p53-Dependent NDRG1 expression induces inhibition of intestinal epithelial cell proliferation but not apoptosis after polyamine depletion

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Submitted 25 October 2006; accepted in final form 16 April 2007

Zhang A-H, Rao JN, Zou T, Liu L, Marasa BS, Xiao L, Chen J, Turner DJ, Wang J-Y. p53-Dependent NDRG1 expression induces inhibition of intestinal epithelial cell proliferation but not apoptosis after polyamine depletion. Am J Physiol Cell Physiol 293: C379–C389, 2007. First published April 18, 2007; doi:10.1152/ajpcell.00547.2006. — Normal intestinal mucosal growth requires polyamines that regulate expression of various genes involved in cell proliferation, growth arrest, and apoptosis. Our previous studies have shown that polyamine depletion stabilizes p53, resulting in inhibition of intestinal epithelial cell (IEC) proliferation, but the exact downstream targets of induced p53 are still unclear. The NDRG1 (N-myc downregulated gene-1) gene encodes a growth-related protein, and its transcription can be induced in response to stress. The current study tests the hypothesis that induced p53 inhibits IEC proliferation by upregulating NDRG1 expression following polyamine depletion. Depletion of cellular polyamines by inhibiting ornithine decarboxylase (ODC) with α-difluoromethylornithine not only induced p53 but also increased NDRG1 transcription as indicated by induction of the NDRG1 promoter activity and increased levels of NDRG1 mRNA and protein, all of which were prevented by using specific p53 siRNA and in cells with a targeted deletion of p53. In contrast, increased levels of cellular polyamines by ectopic expression of the ODC gene decreased p53 and repressed expression of NDRG1. Consistently, polyamine depletion-induced activation of the NDRG1-promoter was decreased when p53-binding sites within the NDRG1 proximal promoter region were deleted. Ectopic expression of the wild-type NDRG1 gene inhibited DNA synthesis and decreased final cell numbers regardless of the presence or absence of endogenous p53, whereas silencing NDRG1 promoted cell growth. However, overexpression of NDRG1 failed to directly induce cell death and to alter susceptibility to apoptosis induced by tumor necrosis factor-α or cycloheximide. These results indicate that NDRG1 is one of the direct mediators of induced p53 following polyamine depletion and that p53-dependent NDRG1 expression plays a critical role in the negative control of IEC proliferation.

mucosal growth; ornithine decarboxylase; gene transcription; small interfering ribonucleic acid; intestinal epithelium

THE INTESTINAL EPITHELIAL INTEGRITY is maintained by continuous replacement of epithelial cells through a stereotyped cycle of cell proliferation, migration, differentiation, and apoptosis (10, 23, 30, 44). Undifferentiated epithelial cells continuously replicate in the proliferative zone within crypts and differentiate as they migrate up the villous tips, where differentiated cells are removed by apoptosis a few days later (10, 30, 33). This self-renewing epithelium of the intestinal mucosa represents an exquisite model for the study of stem cell biology and lineage specification. Although intestinal epithelial stem cells are not currently studied ex vivo, and no unique in vivo markers are available, they can be labeled with [3H]thymidine or bromodeoxyuridine during early postnatal life or in regenerative mucosa after injury induced by irradiation (31). The rapid dynamic turnover rate of intestinal epithelial cells (IECs) is highly regulated and critically controlled by numerous factors, including cellular polyamines (24, 41). The natural polyamines spermidine and spermine and their precursor putrescine are organic cations found in all eukaryotic cells (28, 38). It has been shown that normal IEC proliferation in the mucosa is dependent on the supply of polyamines to the dividing cells in the crypts and that decreasing cellular polyamines inhibits cell renewal in vivo as well as in vitro (24, 44–46). Although few specific functions of polyamines at the molecular level have been identified to date, an increasing body of evidence indicates that polyamines regulate IEC proliferation by virtue of their ability to modulate the expression of various growth-related genes (2, 19–21).

The p53 gene is implicated in regulation of the cell cycle, apoptosis, and the onset of cellular senescence (5, 8, 42), thus playing a critical role in the negative control of normal intestinal mucosal growth (11, 16, 17). The product of the p53 gene is a transcription factor with a sequence-specific DNA binding domain in the central region and a transcriptional activation domain at the NH2 terminus (11). Because p53 triggers diverse cellular processes in IECs, its potent activity demands tight control of its functions. Our previous studies have shown that increased levels of cellular polyamines decrease p53, promoting IEC proliferation in vivo as well as in vitro, whereas polyamine depletion increases p53, resulting in inhibition of IEC proliferation (16, 17). We have further demonstrated that increased p53 in IECs by polyamine depletion results primarily from an increase in the stability of p53 mRNA rather than an increase in its gene transcription (17). Recently, it has been found that polyamine depletion stabilizes p53 mRNA by increasing cytoplasmic levels of RNA-binding protein HuR, which specifically binds to 3’-untranslated region of p53 mRNA (54). However, the direct mediators of increased p53 on cellular events causing IEC growth inhibition following polyamine depletion are still unknown.

