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Lack of guanylyl cyclase C, the receptor for *Escherichia coli* heat-stable enterotoxin, results in reduced polyp formation and increased apoptosis in the multiple intestinal neoplasia (Min) mouse model

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Guanylyl cyclase C (GC-C), a transmembrane receptor for bacterial heat-stable enterotoxin and the mammalian peptides guanylin and uroguanylin, mediates intestinal ion secretion and affects intestinal cell growth via cyclic GMP signaling. In intestinal tumors, GC-C expression is maintained while guanylin and uroguanylin expression is lost, suggesting a role for GC-C activation in tumor formation or growth. We show by *in situ* hybridization that GC-C expression is retained in adenomas from multiple intestinal neoplasia ($Apc^{Min/+}$) mice. In order to determine the *in vivo* role of GC-C in intestinal tumorigenesis, we generated $Apc^{Min/+}$ mice homozygous for a targeted deletion of the gene encoding GC-C and hypothesized that these mice would have increased tumor multiplicity and size compared to wild-type $Apc^{Min/+}$ mice on the same genetic background. In contrast, the absence of GC-C resulted in a reduction of median polyp number by 55%. There was no change in the median diameter of polyps, suggesting no effect on tumor growth. Somatic loss of the wild-type *Apc* allele, an initiating event in intestinal tumorigenesis, also occurred in polyps from GC-C-deficient $Apc^{Min/+}$ mice. We have found increased levels of apoptosis as well as increased caspase-3 and caspase-7 gene expression in the intestines of GC-C-deficient $Apc^{Min/+}$ mice compared with $Apc^{Min/+}$ mice. We propose that these alterations are a possible compensatory mechanism by which loss of GC-C signaling also affects tumorigenesis.
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Key words: APC^{Min}; guanylyl cyclase; colorectal cancer; apoptosis; transgenic mice

Recent evidence has linked the incidence of enterotoxigenic bacterial infections with the incidence of colorectal cancer.[1,2] Well-developed areas of the world with the highest rates of colorectal cancer have the lowest incidence of enterotoxigenic infections, while regions where infections are endemic have the lowest rates of colorectal cancer. Several studies have established that the bacterial heat-stable enterotoxin (ST) as well as the endogenous activators of the guanylyl cyclase C (GC-C) receptor have an antiproliferative effect on the growth of intestinal cancer cells. In this study, we examine the effect of GC-C deficiency in a mouse model of intestinal tumorigenesis.

GC-C is a member of the transmembrane receptor family of guanylyl cyclases that share a common cyclic GMP (cGMP) catalytic domain but differ in their extracellular ligand binding domains.[3] In the intestine, GC-C can be activated by ST to generate high levels of cGMP, mediating a profound diarrhea. The mechanism of fluid loss is coupled to intense chloride secretion via activation of the cystic fibrosis transmembrane conductance regulator by cGMP-dependent protein kinase (PKG) II as well as inhibition of sodium absorption. These data are central to the hypothesis that GC-C is a regulator of ion and fluid balance in the intestine. In addition, it is now becoming clear that activation of GC-C and the resulting increases in cGMP play a role in the proliferation and/or apoptosis of intestinal enterocytes. Addition of exogenous uroguanylin[1] or guanylin,[4] mammalian ligands of GC-C, causes apoptosis in confluent T84 cells. A third study[5] showed that serum-stimulated proliferation was inhibited by ST or uroguanylin via a delay in cell cycle progression in the absence of apoptosis. It has recently been shown that ST exposure activates calcium influx through cyclic nucleotide-gated channels in rat colonocytes[6] and in T84 cells, where it is associated with decreased proliferation.[2] However, mice lacking GC-C, $Gucy2c^{-/-}$, are viable and fertile with no apparent defects in intestinal morphology or growth[7,8] and there are no differences in crypt depth or villus height (data not shown). Functional studies established that while colonic ion transport is similar to that observed in wild-type mice,[9] bicarbonate secretion in the duodenum of GC-C null mice is greatly attenuated in response to acid, a major physiologic stimulus of secretion.[10]

In both human and mouse intestine, GC-C is expressed in villus and crypt enterocytes of the small intestine as well as the surface epithelium and crypts of the colon.[11,12] The endogenous ligands for GC-C are expressed in an overlapping gradient, with uroguanylin expression highest in the proximal intestine and guanylin levels highest in the distal intestine.[13] By both biochemical and molecular methods, expression of GC-C has been widely observed in human primary and metastatic colorectal adenocarcinomas.[14,15] In contrast, expression of both guanylin[16,17] and uroguanylin[1] is absent or markedly decreased in colorectal tumors compared to adjacent normal tissue. Thus, a breakdown in normal signaling between GC-C and its ligands may occur in transformed tissue.

