The persistent activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is oncogenic and involved in colorectal neoplasia. Mutations of both regulatory subunit and catalytic subunit of PI3K have been demonstrated in colon cancers. In the present study, we show that heterozygous disruption of the phosphatase and tensin homolog (PTEN) tumor suppressor gene promoted tumor progression in APC<sup>min/+</sup> mice. Number and size of intestinal tumors were significantly increased in mice bearing both adenomatous polyposis coli (APC) and PTEN mutations. While APC<sup>min/+</sup> PTEN<sup>+/+</sup> mice developed adenomas, invasive carcinomas developed in APC<sup>min/+</sup> PTEN<sup>−/+</sup> mice. Large tumors often resulted in intestinal intussusception and early death of APC<sup>min/+</sup> PTEN<sup>−/+</sup> mice. Targeted array revealed that osteopontin (OPN) was the leading gene whose expression was strongly induced by deficiency of PTEN. In colon cancer cells, gain-of-function mutation of PI3K robustly increased levels of OPN and treatment with OPN reduced growth factor deprivation-induced programmed cell death. Moreover, OPN expression was strongly increased in Ras-induced transformation of intestinal epithelial cells in a PI3K-dependent manner. Inhibition of OPN expression by specific small interfering RNA reduced uncontrolled growth and invasiveness of Ras-transformed intestinal epithelial cells. Thus, our results suggest that the PI3K pathway promotes the transformation of intestinal adenoma to adenocarcinoma. OPN, a downstream effector of PI3K, promotes transformed intestinal epithelial cells from programmed cell death and stimulates their anchorage-independent growth.

Introduction

Colorectal cancer is the second leading cause of cancer-related death in USA. While it is clear that (APC) mutation is an initiating factor in ~85% of colorectal carcinomas, a number of other pathways play critical roles during the adenoma to carcinoma sequence of events involved in the neoplastic transformation of colon epithelium (1); one of these is the PI3K/Akt pathway (2). A number of components of the PI3K pathway are genetically altered in human colon cancers. Somatic mutations of the p53 gene regulatory subunit of the PI3K<sub>a</sub> have been demonstrated in human colon cancer, which lead to constitutive activation of PI3K (3). PIK3CA, which encodes the p110α catalytic subunit, is mutated in ~32% colon cancers, which often cluster in the helical and catalytic domains (4). Studies of PIK3CA mutants suggest that mutations in both helical and catalytic domains increase the lipid kinase activity of the PIK3CA, thereby enhancing its pro-neoplastic actions (5,6). Functionally, activation of PI3K promotes a wide range of cellular processes associated with malignant behavior of cancers. PI3K activity is critical for proliferation of transformed and non-transformed intestinal epithelial cells (7,8). Colon cancer cells that contain mutant PIK3CA possess significant advantage in cell growth and invasion (5). Activation of the PI3K/Akt pathway is important for Ras transformation of mammalian cells and essential in Ras-induced cytoskeletal re-organization (9). We have demonstrated previously the critical role of the PI3K/Akt pathway in Ras-mediated transformation of intestinal epithelial cells (7).

The PI3K/Akt-signaling pathway transmits signals from tyrosine kinase-coupled receptors and plays critical roles in mitogenic signaling (10). Class Ia PI3Ks (PI3K<sub>a</sub>, PI3K<sub>b</sub> and PI3K<sub>d</sub>) consist of an 85 kDa regulatory subunit and a catalytic 110 kDa subunit (p110α, p110β and p110δ). The PI3K catalytic subunit phosphorylates the D3 hydroxyl group of phosphoinositides; products include phosphatidylinositol-3,4,5-P<sub>3</sub> (PtdIns-3,4,5-P<sub>3</sub>) and PtdIns-3,4-P<sub>2</sub>, which then phosphorylate and activate Akt kinase. The serine/threonine kinase, Akt, regulates gene transcription by direct phosphorylation of some of the forkhead transcription factors, such as FKHR, FKHLRL and AFX (11–13) or indirectly by modifying the cAMP-responsive element-binding protein (14,15), E2F (16) or nuclear factor-kB (17). On the other hand, the tumor suppressor PTEN (phosphatase and tensin homolog) dephosphorylates the 3rd position of both PtdIns-3,4,5-P<sub>3</sub> and PtdIns-3,4-P<sub>2</sub>, reverses the reaction catalyzed by PI3K and restrains the activation of Akt (18,19). PTEN is often deleted or mutated in a variety of human tumor types (20,21). Inactivation of the PTEN in mouse models, results in development of a range of tumors, including prostate and breast cancer, as well as hyperplasia of the lymphoid, gynecological and gastrointestinal mucosa (22).

