translation of some target transcripts (Vlasova and Bohjanen, 2008; Zhang *et al.*, 2008; Rattenbacher *et al.*, 2010), although in some instances CUGBP1 also promotes mRNA translation (Iakova *et al.*, 2004). We have recently found that decreasing the levels of cellular polyamines increases cytoplasmic CUGBP1 abundance, induces its association with the cyclin-dependent kinase 4 (CDK4) mRNA, and inhibits CDK4 translation, thus contributing to inhibition of intestinal mucosal growth (Xiao *et al.*, 2011).

Given the multiple posttranscriptional functions of CUGBP1 and the fact that CUGBP1 levels and/or localization are altered in several diseases, including myotonic dystrophy, fragile-X tremor/ataxia syndrome, oculopharyngeal muscular dystrophy, and gut mucosal atrophy (Wang *et al.*, 2007; Vlasova and Bohjanen, 2008; Vlasova *et al.*, 2008; Zhang *et al.*, 2008; Sen *et al.*, 2009; Xiao *et al.*, 2011), we sought to investigate the mechanisms that control CUGBP1 abundance. In this paper, we describe a novel mechanism through which CUGBP1 levels are tightly regulated at the translational level through the action of an miRNA. miRNAs are a family of small (~22 nucleotides), single-stranded, noncoding RNAs that have emerged as major regulators of gene expression (Lal *et al.*, 2009; Siomi and Siomi, 2010). miRNAs are expressed as long, hairpin-forming precursor RNAs that are cleaved into partially double-stranded RNAs and are further processed into mature miRNAs that assemble with members of the Argonaute (Ago) protein family into RNA-induced silencing complex (RISC). The miRNA then directs the RISC to target mRNAs, typically reducing their translation and/or stability (Sontheimer and Carthew, 2004; Djuranovic *et al.*, 2011). It is currently estimated that each miRNA has hundreds of evolutionarily conserved and nonconserved target mRNAs and that at least 50% of all human genes may be regulated by miRNAs (Keene, 2007; Krol *et al.*, 2010). Our study indicates that miR-503 interacts with the CUGBP1 CR and represses its translation by recruiting the CUGBP1 mRNA to processing bodies (P-bodies). Interestingly, the presence of polyamines (critical regulators of mammalian cell proliferation and gut mucosal growth) is necessary for miR-503 expression, while a decrease in cellular polyamines increases CUGBP1 levels at least partially by reducing miR-503. Moreover, the miR-503–mediated reduction in CUGBP1 abundance in turn altered the expression of CUGBP1 target mRNAs and increased the sensitivity of intestinal epithelial cells (IECs) to apoptosis.

RESULTS

miR-503 interacts with CUGBP1 mRNA and represses its translation

Using the program RNA22, we found that there are two predicted binding sites of miR-503 within the CR of the CUGBP1 mRNA and one predicted binding site in the 3′-UTR (Figure 1A), suggesting a potential role for miR-503 in the regulation of CUGBP1 expression. To test this possibility, the following four sets of experiments were performed. First, we examined the association of miR-503 with the CUGBP1 mRNA by RNA pulldown assays using biotin-labeled miR-503 (custom-made by Dharmacon; shown in Figure 1Ba). To assess the transfection efficiency, we examined the levels of miR-503 and small nuclear RNA U6 (which served as control) by real-time quantitative PCR (Q-PCR) analysis 48 h after the transfection. As shown in Figure 1Bb, cells transfected with the biotin-labeled miR-503 exhibited elevated miR-503 levels but displayed no changes in RNA U6 levels (Figure 1Bc). When the presence of CUGBP1 mRNA in the materials pulled down by biotin-miR-503 was examined, the levels of CUGBP1 mRNA were highly enriched in the materials from cells transfected with the biotin-labeled miR-503 but not from cells transfected with scrambled control miRNA (Figure 1Ca). The interaction of miR-503 with the CUGBP1 mRNA is specific, because increasing the levels of biotin-miR-503 failed to increase its binding to the mRNAs that encode CDK2, JunD, and c-Myc. The levels of CDK2, JunD, and c-Myc mRNAs in the pulled-down materials were indistinguishable in cells transfected with biotin-labeled miR-503 compared with cells transfected with scrambled miRNA. In addition, increasing the levels of miR-503 by transfection with biotin-labeled miR-503 did not alter the steady-state levels of CUGBP1, CDK2, JunD, and c-Myc mRNAs (Figure 1Cb). These results strongly suggest that miR-503 directly interacts with the CUGBP1 mRNA and forms the miR-503/CUGBP1–mRNA complex.

Second, we defined the functional consequences of miR-503/CUGBP1–mRNA association. In this study, miR-503 levels were increased by transfection with the miR-503 precursor (pre-miR-503). As shown in Figure 2A, transfection with pre-miR-503 increased miR-503 levels remarkably. Interestingly, increased levels of miR-503 by pre-miR-503 transfection decreased CUGBP1 protein levels (Figure 2B), although it did not reduce the levels of CUGBP1 mRNA as measured by reverse transcription (RT) followed by conventional PCR (Figure 2C, left) or Q-PCR (Figure 2C, right) analyses. To examine whether miR-503 inhibited CUGBP1 expression by repressing its translation, we examined changes in the level of new CUGBP1 protein synthesis after ectopic miR-503 overexpression. Cells were incubated with L-[^{35}S]methionine and of L-[^{35}S]cysteine for 30 min, whereupon newly translated CUGBP1 was visualized by immunoprecipitation (IP) with an antibody against CUGBP1. The brief incubation period was chosen to minimize the contribution of CUGBP1 degradation in our analysis. As shown in Figure 2D, newly synthesized CUGBP1 protein decreased significantly in pre-miR-503–transfected cells compared with cells transfected with scrambled control miRNA. Inhibition of CUGBP1 protein synthesis by miR-503 was specific, since there was no change in nascent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) synthesis after miR-503 overexpression. To determine whether this inhibitory effect was