To test the hypothesis that GC-C and/or its ligands may play roles in tumor formation and/or progression, we have employed the multiple intestinal neoplasia mouse model.[18] These mice carry an inactivating mutation in the *adenomatous polyposis coli* (*Apc*) gene, Apc^{Min}, and spontaneously develop a high number of small intestinal adenomas. Similarly, patients affected by familial adenomatous polyposis (FAP) carry germ line *APC* mutations and develop numerous intestinal adenomas, although primarily in the large bowel, and are at high risk for colorectal cancer. Mutations in the *Apc* gene are also common in sporadic colorectal cancer and appear to occur early in tumorigenesis.[19] APC is a multifunctional protein implicated in a number of diverse processes, including cell proliferation, migration and programmed cell death.[20] Mutation of APC results in the dysregulation of the Wnt signaling pathway by altering levels of β-catenin. One of the ways by which APC appears to work is via transcriptional regulation. In the absence of functional APC, β-catenin translocates to the nucleus, where it forms DNA binding complexes with members of the T-cell lymphocyte factor (TCF) family. The resulting changes in expression of β-catenin/TCF target genes, such as *cyclin D1*, *c-myc* and *mdr1a*,[21–23] play an important role in the tumorigenesis

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process, affecting cell cycle regulation, enterocyte differentiation and apoptosis.

A number of genes have been identified as modifiers of intestinal adenoma multiplicity using the Apc^{Min} model. One strategy has employed naturally occurring mouse strain DNA polymorphisms to identify loci that affect the latency or multiplicity of tumors. This has resulted in the identification of the *Mom1* region on chromosome 4[24] and the *Mom2* locus on chromosome 18.[25] Another approach to investigating modifiers of Apc^{Min} has been to test the effect of the lack of a candidate gene on tumor number. In this report, we demonstrate that crossing the targeted null deletion of *Gucy2c* onto the $Apc^{Min/+}$ mouse background reduces the multiplicity of intestinal polyps by 55% without affecting tumor size.

Material and methods

In situ *hybridization*

Radiolabeled sense and antisense strand riboprobes were synthesized as described[12] from a linearized plasmid template containing 381 bp from the unique 3′ end of murine GC-C cDNA. Intestinal tissues from $Apc^{Min/+}$ mice were prepared, hybridized and processed as performed previously.[17]

Mice

Heterozygous $Gucy2c^{+/-}$ mice,[7] hybrids of 129/Ola (strain of origin of the E14TG2A embryonic stem cells used for targeting) and Black Swiss mice, were backcrossed for 5 generations to C57BL/6J mice (N_5). At this backcross generation, approximately 95% of the loci across the genome are identical to the inbred C57BL/6 partner. Female N_5 heterozygous $Gucy2c^{+/-}$ mice were then bred to male C57BL/6-$Apc^{Min/+}$ mice. Double heterozygotes were mated to $Gucy2c^{+/-}$ to generate $Apc^{Min/+}$; $Gucy2c^{+/+}$ and $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice. DNAs from tail biopsies were used to genotype the mice for the presence of Apc^{Min} and *Gucy2c* by PCR. In addition, a subset was assayed as described[25] for *Mom1*, a known modifier of tumor number.[24] All progeny tested (10/10) were homozygous (data not shown) for the susceptible allele present in the C57BL/6 strain.

Mice of both genotypes were maintained simultaneously under identical specific pathogen-free conditions on a diet containing a minimum of 9% crude fat (5058, LabDiet; PMI Nutrition International, Richmond, IN). They were sacrificed at 6 months of age, at which point genotype was reconfirmed. There was no significant difference in body weight between the 2 genotypes (25.3 ± 1.2 and 27.2 ± 1.3 g, mean ± SE, respectively, for $Apc^{Min/+}$; $Gucy2c^{+/+}$ and $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice; $p > 0.3$, unpaired *t*-test). The entire intestinal tract from duodenum to anus was removed, fixed overnight in 4% paraformaldehyde, then transferred to 70% ethanol. Tumor location, number and diameter (in mm) were independently scored under a dissecting microscope by 2 investigators unaware of the genotype. Polyps were measured to the nearest 0.5 mm; only polyps 1 mm and above were included in this study. The median tumor number and size were compared using the nonparametric Mann-Whitney test (GraphPad Prism Software, Inc., San Diego, CA).

