Phosphatase and Tensin Homolog (PTEN) Represses Colon Cancer Progression through Inhibiting Paxillin Transcription via PI3K/AKT/NF-κB Pathway*

Received for publication, January 28, 2015, and in revised form, April 13, 2015. Published, JBC Papers in Press, April 14, 2015, DOI 10.1074/jbc.M115.641407

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The tumor suppressor gene phosphatase and tensin homolog (PTEN) is frequently mutated in colon cancer. However, the potential contribution of loss of PTEN to colon cancer progression remains unclear. In this study, we demonstrated that PTEN overexpression or knockdown in Lovo colon cancer cells decreased or increased paxillin expression, respectively. Moreover, paxillin reversed PTEN-mediated inhibition of Lovo cell invasion and migration. Overexpression of PTEN in an orthotopic colon cancer nude mice model inhibited tumor formation and progression. In addition, PTEN protein level was negatively correlated with that of paxillin in human colon cancer tissues. Mechanistically, we identified three NF-κB binding sites on paxillin promoter and confirmed that paxillin was a direct transcriptional target of NF-κB. Our findings reveal a novel mechanism by which PTEN inhibits the progression of colon cancer by inhibiting paxillin expression downstream of PI3K/AKT/NF-κB pathway. Thereby, PTEN/PI3K/AKT/NF-κB/paxillin signaling cascade is an attractive therapeutic target for colon cancer progression.

*1 This work was supported by the National Natural Science Foundation of China (Grant 81172350) and China and the Fundamental Research Funds for the Chinese Central Universities (Grant 201130202020016).
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§ The abbreviations used are: PTEN, phosphatase and tensin homolog; AKT, protein kinase B; NF-κB, nuclear factor B; PDTC, ammonium pyrrolidinedithiocarbamate; LUV, lentiviral.

Colon cancer is one of the most common malignant neoplasms in the developed world, and its metastasis leads to a high mortality rate of about 33% (1, 2). Recent evidence suggests that phosphatase and tensin homolog, deleted on chromosome 10 (PTEN), a major tumor suppressor, plays an important role in colon carcinogenesis (3, 4). PTEN is known to regulate cell apoptosis and cell survival through inhibiting oncogenic PI3K/AKT signaling pathway due to its lipid phosphatase activity (5, 6). The phosphoinositol lipids are crucially involved in cell adhesion and migration and tumor metastasis, and thus PTEN as a phosphatase for phosphoinositol lipids plays an important role in the regulation of these processes (7). In particular, PTEN was shown to dephosphorylate one of its substrate focal adhesion kinases to regulate cell migration. The dysregulation of PTEN and focal adhesion kinase is clearly implicated in many kinds of cancers (8). Paxillin is an important adaptor protein to recruit a variety of signaling proteins to plasma membranes including focal adhesion kinase. Paxillin is localized to focal adhesions through its LIM domains, where it provides a platform for protein tyrosine kinases such as focal adhesion kinase and SRC, which are activated due to cell adhesion or growth factor stimulation and then in turn activate downstream effectors to regulate cell motility implicated in tumor progression (9). In the clinical data, the high expression level of paxillin was associated with worse overall survival and lower rates of relapse-free survival in patients with oral cavity squamous cell carcinoma (10). Furthermore, a recent study identified paxillin as a novel interaction partner of PTEN, establishing the direct link between paxillin and PTEN (11). Although the individual roles of PTEN and paxillin in cancer progression are well documented, the functional significance of PTEN-paxillin interaction in cancer progression is still unclear.

In the present study, we aimed to investigate whether PTEN could regulate paxillin expression and to explore the underlying signaling mechanism. Our results showed that PTEN acted as a negative regulator of paxillin expression through inhibiting PI3K/AKT/NF-κB signaling cascade and inhibited colon cancer invasion.

