Inactivating cholecystokinin-2 receptor inhibits progastrin-dependent colonic crypt fission, proliferation, and colorectal cancer in mice

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Hyperproliferation of the colonic epithelium, leading to expansion of colonic crypt progenitors, is a recognized risk factor for colorectal cancer. Overexpression of progastrin, a nonamidated and incompletely processed product of the gastrin gene, has been shown to induce colonic hyperproliferation and promote colorectal cancer in mice, but the mechanism of pathogenesis has not been defined. Cholecystokinin-2 receptor (CCK2R) is the primary receptor for cholecystokinin (CCK) and amidated gastrin. Here, we show that *Cck2r* was expressed in murine colonic crypts and upregulated in the transgenic mice that overexpress human progastrin. Murine deletion of *Cck2r* abrogated progastrin-dependent increases in colonic proliferation, mucosal thickness, and β-catenin and CD44 expression in the colon tumor. In addition, either deletion or antagonism of *Cck2r* resulted in the inhibition of progastrin-dependent increases in progenitors expressing doublecortin and CaM kinase–like-1 (DCAMKL1), stem cells expressing leucine rich repeat–containing G protein–coupled receptor 5 (LgR5), and colonic crypt fission. Furthermore, in the azoxymethane mouse model of colorectal carcinogenesis, *Cck2r* deletion in human progastrin–overexpressing mice resulted in markedly decreased aberrant crypt foci formation and substantially reduced tumor size and multiplicity. Taken together, these observations indicate that progastrin induces proliferative effects, primarily in colonic progenitor cells, through a CCK2R-dependent pathway. Moreover, our data suggest that CCK2R may be a potential target in the treatment or prevention of colorectal cancer.

Introduction

Colorectal cancer is one of the most common cancers and the second leading cause of cancer-related death in the United States (1). The majority of colorectal cancers originate in a multi-step process from adenomatous polyps, acquiring a series of somatic mutations (i.e., p53, KRas, and APCs) (1–4). Colorectal cancer is believed to arise from colonic stem or progenitor cells in association with nuclear localization of β-catenin leading to crypt fission, the development of aberrant crypt foci (ACFs), and the emergence of colorectal cancer stem cells. Hyperproliferation of the colonic epithelium has been recognized as a risk factor for colorectal cancer development and represents an important first step in a sequence of events leading to neoplastic progression (5). Past investigations have focused on dietary factors, hormones, and growth factors that may modulate colonic epithelial cell proliferation and the risk of colon carcinogenesis, but few of these growth pathways have been directly linked to effects on colonic progenitors.

The peptide hormone gastrin is a well-recognized acid secretagogue and growth factor that is produced primarily by gastric G cells. Gastrin exists in a number of molecular forms; the most abundant and well-studied forms of gastrin, G17 and G34, are amidated at the C terminus after posttranslational processing of the 101–amino acid precursor molecule, preprogastrin (6). Prior to its conversion to the amidated forms, preprogastrin undergoes cleavage of a signal peptide to yield progastrin, an 80–amino acid peptide. Progastrin and other nonamidated, incompletely processed forms of gastrin (such as glycine-extended gastrin) typically comprise less than 10% of the total secreted peptide in most individuals, but elevations can occur when processing is impaired.

Although amidated gastrins were for many years thought to be the only biologically active form of gastrin, data derived initially from transgenic mouse models demonstrated unequivocal biological activity for progastrin. Human progastrin (hGAS) transgenic mice (hGAS$^{/+}$ mice) showed increased colonic proliferation and mucosal thickness in 2 independent studies, consistent with a role for progastrin as a trophic growth factor for the colonic epithelium (7, 8). Several in vitro studies with colorectal cancer cell lines showed that progastrin is able to stimulate cellular proliferation (9, 10). Furthermore, progastrin overexpression or exogenous administration could maintain colonic epithelial mitosis after DNA damage by γ irradiation or chemical carcinogens (11). These effects of progastrin were not dependent on other forms of gastrin, since progastrin-expressing hGAS mice showed a similar phenotype when crossed with a gastrin knockout background (12). Nevertheless, there has been a report on increased azoxymethane-induced (AOM-induced) carcinogenesis in GAS knockout mice (13), in contrast to our findings of decreased colonic proliferation, polyposis, and prolonged survival when crossed to Apc/Min mice (14, 15).

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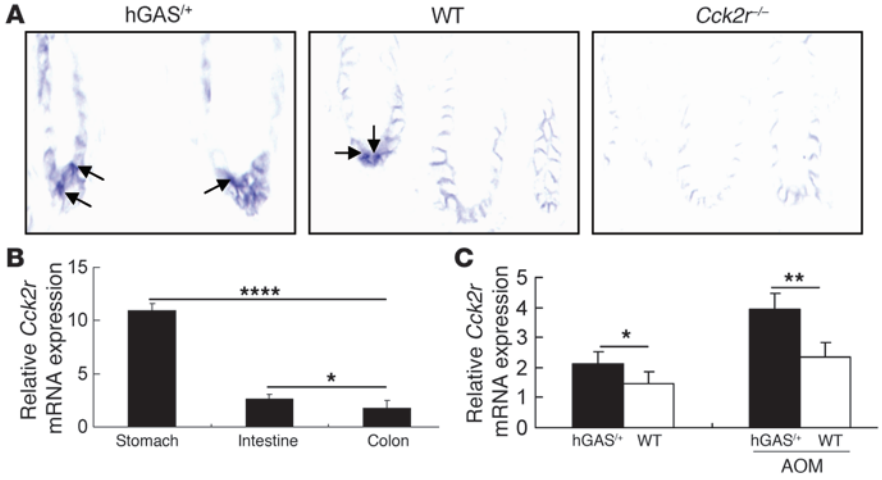

Figure 1
CCK2R is expressed in murine colonic crypts and upregulated in the hGAS$^{/+}$ mouse colon. (**A**) In situ hybridization. Detection of murine *Cck2r* mRNA with a digoxin-labeled antisense cRNA probe in the hGAS$^{/+}$, WT, and *Cck2r*$^{-/-}$ mouse colonic mucosa (original magnification, ×600). The arrows indicate the locations of *Cck2r* mRNA expression. (**B**) qRT-PCR analysis of murine CCK2R expression in WT mouse stomach, small intestine, and colon (n = 4/group). mRNA was prepared, cDNA was synthesized, and qRT-PCR was performed. (**C**) qRT-PCR analysis of murine CCK2R expression in each group of murine colonic mucosa (n = 4 mice/group). The expression levels of *Cck2r* mRNA in the colon were analyzed 2 weeks after 2 weekly injections of AOM (10 mg/kg). The expression levels were normalized to GAPDH expression levels. All values represent the mean ± SD. *P < 0.05, **P < 0.01, ****P < 0.0001.

