Prohibitin Attenuates Colitis-Associated Tumorigenesis in Mice by Modulating p53 and STAT3 Apoptotic Responses


Abstract

Although inflammatory bowel disease (IBD) includes 2 major chronic intestinal disorders, Crohn’s disease and ulcerative colitis (UC), which share related characteristics such as mucosal damage and diarrhea but have distinguishing clinical features. Evidence suggests that in addition to the widely accepted aberrant mucosal immune response, nonimmune cells including epithelial cells play an emerging role in the pathogenesis of disease by modulating mucosal barrier integrity and homeostasis (1). An association between IBD and colorectal cancer (CRC) has been well established with a cumulative risk of developing CRC of 7% after 20 years for UC and 8% for Crohn’s disease (2). Previous studies have shown that production of proinflammatory cytokines in the lamina propria contribute to tumor growth and cancer development during intestinal inflammation (3, 4).

Prohibitin 1 (PHB) is an evolutionarily conserved, multifunctional 32 kDa protein implicated in cellular processes including the regulation of cell cycle progression, apoptosis, and transcription (5–7). Expression of PHB is decreased in intestinal epithelial cells may offer a potential therapeutic approach to prevent colitis-associated carcinogenesis. PHB protects against colitis-associated cancer by modulating p53- and STAT3-mediated apoptosis. Modulation of PHB expression in intestinal epithelial cells may offer a potential therapeutic approach to prevent colitis-associated carcinogenesis.

Introduction

Inflammatory bowel disease (IBD) includes 2 major chronic intestinal disorders, Crohn’s disease and ulcerative colitis (UC), which share related characteristics such as mucosal damage and diarrhea but have distinguishing clinical features. Evidence suggests that in addition to the widely accepted aberrant mucosal immune response, nonimmune cells including epithelial cells play an emerging role in the pathogenesis of disease by modulating mucosal barrier integrity and homeostasis (1). An association between IBD and colorectal cancer (CRC) has been well established with a cumulative risk of developing CRC of 7% after 20 years for UC and 8% for Crohn’s disease (2). Previous studies have shown that production of proinflammatory cytokines in the lamina propria contribute to tumor growth and cancer development during intestinal inflammation (3, 4).

Prohibitin 1 (PHB) is an evolutionarily conserved, multifunctional 32 kDa protein implicated in cellular processes including the regulation of cell cycle progression, apoptosis, and transcription (5–7). Expression of PHB is decreased in mucosal biopsies from UC and Crohn’s disease affected patients and in the dextran sodium sulfate (DSS) and interleukin (IL)-10−/− mouse models of colitis (8, 9). Proinflammatory cytokines such as TNF-α and reactive oxygen species decrease expression of intestinal epithelial PHB in vivo and in vitro (8–10). Restoration of colonic epithelial PHB expression using genetic manipulation [villin-PHB transgenic (Tg) mice] or therapeutic delivery to the colon via nanoparticles or adenovirus protected mice from experimental colitis (11, 12).

The role of PHB in cancer cell proliferation and/or tumor suppression remains controversial especially because PHB expression is increased in many transformed cells and tumors (13–19). Consensus binding sites for the oncoprotein Myc are present in the PHB promoter and likely contribute to the increased PHB levels in many tumors (20). Although somatic mutations in the PHB gene were observed in a few sporadic breast cancers, none were identified in the ovary, hepatic, or...
lung cancers examined (16). Previous studies have shown that PHB has an antitumorogenic role in gastric, prostate, and liver cancers (21–23). Despite the increasing number of studies on PHB, its role in CRC or colitis-associated cancer (CAC) has not been explored. Here, we used mice specifically overexpressing PHB in intestinal epithelial cells (IECs) to assess the role and mechanism of PHB in CAC.

Materials and Methods

Animal models

Wild-type (WT) and villin-prohibitin Tg mice specifically overexpressing prohibitin in IECs (12) were 9 weeks old at the beginning of the experimental protocol. All mice were grouped-housed in standard cages under a controlled temperature (25°C) and photoperiod (12-hour light–dark cycle) and were allowed standard chow and tap water ad libitum. All experiments were approved by the Baylor Research Institute Institutional Animal Care and Use Committee.