PCR analysis of Apc locus

Identification of Apc^{+} and Apc^{Min} was accomplished as described.[26] In brief, intestinal adenomas and surrounding tissue from $Apc^{Min/+}$ and $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice were resected and embedded in paraffin. Using a hematoxylin and eosin-stained slide as a guide, tumor or normal-appearing tissue was scraped into DNA extraction buffer. Primers spanning the Apc^{Min} mutation were used to generate a radiolabeled PCR product, which was then digested with *Hin*dIII. The 2 alleles were separated by 8% polyacrylamide gel denaturing electrophoresis and visualized on exposure to Biomax MS film (Kodak, Rochester, NY). All reactions were performed in duplicate to minimize possible PCR artifacts.

RNA isolation and Northern blot analysis

RNA was isolated from whole thickness proximal jejunum of 8-week-old mice by standard procedures (Tri Reagent; Molecular Research Center, Cincinnati, OH). While this is considerably younger than the time point used to assess polyp multiplicity, the intestine is morphologically and functionally mature at weaning, and since our model is a conventional knockout, any effects of GC-C deficiency should be established. Mouse caspase-3 and caspase-7 cDNA probes were generated by RT-PCR using primers described by Chen *et al*.[27] The PCR fragments were gel-isolated and their identity confirmed by restriction enzyme analysis before use as probes. Northern blots were prepared and hybridized using conditions described previously.[12] Equal loading of RNA was demonstrated by hybridization of an 18S rRNA oligonucleotide probe.

Measurement of apoptosis

Littermates of the appropriate genotype were sacrificed at 4–6 weeks of age. The proximal duodenum was resected and scraped with a glass slide on ice to obtain the mucosa. Scraped mucosa was homogenized according to the procedure of Dunker *et al*.[28] Briefly, each aliquot was placed in phosphate-buffered saline with the addition of protease inhibitors and Dounce homogenized, followed by centrifugation at 17,900*g* for 30 min at 4°C. The protein content of the cytoplasmic fraction was measured with the BCA assay (Pierce, Rockford, IL). Approximately 300 μg was diluted 1:10 in incubation buffer (supplied in the Cell Death Detection ELISA kit; Roche, Mannheim, Germany) and the amount of nucleosomes was quantified in triplicate by ELISA for each aliquot. Absorbance values are corrected for background values and protein content. Statistical significance was evaluated by unpaired *t*-test (GraphPad Prism).

Terminal deoxynucleotidyl transferase-mediated dNTP-biotin nick end labeling (TUNEL) assay

Deparaffinized sections of mouse intestine were digested with proteinase K solution (20 μg/ml; Gibco-BRL, Gaithersburg, MD) for 20 min at room temperature. Slides were rinsed in water and treated with 0.5% H_2O_2 for 10 min at room temperature. Test slides were incubated in terminal deoxytransferase (TdT; 20 U in 100 μl of buffer with 1 μl of biotin-dUTP; Roche) for 1 hr at 37°C. Slides were washed in water, incubated with strepavidin-horseradish peroxidase complex (Dako, Carpinteria, CA) for 30 min at room temperature and detected with AEC (3-amino-9-ethylcarbazole) solution (Sigma, St. Louis, MO) for 10 min. Positive control slides included sections predigested with deoxyribonuclease and negative control slides were run in parallel without TdT.

Determination of cGMP content

$Apc^{Min/+}$ mice, with or without a homozygous deletion of *Gucy2c*, were sacrificed at 8 weeks of age. Mucosa from the proximal half of the duodenum and from the entire colon was obtained separately by scraping the tissue on ice with a glass slide. After homogenization in cold Hank's balanced salt solution (HBSS; Gibco-BRL) containing 5 mM EDTA and 0.1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Calbiochem, San Diego, CA), an aliquot of each homogenate was assayed for protein by the BCA method (Pierce). cGMP was extracted and purified using Amprep SAX anion exchange columns (Amersham Pharmacia Biotech, Cambridge, U.K.) according to manufacturer's protocol. Samples were acetylated and quantified in triplicate using the Biotrak cGMP EIA kit (Amersham Pharmacia Biotech). Data are presented as mean fmol cGMP/μg protein ± SE and were compared using the unpaired *t*-test (GraphPad Prism).