Experimental Procedures

Tissue Specimens—The matched tissue samples of primary colon cancer and adjacent normal colon mucosa from the same patient were collected from 28 colon cancer patients who underwent surgical resection at the Renmin Hospital, Wuhan University (Wuhan, China) from 2010 to 2012. Written in-
formed consent was obtained from each patient. Adjacent normal colon mucosa specimens were obtained from sites 5–10 cm apart from the primary tumors. All tissues were snap-frozen in liquid nitrogen and stored at −80 °C. Each case was reviewed by two experienced histopathologists. All patients had received neither chemotherapy nor radiation therapy before tumor resection.

**Antibodies and Reagents**—Antibodies to β-actin, IßBα, phospho-AKT1(Ser-473), phospho-p65 phospho-p50, and phospho-IßBα were purchased from Santa Cruz Biotechnology (Dallas, TX). mAbs to AKT and PTEN were from Cell Signaling (Danvers, MA). Antibodies to Paxillin, p65, and p50 were from Millipore (Upstate Biotechnology). LY294002, PDTC, and MK2006 were from Sigma. Plasmids of mCherry and Paxillin were gifts from Prof. David D. Schlaepfer (University of California, San Diego). siRNA for human PTEN, p65 and p50, AKT, and control siRNA were from Santa Cruz Biotechnology. Plasmids of GFP, GFP-PTEN, PTEN-C124S, and PTEN-G129E were from Addgene.

**Cell Culture and Transfection**—The colon cancer cell lines Lovo, HT-19, and HCT-116 were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and cultured in 10% CO2 at 37 °C. Cells were seeded in 6-well plates and transfected with plasmids using Lipofectamine 2000 reagent (Invitrogen, Paisley, UK).

**Construction of Tissue Microarrays and Immunohistochemistry**—Colorectal cancer samples and the corresponding adjacent normal tissues and the metastatic lymph nodes (n = 49) were used for the construction of a tissue microarray (Shanghai Biochip Co., Ltd., Shanghai, China). The tissue microarray was stained by Paxillin antibody (Upstate Biotechnology) and PTEN antibody (Cell Signaling Technology), and scored independently by two pathologists. Immunohistochemical staining was performed with the Dako Envision Plus system (Dako, Carpentry, CA) according to the manufacturer’s instructions. The final score of each sample (negative or positive) was assessed by summarization of the results of the intensity and extent of staining. Intensity of staining was scored as 0 (negative), 1 (weak), or 2 (strong). The extent of staining was scored as 0 (negative), 1 (weak), or 2 (strong). The extent of staining was scored as 0 (negative), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). Each case was considered negative if the final score was 0 to 1 (−) or 2 to 3 (+) and positive if the final score was 4 to 5 (+) or 6 to 7 (++), respectively (12).

**Lentiviral Constructs**—Lentiviral vector encoding human PTEN cDNA was constructed by Shanghai GeneChem (Shanghai, China) and designated as LV-PTEN. The empty vector was used as negative control, designated as LV-control. Lovo cells were infected by lentivirus by using enhanced infection solution and cultured in DMEM medium containing 10% FBS.

**Generation, Selection, and Analysis of Transfectants**—Cells were transfected with the plasmids using Lipofectamine 2000 reagent (Invitrogen, Paisley, UK). 24 h after transfection, cells were placed into the selection medium containing 0.5 mg/ml G418 (Life Technologies). 14 days after selection, individual G418-resistant colonies were subcloned. Protein expression was analyzed by immunoblotting.

**Real-time PCR**—The total RNA was extracted by using TRIzol (Invitrogen) according to the manufacturer’s protocol and treated with RNase-free DNase (Promega) to eliminate genomic DNA contamination. cDNA was synthesized as described previously (13). mRNA level was quantified with the real-time PCR system: 1× of iQ SYBR Green supermix (Bio-Rad), 200 nmol/liter of each primer, and 2.5 μl of cDNA in a 25-μl reaction system. The primer sequences were as follows: human paxillin forward primer 5’-GGAGTCTACCCTCCTCACA-3’, reverse primer 5’-CCACTGTCTAAAGGGTCAAA-3’; human PTEN forward primer 5’-CGACGGAGAACAAGTTTCACT-3’, reverse primer 5’-AGGTTCCTCTGGTCCTGGTTG-3’; and β-actin forward primer 5’-CACCATGGAGGGCCGACTCATC-3’, reverse primer 5’-TAAAGACCTTATGCAACACAGT-3’. The reactions were repeated three times.