The N-myc downregulated gene 1 (NDRG1) encodes a 43-kDa protein that contains a NDRG1 core domain and three unique tandem repeats of 10 hydrophilic amino acids near the COOH terminus (35). NDRG1 is ubiquitously expressed in...
different mammalian tissues and modulates cell growth, differen-
tiation, and apoptosis (35, 40). On one hand, expression of the NDRG1 gene is increased in response to various stresses such as DNA damage and hypoxia through distinct signaling pathways (4, 36). For example, the induction of NDRG1 expression by hypoxia is mediated by hypoxia-inducible factor-1 transcription factor (51), whereas activation of PTEN augments NDRG1 expression via an Akt-dependent pathway (1). On the other hand, NDRG1 expression is repressed by overexpression of the N-myc and c-myc genes (25, 34). In N-myc knockout mouse embryos, NDRG1 expression is up-regulated (35). Although the regulation and precise cellular functions of NDRG1 remain obscure, a recent study suggests that NDRG1 is a direct transcriptional target gene of p53 after DNA damage and that increased NDRG1 is necessary for p53-mediated caspase activation and apoptosis (36). The data presented demonstrate that a decrease in cellular polyamines stimulated NDRG1 transcription by increasing p53 and that this p53-dependent NDRG1 expression resulted in inhibition of IEC proliferation but not apoptosis. Some of these results have been published previously in abstract form (50).

MATERIALS AND METHODS

Chemicals and supplies. Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Tissue culture medium and dialyzed fetal bovine serum (dFBS) were obtained from Invitrogen (Carlsbad, CA), and biochemicals were obtained from Sigma (St. Louis, MO). The antibody against NDRG1 was obtained from Zymed Laboratories (San Francisco, CA), and the anti-p53 antibody and the secondary antibody, anti-mouse IgG conjugated to horseradish peroxidase, were purchased from Sigma. α-Difluoromethylornithine (DFMO) was obtained from Ilex Oncology (San Antonio, TX), and [3H]thymidine (2 Ci/mmol) was obtained from New England Nuclear (Boston, MA).

Recombinant viral construction and infection. Adenoviral vectors were constructed using the Adeno-X expression system (Clontech, Mountain View, CA) according to the protocol provided by the manufacturer. Briefly, the full-length cDNA of human NDRG1 was cloned into the pShuttle by digesting the BamHI/HindIII and ligating the resultant fragments into the Xhol site of the pShuttle vector. pAdeno-NDRG1 (AdNDRG1) was constructed by digesting pShuttle construct with PI-SceI/CreT and ligating the resultant fragment into the PI-SceI/CreT sites of the pAdeno-X adenoviral vector. Recombinant adenoviral plasmids were packaged into infectious adenoviral particles by transfecting human embryonic kidney (HEK)-293 cells using Lipofectamine Plus reagent (GIBCO-Bethesda Research Laboratory, Gaithersburg, MD). The adenoviral particles were propagated in HEK-293 cells and purified upon cesium chloride ultracentrifugation. Titers of the adenoviral stock were determined using standard plaque assay. Recombinant adenoviruses were screened for the expression of the introduced gene by Western blot analysis using anti-NDRG1 antibody. pAdeno-X, which was the recombinant replication-defective adenovirus carrying no NDRG1 cDNA insert (Adnull), was grown and purified as described above and served as a control adenovirus. IEC-6 cells were infected with the AdNDRG1 or Adnull, and expression of NDRG1 was assayed by 24 or 48 h after the infection.

Reporter plasmids and luciferase assays. The construct of the full-length wild-type NDRG1-promoter luciferase reporter was purchased from GenHunter (Nashville, TN). The two deletion mutants of NDRG1-promoter, Del-1 and Del-2, as depicted in Fig. 5A, were generated using the GeneEditor in vitro site-directed mutagenesis system and performed according to the manufacturer’s instructions (Promega, Madison, WI). The sequences of deletion mutants of the NDRG1-promoter were verified by DNA sequencing. Transient transfection was performed using the Lipofectamine kit as recommended by the manufacturer (Invitrogen). Cells were collected 48 h after the transfection, and luciferase activity from individual transfection was normalized by the β-gal activity from cotransfected pCMV β-galactosidase plasmid. The experiments were done in triplicate and are reported as means of relative light unit per β-galactosidase.

RNA interference. The small interfering (si)RNA specifically targeting the coding region of p53 mRNA (sip53) was purchased from Invitrogen (Chicago, IL). Scrambled control siRNA (C-siRNA), which had no sequence homology to any known genes, was used as the control. The sip53 and C-siRNA were transfected into cells as described previously (54). Briefly, for each 60-mm cell culture dish, 15 μl of the 20 μM stock sip53 or C-siRNA were mixed with 300 μl of Opti-MEM medium (Invitrogen). This mixture was gently added to a solution containing 15 μl of Lipofectamine 2000 in 300 μl of Opti-MEM. The solution was incubated for 20 min at room temperature and gently overlaid onto monolayers of cells in 3 ml of medium, and cells were harvested for various assays after 48 h of incubation.

