GASTROENTEROLOGY 2002;123:1163–1178

PTEN and TNF-α Regulation of the Intestinal-Specific *Cdx-2* Homeobox Gene Through a PI3K, PKB/Akt, and NF-κB–Dependent Pathway

SUNGHOON KIM,* CLAIRE DOMON–DELL,† QINGDING WANG,* DAI H. CHUNG,* ANTONIO DI CRISTOFANO,§ PIER PAOLO PANDOLFI,§ JEAN–NOEL FREUND,† and B. MARK EVERS*

*Department of Surgery, The University of Texas Medical Branch, Galveston, Texas; †Institut National de la Santé et de la Recherche Médicale, Strasbourg, France; and §Department of Human Genetics, Memorial Sloan-Kettering Cancer Center, New York, New York

See editorial on page 1395.

Background & Aims: **PTEN (phosphatase and tensin homologue deleted from chromosome 10) is a dual-specificity phosphatase implicated in embryonic development, intestinal cell proliferation and differentiation, and tumor suppression. The transcription factor Cdx-2 is critical in intestinal development and homeostasis, and its expression is altered in colorectal cancers. However, the regulation of the *Cdx-2* gene has not been entirely elucidated. Here, we hypothesize that *Cdx-2* may be a target of *PTEN* signaling in the intestine.** ***Methods:*** **The expression patterns for Cdx-2 and PTEN along wild-type mouse colon, as well as in colon tumors occurring in *Pten*$^{+/-}$ mice, were examined. The effect of PTEN or phosphatidylinositol 3-kinase inhibition and tumor necrosis factor α on *Cdx-2* messenger RNA and protein expression, Cdx-2 DNA binding activity, and the promoter activity of the *Cdx-2* gene was analyzed in human colon cancer cell lines.** ***Results:*** **Cdx-2 expression correlates with PTEN along the length of the murine colon and in colonic polyps that develop in *Pten*$^{+/-}$ mice. In colon cancer cells, PTEN stimulates Cdx-2 protein expression and the transcriptional activity of the *Cdx-2* promoter. Phosphatidylinositol 3-kinase inhibition by wortmannin or by a dominant-negative phosphatidylinositol 3-kinase mimics the *Cdx-2* stimulation by PTEN. Inversely, cell treatment by tumor necrosis factor α decreases *Cdx-2* expression. Phosphatidylinositol 3-kinase inhibition by PTEN or wortmannin has an inverse effect compared with tumor necrosis factor α on the balance between the p50 and p65 subunits of nuclear factor κB. p65 inhibits the activity of the *Cdx-2* promoter, whereas p50 prevents p65 action.** ***Conclusions:*** **Our results suggest that the intestinal *Cdx-2* homeobox gene is a target of PTEN/phosphatidylinositol 3-kinase signaling and tumor necrosis factor α signaling via nuclear factor κB–dependent pathways.**

The adult intestinal epithelium is a dynamic tissue system that continuously recapitulates major developmental processes, including cell proliferation, differentiation, migration, and apoptosis.[1] Intestinal homeostasis is dependent on an orchestrated regulation of epithelial cell renewal; perturbation of this equilibrium results in cancer and is associated with inflammatory bowel diseases. The dual-specificity protein phosphatase PTEN (a phosphatase and tensin homologue deleted from chromosome 10), which dephosphorylates serine, threonine, and tyrosine residues and various phosphatidylinositols phosphorylated in the D3 position, has been found to be essential for embryonic development and homeostasis of adult tissues, whereas loss of function contributes to carcinogenesis.[2,3] Homozygous *PTEN* gene inactivation results in early embryonic lethality in mice, and *Pten*$^{-/-}$ embryonic stem cells display altered ability to differentiate into the 3 germ layers. In addition, although *Pten*$^{+/-}$ mice are viable, they show hyperplastic–dysplastic changes and spontaneous tumor formation in various tissues, including the colon.[4] In humans, *PTEN* germline mutations have been found in autosomal dominant cancer syndromes with overlapping clinical features: Cowden disease and Bannayan–Zonana syndrome.[5] Even though each of these syndromes manifests distinct phenotypes, such as thyroid carcinoma,

Abbreviations used in this paper: DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; NF-κB, nuclear factor κB; PCR, polymerase chain reaction; pfu, plaque-forming unit; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted from chromosome 10; RT, reverse-transcription; SIF-1, sucrase-isomaltase footprint site 1; TNF, tumor necrosis factor; UCSF, University of California, San Francisco.

0016-5085/02/$35.00
doi:10.1053/gast.2002.36043

breast cancers, meningiomas, or macrocephaly, they are all characterized by multiple hamartomas in the intestine. Thus, *PTEN*, also identified as *MMAC1* (mutated in multiple advanced cancers), has a tumor suppressor function.[6]

PTEN antagonizes the activity of phosphatidylinositol 3-kinase (PI3K) by dephosphorylating the D3 phosphate group of the lipid second messenger, phosphatidylinositol 3,4,5-triphosphate, that is produced by PI3K.[7] Thus, PTEN-mediated tumor suppression is linked to the inhibition of PI3K signaling (see review[8]). The product of PI3K, phosphatidylinositol 3,4,5-triphosphate, targets Akt/protein kinase B (PKB), Bruton's tyrosine kinase, phosphoinositide-dependent kinases, integrin-linked kinase, atypical protein kinase C, phospholipase Cγ, and others as downstream elements in the cascade, among which are the transcription factors of the nuclear factor κB (NF-κB) family.[9] NF-κB participates in the control of intestinal homeostasis, as evidenced by the increased cell proliferation observed in NF-κB1–deficient mice.[10] Linked to this, NF-κB subunits show a specific distribution pattern in the intestinal epithelium, with p65 being selectively present in the proliferation zone, whereas p50 predominates in the differentiation zone.[10]

The *caudal*-related *Cdx-2* gene encodes an intestinal-specific transcription factor belonging to the homeobox family that has been suggested to be crucial for development and homeostasis of the intestinal epithelium.[11] The homeotic function of *Cdx-2* in the gut is assessed by the phenotypes resulting from *Cdx-2* haploinsufficiency and from gain of function. Indeed, *Cdx-2*$^{+/-}$ mice develop hamartomatous polyps in the ileum and colon that are characterized by gastric heteroplasia,[12,13] whereas ectopic expression of *Cdx-2* in the stomach of transgenic mice triggers intestinal-like heterodifferentiation of the gastric mucosa.[14] Thus, *Cdx-2* has been suggested to participate in the determination of positional information along the antero-posterior axis of the digestive tract, which is reminiscent of the role played by *caudal* during *Drosophila* embryogenesis.[15–17] In the adult intestinal epithelium, the Cdx-2 protein is predominantly expressed in the differentiated cells,[18,19] and forced expression of *Cdx-2* in various intestinal epithelial cell lines inhibits cell proliferation and stimulates cell differentiation and apoptosis.[20–22] *Cdx-2* expression decreases with the tumor grade in human colon cancers and in chemically induced tumors in the rat.[23,24] Moreover, *Cdx-2* overexpression, in combination with the homologue gene *Cdx-1*, reduces malignancy, as reflected by decreased growth rate, reduced cell migration, and increased sensitivity to apoptosis in the HT29 human colon cancer cell line.[21] These observations suggest that *Cdx-2* functions as a tumor suppressor. However, the low rate of genomic alteration reported at the *Cdx-2* locus in human colorectal tumors[25] indicates that down-regulation instead of structural mutation is responsible for its decreased expression in intestinal cancers. To date, little is known about the regulation of *Cdx-2*, except that *Cdx-2* expression is decreased by oncogenic *ras* activation in a human colon cancer cell line.[26]

The multiple-organ disorders observed with the loss of *PTEN* function in humans and in transgenic mice raise the question of whether *PTEN* drives common biological functions in the different targeted organs or whether *PTEN* has tissue-specific effects. To address this question, we were prompted to investigate whether the homeobox gene *Cdx-2* is a target of *PTEN* signaling in the intestine, given the facts that heterozygous *Pten*$^{+/-}$ mice and *Cdx-2*$^{+/-}$ mice develop intestinal hamartomatous polyps and that PI3K inhibition by PTEN,[27] like *Cdx-2* overexpression,[20,22] enhances enterocyte-like differentiation of human colon cancer cell lines. The results indicate that Cdx-2 is stimulated by PTEN through a PI3K/Akt pathway and the p50 NF-κB subunit. Because the PI3K/Akt/NF-κB signaling pathway is also activated by engagement of the proinflammatory cytokine tumor necrosis factor (TNF)-α,[28] we analyzed the effect of TNF-α on Cdx-2. We found that TNF-α has an inverse effect to PTEN on Cdx-2, which was found to be caused by modification in the balance between the p50 and p65 NF-κB subunits.

Materials and Methods

Materials

Wortmannin and TNF-α were purchased from Sigma Chemical Company (St. Louis, MO). The DAKO LSAB2 System kit was obtained from Dako Corporation (Carpinteria, CA). An Avidin Biotin blocking kit was purchased from Vector Laboratories (Burlingame, CA). A Bio-Rad Protein Assay and a Sequi-Blot PVDF Membrane were obtained from Bio-Rad Laboratories (Hercules, CA). An enhanced chemiluminescence detection kit was purchased from Amersham (Arlington Heights, IL). Ultraspec RNA reagent was obtained from Biotech Laboratories (Houston, TX). Nitrocellulose membrane was obtained from Sartorius (Göttingen, Germany). NorthernMax hybridization buffer was obtained from Ambion (Austin, TX). The x-ray film for autoradiography was from Eastman Kodak (Rochester, NY). Lipofectamin-plus was purchased from Life Technologies Inc. (Gaithersburg, MD). Antibodies against PTEN, actin, p50 NF-κB, p65 NF-κB, nor-

Table 1. Sequences of Synthetic Oligonucleotides Used in This Study

Oligonucleotide	Sequence
SIF-1 ([35])	5′-GGCTGGTGAGGGTGCAATAAAACTTTATGAGTA-3′
	5′-TACTCATAAAGTTTTATTGCACCCTCACCAGCC-3′
Cdx-2 NF-κB site 1	
(−22 to −1)	5′-GAGGGA**GGGACTGCCC**GGGAGG-3′
	5′-CCTCCCG**GGGCAGTCCC**TCCCTC-3′
Cdx-2 NF-κB site 2	
(−103 to −82)	5′-TGCGGC**GGGTCATCCCC**GCCTC-3′
	5′-GAGGCG**GGGATGACCC**GCCGCA-3′
Mutated *Cdx-2*	
NF-κB site 1	5′-GAGGGAGTACTGCCCGGGAGG-3′
	5′-CCTCCCGGGCAGTACTCCCTC-3′
Mutated *Cdx-2*	
NF-κB site 2	5′-CAACCTGCGGCCGCCTCTACAG-3′
	5′-CTGTAGAGGCGGCCGCAGGTTG-3′
Consensus	
NF-κB site	5′-AGTTGAGGGGACTTTCCCAGGC-3′
	5′-GCCTGGGAAAGTCCCCTCAACT-3′
Sp1	
oligonucleotide	5′-ATTCGATCGGGGCGGGGCGAGC-3′
	5′-GCTCGCCCCGCCCCGATCGAAT-3′
Oct1	
oligonucleotide	5′-TGTCGAATGCAAATCACTAGAA-3′
	5′-TTCTAGTGATTTGCATTCGACA-3′

NOTE. Bold letters represent the NF-κB binding sites of the NF-κB site 1 and NF-κB site 2 oligonucleotides.

mal rabbit or goat immunoglobulin G, and horseradish peroxidase–conjugated antibody and the purified p52 NF-κB protein were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–β-tubulin was purchased from Boehringer Mannheim (Indianapolis, IN). Concentrated polyclonal rabbit anti-PTEN antibody used for immunohistochemistry was purchased from Zymed Laboratories (South San Francisco, CA). Purified p50 NF-κB protein, pRL-null promoterless plasmid (renilla reporter), pGl3-Basic, a Gene Editor Site Directed Mutagenesis System, and a Dual-Luciferase Assay System were obtained from Promega (Madison, WI). Cdx-2 antiserum was a gift from Dr. Michael German (University of California, San Francisco [UCSF], CA). A second Cdx-2 antibody was purchased from Biogenex (San Ramon, CA). First Strand Buffer (5×) and Superscript II enzyme were purchased from Gibco (Grand Island, NY). PCR Buffer (10×) and Taq polymerase were obtained from Promega. Double-stranded consensus oligonucleotide for NF-κB was purchased from Promega, and Sp1 and Oct1 consensus oligonucleotides were purchased from Stratagene (La Jolla, CA). Oligonucleotides corresponding to the sucrase-isomaltase footprint site 1 (SIF-1), the *Cdx-2* promoter NF-κB sites 1 and 2, mutated NF-κB sites 1 and 2, and the Cdx-2 polymerase chain reaction (PCR) primers were synthesized by Oligos Etc. Inc. (Wilsonville, OR) and are shown in Table 1. [γ^{32}P]Adenosine triphosphate (3000 Ci/mmol) was purchased from NEN Life Science Products, Inc. (Boston, MA). All other reagents were of molecular biology grade and were purchased from Sigma, Stratagene, or Promega.

