

Markedly enhanced colon tumorigenesis in *Apc^{Min}* mice lacking glutathione S-transferase Pi

Kenneth J. Ritchie^a, Shaun Walsh^b, Owen J. Sansom^c, Colin J. Henderson^a, and C. Roland Wolf^{a,1}

^aCancer Research UK Molecular Pharmacology Unit, Biomedical Research Institute, Level 5, and ^bDepartment of Pathology, Ninewells Hospital and Medical School, Dundee DD1 9SY, United Kingdom; and ^cBeatson Institute for Cancer Research, Garscube Estate, Switchback Road, Glasgow G61 1BD, Scotland

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Glutathione transferases are a multigene family of proteins that catalyze the conjugation of toxic electrophiles and carcinogens to glutathione. Glutathione transferase Pi (GSTP) is commonly overexpressed in human tumors and there is emerging evidence that the enzyme has additional cellular functions in addition to its role in drug and carcinogen detoxification. To investigate the unique functions of this enzyme, we have crossed *Gstp* null mice with an initiated model of colon cancer, the *Apc^{Min}* mouse. In contrast to the *Apc^{Min/+}Gstp1/p2^{+/+}* (*Gstp*-wt *Apc^{Min}*) mice, which rarely develop colonic tumours, *Apc^{Min/+}Gstp1/p2^{-/-}* (*Gstp*-null *Apc^{Min}*) mice had a 6-fold increase in colon adenoma incidence, and a 50-fold increase in colorectal adenoma multiplicity, relative to *Gstp*-wt *Apc^{Min}*. This increase was associated with early tumor onset and decreased survival. Analysis of the biochemical changes in the colon tissue of *Gstp*-null *Apc^{Min}* mice demonstrated a marked induction of many inflammatory genes, including IL-6, IL-4, IFN- γ , and inducible nitric oxide synthase. In support of the induction of inducible nitric oxide synthase, a profound induction of nitrotyrosine adducts was observed. *Gstp* therefore appears to play a role in controlling inflammatory responses in the colon, which would explain the change in tumor incidence observed. These data also suggest that individual variation in GSTP levels may be a factor in colon cancer susceptibility.

cancer | colorectal | inflammation

Glutathione S-transferases play a key role in chemical detoxification by catalyzing the conjugation of reduced glutathione to reactive electrophiles (1). In a genetic approach to study GST functions, we have generated mice null at the glutathione transferase Pi (GSTP) *Gstp* gene locus (2). These mice develop normally, are fertile, and show no obvious abnormalities. Topical application of the tumor initiator 7,12-dimethylbenz[*a*]anthracene, followed by the promoting agent 12-*O*-tetradecanoylphorbol-13-acetate, resulted in a significant increase in the number of papillomas in null animals (2). Similarly, increased adenoma formation in the lungs of *Gstp*-null mice relative to wild-type mice was also observed following dosing with benzo[*a*]pyrene, 3-methylcholanthrene and urethane (3). In recent studies we have obtained evidence that this protein can also modulate toxicological or carcinogenic response in a manner distinct from its role in chemical detoxification (4). Further evidence of unique GSTP function is demonstrated by its ability to form protein-protein interactions and regulate the activities of several cellular proteins (JNK and TRAF2) independently of its catalytic function (5, 6). GSTP has also been shown to potentiate S-glutathionylation reactions following oxidative and nitrosative stress in vitro and in vivo (7).

To further evaluate the role of GSTP in disease processes, we have investigated whether GSTP can alter colon cancer susceptibility in the *Apc^{Min}* mouse. Mutations in the *Apc* gene is a major initiating factor in the aetiology of colorectal cancer in humans (8, 9). The *Apc^{Min/+}* mouse model of gastrointestinal tumorigenesis carries an ethylnitrosourea-induced mis-sense mutation of the adenomatous polyposis coli (*Apc*) gene at codon 850, which results in truncation of the APC protein (10). As a consequence, mutant APC is no longer able to bind β -catenin and induce its proteosomal degradation (11). β -Catenin is consequently trans-

ported into the nucleus and drives the Wnt signaling cascade, resulting in adenoma development predominantly in the small intestine of mice (10). Here, we report a profoundly increased adenoma incidence and multiplicity in the distal colon of *Apc^{Min}* mice on a *Gstp*-null background. Furthermore, we also report that the absence of GSTP in the murine colon results in an inflammatory tissue environment consistent with some of the risk factors associated with colon cancer in humans.