Western blot analysis. Western blots of stable NDRG1-transfected IEC-6 cells. The mammalian expression vector pSilencer 3.1-H1 neo (Ambion, Austin, TX) was used in this study, and siRNA that was designed to specially cleave NDRG1 mRNA (siNDRG1) was synthesized. For design of siNDRG1 oligos targeting NDRG1, a DNA sequence of type AA(N19) was selected to correspond to the nt 591–611 located 3′ to the first nucleotide of the start codon of the rat NDRG1 cDNA (GenBank accession no. NM_001011991.1). BLAST search against the rat genome sequence showed that only the NDRG1 gene was targeted. To create pSilencer plasmids, we used the following oligo sequences: 5′-GATCCGGAGGAGATACACAGCAATTTCAAGAGAATTGCTGTTATCCTCCTCTTTTTTGGAATAA3′ (sense) and 5′-AGC TTTCCTAAGAAGGGAGGATACACAGCAATTTCAAGAGAATTGCGAGAGAGATACACAGCAATTTCAAGAGAATTG-3′ (antisense). The corresponding single-stranded sense and antisense oligos (20 μM) were annealed by incubation in annealing buffer [100 mM potassium acetate, 30 mM HEPES-KOH (pH 7.4), and 2 mM magnesium acetate] and cloned into the BamHI and HindIII sites of pSilencer. Orientation and sequence of the insert were confirmed by restriction enzyme digestion and DNA sequencing. The vector was constructed with the insert having no significant homology to any mammalian gene and served as a negative control. IEC-6 cells were transfected with either the siNDRG1 pSilencer or the control vector by using Lipofectamine 2000, and the transfected cells were selected by incubation with the selection medium containing G418 (600 μg/ml). Clones resistant to the selection medium were isolated, cultured, and screened for the inhibition of NDRG1 expression by Western blot analysis using a specific anti-NDRG1 antibody.

Cell culture. The IEC-6 cell line was purchased from the American Type Culture Collection at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. (32). IEC-6 cells originated from intestinal crypt cells as judged by morphological and immunologic criteria. They are nontumorigenic and retain undifferentiated characteristics of intestinal crypt cells. Passages 15–20, which exhibit a stable phenotype (17, 19), were used in experiments. Omithine decarboxylase (ODC)-overexpressing IEC-6 (ODC-IEC) cells were developed as described in previous publications (25, 54) and expressed a more stable ODC variant with full enzyme activity (22). The human colorectal adenocarcinoma cell line HCT-116 p53−/− (with a targeted deletion of p53) (3) was kindly provided by B. Vogelstein (Johns Hopkins University, Baltimore, MD). Stock cells were maintained in T-150 flasks in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated FBS, 10 μg/ml insulin, and 50 μg/ml gentamicin. Flasks were incubated at 37°C in a humidified atmosphere of 90% air-10% CO2.

RT-PCR and real-time PCR. Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). Equal amounts of total RNA

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(2 μg) were transcribed to synthesize single-stranded cDNA with a RT-PCR kit (Invitrogen). The specific sense and antisense primers for NDRG1 included 5′-CATCGGCATGAAACCAGA-3′ and 5′-AG-GCGCTGAT-GAACAGGTG-3′, and the expected size of NDRG1 fragments was ~500 bp. Reverse transcription and PCR were performed as described in our earlier publications (7, 54). Levels of β-actin PCR product were assessed to monitor the even RNA input in RT-PCR samples. Real-time quantitative PCR (Q-PCR) was performed using an Applied Biosystems instrument (Foster City, CA) using specific primers, probes, and software (Applied Biosystems). The levels of NDRG1 mRNA were quantified by Q-PCR analysis and normalized with GAPDH levels.

**Western blot analysis.** Cell samples, placed in SDS sample buffer (250 mM Tris·HCl, pH 6.8, 2% SDS, 20% glycerol, and 5% mercaptoethanol) were sonicated and then centrifuged (10,000 g) at 4°C for 15 min. The supernatant from cell samples was boiled for 5 min and then subjected to electrophoresis on 10% acrylamide gels according to the method of Laemmli (15). After the transfer of protein onto nitrocellulose filters, the filters were incubated for 1 h in 5% nonfat dry milk in 1× phosphate-buffered saline/Tween 20 [PBS-T: 15 mM NaH2PO4, 80 mM Na2HPO4, 1.5 M NaCl, pH 7.5, and 0.5% (vol/vol) Tween 20]. Immunological evaluation was then performed for 1 h in 1% BSA/PBS-T buffer containing 1 μg/ml of the specific antibody against NDRG1 or p53 proteins. The filters were subsequently washed.