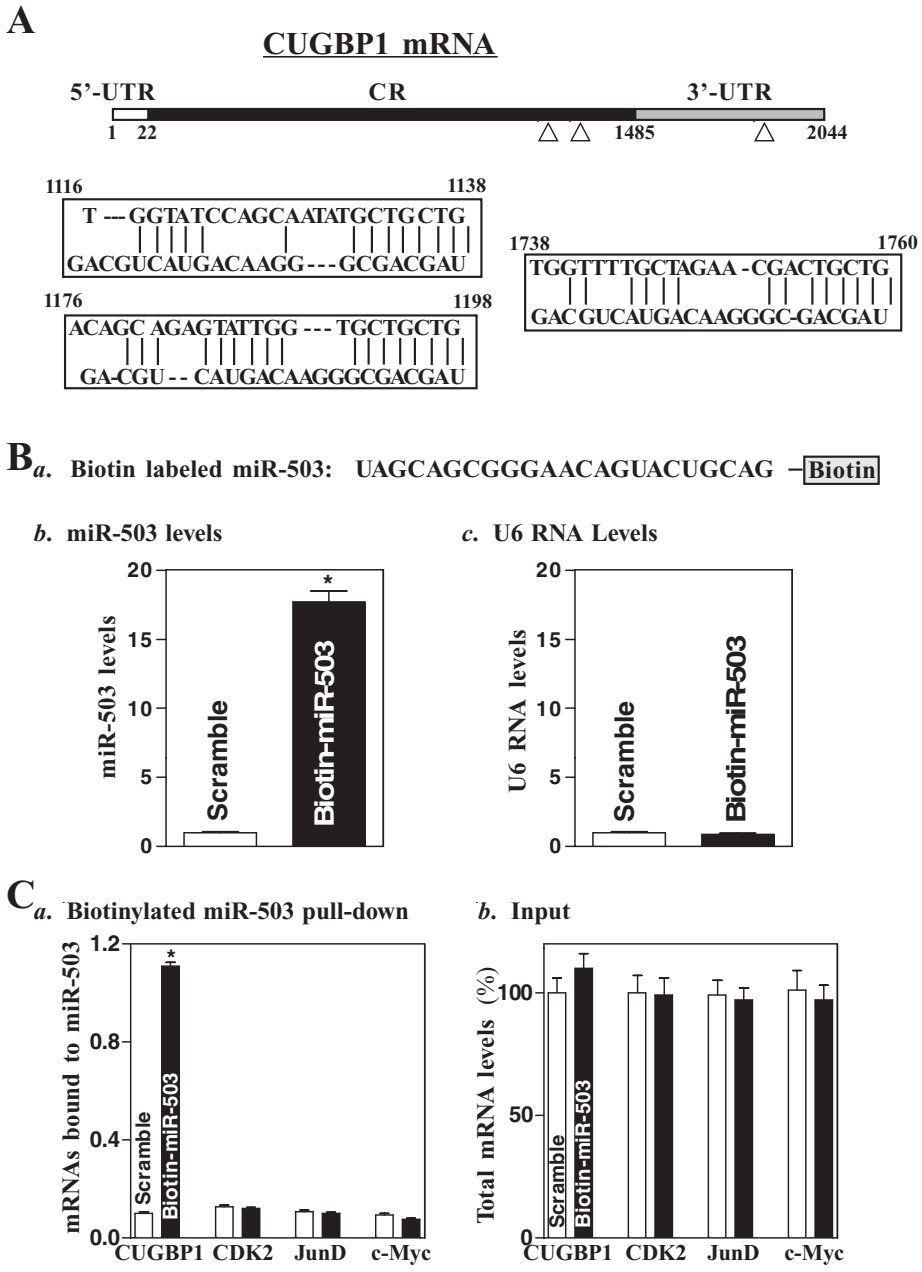

FIGURE 1: miR-503 associates with the CUGBP1 mRNA. (A) Schematic representation of CUGBP1 mRNA depicting predicted target sites for miR-503 in its CR and 3′-UTR. Alignment of the CUGBP1 mRNA sequences with miR-503: top strand, CUGBP1 mRNA; bottom strand, miR-503. (B) Levels of biotinylated miR-503 after transfection for 48 h: (a) schematic representation of biotinylated miR-503, (b) miR-503 levels as measured by Q-PCR analysis, and (c) U6 RNA levels. Values are mean ± SE from three separate experiments. * $p < 0.05$ compared with cells transfected with control scramble oligomer. (C) Binding of biotinylated miR-503 to mRNAs encoding CUGBP1, CDK2, JunD, and c-Myc: (a) levels of mRNAs in the materials pulled down by biotin-miR-503 and (b) levels of total input mRNAs. * $p < 0.05$ compared with cells transfected with scramble oligomer.

mediated through the CUGBP1 CR, 3′-UTR, or both, fractions of the CUGBP1 CR and 3′-UTR were subcloned into the pmirGLO dual-luciferase miRNA target expression vector to generate pmirGLO-CUGBP1-CR and pmirGLO-CUGBP1-3′UTR reporter constructs (Figure 2E, schematic). miR-503 overexpression decreased the levels of CUGBP1-CR luciferase reporter activity (Figure 2E, left), but it failed to inhibit the activity of CUGBP1-3′UTR reporter activity (Figure 2D, right), indicating that increasing the levels of miR-503 represses CUGBP1 mRNA translation through interaction with the CUGBP1 CR rather than with its 3′-UTR.

Third, we further examined the effect of decreasing the level of miR-503 by transfecting the corresponding antisense oligomer (antagomir) targeting miR-503 (anti-miR-503) on CUGBP1 expression. Transfection with anti-miR-503 oligo decreased the levels of miR-503 (Figure 2F) and increased the level of CUGBP1 protein (Figure 2G), although there were no significant changes in the levels of total CUGBP1 mRNA (Figure 2H). Results presented in Figure 2I further show that miR-503 silencing induced the synthesis of new CUGBP1 protein; this stimulatory effect was mediated via the interaction with the CUGBP1 CR, since the levels of luciferase reporter activity were increased in miR-503–silent population of cells only when cells were transfected with the construct of pmirGLO-CUGBP1 CR (Figure 2J). Neither CUGBP1 protein level nor its translation was affected by transfection with a control oligo (C-oligo). These results indicate that decreasing the levels of miR-503 enhances CUGBP1 translation by reducing formation of miR-503/CUGBP1–mRNA complex.

Finally, we characterized the specific binding site of miR-503 in the CUGBP1 CR. Various reporter constructs that expressed chimeric RNA containing the luciferase and partial transcripts spanning the CUGBP1 CR with or without the potential binding site were prepared as indicated in the Figure 3A schematic. Ectopic miR-503 overexpression was found to decrease levels of luciferase reporter gene activity when cells were transfected with the FL-Luc (containing full-length CUGBP1 CR) or F3-Luc (containing a predicted binding site) but not with the F1-Luc (in which the potential binding sites were deleted). When cells were transfected with the F2-Luc (which also contained a potential binding site), increasing the levels of miR-503 just slightly reduced the reporter activity. Internal deletion-mutation of the site located at the F3 of the CUGBP1 CR was also performed, in which the nucleotides spanning positions 1176–1198 of the CUGBP1 CR were eliminated (Figure 3B, schematic). CUGBP1 repression by miR-503 was completely prevented when this specific binding site was deleted from the CUGBP1 CR. Taken together, these results indicate that miR-503 interacts with CUGBP1 mRNA via the specific binding site at 1176–1198, thus repressing CUGBP1 translation.

miR-503 induces recruitment of the CUGBP1 mRNA to P-bodies

Next, we sought to determine whether increased levels of miR-503 repress CUGBP1 translation by increasing the recruitment of the

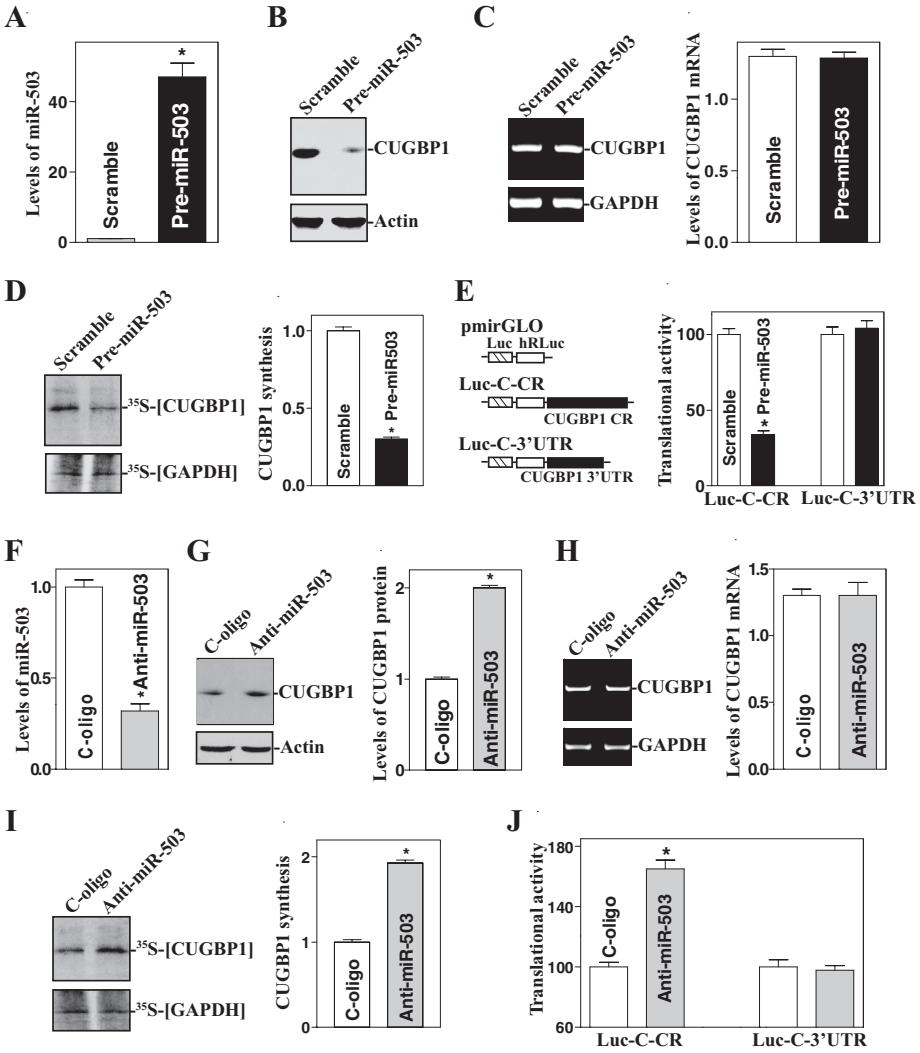

FIGURE 2: miR-503 represses CUGBP1 mRNA translation. (A) Levels of miR-503 in cells transfected with pre-miR-503 for 48 h as measured by Q-PCR analysis. Values are mean ± SE from three separate experiments. * $p < 0.05$ compared with cells transfected with control scramble oligomer. (B) Changes in CUGBP1 protein expression after ectopic miR-503 overexpression. Whole-cell lysates were prepared for Western blotting; equal loading was monitored by assessing β-actin levels. (C) Levels of CUGBP1 mRNA as examined by RT-PCR (left) or Q-PCR (right) analyses. (D) Changes in newly synthesized CUGBP1 protein as measured by IP with anti-CUGBP1 antibody in cells described in (A). Left, representative immunoblots of newly synthesized CUGBP1; right, quantitative analysis of the immunoblotting signals as measured by densitometry. Values are mean ± SE of data from three separate experiments; the relative levels of newly synthesized CUGBP1 were corrected by measuring GAPDH signals. * $p < 0.05$ compared with cells transfected with control scramble oligomer. (E) Changes in CUGBP1 translation efficiency as measured by analysis of CUGBP1-CR or its 3′-UTR luciferase reporter (schematic) after cotransfection with a *Renilla* luciferase reporter. Values are mean ± SE of data from three separate experiments. * $p < 0.05$ compared with cells transfected with control scramble oligomer. (F–J) Effect of miR-503 silencing on CUGBP1 expression. After cells were transfected with the corresponding oligomer targeting miR-503 (anti-miR-503) or C-oligo for 48 h, various measurements were performed as described above. Values are mean ± SE of data from three separate experiments. * $p < 0.05$ compared with cells transfected with C-oligo.

