GPx2 Suppression of H$_2$O$_2$ Stress Links the Formation of Differentiated Tumor Mass to Metastatic Capacity in Colorectal Cancer

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Abstract

Colorectal tumorigenesis is accompanied by the generation of oxidative stress, but how this controls tumor development is poorly understood. Here, we studied how the H$_2$O$_2$-reducing enzyme glutathione peroxidase 2 (GPx2) regulates H$_2$O$_2$ stress and differentiation in patient-derived "colonosphere" cultures. GPx2 silencing caused accumulation of radical oxygen species, sensitization to H$_2$O$_2$-induced apoptosis, and strongly reduced clone- and metastasis-forming capacity. Neutralization of radical oxygen species restored clonogenic capacity. Surprisingly, GPx2-suppressed cells also lacked differentiation potential and formed slow-growing undifferentiated tumors. GPx2 overexpression stimulated multilineage differentiation, proliferation, and tumor growth without reducing the tumor-initiating capacity. Finally, GPx2 expression was inversely correlated with H$_2$O$_2$-stress signatures in human colon tumor cohorts, but positively correlated with differentiation and proliferation. Moreover, high GPx2 expression was associated with early tumor recurrence, particularly in the recently identified aggressive subtype of human colon cancer. We conclude that H$_2$O$_2$ neutralization by GPx2 is essential for maintaining clonogenic and metastatic capacity, but also for the generation of differentiated proliferating tumor mass. The results reveal an unexpected redox-controlled link between tumor mass formation and metastatic capacity. Cancer Res; 74(22): 6717–30. ©2014 AACR.

Introduction

Oxidative stress due to accumulation of reactive oxygen species (ROS) such as superoxide or hydrogen peroxide is commonly observed in many types of cancer cells (1–3). In human colon cancer, several parameters of oxidative stress, such as lipid peroxidation and DNA hydroxylation, are increased with increasing stage (4–6). Specific ROS, like H$_2$O$_2$, can act as second messengers in protumorigenic signaling pathways. For instance, ROS generation is required for the expansion of intestinal stem cells during tumor initiation in the intestine (7). However, excessive ROS generation can also lead to cell-cycle arrest, senescence, and programmed cell death (1, 8, 9). To prevent this, tumor cells have to engage antioxidant programs to ensure redox homeostasis.

Colon tumors consist of tumor cells at different stages of differentiation. Transplantation studies of FACS-separated tumor cell subpopulations have indicated that clonogenic and tumor-initiating potential resides in a subset of undifferentiated cancer cells, frequently referred to as "cancer stem cells" (CSC) or "tumor-initiating cells" (10, 11). The factors and conditions that control maintenance of the undifferentiated clonogenic tumor cell fraction and their differentiation are incompletely defined, but redox homeostasis is likely to play an important role (12–14). How redox homeostasis is achieved in colon tumors and whether this influences differentiation is largely unclear. In the current report, we have identified the H$_2$O$_2$-scavenging enzyme intestinal glutathione peroxidase (GPx2) as a critical determinant of differentiation, tumor growth, and metastasis. GPx2 keeps intracellular H$_2$O$_2$ levels low and thereby maintains the clonogenic and metastatic tumor cell population. In addition, we demonstrate that GPx2 promotes the formation of differentiated proliferating tumor bulk without exhausting the tumor-initiating potential. The results identify redox control by GPx2 as a molecular link between the formation of differentiated tumor mass and metastatic capacity.
Materials and Methods

Collection, isolation, expansion, and tumor formation of colorectal cancer colonospheres

Collection of tumor specimens was performed as described (15). Human colorectal tumor specimens were obtained from patients undergoing a colon or liver resection for respectively primary or metastatic adenocarcinoma, in accordance with the ethical committee on human experimentation. Informed consent was obtained from all patients. The isolation, expansion, and tumor formation of colorectal cancer colonospheres was performed as described (15); for a detailed description of each colonosphere culture used, see Supplementary Methods.