Induction of CAC in mice

Age-matched male and female C57BL/6 WT and Tg mice were intraperitoneally injected with 7.6 mg/kg azoxymethane (AOM; Sigma-Aldrich) on day 0. Colitis was induced 7 days after AOM injection by oral administration of 3% (wt/vol) DSS (molecular weight, 50,000; MP Biomedicals) in drinking water ad libitum for 7 days followed by 14 days of recovery of water alone. After recovery, a second cycle of DSS was repeated and mice were sacrificed after 3 weeks of recovery (day 56) as previously described (24). Three separate groups of mice were used as controls: one group of mice was maintained with regular water ($n=16$ WT; $n=10$ Tg), a second group of mice was injected with AOM and given water for the remainder of the experiment ($n=9$ WT; $n=7$ Tg), and the third group of mice was treated with DSS as described above without AOM injection ($n=20$ WT; $n=8$ Tg). AOM alone represents treatment with the alkylating agent in the absence of inflammation and is expected to result in no tumor formation in WT mice at the dose used (25). DSS alone represents repeated cycles of inflammation and was included to assess whether inflammation alone caused dysplasia in these mice. The study was powered in such a fashion that the control arms required less mice as death before the end of the study was not anticipated for these treatment groups. Body weight, stool consistency, and stool occult blood was monitored during the DSS treatment and recovery phases. Upon sacrifice, colon was excised from the ileocecal junction to anus, cut open longitudinally, and prepared for histologic evaluation.

To assess mortality, the same protocol was followed with the exception of extending through 126 days or until mice developed rectal prolapse and/or more than 20% body weight loss. Colons were assessed macroscopically for polyps using a dissecting microscope.

Clinical score assessment

Assessment of body weight loss, stool consistency, and the presence of occult/gross blood by a guaiac test (Hemoccult SENSA; Beckman Coulter) were determined daily during DSS administration to generate a clinical activity score as described previously (12). During recovery periods, body weight was measured every week for each group.

Endoscopic assessment of polyp formation and polyp count

Colonoscopy was done to assess polyp formation in mice by using the Coloview (Karl Storz Veterinary Endoscopy). Mice were euthanized with 1.5% to 2% isoflurane and approximately 3 cm of the colon proximal to the anus was visualized after inflation of the colon with air. Mice were then sacrificed by cervical dislocation after euthanizing with isoflurane. Colon was excised from ileocolic junction to anus, washed with 0.9% NaCl, cut open longitudinally and preserved in 10% neutral-buffered formalin. The number and size of the polyps were determined using a Jenko dissecting microscope.

Histopathology scoring

Paraffin-embedded sections (5 μm) of colon were analyzed for Ki67 staining as a marker of cell proliferation as previously described (12). A minimum of 15 crypts with normal morphology were counted for Ki67-positive cells per section.

Immunohistochemistry

Paraffin-embedded sections (5 μm) of colon were analyzed for Ki67 staining as a marker of cell proliferation as previously described (12). A minimum of 15 crypts with normal morphology were counted for Ki67-positive cells per section.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining

Immunofluorescent terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was carried out to measure apoptosis from paraffin-embedded sections using the In Situ Cell Death Detection Kit as described by the manufacturer (Roche). Nuclei were stained with 4',6-diamidino-2-phenylindole to count total cells per crypt. A minimum of 10 crypts with normal morphology were counted per section.

p53 mutational analyses

Tumor tissue was microdissected from paraffin-embedded sections (7 μm) of Swiss-rolled mouse colon. Genomic DNA was isolated from microdissected tissues using the QiAamp DNA FFPE Tissue purification kit (Qiagen). The PCR amplicons were generated and sequenced as previously described (26). See the Supplementary Materials and Methods section for primer sequences.

Human tissue samples

Matching normal and tumor tissues were obtained at the time of surgical resection from patients with one or more
UC-associated colorectal neoplasms, consisting of adenocarcinomas or dysplasias. Normal control samples consisted of colonic normal mucosa adjacent to tumors or ileal mucosa. Inflamed UC tissues were obtained from patients without evident dysplasia. All tissues were grossly dissected free of normal surrounding tissue, and parallel sections were used for histologic characterization. Tissue collection was approved by patients according to Institutional Review Board guidelines.