Consistent with a mitogenic effect on the colon, elevated progastrin levels are associated with an increased susceptibility to colon carcinogenesis. Both short-term and long-term studies with AOM treatment in hGAS mice showed a significant increase in the number and multiplicity of ACFs, adenomas, and adenocarcinomas, which all correlated positively with progastrin levels (16, 17). In support of these findings in mice, significantly elevated circulating plasma levels of progastrin can be observed in human patients with colorectal carcinoma (18, 19). Furthermore, gastrin gene expression is upregulated early in the adenoma-carcinoma sequence, since gastrin mRNA and progastrin have been detected in the majority of adenomatous polyps (20). Compared with normal tissue, progastrin has been reported to be synthesized at much higher levels in colorectal cancers and colon cancer cell lines (19, 21). Indeed, numerous types of cancer cell lines and primary tumors express nonamidated gastrin, including gastric, pancreatic, lung, and ovarian cancers (22). The gastrin gene is frequently upregulated in colorectal cancer, as known oncogenic pathways (KRas and β-catenin) have been demonstrated to induce gastrin gene expression in colon cancer cells (23, 24).

Nevertheless, while reproducible biological effects have been demonstrated in vivo for progastrin, the interacting molecules that mediate progastrin's effects have not been well defined. Some high-affinity binding sites for progastrin have been demonstrated by several groups (9, 25, 26), but validated cell surface receptors have remained elusive. A recent report has indicated that annexin II can bind progastrin and mediate growth factor effects (27), although annexin II does not appear to represent a cell surface receptor and genetic evidence for a role in colorectal cancer has been lacking. Our group has confirmed the expression of progastrin binding molecules on the surface of a number of cancer cell lines (28). However, the only known cell surface receptor for gastrin peptides is the cholecystokinin type 2 receptor (CCK2R, originally known as the CCK-B receptor) (29). CCK2R is a member of the G protein–coupled 7 putative transmembrane domains receptor superfamily that binds both amidated gastrin and cholecystokinin (CCK) with equal affinity. With the identification and characterization of CCK2R (29), the receptor was demonstrated to have a negligible affinity for nonamidated gastrins (10). CCK2R has been shown to be widely expressed, with transcripts most abundant in the brain and stomach but also detectable in the colon, kidney, ovary, and pancreas (30–32). It has been shown that overexpression of CCK2R can mediate growth-promoting effects on pancreatic cells both in vitro (33) and in vivo (34). Studies of human adenomatous polyps have demonstrated that CCK2R is upregulated early in the adenoma-carcinoma sequence, as the vast majority of polyps show increased expression of CCK2R. However, the precise location of CCK2R expression is unknown (20).

In order to explore the role for CCK2R in the progastrin pathway, we pursued a genetic approach and crossed progastrin-overexpressing hGAS mice to CCK2R-deficient mice. Our studies suggest an important role for CCK2R in mediating the effects of progastrin on the murine colon and the overall susceptibility to colorectal cancer.

Results

CCK2R is expressed in the murine colonic crypts and upregulated by progastrin transgenic mice. Previous studies have indicated that CCK2R is expressed in the murine colon (30), but the precise location of expression has not been defined. Using quantitative RT-PCR (qRT-PCR) analysis, we confirmed that CCK2R was highly expressed in the stomach and, as expected, expressed at much lower levels in the small intestine and colon in 6-week-old WT FVB/N mice (Figure 1B). Moreover, using in situ hybridization, CCK2R expression could be localized to the most basal colonic crypt cells in hGAS$^{/+}$ and WT mice, while no expression could be observed in *Cck2r*$^{-/-}$ mice (Figure 1A). Consistently, immunofluorescence analysis detected CCK2R-expressing cells predominantly located at the bottom of the colonic crypts (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI38918DS1). CCK2R was upregulated in hGAS$^{/+}$ mouse colonic mucosa compared with that of WT mice (Figure 1C). Furthermore, the carcinogen AOM significantly induced CCK2R upregulation in the hGAS$^{/+}$ mouse colonic mucosa compare with that of WT mice (Figure 1C). These observations suggest that increased progastrin expression in hGAS$^{/+}$ mice leads to marked upregulation of CCK2R expression, with the increased appearance of CCK2R-expressing cells.

Inactivation of the Cck2r gene inhibits progastrin-dependent colonic proliferation and increases colonic apoptosis. In order to clarify the role of CCK2R in progastrin-dependent colonic mucosa proliferation, we crossed hGAS$^{/+}$ mice with CCK2R-deficient mice and

