Glutathione S-Transferase π1 Promotes Tumorigenicity in HCT116 Human Colon Cancer Cells

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Abstract

GSTP1 is a member of the glutathione S-transferase enzyme superfamily, which catalyzes the conjugation of electrophiles with glutathione in the process of detoxification. GSTP1 is widely overexpressed in colorectal cancer, from aberrant crypt foci to advanced carcinomas. Increased expression of GSTP1 is associated with multidrug resistance and a worse clinical prognosis. However, GSTP1-null mice have an increased risk of tumor formation. Thus, the biological function of GSTP1 in colorectal cancer biology remains speculative. In an effort to gain further insights into the role of GSTP1 in tumorigenesis, we disrupted the GSTP1 gene in HCT116 human colorectal cancer cells using targeted homologous recombination. We find that loss of GSTP1 resulted in impaired clonogenic survival and proliferation. Specifically, under growth-limiting conditions, (a) GSTP1 protected HCT116 cells from oxidative stress and associated apoptosis and (b) promoted mitogen-activated protein kinase/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase–mediated G1/S cell cycle progression. In vivo, GSTP1 was critical for engraftment and growth of HCT116 tumor xenografts. These studies directly show that GSTP1 promotes clonogenic survival and proliferation in HCT116 human colon cancer cells. (Cancer Res 2005; 65(20): 9485-94)

Introduction

Glutathione S-transferases (GST) are phase II detoxification enzymes that catalyze the conjugation of electrophilic compounds with reduced glutathione. Based on their biochemical properties, the cytosolic GSTs are divided into α, μ, θ, π, ω, γ, θ, and ξ sub-classes. GST π1 (hereafter called GSTP1) is frequently overexpressed in many cancers, including tumors of the brain, breast, ovary, esophagus, stomach, pancreas, colon, skin, kidney, lung, bile ducts, and lymphatic and hematopoietic systems (1–6). In contrast, loss of GSTP1 expression by promoter hypermethylation is a signature of prostate cancer (7). As such, there has been considerable clinical interest in GSTP1 as a tumor marker and as a therapeutic target (8, 9).

Colorectal cancer is the second leading cause of cancer deaths in the United States. Colorectal cancer develops through multiple steps, with the sequential acquisition of genetic alterations in key tumor suppressors and oncogenes (10). GSTP1 is overexpressed in all stages of colorectal cancer, from aberrant crypt foci to advanced carcinomas (4, 6, 11, 12). However, the biological function of GSTP1 overexpression in colorectal neoplasms remains unclear (13, 14).

In their enzymatic role, GSTP1 dimers catalyze the conjugation of the sulfur atom of glutathione to electrophiles, such as reactive oxygen species (ROS), xenobiotics, and carcinogens (15). In most experimental systems, overexpression of GSTP1 in cancer cells is associated with increased resistance to anticancer agents (16, 17). However, there are contrasting reports that associate GSTP1 expression with protective agents. For example, increased GSTP1 expression is associated with butyrate induction of differentiation and apoptosis in HT29 cells (18, 19). Although these studies have shown the effects of GSTP1 on tumor responses to exogenous compounds, they have not addressed the direct effect of GSTP1 on the growth and survival of cancers.

GSTP1 monomers are also promiscuous ligandins, binding to nonenzymatic substrates, such as steroids, bile acid, and c-Jun NH2-terminal kinase (JNK; refs. 20–22). In this context of binding to the stress signaling protein JNK, there is emerging evidence that GSTP1 may affect the proliferation of cells. GSTP1-null mouse embryonic fibroblasts proliferated faster and had higher JNK levels than their wild-type counterparts, which would suggest that GSTP1 inhibits proliferation (23, 24). Forced overexpression of GSTP1 in NIH 3T3 fibroblasts increased JNK and extracellular signal-regulated kinase (ERK) phosphorylation and protected against hydrogen peroxide (H2O2)–induced cell death, but the direct effect of GSTP1 on cell proliferation was not reported (25). Specific inhibition of GSTP1 by the compound TLK199 enhanced myeloproliferation; however, HL60 leukemia cells treated with noncytotoxic doses of TLK199 had no alterations in growth, albeit increased basal activities of ERK and JNK (23). Thus, the direct role of GSTP1 overexpression in cancer cells remains speculative.

Studies in mice have shown that GSTP1 has a protective role against tumor formation. GSTP1-null mice have increased myeloproliferation and are at increased risk for carcinogen-induced skin papillomas (26). Mice deficient for GSTP1 in a wild-type p53 background developed more lung adenomas (27). Mouse embryonic fibroblasts from GSTP1-null mice exhibit increased proliferation rates compared with their wild-type counterparts and are protected against acetaminophen-induced apoptosis (23, 24, 26). Thus, the data in mice would suggest that GSTP1 is protective against tumors.