Total RNA extraction and quantitative real-time PCR
Total RNA was extracted for human tissues using the RNeasy kit (Qiagen). Quantitative real-time PCR was carried out as previously described (Kathiria and colleagues; submitted for publication). See the Supplementary Materials and Methods section for primer sequences.

Cell culture and transfection
Caco2-BBE cells were used to assess the interaction of PHB with STAT3. As Caco2-BBE cells have mutated p53, WT HCT116 human CRC cells were used to assess PHB interaction with p53. All cell lines were obtained from the American Type Culture Collection. Cells were grown and transfected as previously described (Kathiria and colleagues; submitted for publication).

γ-Irradiation
γ-Irradiation was used to induce DNA damage in WT and p53−/− HCT116 cells (27) after 72 hours of transfection with pEGFPN1 vector or pEGFPN1-PHB. Cells were irradiated by using 137Cs γ-iradiator at 6.5 cGy/s for 32 seconds for a total of 209.4 cGy. Cells were harvested 24 hours after γ-irradiation for subsequent assays.

Protein extraction, Western blot analysis, and immunoprecipitation
Mucosal stripings from Tg and WT mice were obtained for Western blot analysis as described previously (12). Total protein was isolated from cultured cells as described previously (Kathiria and colleagues; submitted for publication). Antibodies used were mouse monoclonal PHB (Thermo Fisher), mouse monoclonal GFP, p53, Bcl-2, Bcl-xL and Bad and rabbit polyclonal BAX, p-Bad (ser 155), STAT3 (Santa Cruz Biotechnology), rabbit polyclonal proliferating cell nuclear antigen (PCNA) antibody (Abcam), rabbit polyclonal caspase-3, p53, pSTAT3 (Cell Signaling Technology), rabbit polyclonal p53 upregulated modulator of apoptosis (PUMA), and mouse monoclonal anti-β-actin (Sigma-Aldrich).

PHB was immunoprecipitated from 0.6 mg total protein lysates from HCT116 or Caco2-BBE cells or 0.20 mg total protein lysates from mouse mucosa with 1 μg mouse anti-PHB, anti-p53, or anti-pSTAT3 antibody and 30 μL 50% protein A sepharose beads (GE Healthcare). Blots were incubated with the rabbit p53 antibody or PHB antibody, respectively. Omission of primary antibody during the immunoprecipitation was carried out as a negative control.

Statistical analysis
Values are expressed as mean ± SEM. Statistical analysis was conducted using 2-way ANOVA and subsequent pairwise comparisons using Bonferroni post hoc tests. A P value less than 0.05 was considered statistically significant in all analyses.

Results
IEC-specific PHB overexpression decreases colonic tumorigenesis in a mouse model of CAC
Previous studies have shown that PHB has an antitumorogenic role in gastric, prostate, and liver cancers (21–23). We used the AOM DSS mouse model to study the role of PHB in CAC. Body weight was measured weekly as one parameter to assess the severity of disease. WT and Tg mice given water only throughout the experiment and mice injected with AOM followed by water alone showed similar body weight gain over the 8-week protocol (Supplementary Fig. S1A) and did not develop polyps as previously reported for this dose of AOM (25). WT mice given 2 cycles of DSS without AOM injection lost more body weight following DSS administration and recovered less weight compared with Tg mice over the 8-week protocol (Supplementary Fig. S1B), similar to our previous findings (12). Although both WT and Tg AOM DSS-treated mice lost weight after the first administration of DSS, Tg mice gained body weight more rapidly during the recovery phase and maintained body weight better than WT mice throughout the remainder of the study (Fig. 1A).

Stool consistency, stool occult blood, and body weight loss were monitored following DSS administration to generate a clinical disease score. AOM DSS-treated Tg mice showed a decreased clinical score after the first administration of DSS compared with WT mice, but this did not reach statistical significance. Tg mice exhibited a significantly lower clinical score after the second administration of DSS (Fig. 1B). Tg mice given DSS without AOM injection had lower clinical scores after DSS administration compared with WT mice (Supplementary Fig. S1C), similar to our previous findings (12).