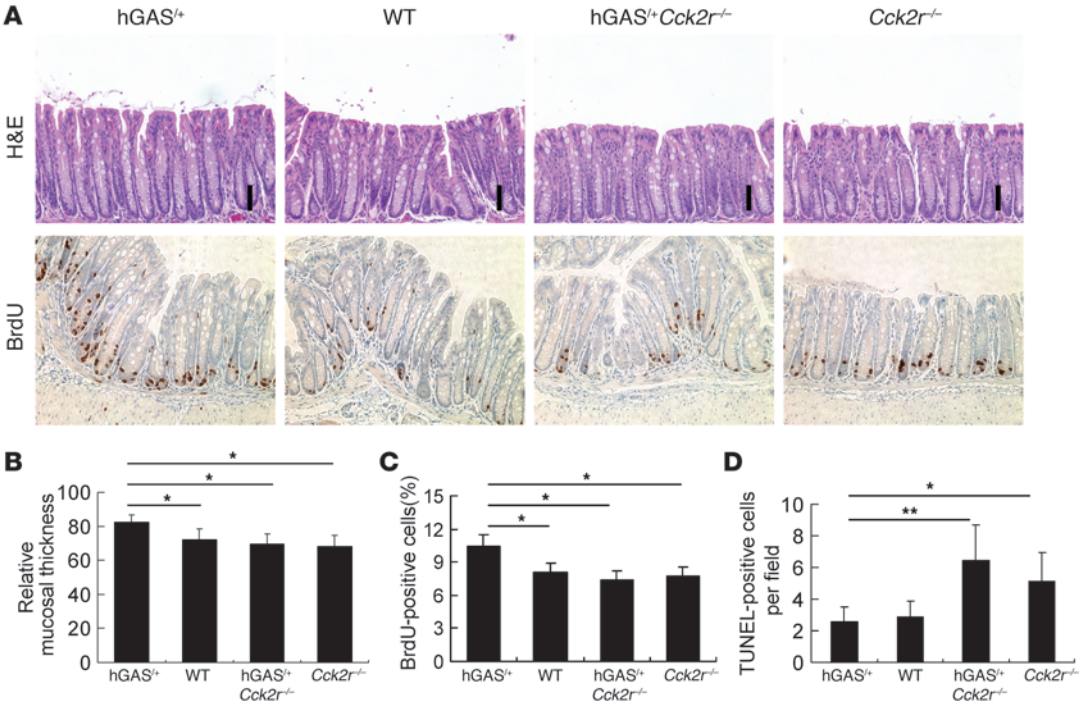

Figure 2

Inactivation of the *Cck2r* gene inhibits progastrin-dependent colonic proliferation and increases colonic apoptosis. (**A**) Top: Hematoxylin and eosin–stained sections from the distal colon show the mucosa thickness in each mouse group (original magnification, ×200). Mucosa thickness was measured with Nikon TE2000 microscope image analysis software. Scale bar: 20 μm. Bottom: Immunohistochemical staining of BrdU-positive cells in the colon from each group of mice after intraperitoneal injection of BrdU (original magnification, ×200). Representative results from 4 different groups are shown. (**B**) Relative colonic mucosal thickness was measured at 3 different locations (proximal, middle, and distal colon) in mice from 4 groups (n = 4/group). (**C**) The percentage of BrdU-positive cells in the colonic crypts was measured at 10 crypts in different locations in mice from each of the 4 groups (n = 4/group). (**D**) Percentage of TUNEL-positive cells in different locations in mice from each of the 4 groups (n = 4/group). All values represent the mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$.

analyzed 4 different groups of mice on a FVB/N background: hGAS$^{/+}$, hGAS$^{/+}$*Cck2r*$^{-/-}$, *Cck2r*$^{-/-}$, and WT. In histopathologic analysis, we observed an increased number of cells per crypt in colons of hGAS$^{/+}$ mice (7), correlating with an increased height of distal colonic crypts compared with the other 3 groups of mice (hGAS$^{/+}$*Cck2r*$^{-/-}$, *Cck2r*$^{-/-}$, and WT mice) (Figure 2A). When measuring the relative colonic mucosal crypt height at 3 different locations (proximal, middle, and distal colon), the height of the hGAS$^{/+}$ mouse colonic crypts was significantly greater than that in the other 3 groups of mice (Figure 2B). Analysis of the BrdU incorporation revealed that most of the proliferating cells were located in the bottom of vertically oriented colonic crypts (Figure 2A). Furthermore, quantification of BrdU-positive cells relative to total epithelial cell number in 20 crypts at randomly chosen sites in each colon showed significantly higher proliferation in hGAS$^{/+}$ mice compared with hGAS$^{/+}$*Cck2r*$^{-/-}$, *Cck2r*$^{-/-}$, and WT mice (Figure 2C). Thus, genetic deletion of CCK2R inhibited colonic epithelial proliferation in progastrin-overexpressing mice but not in WT mice. In addition, the distribution of apoptotic cells along the colonic crypts, analyzed by TUNEL assay, was markedly different in all groups. Interestingly, in hGAS$^{/+}$ mice there were the fewest apoptotic cells and most of these apoptotic cells were observed on the mucosal surface (data not shown). Quantitative analysis throughout the colonic crypts showed significant differences in the amount of apoptotic cells in hGAS$^{/+}$ mice compared with hGAS$^{/+}$*Cck2r*$^{-/-}$ and *Cck2r*$^{-/-}$ mice (Figure 2D). These results were confirmed by gene expression analysis of caspase-3 and FAS, which has been shown to be an important mediator of apoptotic cell death (Supplemental Figure 2). These results suggest that progastrin significantly inhibits colonic mucosa apoptosis.

Inactivation of the Cck2r gene inhibits progastrin-dependent colonic ACF and tumor formation. ACFs are generally regarded as preneoplastic lesions for colorectal cancer (35). In order to investigate the role of CCK2R deficiency in the formation of ACFs, AOM was given in short-term studies to all 4 groups of mice. Two weeks after the last AOM injection, ACFs were found in all 4 groups of AOM-treated mice. ACFs were present primarily in the distal half of the colon, with fewer numbers present in the proximal half of the colon. ACFs could be classified according to multiplicity into 3 different morphologic types of ACF: single crypt, double crypt, or multi-crypt ACFs (Figure 3A). Overall, hGAS$^{/+}$ mice had significantly more ACFs of all 3 types (Figure 3B) — single crypt (Figure 3C), double crypt (Figure 3D), and multi-crypt (Figure 3E) — compared with the other 3 groups of mice. The greatest difference was apparent for multi-crypt ACFs, which were abundant in hGAS$^{/+}$ mice but completely suppressed in *Cck2r*$^{-/-}$ mice (Figure 3E).

