**Tumor Suppressor Function of the Plasma Glutathione Peroxidase Gpx3 in Colitis-Associated Carcinoma**

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Abstract

The glutathione peroxidases, a family of selenocysteine-containing redox enzymes, play pivotal roles in balancing the signaling, immunomodulatory, and deleterious effects of reactive oxygen species (ROS). The glutathione peroxidase GXP3 is the only extracellular member of this family, suggesting it may defend cells against ROS in the extracellular environment. Notably, GXP3 hypermethylated and underexpression occur commonly in prostate, gastric, cerebral, thyroid, and colon cancers. We took a reverse genetics approach to investigate whether GXP3 would augment inflammatory colonic tumorigenesis, a process characterized by oxidative stress and inflammation, comparing Gpx3−/− mice in an established two-stage model of inflammatory colon carcinogenesis. Gpx3-deficient mice exhibited an increased tumor number, though not size, along with a higher degree of dysplasia. In addition, they exhibited increased inflammation with redistribution toward protumorigenic M2 macrophage subsets, increased proliferation, hyperactive WNT signaling, and increased DNA damage. To determine the impact of acute gene loss in an established colon cancer line, we silenced GXP3 in human Caco2 cells, resulting in increased ROS production, DNA damage and apoptosis in response to oxidative stress, combined with decreased contact-independent growth. Taken together, our results suggested an immunomodulatory role for GXP3 that limits the development of colitis-associated carcinoma. *Cancer Res* 73(3); 1245–55. ©2012 AACR.

Introduction

Inflammatory bowel disease (IBD), which affects 1 in 600 Americans, is characterized by severe and chronic inflammation. Such inflammation is a known contributor to cancer as it promotes an environment rich in chemokines, cytokines, and reactive oxygen species (ROS) influencing epithelial proliferation and survival programs and impacting genomic integrity (1, 2). For example, patients with IBD show increased nitric oxide production via activated macrophages and granulocytes and increased plasma levels of the marker for DNA damage 8-hydroxydeoxyguanosine (1, 3). As such, the risk for cancer is increased 6-fold in patients with IBD compared with the general population, and cancer, or risk thereof, is a cause of significant morbidity in IBD (4).

Selenium is a necessary trace element that is present in the primary structure of selenoproteins as selenocysteine. Several epidemiologic studies have inversely correlated nutritional selenium status with colon and prostate cancer risk, although this is somewhat controversial. The effect of selenium status in IBD has not been definitively evaluated. It is proposed that the effect of selenium on carcinogenesis is realized by the selenoproteins into which it is incorporated. In support of this, mouse models with impaired selenoprotein expression show increased aberrant crypt foci, a preneoplastic colon lesion, and breast cancer incidence (5, 6). The selenocysteine present in the catalytic triad of glutathione peroxidases is optimized by hydrogen bonding with glutamate and tryptophan residues, enhancing its activity and, in the case of certain glutathione peroxidases, such as GPX1, this allows it to exert antioxidant activity (7, 8). One mechanism, though by no means the only, by which glutathione peroxidases may reduce cancer risk is by blunting the tumor-promoting effects of oxidative stress.

Gpx3, or plasma glutathione peroxidase, is the only known selenocysteine-containing extracellular form of glutathione peroxidase (Gpx) and accounts for nearly all of the glutathione peroxidase activity in plasma (9). Gpx3 mRNA is expressed in a tissue-specific manner by the kidney, heart, lung, liver, brain, adipose tissue, breast, and gastrointestinal tract, but the majority of plasma Gpx3 is kidney-derived (10–12). It is likely that other organs contribute to plasma Gpx3 as nephrectomized rats retain 30% of original glutathione peroxidase activity.
activity (13). Gpx3 is transported via circulation where it binds to the basement membranes of epithelial cells such as those in the gastrointestinal tract (14). Gpx3 promoter hypermethylation and downregulation is commonly seen in human cancer (15–18). In prostate cancer, hemizygous and homozygous deletions as well as methylation of the first exon of Gpx3 frequently occur (17). Promoter hypermethylation insures decreased expression of Gpx3 in gastric, cervical, thyroid, head and neck, and lung cancers, and in melanoma (15, 16) suggesting that Gpx3 serves as a tumor suppressor in these cancers. Genetic evidence supports a role for GPX enzymes in IBD with Gpx1/Gpx2 double knockout mice developing spontaneous ileocolitis (19) and intestinal carcinoma that is associated with bacteria-induced inflammation (20). In fact, Gpx2 alone may have a protective role in inflammatory carcinogenesis (21). The role of Gpx3 in these processes has never been directly tested.