The overall survival rate for AOM DSS-treated Tg mice was 82% (14 of 17 mice) compared with 60% for WT (12 of 20 mice) (Fig. 1C). Tg mice given DSS without AOM injection exhibited no mortality throughout the 8-week protocol (n = 8) compared with 60% survival rate for WT mice (12 of 20 mice; Supplementary Fig. S1D). Upon sacrifice the entire colon from ileocecal junction to anus was excised and assessed for number and size of polyps. Tg mice showed considerably fewer polyps with the majority being smaller than 3 mm in diameter compared with WT mice (Fig. 1D). Figure 1E shows representative photos of excised colons and endoscopic images of polyp formation in WT and Tg mice following AOM-DSS treatment. After polyps were counted and measured, colons were Swiss-rolled and assessed for histopathologic score by a trained pathologist. WT mice had a higher occurrence of high-grade dysplasia and adenocarcinoma compared with Tg mice, which exhibited more low-grade dysplasia and normal morphology (Fig. 1F). Collectively, these results suggest that PHB Tg mice show less susceptibility to CAC than WT mice.

PHB Tg mice are less susceptible to CAC and mortality
To assess mortality and cancer development, the same protocol was followed with the exception of extending through
126 days or until mice developed rectal prolapse and/or >20% body weight loss. WT mice showed greater mortality than Tg mice throughout the study. The difference in mortality was especially pronounced after the formation of polyps, which occurs at approximately 8 weeks (Supplementary Fig. S2A). Tg mice showed significantly less total number of polyps, and although rare, a significantly less number of large polyps (>6 mm in diameter) compared with WT mice (Supplementary Fig. S2B). Mice that survived from 8 through 18 weeks were assessed for histopathologic score as animals that died before 8 weeks did not yet develop polyps. As shown in Supplementary Fig. S2C, the majority of WT mice developed adenocarcinoma (invasive adenocarcinoma: n = 2, intramucosal adenocarcinoma: n = 7) compared with 5 Tg mice (intramucosal adenocarcinoma). The majority of Tg mice had low- or high-grade dysplasia. Supplementary Fig. S2D shows representative gross anatomy of the colon (top) and representative colonoscopy images (bottom) at the end of the CAC protocol. F, histopathology scores. *, P < 0.05 vs. AOM DSS-treated WT.

IEC-specific PHB overexpression does not affect cell proliferation during CAC

Alteration in cell proliferation can contribute to tumorigenesis (28). Ki67 immunohistochemical staining and PCNA were
assessed to examine the effect of PHB overexpression on cell proliferation in CAC. Both WT and Tg mice showed increased Ki67 staining following AOM-DSS treatment (Fig. 3A, middle panels) compared with mice given water (Supplementary Fig. S3A, top panels and Supplementary Fig. S3B). There was no significant difference in the percent Ki67-positive cells per crypt in normal epithelium (Supplementary Fig. S3A, middle panels and Supplementary Fig. S3B) or areas of dysplasia (Supplementary Fig. S3A, bottom panels) between WT and Tg mice treated with AOM DSS. Similar results were corroborated by PCNA Western blot analysis. PCNA protein expression was increased in both WT and Tg mice following AOM-DSS treatment compared with mice given water (Supplementary Fig. S3C and S3B). There was no significant difference in PCNA protein expression between WT and Tg mice treated with AOM DSS. These results suggest that WT and Tg mice exhibited increased cell proliferation after AOM DSS, and that IEC-specific PHB overexpression does not alter cell proliferation induced by AOM-DSS treatment or in colonic tumors/polyps.

**IEC-specific PHB overexpression is associated with increased apoptosis during CAC**

Suppression of apoptosis plays an important role in tumorigenesis by allowing genetically compromised cells to proliferate (29). TUNEL staining and Western blot analysis of cleaved caspase-3 levels were used to assess IEC apoptosis during CAC. WT and Tg mice exhibited increased number of TUNEL-positive cells per crypt after AOM-DSS treatment (Fig. 2A and B). Tg mice exhibited a higher number of TUNEL-positive cells compared with WT mice after AOM-DSS treatment in normal epithelium (Fig. 2A and B). Figure 2C and D corroborate the TUNEL immunofluorescence staining by showing increased protein expression of cleaved caspase-3 in Tg mice compared with WT mice with CAC. Thus, IEC-specific PHB overexpression may suppress tumor development by promoting IEC apoptosis.
**IEC-specific overexpression of PHB is associated with altered levels of p53, PUMA, Bax, and p-Bad during CAC**