In this article, we report the consequences of genetic disruption of GSTP1 in the human colon cancer cell line HCT116 by targeted homologous recombination. We find that GSTP1 directly mediates clonogenic survival and proliferation under growth-limiting conditions. Consistent with our in vitro observations, we find that GSTP1 promotes in vivo tumor engraftment and growth.
Materials and Methods

Tissue culture. HCT116, RKO, SW480, LOVO, and HT29 cells were acquired from the American Type Culture Collection (Manassas, VA) and cultured in recommended medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (complete medium). For growth-limiting experimental conditions, cells were seeded at densities of 7.5 × 10^4, 2.0 × 10^5, 1.0 × 10^6, or 0.5 × 10^6 per cm² and cultured in medium supplemented with 10% or 1% FBS and 1% penicillin/streptomycin (28, 29).

Real-time reverse transcription-PCR analyses. Total RNA extracted from normal human colon and colon cancer tissues of three different patients were acquired from Clontech (Palo Alto, CA), and total RNA from cell lines was extracted. All total RNA was treated with DNase I and single-stranded cDNA was generated using Moloney murine leukemia virus reverse transcriptase (Bio-Rad, Hercules, CA). Real-time PCR reactions were done in triplicate on reverse transcription–derived cDNA using SYBR Green Supermix (Bio-Rad). Crossing point at which fluorescence increases appreciably above the background fluorescence was determined. Primers used were GSTP1 (5'-CCTACCCCTGTACCAGTCC-3' (forward) and 5'-GAGTATTTCAGCGAGGATG-3' (reverse) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5'-AAAGGCCTGACAACTCTT-3' (forward) and 5'-GGTGGTCCAGGGGTCTTACT-3' (reverse). PCR products met three criteria to be included in the study: (a) the signal from the reverse transcription–derived cDNA was at least 100-fold greater than that of control reactions done without reverse transcription, (b) PCR products from the reactions with reverse transcription had to be the expected size on gel electrophoresis, and (c) melt curve analyses were consistent with specificity of PCR. Relative expression of GSTP1 to GAPDH was calculated using the formula: ratio = 2^[(-crossing point GSTP1) – (crossing point GAPDH)] (30).

Strategy for disruption of the human GSTP1 gene. The endogenous GSTP1 locus, adeno-associated virus (AAV) knockout construct, and resulting targeted locus are shown in Fig. 1B. The targeting strategy is as described previously (31–34). The AAV method of transgene insertion has been shown to result primarily in single-site insertions by Southern blotting (35). The AAV technology has proven useful in the generation of somatic cell knockouts (31–34, 36). Somatic cell knockouts, in turn, have provided added insights into gene function in cancer (31, 36–39). Exon 2 of GSTP1 was targeted for disruption with an AAV cassette containing the Neo resistance gene under the constitutive control of a SV40 promoter flanked by left and right homology arms ~1 kb long. Successful disruption resulted in a 1.0-kb insertion and translation stop codons in all three reading frames. Cells exhibiting neomycin resistance were screened with locus-specific PCR. Once the first allele was successfully targeted, the Neo resistance gene was excised using Cre recombinase (Microbix Biosystems, Inc., Toronto, Ontario, Canada). The same targeting vector was then used to target the second allele. For locus-specific PCR to confirm homologous integration of the targeting vector, genomic DNA was amplified using primers specific for exon 2 of GSTP1. Loss of GSTP1 was confirmed by Western blot analyses.

Western blot analyses. Whole-cell extracts were prepared from various cell lines with radioimmunoprecipitation assay lysis buffer containing protease inhibitors (Roche, Indianapolis, IN). Proteins (50 μg) were separated by electrophoresis, transferred to nitrocellulose membranes, probed with primary and horseradish peroxidase (HRP)–coupled secondary antibodies, and visualized by chemiluminescence reagent (Perkin-Elmer, Norton, OH). At the end of the experiment, membranes were stripped and reprobed for α-tubulin to confirm equal loading. Antibodies were obtained from Oxford Biomedical Research, Inc. (Oxford, MI; rabbit anti-human GSTP1), Cell Signaling Technology [Beverly, MA; cleaved caspase-7, cleaved poly(ADP-ribose) polymerase (PARP), caspase-7, PARP, phospho–stress-activated protein kinase (SAPK)/JNK (Thr183/Tyr185), SAPK/JNK, phospho–mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) 1/2 (Ser271), phospho–ERK p42/p44 (Thr202/Tyr204), MEK1/2, ERK p42/p44, anti-rabbit HRP, and anti-mouse HRP], and Sigma (St. Louis, MO; α-tubulin). Antibody dilutions were as recommended by the manufacturer.