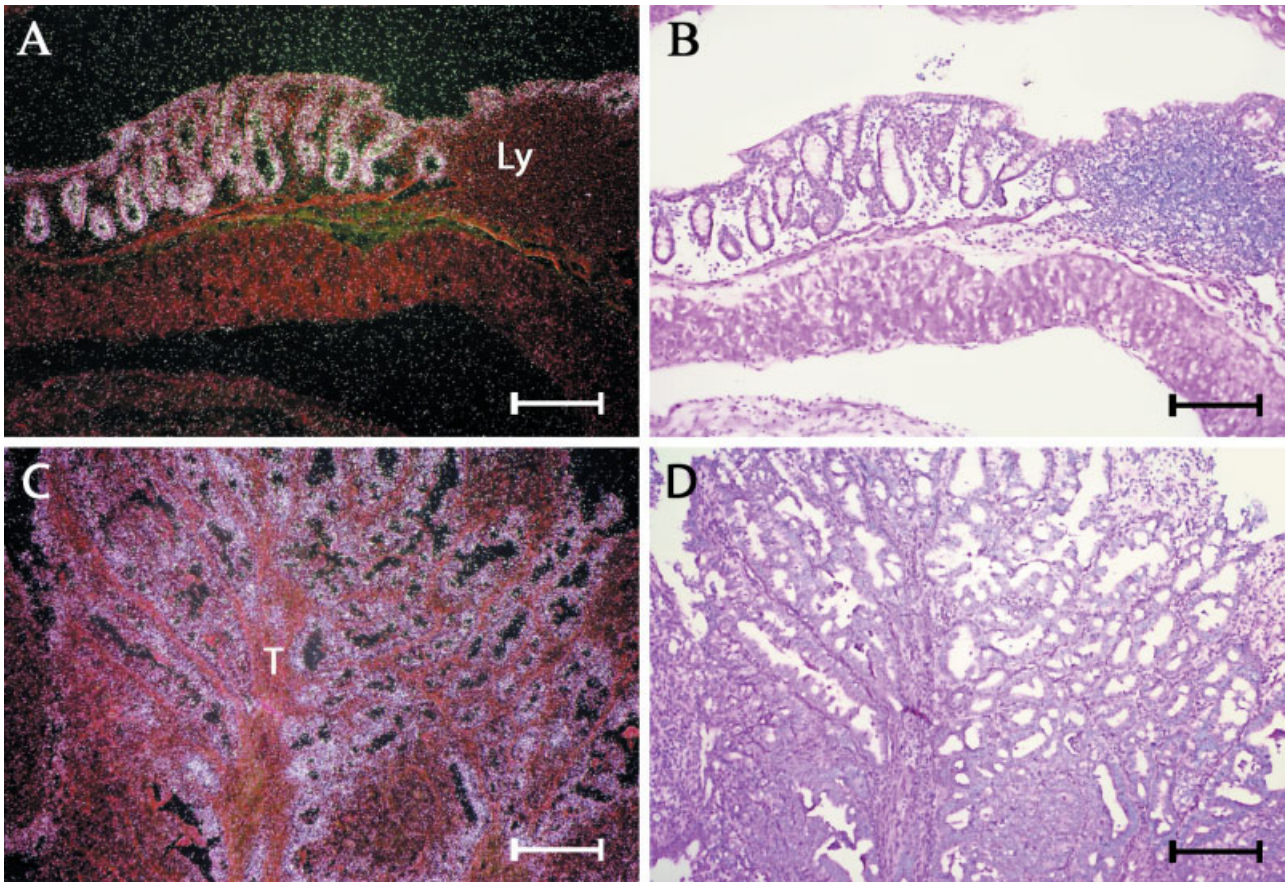

FIGURE 1 – GC-C transcripts are highly expressed in intestinal adenomas. Mouse GC-C antisense riboprobes were hybridized to sections of $Apc^{Min/+}$ intestinal tissue containing normal (*a* and *b*) and transformed adenomatous cells, indicated by T (tumor) in (*c* and *d*). (*a*) and (*c*) represent dark-field images, whereas (*b*) and (*d*) represent identical bright-field hematoxylin and eosin-stained sections. (*a*) Normal intestine with strong signal present in both superficial and deep crypt epithelium. Also visible is a lymphoid follicle (Ly) demonstrating staining of normal overlying superficial epithelial cells. (*c*) Strong signal is preserved in a representative adenomatous tissue. Control hybridization (not shown) indicates that mouse GC-C sense probe does not bind specifically to any cells. The measurement bar is equal to 320 μm.

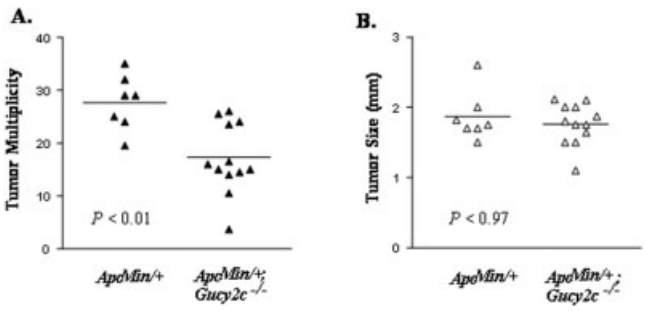

FIGURE 2 – A 55% reduction in the number of adenomas is seen in $Apc^{Min/+}$ mice carrying a homozygous deletion of GC-C while tumor size does not differ. All mice were sacrificed at 6 months of age. (*a*) In $Apc^{Min/+}$; $Gucy2c^{+/+}$ mice, a median of 29 polyps ($n = 7$) was observed throughout the entire intestinal tract while there was a median of 16 ($n = 12$) seen in $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice ($p < 0.006$). (*b*) The median tumor diameter is the same for both genotypes, 1.8 mm. Statistical comparisons were done using the Mann-Whitney test.