Given the effect of PHB overexpression on apoptosis during CAC, we examined levels of p53, a tumor suppressor, and its downstream targets PUMA and Bax as well as the activation of the proapoptotic protein Bad. p53 regulates cell survival through induction of cell cycle arrest or apoptosis. Our previous study showed that PHB protein levels are decreased in mucosal biopsies of active inflammation from Crohn’s disease-affected patients and in the DSS and IL-10−/− mouse models of colitis (9). To determine whether mucosal PHB levels are affected during CAC, Western blot analysis was carried out on total protein isolated from mucosal stippings from WT mice. Figure 3A shows that mucosal PHB protein levels were decreased in WT mice after AOM-DSS treatment compared with water control WT mice (water = 1.0 ± 0.09 vs. AOM DSS = 0.75 ± 0.06, P < 0.05). Tg mice exhibited higher expression of mucosal PHB protein levels compared with WT mice when given water or AOM-DSS treatment (Fig. 3A). Mucosal PHB protein expression in Tg mice was similar to our previous studies using these animals (12). Although induction of CAC increased p53 protein expression in both WT and Tg mice compared with water control mice, p53 levels were significantly higher in Tg mice compared with WT mice after AOM-DSS treatment (Fig. 3B). Sequencing of exons 5 to 8 of p53 from DNA isolated from areas of dysplasia from 5 WT and 5 Tg mice found no mutations. PUMA, a central mediator of the p53 apoptotic response and suppressor of intestinal tumorigenesis, was also analyzed. Mucosal PUMA protein levels were significantly higher in Tg mice compared with WT mice after AOM-DSS treatment (Fig. 3C). Bax, a proapoptotic protein, was also analyzed. Mucosal Bax protein levels were significantly higher in Tg mice compared with WT mice after AOM-DSS treatment (Fig. 3D). p-Bad, a downstream target of p53, was also analyzed. Mucosal p-Bad protein levels were significantly higher in Tg mice compared with WT mice after AOM-DSS treatment (Fig. 3E).
in mice (30), was increased only in Tg mice during CAC (Fig. 3C). Similarly, the proapoptotic protein Bax was also increased in Tg mice after AOM-DSS treatment (Fig. 3D). Phosphorylation of Bad at serine 155 inhibits the proapoptotic function of Bad by disrupting its binding to antiapoptotic Bcl-2 (31). Western blot analysis showed that Tg mice exhibited less phosphorylation of Bad at serine 155 compared with WT mice during CAC (Fig. 3E), suggesting that in Tg mice Bad retains its proapoptotic function. Together, these data suggest that CAC in PHB Tg mice is associated with increased expression of p53, PUMA, and Bax and decreased Bad phosphorylation.

**PHB-induced altered expression of PUMA, Bax, and Bad and requires p53 signaling in HCT116 cells**

HCT116 colorectal carcinoma cells were used as an *in vitro* model to understand the underlying role of PHB in modulating p53 activation and apoptosis. WT and p53−/− HCT116 cells were transiently transfected with GFP-tagged PHB (GFP-PHB) or vector alone and downstream targets of p53 were measured by Western blot analysis. WT HCT116 cells overexpressing PHB (Fig. 4A, lane 2) showed increased PUMA and Bax and decreased phospho-Bad protein expression compared with vector-transfected cells (lane 1). Cleaved caspase-3, a marker of apoptosis, was also increased in PHB overexpressing cells (Fig. 4A, lane 2 vs. lane 1). p53−/− HCT116 cells showed no change in PUMA, Bax, phospho-Bad, or cleaved caspase-3 protein expression after PHB transfection (Fig. 4A, lane 6) compared with vector-transfected cells (lane 5), suggesting that p53 is required for PHB-induced alteration of these proteins. WT and p53−/− HCT116 cells were exposed to γ-irradiation to induce DNA damage. Following γ-irradiation, PHB overexpressing WT HCT116 cells showed significantly.

**Prohibitin Functions as a Tumor Suppressor in CAC**
increased PUMA, Bax, and cleaved caspase-3 protein expression whereas phospho-Bad levels were decreased (Fig. 4A, lane 4) compared with vector-transfected cells (lane 3). PHB overexpressing WT cells exposed to γ-irradiation exhibited a further increase in Bax and cleaved caspase-3 and decrease in phospho-Bad compared with PHB overexpressing cells left untreated (Fig. 4A, lane 4 vs. lane 2). γ-Irradiation did not alter expression of PUMA, Bax, phospho-Bad, or cleaved caspase-3 in p53−/− HCT116 cells regardless of PHB transfection (Fig. 4A, lanes 7 and 8).