Results

Decreased Survival, and Increased Incidence and Multiplicity of Adenomas, in the Large Intestine of *Gstp*-Null *Apc^{Min}* Mice. *Gstp*-null mice were crossed onto *Apc^{Min}* mouse model of colorectal cancer to investigate the susceptibility of mice deficient in *Gstp* to colorectal adenomas. Early indications that *Apc^{Min/+}Gstp1/p2^{-/-}* (*Gstp*-null *Apc^{Min}*) mice had an altered phenotype was seen in the greatly increased frequency of rectal prolapse and bleeding (63% vs. 17%). *Gstp*-null *Apc^{Min}* mice also had significantly decreased survival, based on morbidity relative to *Gstp*-wt *Apc^{Min}* (median 21.5 weeks vs. >30 weeks) (Fig. 1). The colons of *Gstp*-null *Apc^{Min}* mice, killed as a result of ill-health, had a 3.5-fold increase in colorectal adenoma incidence (i.e., mice displaying at least a single adenoma; 71% vs. 20%, $n = 30$), and a 23-fold increase in colorectal adenoma multiplicity (average number of adenomas per mouse; 4.5 vs. 0.2, $n = 30$), relative to *Gstp*-wt *Apc^{Min}* colons (Fig. 2A). *Gstp*-null *Apc^{Min}* mice that survived to 30 weeks had a sixfold increase in adenoma incidence in the distal colon and a 17% increase in the mid-section of the small intestine (Fig. 2B). In contrast, in distal portions of the small intestine, a slight decrease (19%) in adenoma incidence was measured (Fig. 2B). Accompanying the increase in adenoma incidence, a 50-fold increase in adenoma multiplicity (average number of adenomas per mouse) occurred in the distal colon (Fig. 2C). Adenoma multiplicity did not differ significantly between genotypes for any segment of the small intestine investigated. The colonic adenomas in *Gstp*-null *Apc^{Min}* and *Gstp*-wt *Apc^{Min}* groups could not be distinguished on gross morphology (see Fig. 2A and D). The loss of *Gstp* therefore profoundly increased the incidence and multiplicity of colonic adenomas in *Gstp*-null *Apc^{Min}* mice.

Adenoma Pathology. The histopathology of the tumors from each genotype was very similar. Tumors were predominantly tubular adenomas with a pedunculated morphology protruding into the colonic lumen (Fig. 3A and B). There was no evidence of invasion of the underlying submucosa by neoplastic epithelial cells, and therefore none of the adenomas examined were classified as being carcinomas. Adenomas of both genotypes had high-grade dysplasia

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¹To whom correspondence should be addressed. E-mail: c.r.wolf@dundee.ac.uk.

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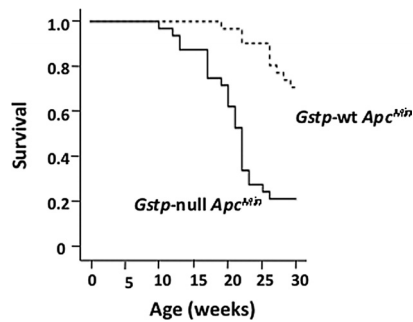


Fig. 1. Decreased survival of *Gstp*-null *Apc*^{Min} mice. Kaplan-Meier survival curve showing *Gstp*-wt *Apc*^{Min} ($n = 30$, 21 males and 9 females) and *Gstp*-null *Apc*^{Min} mice ($n = 32$, 22 males and 10 females). These data are based on observed morbidity: that is, repeated rectal prolapse, rectal bleeding, blood in feces, within the terms of the Animal (Scientific Procedures) Act (1986).

with foci of intraluminal necrosis (Fig. 3 C and D). The epithelial cells within the adenomas had a high nucleus:cytoplasm ratio and moderately prominent nucleoli. Unicryptal adenomas were also found in both genotypes and, again, did not differ in appearance (Fig. 3 E and F). In general, therefore, the adenomas found to arise in the distal colon of both *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} mice were found to be microscopically very similar to high-grade colonic adenomas in humans.

Molecular Analysis of Tumors. In addition to mutations in the *Apc* gene, human colorectal cancer has been associated with muta-