**Western Blot Analysis**—Cells or homogenized tissues were solubilized in ice-cold lysis buffer (1× PBS, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 100 μm sodium orthovanadate, 60 mg/ml aprotinin, 10 mg/ml trypsin inhibitor, and 10 μg/ml leupeptin), and 20 μg of protein was subjected to 10% SDS-polyacrylamide gel electrophoresis analysis. Immunoblot analysis was performed as described previously (13) with the following antibodies: anti-p65, anti-phosphorylated p65, anti-phosphorylated p50, anti-phosphorylated AKT, anti-IßB kinase α, anti-phosphorylated IßB kinase α, anti-β-actin, (Santa Cruz Biotechnology), anti-p50 (1:1000, Upstate Biotechnology), anti-AKT, anti-PTEN (1:1000, Cell Signaling), and anti-PAXILLIN (1:1000, Upstate Biotechnology).

**Migration and Invasion Assays**—The wound-healing cell migration assay was performed as described previously (14). Lovo cells were cultured in 6-well plates in complete medium and replaced with serum-free medium when cells reached a confluent monolayer and then coated with gelatin before wounding. The wound was made by scraping a conventional pipette tip across the monolayer. The migration of cells toward the well center was examined with a light microscope.

For Transwell invasion assays, 1 × 105 cells transfected with different plasmids were seeded in a Matrigel-coated chamber with 8.0-μm pores (BD Biosciences) in a 24-well plate. Then, cells were plated in the top chamber lined with a non-coated membrane. Three randomly selected fields were photographed, and the migrated cells were counted.

**Immunofluorescent Staining**—Preparation of samples for fluorescence microscopy was performed as described previously (15). Cells cultured on gelatin-coated glass coverslips were fixed with 3.7% formaldehyde in phosphate-buffered saline for 10 min at room temperature, permeabilized with 0.5% Triton X-100 for 5 min, and blocked with 5% bovine serum album in PBS for 1 h. Then, coverslips were incubated with primary antibody for paxillin (1:150, Upstate Biotechnology) at 4 °C overnight. Alexa Fluor 488-conjugated phallolidin (Molecular Probes, Cell Signaling Technology) was used to visualize F-actin. After incubation with primary antibody, cells were incubated with Alexa Fluor 488 goat anti-mouse IgG and Texas Red-conjugated anti-rabbit IgG. Cells were analyzed by confocal laser scanning microscopy (CLSM). Immunofluorescent images were analyzed with ImageJ.
Red-phalloidin secondary antibody (Cell Signaling Technology). The nuclei were stained with DAPI, and coverslips were then fixed on slides and imaged using an Olympus fluorescence microscope.

**Luciferase Reporter Assay**—The 1324-bp 5’-UTR of paxillin gene was cloned into the empty luciferase reporter vector pEZx-PG04 (GeneCopoeia, Rockville, MD), generating a wild-type paxillin UTR luciferase reporter construct pEZx-WT-UTR (P-1182, CCS-HPRM10632-PG04, GeneCopoeia). Truncations were made as follows: −982 to +141 bp (P-982, CCS-HPRM10632-PG04-1, GeneCopoeia), −700 to +141 bp (P-700, CCS-HPRM10632-PG04-2, GeneCopoeia), and −350 to +141 bp (P-350, CCS-HPRM10632-PG04-3, GeneCopoeia). Mutations in the 5’-UTR of paxillin were designed for three NF-κB binding sites of P-1182 and named as NF-κB up mutant (CCS-HPRM10632-PG04-4), NF-κB middle mutant (CCS-HPRM10632-PG04-5) and NF-κB down mutant (CCS-HPRM10632-PG04-6), respectively. All constructs were verified institutional animal care and use protocols. All animal procedures were performed with the ChIP assay kit (Millipore, Billerica, MA) following the manufacturer’s instructions. Cells were plated in a 100-mm culture dish, cultured for 24 h, fixed with formaldehyde, and then used to perform the ChIP experiment. After washing with PBS, the cells were resuspended in 300 μl of lysis buffer. DNA was sheared to small fragments by sonication and then incubated with protein G-agarose at 4 °C for 1 h. The recovered supernatants were incubated with p65 and p50 antibody (Upstate Biotechnology) or an isotype control IgG overnight. The immunoprecipitated DNA was retrieved from the beads by incubation with 1% SDS and a 1.1 M NaHCO₃ solution at 65 °C for 6 h, and then purified using a PCR purification kit (Qiagen). PCR was performed using primers (Table 2) to those NF-κB binding sites within the promoter region of paxillin.