**PHB interacts with p53 in HCT116 cells and in colon mucosa**

To determine whether PHB interacts with p53 in IECs, PHB was immunoprecipitated from total cell lysates from WT HCT116 cells overexpressing vector or PHB and exposed to γ-irradiation to induce DNA damage. Following immunoprecipitation and SDS-PAGE, membranes were immunoblotted for p53 and subsequently PHB expression. p53 coimmunoprecipitated with PHB during basal conditions and after γ-irradiation (Fig. 4B and C). To determine whether PHB interacts with p53 in vivo, we conducted coimmunoprecipitation experiments for PHB and p53 in colon mucosal lysates from water- and AOM DSS-treated mice. p53 coimmunoprecipitated with PHB in lysates from both WT and Tg mice (Fig. 4D), with increased levels in Tg mice (Fig. 4D). Collectively, these results suggest that PHB interacts with p53 in cultured human CRC cells and in colon mucosa.

**PHB interacts with phospho-STAT3 and modulates STAT3 responsive genes, Bcl-xl, and Bcl-2 in vivo and in vitro**

The IL-6/STAT3 signaling pathway is known to play a pathogenic role in intestinal inflammation and colonic cancer through the induction of the antiapoptotic genes Bcl-xl and Bcl-2 (32). Caco2-BBE cells, which are responsive to IL-6, show decreased basal and IL-6-induced STAT3 activation, and decreased Bcl-xl and Bcl-2 protein expression when PHB is overexpressed (Fig. 5A). Coinmunoprecipitation experiments using Caco2-BBE cells treated with IL-6 suggest that PHB interacts with phospho-STAT3 (Fig. 5B and C). To determine whether PHB interacts with STAT3 in vivo, we conducted coimmunoprecipitation experiments for PHB and phospho-STAT3 in colon mucosal lysates from water- and AOM DSS-treated mice. Phospho-STAT3 coimmunoprecipitated with PHB in lysates from Tg mice (Fig. 5D). Induction of CAC increased protein expression of STAT3 responsive genes Bcl-2 and Bcl-xl in both WT and Tg mice compared with water control mice. Bcl-2 and Bcl-xl levels were significantly decreased in Tg mice compared with WT mice after AOM-DSS treatment (Fig. 5E).

**PHB mRNA expression is decreased in inflamed UC and UC-associated dysplasia and inversely correlates with STAT3 and p53 mRNA expression**

Quantitative real-time PCR analysis of total RNA isolated from mucosal samples from patients with inflamed UC or UC-associated adenocarcinoma/dysplasia revealed that PHB expression is significantly decreased compared with matched normal samples (Fig. 6). p53 expression was increased in inflamed UC and UC-associated dysplasia tissues, however this increase did not achieve statistical significance in the inflamed tissue samples. STAT3 expression was markedly increased in inflamed UC and UC-associated dysplasia but did not achieve statistical significance.

**Discussion**

The specific mechanism by which IBD leads to CRC is poorly understood, but it is clear that chronic mucosal inflammation plays a causative role in the transition to adenocarcinoma. Our previous studies have shown that restoration of mucosal PHB can ameliorate experimental colitis (11, 12). For this reason, we chose to use the AOM DSS model to assess the role of PHB in CAC. Here, we show that in mice with CAC, IEC-specific PHB overexpression suppresses colonic neoplastic formation in association with increased rates of apoptosis. IEC-specific overexpression of PHB was associated with altered levels of p53, PUMA, Bax, p-Bad, Bcl-xl, and Bcl-2 during CAC. We show, for the first time, that PHB interacts with phospho-STAT3 in addition to p53 in human colon cancer cells and colon mucosa, thereby modulating the apoptotic response (Fig. 7).