**Fig. 1.** Changes in p53 protein levels and N-myc downregulated gene-1 (NDRG1) transcription in control IEC-6 cells and in cells treated with either α-difluoromethylornithine (DFMO; 5 mM) alone or DFMO plus putrescine (PUT; 10 μM) for 4 and 6 days. A: representative immunoblots of p53 protein. Whole cell lysates were harvested, applied to each lane (20 μg) equally, and subjected to electrophoresis on 10% acrylamide gel. Levels of p53 (~53 kDa) were identified by probing nitrocellulose with the specific anti-p53 antibody. After the blot was stripped, actin (~42 kDa) immunoblotting was performed as an internal control for equal loading. Three experiments were performed that showed similar results. B: changes in NDRG1 transcription in cells described in A. a, NDRG1 promoter activity as measured by luciferase reporter gene assays. After cells were grown in cultures containing DFMO or DFMO plus PUT for 2 or 4 days, they were transfected using the NDRG1-promoter luciferase reporter construct via the Lipofectamine technique. Transfected cells were harvested and assayed for luciferase activity after 48 h of incubation in the presence of DFMO or DFMO plus PUT. Data were normalized by Renilla-driven luciferase activity, and values are means ± SE of data from 6 dishes. *P < 0.05 compared with controls and cells treated with DFMO + PUT. b, NDRG1 mRNA as measured by real-time quantitative PCR analysis. Values are means ± SE of data from 3 separate experiments. The lane numbers in B indicate different treatments as described in A. C: representative immunoblots of NDRG1 protein in cells described in A. a, control; b, cells treated with DFMO alone for 6 days; and c, cells treated with DFMO plus PUT for 6 days. Cells were permeabilized and incubated with the anti-NDRG1 antibody and then with anti-IgG conjugated with Alexa Fluor. Nuclei were stained with TO-PRO3. NDRG1 is shown as green, whereas nuclei are shown as red. Original magnification, ×1,000. Three experiments were performed that showed similar results.
with 1× PBS-T and incubated for 1 h with the second antibody conjugated to peroxidase by protein cross-linking with 0.2% glutaraldehyde. After extensive washing with 1× PBS-T, the immunocomplexes on the filters were reacted for 1 min with chemiluminescence reagent (NEL-100; NEN, Boston, MA). Finally, the filters were placed in a plastic sheet protector and exposed to autoradiography film for 30 or 60 s.

**Measurement of DNA synthesis and cell number.** DNA synthesis in cultured cells was measured with the use of [3H]thymidine incorporation techniques as previously described (21, 39). Cells in 24-well plates were pulsed with 1 μCi/ml [3H]thymidine for 4 h before harvest. Cells were washed twice with cold Dulbecco’s PBS (D-PBS) solution and then incubated in cold 10% trichloroacetic acid (TCA) for 30 min at 4°C. After being rinsed twice with 10% TCA, cells were dissolved in 0.5 ml of 0.5 N NaOH at 37°C in humidified air for 90 min. The incorporation of [3H]thymidine into DNA was determined by counting the aliquot of cell lysate in a Beckman liquid scintillation counter. DNA synthesis was expressed as disintegrations per minute per microgram of protein. Cell numbers were counted according to the standard Trypan blue staining method as described previously by our laboratory (27).

**Immunofluorescence staining.** The immunofluorescence staining procedure was carried out according to the method described in our previous publications (19, 54). After the monolayers of control and polyamine-deficient cells were fixed in 3.7% formaldehyde in PBS and rehydrated, they were incubated with the primary antibody against NDRG1 in the block buffer at the concentration of 1:300 dilution at 4°C overnight and then incubated with secondary antibody conjugated with Alexa Fluor-594 (Molecular Probes, Eugene, OR) for 2 h at room temperature. After being rinsed three times, the slides were incubated with TO-PRO3 (Molecular Probes) at the concentration of 1 μM for 10 min to stain cell nuclei. Finally, the slides were washed, mounted, and viewed through a Zeiss confocal microscope (model LSM410). Images were processed using Photoshop software (Adobe, San Jose, CA).

**Polyamine analysis.** The cellular polyamine content was analyzed using high-performance liquid chromatography (HPLC) as previously described (27). Briefly, after 0.5 M perchloric acid was added, the cells were frozen at −80°C until 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.

**Statistics.** All data are means ± SE from 3 or 6 dishes. Autoradiographic and immunoblotting results were repeated three times. The significance of the difference between means was determined using analysis of variance. The level of significance was determined using the Duncan’s multiple-range test (9).

**RESULTS**

**Increased levels of endogenous p53 associate with an activation of NDRG1 transcription following polyamine depletion.** Our previous studies have demonstrated that polyamine depletion stabilizes p53, resulting in the inhibition of IEC proliferation (16, 17). The focus of the current study was to further determine whether increased p53 inhibited cell proliferation by increasing NDRG1 transcription following polyamine depletion. Exposure of IEC-6 cells to 5 mM DFMO for 4 and 6 days completely inhibited ODC (the first rate-limiting step for polyamine biosynthesis) enzyme activity and almost totally depleted cellular polyamines. Levels of putrescine and spermidine were undetectable on days 4 and 6 after treatment with DFMO, and spermine had decreased by ~60% (data not shown). Similar results were reported in our previous publications (19). Consistent with our previous findings (17), polyamine depletion by DFMO significantly increased the steady-state levels of p53 protein, which was completely prevented by exogenous putrescine (10 μM) given together with DFMO (Fig. 1A). Spermidine (5 μM) had an effect equal to that of putrescine on levels of p53 protein when it was added to cultures that contained DFMO (data not shown).