Additionally, colonic tumors were observed in all 4 groups of mice 11 weeks after a 6-week course of AOM treatment. Similar to the localization of ACFs, tumors in all 4 groups of examined mice were located primarily in the distal half of the colon, with

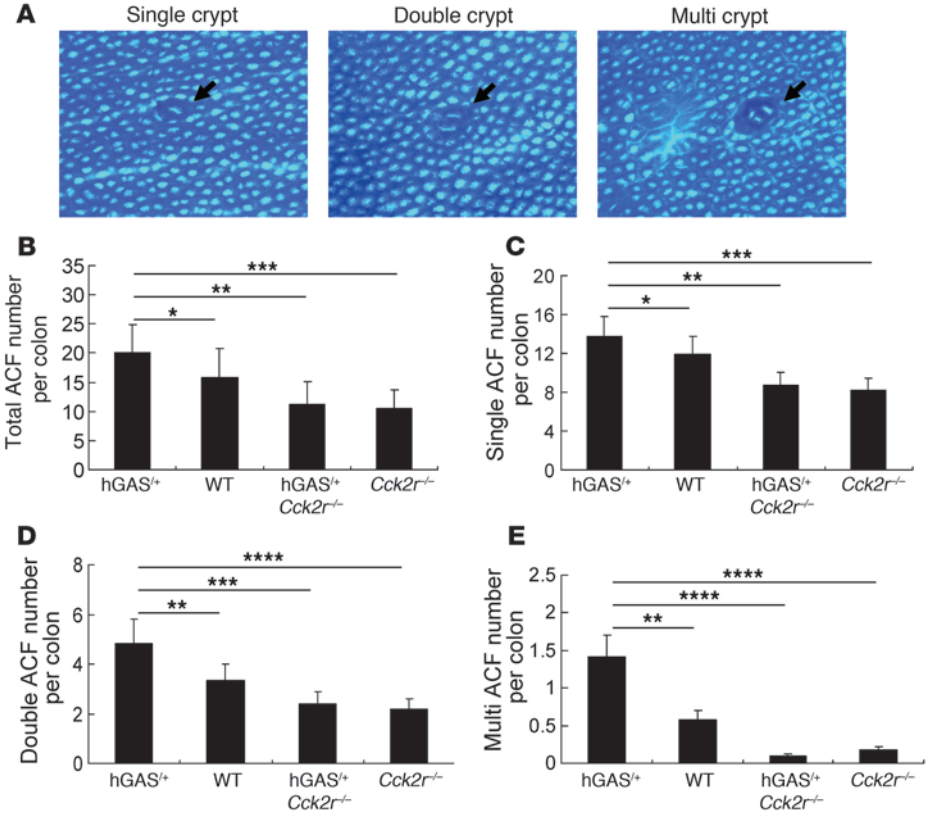

Figure 3
Inactivation of the *Cck2r* gene inhibits progastrin-dependent colonic ACF formation. (**A**) Representative pictures of the 3 different types of ACF (single, double, and multiple crypt) in the colon of AOM-treated mice (original magnification, ×200). The arrows indicate the location of the ACFs. Colons were removed at 2 weeks after 2 weekly injections of AOM and fixed with 70% ethanol overnight, and ACFs were analyzed under a light microscope after methylene blue staining. (**B**–**E**) Total number of ACFs (**B**) and the number of single ACFs (**C**), double ACFs (**D**), and multiple ACFs (**E**) in each colon from mice (n = 12/group). All values represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

only a few tumors present in the proximal half of the colon (Figure 4A). Overall, the tumors were more frequent and much larger in the hGAS$^{/+}$ mice. The average tumor number in hGAS$^{/+}$ mice was significantly higher than in hGAS$^{/+}$*Cck2r*$^{-/-}$, *Cck2r*$^{-/-}$, and WT mice (Figure 4C). Moreover, the tumor size correlated with tumor number, as hGAS$^{/+}$ mice had significantly more tumors measuring less than 2.5 mm (Figure 4D), between 2.5 and 5 mm (Figure 4E), and more than 5 mm (Figure 4F), compared with the other 3 groups. More importantly, CCK2R deficiency markedly suppressed the growth of large tumors, since there were no large (>5 mm) tumors present in hGAS$^{/+}$*Cck2r*$^{-/-}$ or *Cck2r*$^{-/-}$ mice (Figure 4F). In the long-term survival study, following 6 weeks of AOM treatment, tumors in hGAS$^{/+}$ mice appeared to be highly invasive and led to early mortality. The hGAS$^{/+}$ mice began to die at 17 weeks, and more than 90% of mice died before 30 weeks. In contrast, hGAS$^{/+}$*Cck2r*$^{-/-}$ mice began to die only after 27 weeks, and 70% mice remained alive at the 40-week study endpoint (Figure 4B).

In both hGAS$^{/+}$ and hGAS$^{/+}$*Cck2r*$^{-/-}$ mice, progastrin levels were, as expected, highly elevated (about 1,500 fmol/ml) without significant difference. In contrast, progastrin levels were quite low (0.6 fmol/ml) in WT and *Cck2r*$^{-/-}$ mice and showed significant differences from those of hGAS$^{/+}$ mice (Figure 4G). Plasma-amidated gastrin levels were low in hGAS$^{/+}$ and WT mice (about 50 fmol/ml), but were highly elevated in hGAS$^{/+}$*Cck2r*$^{-/-}$ and *Cck2r*$^{-/-}$ mice (about 230 fmol/ml) and clearly related to loss of CCK2R function (Figure 4H), as previously reported (36).