Measurement of ROS

To assay intracellular ROS, cells were incubated with 10 μmol/L CM-H2DCFDA for 25 minutes at 37°C. The increase in fluorescence resulting from the oxidation of DCFDA to DCF was measured by a microplate reader with emission and excitation of 492 to 495 and 517 to 527 nm, respectively. To measure the increase in intracellular ROS levels after H2O2 treatment, cells were treated with 800 μmol/L H2O2, washed with PBS, and loaded with CM-H2DCFDA. To separate ROS viability marker 7-aminoactinomycin D (7-AAD; R&D Systems) made single cell, loaded with CM-H2DCFDA. To separate ROS fluorescence resulting from the oxidation of DCFDA to DCF and 5-CGGGATCCACGCGT GCCGCCACCATGGTGAGCAAGGGCG-3'.

Measurement of ROS

Supplementary Methods.

Transfection of colonosphere cultures

Generation of colonosphere cells stably expressing control scrambled shRNAs, shGpx2#1, or shGpx2#2 was performed as described previously (16). Briefly, 1 × 10^5 cells were transfected with 1 μg of the corresponding plasmids by using the SuperFect Reagent (Qiagen). Stable monoclonal cultures were generated by single-cell cloning and selected with 800 μg/mL Geneticin.

Generation of lentiviral Gpx2-expressing constructs

For cloning YFP-Gpx2 into the PLV-lentiviral vector, PCR-amplified Gpx2 was first cloned into pE-YFP-C1 using the following primers: forward, 5'-CGCGGATCCACGCGT GCCGCCACCATGGTGAGCAAGGGCG-3' and reverse, 5'-CAGAGTGGC-3'. The PCR product was then cloned into the PLV-lentiviral vector with human ubiquitin promoter-driven EGFP (17) into the parenchyma of the spleen of 125-day-old SCID mice using a 27-gauge needle. After 6 weeks, mice were sacrificed and tumor load in the liver was assessed in all the liver lobes. Tumor load was scored as hepatic replacement area (HRA), that is, the percentage of liver tissue that had been replaced by the tumor tissue. A fully detailed description of liver metastasis model and statistical analysis is provided in Supplementary Methods.

Cell viability assay

To measure the viability of H2O2-treated colonosphere lines, 2 × 10^5 cells were seeded in low adherent 24-well plates (Millipore). On the following day, cells were treated with H2O2, and viability was measured over time using the CellTiter 96 Aqueous Non-Radioactive Cell-Proliferation assay or the MultiTox Viability Assay (Promega) according to the manufacturer’s instructions.

Immunofluorescence

Colonospheres growing in suspension were harvested and fixed in PBS containing 4% of formaldehyde and permeabilized with ice-cold (−20°C) methanol. Cells were blocked in PBS containing 0.1% Tween and 5% BSA; cells were incubated with primary antibodies for 2 hours at room temperature or overnight at 4°C. Colonospheres were subsequently washed and incubated with secondary antibodies (goat anti-rabbit Alexa Fluor568, and goat anti-mouse Alexa Fluor647; Invitrogen) for 1 hour at room temperature. DAPI (0.5 μg/mL) was used to stain the nuclei. Images were acquired using a Zeiss LSM510 Meta Confocal microscope and Zeiss LSM5 Software. All images were acquired with identical illumination settings.