Results

GC-C transcripts are present in adenomas of $Apc^{Min/+}$ *mice*

GC-C expression has been documented in human colorectal tumors and metastases by both ligand binding and guanylyl cyclase activation studies and by RT-PCR.[14,15] Here, we used *in situ* hybridization to determine the localization of GC-C mRNA in intestinal tissue obtained from $Apc^{Min/+}$ mice. Robust GC-C expression in histologically normal colon is detectable within the glands of the crypt and on the surface epithelium (Fig. 1*a*) as previously reported.[12] GC-C expression is maintained within the adenomatous epithelium as shown in Figure 1(*c*) for a representative polyp. No signal is seen on hybridization with GC-C sense riboprobe (data not shown).

Polyp number is reduced in $Apc^{Min/+}$ *mice with a homozygous deletion of* Gucy2c

$Gucy2c^{+/-}$ mice on the C57BL/6 background (background generation N_5) were crossed with $Apc^{Min/+}$ mice (C57BL/6) to generate compound heterozygotes, $Apc^{Min/+}$; $Gucy2c^{+/-}$. Their progeny, $Apc^{Min/+}$; $Gucy2c^{+/+}$ and $Apc^{Min/+}$; $Gucy2c^{-/-}$, were sacrificed at 6 months of age and examined for intestinal tumors (Fig. 2). The median number of adenomas present in the intestinal tract of $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice ($n = 12$) was 16 (range, 7–26). This is a decrease of 55% compared to the median of 29 polyps (range, 19–35) observed in $Apc^{Min/+}$; $Gucy2c^{+/+}$ mice ($n = 7$; $p < 0.006$). A third of the $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice (4/12) exhibited polyp numbers within the range of the $Apc^{Min/+}$; $Gucy2c^{+/+}$ group and so may reflect the action of modifier genes contributed by non-C57BL/6 loci still segregating in these mice (see Material and Methods). The median tumor size, indicative of tumor load, was the same (1.8 mm) for both genotypes (Fig. 2*b*). The proportion of large (> 2.5 mm) to smaller polyps was also the

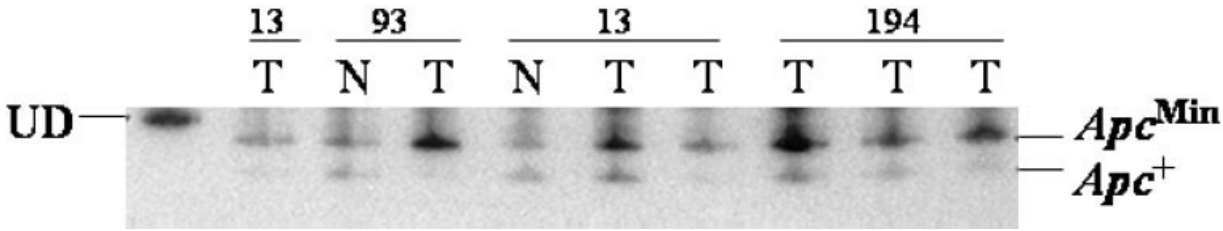

FIGURE 3 – Loss of heterozygosity (LOH) of *Apc* was observed in DNA from $Apc^{Min/+}$; $Gucy2c^{-/-}$ tumors. A 155 bp product was amplified by PCR from DNAs extracted from 7 tumors and 2 areas of histologically normal tissue from 3 different $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice. Digestion with *Hin*dIII resulted in a 144 bp fragment from the Apc^{Min} allele and a 123 bp fragment from the wild-type Apc^{+} allele, which were separated by denaturing 8% acrylamide gel electrophoresis. DNA from normal epithelium (N) exhibited equivalent levels of both alleles as expected. Tumor DNA (T) showed a marked loss of Apc^{+}. DNAs from $Apc^{Min/+}$; $Gucy2c^{+/+}$ tissues gave similar results (not shown). The numbers above the lanes are mouse identifiers. UD, undigested PCR product.