Measurement of cell proliferation, apoptotic index, and clonogenic survival. Cells were trypsinized, counted, and plated at various seeding densities. For cell proliferation studies, cells were harvested after 4 and 8 days and stained with trypan blue, and viable cells were counted on a hemacytometer. Doubling time was calculated as \( t = \frac{\ln 2}{N} \), where \( N \) is the number of cells at 8 days, \( t_b \) is the number of cells initially, \( t \) is time (days), and \( f \) is the frequency of cell cycles per unit time (1/day; ref. 40). For calculations of apoptotic index, cells were harvested at 4 and 8 days and stained with Hoescht 33258, and the number of cells with pyknotic nuclei and intact nuclei was counted as described previously (41). Apoptotic index was calculated as the number of pyknotic nuclei divided by the total number of cells counted per ×100 power field. For clonogenic survival assays, colonies were allowed to grow undisturbed for 2 weeks and stained with crystal violet.

Flow cytometry. For cell cycle analyses, cells were harvested, permeated with 70% ice-cold ethanol, and stained with 50 μg/mL propidium iodide in PBS containing 0.2% Tween 20 and 2.5 μg/mL RNase. DNA contents of 10,000 cells were measured on a FACS-Calibur (Becton Dickinson, Franklin Lakes, NJ) and data were analyzed using ModFit LT software (Verity Software House, Topsham, ME).

Oxidative stress assays. Cellular release of H₂O₂ was measured using the Amplex Red reagent (Molecular Probes, Carlsbad, CA). In the presence of HRP, the Amplex Red reagent (10-acycl-3,7-dihydroxynitrosoamine) reacts with H₂O₂ to produce highly fluorescent resorufin, which can be measured at 560 nm. Equal numbers of cells were harvested and incubated with HRP and Amplex red reagent, and absorbance was read every 5 minutes for 1 hour. At each time point, \( V_{max} \) was calculated, which represented the rate of H₂O₂ production. Fold increase in H₂O₂ production was calculated by dividing the \( V_{max} \) after 30 minutes of incubation by the \( V_{max} \) after 5 minutes of incubation. The lipid peroxidation product malondialdehyde was measured using the thiobarbituric acid–reactive substances (TBARS) assay. Malondialdehyde forms a 1:2 adduct with thiobarbituric acid, which can be measured at 532 nm. Cells were harvested and protein concentrations were equalized. Whole homogenates were incubated with thiobarbituric acid and centrifuged, and absorbance of the supernatant was read (Oxitec, Buffalo, NY). Results were plotted against a standard curve of known malondialdehyde concentrations. For analyses of intracellular 8-oxoguanosine, cells were harvested, fixed, and stained with the FITC-conjugated antibody against the 8-oxoguanine moiety of 8-oxoguanosine (Biotrin, Dublin, Ireland). The fluorescence intensity in 10,000 cells was measured on a FACS-Calibur cytometer. The percentage of cells with 8-oxoguanosine was calculated as the difference in fluorescence of 8-oxoguanine-FITC–stained and unstained cells. The activities of all types of superoxide dismutases (SOD) were measured using the Oxytech SOD-525 Assay (Oxytech Research, Portland, OR). Cells were harvested, disrupted by several freeze-thaw cycles, clarified by centrifugation, and assayed for SOD activity. SOD mediates an increase in the rate of auto-oxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene, which can be measured at 525 nm. Results were plotted against a standard curve of known SOD concentrations.

Treatment with mitogen-activated protein kinase-extracellular signal-regulated kinase inhibitor. Cells were treated with 10 μmol/L of the MEK inhibitor U0126 (Cell Signaling Technology). U0126 is a highly selective inhibitor of MEK1 and MEK2 (42). The concentration of 10 μmol/L is consistent with manufacturer recommendations as well as with previous studies in HCT116 cells (43). Cells were observed during incubation and harvested after 4 days for cell cycle analyses.

In vivo tumorigenesis. GSTP1+/+ and GSTP1−/− cells were grown in complete medium and harvested for in vivo studies as described previously (44, 45). Either 7.5 × 10^4 or 1.0 × 10^5 cells were implanted s.c. into the flanks of 6-week-old female athymic nu/nu mice (Charles River Labs, Wilmington, MA). Tumor sizes in two dimensions were measured with calipers, and volumes were calculated with the formula: \( V = \frac{L \times W^2}{2} \), where \( L \) is length and \( W \) is width. Mice were euthanized once overwhelmed by tumor burden as defined by animal care guidelines. Mice were housed in barrier environments, with food and water provided ad libitum as approved by the University of Michigan Animal Care and Use Committee.
**Statistics.** *In vitro* experiments were done in triplicate and repeated twice. *In vivo* experiments were done with \( n = 10 \) in each group and repeated once. Results are expressed as averages \( \pm \) SD of all experiments. Statistical analyses of data were done using Student’s paired \( t \) test and \( P < 0.05 \) were considered significant.