CUGBP1 mRNA to P-bodies, where mRNAs are thought to be sorted for degradation and/or translational repression (Buchan and Parker, 2009; Kulkarni *et al.*, 2010). First, we tested whether the P-body resident protein Ago2 was functionally linked to the inhibitory activity of miR-503. As shown in Figure 4A, miR-503 overexpression increased the association of CUGBP1 with hemagglutinin (HA)-tagged Ago2 protein. Furthermore, increased levels of both miR-503 and HA-Ago2 by cotransfection with pre-miR-503 and HA-Ago2 expression vector synergistically increased the levels of miR-503/CUGBP1–mRNA complex in the Ago2 IP materials, although HA-Ago2 overexpression alone failed to alter the levels of CUGBP1 mRNA in Ago2 IP materials. Interestingly, repression of CUGBP1 by miR-503 decreased significantly in the absence of P-body resident proteins Ago2 or RCK (Figure 4B). Furthermore, disruption of P-body formation by silencing LSm4 (another P-body resident protein) also prevented miR-503–induced repression of CUGBP1 expression (Figure 4C). On the other hand, silencing Ago2, RCK, or LSm4 alone did not decrease CUGBP1 expression levels.

To determine directly whether the localization of CUGBP1 mRNA in P-bodies is implicated in miR-503–induced repression, we studied the subcytoplasmic localization of CUGBP1 mRNA. The reporter construct pMS2-CUGBP1, which expressed a chimeric RNA (MS2-CUGBP1) comprising the CUGBP1 CR (which contained both binding sites for miR-503) and 12 tandem MS2 RNA hairpins (Figure 4D, schematic), was prepared. Cotransfection of pMS2-CUGBP1 together with plasmid pMS2-yellow fluorescent protein (YFP), which expressed the chimeric fluorescent protein MS2-YFP with a nuclear localization signal (NLS), allowed us to track the subcellular localization of the chimeric MS2-CUGBP1 RNA (as the complex MS2-YFP/MS2-CUGBP1) as well as the control MS2 RNA (as the complex MS2-YFP/MS2) by confocal microscopy. Signals of the P-body marker RCK were detected in the same cells. As shown, the control MS2 RNA appeared to be exclusively nuclear in all of the transfected cells (Figure 4Da) due to the presence of the NLS in the chimeric protein MS2-YFP. As shown in Figure 4Db, some MS2-CUGBP1 RNA was retained in the cytoplasm, colocalizing with some RCK signals in control cells transfected with scrambled miRNA. However, miR-503 overexpression by transfection of pre-miR-503 increased the colocalization of MS2-CUGBP1 RNA and RCK signals (colocalization results in yellow signals in the merged image in Figure 4Dc), suggesting that increasing the levels of miR-503 enhanced the association of CUGBP1 mRNA with P-bodies. Together, these results support the notion that miR-503 represses CUGBP1 mRNA translation at least partially by recruiting the CUGBP1 transcripts to P-bodies.

Polyamines regulate CUGBP1 expression by altering the level of miR-503

The natural polyamines spermidine and spermine and their precursor putrescine are organic cations found in all eukaryotic cells and

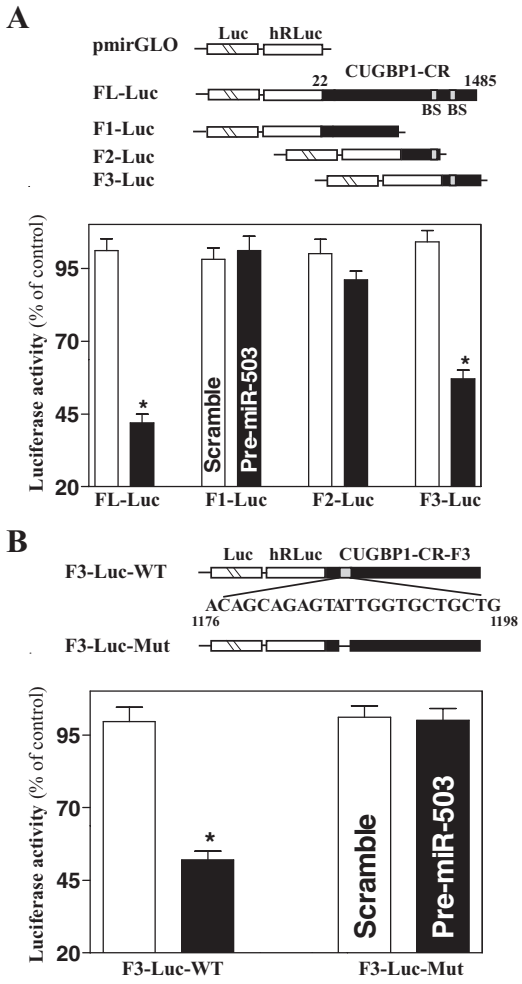

FIGURE 3: Changes in activities of CUGBP1 CR luciferase reporters after deletion of miR-503–binding site. (A) Effect of 5′-deletion of CUGBP1 CR on its luciferase reporter activity. Top, schematic of plasmids of different chimeric firefly luciferase CUGBP1 CR reporters. BS, predicted miR-503–binding site. Bottom, levels of CUGBP1 CR luciferase reporter activity. Twenty-four hours after transfection with pre-miR-503, cells were cotransfected with CUGBP1 CR F-Luc constructs and a *Renilla* luciferase reporter. Levels of firefly and *Renilla* luciferase activities were assayed 24 h later. Results were normalized to the *Renilla* luciferase activity and expressed as the mean ± SE of data from three separate experiments. * $p < 0.05$ compared with cells transfected with control scrambled oligomer. (B) Effect of deletion of specific miR-503–binding site (schematic) in CUGBP1 CR on luciferase reporter activity after ectopic miR-503 overexpression.

are implicated in many aspects of cellular physiology (Gerner and Meyskens, 2004; Casero and Pegg, 2009). Our recent studies showed that polyamines repress CUGBP1 expression in IECs (Xiao *et al.*, 2011), but the exact mechanism underlying this process remains unknown. We examined whether miR-503 plays a role in the regulation of CUGBP1 expression by polyamines. As reported in our previous studies (Liu *et al.*, 2003; Zou *et al.*, 2010), inhibition of ornithine decarboxylase (ODC), a key enzyme for polyamine biosynthesis, by treatment with α-difluoromethylornithine (DFMO) for 4 d almost totally depleted polyamines. Putrescine and spermidine were undetectable by day 4 of continuous treatment with DFMO, and spermine had decreased by ~60% (unpublished data). As shown in Figure 5A, polyamine depletion by DFMO decreased the levels of miR-503, although it increased miR-29b and failed to change levels of U6 RNA. Supplementation with putrescine reversed the DFMO-triggered changes in the levels of miR-503 and miR-29b, as did spermidine supplementation (unpublished data). The reduction in miR-503 in polyamine-deficient cells was associated with an increase in CUGBP1 expression. The steady-state levels of CUGBP1 and de novo synthesized CUGBP1 increased significantly in cells exposed to DFMO for 4 d, but this induction was completely prevented by addition of exogenous putrescine (Figure 5B). Polyamine depletion by DFMO did not alter the levels of CUGBP1 mRNA (Figure 5C) and the stability of CUGBP1 protein (Supplemental Figure S1), suggesting that increased expression of CUGBP1 in polyamine-deficient cells resulted predominantly from the stimulation of CUGBP1 translation. These findings were further supported by the results presented in Figure 5D, which show that polyamine depletion by DFMO treatment induced CUGBP1 translation, as indicated by an increase in CUGBP1 CR luciferase reporter gene activity. The combined treatment with DFMO and putrescine prevented the increase in CUGBP1 translation, rendering the level of CUGBP1 CR–mediated reporter gene activity similar to that observed in control cells. To define the putative role of reduced levels of miR-503 in the induction of CUGBP1 expression following polyamine depletion, pre-miR-503 was used to increase miR-503 levels in DFMO-treated cells. Ectopic miR-503 overexpression attenuated the induced level of CUGBP1 protein in polyamine-deficient cells, although it had no effect on human antigen R (HuR) levels (Figure 5E). Polyamine depletion also increased miR-29b levels, but there are no predicted miR-29b binding sites in the CUGBP1 mRNA. Consistently, increasing the levels of biotin-miR-29b did not increase its association with the CUGBP1 mRNA, although it increased miR-29b/claudin-1 mRNA complex formation (Figure S2). Together, these results indicate that polyamine depletion enhances CUGBP1 translation at least partially by reducing miR-503 levels.