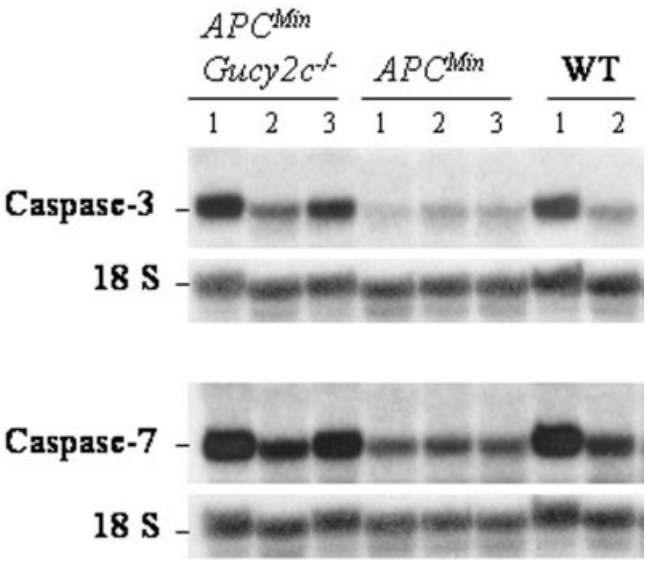

FIGURE 4 – Caspase-3 and caspase-7 mRNA are upregulated in $Apc^{Min/+}$; $Gucy2c^{-/-}$ intestine. Northern analysis of RNA purified from full thickness jejunums of 3 $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice, 3 $Apc^{Min/+}$ mice and 2 wild-type mice. The signal obtained with each caspase probe was normalized to results from hybridization with 18S rRNA probe (shown in the lower panels). There is a decrease of 3- to 4-fold in the expression of both caspase-3 and caspase-7 in RNA from $Apc^{Min/+}$ mice compared to the other genotypes.

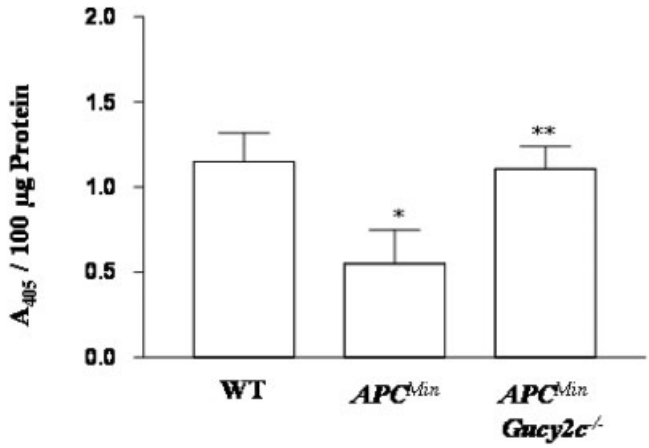

FIGURE 5 – Cell death is significantly increased in the small intestine of $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice. Levels of apoptosis were quantified in intestinal homogenates (Cell Death Detection ELISA). Cell death is reduced in $Apc^{Min/+}$ mouse intestine ($n = 5$) compared to both wild-type ($n = 5$) and $Apc^{Min/+}$; $Gucy2c^{-/-}$ ($n = 6$) intestine. Data are presented as mean ± SE. * $p < 0.05$ *vs*. wild-type; ** $p < 0.04$ *vs*. $Apc^{Min/+}$; $Gucy2c^{-/-}$; unpaired *t*-test.

same (data not shown). In both genotypes, the majority of adenomas were found in the small intestine, as expected in this model. The incidence of colonic polyps (percentage of mice with polyps present in the colon) was 57% in $Apc^{Min/+}$ mice and 25% in the $Apc^{Min/+}$; $Gucy2c^{-/-}$ group but this is not a statistically significant difference (Yates' continuity-corrected chi-square test). Histologic examination revealed that adenoma type and the degree of dysplasia were also similar between genotypes.

Loss of heterozygosity of Apc *gene is found in polyps of* $Apc^{Min/+}$; $Gucy2c^{-/-}$ *mice*

Somatic loss of the wild-type *Apc* allele is thought to initiate tumor formation in $Apc^{Min/+}$ mice.[26] To determine if the same mechanism was involved in GC-C-deficient polyps, several were microdissected from sections of small intestine and colon along with histologically normal tissue and genotyped for *Apc* (Fig. 3). All 7 tumors examined showed a loss of the wild-type *Apc* allele compared to nearly equivalent levels of both alleles present in normal tissue.