**Results**

**GSTP1 is highly expressed in colorectal cancer.** We examined the expression of GSTP1 in normal human colonic epithelia, primary colon cancer tissues, and colorectal cancer cell lines by real-time reverse transcription-PCR (RT-PCR) analyses (Fig. 1A). Consistent with the literature, GSTP1 is highly expressed in colon cancer.

**Targeted disruption of GSTP1 in HCT116 cells.** Somatic cell knockout technology was used to generate clones of HCT116 human colon cancer cells without GSTP1. We chose HCT116 cells because they express GSTP1, are near diploid, and have been shown to be stable in culture (31–34). We targeted exon 2 of GSTP1

Figure 1. Expression and disruption of GSTP1 in colon cancer. **A,** expression of GSTP1 relative to GAPDH by real-time RT-PCR in normal colon, colon cancer, and colon cancer cell lines. \( n = 9 \) for each column. * P < 0.01, Student’s paired \( t \) test comparing colon cancer tissues or cell lines to normal colon tissues. **B,** disruption of GSTP1. The endogenous GSTP1 locus, AAV knockout construct, and resulting targeted locus are shown. Numbered boxes, exons; triangles, loxP sites; ITR, inverted terminal repeats; HA, homology arm; P, SV40 promoter; Neo, neomycin resistance gene; pA, polyA tail. **C,** confirmation of disruption of GSTP1 by locus-specific PCR. Primers P1 and P2, which amplify exon 2, are shown on the endogenous and targeted GSTP1 locus diagrams. Lane 2, the endogenous locus contains a \( \sim \) 200-bp fragment; lanes 3 and 4 after successful targeting of the first allele and treatment with Cre recombinase, the amplification product is a larger, \( \sim \) 300-bp fragment. With successful targeting of the second allele, the amplification product is a \( \sim \) 1,650-bp fragment encompassing the Neo gene. **D,** confirmation of loss of GSTP1 by Western blot analysis with rabbit anti-human GSTP1 and mouse anti-human \( \alpha \)-tubulin (for loading).
Table 1. Cell doubling time in GSTP1+/+ and GSTP1−/− cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Seeding density (cells/cm²), ×10³</th>
<th>Doubling time (h)</th>
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<tbody>
<tr>
<td>GSTP1+/+</td>
<td>7.5</td>
<td>29 ± 4</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>24 ± 2</td>
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<td></td>
<td>1.0</td>
<td>28 ± 8</td>
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<tr>
<td></td>
<td>0.5</td>
<td>40 ± 3</td>
</tr>
<tr>
<td>GSTP1−/− clone 1</td>
<td>7.5</td>
<td>31 ± 5</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>39 ± 8*</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>121 ± 20*</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>&gt;196*</td>
</tr>
<tr>
<td>GSTP1−/− clone 2</td>
<td>7.5</td>
<td>34 ± 4</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>41 ± 7*</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>&gt;196*</td>
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<td></td>
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<td>&gt;196*</td>
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NOTE: Cells were plated at 7.5 × 10³, 2.0 × 10³, 1 × 10³, or 0.5 × 10³ per cm² and counted after 4 and 8 days in culture at 1% FBS, and cell doubling time was calculated. n = 9 for each value.

*P < 0.01, Student’s paired t test comparing GSTP1−/− to GSTP1+/+ cells in the same conditions.

for disruption as outlined in Fig. 1B. Homozygous disruption of GSTP1 was identified by genomic PCR (Fig. 1C). Loss of GSTP1 protein in the knockouts was confirmed by Western blot analysis using GSTP1 antibody (Fig. 1D). The resulting cells (hereafter called GSTP1−/− cells) are similar to their parental HCT116 (GSTP1+/+) cells, with the exception of ablated GSTP1 expression. To control for clonal variation with passage and random vector integration, parental GSTP1+/+ cells were passed in parallel with knockout cells, and one independent clone of GSTP1−/− heterozygous cells as well as two independent clones of GSTP1−/−/− cells were selected for further analyses. The two GSTP1−/− clones were isolated from different plates and were absolutely distinct. The same clones were used for all ensuing studies.