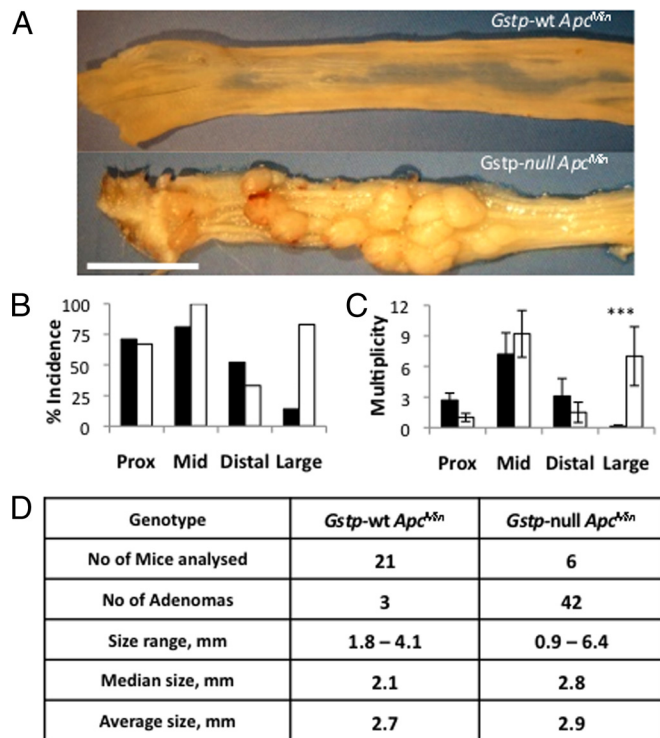


Fig. 2. Increased incidence and multiplicity of adenomas in the distal colon of *Gstp*-null *Apc*^{Min} mice. (A) Gross pathology of polyps in the distal colon of *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} mice. (Scale bar, 1 cm.) (B) Incidence (percentage of mice with one or more adenomas) and (C) multiplicity (number of adenomas per mouse) at week 30. (***, $P < 0.0001$, unpaired t test, $n = 21$ WT, 6 null). Data are mean \pm SEM. Black bars are *Gstp*-wt *Apc*^{Min}, white bars are *Gstp*-null *Apc*^{Min}. (D) Table detailing adenoma size at 30 weeks. For further details, see *Materials and Methods*.

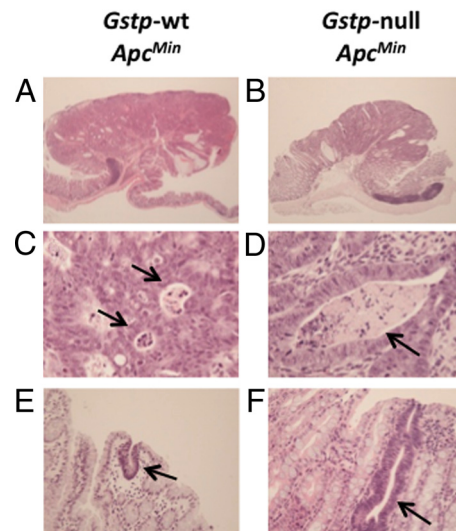


Fig. 3. Histology of colorectal adenomas. Gross histology of colorectal adenomas does not differ between (A) *Gstp*-wt *Apc*^{Min} and (B) *Gstp*-null *Apc*^{Min} genotypes. Magnification $\times 20$. Advanced areas of necrosis (arrows) present in both (C) *Gstp*-wt *Apc*^{Min} and (D) *Gstp*-null *Apc*^{Min}. Magnification $\times 400$. Examples of early adenomas (unicrypt, arrows) present in both (E) *Gstp*-wt *Apc*^{Min} and (F) *Gstp*-null *Apc*^{Min} mice. Magnification $\times 200$. For further details, see *Materials and Methods*.

tions in genes such as *K-ras* and *p53* (8), although the mutations are not always found mutated together in the same tumor (9). In our study we analyzed mutations at codons 12 and 13 of the *K-ras* gene and exons 5, 6, and 8 of the *p53* gene ($n = 19$ for *Gstp*-null *Apc*^{Min} mouse and $n = 5$ from *Gstp*-wt *Apc*^{Min} mice). No mutations in the *K-ras* or *Trp-53* genes were found.

Loss of heterozygosity (LOH) of the normal *Apc* allele is an initiating event in the development of intestinal tumorigenesis in humans and is most commonly caused as a result of mitotic recombination (12, 13). We investigated the frequency of *Apc* LOH in both *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} mice and found that LOH had occurred in all of the *Gstp*-wt *Apc*^{Min} adenomas (4 out of 4) and 94% (17 out of 18) of *Gstp*-null *Apc*^{Min} adenomas [supporting information (SI) Fig. S1]. These data demonstrate that LOH is a key event in adenoma formation in the *Gstp*-null *Apc*^{Min} mouse line.

Studies into the Mechanism of the GSTP Effects. GSTP could influence colon carcinogenesis by a number of different mechanisms. We therefore carried out a series of experiments to gain further insights into the pathways involved.