We hypothesized that loss of Gpx3 would enhance tumorigenesis in inflammatory carcinogenesis, a process rich in ROS production and chemokine/cytokine-driven proliferative stromal signals (1, 2, 22–24). To test this, we applied the azoxymethane/dextran sodium sulfate (AOM/DSS) murine inflammatory carcinogenesis model to Gpx3−/− mice. This model has been used to identify modifiers of colitis-associated carcinoma including NF-kB (25), TLR4 (26), TNF-α (27), and Mtgr1 (28), as well as innate immune responses (29) and intestinal microflora (30). We found a striking increase in tumor number in the absence of Gpx3, indicating a direct impact of Gpx3 on tumor initiation. In support of an initiating role, Gpx3−/− mice showed increased DNA damage. Tumors in Gpx3−/− mice showed increased inflammation as well as increased infiltration of M2 macrophages, proliferation, and nuclear β-catenin. Gpx3−/− tumors were also more advanced.

As expected, knockdown of Gpx3 in the human colon cancer cell line Caco2 resulted in increased ROS production and DNA damage. Thus, we provide genetic evidence that Gpx3 behaves as a potent tumor suppressor during inflammatory carcinogenesis in vivo likely by decreasing the ROS and DNA damage that lead to tumor initiation.

Materials and Methods

Murine inflammatory carcinogenesis and chronic colitis protocols

Seven- to 8-week-old C57Bl/6 wild-type (WT; n = 17) or congenic Gpx3−/− (n = 20) mice were injected with 12.5 mg/kg of AOM (Sigma-Aldrich) intraperitoneally as described in Greten and colleagues (25). After a 3-day recovery period, the animals were started on the first of 4 cycles of 3% DSS ad libitum (see schematic in Fig. 1A). Each cycle lasted 5 days and was separated by a 16-day recovery period. A second DSS-only control arm was included for C57Bl/6 WT (n = 14) and Gpx3−/− (n = 19) that followed the same protocol outlined earlier excluding the AOM injection. After the last cycle, animals were sacrificed following a 26-day recovery period. Tumor counts and measurements were conducted in a blinded fashion under a stereo dissecting microscope. Each colon was then bisected longitudinally and half was Swiss-rolled for microscopic analysis and the other half was partitioned into normal and tumor tissue and saved for protein and RNA analysis. Microscopic analysis was conducted by a gastrointestinal pathologist (M.K. Washington) for severity of inflammation (31) and dysplasia on hematoxylin and eosin (H&E)-stained "Swiss rolled" colons (processed by the Vanderbilt Translational Pathology Shared Resource core). Four days after the third cycle of DSS, murine endoscopy was used to monitor colitis severity (32) and tumor burden. All in vitro procedures...
were carried out in accordance with protocols approved by the Vanderbilt Institutional Animal Care and Use Committee.

**Plasma glutathione peroxidase activity assay**

Blood was collected from mice via the vena cava and plasma was separated by centrifugation (K$_e$,EDTA) at 12,000 rpm for 1 minute. Glutathione peroxidase activity was determined according to a modified protocol (33) based on the method of Paglia and Valentine (34). Briefly, 800 µL reaction cocktail [50 mmol/L potassium phosphate, pH 7.0, 1 mmol/L EDTA, 1 mmol/L NaN$_3$, 0.2 mmol/L NADPH, 1 EU/mL oxidized glutathione (GSSG)-reductase, and 1 mmol/L glutathione (GSH)] was mixed with 20 µL plasma and 80 µL water in a clear plastic cuvette and incubated at room temperature for 5 minutes. Hundred microliter hydrogen peroxide (H$_2$O$_2$) substrate (0.25 mmol/L H$_2$O$_2$ in H$_2$O) was added to the reaction mixture to initiate reaction. Gpx activity was assessed by measuring the rate of change of A$_{340}$ per minute over a 3-minute time period after the addition of H$_2$O$_2$ to the reaction mixture. The enzymatic reaction is linear during this time period. The rate of change was then converted to the number of µmol NADPH oxidized per minute using the extinction coefficient of 6.2 × 10$^3$ mol$^{-1}$ cm$^{-1}$ for NADPH at 340 nm. Blank reactions with plasma replaced by distilled water were subtracted from each assay.