**GSTP1 is required for growth in growth-limiting conditions.**

The GSTP1+/+, GSTP1+/−, and GSTP1−/− cells were found to be indistinguishable with respect to morphology and growth rates under standard culture conditions (data not shown). To rigorously compare growth and survival, we examined the cells under low-density seeding and low-serum supplementation conditions. Low-density seeding and low-serum supplementation are growth-limiting conditions and thus stringently reflect the clonogenic and proliferative potential of cells (28, 29, 46–50). Certainly, one of the hallmarks of cancer is their continued growth in growth-limiting conditions, aptly termed oncogenic resistance to growth-limiting conditions (51).

To quantitate cell proliferation under growth-limiting conditions, cells were seeded at various densities in low serum and counted, and doubling time was calculated (Table 1). Basal doubling time for GSTP1+/+ cells was ~29 hours, which was consistent with previous reports (52). Basal doubling time for GSTP1−/− cells was ~31 to 34 hours, which was not significantly different from their GSTP1+/+ counterparts. When seeding density was decreased from 7.5 × 10³ to 2 × 10³ or 1 × 10³ cells/cm², the doubling time in GSTP1+/+ cells remained roughly the same at 24 to 28 hours. In contrast, the doubling time in both GSTP1−/− clones significantly increased to 39 to 41 hours when seeded at 2 × 10³ cells/cm² and to ≥121 hours when seeded at 1 × 10³ cells/cm². Moreover, at the lowest seeding density of 0.5 × 10³ cells/cm², GSTP1−/− cells never grew to double in number. GSTP1+/− heterozygote cells behaved similarly to GSTP1+/+ cells (data not shown). Our findings are best illustrated with clonogenic survival assays, which again show the significant decreases in colony formation in GSTP1−/− cells compared with GSTP1+/+ cells (Fig. 2A). Strikingly, at the two lowest seeding densities, GSTP1−/− colonies did not form.

Morally, GSTP1−/− cells in growth-limiting conditions seemed to undergo marked cell death, with sporadic live cells that did not form colonies. To confirm morphologic apoptosis in the cells, we stained for pyknotic nuclei and calculated apoptotic indexes. We found no significant differences in apoptotic index in cells seeded at densities of 7.5 × 10³ cells/cm² (Fig. 2B). However, starting with a lower seeding density of 2.0 × 10³ cells/cm², there was a 2-fold increase in the apoptotic index in GSTP1−/− cells compared with GSTP1+/+ cells, particularly after 8 days in culture. Furthermore, at the lowest seeding density of 0.5 × 10³ cells/cm², there was an up to 10-fold increase in the apoptotic index in GSTP1−/− cells compared with GSTP1+/+ cells.

Together, our findings show that GSTP1 is required for cell survival and proliferation in growth-limiting conditions. Notably, the differences in survival and growth at low-density seeding were not evident when the cells were cultured in 10% serum (data not shown). Furthermore, when the cells were cultured at high densities, there were no differences in their proliferation in low-serum conditions (data not shown). Thus, the protective effects of GSTP1 may be replaced by mitogens and nutrients in serum or alternatively by autocrine and/or paracrine factors in high-density seeding (53, 54).

**Effect of GSTP1 on apoptosis and G1-S progression.**

To begin to understand the mechanisms by which GSTP1-mediated cell survival and growth, we analyzed cell cycle variables. As our studies thus far have noted GSTP1-dependent differences in conditions of both low seed density and low serum, we attempted to distinguish the contribution from these two culture conditions. On the one hand, serum deprivation induces growth arrest (55, 56). On the other hand, low seeding density increases apoptosis and decreases cell colonization (47, 57). To assess the effects of low serum alone, we examined cells at a seeding density of 7.5 × 10³ cells/cm² in 10% or 1% serum supplementation. To assess the combined effects of both low serum and low seed density, we compared cells grown in 1% serum at seeding densities of 7.5 × 10³ or 2.0 × 10³ cells/cm².

We found that in both low serum alone and combined with low seed density, GSTP1+/+ cells exhibited small increases in apoptosis, with the sub-G₁ population increasing from 2% to 5% to 7% of the total population of cells (Fig. 2C). Interestingly, serum deprivation did not induce G₁-S arrest in GSTP1+/+ cells, with the G₁-S ratio remaining equivalent in both low serum alone and combined with low seed density. These findings again show that GSTP1+/+ cells have escaped normal growth control mechanisms.

Our findings in GSTP1−/− cells were more dramatic. When exposed to low serum, GSTP1−/− cells underwent G₁-S arrest as evidenced by at least 2-fold increases in the G₁-S ratio (0.8-2.3 in clone 1 and 1.1-2.5 in clone 2; Fig. 2C). In addition, the percentage of cells in the sub-G₁ phase increased from ~4% to ~12%, indicating a significant amount of apoptosis. Furthermore, when cultured in low serum combined with low seed density, the
Figure 2. Clonogenic survival, apoptosis, and cell cycle analyses in GSTP1+/+ and GSTP1-/- cells. 