Liver metastasis model and tumor analysis

Pathogen-free, 10- to 11-week-old female SCID mice were purchased from Taconic. Colorectal liver metastases were induced by injecting single-cell suspensions (7.5 × 10^4 cells/100 μL) into the parenchyma of the spleen of 125-day-old SCID mice using a 27-gauge needle. After 6 weeks, mice were sacrificed and tumor load in the liver was assessed in all the liver lobes. Tumor load was scored as hepatic replacement area (HRA), that is, the percentage of liver tissue that had been replaced by the tumor tissue. A fully detailed description of liver metastasis model and statistical analysis is provided in Supplementary Methods.
Figure 1. GPx2 regulates intracellular H$_2$O$_2$ levels and sensitivity to H$_2$O$_2$-induced stress in colon cancer cells. A, Western blot analysis of GPx2 protein levels in L145 colonospheres following shRNA-mediated GPx2 silencing by two independent shRNAs (#1 and #2). B, FACS analysis of ROS levels using H2DCFDA probe in L145 colonospheres expressing either scrambled or GPx2-targeting shRNAs. C, fluorimetric analysis of ROS levels with H2DCFDA in L145 colonospheres expressing either scrambled or GPx2-targeting shRNAs. Data are shown as means ± SEM and represent data from three independent experiments. *P < 0.05. D, Western blot analysis of GPx2 levels in ROS-low and ROS-high cells (L145 and L167) separated by FACS-sorting on the basis of the H2DCFDA probe. E, fluorimetric analysis of ROS levels with H2DCFDA in L145 colonospheres expressing either scrambled or GPx2-targeting shRNAs after treatment with exogenous H$_2$O$_2$. The data are plotted as H$_2$O$_2$-induced increase of the absorbance values over (untreated) control values. Data are shown as means ± SEM and represent data from three independent experiments. *P < 0.05. F, analysis of cell viability in L145 colonospheres expressing either scrambled or GPx2-targeting shRNAs and rescue vectors encoding the FLAG-tag alone or GPx2-FLAG after treatment with exogenous H$_2$O$_2$ using the MultiTox-fluor Viability Probe (Promega). Data are shown as means ± SD and represent data from three independent experiments. *P < 0.05. G, analysis of cell viability in L145 colonospheres expressing either scrambled or GPx2-targeting shRNAs and rescue vectors encoding the FLAG-tag alone or GPx2-FLAG after treatment with exogenous H$_2$O$_2$ using the MultiTox-fluor Viability Probe. Data are shown as means ± SEM and represent data from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001. See also Supplementary Fig. S1.
Figure 2. GPx2 silencing generates stem-like cancer cells that lack multilineage differentiation capacity. A, FACS-analysis using the Aldefluor assay in L145 colonospheres and in HT29 cell variants expressing either scrambled, shGPx2#1, or shGPx2#2 shRNAs. Cells incubated with the Aldefluor substrate BAAA and the ALDH-specific DEAB inhibitor were used to set the gate. B, qPCR analysis of mRNA levels of GPx2 and the indicated stem-cell markers in L145 and HT29 colonospheres cells stably expressing scrambled shRNAs or shGPx2#1. (Continued on following page.)
Western blot analysis

Lysates of colonospheres were prepared either in NP-40 lysis buffer (20 mmol/L HEPES pH 7.4, 1% Nonidet P-40, 150 mmol/L NaCl, 5 mmol/L MgCl₂, and 10% glycerol), or in laemmli buffer. Equal amounts of protein were loaded on NuPAGE Novex Tris-Acetate Mini Gel (Invitrogen) and were analyzed by Western blotting.

Clone forming assay

Colonosphere cell cultures were dissociated as single cells using Accumax (Innovative Cell Technologies). Single-cell suspensions were then filtered through a 40-µm pore size nylon cell strainer (BD Falcon), and counted. Single cells were then suspended in Matrigel (500 cells in 50 µL; BD Biosciences), and allowed to set in 12-well plates at 37°C in a 5% CO₂ humidified incubator. After setting, stem-cell medium containing fresh growth factors, supplemented with or without 0.2 mmol/L NAC, was added. The number of single clones was analyzed after 2 weeks of culture. Colonies were counted using a Leica DM IRBE microscope (Leica).

Flow cytometry, cell sorting, and immunohistochemistry

The procedures of flow cytometry, cell sorting, and immunohistochemistry have been described (15, 16). A detailed description of methods is provided in Supplementary Methods.

RNA extraction and quantitative RT-qPCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. cDNA from xenografts and colonospheres was synthesized from 1 µg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). The PCR reactions were set up in a volume of 25 µL containing 4 µL diluted cDNA, 12.5 µL 2×iQ SYBR Green SuperMix (Bio-Rad), 8.25 µL H₂O and 300 mmol/L of forward and reverse primers each. The primers (Supplementary Table S5) were mRage specific to prevent signal information from contaminating genomic DNA. RPL13A and HPRT1 were used as housekeeping genes. All cDNA samples were analyzed in triplicate.

Bioinformatic analyses

All analyses were performed using the R2 microarray analysis and visualization platform (http://r2.amc.nl). A fully detailed description of how the GPx2-coexpression and differentiation signature was generated is provided in Supplementary Methods.

Statistical analysis

All values are presented as mean ± SEM. The Student t test (unpaired, two-tailed) was performed to analyze if differences between the groups are statistically significant. Differences with a P value of less than 0.05 were considered statistically significant.