In the present study, we attempted to elucidate the role of the PI3K pathway in malignant transformation of intestinal epithelium initiated by APC mutations in an animal model. We cross-bred PTEN<sup>−/+</sup> mice with APC<sup>min/+</sup> mice. PTEN deficiency significantly promoted tumor development, stimulating tumor growth and invasion. Intestinal obstruction resulting from intussusception was the major cause for the early death of APC<sup>min/+</sup> PTEN<sup>−/+</sup> mice. The observations from animal studies led us to compare the gene expression profile between APC<sup>min/+</sup> PTEN<sup>−/+</sup> and APC<sup>min/+</sup> PTEN<sup>−/−</sup> tumors. Focused oligo array determined that osteopontin (OPN) was the leading gene whose expression was induced by heterozygous mutation of PTEN. Furthermore, we found that OPN was significantly increased in transformed intestinal epithelial cells in a PI3K-dependent manner and played crucial roles in the growth of transformed intestinal epithelial cells.

Materials and methods

Animal experiments

All animals were treated in a manner which complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the study was approved by the Institutional Animal Care and Use Committee of Indiana University. APC<sup>min/+</sup> mice on C57BL/6d background were purchased from Jackson Laboratory (Bar Harbor, ME). PTEN<sup>−/+</sup> mice on 129/C57/BL6 background were kindly provided by Dr Pier Paolo Pandolfi (Memorial Sloan-Kettering Cancer Center, New York, NY) (23). Mice were housed in an animal-holding room under controlled light, temperature and humidity.

Cell culture and reagents

Human colon cancer HCT116 cells containing wild-type (HCT116/PI3K WT) or mutated (HCT116/PI3K MU) PIK3CA were provided by Drs Yarden Samuels and Bert Vogelstein (The Johns Hopkins University Medical Institutions, Baltimore, MD) (5). The rat RIE-iHa-Ras cell line with an inducible Ha-RasV12 cDNA was generated by using LacSwitch eukaryotic expression system (Stratagene, La Jolla, CA) and grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (7.8). The mutated Ras cDNA is under the transcriptional control of the Lac operon. IPTG (isopropyl-1-thio-β-D-galactopyranoside; Gibco BRL, Gaithersburg, MD) at a concentration of 5 mM was used to induce the expression of mutated Ha-Ras. LY 294002 and

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl-1-thio-β-D-galactopyranoside; mRNA, messenger RNA; OPN, osteopontin; RT–PCR, reverse transcription–polymerase chain reaction; siRNA, small interfering RNA.
PD-98059 were purchased from Calbiochem (San Diego, CA). Human OPN was purchased from R&D Systems (Minneapolis, MN).

Cancer PathwayFinder Oligo GEArray
To determine the relative expression levels of cancer-related genes, Mouse Cancer PathwayFinder Oligo GEArray (SuperArray Bioscience Corporation, Frederick, MD) was carried out that profiles the expression of 113 genes representative of six biological pathways involved in transformation and tumorigenesis. Biotin-labeled probe was synthesized from total RNA and hybridized with a nylon membrane printed with proprietary probes. The array image was captured with chemiluminescence detection and analyzed using the software of GEArray Expression Analysis Suite.

Reverse transcription–polymerase chain reaction
Levels of OPN in mouse tumors and rat intestinal epithelial cells were determined using reverse transcription–polymerase chain reaction (RT–PCR) as described previously (24). Primer set for mouse OPN was based on mouse OPN messenger RNA (mRNA) sequence (NM_009263) including forward primer, AAAGTGTAGTATTCTGGACG and reverse primer, GGAAA-CTCCTCAGATTTCGG. PCR products were 421 bp in length. The primers used to amplify 295 bp of the rat OPN (NM_012881) include forward primer, ATAGGCTATCAAGGTATCC and reverse primer, TGTGAAACTCG-TGGCTCT GAT. RT–PCR was carried out using ProStar RT–PCR system (Strategene) according to the manufacturer’s instructions.