A, clonogenic survival assays. Cells were seeded at the noted densities in 1% serum, incubated for 2 weeks, and stained with crystal violet.

B, apoptotic index as calculated by the number of pyknotic nuclei divided by the total number of cells. Cells were seeded at the noted densities in 1% serum and examined by Hoescht 33258 staining after 4 and 8 days in culture.

C, cell cycle analyses. Cells were seeded and cultured at the noted densities and serum supplementation for 4 days, harvested, and analyzed for DNA content. Columns, average percentage of cells in G1, S, G2/M, or sub-G1 phases. Numbers above the brackets, G1/S ratio (average percent of cells in G1 divided by average percent of cells in S phase). 

n = 3, * P < 0.01, Student’s paired t test comparing GSTP1-/- to GSTP1+/+ cells in the same conditions.
percentage of GSTP1−/− cells in the sub-G1 population increased another 2-fold to ~23% in both clones. Altogether, our findings show that GSTP1 protects from cell cycle arrest under serum deprivation and apoptosis under low seeding density conditions. To our knowledge, these are the first data to link GSTP1 to cancer cells’ resistance to growth-limiting conditions and again point to the critical role of GSTP1 in the clonogenic survival and proliferation of HCT116 cancer cells.

Effect of GSTP1 on cellular oxidative stress. As GSTP1 is important for maintaining cellular redox status (58–60), and increased cellular oxidative stress has been associated with apoptosis under low seeding densities (53, 57), we examined the effect of GSTP1 on cellular oxidative stress under growth-limiting conditions. We used four independent assays as indicators of cellular oxidative stress: cellular release of H2O2, production of the lipid peroxidation product malondialdehyde, the presence of the oxidized DNA product 8-oxoguanosine, and total SOD activity. H2O2 is a ROS generated from the breakdown of superoxide anions (61). Malondialdehyde is an aldehyde by-product of lipid peroxidation, a major mechanism of ROS-mediated cellular injury (61). ROS can also generate nucleic acids adducts, for example, the hydroxylation of 2′-deoxyguanosine to form 8-oxoguanosine (61). 8-Oxoguanosine is biologically significant, as it can induce G:C to T:A transversions in DNA replication. Thus, lipid peroxidation and DNA adduct products reflect oxidative stress and damage to the cell. SODs are metalloenzymes that catalyze the dismutation of superoxide anions into oxygen and H2O2 and thus are an indicator of cellular oxidative stress and antioxidant capacity (62).

We found that serum deprivation alone did not alter oxidative stress in both cell lines. Cellular production of H2O2 in both cell lines was not altered with serum deprivation (Fig. 3A). To determine cellular damage from oxidative stress, we measured levels of the lipid peroxidation product malondialdehyde. We found comparable basal levels of malondialdehyde in GSTP1+/+ and GSTP1+/− cells, with no significant changes after treatment with serum deprivation (Fig. 3B). In the same manner, there were no significant changes in 8-oxoguanosine after treatment with serum deprivation (Fig. 3C). Interestingly, basal SOD activity was up to 40 units/mg higher in GSTP1−/− compared with GSTP1+/− cells (Fig. 3D). With serum deprivation, both cell lines had similar increases in SOD activity (by ~5 units/mg protein in both cell lines). These findings would suggest that basal SOD antioxidant activity is up-regulated in GSTP1−/− cells, but the response to serum starvation is quantitatively similar in GSTP1−/− compared with GSTP1+/− cell lines. Altogether, our results would suggest that loss of GSTP1 did not significantly alter cellular oxidative stress under serum deprivation. As such, although cleavage of the apoptosis mediators PARP and caspase-7 were increased with serum deprivation, the increases were similar between GSTP1+/− and GSTP1−/− cells (Fig. 3E). The finding of increased basal levels of SOD in GSTP1−/− cells suggests a potential compensatory mechanism when GSTP1 is ablated.

In contrast, loss of GSTP1 resulted in marked increases in oxidative stress when the cells were cultured at low seeding density combined with low-serum conditions. The combined treatments induced significant increases in H2O2 production, malondialdehyde, 8-oxoguanosine, and SOD activity in GSTP1−/− cells compared with GSTP1+/− cells (Fig. 3A-D, last columns). This was associated with marked cleavage of the apoptosis mediators caspase-7 and PARP in GSTP1−/− cells compared with GSTP1+/− cells (Fig. 3E). Altogether, these experiments show that GSTP1 is critical for cytoprotection against oxidative stress and associated apoptosis under low-serum and low-density seeding conditions.