Caspase gene expression and apoptosis in $Apc^{Min/+}$; $Gucy2c^{-/-}$ *intestine are increased compared to* $Apc^{Min/+}$ *intestine while cGMP levels are reduced*

A recent report has demonstrated that expression levels of caspase-3 and caspase-7 are downregulated in both normal epithelium and polyps of $Apc^{Min/+}$ intestine[27] and that this is correlated with reduced apoptosis *in vitro*. We used Northern blot analysis (Fig. 4) to examine the expression of caspase-3 and caspase-7 in jejunum from $Apc^{Min/+}$; $Gucy2c^{-/-}$, $Apc^{Min/+}$ and wild-type mice. As expected, there is a marked decrease in expression of both these caspases in $Apc^{Min/+}$ RNA compared to wild-type. However, the levels of both caspase-3 and -7 in RNA from $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice are similar to the expression seen with wild-type intestinal RNA. Densitometric analysis of the bands, normalized to 18 S rRNA content, showed a 3- to 4-fold increase over levels in $Apc^{Min/+}$ intestine.

To assess whether there was a corresponding effect on apoptosis levels, we examined sections of small intestine using the terminal deoxynucleotidyl transferase-mediated dNTP-biotin nick end labeling (TUNEL) assay. Results (data not shown) showed considerable variation in the number of positively stained cells in sections from individual mice of the same genotype. As this obscured any possible differences between genotypes, we employed an immunoassay to quantify the amount of cytoplasmic histones, indicative of programmed cell death, present in intestinal homogenates. As seen in Figure 5, these assays show decreased apoptosis in mucosa from the small

TABLE I – LEVELS OF cGMP PRESENT WITHIN MUCOSA OF $APC^{MIN/+}$ MICE

	Duodenum (fmol/μg; mean ± SE)	Colon (fmol/μg; mean ± SE)
$Apc^{Min/+}$[1]	0.268 ± 0.035	0.083 ± 0.009
$Apc^{Min/+}$; $Gucy2c^{-/-}$[1]	0.086 ± 0.005[2]	0.047 ± 0.007[3]

[1] $n = 3$.–[2] $p < 0.01$ *vs.* $Apc^{Min/+}$.–[3] $p < 0.05$ *vs.* $Apc^{Min/+}$, unpaired *t*-test.

intestine of $Apc^{Min/+}$ mice (0.55 ± 0.19, mean ± SE) compared to wild-type mice (1.15 ± 0.17; $p < 0.05$, *t*-test). The level in $Apc^{Min/+}$; $Gucy2c^{-/-}$ intestine (1.11 ± 0.13) is similar to wild-type but is significantly higher than the $Apc^{Min/+}$ level ($p < 0.04$). This was surprising considering that cGMP has been implicated as a proapoptotic agent in intestinal cancer cell lines.[1,29,30] As expected, levels of cGMP from both duodenal and colonic mucosa of $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice were significantly lower ($p \leq 0.05$) than from $Apc^{Min/+}$; $Gucy2c^{+/+}$ mice (Table I). To examine possible effects on crypt cell proliferation, we used immunohistochemical detection of the number of nuclei positive for bromodeoxyuridine at 2 hr postinjection, but saw no differences between the 3 genotypes (data not shown).

Discussion

Here we report that lack of GC-C expression is associated with a 55% reduction in the median number of adenomas in $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice. All GC-C null tumors showed the expected loss of heterozygosity for *Apc*, indicating no change in the somatic DNA mutations necessary for tumor initiation. One interpretation of these data is that GC-C deficiency reduces the likelihood of successful polyp initiation. Because there was not a significant difference in tumor size between $Apc^{Min/+}$; $Gucy2c^{+/+}$ and $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice, we conclude that tumor growth is not dependent on GC-C. A factor in interpreting our data, however, is that due to our breeding design, both $Apc^{Min/+}$; $Gucy2c^{+/+}$ and $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice carry approximately 5% non-C57BL/6 loci, which may act as modifiers of both polyp incidence and size.

Our results contrast with the work of Shailubhai *et al.*,[1] who used the $Apc^{Min/+}$ model to assess the function of uroguanylin/GC-C signaling. After long-term uroguanylin feeding (for 17 weeks, beginning at approximately 6 weeks of age) they found a 50% reduction in tumor number as well as a marked decrease in tumor size. GC-C activity and cGMP are probably reduced due to loss of guanylin and uroguanylin expression[1,17] within polyps in the wild-type $APC^{Min/+}$ intestine. Shailubhai *et al.*[1] speculate that adding exogenous uroguanylin increases GC-C activity, raising the cellular cGMP level and so leading to apoptosis of enterocytes that otherwise may have given rise to adenomas. Although we also observed a reduction in the number of polyps, our studies differ regarding the presence of GC-C receptor and therefore most likely differ in mechanism as well.