**Orthotopic Colon Cancer in Mouse Model**—6-week-old female nude (nu/nu) mice (Beijing HFK Bioscience, Beijing, China) were housed in pathogen-free conditions under approved institutional animal care and use protocols. All animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR), Renmin Hospital of Wuhan University. Ten million Lovo cells infected with LV-PTEN or LV-control were mixed with growth factor-reduced Matrigel (BD Biosciences) in 50 μl and were inoculated into the distal posterior rectal wall as described previously (15). 8 weeks after the tumor cell inoculation, the peritoneal cavity was opened, tumors were resected and weighed, and peritoneal dissemination was evaluated by counting the number of tumor nodules in the mesenterium. The body organs were examined for metastasis and various tissues were prepared for standard histological examination. Survival Analysis—Colon cancer patients’ gene profiling data and clinical information (GSE17536 and GSE39582) were obtained from Gene Expression Omnibus (GO) site. The patients were divided into two groups according to their paxillin expression level (top 25%: high versus bottom 75%: low), and Kaplan-Meier analysis was conducted.

**Data Analysis**—For cell culture experiments, mean values and S.D. of at least triplicate experiments were calculated. Student’s t test was used to compare mean value differences.

### Table 1

| NF-κB probes used in EMSA assay | NF-κB probes used in EMSA assay |
|---------------------------------|---------------------------------|
| **NF-κB up**                    | Wild: 5'-TTTGGTCTGAGAATCCCTGTATGGTT-3' |
|                                 | 5'-AAACCATGAGGAAATCCAGGACAAA-3' |
|                                 | Mutant: 5'-TTTGGTCTGAGAATCCCTGTATGGTT-3' |
|                                 | 5'-AAACCATGAGGAAATCCAGGACAAA-3' |
| **NF-κB middle**                | Wild: 5'-TTTGGTCTGAGAATCCCTGTATGGTT-3' |
|                                 | 5'-CTCCTAGAGGTATTCTTCCCCCTGT-3' |
|                                 | Mutant: 5'-TTTGGTCTGAGAATCCCTGTATGGTT-3' |
|                                 | 5'-CTCCTAGAGGTATTCTTCCCCCTGT-3' |
| **NF-κB low**                   | Wild: 5'-CTACGGGTGTACCTTTCTTGATTT-3' |
|                                 | 5'-AATACAGAGAAAGTACCCAGTG-3' |
|                                 | Mutant: 5'-CTACGGGTGTACCTTTCTTGATTT-3' |
|                                 | 5'-AATACAGAGAAAGTACCCAGTG-3' |
| **NF-κB consensus**             | 5'-AGTGGAGGGAATTTCTCCACGGC-3' |
|                                 | 5'-GCCAGGAAATGCTCCAATCCT-3' |