GSTP1 is required for mitogen-activated protein kinase-extracellular signal-regulated kinase/extracellular signal-regulated kinase signaling in growth-limiting conditions. Although our studies have shown that GSTP1 is critical for protection against oxidative stress and associated apoptosis under low-serum and low-density seeding, it remains unclear how GSTP1 mediates continued cell cycle progression under low-serum conditions. We next examined JNK and ERK phosphorylation in GSTP1+/+ and GSTP1−/− cells. The GSTP1 monomer is a direct endogenous inhibitor of the stress-activated kinase JNK in nonstressed fibroblasts (22). Furthermore, forced expression of GSTP1 has been associated with altered regulation of ERK kinase (25).

Under the least stressful culture conditions, phospho–SAPK/JNK was increased in GSTP1−/− cells compared with GSTP1+/+ cells (Fig. 4A, column 1). These data support previous findings that GSTP1 inhibits JNK phosphorylation in nonstressed cells (22). However, under the more stressful growth-limiting conditions, phospho–SAPK/JNK was equivalent in GSTP1−/− cells compared with GSTP1+/+ cells (Fig. 4A, columns 2 and 3). These data support previous findings that cellular stress induces GSTP1 dimerization and abolishes the inhibitory interaction between GSTP1 monomers and JNK (22). Altogether, our data are consistent with the published concepts that (a) GSTP1 inhibition of JNK phosphorylation occurs primarily in nonstressed cells and (b) phosphorylation of JNK becomes largely independent of GSTP1 expression in stressed cells (22, 63). Thus, in HCT116 cells, altered JNK phosphorylation is unlikely a mechanism by which GSTP1 mediates continued cell cycle progression under growth-limiting conditions.

In contrast, under growth-limiting conditions, GSTP1−/− cells exhibited significant decreases in phospho-ERK compared with GSTP1+/+ cells (Fig. 4B). ERK is a component of the MAPK pathway that controls the growth and survival of tumors (64). In fact, signals from a multitude of growth factors, cytokines, and proto-oncogenes converge on the G protein RAS, which activates the serine/threonine kinase RAF, which activates the MAPK kinase MEK, which in turn activates ERK. The MEK-ERK cascade is critical in tumor growth and progression (65). As ERK is phosphorylated by MEK, we next examined phosphorylation of MEK1 and MEK2 in our model system. We found significantly decreased phospho-MEK1/2 in GSTP1−/− cells compared with GSTP1+/+ cells (Fig. 4B). Together, these data suggest that GSTP1 is important for MEK-ERK survival signaling under growth-limiting conditions. Although it is not surprising that activation of the MEK-ERK cascade is important for tumorigenesis, the association of GSTP1 to MEK-ERK phosphorylation and clonogenic survival is novel.

To determine whether GSTP1-dependent augmentation of MEK phosphorylation was functionally contributing to the growth and survival of HCT116 cancer cells, we treated GSTP1+/+ and GSTP1−/− cells with the MEK inhibitor U0126. We reasoned that if the decreases in MEK-ERK phosphorylation in GSTP1−/− cells functionally impaired their growth in growth-limiting conditions, then (a) treatment of parental GSTP1+/+ cells with MEK inhibitors would render them more sensitive to G1-S arrest and apoptosis under growth-limiting conditions and (b) GSTP1−/− cells would be more sensitive to the effects of MEK inhibition. Indeed,
GSTP1+/+ cells treated with U0126 underwent significant G1-S arrest under serum deprivation, with a 3-fold increase in the ratio of cells in the G1 phase compared with the S phase (Fig. 4C). Under the same conditions, GSTP1−/− cells were significantly more sensitive to the effects of U0126, developing an 8-fold increase in the ratio of cells in the G1 phase compared with S phase. Thus, GSTP1-dependent MEK activation contributes to oncogenic G1-S progression in growth-limiting conditions.

Treatment with U0126 also induced significant apoptosis (Fig. 4D). In both cell lines, the percentage of the cell population in sub-G1 dramatically increased to ~10% when treated with U0126 and serum deprivation. Thus, MEK signaling is important for the survival of cells under serum deprivation. However, this aspect of MEK signaling did not seem to be dependent on GSTP1, as GSTP1−/− cells were no more sensitive to apoptosis than GSTP1+/+ cells. We were not able to address the effects of U0126 in conditions of low serum combined with low seeding density, as the cells were not viable under these conditions (data not shown). Altogether, these data point to a role for GSTP1 in mediating MEK/ERK–dependent cell cycle progression.