Plasmids, Adenoviruses, and Cdx-2 Promoter Constructions

A dominant-negative mutant plasmid of p85, pCGNN-Δp85, was a gift from Dr. Warren G. King (Harvard Medical School, Boston, MA). The p50 NF-κB and p65 NF-κB plasmids encoding the human p50 and p65 subunits of NF-κB were a gift from Dr. Catherine Regnier (Institut National de la Santé et de la Recherche Médicale Unit 255, Paris, France). Adenovirus vectors encoding hemagglutinin-tagged dominant-negative Akt (AxCA-Akt-AA) and the myristoylated active form of Akt (AxCA-Myr-Akt) were gifts from Dr. Wataru Ogawa (Kobe University School of Medicine, Kobe, Japan). The recombinant adenoviruses containing the wild-type PTEN (AxCA-PTEN) and the control adenovirus encoding galactosidase (AxCA-lacZ) were gifts from Dr. Akira Horii (Tohoku University School of Medicine, Sendai, Japan). An adenovirus vector encoding p65 NF-κB (Ad5p65) and its control vector (Ad5GFP) were gifts from Dr. Christian Jobin (University of North Carolina, Chapel Hill). A series of progressive 5′-promoter deletions of the murine *Cdx-2* gene promoter linked to the firefly luciferase reporter were constructed: −908/+117, −693/+117, −552/+117, −392/+117, −338/+117, and −171/+117. The position +1 refers to the major transcription start site identified in the *Cdx-2* gene.[16] The −908/+117 plasmid is equivalent to pCdx2-1Luc previously described.[26] The −171/+117 construct is designated as Δ-171 in this study. Each fragment was cloned into the unique *Sac*I and *Nco*I sites of luciferase reporter pGl3-Basic. Mutations of 2 putative NF-κB binding sites within the −908/+117 and Δ-171 promoter fragments were made by using the Gene Editor Site Directed Mutagenesis System (see Table 1 for mutated sequences). The Δ-171 reporter with the site 1 and 2 mutations was designated Δ-171-1/2M. The sequences were confirmed by automated sequencing.

Immunohistochemical Analysis of Mouse Colon

Heterozygous *Pten*$^{+/-}$ mice and their wild-type littermates were genotyped as described previously.[4] The DAKO LSAB2 System kit was used for the immunohistochemical staining of the sections. Briefly, tissue sections (3–5 μm) were mounted on positively charged slides. After the sections were deparaffinized with xylene and rehydrated through a series of graded alcohol, endogenous peroxides were quenched by soaking in 2 changes of 3% methanol H_2O_2. Both avidin and biotin in the Avidin Biotin blocking kit were diluted in Antibody Diluent (Dako) at a ratio of 1 mL of avidin or biotin to 5 mL of diluent. The primary antibody (Cdx-2 antiserum from UCSF, 1:1000 dilution; PTEN antiserum, 1:300 dilution) in biotin was applied for 1 hour. Sections were then incubated in rabbit-specific antibody from Vector Laboratories for 15 minutes and then in Chromagen liquid 3,3′-diaminobenzidine tetrahydrochloride (Dako) for 5 minutes. Primary antibodies were omitted for control slides.

Cell Culture and Viral Infections

The human colon cancer cell lines HT29, Caco-2, and HCT-116 were obtained from the American Type Culture Collection (Manassas, VA). HT29 and HCT-116 cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS). Caco-2 cells were grown in minimum essential medium with 15% FBS, 10 mL/L of pyruvic acid, and 10 mL/L of nonessential amino acid. In some experiments, cells were treated with the PI3K inhibitor wortmannin, dissolved in dimethyl sulfoxide (DMSO). Because of the instability of wortmannin in aqueous solution,[29] medium from wortmannin-treated cells and control cells was replaced every 4 to 5 hours. In other experiments, 1 nmol/L of TNF-α was added in the culture medium for 24 hours. Adenoviruses were amplified in HEK293 cells, and titer was determined by standard plaque assays. Cells were infected with adenovirus vectors at 10–20 plaque-forming units (pfu) per cell, as previously described.[27,30]

Protein Preparation and Western Immunoblots

Cells were lysed with TNN buffer (50 mmol/L of Tris HCl, pH 7.5, 150 mmol/L of NaCl, 0.5 mmol/L of Nonidet P-40, 50 mmol/L of NaF, 1 mmol/L of sodium orthovanadate, 1 mmol/L of dithiothreitol [DTT], and 1 mmol/L of phenymethylsulfonyl fluoride) and 25 μg/mL each of aprotinin, leupeptin, and pepstatin A at 4°C for 30 minutes. Lysates were clarified by centrifugation at 10,000*g* for 30 minutes at 4°C, and protein concentrations were determined with the Bio-Rad Protein Assay reagent. Western immunoblot analyses were performed as described previously.[27] Briefly, total protein (100 or 150 μg) was resolved on a 10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Filters were incubated overnight at 4°C in blotting solution (Tris-buffer saline containing 5% nonfat dried milk and 0.1% Tween 20). Cdx-2 protein was detected with Cdx-2 antiserum (dilution 1:1000 for UCSF antiserum and 1:100 for Biogenex antibody) after blotting with a horseradish peroxidase–conjugated second antibody (dilution 1:4000) and visualizing with the enhanced chemiluminescence detection kit.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated with Ultraspec RNA reagent. Polyadenylated RNA was extracted from HT29 cells by using oligo(deoxythymidine) cellulose, fractionated by electrophoresis on a 1.2% agarose–formaldehyde gel, and transferred to a nitrocellulose membrane as previously described.[31] Membranes were hybridized to a ^{32}P-labeled *Cdx-2* complementary DNA (cDNA) probe overnight at 43°C in NorthernMax hybridization buffer and then washed 3 times at 43°C for 20 minutes with 2× standard saline citrate/0.5% sodium dodecyl sulfate. Blots were reprobed with the glyceraldehyde-3-phosphate dehydrogenase cDNA to ensure equal loading. Signals were detected by autoradiography of x-ray films by using an intensifying screen at −70°C.

Reverse-Transcription Polymerase Chain Reaction

For the reverse-transcription (RT) reaction, random primers were mixed with 5 μg of total RNA. After 70°C incubation, appropriate amounts of 5× First Strand Buffer, 0.1 mol/L of DTT, and 10 mmol/L of deoxynucleoside triphosphate were added and incubated at 42°C for 2 minutes, followed by Superscript II enzyme, as recommended by the manufacturer. After the addition of Superscript II enzyme, the mixture was heated at 42°C for 50 minutes and then at 70°C for 15 minutes. The PCR reaction was performed by adding the cDNA generated from the RT reaction with appropriate amounts of 10× PCR Buffer, 25 mmol/L of $MgCl_2$, 10 mmol/L of deoxynucleoside triphosphate, 40 pmol of sense (TATTTGTCTTTTGTCCTGGTTTTCA) and antisense (CCACCATGTACGTGAGCTACCTTCT) primers, and Taq polymerase and cycled in a Stratagene PCR Robocycler at 94°C for 30 seconds, 50°C for 45 seconds, and 72°C for 45 seconds (total of 30 cycles) to produce a 575–base pair *Cdx-2* PCR product. Glyceraldehyde-3-phosphate dehydrogenase primers were used in separate reactions to confirm intact RNA.

Electrophoretic Mobility Shift Assays

Nuclear protein fractionation of HT29 cells was performed by using sucrose density-purification[32] and was quantified with the protein assay dye reagent. The method of electrophoretic mobility shift assay (EMSA) has been described previously.[33] Briefly, nuclear extracts (10 μg) were incubated with 50,000 counts per minute of ^{32}P-labeled SIF-1 or NF-κB double-stranded oligonucleotide probes and 1 μg of poly(deoxyinosinate · deoxycytidylate) in a buffer containing 10% glycerol, 12.5 mmol/L of HEPES, pH 7.9, 100 mmol/L of KCl, 1 mmol/L of DTT, and 1 mmol/L of EDTA in a final volume of 20 μL for 20 minutes at room temperature. The DNA/protein complexes were fractionated on a 6% acrylamide gel, run in 0.5× Tris-borate-EDTA buffer, dried, and exposed to Kodak X-AR film at −70°C. Competition experiments were performed by the addition of 100-fold molar excess of the unlabeled oligonucleotide before the addition of radioactive probe. Supershift experiments were performed by adding antibodies Cdx-2 or NF-κB subunits (p50 or p65) (2–4 μg) and incubating on ice for 2 hours before the addition of labeled probe. The specificity of the antibodies used was compared with preimmune immunoglobulin G.

Transient Transfection and Luciferase Assays

HT29 cells were seeded at 1×10^5 cells into 24-well plates in triplicate 24 hours before transfection. Cells were then transiently transfected with 0.4 μg of a firefly luciferase reporter plasmid by using the Lipofectamin-plus transfection agent and following the manufacturer's recommended proto-

col. The amounts of plasmids p50 NF-κB and p65 NF-κB used for transfections ranged between 0.1 μg and 1 μg, as indicated in the text. The renilla reporter pRL-null (0.04 μg per well) was co-transfected to normalize for variation in transfection efficiency. The effect of dominant-negative PI3K expression plasmid pCGNN-Δp85 (0.4 μg) was compared with that of its empty vector, pCGNN, by co-transfecting the expression plasmid with the Cdx-2 luciferase reporter plasmids. After 48 hours of incubation, the cells were harvested for measurement of firefly and renilla luciferase activities by using the Dual-Luciferase Assay System. Firefly luciferase activity was determined by subtracting background signal and normalized to the renilla activity. Fold induction or inhibition was calculated by dividing treatment values by control values. Infection experiments using the adenoviruses and plasmid reporters were performed by the addition of adenoviruses (10 pfu per cell) to the cells 5 hours after the initial transfection. After 1 hour of infection, fresh medium containing FBS was added to make the incubating medium consistent with 10% serum. Light emissions were integrated for the initial 10 seconds of emission by using a Monolight 2010 Luminometer as described previously[31] (Analytical Luminescence Laboratory, San Diego, CA). Samples were run in triplicate, and all experiments were performed on at least 2 separate occasions.