Upregulation of β-catenin and CD44 expression in colon tumors is CCK2R dependent. Activation of β-catenin, an integral component of Wnt signaling pathway, is a critical early step in colonic proliferation and progression to colon cancer carcinogenesis (37). In a recent study, it was demonstrated that β-catenin/Tcf-4 inhibition after progastrin targeting reduces growth and drives differentiation of intestinal tumors (38). Immunohistochemistry analysis of the untreated colons of the 4 groups of mice (hGAS$^{/+}$, hGAS$^{/+}$*Cck2r*$^{-/-}$, *Cck2r*$^{-/-}$, and WT) showed no significant difference in β-catenin expression (Figure 5A). Contrarily, β-catenin expression was markedly increased in colonic tumor cells in all 4 groups of mice after AOM treatment. Interestingly, in tumors from hGAS$^{/+}$ mice, β-catenin cytoplasmic accumulation and nuclear localization was much higher than in tumors from the other 3 groups of mice (Figure 5A), and this was consistent with the more aggressive nature of tumors in these animals. While cytoplasmic β-catenin was present in tumors from all 4 groups of mice, we only observed nuclear localization in hGAS$^{/+}$ mouse tumor cells.

CD44 is a known downstream target gene of β-catenin (39), a cancer stem cell marker and functionally relevant with regard to colon cancer metastasis (40). Immunohistochemistry revealed focal CD44 expression in both colonic crypt and stromal cells, with no significant difference in epithelial CD44 expression among the 4 groups of mice (Figure 5B). As expected, CD44 was expressed in colonic tumors in all 4 groups but showed much higher expression in hGAS$^{/+}$ mice tumors compared with the other 3 groups (Figure 5B). qRT-PCR analysis demonstrated that *Cd44* gene expression was significantly upregulated in the hGAS$^{/+}$ mice colon tumors compared with the other 3 groups (Figure 5C). Cyclin D1, another downstream target of β-catenin, is an important cell regulator of the G1- to S-phase transition that also functions as a cofactor for several transcription factors in numerous cell types (41). qRT-PCR analysis revealed a significant upregulation of cyclin D1 in hGAS$^{/+}$ mice compared with the other 3 mouse groups (Supplemental Figure 3).