Antibodies and reagents

A detailed description of all the products used is provided in Supplementary Methods.

Results

Identification of GPx2 as a key regulator of intracellular H₂O₂ levels and sensitivity to H₂O₂ stress.

Multiple antioxidant enzymes control the levels of ROS in (tumor) cells. To identify the factors regulating redox status in colon cancer, we used three independent H₂O₂-stress–reflecting gene signatures (refs. 18, 19; and http://speed.sys-bio.net) and combined them into a single H₂O₂-stress ‘metagene’ (Supplementary Table S1). Although there was little overlap between the gene sets, their expression was strongly correlated (Supplementary Table S1). Expression of the metagene was compared with the expression of the genes encoding major ROS-neutralizing enzymes in three large cohorts of patients with colon cancer (20–23). Of all the genes analyzed, only one was significantly inversely correlated with the expression of the H₂O₂-stress metagene in all the three datasets: GPx2(Supplementary Table S2).

GPx2 reduces H₂O₂ by using glutathione as a thiol substrate (24). It is mainly expressed in the epithelial cells of the gastrointestinal tract, particularly those at the proliferating crypt base (25–27) and is also highly expressed in intestinal adenomas and in colon tumors (27). This suggests that GPx2 may regulate redox homeostasis in colon cancer. To assess its function in colon cancer cells, we suppressed GPx2 expression by using two independent short-hairpin RNAs (shRNA) in patient-derived colonosphere cultures (Fig. 1A and Supplementary Fig. S1A and S2A). First, we assessed how the depletion of GPx2 would affect intracellular redox status by using the 2′, 7′dichlorofluorescein (CM-H2DCFDA) probe. GPx2-depleted cells contained significantly higher basal levels of ROS than control cells (Fig. 1B and 1C). Furthermore, cell sorting on the basis of intracellular ROS levels with CM-H2DCFDA showed that ROShigh cells express far lower levels of GPx2 than ROSlow cells (Fig. 1D). Upon treatment with increasing concentrations of H₂O₂, GPx2-depleted cells showed a significantly more pronounced increase in intracellular ROS levels when compared with the control cells (Fig. 1E) and this resulted in a concomitant sensitization to H₂O₂-induced cell death (Fig. 1F). Rescue with an shRNA-insensitive GPx2 mutant completely restored tumor cell resistance to H₂O₂-induced...
cell death (Fig. 1G). Moreover, loss of GPx2 greatly augmented apoptosis induced by loss of cell–cell adhesion (Supplementary Fig. S1B), which is known to be ROS mediated (28). In addition, the treatment of colonospheres with elesclomol or cisplatin, two drugs known to induce high intracellular ROS levels, resulted in a clear sensitization of
the GPx2-depleted cells to both the drugs (Supplementary Fig. S1C). Next, we tested how GPx2-depletion affects in vitro clone-forming potential. GPx2 silencing strongly reduced the capacity of colonospheres cells to proliferate and to form single cell-derived clones (Supplementary Fig. S1D). Next, we examined whether elevated ROS levels were the cause of reduced proliferation and clonogenic potential in GPx2-depleted tumor cells, by using the ROS scavenger N-acetylcysteine (NAC). NAC treatment largely restored the clone-forming capacity of GPx2-suppressed cells (Fig. 1H) and significantly increased the expression of the proliferation marker Ki67 (Supplementary Fig. S1E). Consistent with this observation, H2O2 treatment decreased the expression of Ki67 in control and GPx2-depleted cells (Supplementary Fig. S1F). These results identify redox control by GPx2 as an important determinant of proliferation and clonogenic capacity in colon cancer cells.

**GPx2 silencing generates stem-like cancer cells that lack multilineage differentiation capacity.**

Increased ROS levels in GPx2-suppressed colonospheres may lead to exhaustion of the fraction of tumor-initiating cells. Therefore, we analyzed how GPx2 silencing would affect the expression of markers reflecting the presence of stem-like cells and differentiation. Surprisingly, despite reducing clone-forming capacity, GPx2 silencing led to a marked increase in aldehyde dehydrogenase (ALDH) activity, which marks the clonogenic tumor cell population in these colonosphere lines (Fig. 2A; ref. 15). In addition, loss of GPx2 resulted in a strongly increased expression of several stem cell genes, including OLFM4, Nanog, Sox2, and EphB2 in three independent colonosphere cell lines (Fig. 2B and Supplementary Fig. S2B). Add-back of an shRNA-insensitive form of GPx2, or ROS neutralization with NAC, reduced stem-cell marker expression to (near) normal levels while H2O2 treatment further augmented it (Fig. 2C and Supplementary Fig. S2C). H2O2 and NAC treatment had no effect on GPx2 gene expression (Fig. 2D).