Statistical Analysis

Data in Figure 5*A* and *B* were analyzed with a 2-sample *t* test. Data in Figure 6*B* and *C* were analyzed with analysis of variance for a 2-factor factorial experiment. Data in Figure 5*C* were analyzed with the 1-way classification analysis of variance with Duncan's multiple range test for the multiple comparisons. All tests were assessed at the 0.05 level of significance.

Results

Expression Pattern of PTEN and Cdx-2 in the Colon

To determine whether a possible link between *PTEN* and *Cdx-2* exists in the intestine, we first compared the pattern of expression of the corresponding proteins in the colon epithelium of wild-type and heterozygous *Pten*$^{+/-}$ mice. Immunohistochemical analysis of colonic sections of wild-type mice indicated that the level of PTEN protein gradually increased in the epithelial cells from the crypt base to the tip of the colonic cuffs; this pattern was noted along the entire length of mouse colon (Figure 1*A*: from the cecal section of mouse colon). As already reported,[16,19] Cdx-2 immunostaining is nuclear, and, similar to PTEN, the signal also increased along the colonic gland axis; this pattern was noted along the entire length of mouse colon (Figure 1*B*: from cecal section). Western blot analysis of wild-type colon showed higher PTEN expression in the proximal colon compared with the distal segment (not shown). Like PTEN, Cdx-2 protein was also more abundant in the proximal colon compared with the distal colon, which is consistent with the longitudinal pattern of the *Cdx-2* messenger RNA (mRNA) and protein previously reported.[16,19]

Heterozygous *Pten*$^{+/-}$ mice develop polyps scattered at the surface of the colonic epithelium with an otherwise normal phenotype. Western blot analysis between 2 sets of wild-type and *Pten*$^{+/-}$ animals showed that the level of PTEN protein was reduced in the distal colon of the mutant mice (Figure 1*E*). It is noteworthy that a decrease in Cdx-2 protein accompanied the reduction in PTEN in the distal colon of mutant mice as compared with the wild-types (Figure 1*E*). Unlike in the distal colon, we failed to detect any significant reduction of either PTEN or Cdx-2 in mutant compared with wild-type animals in the proximal colon (not shown). In the area with normal phenotype in *Pten*$^{+/-}$ animals, the distribution of PTEN and Cdx-2 proteins was similar to that observed in wild-type mice (Figure 1*C* and *D*, respectively: from midsection of mouse colon). In the adenomatous polyps, we observed minimal PTEN expression, and the regions where a faint expression was still detected were principally localized to the surface epithelium, as compared with the glandular structures located in central portions of the tumors in which we failed to show PTEN by immunohistochemistry (Figure 2*A–C*). Cdx-2 immunostaining was also strongly reduced in the adenomas, and the pattern with anti–Cdx-2 antibody coincided with the area of residual PTEN expression at the surface of the polyps (Figure 2*D–F*).

Taken together, these data indicate that PTEN and Cdx-2 proteins are expressed in a similar pattern along the longitudinal and vertical axes of the colon in wild-type and *Pten*$^{+/-}$ mice, that the level of both proteins is reduced in the distal colon of *Pten*$^{+/-}$ mice, and that PTEN and Cdx-2 also show a similar distribution in the colonic adenomatous polyps resulting from *PTEN* haploinsufficiency.

Cdx-2 expression is stimulated by PTEN overexpression in colon cancer cell lines. The similar patterns of PTEN and Cdx-2 observed in the murine intestine prompted us to investigate, at the functional level, whether Cdx-2 expression can be regulated by PTEN. For this purpose, the human colon cancer cell line HT29, a widely used model for studies of intestinal cell differentiation,[34–36] was infected with either the control AxCA-LacZ adenovirus or the AxCA-PTEN adenovirus that encodes PTEN at a multiplicity of infection of 10 pfu per cell. After 2 days, cells were harvested and lysed,

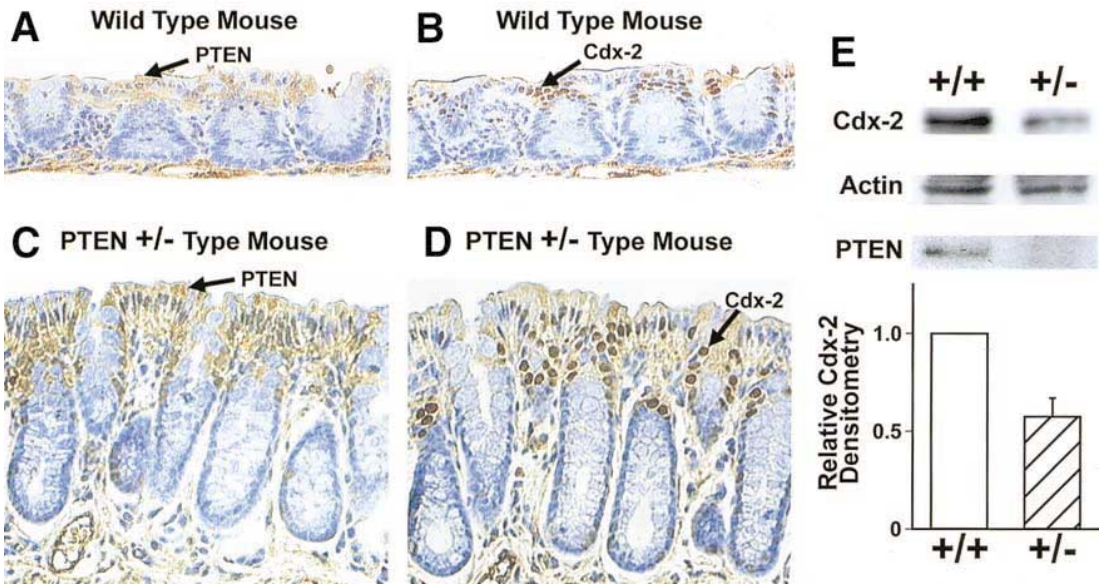

Figure 1.

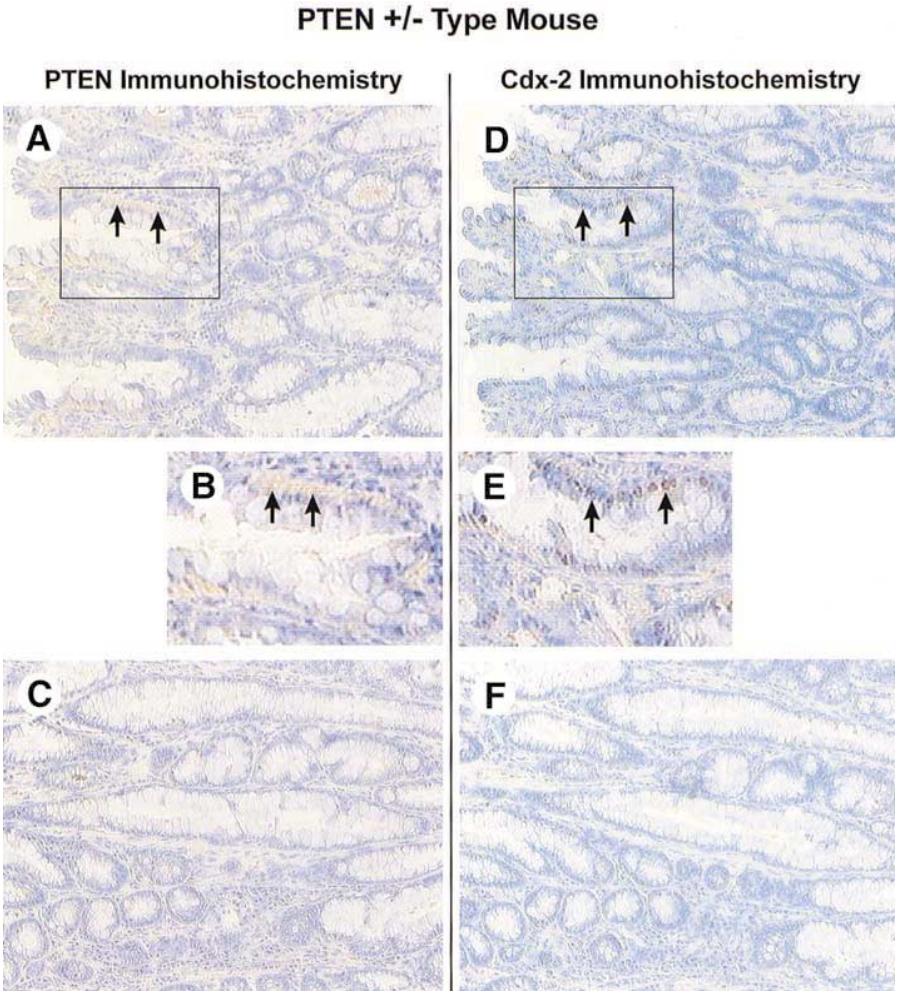

Figure 2.

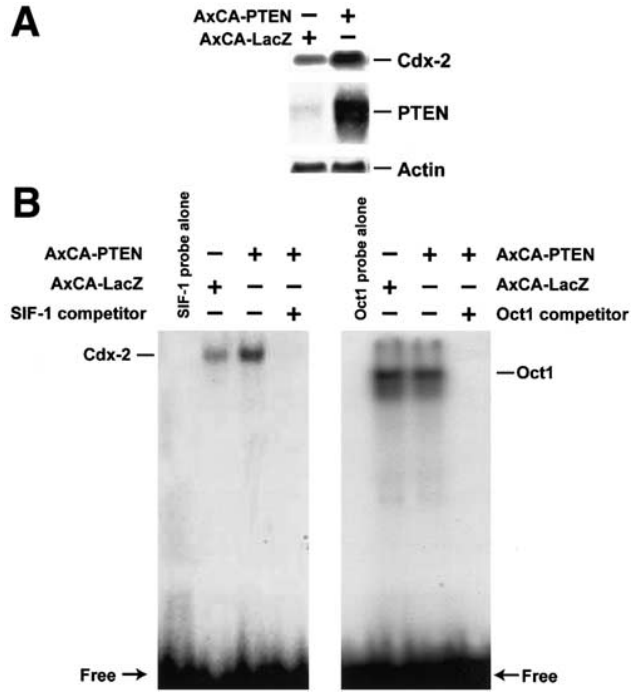

Figure 3. Stimulation of Cdx-2 protein expression by PTEN overexpression. (*A*) HT29 cells were infected for 1 hour with AxCA-PTEN encoding PTEN or with the control AxCA-LacZ (multiplicity of infection, 10). Two days later, whole-cell protein was extracted. Protein (150 μg) was fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted with anti–Cdx-2, -PTEN, and –β-actin antibodies. β-Actin immunodetection was used to assess equal protein loading. HT29 cells infected with AxCA-PTEN showed increased expression of Cdx-2 compared with control. In AxCA-PTEN–infected cells, PTEN overexpression was assessed with PTEN immunoblotting. (*B*) HT29 cells were infected with the AxCA-PTEN or control AxCA-LacZ adenovirus as in Figure 5*A*. Cells were harvested 2 days later, and the nuclear protein was extracted for EMSA. Protein (10 μg) was incubated either with labeled SIF-1 or Oct1 oligonucleotide probes. Competition experiments were performed with either unlabeled SIF-1 or Oct1 oligonucleotide in 100-fold molar excess. Increased Cdx-2 binding to the SIF-1 promoter element was noted for the AxCA-PTEN–infected HT29 cells. No difference in Oct1 binding activity was noted.

and total cellular protein was extracted. Western blot analysis of the protein extracts showed an increased expression of the Cdx-2 protein in cells infected with AxCA-PTEN compared with control infected cells (Figure 3*A*; upper panel). The blot was stripped and reprobed with an anti-PTEN antibody to confirm overexpression in AxCA-PTEN–infected cells and with an anti–β-actin antibody to assess equal protein loading for electrophoresis (Figure 3*A*; middle and lower panels).