Increased levels of endogenous p53 in the polyamine-deficient cells were associated with a stimulation of NDRG1 transcription as demonstrated by increases in NDRG1-promoter activity and NDRG1 mRNA (Fig. 1B). Levels of NDRG1-promoter activity in cells treated with DFMO for 4 and 6 days were 2.2 and 3.8 times the control value (Fig. 1Ba), whereas levels of NDRG1 mRNA, as measured using conventional PCR (data not shown) and Q-PCR analysis (Fig. 1Bb), were 2.8 and 3.9 times the control value, respectively. Consistently, induced levels of NDRG1 mRNA were associated with a significant increase in NDRG1 protein (Fig. 1C). Levels of NDRG1 protein in cells exposed to DFMO for 4 and 6 days were more than two and three times the control values. The increased NDRG1 expression in DFMO-treated cells was completely prevented when cells were treated with putrescine. Levels of NDRG1-promoter activity and NDRG1 mRNA and protein in cells grown in the presence of DFMO plus putrescine were indistinguishable from those of control cells. To extend these positive findings of increased NDRG1 protein after polyamine depletion, we performed immunofluorescence staining to determine cellular distribution of NDRG1 in the presence or absence of cellular polyamines. In control cells (Fig. 1Da), immunoreactivities of NDRG1 were primarily localized in the nucleus, although there was slight staining in the cytoplasm. Consistent with the Western blotting results, NDRG1

![Fig. 2. Changes in expression of p53 and NDRG1 in stable ornithine decarboxylase (ODC)-overexpressing IEC-6 (ODC-IEC) cells. A: representative immunoblots for p53 and NDRG1 proteins. IEC-6 cells were infected with either the retroviral vector containing the sequence encoding mouse ODC or control retroviral vector lacking ODC cDNA, and stable clones highly expressing ODC were isolated and characterized. Whole cell lysates from stable ODC-IEC cells, including clone (C)1 and C2, and cells infected with the control vector were harvested. Levels of p53 and NDRG1 proteins were measured by Western blot analysis, and equal loading was monitored by immunoblotting of actin. B: quantitative analysis derived from densitometric scans of Western blots of p53 (a) and NDRG1 proteins (b) from cells described in A. Values are means ± SE of data from 3 separate experiments. *P < 0.05 compared with vector alone.](http://ajpcell.physiology.org/)

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NDRG1 protein was inhibited by with a significant inhibition of NDRG1 expression. The level of cellular polyamines in stable ODC-IEC cells was associated with a 2-fold increase in ODC enzyme activity. The levels of putrescine, spermidine, and spermine in stable ODC-IEC cells were increased ~12-fold, ~2-fold, and ~25% compared with cells transfected with the control vector lacking ODC cDNA (data not shown). Similar results were reported in our previous publication (22). As shown in Fig. 2A, stable ODC-IEC cells exhibited a substantial decrease in p53 expression. The levels of p53 protein were decreased by >75% in stable ODC-IEC cells compared with those observed in cells transfected with the control vector (Fig. 2Ba). Interestingly, reduced p53 following increased levels of cellular polyamines in stable ODC-IEC cells was associated with a significant inhibition of NDRG1 expression. Level of NDRG1 protein was inhibited by ~60% in stable ODC-IEC cells (Fig. 2Bb). The effect of ODC overexpression on expression of p53 and NDRG1 was not simply due to clonal variation, since two stable clones, ODC-IEC-C1 and ODC-IEC-C2, showed similar responses. These results indicate that increasing cellular polyamines decreases p53 and represses NDRG1 expression in IECs.

Inhibition of p53 prevents the increased expression of NDRG1 in polyamine-deficient cells. To determine the causal relationship between increased endogenous p53 and NDRG1 induction, we examined the effect of inhibition of p53 by using specific siRNA targeting the coding region of p53 mRNA (sip53) on NDRG1 expression in polyamine-deficient cells. This specific sip53 was designed to cleave rat p53 mRNA by activating endogenous RNase H and was shown to have a unique combination of specificity, efficacy, and reduced toxicity (17, 53). Initially, we determined the transfection efficiency of the siRNA nucleotides in IEC-6 cells and have demonstrated that >95% of cells were positive when they were transfected with a fluorescent FITC-conjugated control siRNA for 24 h (data not shown). Cells were grown in the presence of 5 mM DFMO for 4 days and then transfected with sip53 or control siRNA (C-siRNA). Levels of p53 and NDRG1 proteins were measured 48 h after the transfection. Results presented in Fig. 3, A and B, clearly show that inhibition of p53 expression by sip53 prevented the increase in NDRG1 protein expression. When the DFMO-treated cells were exposed to sip53 for 48 h, NDRG1 protein expression was not increased. In addition, inhibition of p53 expression by the transfection with sip53 also promoted cell proliferation in polyamine-deficient cells (data...
not shown). Similar results were published in our previous studies (17). On the other hand, transfection with C-siRNA had no effect on expression of both p53 and NDRG1 proteins. Furthermore, increased NDRG1 expression following polyamine depletion was completely abolished in cells lacking endogenous p53 (Fig. 3C). No significant change in the steady-state levels of NDRG1 protein was obtained in HCT-116 p53−/− cells exposed to DFMO alone or DFMO plus putrescine for 4 and 6 days. These results indicate that induced NDRG1 in IECs is dependent on the increased levels of endogenous p53 following polyamine depletion.