Real-time RT–PCR
OPN expression was quantified using real-time quantitative PCR (Applied Biosystems, Foster City, CA). The sequence of the primer/probe set was based on rat OPN mRNA sequence (GeneBank NM_012881) and includes forward primer, CCGCCTGTCTATGCGATTCGCG, and reverse primer, TGTGA-AACTCTGGCTCTCGATG. An 18S ribosomal RNA TaqMan® assay reagent was used for internal control. One-step RT–PCR was performed with 10 ng RNA for both target gene and endogenous controls. Duplicate Ct values were analyzed in Microsoft Excel using the comparative Ct (ΔΔCt) method as described by the manufacturer (Applied Biosystems). The expression of OPN was normalized to the levels of 18S and relative to the OPN levels in non-transformed RIE-i-Ha-Ras cells.

Immunoblot analysis
Immunoblot analysis was performed as described previously (25). Anti-OPN antibodies were purchased from Developmental Studies Hybridoma Bank (Iowa, IA).

Immunohistochemistry
Tumors were fixed in 10% formalin, paraffin-embedded and sectioned. Endogenous peroxidase activity was quenched by incubating the sections in 0.3% hydrogen peroxide for 20 min at room temperature. After blocking in 1.5% normal serum in phosphate-buffered saline for 1 h, 2-opN antibody (Developmental Studies Hybridoma Bank, 1:200) or mouse pre-immune IgG (negative control) was added to the sections and incubated overnight at 4 °C. The sections were then incubated with biotinylated secondary antibody and ABC-reverse primer, TGTGAAACTCG-TGGCTCT GAT. RT–PCR was carried out using ProStar RT–PCR system (Strategene) according to the manufacturer’s instructions.

Enzyme-linked immunosorbent assay
Levels of human or rat OPN protein in cell culture media were quantified using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems). Cells (5 × 10⁵) were seeded in 60 mm plates and conditioned media were collected at the indicated time points for ELISA assays. Cells were then lysed in lysis buffer; protein concentrations were determined by Bio-Rad protein assay and used to standardize the levels of OPN in conditioned media. The results were expressed as the amount of OPN in conditioned media per milligram of cellular protein.

Cellular DNA fragmentation
DNA fragmentation was determined by either electrophoresis or ELISA. HCT116 cells were grown to confluence in 60 mm plates. After a 48 h serum deprivation, floating and attached cells were collected and lysed in lysis buffer (1% NP-40 in 20 mM ethylenediaminetetraacetic acid and 50 mM Tris, pH 7.5). The supernatant containing fragmented DNA was clarified by centrifugation for 5 min at 1600g. The cell lysates were treated with a solution containing RNase A (5 mg/ml) and protease K (2.5 mg/ml). The DNA was then separated on 1.6% agarose gels. For ELISA assay, Cellular DNA Fragmentation ELISA kit from Roche (Mannheim, Germany) was used according to the manufacturer’s instruction. Cells were seeded in 96-well plates and labeled with BrdU. BrdU-labeled DNA fragments in cytosolic fraction were detected by ELISA.

RNA interference
OPN-specific small interfering RNA (siRNA) sequences were purchased from Ambion, Austin, TX (GeneBank accession number NM_012881, siRNA ID 197203 and 197204). Transfection was accomplished using X-tremeGene transfection reagent (Roche) according to the supplier’s instruction. Conditioned media were collected 24 h after transfection and levels of OPN were determined by ELISA assays.

Anchorage-independent growth assay
RIE-i-Ha-Ras cells were placed in 24-well plates and transfected with siRNA. After cells were trypsinized, 1 × 10⁴ cells were mixed with Sea Plaque Agarose (Hoeffer Scientific Instrument, San Francisco, CA) at a final concentration of 0.4% in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cell mixtures were then placed on top of a 0.8% agarose layer in 35 mm plates. One milliliter of cover medium containing IPTG was added after the gel was polymerized. Colonies were photographed after a 10 day incubation using a computerized digital camera. The size of colonies was measured manually on a computer screen.

Data analysis
All statistical analyses were performed on a personal computer with the StatView 5.0.1 software (SAS Institute, Cary, NC). Analyses between two groups were determined using the unpaired Student’s t-test. Differences with a P value of <0.05 were considered as statistically significant.