We next determined whether PTEN overexpression results in enhanced Cdx-2 DNA binding activity by EMSA using the SIF-1 oligonucleotide, which corresponds to the Cdx-2 binding site located in the proximal promoter region of the intestinal-specific sucrase-isomaltase gene.[37] EMSA revealed increased binding activity for HT29 cells infected with the AxCA-PTEN adenovirus compared with cells infected with the control adenovirus (Figure 3*B*; left panel). Competition with a molar excess of unlabeled probe effectively inhibited protein binding, indicating sequence-specific binding of the complex. As a control to ensure that AxCA-PTEN infection did not produce a generalized induction in DNA binding activity, we performed EMSA with oligonucleotides containing a consensus Oct1 or Sp1 binding site. No increase in Oct1 binding activity (Figure 3*B*; right panel) or in Sp1 binding activity (not shown) was noted after AxCA-PTEN infection, indicating that the increase in SIF-1 binding activity was a specific effect of PTEN.

The effect of PTEN on Cdx-2 expression was also analyzed in 2 other human colon adenocarcinoma cell lines, Caco-2 and HCT-116. As in HT29, infection of these cell lines with AxCA-PTEN adenovirus led to an increased level of Cdx-2 protein, visualized on Western blots and by EMSA by using the SIF-1 radiolabeled probe (data not shown). Taken together, these findings show induction of Cdx-2 protein expression and Cdx-2 DNA binding activity by overexpression of PTEN in intestinal cells.

Figure 1. *PTEN* and *Cdx-2* expression patterns in the mouse colon. Immunohistochemical analysis of mouse colon sections were performed as described in Materials and Methods. Colonic sections from the wild-type and *Pten*$^{+/-}$ (normal mucosa) mice displayed similar immunohistochemical patterns. (*A*) PTEN staining of the cecal area in wild-type mouse shows brown stains representing PTEN protein predominantly in the cuffs of the crypts, as indicated by the arrow (original magnification, 10×). (*B*) Cdx-2 staining (brown) of the same area as shown in (*A*) shows increased Cdx-2 protein expression in the nuclei along the crypt-to-cuff axis. (*C* and *D*) Similar expression patterns of PTEN and Cdx-2, respectively, are seen in an area showing a normal phenotype in *Pten*$^{+/-}$ proximal colon (original magnification, 20×). (*E*) Protein from the distal colon of wild-type (+/+) and *Pten*$^{+/-}$ mice (+/−) was analyzed by Western blotting by using antibodies for Cdx-2, β-actin, and PTEN. The heterozygous *Pten*$^{+/-}$ mouse shows decreased Cdx-2 and PTEN levels in the distal colon compared with the wild-type mouse.

Figure 2. *PTEN* and *Cdx-2* expression patterns in the colonic polyps of *Pten*$^{+/-}$ mice. (*A*) In the colon adenomatous polyps of *Pten*$^{+/-}$ mice, PTEN immunostaining is largely reduced compared with the areas with normal phenotype (original magnification, 10×); a faint immunostaining was hardly detected in the epithelial cells at the surface of the polyps (arrows). (*B*) Higher magnification of the area delineated in (*A*) to better visualize PTEN immunostaining. (*C*) The inner region of the polyps shows no PTEN staining. (*D*) Staining of a serial section of the adenoma area shown in (*A*) shows a low expression of Cdx-2 protein in the nuclei at the surface of the polyps (arrows). (*E*) Higher magnification of the area delineated in (*D*). (*F*) Cdx-2 was also absent in the inner region of the polyps, with the exception of a faint and occasional staining of some scattered nuclei.

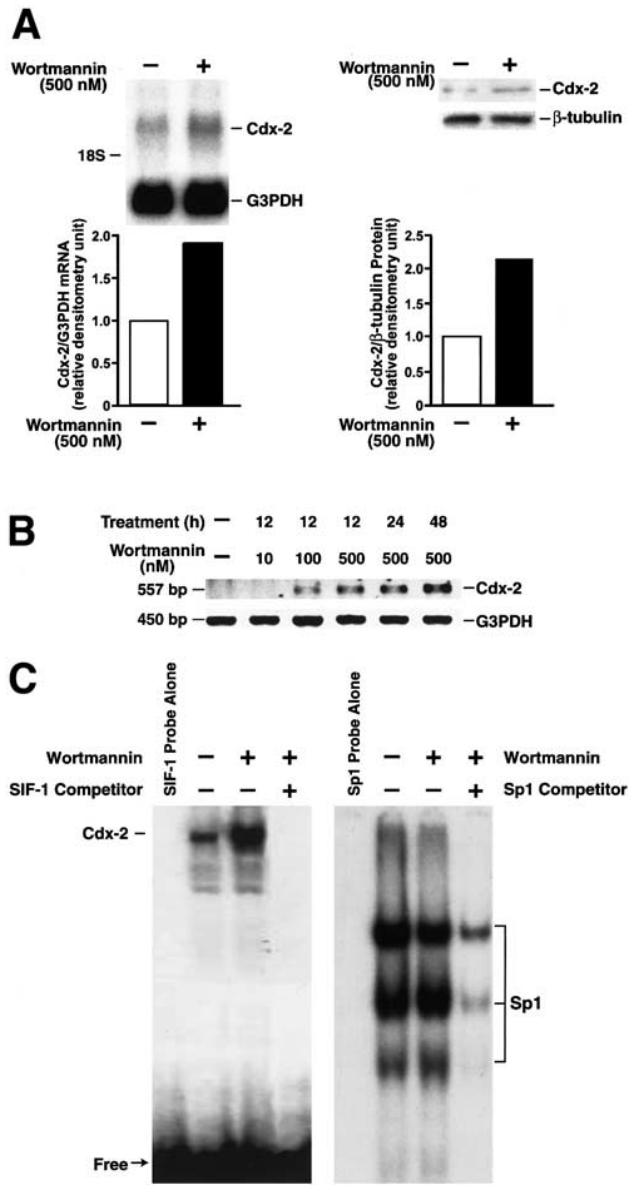

Figure 4. Simulation of Cdx-2 protein and gene expression by PI3K inhibition with wortmannin. (*A*) HT29 cells were treated with wortmannin (500 nmol/L) or DMSO (control) for 48 hours and then harvested for polyadenylated RNA and nuclear proteins. RNA (10 μg) was fractionated, transferred to nitrocellulose membranes, and probed with labeled *Cdx-2* cDNA; blots were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) to ensure equal loading. Wortmannin treatment increased *Cdx-2* mRNA levels by 2-fold as evaluated by densitometry (left panel). Nuclear proteins (50 μg) were fractionated, and Western blot was performed with anti–Cdx-2 and –β-tubulin antibodies. Equal loading was confirmed with β-tubulin. Densitometric analysis indicated a 2.3-fold increase of Cdx-2 protein in wortmannin-treated cells (right panel). (*B*) The wortmannin dose response was evaluated by using HT29 cells treated with increasing doses of wortmannin for variable time periods. Total RNA was extracted and analyzed by RT-PCR with specific *Cdx-2* and *G3PDH* primers. Increasing *Cdx-2* signals were noted with higher doses and treatment periods of wortmannin. G3PDH signals confirmed equal loading. (*C*) Binding activity of Cdx-2 was assessed with EMSA by using 10 μg of nuclear protein with labeled SIF-1 oligonucleotide (left panel). Competition experiments were performed with unlabeled SIF-1 in 100-fold molar excess. Wortmannin treatment increased Cdx-2 binding to the SIF-1 promoter element. Unlike Cdx-2 DNA binding activity, Sp1 DNA binding activity to the corresponding labeled probe was unchanged by wortmannin treatment (right panel).

PI3K inhibition mimics the stimulatory effect of PTEN on Cdx-2 expression. Because PTEN antagonizes PI3K activity,[7] we next determined whether inhibition of PI3K can stimulate Cdx-2 expression. For this purpose, HT29 cells were treated with wortmannin, a specific covalent inhibitor of the catalytic p110 subunit of PI3K that mimics the effect exerted by PTEN.[38] Polyadenylated RNA and nuclear proteins were extracted from HT29 cells treated for 24 hours with 500 nmol/L of wortmannin or with DMSO and were used for Northern and Western blot analyses. The densitometric analysis showed that wortmannin treatment led to a 2-fold increase of *Cdx-2* mRNA and to a 2.3-fold increase of Cdx-2 protein (Figure 4*A*).

To examine time and dose response to wortmannin, RT-PCR was performed for the *Cdx-2* transcript by using total RNA extracted from HT29 cells treated with 10–500 nmol/L of wortmannin for 24 and 48 hours. The stimulation by wortmannin was dose and time dependent, with a maximal effect noted after 48 hours of treatment with 500 nmol/L of wortmannin (Figure 4*B*). By EMSA, we also showed that the Cdx-2 DNA binding activity increased for wortmannin-treated cells compared with control DMSO-treated cells (Figure 4*C*; left panel). The band obtained by EMSA was competed with a molar excess of unlabeled probe. In these experiments, no increase in Sp1 DNA binding activity (Figure 4*C*; right panel) or in Oct1 DNA binding activity (not shown) was observed, indicating that the increased SIF-1 binding activity was a specific effect of wortmannin.

These data indicate that PI3K inhibition with the covalent inhibitor wortmannin stimulates *Cdx-2* gene expression and DNA binding activity in HT29 cells, thus recapitulating the stimulatory effect exerted by overexpression of PTEN. This supports the role of the PTEN/PI3K balance in the regulation of *Cdx-2*.

PTEN overexpression or PI3K inhibition stimulates the activity of the Cdx-2 promoter and is dependent on the kinase Akt. To determine whether the increase in *Cdx-2* gene expression is due to increased *Cdx-2* promoter activity and to assess the *cis*-regulatory elements responsible for this induction, we constructed a series of reporter plasmids containing different lengths of the *Cdx-2* promoter linked to the luciferase reporter gene. These *Cdx-2* reporter constructs were transfected into HT29 cells, which were then either infected with the