The increase in stem-cell marker expression was accompanied by a concomitant loss of differentiation markers, including cytokeratin 20 (CK20), Mucin2 (MUC2; goblet cells), and FABP1/2 (enterocytes; Fig. 2D and Supplementary Fig. S2E and S2F). Normally, differentiation marker expression increases over time as colonospheres grow from single cells, but this was completely prevented in the absence of GPx2 (Fig. 2E). Furthermore, GPx2 suppression greatly decreased basal and butyrate-induced alkaline phosphatase (ALP) activity, a marker for differentiation (Fig. 2F). Add-back of the shRNA-insensitive form of GPx2 restored ALP activity and differentiation marker expression (Fig. 2G and Supplementary Fig. S2G).

To assess whether the reduced differentiation capacity of GPx2-suppressed cells was the result of a selection process due to their apoptosis sensitivity (Fig. 1F, Supplementary Fig. S1B and S2H), we tested whether blocking apoptosis would rescue their differentiation capacity. Supplementary Fig. S2H shows that blocking apoptosis with the z-VAD peptide did not rescue differentiation in GPx2-suppressed cells. There was no effect of GPx2 suppression on markers of senescence, including the expression of p16 and nuclear heterochromatin foci (Supplementary Fig. S2H and S2I, and data not shown).

**Figure 4.** GPx2-suppressed cells lack metastatic capacity. A, photograph showing liver metastasis derived from scrambled-YFP-HT29 or shGPx2#1-YFP-HT29 cells after injection of 7.5 × 10^4 cells into the spleen of SCID mice. B, measurement of organ weight of livers and spleens derived from mice injected with scrambled-YFP-HT29 or shGPx2#1-YFP-HT29 cells. Graph, mean ± SEM; *; P < 0.05. C, graph, measurement of the HRA for each group. Graph, mean ± SEM; *; P < 0.05. D, immunostaining of paraffin-embedded tissue sections of spleen tumors and liver metastases derived from YFP-HT29 shGPx2#1-expressing cells using anti-GPx2 and anti-YFP antibodies. Dashed line, GPx2-positive patch in a shGPx2#1 spleen tumor. E, quantification of GPx2 expression (% positive tumor area and intensity of staining) in spleen and liver tumors formed by YFP-HT-29 shGPx2#1 cells.
Together, the above findings show that GPx2 loss generates a population of stem-like cancer cells that are prone to H2O2-induced stress and that have lost multilineage differentiation capacity.

**GPx2-suppressed cells form slow-growing tumors with a stem-like phenotype but lack metastatic capacity**

The above results show that GPx2 regulates both stress sensitivity and differentiation potential and may therefore affect tumor and metastasis formation. To test this, we injected control and GPx2-depleted colonospheres into immunodeficient mice. The equivalent of 1,000 cells was inoculated subcutaneously into immunodeficient mice (n = 6). Graph, mean ± SEM; *, P < 0.05.