To determine the effect of increasing the levels of cellular polyamines on miR-503 and CUGBP1 expression, we used two clonal populations of IECs stably expressing ODC (ODC-IEC) in this study. As reported previously (Liu *et al.*, 2006; Zou *et al.*, 2006), ODC-IEC cells exhibited very high levels of ODC protein and a greater than 50-fold increase in ODC enzyme activity. Consistently, the levels of putrescine, spermidine, and spermine in IEC cells stably overexpressing ODC were increased by ~12-fold, ~twofold, and ~25% when compared with cells transfected with the control vector lacking ODC cDNA, as reported previously (Liu *et al.*, 2006). The results in Figure 6A show that increasing cellular polyamines by ODC overexpression induced the levels of miR-503, but it decreased miR-29b and failed to alter the levels of U6 RNA. The increased levels of miR-503 were associated with a repression of CUGBP1 translation, as shown by a decrease in the levels of CUGBP1 protein in ODC-IEC cells as compared with those observed in cells infected with control vector (Figure 6Ba). Although increasing cellular polyamines did not reduce total CUGBP1 mRNA levels (Figure 6C), it did decrease the levels of newly synthesized CUGBP1 protein (Figure 6Bb) and the activity of CUGBP1-CR luciferase reporter gene (Figure 6D), indicating that this inhibition of CUGBP1 expression was primarily due to a reduction in CUGBP1 mRNA translation. The inhibitory effects of ODC overexpression on CUGBP1 translation were not simply due to clonal variation, since two different clonal populations, ODC-IEC-C1 and ODC-IEC-C2, showed similar responses. In addition, increasing the levels of cellular polyamines failed to alter the stability of CUGBP1 protein (Figure S3). The results presented in Figure 6E further showed that silencing miR-503 by transfection with anti-miR-503 oligomer promoted CUGBP1 expression in stable ODC-IEC cells, indicating that repression of CUGBP1

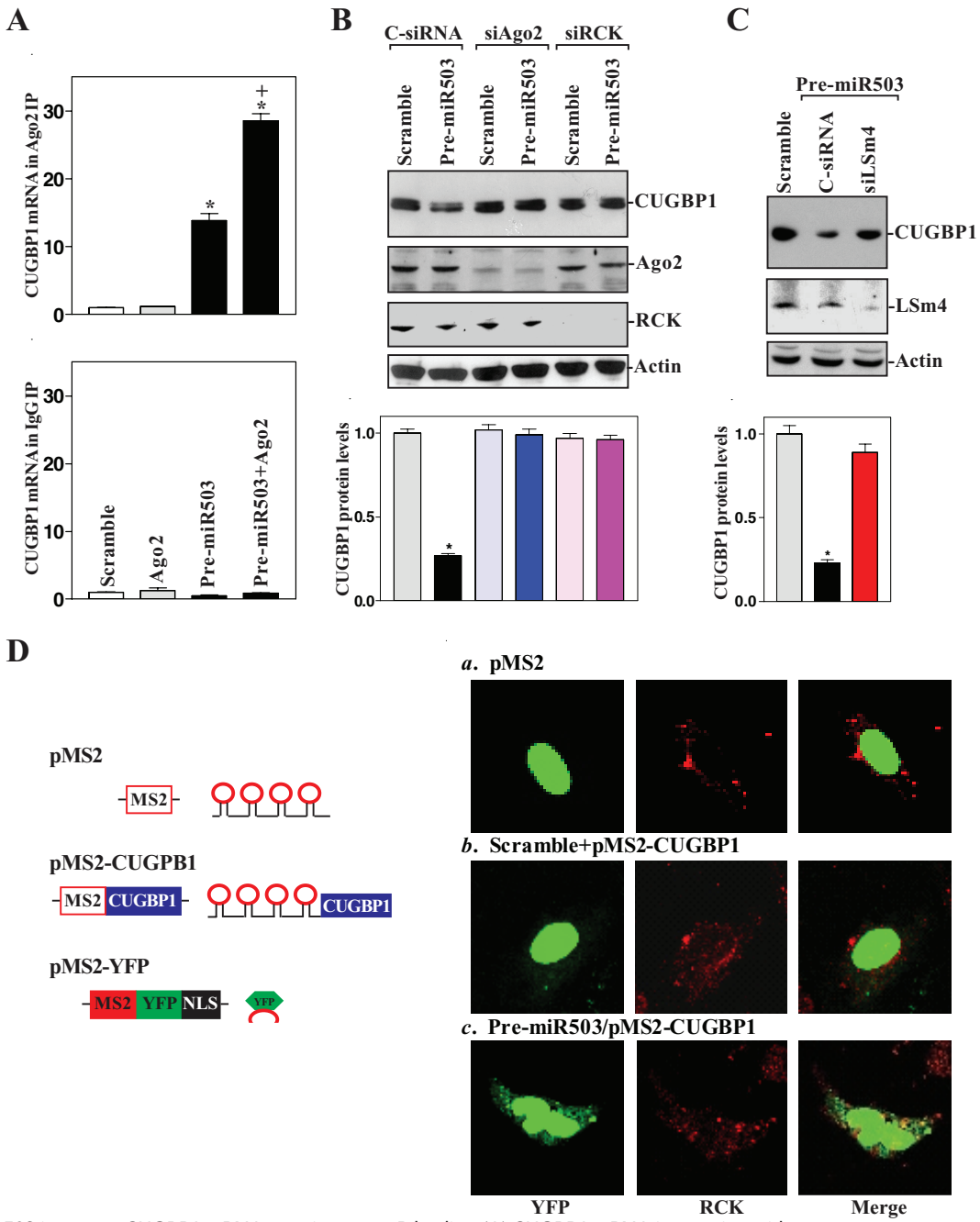

FIGURE 4: miR-503 increases CUGBP1 mRNA recruitment to P-bodies. (A) CUGBP1 mRNA interaction with components of P-bodies. After cells were transfected with the Ago2 expression vector (Ago2) or pre-miR-503 alone or cotransfected with Ago2 and pre-miR-503 for 48 h, the association of CUGBP1 mRNA with HA-Ago2 was measured by RNP IP using anti-Ago2 (top) or control IgG (bottom) antibodies, which was followed by Q-PCR analysis. Values are mean ± SE of data from three separate experiments. $^{*,+}$ $p < 0.05$ compared with cells transfected with scramble or Ago2 and cells transfected with pre-miR-503, respectively. (B and C) Effect of silencing Ago2, RCK, and LSm4 on CUGBP1 expression in cells overexpressing miR-503. Top, representative immunoblots of CUGBP1 protein; bottom, quantitative analysis of the immunoblotting signals as measured by densitometry. Cells were transfected with pre-miR-503 or cotransfected with pre-miR-503 and specific siRNA targeting Ago2 (siAgo2), RCK (siRCK), or LSm4 (siLSm4) for 48 h; the levels of CUGBP1, Ago2, and RCK proteins (B), CUGPB1 and LSm4 proteins (C), and loading control β-actin (B and C) were assessed by Western blot analysis. Values are mean ± SE of data from three separate experiments; the relative levels of CUGBP1 were corrected for protein loading by measuring β-actin signals. * $p < 0.05$ compared with cells transfected with scramble or cells cotransfected with pre-miR-503 and siAgo2, siRCK or siLSm4. (D) Colocalization of CUGBP1 mRNA with P-bodies after miR-503 overexpression. Left, schematic of the plasmids used for the visualization of CUGBP1 mRNA. pMS2 and pMS2-CUGBP1 expressed MS2 and MS2-CUGBP1 RNAs, each containing 12 tandem MS2 hairpins; pMS2-YFP expressed a fusion fluorescent protein (MS2-YFP) capable of detecting MS2-containing RNA. Right, images of CUGBP1 mRNA colocalization with P-bodies in cells overexpressing miR-503. Confocal microscopy was used to visualize MS2 and MS2-CUGBP1 mRNA using MS2-YFP (green fluorescence); red, RCK (P-body marker) signals; yellow, colocalized red and green signals. Three experiments were performed that showed similar results.