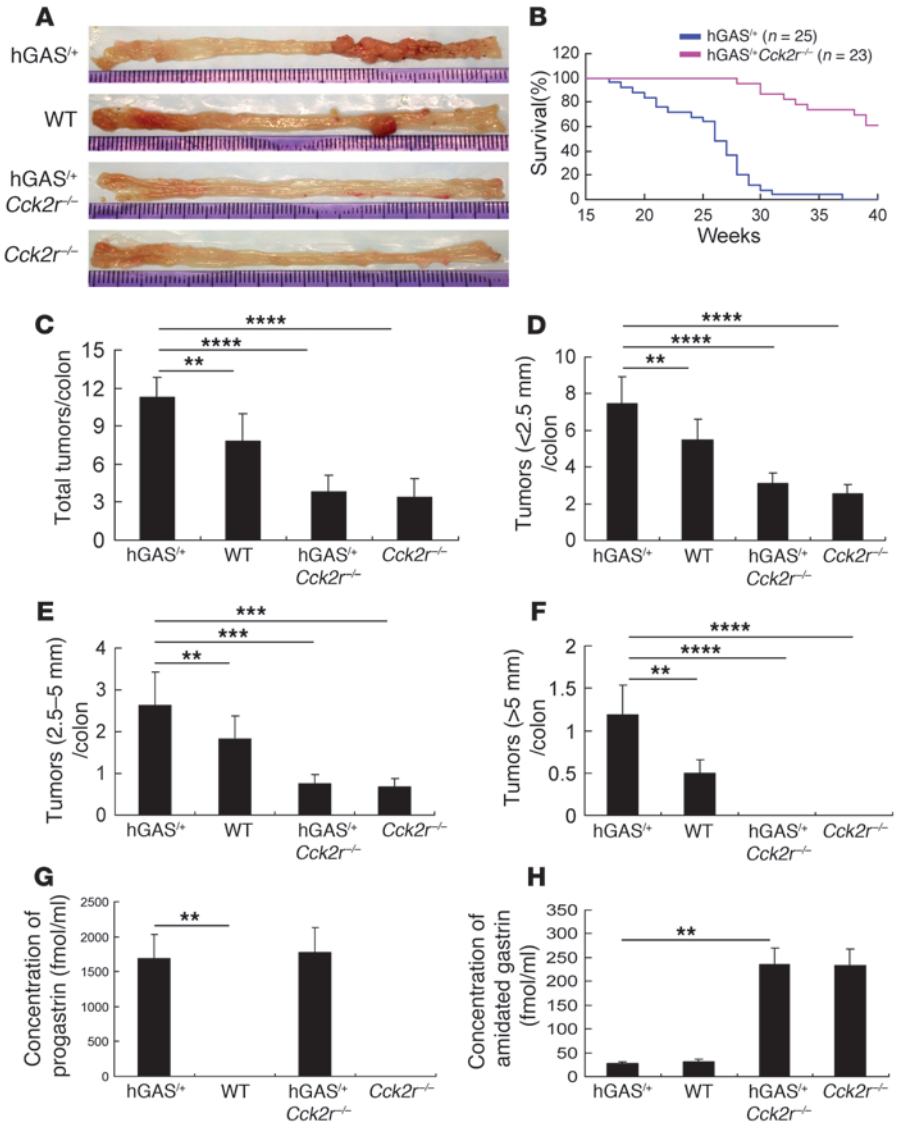

Figure 4
Inactivation of the *Cck2r* gene inhibits progastrin-dependent colon tumor formation. (**A**) Macroscopic changes and tumor formation in colons of mice from each of 4 groups. Colons were removed at 11 weeks after 6 weeks of AOM treatment. Representative results from 4 independent animals are shown. (**B**) Effect of CCK2R deficiency on mortality after AOM treatment in hGAS$^{/+}$ mice (n = 25) and hGAS$^{/+}$*Cck2r*$^{-/-}$ mice (n = 23). The difference in survival was found to be significant using the Mantel-Haenszel/log-rank test. (**C**–**F**) Total number of visible tumors (**C**), tumors less than 2.5 mm in diameter (**D**), tumors measuring 2.5–5 mm in diameter (**E**), and tumors measuring more than 5 mm in diameter (**F**) in colons of mice from each group (n = 12/group). (**G** and **H**) Average (n = 12/group) serum levels of hGAS (**G**) and murine amidated gastrin (**H**) were analyzed. All values represent the mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Progastrin overexpression promotes expansion of colonic progenitors and crypt fission via CCK2R. Recently, doublecortin and CaM kinase–like-1 (DCAMKL1) was reported to be a transcript specifically upregulated in gut epithelial progenitors (42) and shown to be a putative progenitor or stem cell marker (43). Furthermore, leucine rich repeat–containing G protein–coupled receptor 5 (LgR5) has been well validated as a marker for both intestinal and colonic stem cells (44). Immunostaining of colons from WT mice confirmed that DCAMKL1 expression was highly localized to the +4 position and close to the isthmus in the colonic crypts. In hGAS$^{/+}$ mice, additional DCAMKL1-positive cells appeared to migrate toward the bottom of the colonic crypts, in contrast to the crypts of hGAS$^{/+}$*Cck2r*$^{-/-}$ and *Cck2r*$^{-/-}$ mice, in which DCAMKL1-positive cells tended to be located more in the upper part of the colon crypts (Figure 6A). Interestingly, there were many more DCAMKL1-positive cells in hGAS$^{/+}$ mouse colonic crypts compared with the other 3 groups of mice (Figure 6B). Within tumors, we observed a widely distributed pattern of expression of DCAMKL1-positive cells in hGAS$^{/+}$ mouse colon tumors, compared with WT mice tumors, and only very few DCAMKL1-positive cells in hGAS$^{/+}$*Cck2r*$^{-/-}$ and *Cck2r*$^{-/-}$ mouse tumors (Figure 6C). Therefore, DCAMKL1 expression seems to correlate with progastrin- and CCK2R-dependent tumor growth. Similar results were obtained using LgR5 immunostaining. While the available antibodies do not precisely reflect the pattern of expression reported using transgenic tagging (44), we confirmed that LgR5 cells are expressed at the bottom of WT colonic crypts, with increased expression evident in hGAS$^{/+}$ colonic crypts (Supplemental Figure 5).

Expansion of colonic stem cells is thought to promote colonic growth in part through crypt fission, and it has been reported that intestinal crypts divide in response to a doubling of stem cell number (45). Crypt fission is a physiologic mechanism of crypt reproduction that increases in pathophysiologic situations in which crypt regeneration is required. Increased crypt fission is thus another indicator of high cell turnover and a marker of colorectal cancer risk. We found a significant increase in crypt fission in hGAS$^{/+}$ mice (Figure 7A), with predominance in the distal