Induced p53 increases NDRG1 transcription by interacting with p53-binding sites within the proximal region of the NDRG1-promoter following polyamine depletion. To define the mechanism by which induced p53 stimulates NDRG1 transcription, we removed the p53-binding sites within the NDRG1-promoter and constructed two deletions in the NDRG1-promoter luciferase reporter genes as illustrated in Fig. 4A. Consistent with the findings from others (36), the elements that contained a 541-bp region of the NDRG1-promoter and 60 bp of the exon are required for basal and regulatory NDRG1 expression in IEC-6 cells. These deletion mutants of the NDRG1-promoter exhibited substantial differences in their basal expression after transfection in IEC-6 cells. Basal levels were 7.6 ± 0.5 (n = 6) at the wild type (WT), 2.3 ± 0.2 (n = 6) at the Del-1, and 2.7 ± 0.3 (n = 6) at the Del-2. Results presented in Fig. 4B, left, show that NDRG1-promoter activity was increased significantly when polyamine-deficient cells were transfected with the WT-Luc construct that contained p53-binding sites. However, deletion of nucleotides from the base pair positions −541 to −343 (Del-1) relative to the transcriptional start site or internal deletion from −373 to −341 in the NDRG1-promoter (Del-2) significantly prevented the stimulatory effect of induced p53 on NDRG1-promoter activity in polyamine-deficient cells. The stimulatory rates of NDRG1-promoter were decreased ~50% when polyamine-deficient cells were transfected with the Del-1 or Del-2 construct lacking p53-binding sites. These results indicate that increased p53 levels following polyamine depletion stimulate NDRG1 transcription through their interaction with the p53-binding sites within the proximal region of the NDRG1-promoter.

Overexpression of the NDRG1 gene inhibits IEC proliferation. To define the function of induced NDRG1 by p53 in IECs, we examined the effect of overexpression of the wild-type NDRG1 gene on cell proliferation in both lines of IEC-6 and HCT-116 p53−/− cells. The adenoviral vector containing the corresponding NDRG1 cDNA under the control of the human cytomegalovirus immediate-early gene promoter (AdNDRG1) was constructed (Fig. 5Aa) and used in this study. The reason we have chosen adenoviral vectors over other methods of transfection is that adenoviral vectors have been shown to infect a variety of cultured rat and human epithelial cells with nearly 100% efficiency (21, 22). We have demonstrated that >95% of IEC-6 cells and HCT-116 p53−/− cells were positive when they were infected with the adenoviral vector encoding green fluorescent protein (GFP), which served as the marker for 24 h. Consistently, the NDRG1 protein was expressed in amounts increasing with the viral load, and levels of NDRG1 protein in IEC-6 cells infected with the AdNDRG1 at concentrations ranging from 10, 50, and 100 plaque-forming units/cell for 48 h were increased by ~4, ~20, and ~30 times the control level, respectively (Fig. 5Ab). Overexpression of the NDRG1 gene significantly inhibited IEC-6 cell proliferation as indicated by decreases in DNA synthesis (Fig. 5B) and final cell numbers (Fig. 5C). The rate of DNA synthesis was inhibited by ~45% at 48 h after the infection, whereas cell numbers were decreased by ~55% at 48 and ~65% at 72 h after the infection, respectively. An adenovirus that lacked exogenous NDRG1 cDNA (Adnull) was used as negative control in this experiment and did not alter NDRG1 levels and cell growth.

In HCT-116 p53−/− cells, levels of NDRG1 protein were also dramatically increased after infection with the AdNDRG1 (Fig. 6A). Consistent with the observation from IEC-6 cells, ectopic expression of NDRG1 significantly inhibited cell proliferation in HCT-116 p53−/− cells. Levels of DNA synthesis were inhibited by ~50% on days 4 and 5 after the infection (Fig. 6B), whereas cell numbers were decreased by ~45% on day 4 and ~52% on day 5, respectively (Fig. 6C). These results indicate that increased NDRG1 expression suppresses IEC proliferation regardless of the presence or absence of endogenous p53.

Inhibition of NDRG1 expression by stable transfection with siNDRG1 promotes IEC cell growth in polyamine-deficient cells. To further define the role of induced NDRG1 in the regulation of IEC proliferation following polyamine depletion,
endogenous NDRG1 was knocked down by stable transfection with siNDRG1. As shown in Fig. 7A, stable siNDRG1-transfected IEC (siNDRG1-IEC) cells exhibited a significant decrease in expression of NDRG1 after polyamine depletion by DFMO. The level of NDRG1 protein was inhibited by $80\%$ in stable siNDRG1-IEC cells when exposed to DFMO for 6 days compared with those observed in parental IEC-6 cells or cells transfected with the control vector lacking siNDRG1.