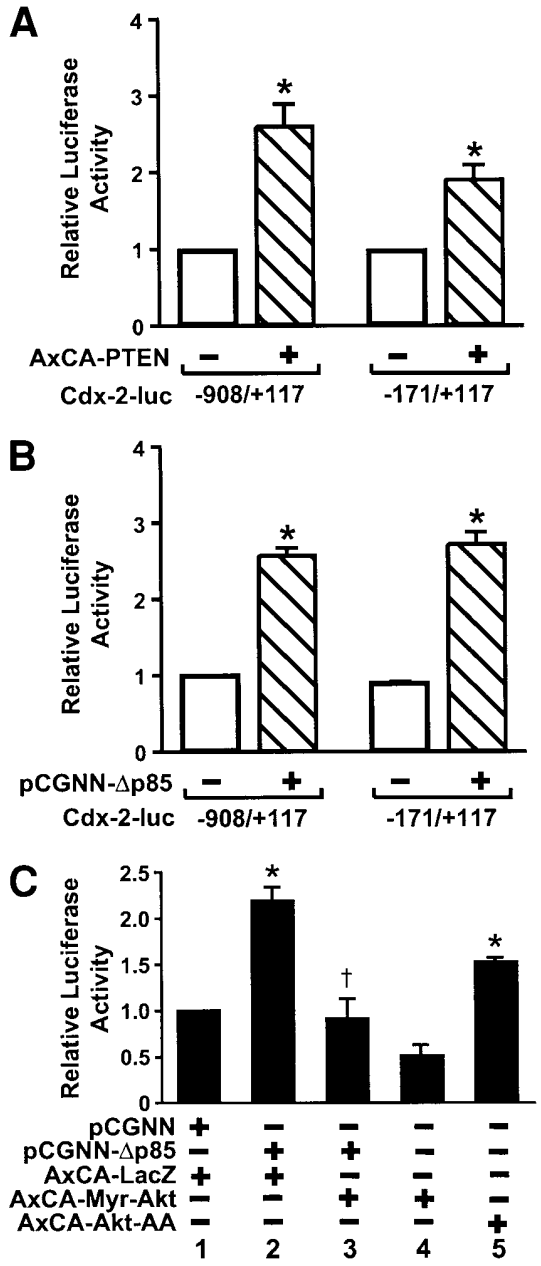

Figure 5. *Cdx-2* promoter activity is increased by PTEN expression or dominant-negative p85 expression and is dependent on Akt. (*A*) HT29 cells (1×10^5) were transfected with the −908/+117 or Δ-171 *Cdx-2* luciferase reporter plasmids (0.4 μg) together with pRL-null (0.04 μg) for normalization. The cells were then infected with AxCA-PTEN or the control adenovirus AxCA-lacZ at a multiplicity of infection of 10. Luciferase assays were performed after 48 hours of incubation. Infection with AxCA-PTEN stimulated both −908/+117 and −171/+117 (Δ-171) *Cdx-2* luciferase reporter activities compared with control. (*B*) Similar experiments performed with the pCGNN-Δp85 plasmid instead of the AxCA-PTEN adenovirus produced a similar effect on the 2 *Cdx-2* reporter plasmids. Data are expressed as mean ± SD; $*P <$ 0.05 compared with control. (*C*) HT29 cells were co-transfected with the Δ-171 *Cdx-2* luciferase reporter in all experiments. Cells were co-transfected with the control pCGNN vector (*lane 1*) or with the pCGNN-Δp85 plasmid to stimulate the activity of the *Cdx-2* promoter (*lane 2*). pRL-null was used for normalization. Infection of the Δ-171– and pCGNN-Δp85–transfected cells with AxCA-Myr-Akt encoding the adenovirus expressing PTEN or cotransfected with the plasmid pCGNN-Δp85, which encodes a dominant-negative form of the regulatory subunit of PI3K to antagonize PI3K activity.

Infection of HT29 cells with the PTEN-encoding adenovirus and co-transfection with the series of reporter *Cdx-2* promoter plasmids resulted in a 2–3-fold stimulation of *Cdx-2* promoter activity compared with cells infected with the control adenovirus (Figure 5*A*). This effect was noted with all of the promoter constructs used for this study, including the shortest fragment corresponding to the segment −171/+117 (Δ-171) of the *Cdx-2* gene promoter. No stimulation of luciferase activity was observed with the empty vector. Consistent with the effect produced by PTEN overexpression, inhibition of PI3K by cotransfection with pCGNN-Δp85 also resulted in a 2–3-fold induction of *Cdx-2* promoter activity with the various *Cdx-2* promoter constructs (Figure 5*B*). PI3K activates several downstream targets, including PKB/Akt.[9] To determine whether PKB/Akt mediates the PI3K effect on the *Cdx-2* promoter, HT29 cells were cotransfected with the Δ-171 *Cdx-2* reporter plasmid and control pCGNN or pCGNN-Δp85 to activate the transcriptional activity of the Cdx-2 promoter (Figure 5*C*; lanes 1 and 2), and the cells were then infected with either the control AxCA-LacZ adenovirus or with the AxCA-Myr-Akt adenovirus encoding the activated myristoylated form of Akt (lane 3). Introduction of the myristoylated form of Akt in HT29 cells previously transfected with pCGNN-Δp85 abolished the 2-fold stimulatory effect of the *Cdx-2* promoter activity exerted by PI3K inhibition. In addition, in the absence of PI3K inhibition by pCGNN-Δp85, the activity of the *Cdx-2* promoter was reduced in cells infected with the AxCA-Myr-Akt adenovirus compared with cells infected with control AxCA-LacZ (Figure 5*C*; lane 4). Conversely, HT29 cell infection with the AxCA-Akt-AA adenovirus to express a mutant form of Akt stimulated the activity of the *Cdx-2* promoter (lane 5). Similar results were obtained when the −908/+117 *Cdx-2* reporter plasmid

myristoylated activated form of Akt blunted the stimulatory effect of the dominant-negative p85 on the *Cdx-2* promoter (*lane 3*). In the absence of stimulation by pGCNN-Δp85, the expression of the myristoylated activated form of Akt in HT29 cells infected by AxCA-Myr-Akt reduced the activity of the Δ-171 Cdx-2 reporter (*lane 4*) compared with control cells infected with the control AxCA-LacZ adenovirus, whereas infection with AxCA-Akt-AA, encoding the dominant-negative form of Akt, stimulated Δ-171 activity (*lane 5*). Data are expressed as mean ± SD; $*P < 0.05$ compared with co-transfection with the empty plasmids (control, *lane 1*); $\dagger P < 0.05$ compared with *lane 2* (co-transfection with pCGNN-Δp85 and AxCA-LacZ).

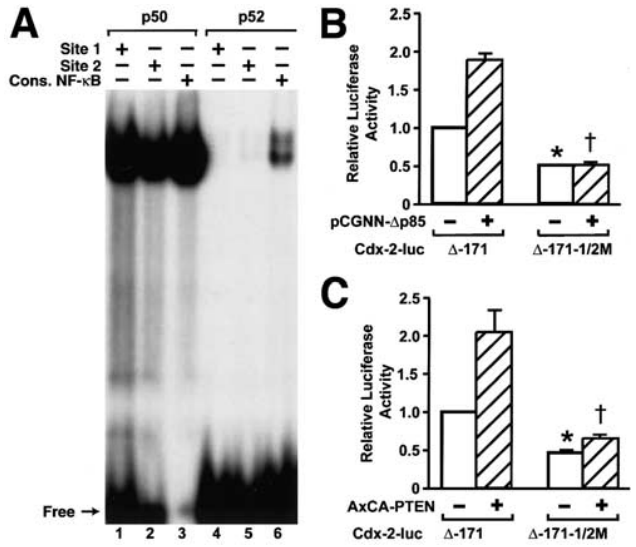

Figure 6. NF-κB DNA binding sites in the Cdx-2 promoter. (*A*) Commercially available p50 and p52 NF-κB proteins were used for EMSA with the labeled NF-κB consensus oligonucleotide or the oligonucleotides corresponding to the NF-κB site 1 and NF-κB site 2 of the *Cdx-2* promoter. The p50 protein efficiently bound to the 3 oligonucleotide probes (lanes 1–3). The p52 protein showed a lower affinity to the consensus NF-κB oligonucleotide than p50, and it did not bind to the NF-κB site 1 or NF-κB site 2 of the *Cdx-2* promoter (lanes 4–6). (*B* and *C*) HT29 cells transfected with Δ-171 or Δ-171-1/2M luciferase reporter plasmids were co-transfected with the plasmid pGCNN-Δp85 encoding dominant-negative p85 (*B*), with the AxCA-PTEN adenovirus encoding PTEN (*C*), or with the control adenoviruses. Mutation of both NF-κB sites of the *Cdx-2* promoter eliminated the stimulatory effect exerted by PTEN or by dominant-negative p85. Data are expressed as mean ± SD; $*P < 0.05$ compared with Δ-171 co-transfected with empty vector (control); $\dagger P < 0.05$ compared with Δ-171 co-transfected with either pCGNN-Δp85 or AxCA-PTEN. No statistical difference was found between the control and experimental treatment for Δ-171-1/2M reporter in *B* and *C*.

was used instead of the Δ-171 construct (data not shown).

Together, these results show that inhibition of PI3K by PTEN overexpression or transfection of a dominant-negative p85 stimulates *Cdx-2* promoter activity, thus suggesting that the increased Cdx-2 protein and mRNA levels after PTEN overexpression or wortmannin treatment are dependent on transcriptional regulation of the *Cdx-2* gene. These effects are dependent on the kinase Akt, a common downstream target of PI3K. Furthermore, these findings identify the −171/+117 region of the *Cdx-2* gene as critical for regulation by PTEN/PI3K/Akt.

The effect of PI3K inhibition by PTEN or by Δp85 on the Cdx-2 promoter is mediated by 2 NF-κB binding sites. Several studies have linked the PI3K/Akt pathway to NF-κB regulation.[28,39] In addition, we have recently reported that PI3K inhibition by wortmannin increased NF-κB binding activity in HT29 cells.[40] Therefore, we have investigated whether putative NF-κB binding sites exist in the promoter of the *Cdx-2* gene. Computer-based analysis of the −171/+117 region of the *Cdx-2* promoter using MatInspector (Genomatix, München, Germany) identified 2 putative NF-κB binding sites located at positions −16/−7 (site 1) and −97/−88 (site 2) upstream of the minor *Cdx-2* transcription start site (site 1). We then investigated whether the 2 putative NF-κB sites in the *Cdx-2* promoter bind to NF-κB proteins in vitro. To test the NF-κB binding ability of these sites, commercially available purified proteins were used. EMSA showed p50 NF-κB protein efficiently bound to the 2 binding sites and to the consensus NF-κB–binding oligonucleotides, whereas minimal binding was detected with purified p52 NF-κB protein (Figure 6*A*).

We next determined whether the putative NF-κB binding sites 1 and 2 identified in the *Cdx-2* gene promoter actually participate in the stimulation caused by PI3K inhibition. Mutations of both NF-κB sites 1 and 2 were introduced into the −171/+117 (Δ-171) Cdx-2 luciferase reporter plasmid. The wild-type and mutated constructs were cotransfected into HT29 cells along with pCGNN-Δp85 encoding dominant-negative p85 or with the empty vector. As previously shown, cotransfection of the dominant-negative p85 results in an approximately 2-fold increase in promoter activity of the wild-type *Cdx-2* promoter. Mutations of the NF-κB sites reduced by 2-fold its basal transcriptional activity. In addition, these mutations abolished the stimulation of the promoter activity caused by dominant-negative p85 (Figure 6*B*). Similar results were obtained when AxCA-PTEN was substituted for pCGNN-Δp85 (Figure 6*C*). Mutation of both NF-κB sites was required for the inhibition of *Cdx-2* promoter activity, as noted by the fact that cotransfection of dominant-negative p85 stimulated *Cdx-2* promoter activity when only one of the sites was mutated (data not shown). These results suggest a cooperation between sites 1 and 2 for the regulation of *Cdx-2* by the PTEN/PI3K pathway.