It has been reported that deletion of GSTP results in the increased proliferation of both bone marrow-derived mast cells and embryonic fibroblasts (14, 15). To investigate if there was increased proliferation in the colons of *Gstp*-null *Apc*^{Min} mice, migration of enterocytes along the crypt-villus axis was scored after exposure to BrdU for either 2 h or 48 h. No difference between *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} mice was noted in the migration rates of cells along the crypt-villus axis (Fig. 4), as noted by an equivalent shift to the right for both genotypes, indicating similar cellular proliferation rates.

Increased Inflammation in the Distal Colon of *Gstp*-Null *Apc*^{Min} Mice. mRNA expression profiles within the distal colon were determined by microarray analyses on both *Gstp1/p2*^{+/+} and *Gstp1/p2*^{-/-} animals. In *Gstp1/p2*^{-/-} colons, the expression of 308 genes was significantly increased and the expression of 202 genes significantly decreased by a factor of twofold or greater. *Gstp1* mRNA was found to be absent from *Gstp1/p2*^{-/-} colon tissue, consistent with deletion

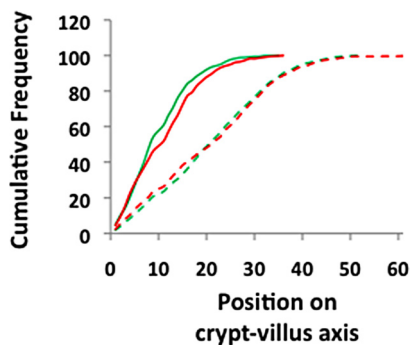


Fig. 4. No difference in migration of enterocytes in the colons of *Gstp*-null *Apc*^{Min} mice. Cumulative frequency plot showing distribution of BrdU-labeled cells along the length of the crypt-villus axis 2 and 48 h after exposure to BrdU. At 2 h there is no difference in distribution between *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} mice. At 48 h, enterocytes in both *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} mice have moved equivalent distances along the axis, indicated by a shift in the graph to the right. For all data, a minimum of three mice were used of each genotype. Solid green and red lines represent *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} mice at 2 h, respectively, while the dashed green and red lines represent *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} mice at 48 h, respectively. For further details, see *Materials and Methods*.

of the gene (Table S1). Interestingly, of the overexpressed genes, a significant number were associated with an inflammatory response, including *Saa2*, *Saa1*, *STAT1*, and *IL-4*, as well as genes involved in immune response, mast-cell proteases *Mcpt1*, 2, 4, and 9 (see Table S1). Unbiased pathway enrichment profiling of the up-regulated genes confirmed the induction of inflammation-related changes (Table S2). Genes whose expression was reduced in the distal colon of *Gstp1/p2*^{-/-} mice included genes involved in development, cell adhesion, and the cytoskeleton.

The majority of the inflammation-related changes were also found in the colons of *Gstp*-null mice carrying the *Apc*^{Min} mutation (Table S3 and S4). Particular to *Gstp*-null *Apc*^{Min} mice, however, were increases in *IL-6* (3.2-fold induction) and a 2.8-fold induction in inducible nitric oxide synthase (*iNOS*, *NOS2*), both proteins being implicated in inflammation-mediated carcinogenesis. Repression of the cryptdin related-sequences 2 and 10 (18- and 8-fold, respectively), key molecules in the prevention of bacterial damage to the epithelial cells of the intestine, was also noted. The differences in the profiles between the *Gstp*-null and *Gstp*-null *Apc*^{Min} suggests that there is some crosstalk between the *GSTP* effects and the *Apc*^{Min} phenotypes.

To investigate the inflammatory changes further, we analyzed colon tissue for the presence of mast cells, neutrophils, and macrophages by immunohistochemistry. Substantial numbers of both macrophages (Fig. 5 *A* and *B*) and neutrophils (Fig. 5 *C* and *D*) were detected in both *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} mice. Consistent with the microarray data (see Table S3), substantially increased levels of mast cells were found in the distal colon of *Gstp*-null *Apc*^{Min} mice relative to *Gstp*-wt *Apc*^{Min} colons (Fig. 5 *E* and *F*).

In addition to analysis in normal tissue, mRNA profiles were also determined in the colonic adenomas from both *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} mice (Table S5). Although genes were expressed at higher levels in *Gstp*-null *Apc*^{Min} vs. *Gstp*-wt *Apc*^{Min} adenomas, the differences were relatively small and no differences in activated pathways were clearly identifiable. Interestingly, the differences in cytokines and inflammatory-mediated expression observed in the *Gstp*-null colon were not found between the adenoma samples, suggesting that the changes arose from the infiltration of inflammatory cells rather than from the epithelial cells themselves. A striking difference between the adenoma samples was the reduction in expression