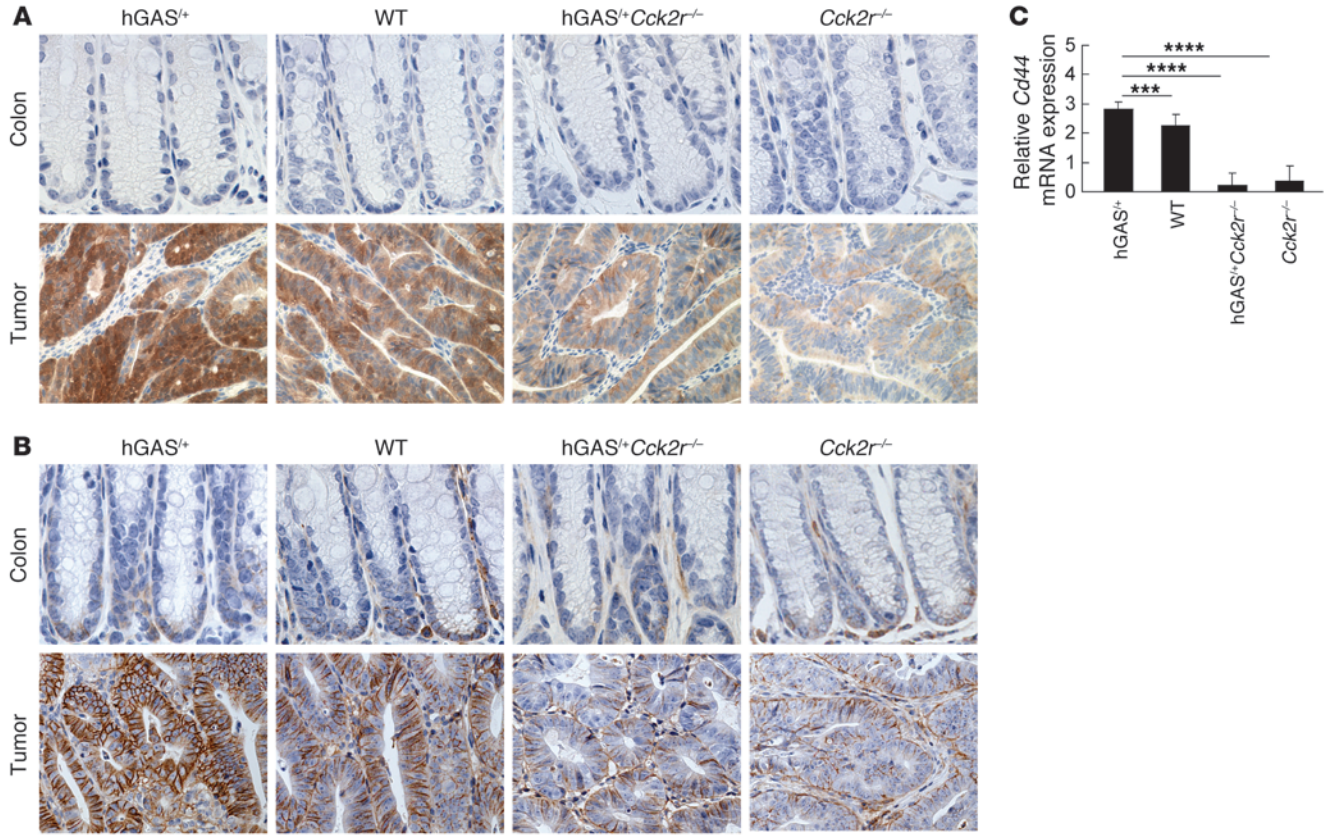

Figure 5

Upregulation of β-catenin and CD44 expression in colon tumors is CCK2R dependent. Immunohistochemistry for β-catenin (**A**) and CD44 (**B**) in colon mucosa (upper panels) and colon tumors (lower panels) of mice from each group. Representative results from 4 different groups are shown (original magnification, ×600). (**C**) qRT-PCR analysis of *Cd44* mRNA expression in each group of colon tumors (n = 4/group). mRNA was prepared, and cDNA was synthesized. The expression levels were normalized to GAPDH expression levels. All values represent the mean ± SD. ***$P < 0.001$, ****$P < 0.0001$.

colon. In contrast, we observed a much lower rate of crypt fission in hGAS$^{/+}$*Cck2r*$^{-/-}$, *Cck2r*$^{-/-}$, and WT mice (Figure 7B).

In order to confirm the importance of progastrin for colonic stem cell division, we investigated the response of progenitor cells in hGAS$^{/+}$ and WT mouse colonic mucosa to γ irradiation. Previous studies by our group had shown that hGAS$^{/+}$ mice are markedly resistant to γ irradiation–induced cell cycle arrest, and studies by others had shown that γ irradiation results in a marked decrease in DCAMKL1 progenitors in WT mice (43). We confirmed that DCAMKL1-positive cells were significantly decreased after irradiation in WT mice (Supplemental Figure 4). In contrast, in hGAS$^{/+}$ mouse colonic crypts, we did not observe any significant decrease in DCAMKL1 cells after γ irradiation, suggesting a specific protective role of progastrin on DCAMKL1 progenitors in the colon.

The CCK2R antagonist YM022 blocks progastrin-dependent colonic mucosa proliferation, increases colonic apoptosis, and inhibits ACF formation. In order to confirm that colonic proliferation in the hGAS transgenic mice was CCK2R dependent, we treated hGAS$^{/+}$ mice with the highly specific CCK2R antagonist, YM022 (46). Similar to our observations regarding hGAS$^{/+}$*Cck2r*$^{-/-}$ mice, cellular proliferation as reflected by the BrdU-labeling index (Figure 8A) was significantly decreased in hGAS$^{/+}$ mice treated with YM022 compared with saline-treated hGAS$^{/+}$ mice (Figure 8B). Furthermore, the number of DCAMKL1-positive cells in the colon crypts (Figure 8A) was significantly decreased in hGAS$^{/+}$ mice treated with YM022 and similar to that in hGAS$^{/+}$*Cck2r*$^{-/-}$ mice (Figure 8C). These findings were confirmed by decreased crypt fission rates in hGAS$^{/+}$ mice treated with YM022, which were similar in range to hGAS$^{/+}$*Cck2r*$^{-/-}$ mice (Figure 8D). Furthermore, TUNEL assay showed a higher number of apoptotic cells in YM022-treated hGAS$^{/+}$ mice and hGAS$^{/+}$*Cck2r*$^{-/-}$ mice compared with untreated hGAS$^{/+}$ mice (Figure 8E). Finally, ACF numbers in the YM022-treated hGAS$^{/+}$ mice were significantly decreased compared with untreated hGAS$^{/+}$ mice and were identical to those in hGAS$^{/+}$*Cck2r*$^{-/-}$ mice (Supplemental Figure 6).

Discussion

Previous studies have shown that progastrin, the precursor of amidated gastrin, could stimulate colonic proliferation and increase susceptibility to colorectal cancer (7, 9, 11). In the current study, we link the proliferative effects of progastrin and colorectal cancer progression to a genetic pathway that involves CCK2R, the only known receptor for amidated gastrin. We show that CCK2R is expressed in colon crypt cells adjacent to the proliferative zone and that elevated levels of progastrin lead to an expansion of CCK2R-expressing cells. Deletion or antagonism of CCK2R blocked the proliferative effects of progastrin and resulted in increased epithe-