PI3K inhibition by PTEN overexpression or wortmannin preferentially increases the in vitro DNA binding activity of the p50 NF-κB subunit. We have determined the effect of PTEN overexpression on NF-κB DNA binding activity. EMSA with a double-stranded oligonucleotide containing a consensus NF-κB binding site resulted in 2 retarded bands in HT29 cells, corresponding to the p50/p50 and p65/p50 NF-κB isoforms. Using this probe, we found increased DNA binding activity in cells infected with AxCA-PTEN compared with cells infected with control adenovirus (Figure 7*A*; lanes 2 and 3), which is consistent with our results reported previ-

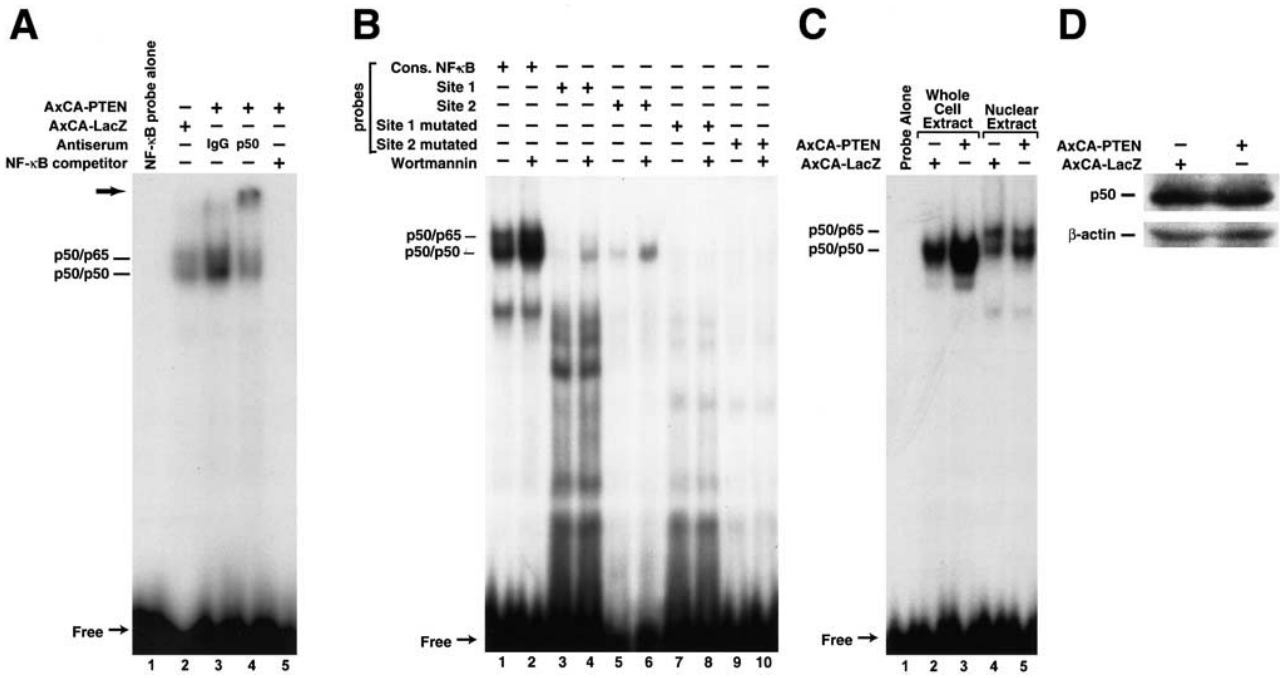

Figure 7. PI3K inhibition by PTEN or wortmannin stimulates NF-κB p50/p50 DNA binding activity on sites 1 and 2 of the Cdx-2 promoter. (*A*) HT29 cells were infected with AxCA-PTEN or control AxCA-LacZ adenovirus, and nuclear protein was extracted 2 days later. Protein (10 μg) was incubated with labeled oligonucleotide containing a consensus binding site for NF-κB (*lane 2*). Overexpression of PTEN caused increased NF-κB DNA binding activity (*lane 3*). The presence of p50 NF-κB protein in the DNA/protein complex was assessed by supershift with anti-p50 antibody (lane 4). Competition experiments were performed with a 100-fold molar excess of consensus unlabeled NF-κB oligonucleotide (*lane 5*). (*B*) HT29 cells were treated with wortmannin (500 nmol/L) or DMSO (control) for 48 hours, and protein was extracted. EMSA was performed with 10 μg of protein by using the NF-κB consensus oligonucleotide, the oligonucleotides containing the NF-κB site 1 and site 2 of the *Cdx-2* promoter, or mutated forms of these oligonucleotides (see Table 1 for the sequences of the oligonucleotides). Wortmannin-treated samples showed increased binding activity for the consensus NF-κB (*lanes 1* and *2*), NF-κB site 1 (*lanes 3* and *4*), and NF-κB site 2 probes (*lanes 5* and *6*), as compared with control DMSO-treated samples. Sites 1 and 2 predominantly bound to the p50/p50 homodimer, and the binding was increased by wortmannin. No DNA/protein complex was observed with the mutated forms of NF-κB site 1 and site 2 (*lanes 7–10*). (*C*) To determine whether the nuclear protein separation affected the NF-κB protein distribution, whole-cell extracts, as well as nuclear extract from AxCA-PTEN–infected HT29 cells, were analyzed by EMSA. Infection with AxCA-PTEN increased NF-κB binding activity, particularly for the p50 protein shift, in both whole-cell and nuclear extracts. (*D*) Whole-cell protein extracts (as in *C*) were then used for Western blotting. Blotting with anti-p50 NF-κB antibody showed equal levels of p50 protein, suggesting that the increased p50 NF-κB binding activity noted in (*C*) was not due to increased p50 protein expression.

ously with wortmannin-treated cells.[40] The presence of p50 NF-κB protein in these DNA/protein complexes was confirmed by supershift experiment with anti-p50 antibody (Figure 7*A*; lane 4). Stimulation of NF-κB DNA binding activity by PTEN overexpression was also found in Caco-2 and HCT-116 cells (data not shown). It is noteworthy that careful examination of the EMSA results showed throughout these experiments in every cell line that the faster migrating band corresponding to the p50/p50 homodimer was always more stimulated than the slow migrating band corresponding to the p65/p50 heterodimer, with a densitometric ratio of approximately 2-fold.

A similar result as above was obtained by EMSA with the consensus NF-κB oligonucleotide when wortmannin-treated cell extracts were used instead of PTEN-overexpressing cell extracts (Figure 7*B*; lanes 1 and 2). However, unlike the oligonucleotide comprising the consensus NF-κB site, sites 1 and 2 of the *Cdx-2* promoter predominantly bound to the p50/p50 homodimer, whereas the p50/p65 heterodimer was barely detected by EMSA in either control or wortmannin-treated cell extracts. In addition, increased DNA binding activity of the p50/p50 homodimer to these sites was observed after wortmannin treatment compared with untreated cell extracts (Figure 7*B*; lanes 3–6). To further show the specificity of protein binding, oligonucleotides containing mutations of either site 1 or 2 (Table 1) were synthesized and used in the EMSA. Mutation of these sites effectively prevented the electromobility shift (lanes 7–10). In addition, a molar excess of unlabeled oligonucleotide containing the consensus NF-κB site competed protein binding to either labeled probe containing NF-κB sites 1 and 2 of the *Cdx-2* promoter (not shown).

In an attempt to obtain further insight into the stimulation of the p50/p50 complex by PTEN, we have compared by EMSA the NF-κB binding activity in whole-cell extracts and in nuclear extracts (Figure 7*C*). As shown previously in Figure 7*A*, EMSA with whole-cell extract showed increased NF-κB binding activity, particularly for the p50/p50 protein complex with AxCA-PTEN infection. Induction of NF-κB binding was likewise noted in the nuclear extract (Figure 7*C*). However, it is worth noting that Western blot analysis with the same whole-cell extracts showed that p50 protein levels were unchanged in control and AxCA-PTEN–infected cells (Figure 7*D*). These results suggest that PTEN induction of NF-κB binding activity is not due to increased expression of NF-κB p50 proteins.

These results show that the sites 1 and 2 of the Cdx-2 promoter preferentially bind to the p50/p50 NF-κB homodimer under control conditions and that the activation of the Cdx-2 promoter by PTEN- or wortmannin-dependent PI3K inhibition is associated with increased in vitro DNA binding activity of the p50/p50 homodimer to sites 1 and 2.

TNF-α inhibits Cdx-2 expression while stimulating the NF-κB p65 subunit. The previous results indicate that Cdx-2 is regulated by the PTEN/PI3K/Akt/NF-κB pathway. Because NF-κB is also a target of the proinflammatory cytokine TNF-α through PI3K and Akt,[28] we have investigated the effect of TNF-α on Cdx-2 expression in HT29 cells. We found that cell treatment with 1 nmol/L of TNF-α for 24 hours decreased the amount of Cdx-2 protein (Figure 8*A*). A similar effect was observed when cells were infected with the recombinant adenovirus encoding the NF-κB p65 subunit, whereas cell infection with the adenovirus together with TNF-α treatment completely abolished Cdx-2 expression. The cell extracts were then used for DNA binding activity analysis by EMSA using the oligonucleotide containing the consensus NF-κB binding site and the 2 oligonucleotides containing binding sites 1 and 2 of the Cdx-2 promoter (Figure 8*B*). As expected, 1 nmol/L of TNF-α treatment increased the DNA binding activity to the consensus NF-κB site with a predominant increase of the p65/p50 heterodimer. As shown previously (Figure 7), only the p50/p50 homodimer bound to sites 1 and 2 of the Cdx-2 promoter in control untreated cells. However, TNF-α treatment led to 2 retarded bands, one corresponding to the p50/p50 homodimer and the other one corresponding to the p65/p50 heterodimer. Therefore, unlike wortmannin treatment, which stimulated Cdx-2 expression and p50/p50 NF-κB DNA binding activity, TNF-α treatment decreased Cdx-2 expression while increasing p65/p50 NF-κB DNA binding activity.

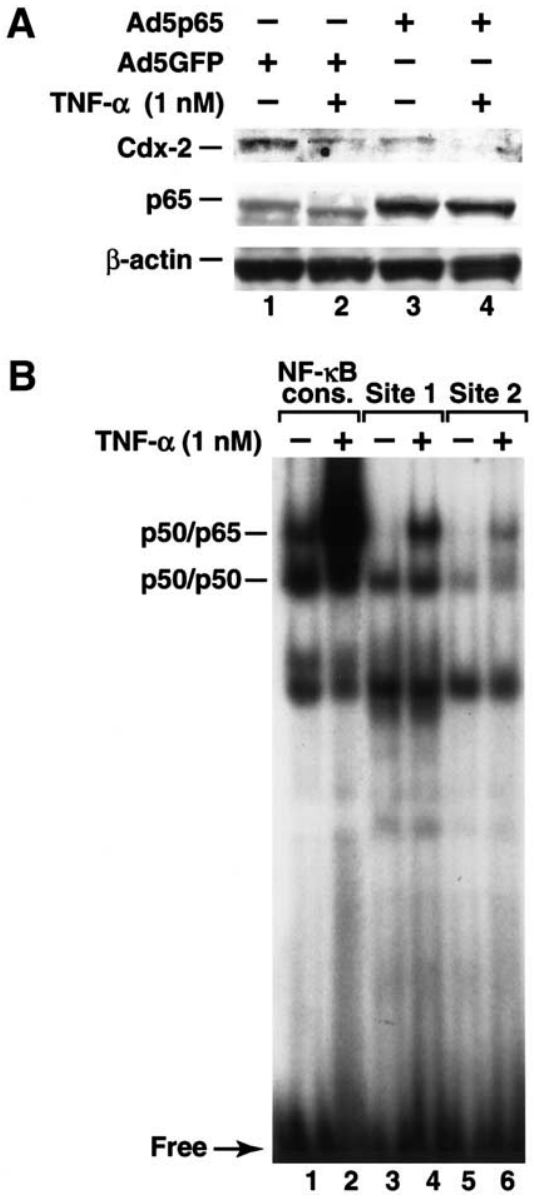

Figure 8. TNF-α and NF-κB subunit p65 reduces Cdx-2 expression. (*A*) HT29 cells were treated with 1 nmol/L of TNF-α for 24 hours, and the expression of Cdx-2 protein was analyzed by Western blot and compared with untreated cells (*lanes 1* and *2*). Alternatively, cells were infected either with the adenovirus encoding the p65 NF-κB subunit Ad5p65 or with the adenovirus encoding green fluorescent protein as control (compare *lanes 1* and *3*). In *lane 4*, cells were simultaneously treated with TNF-α and infected with Ad5p65. Overexpression of p65 in the infected cells was confirmed by immunodetection with anti-p65. β-Actin was used to normalize the amount of protein. (*B*) Nuclear extracts from untreated HT29 cells or cells treated for 24 hours with 1 nmol/L of TNF-α were used for EMSA with the oligonucleotides containing the consensus NF-κB binding site or binding sites 1 and 2 of the Cdx-2 promoter. The position of the p50/p50 homodimer and p65/p50 heterodimer is indicated.