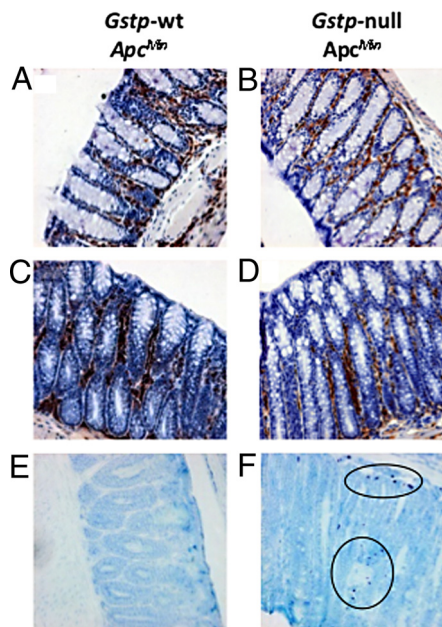


Fig. 5. Increased mast cell infiltrates in the *Gstp*-null *Apc*^{Min} colon. Immunohistochemical staining of (*A*, *C*, and *E*) *Gstp*-wt *Apc*^{Min} and (*B*, *D*, and *F*) *Gstp*-null *Apc*^{Min} was carried out to detect immune cell infiltrates. Tissues from both genotypes were found to have substantial infiltration of macrophages (*A* and *B*, magnification $\times 200$) and large numbers of neutrophils were also present in both genotypes (*C* and *D*, magnification $\times 200$). Greatly increased numbers of mast cell infiltrates were found in the colon of (*F*) *Gstp*-null *Apc*^{Min} mice relative to (*E*) *Gstp*-wt *Apc*^{Min} mice (circles indicate mast cells; magnification $\times 200$). For further details, see *Materials and Methods*.

of cryptdin related-sequences. *Defcr*-rs 2, 10, 7, and 12 were particularly prominent, and all repressed by more than a 100-fold in adenomas from *Gstp*-null *Apc*^{Min} mice.

We have previously described the altered expression of genes involved in mucin production (*Gob-5* and *Gob-4*) in the lungs of *Gstp1/p2*^{-/-} mice (3). Reduced mucin production in the intestine accompanies both inflammatory bowel disease and colon cancer and has been postulated as allowing irritation of the underlying epithelial cells by the intestinal microbiota (16). To investigate the possibility that altered mucin production, as a consequence of *Gstp* deletion, was a possible mechanism for the observed increase in inflammation noted in the distal colons of *Gstp*-null *Apc*^{Min} mice, we stained colon tissue with alcian blue to detect mucous-producing goblet cells. No difference in goblet cell numbers was noted between *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} distal colon (Fig. 6 *A* and *B*).

Given the increased expression of *iNOS* in the colon of *Gstp*-null *Apc*^{Min} mice (see Table S3), and because changes in nitric oxide production could account for the some of the inflammatory changes observed and the alteration in colon carcinogenesis, we investigated nitrotyrosine levels in the colons of *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} mice. In the absence of *Gstp*, a profound increase in nitrotyrosine levels was observed (Fig. 6 *C* and *D*). The increased production of nitric oxide could also be a consequence of an increased level of oxidative stress; however, no changes in the expression of proteins commonly induced by oxidative stress, including heme oxygenase 1, and *NQO1*, were found (Fig. S2). We also measured 8-oxo-7,8-dihydro-20-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydro-20-deoxyadenosine (8-oxodA), two of the most common DNA adduct biomarkers for oxidative stress. No difference in either of these DNA adducts was noted between *Gstp*-wt *Apc*^{Min} and *Gstp*-null *Apc*^{Min} mice (Fig. S3).

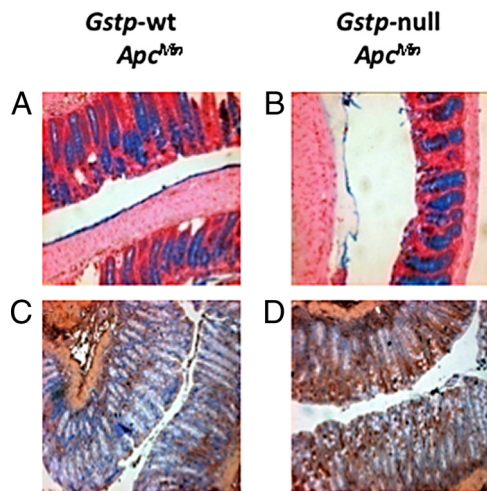


Fig. 6. Evidence of increased nitrosative stress in colons of *Gstp*-null *Apc*^{Min} mice. Alcian blue staining of goblet cells shows equivalent numbers of goblet cells in the colons of both (A) *Gstp*-wt *Apc*^{Min} and (B) *Gstp*-null *Apc*^{Min} mice (magnification $\times 200$). Significantly increased levels of nitrotyrosine staining was present in the colon of (D) *Gstp*-null *Apc*^{Min} relative to (C) *Gstp*-wt *Apc*^{Min} mice following immunohistochemical staining.