### Table 2

| Paxillin primers used in ChIP assay |
|------------------------------------|
| NF-κB Forward: 5'-CCATCACCCGCCCCACG-3' |
| NF-κB Reverse: 5'-CCACCTCCCTAGACCTTT-3' |
| NF-κB middle Forward: 5'-CTCCCTGCTCCACCCTGCT-3' |
| NF-κB middle Reverse: 5'-CTAGAGGATCTCCCTACCTCCTG-3' |
| NF-κB low Forward: 5'-TACACAGAAAGATACCCAGTG-3' |
| NF-κB low Reverse: 5'-CCCAGGAGCTTGTGCCCCG-3' |
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PTEN Represses Paxillin Expression in Colon Cancer Cells through PI3K/AKT/NF-κB Pathway—Next we investigated the mechanism by which PTEN represses paxillin expression. We focused on PI3K/AKT/NF-κB pathway because NF-κB is an important transcription factor that regulates genes involved in cancer cell migration and invasion (18, 19). First, we employed the PI3K inhibitor LY294002 or the NF-κB inhibitor PDTC to treat Lovo cells transfected with PTEN siRNA or control siRNA. Real-time PCR analysis showed that PTEN knock-down-mediated up-regulation of paxillin mRNA expression was inhibited by both LY294002 and PDTC (Fig. 4A). To elucidate the regulatory function of AKT on NF-κB, we conducted Western blot analysis, and the results showed that the levels of phosphorylated NF-κB subunits p65 and p50 in Lovo cells were reduced after treating with AKT specific inhibitor MK2206 or AKT siRNA. Furthermore, Western blot analysis showed that paxillin protein level and phosphorylated p65 and p50 levels were decreased in cells treated with PDTC, but were increased in cells treated with PTEN siRNA (Fig. 4B). These data demonstrated that the inhibition of PI3K/AKT and NF-κB decreased the up-regulation of paxillin mRNA and protein expression upon loss of PTEN.

To confirm that NF-κB mediates the up-regulation of paxillin expression, we performed a ChIP assay and an EMSA assay. The results showed that overexpression of wild-type PTEN inhibited NF-κB binding activity on paxillin promoter (Fig. 4, C and D). Taken together, our data suggest that NF-κB is a potential regulator of paxillin transcription downstream of PTEN/PI3K/AKT pathway.

NF-κB Subunits Bind Paxillin Promoter and Regulate the Transcription of Paxillin in Lovo Cells—To provide further evidence that NF-κB regulates paxillin transcription, the 5’-flanking 1.3-kb region of human wild-type paxillin gene was cloned into a luciferase reporter. Three potential NF-κB binding sites were identified in the promoter region of paxillin gene by TFASARCH program as follows: NF-κB-up: from −982 to −971; NF-κB-middle: from −738 to −729; and NF-κB-down: from −619 to −610 (Fig. 5A). Therefore, we made four deletion luciferase reporter constructs (Fig. 5B), which were co-transfected with control siRNA, p65 siRNA, p50 siRNA, or PTEN siRNA into Lovo cells. Luciferase assay showed that paxillin promoter activity was significantly decreased in cells treated with p65 siRNA or p50 siRNA, but was increased in cells treated with PTEN siRNA (Fig. 5C, left panel). Consistently, overexpression of PTEN significantly inhibited paxillin promoter activity (Fig. 5C, right panel). As the negative control, the luciferase activity was not significantly changed in cells transfected with P-350 construct reporter containing no NF-κB binding site. Next we made site-directed mutagenesis of three NF-κB binding sites in paxillin promoter. Luciferase assay showed that mutation of any one of NF-κB binding sites significantly decreased the luciferase activity of paxillin promoter (Fig. 5D).

To further analyze NF-κB binding to paxillin promoter, we performed EMSA with oligonucleotides containing the three binding sites (NF-κB-up, NF-κB-middle, and NF-κB-down) and oligonucleotides containing point mutations of the sites. We observed that NF-κB effectively bound to wild-type oligo-