These data prompted us to analyze the effect of p50 and p65 NF-κB subunits on the transcriptional activity

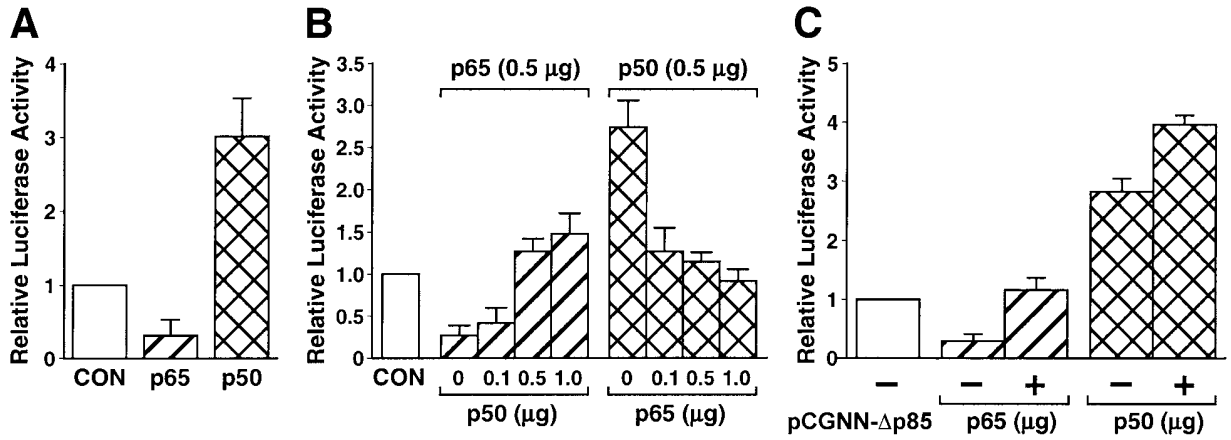

Figure 9. The p50 and p65 subunits of NF-κB have opposite effects on the Cdx-2 promoter. (*A*) HT29 cells were transfected with the reporter plasmid Δ-171 containing the Cdx-2 promoter, together with p50 NF-κB or p65 NF-κB. p50 stimulates the activity of the Cdx-2 promoter, whereas p65 has an inhibitory effect. (*B*) HT29 cells were co-transfected with Δ-171, 0.5 μg of p50 NF-κB, and increasing amounts of p65 NF-κB (0, 0.1, 0.5, and 1 μg). p65 reduced the stimulatory effect of p50. Inversely, cells were co-transfected with Δ-171 and 0.5 μg of p65 NF-κB and increasing amounts of p50 NF-κB (0, 0.1, 0.5, and 1 μg). p50 prevented the stimulatory effect of p65. (*C*) HT29 cells were transfected with Δ-171 and either 0.5 μg of p65 NF-κB plus 0.5 μg of pCGNN or 0.5 μg of p65 NF-κB plus 0.5 μg of pCGNN-Δp85. Inhibiting PI3K by the dominant-negative form of p85 prevented the inhibitory effect of p65 NF-κB on the Cdx-2 promoter. Inversely, HT29 cells were transfected with Δ-171 and either 0.5 μg of p50 NF-κB plus 0.5 μg of pCGNN or 0.5 μg of p50 NF-κB plus 0.5 μg of pCGNN-Δp85. Inhibiting PI3K by the dominant-negative form of p85 increased the stimulatory effect of p65 NF-κB on the Cdx-2 promoter. Data are expressed as mean ± SD.

of the Cdx-2 promoter. In cells transfected with the Δ-171 reporter, co-transfection with the plasmid encoding p50 led to a 3-fold stimulation of the transcriptional activity of the Cdx-2 promoter fragment, whereas co-transfection with the p65-encoding plasmid resulted in a 3-fold decrease of transcriptional activity (Figure 9*A*). Moreover, when the amount of p50-encoding plasmid was kept constant, transfection with increasing amounts of p65 NF-κB progressively inhibited the p50-evoked stimulatory effect, whereas the inhibitory effect of p65 NF-κB was prevented by co-transfection with increasing amounts of p50 NF-κB (Figure 9*B*). Because we showed previously that PI3K inhibition stimulated the activity of the p50 NF-κB subunit, we investigated the effect of PI3K inhibition on Cdx-2 changes caused by p65 and p50. For this purpose, HT29 cells were co-transfected with the Δ-171 Cdx-2 reporter plasmid and p65 NF-κB or p50 NF-κB and were additionally co-transfected with pCGNN-Δp85 to inhibit PI3K. Figure 9*C* shows that the inhibitory effect of p65 on the Δ-171 Cdx-2 promoter was prevented by co-transfection with pCGNN-Δp85, whereas pCGNN-Δp85 increased the stimulatory effect of p50. A similar effect was obtained when PI3K was inhibited by wortmannin instead of pCGNN-Δp85 (not shown).

Discussion

The mechanisms that regulate cell proliferation and differentiation in the continuously renewed intestinal epithelium are far from being elucidated. Recent studies using knockout mouse and cell transfection models have pointed out the role of the 2 intestinal-specific homeobox genes, *Cdx-1* and *Cdx-2*, in the control of intestinal homeostasis.[11] *Cdx-1*, which is expressed at the level of the proliferative cell compartment, stimulates cell growth and initiates cell differentiation,[41] whereas *Cdx-2*, which is predominantly active in the differentiated cell compartment, inhibits cell growth and stimulates cell differentiation and apoptosis.[20–22] The consequence of *Cdx-2* perturbation is highlighted by the heteroplastic hamartoma and adenoma formation found in the colons of $Cdx\text{-}2^{+/-}$ mice.[12,13] For this study, we were prompted by the fact that heterozygous mice deficient for 1 copy of the phosphatase-encoding tumor suppressor gene *PTEN* develop dysplastic/hyperplastic polyps in the colon, along with numerous disorders in several organs, consistent with the wide variety of malignancies reported in humans harboring *PTEN* mutations.[4] Here, we show that PTEN and Cdx-*2* proteins show a similar cell pattern in the murine colon and that Cdx-2 is decreased in relation to the decrease of PTEN in heterozygous $Pten^{+/-}$ mice, and we further provide evidence that PTEN positively regulates *Cdx-2* gene expression through its antagonist effect on the PI3K-PKB/Akt pathway, via 2 NF-κB binding sites located near the *Cdx-2* transcription start site. Thus, we surmise that many disorders resulting from *PTEN* haploinsufficiency are related, at

least in part, to the fact that *PTEN* regulates tissue-specific genes. This study also provides evidence for a link between TNF-α signaling and Cdx-2 expression through NF-κB.

When colonocytes migrate up the vertical crypt axis during the continuous renewal of the epithelium, they progressively lose their proliferative capacity, differentiate, and exfoliate into the intestinal lumen. The increasing expression of PTEN reported here along the crypt-to-cuff axis is consistent with the function attributed to this phosphatase as an inhibitor of cell growth[30] and as an antagonist of the PI3K-PKB/Akt pathway that promotes cell growth and survival.[9] In addition, the fact that *Cdx-2* expression is downregulated by the cell growth/survival PI3K-PKB/Akt pathway is in line with the antiproliferative, differentiating, and proapoptotic functions exerted by Cdx-2.[20–22] Also worth noting is the fact that *Cdx-2* is a target of extracellular signaling by basement membrane molecules, including laminins,[20] whereas another action of the *PTEN* pathway is directed toward integrin signaling via the focal adhesion kinase.[42] Taken together, our results suggest that the function of the PTEN/PI3K pathway in the regulation of intestinal homeostasis is mediated, at least in part, by the *Cdx-2* homeobox gene. *PTEN* may also participate in the regulation of *Cdx-2* during development, because both genes are expressed in a number of embryonic and extraembryonic tissues[43,44] and because early lethality occurs in homozygous *Cdx-2*$^{-/-}$ mutants, whereas *PTEN*-deficient embryonic stem cells fail to differentiate properly into the 3 germ layers.[4,45] Unlike with *Cdx-2*, we did not find any evidence that PTEN regulates the activity of the *Cdx-1* promoter (data not shown). Conversely, the fact that *Cdx-1*, but not *Cdx-2*, is targeted by Wnt/β-catenin/Tcf signaling[46] indicates that these 2 homeobox genes specifically respond to distinct regulatory pathways in the intestinal epithelium.

In this study, we report that PTEN and TNF-α regulate Cdx-2 by signaling pathways that target the transcription factors of the NF-κB family. This family comprises several members that interact as homodimers or heterodimers and function as key regulators of both developmental and pathologic processes.[47,48] It is interesting to note that the p65 and p50 NF-κB subunits show different patterns in the intestinal epithelium because p65 is restricted to the proliferative zone, whereas p50 is found in both proliferative and differentiation compartments.[10] Here we show that PI3K inhibition by PTEN stimulated the DNA binding activity of the p50/p50 homodimer and increased Cdx-2 expression, whereas TNF-α at the concentration of 1 nmol/L stimulated the DNA binding activity of the p65/p50 heterodimer and decreased Cdx-2 expression. Transfection assays further provide evidence for the opposing effects of NF-κB p50 and p65 subunits on the Cdx-2 promoter. Activation of NF-κB by a low concentration of TNF-α has already been shown to be mediated by both PI3K/Akt and NF-κB–inducing kinase pathways that converge on IκB kinase phosphorylation.[28] In HT29 cells, NF-κB–inducing kinase overexpression decreases Cdx-2 expression (unpublished data). Although a high concentration of TNF-α inhibits cell proliferation and activates the proapoptotic pathway, a low concentration stimulates cell proliferation.[49] This is consistent with our finding of reduced Cdx-2 expression by low TNF-α, because Cdx-2 inhibits cell growth. Although other reports indicate a stimulatory role of PI3K/Akt in different cell systems,[30,39] our data confirm our previous findings[39] and corroborate a recent report with a DNA microarray technique that showed that PTEN overexpression in lung cancer cell lines up-regulated NF-κB.[50] Although the mechanism of activation of NF-κB by PTEN is not elucidated, all the data shown here suggest that NF-κB regulation is more complex than already described. Complexity is also illustrated upstream to NF-κB by the fact that PTEN haploinsufficiency triggers colonic tumorigenesis,[4] whereas inactivation of the p110γ catalytic subunit of PI3K also promotes the development of colon adenocarcinoma in mice.[51] Thus, it suggests that relative levels of PI3K subunits may determine the overall influence of the PI3K effect within a cell and allow for fine-tuning the cellular effects exerted by PI3K.

In summary, this study shows that the dual-specificity phosphatase encoded by the *PTEN* tumor suppressor gene and the proinflammatory cytokine TNF-α have opposite effects on the intestinal *Cdx-2* homeobox gene through a balance between p50 and p65 NF-κB subunits. The contrasting effects of p50 and p65 on Cdx-2 expression are in agreement with the respective distribution of these NF-κB subunits along the proliferation–differentiation compartments,[10] as compared with Cdx-2. Because *Cdx-2* expression decreases in colorectal cancers in relation to the tumor grade, these results suggest that PI3K activation during tumor progression may participate in this decrease. The data also suggest that a modification of *Cdx-2* may occur during inflammatory bowel diseases because of the link between this homeobox gene and TNF-α.