Discussion

We have investigated whether GSTP modulates pathways of colon tumorigenesis in a manner which is distinct from its role in the detoxification of carcinogenic electrophiles. The *Gstp*-null phenotype profoundly increased the incidence, and particularly the multiplicity, of colorectal adenomas in the *Apc*^{Min} mouse, providing strong evidence that this gene can alter cancer susceptibility by previously unrecorded mechanisms. This *in vivo* evidence is unique in showing that *Gstp* can alter tumorigenicity in a model that does not involve carcinogens, and suggests that variation in GSTP expression could influence colon cancer susceptibility. Human GSTP can be regulated by a number of pathways including AP1, retinoic acid, and p53 signaling (17–19). Using a mouse humanized for the *Gstp* gene, we are currently investigating whether GSTP can be regulated in the colon by chemopreventative agents such as ethoxyquin, butylated hydroxyanisole, and sulforaphane. On this basis, it would be very interesting to evaluate any changes in the incidence of colon cancer in populations receiving chemopreventative agents; for example, there is the trial in Qidong city, China, investigating the potential of Oltipraz to prevent liver cancer (20).

A number of studies have reported an increase in colon adenomas on an *Apc*^{Min} genetic background; *Apc*^{Min}: *BubR1*^{+/-} *Apc*^{Min/+} (10-fold increase), *Ephb3*^{+/-} *Apc*^{Min/+} (2-fold increase) and *Erβ*^{-/-} *Min*^{+/-} (2.5-fold) (21–23). As reported in the present study, the effects of the absence of *Gstp* on the *Apc*^{Min} background appear to be particularly profound with regards to tumor multiplicity (50-fold increase). We have carried out a number of experiments to establish the basis of the increased tumor incidence in *Gstp*-null *Apc*^{Min} mice. The absence of *Gstp* does not appear to alter the level of DNA adducts, or other markers such as heme oxygenase 1 or quinone reductase, resulting from oxidative stress, nor does it increase cell proliferation rates or mucin production in the colonic epithelium. Indeed, the most striking change in the normal colon of *Gstp*-null *Apc*^{Min} mice was in pathways linked with inflammatory response, appearing, at least in part, to be related to a significant increase in mast cell infiltration. Mast cells have also been reported as being an essential hematopoietic component of the development of adenomatous polyps (24), and microarray data from this study provided further support for their involvement with significantly increased expression of mast cell-

related genes. Increased mast cell numbers have also been observed in patients with ulcerative colitis and Crohn disease, both of which are risk factors in colon cancer susceptibility (25, 26).

Unbiased pathway enrichment analysis of the mRNA profiles suggested significant changes in pathways associated with inflammation. Related to this, normal colonic tissue from *Gstp*-null *Apc*^{Min} mice had significantly increased expression of several interleukins (IL-4 and IL-6) and interleukin receptors. Of particular note was the expression of IL-6 (3.2-fold induction), a major proinflammatory cytokine that participates in inflammation-associated carcinogenesis and which has been found to positively correlate with tumor load in colon cancer patients, and promote anchorage-independent growth of human colon carcinoma cells (27, 28). Indicative that this increase in IL-6 gene expression is of biological significance, several pathways known to be modulated by IL-6 signaling, including major inflammatory pathways such as STAT1, IFN- γ , and TGF- β (29–31), were also found to be modulated in the colon of *Gstp*-null *Apc*^{Min} mice. Interestingly, such changes in cytokine expression were not observed in the adenomas, suggesting that the changes observed arise—directly or indirectly—from the presence of infiltrating immune cells. There is increasing evidence that GSTP is involved in the regulation of inflammation (32, 33), and recent reports suggest that the Phase-2 family of drug metabolizing enzymes as a whole have a significant role to play in the suppression of inflammation (34).

Inducible nitric oxide synthase (iNOS, NOS2), was increased in the colon of *Gstp*-null *Apc*^{Min} mice, and this was reflected in a marked increase in nitrotyrosine protein adducts. There is now strong evidence that NO itself, through its proinflammatory effects, may play an important role in carcinogenesis (35). The increase in iNOS activity may well be a consequence of the proinflammatory environment in the colon of *Gstp*-null *Apc*^{Min} mice.