Results

PTEN Represses Paxillin Expression in Colon Cancer Cells in a Phosphatase Activity-dependent Manner—To determine whether PTEN could regulate paxillin expression, we established stable Lovo colon cancer cell lines that were transfected with GFP-PTEN (wild type), GFP-PTEN-G129E (a protein phosphatase-active, lipid phosphatase-inactive mutant of PTEN), GFP-PTEN-C124S (a protein phosphatase-inactive mutant of PTEN) (16), or GFP as control. After selection with puromycin for 30 days, cells were analyzed for PTEN and paxillin expression by real-time PCR and Western blot analysis. The results showed that paxillin expression at both mRNA and protein levels was significantly down-regulated in Lovo cells transfected with GFP-PTEN exhibited reduced cell invasion, compared with those transfected with wild-type PTEN expression vector but not in cells transfected with mutant PTEN expression vector (G129E or C124S) (Fig. 1A). Furthermore, Lovo cells were transfected with PTEN siRNA or control siRNA. The results showed that paxillin expression was significantly up-regulated in cells transfected with PTEN siRNA, when compared with those transfected with control siRNA (Fig. 1B). Similar findings were made in two other colon cancer cell lines (HT-29 and HCT-116) (Fig. 1C). Collectively, these data suggest that PTEN acts as a negative regulator of paxillin expression in a phosphatase activity-dependent manner.

Paxillin Reverses PTEN-mediated Inhibition of Colon Cancer Cell Invasion and Migration—Next we examined the functional significance of the down-regulation of paxillin by PTEN. By Transwell invasion assay, we found that Lovo cells transiently transfected with GFP-PTEN exhibited reduced cell invasion, when compared with those transfected with GFP. However, co-transfection of paxillin significantly relieved the inhibition of Lovo cancer cell invasion by PTEN (Fig. 2A). Furthermore, by wound healing assay, we found that Lovo cells transiently transfected with GFP-PTEN exhibited reduced cell migration, when compared with those transfected with GFP. Similarly, co-transfection of paxillin significantly relieved the inhibition of Lovo cancer cell migration by PTEN (Fig. 2B). These data demonstrate that PTEN and paxillin have antagonistic roles in the regulation of colon cancer cell invasion and migration.

PTEN Inhibits the Formation of Paxillin Focal Adhesions in Colon Cancer Cells—Protrusion of the plasma membrane at the leading edge is considered to be the first step of cell migration (17). To provide direct evidence for the inhibitory effect of PTEN on colon cancer cell migration, levels of paxillin were visualized in Lovo cells. Immunofluorescent staining showed that the intensity and number of focal adhesions of paxillin were lower in cells transfected with GFP-PTEN than in those transfected with mutant PTEN and GFP control (Fig. 3A). This result suggests that PTEN reduces the number and size of the membrane protrusions in Lovo cells by inhibiting paxillin’s focal adhesions location.

Differences between pairs of data were determined using an unpaired two-tailed Student’s t test. Statistical analyses were performed using GraphPad Prism (version 5.0b). p < 0.05 was considered significantly different.
nucleotides but not to those with mutations. In addition, both p65 and p50 antibodies induced supershift of the probes in LoVo cells (Fig. 5E). To expand this observation, we performed a ChIP assay and found that either p65 or p50 could bind to the three NF-κB binding sites in paxillin promoter region (Fig. 5F). Collectively, these results showed that NF-κB subunits p65 and
p50 bind paxillin promoter and regulate the transcription of paxillin in Lovo cells.

**PTEN Inhibits Orthotopic Tumor in Nude Mice, and PTEN Expression Level Is Negatively Correlated with Paxillin in Human Colon Cancer Tissues**—Lovo cells infected with PTEN lentivirus or control vector were injected into the distal posterior rectum of nude mice and allowed to grow as orthotopic tumors. After 8 weeks, the peritoneal cavity was opened, and the tumors were visualized prior to and after surgical resection. We found that primary tumors and metastatic nodules of peritoneum were fewer and smaller in nude mice injected with cells overexpressing PTEN than in those injected with control cells (Fig. 6A). Immunohistochemistry analysis showed that paxillin expression levels were significantly inhibited in tumors overexpressing PTEN, when compared with control tumors (Fig. 6B).

To further confirm the in vivo results, we examined the expression of PTEN and paxillin in human colon cancer tissues. Immunohistochemistry analysis showed that PTEN and paxillin expression levels were negatively correlated in matched samples of human colorectal cancer and adjacent normal mucosa. Paxillin staining was markedly increased, whereas PTEN staining was decreased in normal mucosa specimens, when compared with matched primary adenocarcinoma specimens (Fig. 6C). Western blot analysis of 28 paired colon cancer samples showed that PTEN protein was down-regulated, whereas paxillin protein was up-regulated in colon cancer tissues, when compared with the corresponding normal samples (Fig. 6D). These data demonstrate that PTEN expression level is negatively correlated with that of paxillin in human colon cancer tissues.