IBD patients with longstanding colitis require surveillance colonoscopy after 8 years of disease duration because of the high risk of cancer development (33). Several features make CAC distinct from sporadic colon cancer. CAC is often anaplastic, broadly infiltrating, rapidly growing, develops in flat dysplastic tissue, and occurs at a younger age. While genetic alterations are similar to those in sporadic colon cancer, the frequency and sequence of these events differ in CAC (34). Loss of p53 function is an early critical event in colitis-associated neoplasia in humans in the pathway from dysplasia to cancer (35). Furthermore, aberrant IL-6/STAT3 signaling is associated with IBD and CAC (32). Activated STAT3 is enhanced in CRC patients and has been shown to induce the antiapoptotic proteins Bcl-2 and Bcl-xl (36). Inhibition of STAT3 signaling induces apoptosis of CRC cells via the mitochondrial pathway by modulating Bcl-2 and Bcl-xl (37). Here, we show that in mice with CAC, PHB suppresses colonic tumor formation in association with increased rates of apoptosis. In human colon cancer cells, PHB interacts with p53 and phospho-STAT3 and modulates their downstream apoptotic proteins. These findings suggest that PHB protects against CAC by modulating p53 and STAT3 apoptotic responses.

The best-characterized function of PHB is as a chaperone involved in the stabilization of mitochondrial proteins thereby maintaining normal mitochondrial function and morphology (reviewed in ref. 38). In most cell types studied, including IECs, PHB is predominantly localized to the mitochondria (9). However, it has been localized to the cell membrane, cytoplasm, or the nucleus in some cell types (6, 39, 40). Although PHB expression is increased in many transformed cells and tumors (13–19), in our study, colonic mucosal PHB protein expression was reduced after the development of colitis-associated tumors in WT mice. PHB expression was decreased in
mucosal samples from human patients with inflamed UC or UC-associated adenocarcinoma/dysplasia. Proinflammatory cytokines such as TNFα and reactive oxygen species decrease expression of intestinal epithelial PHB in vivo and in vitro (8–10). Thus, it is likely that PHB levels are decreased in the colonic mucosa following AOM-DSS treatment due to the inflammatory process. Tg mice with forced overexpression of PHB in IECs exhibited no inflammation-induced loss of PHB expression and showed decreased susceptibility to CAC and mortality.

PHB has been reported to have an antitumorigenic role in prostate cancer (21), gastric cancer (23), and liver cancer (22). Mice with hepatocyte-specific deletion of PHB exhibited liver injury, fibrosis, oxidative stress, and hepatocellular carcinoma developing by 8 months of age (22). Mitochondrial abnormalities in hepatocytes are evident by 3 weeks of age including no cristae formation (22). Knockdown of PHB expression by siRNA in Caco2-BBE IECs caused mitochondrial depolarization and increased intracellular reactive oxygen species (41). Interestingly, mitochondrial dysfunction is a common feature of cancer cells and multiple studies suggest that mitochondrial chaperone proteins, including PHB, modulate tumorigenesis (reviewed in ref. 42).

A functional role for PHB as a regulator of transcription has become evident due to its interactions with p53, E2F, and Rb (6, 7, 43). Here, we show that in addition to p53, PHB interacts with STAT3 in vivo and in vitro. Studies in breast cancer, prostate cancer, and retinal pigment epithelial cell lines indicate that PHB increases the transcriptional activity and prevents denaturation of p53, thereby regulating apoptosis (44–46). Our study showed that PHB suppresses colonic tumor formation in association with increased rates of apoptosis and p53 apoptotic responses. Sequencing of