Figure 5. Overexpression of GPx2 stimulates the formation of proliferating differentiated tumor mass. A, immunoblot analysis of GPx2 protein and the indicated differentiation markers in L169 colonospheres expressing YFP or YFP-GPx2. ‘‘, endogenous GPx2 protein. B, qPCR analysis of mRNA levels of the indicated differentiation markers was performed on L169 colonospheres expressing YFP-GPx2 protein. Data, means ± SEM and represent data from three independent experiments. ‘‘, P < 0.05. C, immunofluorescence staining of the differentiation markers MUC2, FABP1, CK20, and ChkA on control L169 colonospheres and those stably expressing YFP-GPx2. D, qPCR analysis of mRNA levels of the indicated stem-cell genes in L169 colonosphere line stably expressing YFP-GPx2 protein. Data, means ± SEM and represent data from three independent experiments. ‘‘, P < 0.05. E, tumor formation by L169 colonospheres expressing YFP or YFP-GPx2 injected subcutaneously into immunodeficient mice. n = 10. Graph, mean ± SEM; ‘‘, P < 0.05. F, subcutaneous xenografts derived from L169 colonospheres expressing YFP or YFP-GPx2 as described in E were digested into single cells, retransplanted into immunodeficient mice, and measured for tumor growth. Graph, mean ± SEM; ‘‘, P < 0.05. G, measurement of ALP activity in xenografts derived from L169 colonospheres expressing YFP or YFP-GPx2 as described in E. Graph, mean ± SEM; ‘‘, P < 0.05. H, qPCR analysis of mRNA levels of GPx2, the indicated differentiation genes, and the proliferation gene Ki67 in L169 xenografts stably expressing YFP-GPx2 protein. Graphs, mean ± SEM; ‘‘, P < 0.05. I, qPCR analysis of mRNA levels of the indicated stem-cell genes in L169 xenografts stably expressing the YFP-GPx2 protein. Graphs, mean ± SEM; ‘‘, P < 0.05. See also Supplementary Fig. S4.
Next, we investigated the requirement for GPx2 during metastasis formation. The most common site for colon cancer metastasis is the liver. To induce liver metastases, we injected control and GPx2-depleted tumor cells into the spleen, from where they reach the liver through the portal vein. Despite their stem-like phenotype, GPx2-depleted cells displayed a dramatic reduction in metastatic capacity (Fig. 4A–4C). The few metastatic nodules that did develop from GPx2-depleted cell populations had retained expression of GPx2 (Fig. 4D and 4E). These GPx2-positive cell populations were preexistent as demonstrated by the presence of GPx2-positive patches in the developing spleen tumors (Fig. 4D and 4E).

Taken together, GPx2-suppressed colonospheres form slowly proliferating tumors with an undifferentiated CSC-like phenotype, but they are unable to establish liver metastases.

Overexpression of GPx2 stimulates the formation of proliferating differentiated tumor mass

The above results suggest that GPx2 is required for the formation of differentiated proliferating tumor mass. To determine whether GPx2 levels regulate differentiation and drive tumor mass formation in vivo, we expressed flag- or YFP-tagged GPx2 in colonosphere cell lines with relatively low levels of endogenous GPx2 (Fig. 5A and Supplementary Fig. S4A). Overexpression of YFP-GPx2 caused a marked increase in the expression of markers associated with various differentiation lineages present in the colon, including MUC2 (goblet cells), FABP1 (enterocytes), and Chromogranin A (enteroendocrine cells; Fig. 5B and Supplementary Fig. S4B and S4C). In contrast, stem-cell markers were suppressed following GPx2 expression (Fig. 5D and Supplementary Fig. S4D). Similar results were obtained with Flag-GPx2–expressing colonosphere lines (data not shown). Importantly, when injected subcutaneously into immunodeficient mice, YFP-GPx2 or flag-GPx2–overexpressing colonospheres (three independent lines) grew more rapidly when compared with control (YFP-expressing) cells and with a reduced latency (Fig. 5E and Supplementary Fig. S4E). This was even more pronounced in a secondary transplantation experiment (Fig. 5F), indicating that GPx2 overexpression does not lead to exhaustion of the tumor-initiating cell fraction but rather that these cells are more prone to form rapidly proliferating and differentiating off-spring expanding the tumor mass. Tumors derived from GPx2-overexpressing colonospheres also displayed increased basal ALP activity and elevated expression of multilineage differentiation markers and the proliferation marker Ki67 (Fig. 5H and 5G), whereas stem-cell genes were strongly suppressed (Fig. 5I).

To assess whether endogenous expression levels of GPx2 correlate with proliferation and differentiation potential, we generated a lentiviral construct in which the GPx2 promoter drives the expression of GFP. Colonospheres were transduced with this construct and monoclonal cultures were generated by single-cell cloning to exclude variations in promoter activity due to differential transduction efficiency. FACS analysis showed heterogeneous expression of GFP in these cultures (Fig. 6A). Next, we used FACS sorting to separate GFP-high from GFP-low cells. This generated subpopulations of GPx2-high and GPx2-low tumor cells (Fig. 6A). GPx2-high cells expressed high levels of differentiation and proliferation markers, whereas GPx2-low cells expressed high levels of stem-cell genes (Fig. 6B and 6C). In addition, GPx2-high cells had a significantly higher tumor-initiating potential than GPx2-low cells (Fig. 6D). Collectively, our results identify GPx2 as a critical regulator of the formation of proliferating differentiated tumor mass.