To confirm the correlation between the paxillin and clinical prognosis, we downloaded and analyzed the data of two inde-
pendent colon cancer cohorts (GSE17536 and GSE39582) from GEO datasets. The Kaplan-Meier plot demonstrated that higher paxillin expression was associated with shorter disease-specific survival and lower rates of recurrence-free survival of colon cancer (Fig. 6, E and F), implying that paxillin functioned in colon cancer progression. From these results above, we conclude that paxillin promotes colon cancer progression, at least partly modulated by PTEN/PI3K/AKT/NF-κB signaling.

Discussion

PTEN has been considered as a tumor suppressor (20–22). However, the mechanism by which PTEN suppresses tumor
FIGURE 4. PTEN-induced suppression of paxillin expression is mediated by PI3K/NF-κB pathway. A, Lovo cells were transfected with control siRNA or PTEN siRNA and treated with 10 μM NF-κB inhibitor PDTC or with 10 μM PI3K inhibitor LY294002 for 12 h, respectively. The mRNA levels of PTEN and paxillin were detected by real-time PCR. B, Lovo cells were transfected with control siRNA or PTEN siRNA or treated with 10 μM NF-κB inhibitor PDTC or treated with AKT siRNA or treated with 0.5 μM AKT inhibitor MK2206 for 24 h, and the indicated protein levels were detected by Western blot analysis. p, phospho form. C and D, cells were transfected with GFP, GFP-PTEN, GFP-PTEN-C124S, and GFP-PTEN-G129E, respectively. The DNA binding capability of NF-κB on paxillin promoter was detected by ChIP (C) and EMSA (D). Values are the means ± S.D. from 3 different experiments. *, p < 0.05, **, p < 0.01.
progression remains largely unknown. In this study, we provide new evidence that PTEN inhibits colon cancer cell invasion and migration by suppressing paxillin expression through PI3K/AKT/NF-κB pathway.

Previous studies have shown that PTEN hydrolyzes the 3-phosphate of inositol 1,4,5-trisphosphate (PIP3) to generate phosphatidylinositol 4,5-bisphosphate (PIP2), and thereby negatively regulates inositol 1,4,5-trisphosphate-mediated down-

FIGURE 5. NF-κB subunits p65 and p50 bind paxillin promoter and regulate the transcription of paxillin. A, the human paxillin promoter sequence. B, a schematic diagram of reporter constructs with different deletions. C, bar graphs representing the relative luciferase activity in each of the transfected samples (n = 3). D, bar graphs representing the relative luciferase activity in Lovo cells transfected with reporter constructs harboring different mutations in NF-κB binding sites in the paxillin promoter (n = 3). E, EMSA assay. F, ChIP assay showing that NF-κB subunits p65 and p50 bound to the three NF-κB binding sites of paxillin promoter in Lovo cells. Values are the means ± S.D. from 3 independent experiments. *, p < 0.05, **, p < 0.01.
stream signaling. PI3K and its product inositol 1,4,5-trisphosphate, mediated by PTEN, regulate a variety of cellular processes such as cell survival, proliferation, migration, transcriptional regulation, and protein synthesis. In this study, we found that the overexpression of wild-type PTEN but not mutant PTEN (either G129E or C124S) inhibited paxillin expression at both mRNA and protein levels. Thus, our study reveals a novel mechanism by which PTEN regulates tumor cell invasion via transcriptional regulation of paxillin, a well-known regulator of tumor metastasis. It is well known that the loss of PTEN contributes to the activation of the PI3K/AKT signaling pathway and consequent tumorigenesis (23). In addition, the relevance of PTEN/PI3K/AKT pathway to focal adhesions has traditionally been thought to be via the dephosphorylation of focal adhesion proteins such as focal adhesion kinase (24, 25).