Inhibition of endogenous NDRG1 by ectopic expression of siNDRG1 also significantly promoted cell proliferation in polyamine-deficient cells (Fig. 7B). Cell numbers in stable
siNDRG1-IEC cells were increased by ~35% on day 3, ~40% on day 4, ~65% on day 5, and ~73% on day 6 after polyamine depletion, respectively. These results indicate that decreased levels of endogenous NDRG1 promote IEC proliferation in the absence of cellular polyamines.

Effect of ectopic expression of the NDRG1 gene on apoptosis. To investigate the involvement of induced NDRG1 in apoptosis, we performed the following two experiments in IEC-6 cells. First, we examined changes in spontaneous apoptotic cell death without any challenge of apoptotic stimulators and demonstrated that forced expression of the NDRG1 gene in IEC-6 cells failed to directly induce apoptosis. There were no apparent differences in cell viability between control cells and cells infected with the Adnull or AdNDRG1 as measured by Trypan blue staining assay. No typical morphological features of apoptosis (Fig. 8A) and detectable levels of active caspase-3 protein (Fig. 8C) were obtained in cells overexpressing NDRG1. Second, we examined changes in the susceptibility to apoptotic stimulators tumor necrosis factor-α (TNF-α) plus cycloheximide (CHX). This apoptotic model was chosen in this study because TNF-α/CHX-induced apoptosis is widely accepted as a form of programmed cell death induced by a biological apoptotic inducer (17, 48). As shown in Fig. 8A, when control cells were exposed to TNF-α/CHX for 4 h, typical morphological features of programmed cell death were identified. Morphological assessments of apoptosis were confirmed by an increase in levels of active caspase-3 protein after treatment with TNF-α/CHX (Fig. 8C, left). However, forced expression of NDRG1 did not alter the susceptibility of IEC-6 cells to TNF-α/CHX-induced apoptosis. There were no significant differences in percentages of apoptotic cells (Fig. 8B) and levels of active caspase-3 protein (Fig. 8C) between control cells and cells preinfected with the Adnull or AdNDRG1 after exposure to TNF-α/CHX. We also determined the effect of ectopic expression of the NDRG1 on apoptosis when apoptotic agents were used separately and demonstrated that increased NDRG1 did not alter the sensitivity of IEC-6 cells to TNF-α or CHX as measured by morphological assessments and caspase-3 activity (data not shown). These results indicate that induced NDRG1 plays a little role in the regulation of apoptosis in IECs.

DISCUSSION

p53 is a transcription factor that specifically binds to a consensus DNA sequence and efficiently transactivates expression of the target genes (5, 8, 11). In addition, p53 also regulates a wide variety of cellular and viral promoters that lack specific p53-binding sites (37, 47). Because increased expression of p53 exerts distinct cellular outcomes such as growth arrest, apoptosis, or cellular senescence, depending on activation or repression of specific p53 target genes, cell type, and the nature of stress (5, 11), it is important and interesting to define the direct mediators of polyamine depletion-induced p53 in the normal epithelial cells of the intestinal mucosa. Our previous studies have found that polyamine depletion increases cytoplasmic HuR levels, in turn controlling the stability of the p53 and nucleophosmin (NPM) mRNAs and increasing p53 and NPM protein levels (54). Increased NPM also interacts with and stabilizes p53 protein in polyamine-deficient cells (53). Although increased p53 is shown to play a critical role in the negative control of cell cycle progression following polyamine depletion (16, 17), the exact downstream targets of induced p53 remain still unclear. To date, p21 is the only gene identified as a p53 target in polyamine-deficient IECs and works in concert to mediate the growth arrest (12, 53). The particular interest of the current study was focused on further defining the mechanism by which polyamine depletion-in-
The results reported clearly show that increased levels of endogenous p53 in response to polyamine depletion were associated with a significant activation of NDRG1 transcription as indicated by stimulation of NDRG1-promoter activity and increases in NDRG1 mRNA and protein. This increased NDRG1 protein following polyamine depletion localized to both the cytoplasm and the nucleus, which is consistent with previous findings from others (40, 43). However, no nuclear localization signal sequence has been identified for NDRG1 to date (13, 14). This enhanced NDRG1 expression was completely prevented in polyamine-deficient IECs when increased p53 was abolished by using specific p53 siRNA, and in HCT-116 p53<sup>−/−</sup> cells (with a targeted deletion of p53). On the other hand, reducing p53 by increasing cellular polyamines with ODC overexpression decreased NDRG1 expression. Our data further show that increased NDRG1 expression is likely the direct result of p53 binding to the NDRG1-promoter, because polyamine depletion-induced NDRG1-promoter activity was significantly eliminated when p53-binding sites within the NDRG1 proximal promoter region were deleted. This finding is supported by previous observations from others (36), who have demonstrated that the identified p53-binding sites in the NDRG1-promoter are functional and could confer p53 responsiveness in transcriptional activation.