GPx2 expression defines a subgroup of colon tumors displaying differentiation, proliferation, and low H2O2 stress

The data so far link GPx2 to differentiation and proliferation of patient-derived colonosphere cultures and the experimental tumors that are derived from them. We next assessed the relevance of these findings for human colon cancer. To this end, we used transcriptomic profiles of two distinct cohorts (20, 23) of colorectal tumors and identified the genes whose expression was significantly correlated with GPx2. The 100 genes that were most significantly coexpressed with GPx2 in both cohorts were identified. The overlapping gene set (53 genes; Supplementary Table S3) was then used to cluster the tumors of three large cohorts (20–23) into GPx2-expression subgroups with low, median, and high expression of GPx2 (Fig. 7A and B). GPx2-high tumors were characterized by high expression of Wnt target genes and markers of epithelial differentiation, while being inversely correlated with mesenchymal gene expression (Fig. 7B). The GPx2-high group almost completely overlapped with the well-differentiated Wnt-high colon cancer subtype 1 (CCS1; Supplementary Fig. S5A; ref. 22). Gene Set Enrichment Analysis (GSEA) shows that signatures reflecting epithelial differentiation (Supplementary Methods and Table S4), WNT pathway activity (29) and proliferation (30) are strongly enriched in GPx2-high tumors (Fig. 7C). Indeed, histologically well-differentiated human colon tumors express significantly higher levels of GPx2 (31) as well as the GPx2 coexpression signature (Supplementary Fig. S6A). Conversely, GPx2 was inversely correlated with a signature for primitive undifferentiated tumors [Nanog/Oct4/Sox2 (NOS)-target genes; Fig. 7D; ref. 32]. In line with its function as an H2O2 scavenger, GPx2 expression was also inversely correlated with expression of the H2O2-metagene (Supplementary Table S1; Fig. 7D; Supplementary Fig. S5C). Indeed, NOS-high/GPx2-low (stem cell-like) tumors displayed far higher expression of the H2O2 metagene than differentiated NOS-high/GPx2-high tumors (Fig. 7D).

While GPx2 is clearly more highly expressed in the less aggressive CCS1 subtype, its expression is also considerable in the aggressive CCS3 subtype (median 2log expression, −9). In fact, the HT29 cell line that was used for the metastasis experiments belongs to this CCS3 subgroup (22). Strikingly, when only considering the CCS3 subtype, high GPx2 expression levels were associated with a
Figure 7. GPx2 expression defines a subgroup of colon tumors characterized by epithelial differentiation, expression of Wnt targets, and proliferation. 
A, K-means clustering of tumors in the MVRM meta-cohort (n = 345) into three subgroups with high, intermediate, and low expression of GPx2 coexpressed genes. B, analysis of the expression of single genes (Wnt targets, epithelial, and mesenchymal) in the GPx2-low, -intermediate, and -high groups. C, GSEA of the enrichment of the indicated signatures in GPx2-high versus low tumors. D, correlation of expression of the H2O2 metagene with the GPx2 coexpression signature and with the Nanog/Oct4/Sox2 (NOS) signature for primitive undifferentiated tumors. The XY plot shows the inverse correlation of H2O2 metagene with the GPx2 coexpression signature. NOS<sup>high</sup> tumors are depicted in blue. GSEA shows enrichment of the GPx2 signature in NOS<sup>low</sup> tumors. See also Supplementary Fig. S5.
significantly increased risk of metastasis formation in two independent large human colon tumor cohorts (AMC-90, ref. 22; MVRM-345, refs. 20, 23; Supplementary Fig. S6B and S6C).