Results
PTEN deficiency stimulated tumor progression in APCmin/þ mice
The APCmin/þ mouse serves as a murine model for familial adenomatous polyposis (26). Mice heterozygous for PTEN (PTEN +/-/ mice), which have increased PI3K/Akt activity, spontaneously develop malignant tumors in a variety of organs (23,27). In order to determine whether elevated PI3K activity stimulated tumor progression in APCmin/þ mice, we cross-bred PTEN-deficient mice with APCmin/þ mice to generate double-heterozygous offspring. To control for the variation of genetic backgrounds, littersmates were used for this study. Since most of animals carrying both APC and PTEN mutations died within 5 months, we killed all F1 mice at age between 4 and 4.5 months. The results of tumor growth in mouse intestine were summarized in Table I. There was no intestinal tumor in both APC+/+/PTEN+/+/ and APC+/−/PTEN+/−/ mice at this age. While approximately six tumors were found in the gut of APCmin/þ/PTEN+/−/ mice, on average, 22 tumors were observed in the intestine of APCmin/þ/PTEN+/−/ littersmates. The size of tumors in APCmin/þ/PTEN+/−/ mice were significantly larger than those in APCmin/þ/PTEN+/−/ mice (P < 0.0001) (Figure 1A).

Histologic analysis showed that adenomas of APCmin/þ/PTEN+/−/ mouse intestine were similar to APCmin/þ tumors. The irregular crypts comprising the adenoma are lined by columnar cells that are larger than normal intestinal epithelial cells, with increased nuclear to cytoplasmic ratio (Figure 1B, left panel). Most adenomas show changes of low-grade dysplasia; in more advanced lesions with high-grade dysplasia, complex crypt outlines with cribriform glands and loss of nuclear polarity are seen. In contrast, adenomas from PTEN-deficient

<table>
<thead>
<tr>
<th>Table I. Comparison of tumor number, tumor size and carcinoma incidence</th>
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<tbody>
<tr>
<td>Strain</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>APC+/+/PTEN+/+</td>
</tr>
<tr>
<td>APC+/−/PTEN+/+</td>
</tr>
<tr>
<td>APCmin/−/PTEN+/+</td>
</tr>
<tr>
<td>APCmin/−/PTEN+/−</td>
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P value, versus APCmin/−/PTEN+/− mice.
APC\textsuperscript{min+} mice showed a much more advanced growth pattern which were more solid with higher grade of dysplasia (Figure 1B, middle panel). Stromal desmoplasia and infiltrative appearance of glands at the base of lesions suggested early invasion. Moreover, invasive carcinomas were identified in 8/10 of \textit{APC\textsuperscript{min+} PTEN\textsuperscript{++}}/C\textsuperscript{0} mouse intestine (Figure 1B, right panel). Intestinal intussusception resulted from large tumors was observed in 50% of \textit{APC\textsuperscript{min+} PTEN\textsuperscript{++}/C\textsuperscript{0}} mice which led to chronic intestinal obstruction (Figure 1C). As a result, most \textit{APC\textsuperscript{min+} PTEN\textsuperscript{++}/C\textsuperscript{0}} mice were ill and inactive by 4.5 months and the life span for these mice was \~5 months. In contrast, \textit{APC\textsuperscript{min+} PTEN\textsuperscript{++}/C\textsuperscript{0}} mice were healthy and active at the end of the experiment. These results suggest that increased PI3K activity, which resulted from PTEN deficiency, promoted \textit{APC} mutation-initiated intestinal neoplasia in mice.
Table II. Results of Cancer PathwayFinder Array