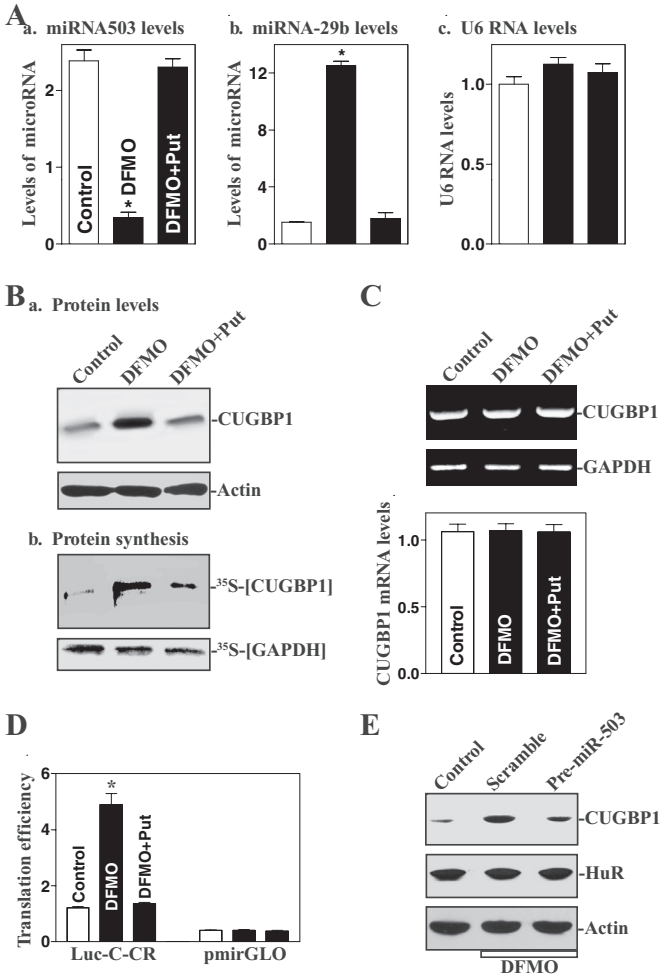

FIGURE 5: Decreasing cellular polyamines by inhibiting ODC with DFMO enhances CUGBP1 mRNA translation by reducing miR-503. (A) Changes in the levels of miR-503, miR-29b, and U6 RNA as examined by Q-PCR analysis in cells exposed to DFMO or DFMO plus putrescine (Put) for 4 d. Values are mean ± SE of data from three separate experiments. * $p < 0.05$ compared with control cells and cells exposed to DFMO plus putrescine. (B) Changes in the level of CUGBP1 expression after polyamine depletion: (a) representative immunoblots of CUGBP1 protein from Western blot analysis and (b) newly synthesized CUGBP1 protein as measured by IP with anti-CUGBP1 antibody. (C) Changes in CUGBP1 mRNA levels as measured by RT- (top) or Q-PCR (bottom) analysis. (D) Changes in CUGBP1 translation efficiency as measured by analysis of CUGBP1-CR luciferase reporter in cells described in (A). Values are mean ± SE of data from three separate experiments. * $p < 0.05$ compared with controls and cells exposed to DFMO plus Put. (E) Effect of ectopic overexpression of miR-503 on CUGBP1 expression in polyamine-deficient cells. After cells were exposed to DFMO for 2 d, they were transfected with pre-miR-503 or control scramble oligomer and then maintained in the medium containing DFMO for an additional 2 d. Levels of CUGBP1 and HuR were examined by Western blot analysis; three separate experiments were performed that showed similar results.

expression by increasing cellular polyamines results from an induction in miR-503.

miR-503/CUGBP1 signaling plays an important role in the regulation of apoptosis

To investigate the biological significance of miR-503, we examined its possible involvement in the regulation of apoptosis in IECs. We first examined spontaneous apoptotic cell death without any challenge of apoptotic stimulators after inhibition of miR-503 expression with anti-miR-503 oligomer. Transfection with anti-miR-503 oligomer for 48 h increased CUGBP1 expression (Figure 2) but failed to directly induce apoptosis (Figure 7, Aa and Ab, left). There were no apparent differences in cell viability between miR-503–antagonized populations and control cells; no morphological features of apoptosis or detectable levels of active caspase-3 protein were obtained with or without the miR-503 antagomir. Second, we determined whether antagonizing miR-503 altered the susceptibility of IECs to apoptosis after exposure to tumor necrosis factor-α (TNF-α) plus cycloheximide (CHX). This apoptotic model was chosen, because TNF-α/CHX–induced apoptosis is widely accepted as a form of programmed cell death induced by a biological apoptotic inducer in the gut mucosa (Cardone *et al.*, 1998; Xiao *et al.*, 2007). When control cells were exposed to TNF-α/CHX for 4 h, morphological features characteristic of programmed cell death were observed, and annexin V staining showed significant phosphatidylserine presence in the cell membrane, a classic indicator of apoptotic cells (Figure 7Aa, right, and B, left). The assessments of apoptosis were confirmed by an increase in the levels of active caspase-3 (Figure 7C) after treatment with TNF-α/CHX. Interestingly, miR-503 inactivation protected cells against TNF-α/CHX–induced apoptosis, as indicated by decreased percentages of apoptotic cells. This protective effect was not altered when cells were transfected with control siRNA, but it was lost when CUGBP1 expression was silenced by small-interfering RNA (siRNA) targeting the CUGBP1 (siCUGBP1). The percentages of apoptotic cells (Figure 7B, right) and levels of active caspase-3 protein (Figure 7C) in miR-503–silent cells transfected with siCUGBP1 were increased, compared with those observed in miR-503–antagonized cells transfected with C-siRNA after exposure to TNF-α/CHX. In addition, CUGBP1 silencing alone did not directly induce cell death, but it increased the sensitivity of IEC-6 cells to TNF-α/CHX–induced apoptosis (Figure S4). To investigate the downstream targets of miR-503 in the regulation of apoptosis, we examined changes in the levels of the inhibitors of apoptosis (IAP) proteins c-IAP1 and c-IAP2, and the NF-κB subunit p65 in cells overexpressing miR-503 and in cells antagonizing miR-503. Increased levels of miR-503 resulting from pre-miR-503 transfection decreased levels of c-IAP1 and c-IAP2 proteins, although it did not alter the level of p65 protein (Figure 7D, left). In contrast, in cells transfected with anti-miR-503 the levels of c-IAP1 and c-IAP2 were higher (Figure 7D, right). This increase in c-IAP expression by miR-503 silencing resulted from the increase in CUGPB1 levels, because this stimulatory effect was prevented by silencing CUGPB1. These results strongly suggest that miR-503 functions as a proapoptotic factor in IECs partially by altering the expression of c-IAP1 and c-IAP2 in a CUGBP1-dependent manner.