**Oxidative stress gene RT-PCR**

RNA was made from 30 mg WT water treated colons and normal adjacent tissue from AOM/DSS colonos using the RNeasy Mini kit (Qiagen), according to the manufacturer's directions. cDNA was then made using the SuperScript cDNA kit (Invitrogen). Real-time PCR (RT-PCR) was then conducted using an oxidative stress primer library (HOSL-1, Realtimeprimers.com). Reactions were conducted according to the manufacturer's recommendations. Analysis was conducted using the ΔΔCt method.

**Immunohistochemistry and immunofluorescence**

Five-micrometer sections were cut, dewaxed, hydrated, and endogenous peroxidase activity quenched with 0.03% H$_2$O$_2$ in MeOH. Antigen retrieval was conducted using the boiling deoxyguanosine (Abcam) 1:50; -interleukin (IL)-1a (R&D Systems), 1:50; -arginase I (Arg1; Santa Cruz), 1:500; -interleukin (IL)-1β (R&D Systems), 1:50; fluorescein isothiocyanate (FITC)-α-F4/80 (eBioscience), 1:1,000] and incubated overnight at 4°C. Isotype-matched antibodies were included as negative controls. The Vectastain ABC Elite System (Vector Laboratories) was used to visualize staining for immunohistochemistry. Identification of intratumoral apoptotic cells was conducted using the Apoptag Plus Peroxidase In Situ Apoptosis Kit (Chemicon) according to the manufacturer's protocol. Control H$_2$O$_2$ was obtained by omitting the terminal transferase (TdT) enzyme. For immunofluorescence staining of macrophage markers and DNA damage, slides were counterstained and mounted with ProLong Gold antifade including 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Gpx3 staining was conducted as described (14). Immune cell, apoptosis, and proliferation indices were generated by counting the number of positive cells per high-powered field (HPF; 40 objective) within each tumor by a blinded observer. Aβ-catenin index was used, as previously reported (28). This index is generated by multiplying the staining intensity (on a scale of 1–4) by percentage of the cells showing nuclear staining. The average score was then calculated for each Swiss-rolled colon.

**Cell culture and short hairpin RNA knockdown**

Caco2 human colon tumor cells were obtained from American Type Culture Collection (HTB-37) and had morphologic characteristics consistent with their known identity. Formal authentication was not conducted. Caco2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin. HEK293T cells were maintained in RPMI-1640 supplemented with 10% FBS and penicillin/streptomycin. HEK293T packaging cells were transfected with Mission short hairpin RNA (shRNA) constructs (Sigma-Aldrich) specific for human Gpx3 (clone ID: TRCN0000273651-NM, TRCN0000273684, and TRCN0000008678) as well as the PAX2 and pMD2.G plasmids using the calcium phosphate transfection method. Twenty-four hours posttransfection, medium was removed from the HEK293T cells and passed through a 0.45 µm filter. A total of 4 µg/mL polybrene (Millipore) was added to the filtered media. Growth medium was removed from Caco2 cells and replaced with infection medium. After 6 hours, the infection medium was replaced with normal growth medium and the infection process was repeated 24 hours later. Cells were selected for knockdown using 5 µg/mL puromycin (Invitrogen) over a 3-day period. Knockdown was analyzed by the ΔΔCt method following RT-PCR using Gpx3-specific TaqMan probes (Invitrogen) and by Western blotting using rabbit anti-mouse Gpx3 8096 (dilution 1:5,000; custom-made by Rockland).