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt1</td>
<td>Thymoma viral proto-oncogene 1</td>
<td>1.0</td>
</tr>
<tr>
<td>Akt2</td>
<td>Thymoma viral proto-oncogene 2</td>
<td>1.0</td>
</tr>
<tr>
<td>Apaf1</td>
<td>Apoptotic peptidase activating factor 1</td>
<td>1.3</td>
</tr>
<tr>
<td>Becl2/1</td>
<td>Becl2-like 1</td>
<td>1.5</td>
</tr>
<tr>
<td>Casp8</td>
<td>Caspase 8</td>
<td>1.5</td>
</tr>
<tr>
<td>Cnmnb1</td>
<td>Catenin, beta 1</td>
<td>1.5</td>
</tr>
<tr>
<td>Cnd1</td>
<td>Cyclin D1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ccne1</td>
<td>Cyclin El</td>
<td>0.8</td>
</tr>
<tr>
<td>CD44</td>
<td>CD44 antigen</td>
<td>0.9</td>
</tr>
<tr>
<td>Cadh1</td>
<td>Cadherin 1</td>
<td>1.0</td>
</tr>
<tr>
<td>Cdk4</td>
<td>Cyclin-dependent kinase 4</td>
<td>1.8</td>
</tr>
<tr>
<td>Cdkra1a</td>
<td>Cyclin-dependent kinase inhibitor 1A (P21)</td>
<td>0.6</td>
</tr>
<tr>
<td>Cdkra1b</td>
<td>Cyclin-dependent kinase inhibitor 1B</td>
<td>1.3</td>
</tr>
<tr>
<td>Coll1a1</td>
<td>Procollagen, type XVIII, alpha 1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ets2</td>
<td>E2f avian leukemia oncogene 2, 3’ domain</td>
<td>0.9</td>
</tr>
<tr>
<td>Fos</td>
<td>FBJ osteosarcoma oncogene</td>
<td>0.8</td>
</tr>
<tr>
<td>Igα6</td>
<td>Integrin alpha 6</td>
<td>1.9</td>
</tr>
<tr>
<td>Igβ1</td>
<td>Integrin beta 1</td>
<td>1.3</td>
</tr>
<tr>
<td>Jun</td>
<td>Jun oncogene</td>
<td>0.8</td>
</tr>
<tr>
<td>Map2k1</td>
<td>Mitogen-activated protein kinase kinase 1</td>
<td>0.7</td>
</tr>
<tr>
<td>Mapk14</td>
<td>Mitogen-activated protein kinase 14</td>
<td>1.1</td>
</tr>
<tr>
<td>Mib2</td>
<td>Transformed mouse 3T3 cell double minute 2</td>
<td>0.7</td>
</tr>
<tr>
<td>Met</td>
<td>Met proto-oncogene</td>
<td>1.0</td>
</tr>
<tr>
<td>Mmp2</td>
<td>Matrix metallopeptidase 2</td>
<td>1.5</td>
</tr>
<tr>
<td>Mmp9</td>
<td>Matrix metallopeptidase 9</td>
<td>1.9</td>
</tr>
<tr>
<td>Mvc</td>
<td>Myelocytomatosis oncogene</td>
<td>0.6</td>
</tr>
<tr>
<td>Nfkβ1</td>
<td>Nuclear factor of κ light chain gene enhancer in B-cells, p105</td>
<td>0.7</td>
</tr>
<tr>
<td>Nfkbia</td>
<td>Nuclear factor of κ light chain gene enhancer in B-cells, α</td>
<td>0.7</td>
</tr>
<tr>
<td>Pdgfa</td>
<td>Platelet-derived growth factor, α</td>
<td>1.3</td>
</tr>
<tr>
<td>Rb1</td>
<td>Retinoblastoma 1</td>
<td>0.6</td>
</tr>
<tr>
<td>Spp1</td>
<td>Secreted phosphoprotein 1</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Listed are relative levels of genes expressed in the intestinal tumors of APC\(^{min/-}\)-PTEN\(^{+/+}\) and APC\(^{min/-}\)-PTEN\(^{+/-}\) mice. Changes of differential expression are expressed as levels in APC\(^{min/-}\)-PTEN\(^{+/-}\) mice/levels in APC\(^{min/-}\)-PTEN\(^{+/-}\) mice.

PTEN deficiency induced OPN

The PI3K pathway regulates the expression of a large number of genes. In order to determine the mechanism by which PTEN deficiency promoted the growth and invasion of intestinal tumors, we next compared the gene expression profile between APC\(^{min/-}\)-PTEN\(^{+/+}\) tumors and APC\(^{min/-}\)-PTEN\(^{+/-}\) tumors, using Mouse Cancer PathwayFinder Oligo GEArray. The expression of 113 genes representative of six biological pathways involved in cell cycle control, apoptosis, signal transduction molecules, cell invasion and angiogenesis was analyzed. Three independent assays were conducted to compare differential gene expression profile between APC\(^{min/-}\)-PTEN\(^{+/+}\) and APC\(^{min/-}\)-PTEN\(^{+/-}\) tumors. Changes of differential expression are expressed as levels in APC\(^{min/-}\)-PTEN\(^{+/-}\) mice/levels in APC\(^{min/-}\)-PTEN\(^{+/-}\) mice.

Levels of OPN protein were increased in APC\(^{min/-}\)-PTEN\(^{+/-}\) adenomas (Figure 2C, panel b). However, abundant OPN was detected in APC\(^{min/-}\)-PTEN\(^{+/-}\) adenocarcinomas, which was predominantly localized to the cytoplasm of epithelial cells (Figure 2C, panel d and e). However, immunoreactivity of OPN was clearly detected in the stroma surrounding tumor cells (Figure 2C, panel e). Since OPN has been identified as lead marker of colon cancer progression (28), we decided to further investigate the roles of OPN in PI3K pro-neoplastic actions.