DISCUSSION

Our findings demonstrate the translational repression of CUGBP1 by miR-503 and provide insight into the regulation of one type of posttranscriptional regulator (an RBP) by another type of posttranscriptional regulator (an miRNA). miR-503 directly interacts with and represses CUGBP1 mRNA translation but does not appear to affect CUGBP1 mRNA stability, since miR-503 overexpression specifically reduced CUGBP1 nascent translation and miR-503 acting as an antagonist elevated it, but neither intervention affected total CUGBP1 mRNA levels (Figure 2). Unlike the more common observations showing that miRNAs often exert their regulatory actions through interactions with the 3′-UTRs of target transcripts (Baltimore *et al.*, 2008; Lal *et al.*, 2009), the miR-503 binding site in the CUGBP1 CR

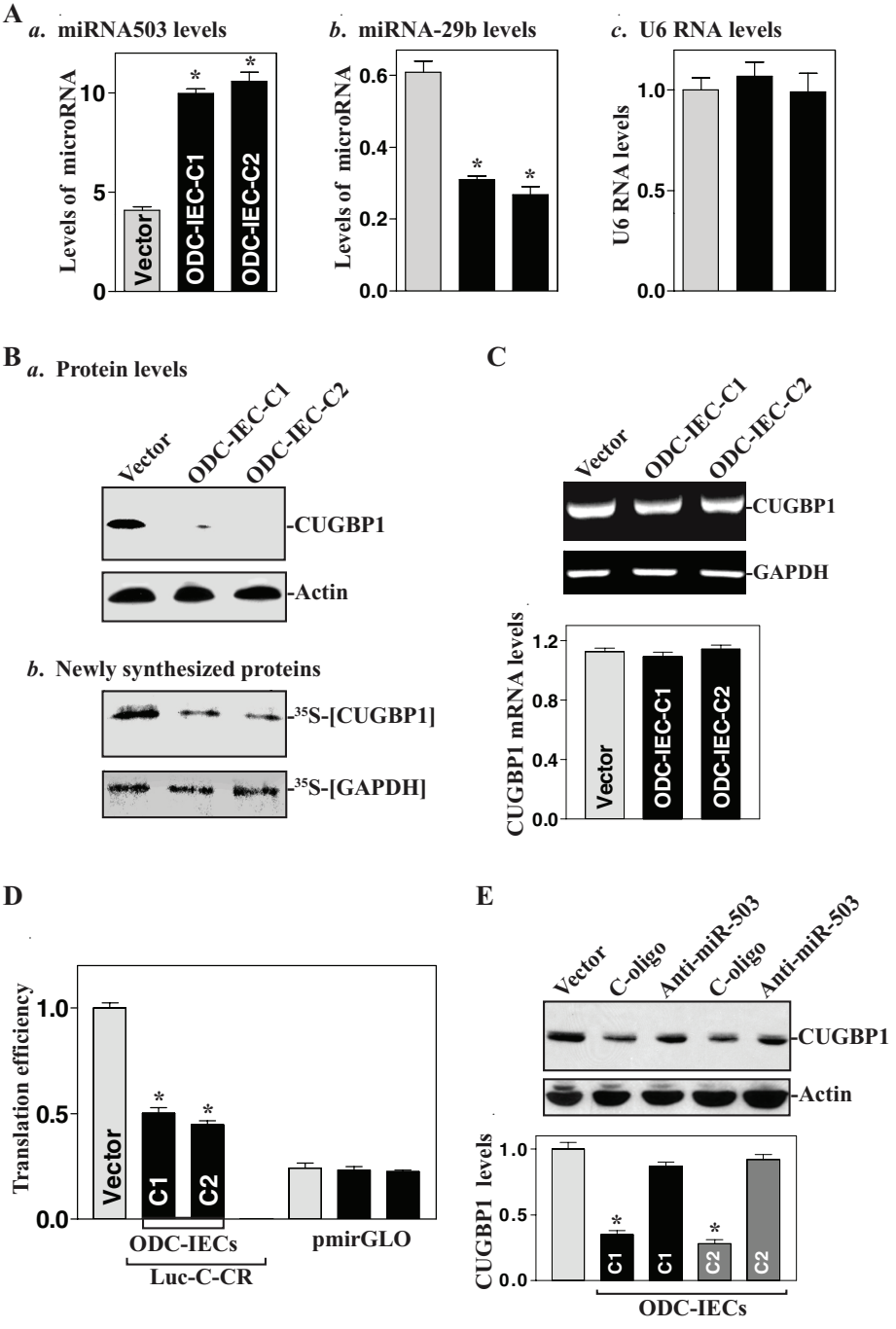

FIGURE 6: Increasing cellular polyamines by ectopic ODC overexpression represses CUGBP1 translation by increasing miR-503. (A) Changes in the levels of miR-503, miR-29b, and U6 RNA as examined by Q-PCR analysis in stable ODC-IEC cells. Values are mean ± SE of data from three separate experiments. * $p < 0.05$ compared with cells transfected with vector containing no ODC cDNA. (B) Changes in the level of CUGBP1 expression in stable ODC-IEC cells: (a) representative immunoblots of CUGBP1 and (b) newly synthesized CUGBP1 protein as measured by IP with anti-CUGBP1 antibody. (C) Changes in CUGBP1 mRNA levels as measured by RT- (top) or Q-PCR (bottom) analyses. (D) CUGBP1 translation efficiency as measured by analysis of CUGBP1-CR luciferase reporter in stable ODC-IEC cells. Values are mean ± SE of data from three separate experiments. * $p < 0.05$ compared with cells transfected with vector alone. (E) Effect of miR-503 silencing on CUGBP1 expression in stable ODC-IEC cells. Top, representative immunoblots of CUGBP1 protein; bottom, quantitative analysis of the immunoblotting signals as measured by densitometry. Cells were transfected with the corresponding oligomer targeting miR-503 (anti-miR-503) or C-oligo for 48 h; levels of CUGBP1 protein were examined by Western blot analysis. Values are mean ± SE of data from three separate experiments; the relative levels of CUGBP1 were corrected for protein loading by measuring β-actin signals. * $p < 0.05$ compared with vector alone or ODC-IEC cells transfected with C-oligo.

appeared to be the predominant site through which miR-503 repressed CUGBP1 translation. Through the use of ectopic various reporters bearing partial transcripts spanning the CUGBP1 CR with or without the miR-503 site (Figure 3), our results further show that the 1176–1198 site of the CUGBP1 CR was primarily used by miR-503 for its inhibitory effect, whereas another predicted site at 1116–1138 exhibited a lesser effect. In an earlier report, miR-519 was shown to repress translation of the RBP HuR (Abdelmohsen *et al.*, 2008) by interacting with the HuR coding region, rather than the 3′-UTR.

The levels of miR-503 and its associated cluster of miRNAs increase during muscle differentiation and promote cell cycle quiescence and differentiation by down-regulating Cdc25A (Sarkar *et al.*, 2010), although the exact mechanism whereby miR-503 reduces Cdc25A expression remains unclear. Deregulation of miR-503 is also found to contribute to diabetes mellitus–induced impairment of endothelial function and reparative angiogenesis after limb ischemia (Caporali *et al.*, 2011). The present study provides new evidence that miR-503 suppresses CUGBP1 translation and is implicated in the regulation of apoptosis in IECs. The fact that CUGBP1 destabilizes many target mRNAs and represses translation (Vlasova and Bohjanen, 2008; Vlasova *et al.*, 2008; Zhang *et al.*, 2008) raises an intriguing possibility that should be considered. If miR-503 enhances the stability or translation of a putative target mRNA, it is possible that the effect is indirect, should CUGBP1 be a destabilizing RBP for that particular mRNA. In broad terms, our findings advise caution regarding the stimulatory functions of some miRNAs upon some target mRNAs, since they could result from an indirect, suppressive effect of the miRNA upon a destabilizing or translational inhibitory RBP, such as CUGBP1, which may control the same target mRNA.

The results presented here also show that miR-503 represses CUGBP1 translation at least partially by recruiting the CUGBP1 mRNA to P-bodies. miR-503 overexpression increased CUGBP1 mRNA levels in P-bodies as measured by RNP IP using an antibody specific for the P-body resident protein Ago2 and by measuring colocalization of the CUGBP1 mRNA with the P-bodies (Figure 4). Furthermore, silencing Ago2, RCK, or LSm4 decreased miR-503–mediated repression of CUGBP1 expression. P-bodies are small cytoplasmic foci visible in somatic cells and are believed to have the dual functions of: 1) harboring nontranslating mRNAs