The mechanisms responsible for the reduction in polyp number in $Apc^{Min/+}$; $Gucy2c^{-/-}$ intestine are not known. GC-C is known to signal via both cGMP and calcium,[2,6,31–33] although these pathways have not been fully explored with regard to proliferation and apoptosis. We have shown that there is an approximately 2-fold decrease in mucosal cGMP levels in GC-C-deficient $Apc^{Min/+}$ mice compared to $Apc^{Min/+}$; $Gucy2c^{+/+}$ mice (Table I). The magnitude of the cGMP decrease is the least in the colon (1.8-fold), where there was no difference in the number of polyps observed and where only a small fraction of the total polyp number in both genotypes is located. Conversely, both the location of most polyps and the greatest decrease in cGMP levels (3.1-fold) occur in the small intestine. This may suggest a greater influence of cGMP on polyp development in the small intestine in this mouse model and also suggests that in $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice, low levels of cGMP correlate with lowered polyp number. However, it is difficult to know how these differences impact the function of cGMP-dependent proteins, such as PKG or cyclic nucleotide-gated channels, on the cellular level. It has been reported that PKG activation *in vitro* can lead to the stimulation of the proapoptotic Jun kinase pathway[30] as well as the phosphorylation and increased proteosomal degradation of β-catenin.[4,29,34] Lowered β-catenin levels may mean decreased signaling of the antiapoptotic and proliferative genes upregulated as a result of *Apc* mutation. These have been proposed as possible mechanisms for the action of exisulind (sulindac sulfone), a cGMP-specific phosphodiesterase inhibitor, that causes regression of polyps in patients with familial adenomatous polyposis, who carry a mutated *APC* allele.[35] A recent report[36] has also demonstrated that treatment with sulindac metabolites *in vitro* results in increased degradation of β-catenin via both the proteasomal route and caspase-mediated cleavage.

If GC-C activation and thus increased cGMP generation can function as a negative regulator of cell growth, then in the simplest model, a consequence of GC-C deficiency and lowered cGMP level in the context of the $Apc^{Min/+}$ mouse would mean an increase in the number/size of polyps, not the decrease we report here. Instead, we hypothesize that the absence of normal GC-C expression during development and into adulthood and the corresponding reduction in cGMP/calcium signaling have caused alterations in cell growth regulatory pathway(s). It is the action, then, of these altered pathways that results in partial circumvention of the tumorigenesis process in the $Apc^{Min/+}$; $Gucy2c^{-/-}$ mouse. In particular, we have demonstrated a reversal of the low level of apoptosis found in the intestine of $Apc^{Min/+}$ mice.

Previous studies have demonstrated low levels of apoptosis in the intestinal epithelium of both $Apc^{Min/+}$ mice and of patients with FAP, which are increased upon treatment with sulindac.[37,38] Another study looking at rectal biopsies of patients with colorectal adenomas found that high levels of apoptosis correlated with lower incidence of adenomas.[39] We examined apoptosis by means of an ELISA that measures the content of cytoplasmic nucleosomes, a defining feature of apoptosis. We found significantly decreased apoptosis in mucosal homogenates from $Apc^{Min/+}$ mice compared to both wild-type and $Apc^{Min/+}$; $Gucy2c^{-/-}$ mucosa. This was accompanied by a similar pattern in gene expression of both caspase-3 and caspase-7. These results confirm the recent study of Chen *et al.*,[27] who first identified a reduction in expression of these caspases as well as other apoptosis-related genes in the $Apc^{Min/+}$ model and in human colon cancer samples. In addition, they demonstrated increased caspase expression as a function of inhibition of β-catenin/TCF signaling *in vitro*. Based on these data, further exploration of the apoptotic process in the $Apc^{Min/+}$; $Gucy2c^{-/-}$ intestine is warranted.

We have previously demonstrated a defect in phosphorylation of extracellular-regulated kinase 1 and 2 (ERK1/2) in mice lacking GC-C.[10] Work with colon cancer cell lines has linked inhibition of epidermal growth factor-induced phosphorylation of ERK1/2 with apoptosis.[40,41] In $Apc^{Min/+}$ mice expressing a mutant epidermal growth factor receptor, polyp number was reduced 90% compared to mice with wild-type receptor.[42] In a mouse xenograft model, treatment with an inhibitor of the upstream kinase MEK suppressed tumor growth,[43] providing further evidence of the importance of the ERK signaling pathway in colon cancer. The impact of GC-C deficiency on this pathway may be one of the alterations leading to increased apoptosis in the intestine of $Apc^{Min/+}$; $Gucy2c^{-/-}$ mice.

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