A further potential mechanism for the source of the increased inflammation observed in colonic tissue lacking GSTP is seen in the microarray data, which shows a striking reduction in the expression of cryptdin-related genes, both in normal tissue and in adenomas derived from *Gstp*-null mice. Cryptdins, known also as α -defensins in humans, are important antibacterial peptides secreted from the Paneth cells in the intestinal crypts, which selectively insert into the membranes of bacteria to create pores and instigate lysis (36). Significantly, a reduction in cryptdin expression is consistently seen in sufferers of inflammatory bowel disease and is thought to result in an imbalance in the amount of virulent bacteria present in the gut, leaving the area prone to immune-mediated intestinal injury (37).

GSTP therefore appears to protect the colon against the development of a pro-inflammatory environment, although the mechanisms by which GSTP may influence such pathways or the inflammatory response remains unclear. However, GSTP can modulate down-stream effector pathways of reactive nitrogen species, including the MAPK signaling cascade, in which JNK, a protein inhibited by GSTP, is an important mediator (5, 38, 39). Another intriguing possibility is the emerging role for GSTP in controlling protein glutathionylation (7).

In conclusion, the *Gstp*-null *Apc*^{Min} mouse provides an excellent model for understanding environmental influences on the aetiology of colorectal cancer and could also offer a useful system for investigating the efficacy of novel therapies for the prevention and treatment of this disease.

Methods

Reagents. All chemicals were purchased from Sigma or Fisher Scientific Ltd..

Animals. Experiments were undertaken in accordance with the Animals (Scientific Procedures) Act of 1986 and approved by the Animal Ethics Committees of the University of Dundee and Cancer Research U.K. *Gstp1/p2* null and wild-type mouse lines, on a 129 \times MF1 background, were generated and maintained by random inter-crossing to sustain a heterogeneous mixed genetic background as

reported in ref. 2. To produce *Apc^{Min/+}Gstp1/p2^{-/-}* mice (referred to as *Gstp*-null *Apc^{Min}*), *Gstp1/p2^{-/-}* mice were bred with *Apc^{Min}* mice on a C57BL/6 background to produce *Apc^{Min/+}Gstp1/p^{+/+}* mice. These mice were then crossed onto either *Gstp1/p2^{-/-}* or *Gstp1/p2^{+/+}* mice to produce *Apc^{Min/+}Gstp1/p2^{-/-}*, *Apc^{+/+}Gstp1/p2^{-/-}*, and *Apc^{+/+}Gstp1/p2^{+/+}*, *Apc^{Min/+}Gstp1/p2^{+/+}* (referred to as *Gstp*-wt *Apc^{Min}*) respectively. Animals were then either used for experiments or crossed again into *Gstp1/p2^{-/-}* or *Gstp1/p2^{+/+}* mouse genotypes to generate further *Gstp*-null *Apc^{Min}* and *Gstp*-wt *Apc^{Min}* mice.

Genotyping. For determination of *Gstp* genotype PCR was carried-out using tail DNA using the following primers:

For *Gstp1*, 5'-GGC CAC CCA ACT ACT GTG AT-3' and 5'-AGA AGG CCA GGT CCT AAA GC-3', giving a 200-bp band.

For *Lac-Z* insert, 5'-CTG TAG CGG CTG ATG TTG AA-3' and 5'-ATG GCG ATT ACC GTT GAT GT-3', giving a 309-bp band.

For detection of the *Apc^{Min}* mutation, tail DNA was genotyped using published conditions in ref. 40.

Intestinal Histopathology. Mice were killed at defined time-points or when showing signs of intestinal neoplasia, such as hunching, pale feet, swollen abdomen, and prolapse. The entire intestine was removed and the small intestine divided into three equal sections, with a fourth section comprising the entire large intestine. Intestine segments were flushed with PBS and opened longitudinally onto Whatmann 3MM paper or made into "Swiss-roll" preparations for detailed histological analyses. Both preparations were fixed overnight at 4 °C in 10% neutral buffered formalin. Adenomas were visualized by staining with 0.2% methylene blue, and identified, counted, and measured with the aid of a Zeiss dissecting microscope with Axiovision imaging software. For detailed histological analysis, H&E sections were prepared from Swiss-roll specimens by standard techniques. To assess mucin production, slides were treated with Alcian Blue (pH 2.5) for 5 min, then mounted using standard techniques.

In Vivo Migration Assays. In vivo migration was scored as described in ref. 41. To assess rates of in vivo proliferation, cellular proliferation labeling agent (Amersham) containing BrdU was given i.p at 10 μl per gram body weight. Mice were then killed at 2 h and 48 h and Swiss-roll gut preparations produced and fixed as previously described. For each analysis, 25 full crypts were scored from at least three mice of each genotype.