**ROS determination**

Caco2 cells infected with either scrambled or Gpx3-specific shRNA were pretreated with 500 U/mL PEGylated catalase (PEG-CAT, Sigma) 1 hour before 1.5-hour treatment with 200 µmol/L H$_2$O$_2$. Either ROS detection reagent 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxyethyl ester; DCFH2; Invitrogen) or the oxidation insensitive analog of DCFH2 (DCF) was used to stain treated cells according to the manufacturer's protocol for flow cytometry analysis. In short, cells plated on a 6-well plate in phenol red-free medium were washed with 500 µL PBS. Cells were then resuspended in 1 µmol/L DCFH2 or 0.5 µmol/L DCF in prewarmed PBS and incubated for either 20 or 5 minutes at 37°C, respectively. Cells were then washed twice with PBS and centrifuged at 1,600 rpm for 6 minutes between washes. Cells were analyzed for positivity by flow cytometry.

**Detection of DNA damage and active caspase-3 by flow cytometry**

Caco2 (sh-scrambled) and Caco2 (sh-hGpx3) cells were treated with H$_2$O$_2$ (100 µmol/L) for 4 hours and washed with

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PBS, fixed in 0.1% paraformaldehyde, and permeabilized with 100% methanol on ice. Levels of DNA damage and active caspase-3 were assessed as described previously (35). In brief, cells were incubated with rabbit antiactive caspase-3 antibody conjugated with phycoerythrin (PE; dilution 1:50; BD Biosciences) and 8-oxoguanosine–binding peptide conjugated with FITC (dilution 1:100) for 30 minutes at room temperature. Cells were acquired with a BD LSRII system (BD Biosciences) and analyzed using FlowJo software (Star tree).

**Matrigel colony formation assay**

The day before Matrigel plating, Matrigel (BD, 354234) at 9 mg/mL was thawed overnight in ice at 4°C. For each well of a 48-well plate, 50 μL ice-cold Caco2 media (DMEM supplemented with 10% FBS and penicillin/streptomycin) was mixed with 50 μL Matrigel. Plates were then allowed to solidify at 37°C for 30 minutes. Caco2 cells were trypsinized, washed with PBS, and diluted to 10,000 cells/mL in 2 mL media. A 1:1 mixture of cells and Matrigel was added to the wells in the prepared 48-well plate and incubated at 37°C for 30 minutes. To avoid drying, 200 μL complete Caco2 media was added to each Matrigel-filled well. Cells were maintained at their normal growth conditions and media was changed every 2 to 3 days. On day 22, cells were counted using GelCount (Oxford Optronix).

**Results**

**Glutathione peroxidase activity is decreased and oxidative stress is increased in AOM/DSS-treated mice**

Gpx3 is often downregulated in cancer (15–18) and we sought to determine whether its expression or activity was altered in the AOM/DSS model of inflammatory carcinogenesis. For these experiments, 7- to 8-week-old mice were injected with AOM and treated with 4 cycles of DSS as defined in Materials and Methods and shown in Fig. 1A. During the course of this protocol, 6 Gpx3<sup>−/−</sup> mice died (lost over 20% of original body weight and were sacrificed), 2 after cycle 1, 2 after cycle 2, and 2 during cycle 4 of DSS. Although, as is typical with this protocol, 2 WT mice died, both after cycle 4, a time when we usually see mortality. These mice were not included in the final analysis. This increased morbidity suggests that Gpx3 may protect the mucosa from DSS induced injury.