Figure 3. Oxidative stress analyses in GSTP1+/+ and GSTP1−/− cells. Cells were seeded and harvested after 4 days in culture for respective assays. A, Amplex red assay to determine the cellular release of H2O2. Columns, average fold increase in H2O2 production rate: Vmax at 30 minutes divided by Vmax at 5 minutes. B, TBARS assay to show malondialdehyde lipid peroxidation products. Columns, average amount of TBARS detected (nmol/mg). C, 8-oxoguanine-FITC binding protein fluorescence to show the presence of 8-oxoguanosine DNA adducts. Columns, average percent of 8-oxoguanine-FITC–stained cells that were fluorescent minus the percent of unstained cells that were fluorescent. D, SOD activity in cells. Columns, average SOD activity (units/mg protein). E, Western blots for cleaved PARP and cleaved caspase-7. Cell type, serum supplementation, and density seeding are as labeled. For 3A-3D, n = 9 for each column, with the exception of (C), where n = 3 for each columns. *, P < 0.01, Student’s paired t test comparing GSTP1−/− to GSTP1+/+ cells in the same conditions.
GSTP1 promotes in vivo tumorigenesis. Thus far, our in vitro data have shown that, under growth-limiting conditions, GSTP1 promotes continued HCT116 oncogenic cell survival and growth by (a) protecting cells from oxidative stress and associated apoptosis and (b) promoting MEK/ERK mitogenic signaling for continued G1-S progression. These cell culture–based findings prompted us to test the effects of GSTP1 in vivo.

We proceeded to implant the GSTP1+/+ cells and the two GSTP1−/− clones used in the preceding studies into the flanks of athymic nude mice. When high numbers of tumor cells (7.5 × 10⁶) were implanted, all of the tumors engrafted. However, loss of GSTP1 significantly hindered xenograft growth (Fig. 5A). After 21 days, GSTP1+/+ xenografts attained volumes that were ~3-fold that of both clones of GSTP1−/− xenografts. Interestingly, loss of GSTP1 seems to preferentially affect the lag phase rather than the log phase of tumor growth. These in vivo data are reminiscent of our in vitro findings, in which no differences were noted between GSTP1−/− and GSTP1+/+ cells at higher seeding density. Thus, the presence of GSTP1 may be particularly critical in early HCT116 xenograft growth.

We next implanted low numbers (1 × 10⁶) of tumor cells into the flanks of athymic nude mice. Based on our in vitro data, we reasoned that the presence or absence of GSTP1 would be critical in low seeding conditions. We found that all of the GSTP1+/+ cells engrafted and grew as xenografts over 35 days (Fig. 5B). In contrast, most of the GSTP1−/− cells did not engraft. Specifically, 100% of GSTP1−/− clone 1 and 60% of GSTP1−/− clone 2 did not engraft after 77 days. These results are consistent with our in vitro data and show that GSTP1 is critical for HCT116 tumor engraftment and early growth.

Discussion

Our results directly show that GSTP1 is critical for the clonogenic survival and proliferation of HCT116 human colon cancer cells. Specifically, GSTP1 promotes HCT116 tumor engraftment and growth by (a) protecting against oxidative stress and associated apoptosis and (b) augmenting MEK/ERK mitogenic signaling in growth-limiting conditions. Thus, GSTP1 mediates oncogenic survival and growth under physiologically stressful conditions.

Surprisingly, we found that GSTP1 was not critical for HCT116 cancer cell survival and growth in standard culture conditions. Furthermore, in mice xenograft studies, once a tumor mass had
formed, the presence or absence of GSTP1 did not significantly alter its growth curve. We can speculate that GSTP1 overexpression is sustained in a HCT116 tumor mass because it continues to support proliferation and detoxification; however, these functions in a larger tumor mass may be redundant.

Clonal cell survival and expansion are reminiscent of small, indolent growths in the clinical setting, such as those found in precancerous lesions, remain postoperatively, become drug resistant, or metastasize to a new site (66). It is notable that GSTP1 overexpression has been associated with these stages in colorectal cancer (6, 12, 67). Our data, integrated with the literature, would suggest that GSTP1 may contribute to clonal survival in these "low-tumor burden" stages. It is notable that GSTP1 inhibitors, such as ethacrynic acid and TLK199, have been shown to modulate tumor drug resistance (16). Based on our findings, we may postulate that an additional setting for a GSTP1 inhibitor would be low-tumor burden settings. These approaches would have to be extensively explored first, as hypermethylation of the GSTP1 promoter and loss of GSTP1 expression have been reported in prostate cancer, among other cancers (7, 68).

Certainly, further investigations are warranted and ongoing. As we were seeking to determine the effects of GSTP1 on tumorigenicity, we have yet to determine the effects of GSTP1 on drug response and metastasis in this experimental model. Furthermore, this model could be used to characterize the molecular interactions of GSTP1 with signaling proteins and for drug discovery efforts. Nevertheless, these are the first data to conclusively show that GSTP1 promotes clonogenic survival and proliferation in HCT116 human colon cancer cells.

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