References

1. Booth C, Potten CS. Gut instincts: thoughts on intestinal epithelial stem cells. J Clin Invest 2000;105:1493–1499.
2. Ali IU, Schriml LM, Dean M. Mutational spectra of PTEN/MMAC1

gene: a tumor suppressor with lipid phosphatase activity. J Natl Cancer Inst 1999;91:1922–1932.
3. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 1997;275:1943–1947.
4. Di Cristofano A, Pesce B, Cordon-Cardo C, Pandolfi PP. Pten is essential for embryonic development and tumour suppression. Nat Genet 1998;19:348–355.
5. Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, Parsons R. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet 1997;16:64–67.
6. Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH, Tavtigian SV. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 1997;15:356–362.
7. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-triphosphate. J Biol Chem 1998;273:13375–13378.
8. Vazquez F, Sellers WR. The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3-kinase signaling. Biochim Biophys Acta 2000;1470:M21–M35.
9. Wymann MP, Pirola L. Structure and function of phosphoinositide 3-kinases. Biochim Biophys Acta 1998;1436:127–150.
10. Inan MS, Tolmacheva V, Wang QS, Rosenberg DW, Giardina C. Transcription factor NF-κB participates in regulation of epithelial cell turnover in the colon. Am J Physiol Gastrointest Liver Physiol 2000;279:G1282–G1291.
11. Freund JN, Domon-Dell C, Kedinger M, Duluc I. The Cdx-1 and Cdx-2 homeobox genes in the intestine. Biochem Cell Biol 1998;76:957–969.
12. Beck F, Chawengsaksophak K, Waring P, Playford RJ, Furness JB. Reprogramming of intestinal cell differentiation and intercalaryregeneration in Cdx2 mutant mice. Proc Natl Acad Sci U S A 1999;96:7318–7323.
13. Tamai Y, Nakajima R, Ishikawa T, Takaku K, Seldin MF, Taketo MM. Colonic hamartoma development by anomalous duplication in Cdx2 knockout mice. Cancer Res 1999;59:2965–2970.
14. Silberg DG, Sullivan J, Kahn E, Swain GP, Moffett J, Sund NJ, Sackett SD, Kaestner KH. Cdx2 ectopic expression induces gastric intestinal metaplasia in transgenic mice. Gastroenterology 2002;122:689–696.
15. Duluc I, Lorentz O, Fritsch C, Leberquier C, Kedinger M, Freund JN. Changing intestinal connective tissue interactions alters homeobox gene expression in epithelial cells. J Cell Sci 1997;110:1317–1324.
16. James R, Erler T, Kazenwadel J. Structure of the murine homeobox gene cdx-2. Expression in embryonic and adult intestinal epithelium. J Biol Chem 1994;269:15229–15237.
17. Macdonald PM, Struhl G. A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. Nature 1986;324:537–545.
18. James R, Kazenwadel J. Homeobox gene expression in the intestinal epithelium of adult mice. J Biol Chem 1991;266:3246–3251.
19. Silberg DG, Swain GP, Suh ER, Traber PG. Cdx1 and cdx2 expression during intestinal development. Gastroenterology 2000;119:961–971.
20. Lorentz O, Duluc I, Arcangelis AD, Simon-Assmann P, Kedinger M, Freund JN. Key role of the Cdx2 homeobox gene in extracellular matrix-mediated intestinal cell differentiation. J Cell Biol 1997;139:1553–1565.
21. Mallo GV, Soubeyran P, Lissitzky JC, Andre F, Farnarier C, Marvaldi J, Dagorn JC, Iovanna JL. Expression of the Cdx1 and Cdx2 homeotic genes leads to reduced malignancy in colon cancer-derived cells. J Biol Chem 1998;273:14030–14036.
22. Suh E, Traber PG. An intestine-specific homeobox gene regulates proliferation and differentiation. Mol Cell Biol 1996;16:619–625.
23. Ee HC, Erler T, Bhathal PS, Young GP, James RJ. Cdx-2 homeodomain protein expression in human and rat colorectal adenoma and carcinoma. Am J Pathol 1995;147:586–592.
24. Mallo GV, Rechreche H, Frigerio JM, Rocha D, Zweibaum A, Lacasa M, Jordan BR, Dusetti NJ, Dagorn JC, Iovanna JL. Molecular cloning, sequencing and expression of the mRNA encoding human Cdx1 and Cdx2 homeobox. Down-regulation of Cdx1 and Cdx2 mRNA expression during colorectal carcinogenesis. Int J Cancer 1997;74:35–44.
25. Yagi OK, Akiyama Y, Yuasa Y. Genomic structure and alterations of homeobox gene CDX2 in colorectal carcinomas. Br J Cancer 1999;79:440–444.
26. Lorentz O, Cadoret A, Duluc I, Capeau J, Gespach C, Cherqui G, Freund JN. Downregulation of the colon tumor-suppressor homeobox gene Cdx-2 by oncogenic ras. Oncogene 1999;18:87–92.
27. Wang Q, Wang X, Hernandez A, Kim S, Evers BM. Inhibition of the phosphatidylinositol 3-kinase pathway contributes to HT29 and Caco-2 intestinal cell differentiation. Gastroenterology 2001;120:1381–1392.
28. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-κB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 1999;401:82–85.
29. Kimura K, Hattori S, Kabuyama Y, Shizawa Y, Takayanagi J, Nakamura S, Toki S, Matsuda Y, Onodera K, Fukui Y. Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. J Biol Chem 1994;269:18961–18967.
30. Sakurada A, Hamada H, Fukushige S, Yokoyama T, Yoshinaga K, Furukawa T, Sato S, Yajima A, Sato M, Fujimura S, Horii A. Adenovirus-mediated delivery of the PTEN gene inhibits cell growth by induction of apoptosis in endometrial cancer. Int J Oncol 1999;15:1069–1074.
31. Evers BM, Zhou Z, Celano P, Li J. The neurotensin gene is a downstream target for Ras activation. J Clin Invest 1995;95:2822–2830.
32. Han Y, Meng T, Murray NR, Fields AP, Brasier AR. Interleukin-1–induced nuclear factor-κB-IκBα autoregulatory feedback loop in hepatocytes. A role for protein kinase cα in post-transcriptional regulation of IκBα resynthesis. J Biol Chem 1999;274:939–947.
33. Evers BM, Wang X, Zhou Z, Townsend CM Jr, McNeil GP, Dobner PR. Characterization of promoter elements required for cell-specific expression of the neurotensin/neuromedin N gene in a human endocrine cell line. Mol Cell Biol 1995;15:3870–3881.
34. Cohen E, Ophir I, Shaul YB. Induced differentiation in HT29, a human colon adenocarcinoma cell line. J Cell Sci 1999;112:2657–2666.
35. Heerdt BG, Houston MA, Augenlicht LH. Potentiation by specific short-chain fatty acids of differentiation and apoptosis in human colonic carcinoma cell lines. Cancer Res 1994;54:3288–3293.
36. Taupin D, Podolsky DK. Mitogen-activated protein kinase activation regulates intestinal epithelial differentiation. Gastroenterology 1999;116:1072–1080.
37. Suh E, Chen L, Taylor J, Traber PG. A homeodomain protein related to caudal regulates intestine-specific gene transcription. Mol Cell Biol 1994;14:7340–7351.
38. Yano H, Nakanishi S, Kimura K, Hanai N, Saitoh Y, Fukui Y, Nonomura Y, Matsuda Y. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. J Biol Chem 1993;268:25846–25856.

39. Romashkova JA, Makarov SS. NF-κB is a target of AKT in anti-apoptotic PDGF signalling. Nature 1999;401:86–90.
40. Wang Q, Kim S, Wang X, Evers BM. Activation of NF-κB binding in HT-29 colon cancer cells by inhibition of phosphatidylinositol 3-kinase. Biochem Biophys Res Commun 2000;273:853–858.
41. Soubeyran P, Andre F, Lissitzky JC, Mallo GV, Moucadel V, Roccabianca M, Rechreche H, Marvaldi J, Dikic I, Dagorn JC, Iovanna JL. Cdx1 promotes differentiation in a rat intestinal epithelial cell line. Gastroenterology 1999;117:1326–1338.
42. Tamura M, Gu J, Matsumoto K, Aota S, Parsons R, Yamada KM. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science 1998;280:1614–1617.
43. Beck F, Erler T, Russell A, James R. Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. Dev Dyn 1995;204:219–227.
44. Luukko K, Ylikorkala A, Tiainen M, Makela TP. Expression of LKB1 and PTEN tumor suppressor genes during mouse embryonic development. Mech Dev 1999;83:187–190.
45. Chawengsaksophak K, James R, Hammond VE, Kontgen F, Beck F. Homeosis and intestinal tumours in Cdx2 mutant mice. Nature 1997;386:84–87.
46. Lickert H, Domon C, Huls G, Wehrle C, Duluc I, Clevers H, Meyer B, Freund JN, Kemler R. Wnt/β-catenin signaling regulates the expression of the homeobox gene Cdx1 in embryonic intestine. Development 2000;127:3805–3813.
47. Bushdid PB, Brantley DM, Yull FE, Blaeuer GL, Hoffman LH, Niswander L, Kerr LD. Inhibition of NF-κB activity results in disruption of the apical ectodermal ridge and aberrant limb morphogenesis. Nature 1998;392:615–618.
48. Kanegae Y, Tavares AT, Izpisua Belmonte JC, Verma IM. Role of Rel/NF-κB transcription factors during the outgrowth of the vertebrate limb. Nature 1998;392:611–614.
49. Kaiser GC, Polk BD. Tumor necrosis factor alpha regulates proliferation in a mouse intestinal cell line. Gastroenterology 1997; 112:1231–1240.
50. Hong TM, Yang PC, Peck K, Chen JJ, Yang SC, Chen YC, Wu CW. Profiling the downstream genes of tumor suppressor PTEN in lung cancer cells by complementary DNA microarray. Am J Respir Cell Mol Biol 2000;23:355–363.
51. Sasaki T, Irie-Sasaki J, Horie Y, Bachmaier K, Fata JE, Li M, Suzuki A, Bouchard D, Ho A, Redston M, Gallinger S, Khokha R, Mak TW, Hawkins PT, Stephens L, Scherer SW, Tsao M, Penninger JM. Colorectal carcinomas in mice lacking the catalytic subunit of PI(3)Kγ. Nature 2000;406:897–902.

Received December 20, 2001. Accepted July 11, 2002.

Address requests for reprints to: B. Mark Evers, M.D., Department of Surgery, The University of Texas Medical Branch, 301 University Boulevard, Galveston, Texas 77555-0536. e-mail: mevers@utmb.edu; fax: (409) 747-4819.

S.K. and C.D.-D. contributed equally to this work.

Supported by grants R01DK48498, R37AG10885, P01DK35608, and T32DK07639 from the National Institutes of Health; a grant of the Association pour la Recherche sur le Cancer; a pilot feasibility grant (P30 DK56338) from the Texas Gulf Coast Digestive Disease Center (to S.K.); and a fellowship of the Ligue Nationale Contre le Cancer (to C.D.).

The authors thank Dr. W. G. King (Harvard Medical School, Boston, Massachusetts) for plasmid pCGNN-Δp85, Dr. A. Horii (Tohoku University School of Medicine, Sendai, Japan) for the adenoviruses AxCA-PTEN and AxCA-lacZ, Dr. W. Ogawa (Kobe University School of Medicine, Kobe, Japan) for the adenoviruses AxCA-Akt-AA and AxCA-Myr-Akt, Dr. C. Regnier for the p50 NF-κB and p65 NF-κB plasmids (INSERM U255, Paris, France), and Dr. M. German (University of California, San Francisco, California) for the gift of anti–Cdx-2/3 antibody. We also thank Dr. Christian Jobin (University of North Carolina, Chapel Hill) for the adenoviruses Ad5GFP and Ad5p65 and for his thoughtful discussions and review of the manuscript; Tatsuo Uchida for statistical analysis; Jell Hsieh, Junghee Kang, Wanqin Hu, and Xiao-fu Wang for technical assistance; and Eileen Figueroa, Karen Martin, and Steve Schuenke for manuscript preparation.