Immunohistochemistry. To detect BrdU incorporation and nitrotyrosine levels, sections were dewaxed and rehydrated using standard techniques and then underwent microwave antigen retrieval using 10-mM citrate buffer for 10 min. Immunohistochemistry was then carried-out using the Dako Envision staining system with a 1:50 dilution of anti-BrdU antibody (Becton Dickinson) or anti-nitrotyrosine antibody (Millipore). For detection of mast cells, neutrophils and macrophage gut tissue was fixed in methacarn (4:2:1 methanol, chloroform and glacial acetic acid) overnight at 4 °C and in 4% formalin for an additional 24 h. For the detection of mast cells, slides were stained with toluidine blue (pH 2.3) for 5 min. Macrophages were detected by probing appropriately prepared sections with a 1:1000 dilution of anti-F4/80 antibody (Abcam) and, similarly, neutrophils detected by probing sections with a 1:1,000 dilution of anti-myeloperoxidase antibody (Dako). Signals were developed by standard techniques.

Isolation of RNA. To isolate RNA from colon tissue, week-10 mice were killed by cervical dislocation and the last 5 cm of the colon flushed with PBS and immediately snap-frozen. RNA was then isolated using TRIzol (Invitrogen) and

further purified with an RNeasy Mini Kit (Qiagen). The A260/280 ratio of total RNA used was typically ≥1.9. The quality of RNA was assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies).

Microarray Analysis. Total RNA (1 μg) was labeled with Cyanine 3 (Cy3)-CTP according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol version 5.0.1 using the Low Input RNA Fluorescent Linear Amplification Kit (Agilent). Agilent 4 × 44K Whole Mouse Genome Oligo Microarray slides were hybridized and washed using Agilent hybridization and wash buffers, and scanned at 5 μm resolution on an Agilent Microarray Scanner. Images from the scanner were processed using Agilent Feature Extraction Software v9.1. The microarray scanned image and intensity files were imported into Rosetta Resolver gene-expression analysis software version 6.0.0.0.1. Individual expression profiles from the various genotypes (*Apc^{Min/+}Gstp1/p2^{-/-}*, *Apc^{+/+}Gstp1/p2^{-/-}*, and *Apc^{+/+}Gstp1/p2^{+/+}*, *Apc^{Min/+}Gstp1/p2^{+/+}*) were pooled in silico by calculating an error-weighted mean and compared to build ratios. Data were then analyzed using Bioconductor 2.2 and normalized using quantile normalization. Differential gene expression was assessed between comparison groups using an empirical Bayes *t* test. Probes that exhibited an adjusted *P* value of <0.05 and an absolute fold-change of >2 were called differentially expressed. Probes that exhibited an adjusted *P* value of <0.05 were also used for enrichment analysis using Genego's Metcore pathway tool to identify enriched pathways and biological processes.

Molecular Analysis of Adenomas. To enable molecular analysis of adenomas, small portions of colorectal adenomas were carefully harvested with the aid of a dissecting microscope to minimize stromal contamination. DNA was then isolated from each adenoma sample using the Wizard SV Genomic Purification System (Promega) according to the manufacturer's instructions. For the mutation analysis, selected regions in KRAS and p53 were amplified using the following specific oligonucleotide primers. For p53 mutational analysis, exons 5, 6, and 8 were probed for mutations using the following primers:

- Trp53E5-S (5-TCCAATGGTGTGGACAATGTG-3),
- Trp53E5-AS (5-CCTAAGAGCAAGAATAAGTCAG-3),
- Trp53E6-S (5-CTGCTCCGATGGTGATGGTAAG-3),
- Trp53E6-AS (5-CTCTAAGCCTAGCTAGCACTAG-3),
- Trp53E8-S (5-CCTTTGGCTGCAGATATGACCAAG-3),
- Trp53E8-AS (5-TGTGGAAGGAGAGAGCAAGAGGTG-3).

For mutational analysis of KRAS codons 12 or 13, the following primers were used:

- KRAS 1S (5-TTATTGTAAGGCCTGCTGAA-3),
- KRAS 1AS (5-GCAGCGTTACCTCTATCGTA-3).

PCR products for KRAS and p53 were purified using the QIAGEN PCR purification kit before sequencing. Mutations in codons were detected by direct sequencing using the previously described amplification primers. The sequencing was performed by the DNA Analysis Facility at Ninewells Hospital, Dundee, United Kingdom.

Statistical Analysis. We performed statistical analyses using GraphPad Quick-cals Online statistical calculator or SPSS. Significant differences when comparing two groups were determined by unpaired *t* test. *P* values were considered significant if they were <0.05.

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