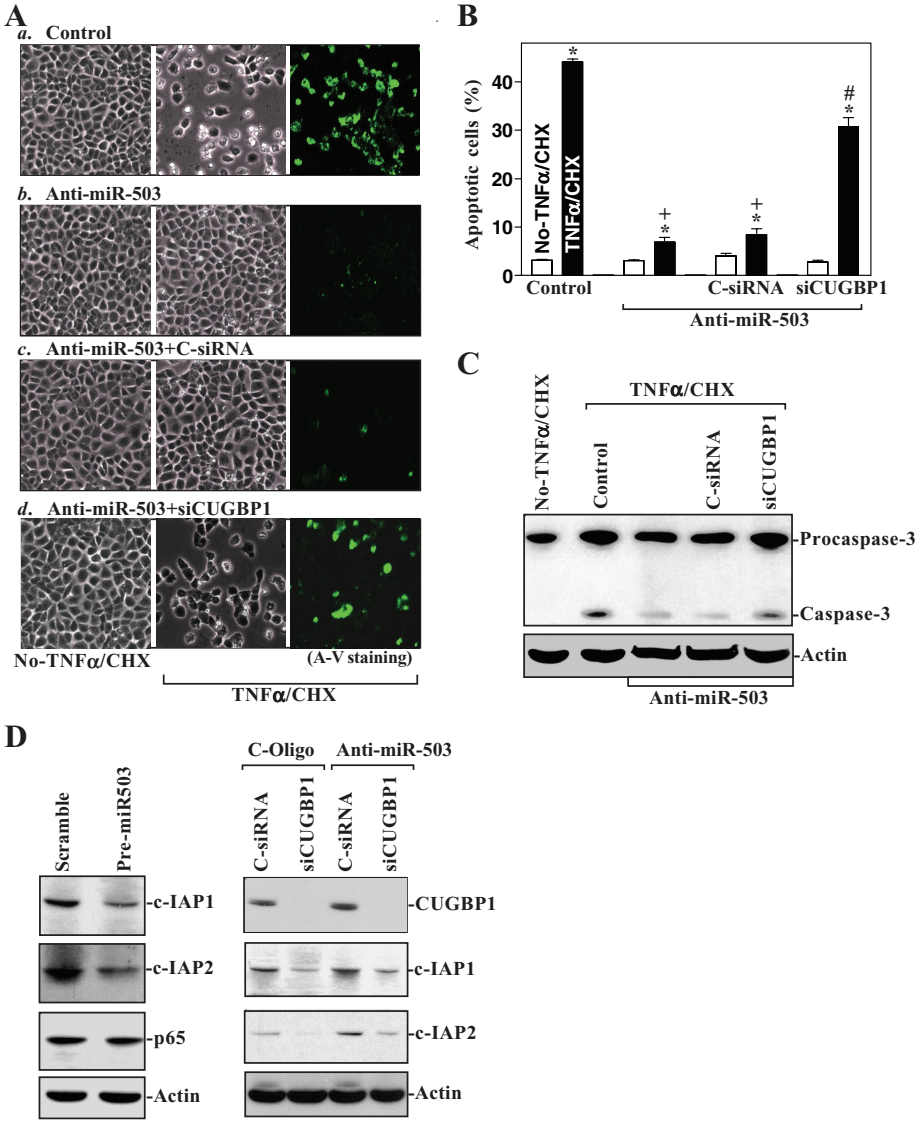

FIGURE 7: miR-503 silencing protects IEC cells against apoptosis through induction in CUGBP1. (A) TNF-α/CHX–induced apoptosis after various treatments. Cells were transfected with the anti-miR-503 or C-oligo (Control) for 48 h; apoptosis was measured 4 h after treatment with TNF-α/CHX: (a) cells transfected with C-oligo, (b) cells transfected with anti-miR-503, (c) cells transfected with anti-miR-503 and control siRNA (C-siRNA), and (d) cells transfected with anti-miR-503 and siRNA targeting CUGBP1 (siCUGBP1). Original magnification: 150×. (B) Percentages of apoptotic cells after different treatments described in (A). Values are mean ± SE of data from three experiments. * $p < 0.05$ compared with cells untreated with TNF-α/CHX; $^{+}$ $p < 0.05$ compared with cells exposed to TNF-α/CHX; $^{\#}$ $p < 0.05$ compared with cells cotransfected with anti-miR-503 and C-siRNA and then exposed to TNF-α/CHX. (C) Changes in levels of caspase-3 in cells described in (A). Whole-cell lysates were harvested, and the levels of procaspase-3 and caspase-3 were assessed by Western blot analysis. β-actin immunoblotting was performed as an internal control for equal loading. (D) Effect of miR-503 on the expression of c-IAP and p65 proteins. Left, changes in the levels of c-IAP1, c-IAP2 and p65 proteins in cells overexpressing miR-503. Cells were transfected with pre-miR-503 for 48 h, whereupon whole-cell lysates were prepared, and the levels of various proteins were measured. Three separate experiments were performed that showed similar results. Right, the levels of CUGBP1 and c-IAP proteins after transfecting anti-miR-503 alone or transfecting both anti-miR-503 and siCUGBP1 as described in (A).

that can exit again from P-bodies to reengage in translation and 2) recruiting mRNAs that are targeted for deadenylation and degradation by the decapping/Xrn1 pathway (Zhai *et al.*, 2008; Carbonaro *et al.*, 2011; Kurischko *et al.*, 2011; Saito *et al.*, 2011). Many proteins associated with RISC and implicated in miRNA-mediated suppression are concentrated in P-bodies. These include all four human Ago proteins, GW182 (TNRC6A) and its two human paralogues (TNRC6B and TNRC6C), two RNA helicases (RCK and MOV10), decapping enzymes (DCP1 and DCP2), mRNA deadenylation factors (such as the CCR4-CAF-1-Not complex), activators of decapping (Dhh1/RCK/p54, Pat1, Scd6/RAP55, Edc3, and the LSm1-7 complex), and exonucleases (such as XRN-1; Kulkarni *et al.*, 2010). Among them, both RCK and GW182 are essential for P-body formation (Yang *et al.*, 2004; Chu and Rana, 2006; Saito *et al.*, 2011). A compelling example for the transient association of an miRNA target was provided by the study from Bhattacharyya *et al.* (2006), which demonstrated that the endogenous cationic amino acid transporter-1 (CAT-1) mRNA and miR-122 localize to P-bodies in liver cells and are linked to the inhibition of CAT-1 translation. The same mRNA is released from P-bodies when its translation is activated by amino acid starvation. However, the dynamic turnover of miR-503 association with the CUGBP1 mRNA in P-bodies in IECs under various pathophysiological conditions remains to be investigated.

The data obtained in the present study also indicate that miR-503 biogenesis is tightly regulated by cellular polyamines in IECs. Polyamines have been recognized for many years as key molecules that control multiple signaling pathways and have distinct cellular functions (Gerner and Meyskens, 2004; Casero and Marton, 2007). The levels of cellular polyamines are highly regulated and depend on the dynamic balance among polyamine biosynthesis, degradation, and transport. We have recently demonstrated that decreasing cellular polyamines increased CUGBP1 protein, whereas CUGBP1 expression level decreased in cells containing high polyamines (Xiao *et al.*, 2011). Although the full mechanisms by which polyamines regulate CUGBP1 expression are not yet understood, our current studies show that decreasing the levels of cellular polyamines decreased miR-503, which in turn induced CUGBP1 translation (Figure 5). This stimulatory effect was completely prevented by addition of exogenous polyamine putrescine or ectopic miR-503 overexpression. In contrast, an increase in polyamines due to ODC gene overexpression increased miR-503 levels, thus suppressing CUGBP1 translation (Figure 6). Polyamine depletion also increased the cytoplasmic abundance of HuR that directly interacts with the CUGBP1 3′-UTR and induces CUGBP1 translation (Xiao *et al.*, 2011). It is unknown at this time whether HuR enhances CUGBP1 expression by blocking the miR-503–induced recruitment of CUGBP1 mRNA to P-bodies.

The miR-503–mediated repression of CUGBP1 expression is of biological significance, because it plays an important role in the regulation of IEC apoptosis and is thus implicated in maintaining homoeostasis of the intestinal epithelium. The epithelium of the intestinal mucosa is continuously renewed from the proliferative zone within the crypts, and this dynamic process is counterbalanced by apoptosis (Rao and Wang, 2011). 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 intestinal mucosa, where differentiated cells are lost. Our previous studies and studies from other laboratories (Seiler and Raul, 2007) have demonstrated that NF-κB (Li *et al.*, 2001), Akt kinase (Zhang *et al.*, 2004), ATF-2 (Xiao *et al.*, 2007), XIAP (Zhang *et al.*, 2009), and MEK1 (Wang *et al.*, 2010) are involved in the control of IEC apoptosis, and these signals are highly regulated by cellular polyamines. Our current studies provide additional new evidence that polyamine-reduced miR-503 is crucial for the maintenance of intestinal epithelial integrity and tissue homoeostasis. Our results show that increased levels of endogenous CUGBP1 (Figure 2F) protected cells against TNF-α/CHX–induced apoptosis by silencing miR-503, and this effect was abolished when CUGBP1 expression was inhibited by transfection with siCUGBP1 in miR-503–silent populations of IEC cells (Figure 7). Although the exact downstream targets of miR-503 in the regulation of apoptosis remain to be fully defined, our results indicate that c-IAP1 and c-IAP2 expression was also repressed by miR-503 through a process involving CUGBP1. In addition, CUGBP1 represses the expression of c-Myc, which functions as a proapoptotic factor in epithelial cells (You *et al.*, 2002; Xiao *et al.*, 2011).