Through its role in phosphatidylinositol homeostasis, PTEN could regulate cell polarity and migration, thereby providing a potential link between cell membrane phospholipids and cytoskeletal reorganization (26). Previous studies have shown that paxillin activation is mediated by integrin β1 via AKT pathway (27–29). Here we reported the new findings that paxillin expression was blocked by the inhibition of NF-κB activity or PI3K pathway; thus we speculated that PTEN represses paxillin transcription via inhibiting NF-κB downstream of PI3K/AKT pathway.

To prove our hypothesis, we analyzed paxillin promoter sequence and identified three putative NF-κB binding motifs in the promoter region. By dual-luciferase assay, we demonstrated that fragments containing these motifs could induce luciferase activity, whereas mutation of these motifs abrogated luciferase activity in the presence of PI3K inhibitors.

**FIGURE 6.** PTEN inhibits orthotopic tumor in nude mice, and its expression is negatively associated with paxillin expression in human colon cancer tissues. A, the weight of primary tumor and the number of peritoneal lymph nodes metastasis were lower in the LV-PTEN group than in the LV-control group. B, immunohistochemistry staining and Western blot of PTEN and paxillin in dissected primary tumors from the representative mice orthotopically inoculated with LV-PTEN cells or with LV-control (original magnification ×100). C, immunohistochemical staining patterns of PTEN and paxillin in a matched sample of human colon cancer and adjacent normal mucosa. D, Western blot analysis of PTEN and paxillin protein expression in matched samples. p, phospho form. *, p < 0.05. E, high expression of paxillin (POX) reduces disease-specific survival of colon cancer patients in data set GSE17536. F, high expression of paxillin reduces recurrence-specific survival of colon cancer patients in data set GSE39582. The p values in the graphs were obtained from a log-rank test. Values are the means ± S.D. from 3 independent experiments.
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activity. ChIP and EMSA assays further confirmed that NF-κB subunits p65 and p50 both bound to paxillin promoter in Lovo cells. Taken together, these data indicate that NF-κB is a new transcription factor that regulates paxillin expression. Paxillin overexpression has been reported recently in several types of cancer including colon cancer, but the role of NF-κB in the regulation of paxillin overexpression remains elusive (30, 31). To our knowledge, this is the first study that identified paxillin as a direct downstream target of NF-κB.

To investigate the significance of down-regulation of paxillin expression by PTEN in colon carcinogenesis, first we made an orthotopic colon tumor model in nude mice and found that PTEN overexpression inhibited primary tumors and metastatic nodules of peritoneum in nude mice, and this was correlated with decreased paxillin expression level in the tumors. These data suggest that PTEN inhibits colon cancer development and progression by inhibiting paxillin expression. Next we examined the expression of PTEN and paxillin in human colon cancer tissues by immunohistochemical and Western blot analysis, and the results showed that PTEN protein was down-regulated, whereas paxillin protein was up-regulated in colon cancer tissues, when compared with the corresponding normal samples. These data confirm that the negative correlation between PTEN and paxillin expression contributes to colon tumorigenesis. Indeed, a recent study reported that the expression of PTEN was down-regulated, whereas the expression of paxillin was up-regulated in cervical cancer (32).

In summary, our present study demonstrated that the down-regulation of paxillin expression by NF-κB is abrogated upon PTEN overexpression in colon cancer. More specifically, NF-κB is directly involved in transcription regulation of paxillin. Our findings reveal a novel mechanism by which PTEN inhibits the progression of colon cancer by inhibiting paxillin expression downstream of PI3K/AKT/NF-κB pathway. PTEN mutation is prevalent in colon cancer, and consequently, loss of PTEN function leads to the activation of PI3K/AKT/NF-κB pathway. The activated NF-κB then drives the transcription of paxillin to promote cell invasion and migration, leading to colon cancer progression. Thereby, PTEN/PI3K/AKT/NF-κB/paxillin signaling cascade is an attractive therapeutic target for colon cancer progression.

Acknowledgments—We thank Prof. David D. Schlaepfer for the generous gifts of plasmids (mCherry and paxillin) and Hong Xiu for excellent technical assistance in this work.

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