As decreased Gpx3 levels are commonly seen in cancer, we first tested plasma Gpx3 activity in mice receiving water only versus mice after the AOM/DSS protocol to determine if Gpx3 activity might be altered. Measurement of oxidation of NADPH allowed us to determine that AOM/DSS leads to diminished plasma glutathione peroxidase activity (497.7 ± 56.9 vs. 239.4 ± 40.8 nmol NADPH oxidized/mL plasma/min; \( P = 0.0013 \); Fig. 1B). As this indicated that there was a systemic decrease in glutathione peroxidase activity and protein levels of Gpx3 in the plasma validated glutathione peroxidase activity assays (Supplementary Fig. S1), we next wanted to determine if local Gpx3 mRNA levels were affected in AOM/DSS tumorigenesis. There was no difference in Gpx3 mRNA expression between matched tumor and adjacent normal mucosa samples (Supplementary Fig. S2), but other oxidative stress response genes were upregulated in normal adjacent colon tissue from AOM/DSS mice (Fig. 1C) suggesting an increase in oxidant stress resulting from the protocol. Finally, we wanted to determine if Gpx3 expression varied in a large colorectal cancer expression array dataset, and in this case Gpx3 was markedly and consistently underexpressed as early as the adenoma stage (Supplementary Fig. S3A). We confirmed this observation with 10 matched normal/colon cancer samples by RT-PCR, and in a second set of 10 matched samples by protein analysis, finding reduced Gpx3 levels in 80% of the samples (Supplementary Fig. S3B and S3C). These results support previous studies (18) and suggest that Gpx3 may serve as a tumor suppressor in colorectal cancer.

**Gpx3 is a tumor suppressor in inflammatory tumorigenesis**

Using the Gpx3<sup>−/−</sup> mouse, we directly tested whether Gpx3 was a tumor suppressor in the AOM/DSS model. Seven- to 8-week-old WT or Gpx3<sup>−/−</sup> mice were placed on the AOM/DSS protocol (Fig. 1A). As expected, mice lost weight and had looser stools as the result of DSS administration in both the DSS and AOM/DSS arms of the experiment (Supplementary Fig. S4), though there was no significant difference between genotypes in these parameters. Gpx3 protein is normally seen in the colon binding the basement membrane on the basolateral surface (ref. 14; Fig. 2A). Gpx3<sup>−/−</sup> mice showed an increased endoscopic colitis score (36) during both DSS and AOM/DSS protocols (Fig. 2B) with colonic thickening, vascular changes, evidence of fibrin, increased granularity, and softer stools than wild-type mice (DSS: 12.1 ± 1.4, \( N = 6 \) vs. 6.8 ± 1.0, \( N = 6 \); AOM/ DSS: 12.1 ± 1.2, \( N = 4 \) vs. 6.9 ± 0.8, \( N = 6 \); \( P = 0.01 \) for both comparisons). Twenty-six days after the last DSS cycle the animals were sacrificed. Consistent with a role for Gpx3 in tumor suppression, quantification of tumor number in AOM/ DSS mice showed increased tumor multiplicity in Gpx3<sup>−/−</sup> mice (21.1 ± 1.6 tumors/mouse \( N = 11 \) vs. 10.8 ± 1.5 tumors/mouse \( N = 12 \); \( P < 0.0001 \)). In addition, there was a trend toward increased tumor number and size in Gpx3<sup>−/−</sup> mice receiving DSS only without AOM when compared with wild-type mice (1.0 ± 0.3 tumors/mouse \( N = 18 \) vs. 0.4 ± 0.2 tumors/mouse, \( N = 13 \); \( P = 0.1 \), Fig. 2C).

Histologic examination by a gastrointestinal pathologist (M.K. Washington) of H&E-stained sections from colons prepared as “Swiss Rolls” revealed increased injury severity based on a multiparameter histologic injury score (31) in Gpx3<sup>−/−</sup> AOM/DSS mice (16.2 ± 2.6 \( N = 14 \) vs. 7.8 ± 0.92 \( N = 16 \); \( P = 0.003 \), Fig. 3A and B) with significant increases in inflammation, inflammation extent, and crypt damage/ regeneration (Fig. 3C). Tumors were also analyzed for grade and Gpx3<sup>−/−</sup> adenomas showed increased propensity for high-grade dysplasia characterized by loss of polarity and a more complex tumor growth pattern than WT tumors (Fig. 3D). In fact, one Gpx3<sup>−/−</sup> tumor showed local invasion (Fig. 3A and D), a property rarely seen in response to AOM/DSS. These results suggest that Gpx3 loss results in increased colonic injury and inflammation and contributes to tumor promotion and progression in the AOM/DSS model of inflammatory carcinogenesis.
Altered intratumoral proliferation in the absence of Gpx3

Tumor number may be modified by increased cellular proliferation and/or survival or decreased apoptosis of transformed cells. Intratumoral proliferation rates were analyzed using in situ Ki67 staining. Gpx3−/− mice showed an average of 163.9 ± 14.1 Ki67+ cells per tumor HPF. WT mice, on the other hand, displayed 73.9 ± 15.1 Ki67+ cells per tumor HPF (P = 0.0003; Fig. 4A and B) indicating a significant increase in proliferation in Gpx3−/− tumors. No differences were seen in apoptotic indices as determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (Supplementary Fig. S5).