---

**Figure 5.** PHB interacts with phospho-STAT3 and modulates STAT3 responsive genes, Bcl-xL and Bcl-2 in vivo and in vitro. A, representative Western blots of pSTAT3, Bcl-2, Bcl-xL protein expression in Caco2-BBE cells stably overexpressing pEGFPN1-PHB (GFP-PHB) or empty vector and treated with 100 ng/mL IL-6 basolaterally for 1 hour. Blots were subsequently probed with anti-GFP to ensure transfection efficiency. B and C, coimmunoprecipitation of PHB and pSTAT3 using cell lysates described in A. D, PHB was immunoprecipitated from colon mucosal protein lysates from water- and AOM DSS-treated mice, followed by immunoblot for pSTAT3 expression. E, representative Western blots of total protein isolated from colonic mucosal strips were probed with anti-Bcl-2, anti-Bcl-xL, anti-β-actin (loading control). Histograms show mean band densitometry ± SEM. n ≥ 5 per treatment. *, P < 0.05.
exons 5, 6, 7, and 8 of p53 from DNA isolated from areas of dysplasia from 5 WT and 5 Tg mice found no mutations. Unlike human UC-associated cancers, DSS-induced colon tumors in mice do not commonly harbor p53 mutations (26). Therefore, it is likely that the increased p53 in Tg mice is functional, unlike the increased, but mutant p53, in human IBD patients. PHB interaction with phospho-STAT3 was associated with decreased Bcl-xL and Bcl-2 expression in cultured IECs and colon mucosa. The localization of PHB in the mitochondria and nucleus makes it an ideal candidate to modulate the mitochondrial apoptotic pathway and transcription activation. Further studies will assess the mechanism of PHB modulation of STAT3 signaling. PHB interacts with p53 and STAT3 in colon mucosa from mice treated with DSS alone without AOM injection (Supplementary Fig. S1E), suggesting interaction is not dependent upon the presence of tumors. Furthermore, Tg mice treated with DSS alone without AOM injection exhibited altered mucosal p53, PUMA, Bcl-2, and Bcl-xL protein expression (Supplementary Fig. S1F). Because the development of CAC is dependent on the severity of chronic inflammation, PHB transgene expression likely decreases the occurrence of CAC by decreasing the severity of DSS-induced inflammation and enhancing p53-mediated apoptosis and inhibiting STAT3 induction of antiapoptotic genes during tumor formation. Early loss of PHB expression during inflammation may make genetically compromised IECs more resistant to cell death, thereby promoting tumorigenesis. In conclusion, PHB suppresses colonic tumor formation in association with increased rates of apoptosis in mice with CAC. PHB interacts with p53 and phospho-STAT3 in IECs in vivo and in vitro and modulates downstream apoptotic responses. Reduced levels of PHB during chronic intestinal inflammation may be an underlying factor promoting tumorigenesis. Modulation of PHB expression in IECs represents a potential therapeutic approach to prevent colitis-associated tumorigenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A.S. Kathiria, A.L. Theiss
Development of methodology: A.S. Kathiria, W.L. Neumann
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.S. Kathiria, W.L. Neumann, Y.Cheng, S.J. Meltzer, A.L. Theiss
Analysis and interpretation of data (e.g., statistical analysis, biosystems, computational analysis): A.S. Kathiria, R.M. Genta, R.F. Souza, A.L. Theiss
Writing, review, and/or revision of the manuscript: A.S. Kathiria, W.L. Neumann, S.J. Meltzer, R.F. Souza, A.L. Theiss
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Rhees, E. Hotchkiss, R.M. Genta, S.J. Meltzer
Study supervision: A.L. Theiss
Interpreting pathology slides: R.M. Genta

Acknowledgments
The authors thank the late Dr. Shanthi V. Sitaraman from Emory University, Atlanta, GA for her mentoring role with this project; Dr. Ajay Goel from Baylor Research Institute, Baylor University Medical Center, Dallas, TX for providing the HCT116 cell line as well as Bax, Bcl-2, and Bcl-xL antibodies; Dr. Linda A. Feagins from Veterans Affairs North Texas Health Care System, University of Texas Southwestern Medical Center, Dallas, TX for helpful scientific discussions. The authors also thank Dr. Shinichi Matsumoto, Ana Rahman, Jeff Stolle, and Mazhar Kanak for their assistance with tissue sectioning through the Baylor Islet Cell Transplant Program, Baylor Research Institute as well as Dr. Steven Phillips from Baylor Research Institute for his assistance with the $\gamma$-irradiator.
Prohibitin Functions as a Tumor Suppressor in CAC

Grant Support
This work was supported by NIH grants K01-DK085222 (A.L. Theiss) and R01-CA133012 (S.J. Meltzer) and funds from the Baylor Research Institute (A.L. Theiss).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 16, 2012; revised June 6, 2012; accepted June 26, 2012; published OnlineFirst August 6, 2012.

References


Published OnlineFirst August 6, 2012; DOI: 10.1158/0008-5472.CAN-12-0603


Prohibitin Attenuates Colitis-Associated Tumorigenesis in Mice by Modulating p53 and STAT3 Apoptotic Responses


Cancer Res  Published OnlineFirst August 6, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-0603

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2012/08/06/0008-5472.CAN-12-0603.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.