**OPN promoted the survival of colon cancer cells**

HCT116 colon cancer cell line contains one wild-type PIK3CA allele and one kinase domain mutated PIK3CA allele. Samuels et al. (5) disrupted either the wild-type or mutant alleles and established HCT116 cell lines which express either wild-type PIK3CA (HCT116/PIK3CAWT) or mutant PIK3CA (HCT116/PIK3CAMU) (5). These isogenic cell lines provide excellent models for studying roles of PI3K in colorectal neoplasia. HCT116/PIK3CAMUT and HCT116/PIK3CAMU cells grow at similar rates in the presence of serum; deprivation of growth factors results in strong apoptosis in HCT116/PIK3CAMUT cells. Large number of floating cells was observed in HCT116/PIK3CAMUT cells 48 h after withdrawal of serum (Figure 3A). DNA fragmentation assay revealed that serum deprivation-induced programmed cell death (Figure 3B). In contrast, withdrawal of serum did not lead to significant cell death in HCT116/PIK3CAMU cells. Levels of OPN in HCT116/PIK3CAMU cell-conditioned media were modestly higher than those in HCT116/PIK3CAWT cell-conditioned media when grown in the medium containing 10% fetal bovine serum. Serum deprivation increased the secretion of OPN, particularly in PI3K-mutated HCT116 cells. After a 72 h serum starvation, levels of OPN in HCT116/PIK3CAMU conditioned media were significantly higher than those in HCT116/PIK3CAWT culture media (Figure 3C). In order to determine whether OPN played any roles in HCT116 cell survival, we pre-coated cell culture plates with OPN. Serum deprivation-induced apoptosis was significantly reduced when HCT116/PIK3CAWT cells were grown on OPN-treated plates (Figure 3D). These results suggest that the expression of OPN was correlated to PI3K activity and OPN promoted the survival of colon cancer cells.

**Ras induced OPN in a PI3K- and MAPK-dependent manner**

Ectopic expression of mutated Ras leads to transformation of intestinal epithelial cells and activation of the PI3K pathway is essential for this transformation (7). In order to elucidate the roles of OPN in Ras-mediated transformation of intestinal epithelial cells, RIE-i-Ha-Ras cell line with an inducible Ha-RasVal12 cDNA was employed (7). Non-transformed RIE-i-Ha-Ras cells grew as a monolayer and underwent apoptosis when they reached confluence, noted by floating cells (Figure 4A). Following Ras induction, RIE cells acquired a transformed appearance characterized by spindle morphology and growth in overlapping clusters. Addition of a specific inhibitor for PI3K, LY 294002 (20 μM), significantly inhibited the overgrowth and morphologic transformation of Ras-induced RIE cells. On the other hand, a specific inhibitor for MAPK/ERK Kinase (MEK), PD-98059 (25 μM), completely attenuated Ras-mediated transformation, restoring epithelial morphology and inducing apoptosis in RIE-i-Ha-Ras cells. Interestingly, the levels of OPN were low in non-transformed RIE-i-Ha-Ras cells; induction of Ras by IPTG robustly increased levels of OPN mRNA by 48 h. Real-time PCR assay demonstrated that levels of OPN mRNA were increased ~100-fold in Ras-transformed RIE cells compared with non-transformed RIE-i-Ha-Ras cells (Figure 4B, left panel). While OPN protein was barely detected in non-transformed RIE-i-Ha-Ras cell culture media, levels of OPN increased ~16-fold in RIE-i-Ha-Ras conditioned media by 72 h after addition of IPTG (Figure 4B, right panel). Activation of PI3K was required for Ras induction of OPN, since the presence of LY 294002 completely attenuated the induction of OPN mRNA and protein (Figure 4C). LY 294002 concentration dependently inhibited the secretion of OPN by...
Ras-transformed RIE cells. Additionally, MEK/extracellular signal-regulated kinase activity was also essential for Ras-induced OPN production; PD-98059 completely blocked the induction of OPN as well.