Several studies have suggested that induction of NDRG1 expression by activated p53 is cell type specific, although there is a ubiquitous expression pattern for NDRG1 in a variety of tissues (40). For example, ectopic expression of the wild-type p53 gene or increased levels of endogenous p53 by treatment with DNA-damaging chemotherapeutic drug doxorubicin dramatically stimulate NDRG1 transcription through direct interaction with p53-binding sites within the NDRG1-promoter in the nonmetastatic human colon cancer DLD-1-p53 and HCT-116 p53<sup>+/−</sup> cells, but not in the metastatic human lung cancer H1299-p53 cells (36). In contrast, forced expression of the wild-type p53 enhances p21 expression in all three cancer cell lines. Similar results also have been reported with the use of a differential screening technique (13). Interestingly, metastatic human colon cancer SW620 cells also do not express NDRG1 (6), suggesting that NDRG1 plays different roles in metastatic versus nonmetastatic cells. The results from the current study and our previous work (17, 53) have demonstrated that increased endogenous p53 following polyamine depletion activates transcription of both the NDRG1 and p21 genes in normal IECs (IEC-6 line).

The data from the current studies also indicate that p53-dependent NDRG1 expression plays a critical role in the inhibition of normal IEC proliferation following polyamine depletion. Increased levels of p53 and NDRG1 were associated with a significant increase in G<sub>1</sub> phase growth arrest in polyamine-deficient cells (16, 17, 19), whereas decreased levels of NDRG1 resulting from the stable transfection with siNDRG1 significantly promoted cell growth in the absence of cellular polyamines. Increased levels of NDRG1 caused by infection with the recombinant adenoviral vector containing wild-type NDRG1 cDNA also repressed DNA synthesis and inhibited cell proliferation regardless of the presence (in IEC-6 cells) or absence (HCT-116 p53<sup>+/−</sup> cells) of endogenous p53, suggesting that NDRG1 is a downstream mediator of p53 and results in the inhibition of IEC proliferation. These results are consistent with others showing that expression of the NDRG1 gene is...
increased during the growth arrest and cell differentiation but repressed in cell transformation (6, 13, 26), and that NDRG1 induction inhibits cell proliferation of the metastatic lung cancer H1299 cells (36). To our knowledge, this is the first study showing the biological function and involvement of NDRG1 in the regulation of normal IEC proliferation. Because IECs highly express NDRG1, and its cellular levels are negatively regulated by polyamines via decreasing p53, the current findings suggest that NDRG1 is a biological regulator for IEC proliferation under physiological conditions.

The results presented in Fig. 8 provide direct evidence that ectopic expression of the NDRG1 gene neither directly elicited cell death nor sensitized IECs to apoptosis induced by exposure to TNF-α/CHX together or TNF-α or CHX separately (data not shown). To date, the functions of NDRG1 in the regulation of apoptosis are paradoxical, and available results suggest that its role is cell type dependent. Consistent with our results, it has been reported that enhanced NDRG1 expression fails to induce any characteristic signs of apoptosis in the metastatic lung cancer H1299-NDRG1 cells and the nonmetastatic colon cancer DLD-NDRG1 cells (36). However, the forced expression of NDRG1 in H1299-p53 enhances p53-dependent caspase activation as well as apoptosis. In contrast, hypoxia-induced NDRG1 expression protects trophoblasts from hypoxic injury by inhibiting p53-mediated apoptosis (4). Our previous observations (16) and present studies have shown that polyamine depletion induces expression of p53 and NDRG1 but not apoptosis in IEC-6 cells. In fact, polyamine-deficient cells exhibit a tolerance to apoptosis when they are exposed to various apoptotic inducers (17). It has been shown that the increased resistance of polyamine-deficient IECs to apoptosis results primarily from the activation of NF-κB signaling (29, 52) and increases in activities of Akt kinase (48) and focal adhesion kinase (49). The findings from present studies suggest that NDRG1 has a little role in the regulation of apoptosis in normal IECs.

In summary, these results indicate that NDRG1 is a direct transcriptional target of induced p53 following polyamine depletion and that p53 activates NDRG1, probably through the p53-binding sites within the NDRG1-promoter in IECs. Importantly, this study also provides new evidence showing that p53-dependent NDRG1 expression plays a critical role in the negative control of IEC growth but not in apoptosis, because forced expression of wild-type NDRG1 inhibited DNA synthesis and decreased final cell numbers but failed to induce cell death and alter susceptibility to apoptotic inducers. IECs contain high concentrations of cellular polyamines that are involved in expression of various genes, and levels of cellular polyamines are tightly regulated under physiological conditions. Our previous studies (17, 54) have demonstrated that polyamine depletion stabilizes p53, thus resulting in inhibition of normal IEC proliferation. Together, these findings suggest that p53-dependent NDRG1 expression plays an important role in the negative control of normal intestinal mucosal growth and is crucial for maintenance of homeostasis in rapidly self-renewing mucosal tissue.

GRANTS
This work was supported by a Merit Review Grant from the Department of Veterans Affairs and by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-57819, DK-61972, and DK-68491. J.-Y. Wang is a Research Career Scientist, Medical Research Service, U.S. Department of Veterans Affairs.

REFERENCES
NDRG1 EXPRESSION AND POLYMINES