In summary, these results indicate that miR-503 suppresses CUGBP1 mRNA translation through direct interaction with the CUGBP1 CR rather than its 3′-UTR. Since the level of CUGBP1 mRNA in the P-body was increased by ectopic miR-503 overexpression and because silencing P-body resident proteins reduced miR-503–mediated suppression of CUGBP1 expression, we propose that miR-503 inhibits CUGBP1 translation at least partially by increasing CUGBP1 mRNA recruitment to P-bodies, where nontranslating mRNAs accumulate. The present study also shows that polyamines are necessary for miR-503 biogenesis and that depletion of cellular polyamines decreases miR-503, thus increasing CUGBP1 expression. In contrast, increasing the levels of cellular polyamines represses CUGBP1 expression by inducing miR-503. By reducing CUGBP1 protein levels, miR-503 indirectly alters the expression of CUGBP1 target mRNAs, many of which encode apoptosis-associated proteins, thereby regulating IEC apoptosis and intestinal epithelial homoeostasis.

MATERIALS AND METHODS

Chemicals and cell culture

Tissue culture medium and dialyzed fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA) and biochemicals were from Sigma (St. Louis, MO). The antibodies recognizing CUGBP1, HuR, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Biosciences (San Diego, CA), and the secondary antibody conjugated to horseradish peroxidase was from Sigma. DFMO was purchased from Genzyme (Cambridge, MA). Pre-miR miRNA precursor and anti-miR miRNA inhibitor of miR-503 were purchased from Ambion (Austin, TX). Biotin-labeled miRNA-503 was custom made by Dharmacon (Lafayette, CO).

The IEC-6 cell line, derived from normal rat intestinal crypt cells (Quaroni *et al.*, 1979), was used at passages 15–20 and was cultured in DMEM supplemented with 5% heat-inactivated FBS. ODC-overexpressing IEC-6 (ODC-IEC) cells were developed and described in our previous studies (Zou *et al.*, 2006; Wang *et al.*, 2010) and expressed a more stable ODC variant with full enzyme activity.

Plasmid construction

The chimeric firefly luciferase reporter construct of the CUGBP1 CR or 3′-UTR was generated as described previously (Zou *et al.*, 2010). The full-length CUGBP1 CR or its 3′-UTR and different CR fragments with or without predicted miR-503 binding site were amplified and subcloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI) to generate the pmirGLO-Luc-CUGBP1-CR and pmirGLO-CUGBP1-3′UTR. The sequence and orientation of the fragment in the luciferase reporter were confirmed by DNA sequencing and enzyme digestion. Transient transfections were performed using Lipofectamine reagent according to the manufacturer's recommendations (Invitrogen). The luciferase reporter constructs were transfected into cells along with phRL-null, a *Renilla* luciferase control reporter vector from Promega, to monitor transfection efficiencies as described previously (Liu *et al.*, 2009). Luciferase activity was measured using the Dual-Luciferase Assay System, and the levels of pmirGLO-Luc-CUGBP1-CR or CUGBP1-3′UTR luciferase activity were normalized to *Renilla* luciferase activity and were further compared with the levels of luciferase mRNA in every experiment. Both pcDNA-MS2 and pcDNA-MS2-YFP plasmids were described previously (Lee *et al.*, 2010a), and the fragment of CUGBP1 CR was inserted into pcDNA-MS2 at the *Xho*I site. The expression vector containing HA-tagged Ago2 protein was constructed as described previously (Schaefer *et al.*, 2010). All primer sequences for generating these constructs are provided in Supplemental Table S1.

RT and real-time Q-PCR analyses

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and was used in RT and PCR amplification reactions as previously described (Zou *et al.*, 2010). The levels of GAPDH PCR product were assessed to monitor the evenness in RNA input in RT-PCR samples. Real-time Q-PCR analysis was performed using 7500-Fast Real-Time PCR Systems with specific primers, probes, and software (Applied Biosystems, Foster City, CA). For miRNA studies, the levels of miRNA-503 were also quantified by Q-PCR by using TaqMan MicroRNA assay (Applied Biosystems); small nuclear RNA U6 was used as endogenous control.

Western blotting analysis

Whole-cell lysates were prepared using 2% SDS, sonicated, and centrifuged (12,000 rpm) at 4°C for 15 min. The supernatants were boiled for 5 min and size-fractionated by SDS–PAGE (7.5% acrylamide). After proteins were transferred onto nitrocellulose filters, the blots were incubated with primary antibodies recognizing CUGBP1, HuR, Ago2, or RCK; following incubations with secondary antibodies, immunocomplexes were developed by using chemiluminescence.

Analysis of newly translated protein

New synthesis of CUGBP1 protein was measured by L-[^{35}S]methionine and L-[^{35}S]cysteine incorporation assays as previously described (Liu *et al.*, 2009). Cells were incubated with 1 mCi L-[^{35}S]methionine and L-[^{35}S]cysteine per 60-mm plate for 20 min, whereupon cells were lysed using RIPA buffer. IPs were carried out for 1 h at 4°C using either a polyclonal antibody recognizing CUGBP1 or IgG1 (PharMingen, BD Biosciences, San Diego, CA). Following extensive washes in TNN buffer (50 mM Tris-HCl, pH 7.5, 250 mM

NaCl, 5 mM EDTA, 0.5% NP-40), the immunoprecipitated material was resolved by 10% SDS–PAGE, transferred onto polyvinylidene fluoride filters, and visualized with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Biotin-labeled miR-503 pulldown assays

Binding of miR-503 to target mRNAs was examined by biotin-labeled miR-503 as described previously (Orom and Lund, 2007; Nonne *et al.*, 2010). Briefly, biotin-labeled miR-503 was transfected into cells for 48 h, and then whole-cell lysate was collected. Cell lysates were mixed with streptavidin-coupled Dynabeads (Invitrogen, Carlsbad, CA) and incubated at 4°C on rotator overnight. After the beads were washed thoroughly, the bead-bound RNA was isolated and subjected to RT followed by Q-PCR analysis. Input RNA was extracted and served as a control.

Immunofluorescence staining

Immunofluorescence was performed as previously described (Lee *et al.*, 2010b). Cells were fixed using 3.7% formaldehyde, and the rehydrated samples were incubated overnight at 4°C with primary antibody anti-RCK diluted 1:300 in blocking buffer and then incubated with secondary antibody conjugated with Alexa Fluor-594 (Molecular Probes, Eugene, OR) for 2 h at room temperature. Images were processed using Axio Observer microscope (Zeiss, Jena, Germany) with LSM 510 Meta (Zeiss) image-processing software.

Assays for ODC enzyme activity and cellular polyamine content

ODC activity was determined by a radiometric technique in which the amount of $^{14}CO_2$ liberated from L-[1-^{14}C]ornithine was estimated (Liu *et al.*, 2006), and enzymatic activity was expressed as picomoles of CO_2 per milligram of protein per hour. The cellular polyamine content was analyzed by high-performance liquid chromatography (HPLC) analysis as described previously (Li *et al.*, 1999). After 0.5 M perchloric acid was added, the cells were frozen at –80°C until they were ready for extraction, dansylation, and HPLC analysis. The standard curve encompassed 0.31–10 µM. Values that fell >25% below the curve were considered undetectable. The results are expressed as nanomoles of polyamines per milligram of protein.

Determination of apoptosis

Apoptosis was induced by TNF-α in combination with CHX as described previously (Li *et al.*, 2001). After various experimental treatments, cells were photographed with a Nikon inverted microscope before fixation. Annexin V staining of apoptosis was carried out by using a commercial apoptosis kit (Clontech Laboratories, Palo Alto, CA) and was performed according to the protocol recommended by the manufacturer. Briefly, cells were rinsed with 1X binding buffer, and resuspended in 200 µl of 1X binding buffer. Five µl of annexin V was added on slide and incubated at room temperature in the dark for 10 min. Annexin-stained cells were visualized and photographed under a fluorescence microscope using a dual filter set for fluorescein isothiocyanate and rhodamine, and the percentage of "apoptotic" cells was determined.

Statistics

Values are mean ± SE from three to six samples. Autoradiographic results were repeated three times. The significance of the difference between means was determined by analysis of variance. The level of significance was determined by using Duncan's multiple-range test (Harter, 1960).

ACKNOWLEDGMENTS

We thank J. L. Martindale for experimental support. This work was supported by Merit Review Grants (to J.-Y.W. and J.N.R.) from the Department of Veterans Affairs and by National Institutes of Health (NIH) grants DK-57819, DK-61972, DK-68491 (to J.-Y.W.). J.-Y.W. is a Senior Research Career Scientist, Medical Research Service, U.S. Department of Veterans Affairs. M.G. is supported by the National Institute on Aging–Intramural Research Program, NIH.

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