OPN played important roles in intestinal epithelial transformation

In order to elucidate the functional roles of Ras-induced OPN in intestinal epithelial transformation, we performed an acute knock-down of OPN expression using siRNA. RIE-i-Ha-Ras cells were transfected with OPN siRNA prior to IPTG treatment. Efficient down-regulation of OPN protein was achieved 72 h after siRNA transfection. As a result, Ras-induced OPN expression was significantly inhibited by two independent siRNA sequences in RIE-i-Ha-Ras cells, reducing Ras-induced OPN by 50–70% (Figure 5A). Reduction of OPN expression did not attenuate Ras-mediated morphologic transformation of RIE cells; however, it reduced uncontrolled cell growth. Cell number was increased ~3-fold 72 h after addition of IPTG in negative siRNA transfected RIE-i-Ha-Ras cells (Figure 5B). Transfection with OPN siRNA inhibited Ras-stimulated cell growth and reduced the cell number by 30%. Next, we placed RIE-i-Ha-Ras cells, which were transfected with either negative siRNA or OPN siRNA into soft agarose. While non-transformed RIE cells did not grow colonies in soft agarose; RIE-i-Ha-Ras cells form colonies in the presence of IPTG. Acute knock-down of OPN expression did not significantly alter the number of colonies formed by IPTG-induced RIE-i-Ha-Ras cells. However, the size of colonies formed by OPN siRNA transfected cells was significantly smaller than those formed by negative siRNA transfected RIE-i-Ha-Ras cells (Figure 5C). In addition, negative siRNA transfected RIE-i-Ha-Ras cells formed invasive colonies; cells migrated from colonies into surrounding agarose (Figure 5D). In contrast, OPN siRNA transfected RIE-i-Ha-Ras cells formed non-invasive colonies, which were surrounded by cell debris.
Discussion

Aberrant activation of the PI3K pathway is oncogenic and plays critical roles in a variety of cancers. In contrast, PTEN dephosphorylates and inactivates the lipid second messenger phosphorylated by PI3K, therefore, acting as a tumor suppressor (29). Disruption of PTEN activity results in increased growth signals and promotion of tumor formation (23,30). Numerous in vitro studies demonstrate the important role of PI3K in colorectal neoplasia. Activation of the PI3K pathway is essential for Ras-mediated transformation of intestinal epithelial cells (7). Colon cancer HCT116 cells which contain mutant PI3KCA display significant growth advantage compared with the cells in which mutant PI3KCA is disrupted (5). Our data from animal experiments suggest that increase in PI3K activity by reduction of PTEN expression promotes intestinal tumor progression. While low-grade dysplasia was observed in most APC−/−; PTEN−/− mice by age 5 months, advanced adenoma and invasive adenocarcinoma developed in animals bearing both mutated APC and PTEN, suggesting the critical roles of the PI3K pathway in malignant transformation of colonic epithelium.

An interesting finding from the present study is a high incidence of intestinal intussusception, which often led to the death of APC−/−; PTEN−/− mice by 5 months. Intussusception is the invagination of a bowel loop with its mesenteric fold into the lumen of a contiguous portion of bowel as a result of peristalsis. Intussusception in APC−/−; PTEN−/− mouse was caused by large adenomas or adenocarcinomas, which acted as the lead point of the invaginating bowel. Additionally, PI3K plays important role in intestinal smooth muscle growth and increases intestinal motility (31,32). Thus, PTEN deficiency may stimulate intestinal motility and result in dysrhythmic contraction further promoting intestinal intussusception.

Agrawal et al. (28) analyzed gene profile of colon tumors at multiple stages using oligonucleotide expression arrays and found that OPN is most consistently and differentially expressed in conjunction with tumor progression. Expression of OPN is linked to survival of colorectal cancer patients; profiling a core set of 43 genes including OPN was 90% accurate in predicting 36 month overall survival in these patients (33). Our results of targeted microarray determined OPN as a leading marker in intestinal carcinoma of APC−/− mice, suggesting that OPN is a PI3K target gene and may play crucial roles in relatively early stage of colorectal carcinogenesis, promoting the transformation of benign adenoma to invasive adenocarcinoma. OPN is a downstream effector of the PI3K pathway in melanomas; PTEN aberrations significantly increase the expression of OPN (34). We found that HCT116 cells that contain kinase domain mutated PI3KCA allele secreted significantly higher levels of OPN in culture media compared with cells carrying wild-type PI3KCA allele when grown in growth factor-deprived media. OPN is a secreted glycosylated phosphoprotein and involved in a number of biological functions including cell adhesion, survival, migration and bone calcification (35). Numerous studies suggest that OPN plays critical roles in tumor progression (36). OPN promotes tumor cell growth; down-regulation of