Increased nuclear and total β-catenin in Gpx3−/− tumors
Selenium deficiency in mice is predicted to stimulate the Wnt pathway (37). As the Wnt pathway is known to drive proproliferative and survival processes and is often hyperactive in the AOM/DSS model (38), we hypothesized that Wnt signaling, as evidenced by altered β-catenin localization, might be further perturbed in the absence of Gpx3. Therefore, we stained Gpx3−/− tumors for β-catenin localization. Gpx3−/− tumors showed a significant increase in both nuclear and total β-catenin, suggesting that the Wnt pathway is upregulated in these mice, even above that typically seen in AOM/DSS tumors (Fig. 4C and D; P = 0.03).

Increased M2 macrophage infiltrate and DNA damage in Gpx3−/− tumors
Macrophages are major modifiers of inflammation and resolution in response to injury. Their activity and motility is also significantly modified by selenium and selenoprotein modulation (39). Because Gpx3−/− mice show increased injury and inflammation in response to AOM and DSS (Fig. 3B and C), Gpx3 loss may influence intratumoral macrophage composition. Gpx3−/− tumors showed increased F4/80+ and Arg1+ protumorigenic M2 macrophages (17.1 ± 1.7 vs. 9.7 ± 1.5 F4/80+/Arg1+ cells per tumor HPF; P = 0.005; Fig. 5A and B, bottom) and decreased F4/80+ and IL-1β+ antitumorigenic M1 macrophages (10.1 ± 1.7 vs. 18.6 ± 1.8 F4/80+/IL-1β+ cells per tumor HPF; P = 0.003; Fig. 5A and B, top) compared with WT mice, suggesting that Gpx3 may have an impact on...
macrophage polarization, promoting a skewing toward protumorigenic macrophage distribution.

ROS is produced during the AOM/DSS protocol as evidenced by increased expression of oxidative stress response genes (Fig. 1C and ref. 40). Because ROS can directly induce DNA damage via oxidation and deamination of DNA bases (41), and because DNA damage is an indicator of tumorigenesis risk, we wanted to determine whether loss of Gpx3...
would lead to increased DNA damage. 8-Hydroxguanine (8-OHdG) staining was conducted to determine intratumoral DNA damage. In fact, Gpx3−/− tumors showed evidence for increased DNA damage compared with WT tumors (10.0 ± 0.8 vs. 7.1 ± 0.4 8-OHdG+ cells per HPF; \( P = 0.002 \); Fig. 5C).

**GPX3 knockdown in vitro leads to increased ROS levels and DNA damage**

To determine whether knockdown of GPX3 in vitro would recapitulate results seen in vivo we used shRNA to GPX3 in the human colon cancer cell line Caco2. Caco2 cells were used as a model cell line because they express relatively higher levels of endogenous GPX3 compared with other colon cancer cell lines (Supplementary Fig. S6A and S6B). shRNA-mediated knockdown resulted in decreased GPX3 expression both at the mRNA and protein levels (Fig. 6A). At baseline, knockdown of GPX3 had no impact on ROS production or DNA damage, though stimulation with \( \text{H}_2\text{O}_2 \) resulted in a significant increase in ROS (16; 1% vs. 7.5%; \( P = 0.0019 \)), which was abolished with pretreatment with the \( \text{H}_2\text{O}_2 \) scavenger PEG-CAT. Furthermore, GPX3 knockdown had no effect on the activity of the oxidation-insensitive DCFH2+ analog, DCF+ (Fig. 6B, right graph, compare white with black bars), indicating the results obtained with DCFH2+ were due to changes in \( \text{H}_2\text{O}_2 \) concentrations as opposed to changes in uptake, ester cleavage, or efflux of the probe. There was also a concomitant increase in DNA damage (1,401 vs. 766 8-oxoguanosine mean fluorescence units; \( P < 0.05 \); Fig. 6C) in GPX3 knockdown cells, consistent with data seen in tumors of Gpx3−/− mice. Thus, GPX3 does not seem to impact baseline oxidant parameters in vitro without application of a stressor, suggesting that its primary role is to buffer excessive \( \text{H}_2\text{O}_2 \) concentrations.