Discussion

In the present report, we show that GPx2 regulates two aspects of colon tumor growth. First, it is required for the survival of clonogenic tumor cells in response to H2O2 stress. Second, it is essential for the generation of proliferating differentiated tumor mass. Thus, by maintaining reductive homeostasis, GPx2 controls the balance between survival, proliferation, and differentiation. These results are in line with the general idea that ROS generation supports tumor growth, but also creates a vulnerability that may be exploited therapeutically (8, 33, 34). Although high ROS level may be associated with and contribute to tumor survival, proliferation, and differentiation. These results are required for the formation of proliferative progenitors during tumor initiation (7). However, intestinal adenoma formation also requires neutralization of excessive ROS via the metabolic enzyme TIGAR (36). In addition, the Nrf2 antioxidant program is essential for the development of lung and pancreas carcinomas in mice (37). Nrf2 induces expression of GPx2 (38) and other antioxidant enzymes, including thioredoxin reductase 1 (TR1) and peroxiredoxins. Interestingly, like GPx2-deficient tumor cells (this report), TR1-deficient tumor cells show strongly reduced tumor- and metastasis-forming capacity (39). Together, these studies highlight the importance of maintaining reductive balance during tumor initiation and progression. We propose that GPx2, possibly in concert with other anti-oxidant enzymes such as GPx1 (40), TIGAR (36), and TR1 (39), promotes survival and tumor growth in colon tumor cells by neutralizing the overproduction of ROS that is associated with tumor development. This ensures maintenance of clonogenic tumor cells and differentiation potential, resulting in tumor mass formation.

Another important aspect of the regulation of tumor development by GPx2 is inflammation. Mice deficient for both GPx1 and GPx2 show increased intestinal inflammation and spontaneous development of ileocolitis and intestinal cancer. The restoration of one GPx2 (but not GPx1) allele almost fully rescued these phenotypes (41). Furthermore, GPx2-knockout mice show severe inflammation and an increased number of tumors in a mouse model for inflammation-triggered colon cancer (42). GPx2 may suppress inflammation by inhibiting cyclooxygenase-2 (COX-2) expression or activity (16, 31). Interestingly, although tumor multiplicity was higher in the GPx2-knockout mice, presumably due to more severe inflammation, tumor size was significantly smaller (42). This is consistent with a model in which GPx2 prevents colon tumor initiation by limiting inflammation, but promotes the growth of established tumors by stimulating the formation of differentiated tumor mass (this report). In our experiments, GPx2 knockdown had no measurable effect on tumor inflammation score or tumor stroma content, as judged by histology. However, these experiments were performed in immune-deficient mice, which lack a large part of their immune cell repertoire. Therefore, the absence of differences relating to immune infiltrate and other stromal parameters in our experiments has to be interpreted carefully.

The observed requirement for GPx2 in generating differentiated tumor mass is supported by analysis of transcriptomics data, demonstrating a strong positive correlation between GPx2 expression and epithelial differentiation, proliferation, and Wnt activity. Vice versa, we found a strong negative correlation between GPx2 expression and expression of a signature for undifferentiated tumor cells and the H2O2–response metagene. Interestingly, the epithelial-type Wnt^{high} subgroup of colon tumors has a reduced propensity to develop metastases (22, 43). This seems to be at odds with our finding that GPx2 is required for metastasis formation. Strikingly, high expression of Wnt target genes is also associated with low metastatic potential (43), while being essential for the maintenance of colon cancer subgroups when analyzing gene associations with clinical outcome. Indeed, we found that GPx2 is required for metastasis formation by CCS3-type tumor cells (HT-29) and that high GPx2 levels were significantly correlated with increased risk of metastasis within that aggressive CCS3 subtype of colon tumors.

Together, the data identify GPx2 as an enzyme that is required to protect colon cancer cells against a variety of stress sources (H2O2, loss of cell–cell adhesion, and chemotherapeutic drugs). GPx2 levels are associated with metastasis formation, particularly in the aggressive CCS3 subtype, which has a primitive stem-like phenotype (22, 45) and is relatively resistant to current systemic therapy (22). We therefore propose that therapeutically increasing ROS levels, either through targeting GPx2 or by other means, may be particularly effective in sensitizing the aggressive colon tumor subtype to chemotherapy and/or in reducing their propensity to form metastases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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GPx2 Suppression of H₂O₂ Stress Links the Formation of Differentiated Tumor Mass to Metastatic Capacity in Colorectal Cancer

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