![Fig. 4. Expression of OPN in transformation of RIE-i-Ha-Ras cells. (A) RIE-i-Ha-Ras cells were treated with vehicle (a), 5 mM IPTG (b), IPTG plus 20 μM LY 294002 (c) or IPTG plus 25 μM PD-98059 (d) for 72 h and photographed (×100). (B) RIE-i-Ha-Ras cells were treated with vehicle (V) or 5 mM IPTG (I) for the indicated times. Relative levels of OPN mRNA were determined by real-time RT-PCR (left panel). The quantity of OPN protein in conditioned media was determined by ELISA. Results were normalized to cellular protein content and expressed as OPN per milligram protein, *P < 0.05. (C) RIE-i-Ha-Ras cells were treated with vehicle (V), 5 mM IPTG (I), IPTG (I) plus 20 μM LY 294002 (LY) or IPTG (I) plus 25 μM PD-98059 (PD) for indicated times. Total RNA was extracted. Levels of OPN mRNA were determined by RT–PCR (left panel). RIE-i-Ha-Ras cells were treated with vehicle (V), 25 μM PD-98059 (PD) or 20 μM LY 294002 (LY) in the presence (IPTG) or absence (C) of 5 mM IPTG. Relative levels of OPN mRNA were determined by real-time RT–PCR after a 48 h treatment (middle panel). The amount of OPN protein in conditioned media was quantitated by ELISA after cells were treated by vehicle (V), 25 μM PD-98059 (PD) or LY 294002 (5, 10 or 20 μM) in the presence (IPTG) of 5 mM IPTG for 72 h (right panel). Results were normalized to cellular protein content and expressed as OPN per milligram protein, *P < 0.05.](image)
OPN expression reduced the growth of Ras-transformed 3T3 cells in soft agarose and in animal implants (37). OPN protein is activated both by interleukin-3 and granulocyte-macrophage colony-stimulating factor and contributes to their pro-survival activities (38). Our data show that growth factor deprivation induced strong apoptosis in HCT116/PIK3CAWT cells, which produced low levels of OPN. Pre-coating the surface of the culture with OPN significantly reduced programmed cell death. These results suggest that promoting cell survival by OPN may be an important mechanism for tumor progression and metastasis.

About 50% of colorectal carcinomas contain K-Ras mutations (39) and the K-Ras oncogene plays key roles in colorectal neoplasia (1). The mechanism governing Ras-mediated transformation is complex and involves a number of downstream signaling pathways. Previous studies show that PI3K catalytic subunit can be directly activated by Ras (40) and the PI3K/Akt pathway is a key mediator for Ras-mediated carcinogenesis in intestinal epithelial cells (7). Ras rapidly induces the expression of a number of pro-neoplastic genes in intestinal epithelial cells, including cyclin D1, cycooxygenase-2, Bcl-2 and Bcl-xL (41–43). In the present study, we found that expression of oncogenic Ras increased levels of OPN mRNA in RIE cells; a ~100-fold induction was observed after Ras was induced for 48 h. Additional experiments are required to address mechanisms by which Ras increases OPN expression. Increase in OPN expression was closely associated with the transformation of RIE cells. Inhibitors for MEK and PI3K completely attenuated Ras-mediated morphological transformation as well as the induction of OPN expression. OPN-specific siRNA reduced the levels of OPN in cell culture media by 50–70%, which modestly inhibited the growth of Ras-transformed RIE cells. An interesting observation was that Ras-transformed RIE cells were able to penetrate and survive in surrounding agarose forming invasive colonies. On the other hand, RIE-i-Ha-Ras cells in which OPN was knocked down underwent cell death when they migrated into agarose, as evidenced by cell debris surrounding the colonies. These results suggest that OPN plays critical roles in anchorage-independent growth of transformed intestinal epithelial cell through promoting cell survival.

Although mutations of PI3K catalytic subunit are detected in ~30% of colon cancers (4), many oncogenic signaling pathways involved in colorectal carcinogenesis activate the PI3K pathway. For example, the epidermal growth factor receptor signaling system, the Ras oncogene and the cycooxygenase-2/prostaglandin E2-signaling pathway directly or indirectly increase PI3K activity, suggesting that PI3K plays a central role during the adenoma to carcinoma transformation in colonic epithelium. The APC<sup>min−/−</sup>/PTEN<sup>+/−</sup> mouse which develops invasive carcinoma in the gut provides a novel animal model for mechanistic and therapeutic research in colorectal carcinogenesis. In addition, our data show that OPN is up-regulated by PI3K and contributes to PI3K pro-neoplastic actions in colonic neoplasia. OPN regulates cell adhesion, migration and survival through interacting with CD44, integrins and matrix metalloprotease. The expression of OPN has been repeatedly shown to correlate with tumor progression and metastasis in a variety of carcinomas. Further evaluation of the clinical significance of OPN in colorectal cancer patients is warranted.

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![Fig. 5. OPN promoted RIE cell transformation.](image-url)
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PI3K induction of osteopontin

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