**Knockdown of GPX3 in vitro leads to increased apoptosis and decreased contact-independent growth**

Because our data suggest that Gpx3 functions as a tumor suppressor in colitis-associated carcinoma in vivo, we wanted to determine whether it modified cellular growth properties in vitro in the acute setting in an established colon cancer cell line. In contrast to in vivo modeling, in which there was no effect of Gpx3 deletion on apoptosis, knockdown of GPX3 in Caco2 cells led to increased apoptosis (26.8% vs. 15.6%; \( P < 0.001 \); Fig. 6D) and no difference in proliferation when exposed to the stressor \( \text{H}_2\text{O}_2 \). Also of note, knockdown of GPX3 led to decreased contact-independent growth in Matrigel colony formation assays (7.3 vs. 16.1 colonies; \( P < 0.001 \); Fig. 6E), indicating the potential for a dual role for Gpx3 as a tumor suppressor in chronic inflammatory carcinogenesis, whereas its loss in already established tumor cells (such as Caco2 cells) is detrimental to them.
Discussion

Chronic inflammation, as seen in ulcerative colitis and Crohn’s colitis, predisposes to malignancy (2). In fact, the risk for cancer is increased 6-fold in patients with IBD compared with the general population (4). Although the relationship between chronic inflammation and carcinogenesis is complicated and imprecisely understood, it is hypothesized that the excess ROS and reactive nitrogen species (RNS) accompanying inflammation contribute to malignancy. During chronic inflammation superoxide is produced at rates that overwhelm antioxidant systems (42, 43). When reactive oxygen and nitrogen species are not cleared efficiently, they begin to react with lipids, proteins, and DNA (44). More specifically, oxidative DNA damage leads to C/G base pair mutations, which lead to an increased frequency of C:G→T:A transversion (45), a mutation that is most frequently observed in mutated proto-oncogenes and tumor suppressor genes (46). GPX3 is an extracellular glutathione peroxidase that is able to abrogate ROS and is postulated to protect from carcinogenesis via these activities, although this has up to now not been rigorously tested using reverse genetics. We applied this approach to define the role of Gpx3 in inflammatory carcinogenesis and now show that Gpx3 is indeed a potent inhibitor of tumor promotion and progression. The impact of Gpx3 loss on tumorigenesis is likely mediated by increased inflammation and DNA damage as well as increased intratumoral proliferation that probably results in increased ROS, DNA damage, and apoptosis and decreased soft agar colony formation post-GPX3 knockdown. A, GPX3 mRNA and protein (inset) expression after knockdown with GPX3-specific shRNA constructs in Caco2 cells. **, \( P < 0.01 \). B, quantification of percentage of ROS-positive cells (DCFH2+ ) post-GPX3 knockdown in untreated or 200 μmol/L H2O2-treated Caco2 cells after pretreatment with the H2O2 scavenger PEG-CAT (left). *, \( P < 0.01 \). C, DNA damage as measured by 8-oxoguanosine-FITC flow cytometry in cells treated with scrambled or Gpx3-specific shRNAs and with or without H2O2 treatment. *, \( P < 0.05 \); $, \( P < 0.05 \). D, apoptosis, assessed as staining for active caspase-3 by flow cytometry (sidescatter-A; SSC-A). The percentage positive cells are shown. **, \( P < 0.01 \); $, \( P < 0.01 \). E, colony formation assay in Matrigel in Gpx3 knockdown and scrambled control Caco2 cells with and without H2O2 treatment. **, \( P < 0.001 \) Scr versus shGPX3 control.
from an increase in β-catenin nuclear translocation and Wnt stimulation. Loss of Gpx3 in the AOM/DSS colitis-associated carcinoma model leads to increased tumor number but not size. Gpx3−/− tumors also showed increased tumor staging suggesting that its loss also contributes to progression. In fact, one Gpx3−/− tumor showed local invasion, a characteristic that is not normally seen as the result of the AOM/DSS protocol. This is significant because it supports epidemiologic data that show that throughout the course of colorectal cancer progression, GPX3 expression is decreased (18).

While the in vivo reverse genetic experiments using the Gpx3−/− mice in the AOM/DSS protocol model allowed us to decipher the cumulative affects of the absence of Gpx3 throughout the full spectrum of transformation from normal cells to tumor cells, in vitro experiments were conducted in established cancer cells (Caco2) to isolate the contribution of Gpx3 to maintenance or promotion. As noted in vivo, Gpx3 loss resulted in increased DNA damage. Of interest, knockdown of Gpx3 in Caco2 cells resulted in a decrease in contact-independent growth and increase in apoptosis in response to H2O2, indicating that the acute absence of Gpx3 is detrimental to established carcinoma growth.

Our data show that there is increased inflammation and crypt damage and regeneration in Gpx3−/− AOM/DSS mice as compared with WT mice. Furthermore, this inflammation occurred despite a decrease in M1 and increase in M2 macrophages. It is theorized that M1 macrophages, which normally serve as proinflammatory cells, are tumoricidal and downregulated during cancer development, whereas M2 macrophages, which normally aid in the resolution of the inflammatory response, are protumorigenic and upregulated within the tumor microenvironment (47). Thus Gpx3 loss likely contributes to differential macrophage activation or recruitment that further supports tumor growth and survival. This is an interesting finding as selenoprotein deficiency leads to diminished macrophage migration (39) and selenium enhances the capacity of a host to generate cytotoxic lymphocytes and macrophages to destroy tumor cells (48, 49). Thus, our data suggest that at least part of the immune cell modifying properties of selenium are actuated by Gpx3.

Thus far, our data support a tumor suppressor role for Gpx3 via clearance of ROS. Upon knockdown of Gpx3 in the human colorectal cancer cell line Caco2, we saw an induction of ROS as well as an increase in DNA damage resulting from H2O2 treatment that supports our in vivo data. Contrary to what was originally expected, knockdown of Gpx3 did not alter proliferation at baseline or during stress as was seen in vivo. Instead, apoptosis was increased in response to H2O2 administration, a result that would be expected in cells that do not harbor the capacity to deal with oxidative stress due to the loss of the antioxidant Gpx3. Even at baseline, knockdown of Gpx3 in Caco2 cells resulted in a decrease in contact-independent cell growth, a marker of tumorigenicity. This is contrary to our in vivo findings in which Gpx3 served as a tumor suppressor. Although initially surprising, we hypothesize that the tumor suppressive role of Gpx3 exists in its ability to prevent the initiation and malignant transformation in vivo, but in cells that are already malignant, Gpx3 is able to promote tumorigenesis by protecting them from apoptosis. This also likely explains why, despite having higher proliferation rates, the tumors of Gpx3−/− mice are not larger than those of WT mice. It may be that, upon the application of a stressor such as DSS, malignant cells in Gpx3−/− mice are dying more rapidly than those in WT mice and stabilizing the tumor size.

In conclusion, we show that removal of Gpx3 enhances inflammation and injury, proliferation, nuclear β-catenin, and DNA damage in tumors of mice subjected to an inflammatory carcinogenesis protocol. The net effect of these changes leads to an increase in tumor promotion in response to Gpx3 loss. Thus, Gpx3 seems to serve as a tumor suppressor in colitis-associated carcinoma. These studies provide insight into disease pathogenesis and indicate that at least one of the selenoproteins that modifies carcinogenesis is Gpx3 and that it may serve as a substrate for translational investigations in colitis-associated carcinoma.

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

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.W. Barrett, W. Ning, J.J. Smith, M.K. Washington, K.E. Hill, R. Chaturvedi, C.S. Williams
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