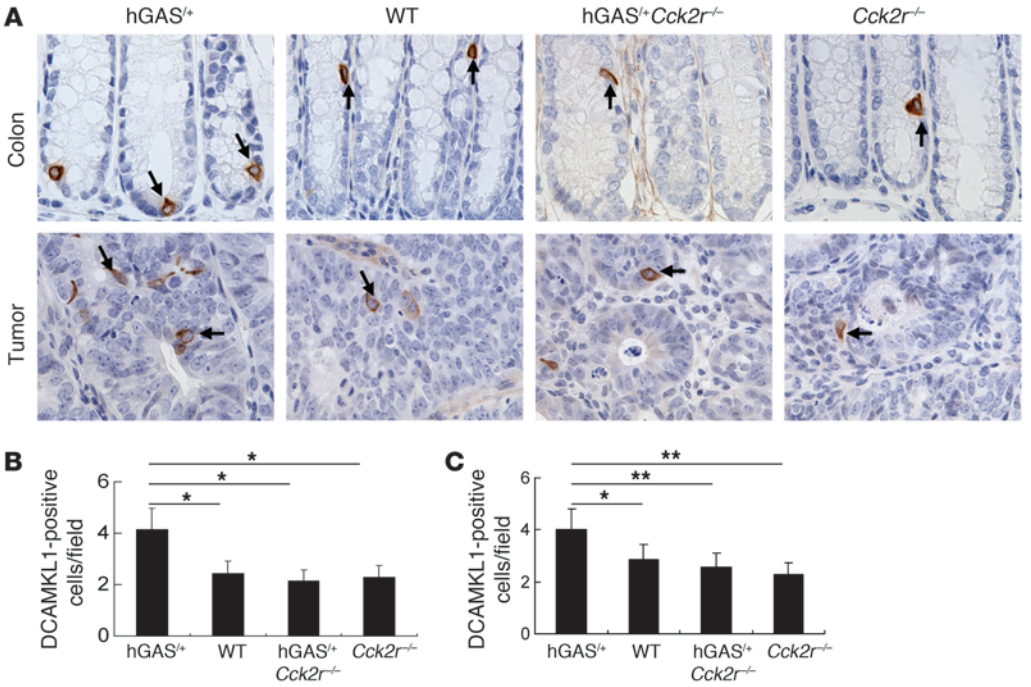

Figure 6

Progastrin overexpression promotes the expansion of colonic progenitors in a CCK2R-dependent manner. (**A**) Immunohistochemistry of DCAMKL1 expression in colonic mucosa (upper panels) and colon tumors (lower panels) in each group of mice. Representative results from 4 different groups are shown (original magnification, ×600). The arrows indicate the locations of DCAMKL1-expressing cells. (**B** and **C**) The average percentage of DCAMKL1-positive cells in colonic crypts (**B**) and tumors (**C**) observed in the 4 groups of mice (n = 4/group). All values represent the mean ± SD. *P < 0.05; **P < 0.01.

lial apoptosis. While progastrin overexpression resulted in acceleration of AOM-dependent colonic carcinogenesis, CCK2R deletion led to a reduction in both ACFs and tumors in the AOM model. Progastrin-dependent colon carcinogenesis was associated with an increase in β-catenin, CD44, and cyclin D1 expression, and these increases were abrogated by deletion of CCK2R. Finally, progastrin overexpression led to a CCK2R-dependent expansion of colonic DCAMKL1 progenitors and LgR5 stem cells and increased colonic crypt fission. Overall, these findings suggest a role for CCK2R in the proliferation and survival of colonic stem cells, the presumed target of carcinogens.

The biological effects of progastrin on colonic proliferation and colon cancer susceptibility have been firmly established in the past, although a different report revealed increased proliferation in gastrin knockout mice (13), which we did not find in our previous studies (14, 15). Nevertheless, the cell surface molecules mediating the effects of progastrin have been elusive. Although several progastrin-interacting proteins have been reported (e.g., annexin II) (27), none of these have represented authentic cell surface receptors or have been localized specifically to the colonic crypts, where progastrin is known to have its primary effects. CCK2R was the first and only gastrin receptor identified (29) but was thought to be highly specific for amidated gastrin, since CCK2R was shown not to bind or directly interact with glycine-extended gastrin (47) or with recombinant progastrin (9). In addition, in vitro studies from other labs have suggested that progastrin's proliferative effects were likely to be independent of CCK2R, since they could not be blocked with CCK2R antagonists such as L365,260 (9, 29).

However, based on the genetic evidence in this study and our localization of CCK2R to the colonic crypts, the site of progastrin's activity, it appears that CCK2R is required for progastrin's proliferative and anti-apoptotic effects in vivo. One possibility is that CCK2R is a direct downstream target of progastrin, and indeed receptor mRNA expression was upregulated in hGAS$^{/+}$ colon. On the other hand, our previous studies with hGAS$^{/+}$/GAS knockout mice have shown that progastrin is able to stimulate colonic proliferation in the absence of amidated gastrin (12), which is contradictory to another report (13). Formally, another possibility is that CCK2R deficiency results in a dominant inhibitory cancer phenotype independent of progastrin effects. Thus, despite the negative results of in vitro studies to date, it remains a possibility that CCK2R is part of the receptor complex that directly mediates progastrin's effect in vivo, possibly binding progastrin directly or else involved in downstream signaling. Previous studies have shown that carboxyterminal-extended gastrins resembling progastrin can induce histamine release in a vascularly perfused rat stomach through a CCK2R-dependent pathway (48). Progastrin's interaction, if any, with CCK2R must be quite different from classical G-17/CCK2R interactions, and amidated G-17, which binds CCK2R, does not stimulate colonic proliferation.

However, in addition to mediating the proliferative effects of progastrin on the colon, our study demonstrates an important role for CCK2R in the progression of colorectal cancer. AOM-induced ACFs and colon tumors were significantly decreased in the hGAS$^{/+}$*Cck2r*$^{-/-}$ mice compared with hGAS$^{/+}$ mice, and the effect of CCK2R deletion appeared to be greatest in reducing

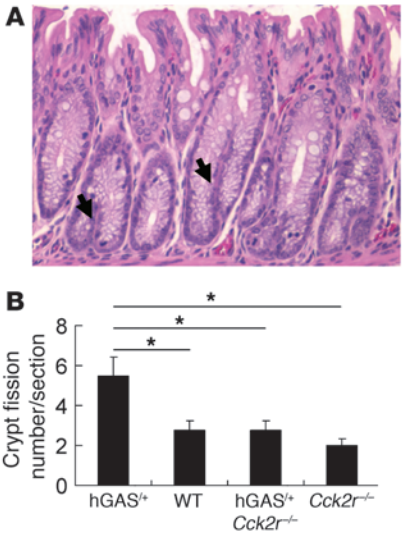

Figure 7
Progastrin overexpression promotes the expansion of colonic crypt fission. (**A**) Representative hematoxylin and eosin staining of 6-week-old mouse distal colonic mucosa with crypt fission (arrows) (original magnification, ×300). (**B**) Relative number of mouse colonic mucosa crypt fissions per section of slide (n = 4/group). All values represent the mean ± SD. *P < 0.05.

the multiplicity of ACFs and large colon tumors that measured more than 5 mm in diameter. Nevertheless, it seems to be important to distinguish dysplastic ACFs from non-dysplastic ACFs, as a recent study showed a lack of correlation between ACF and colon cancer carcinogenesis (49). Previous studies have shown that CCK2R is expressed in many colorectal cancers (50, 51), although the frequency reported varies depending on the methods of detection and whether isoforms are present (52). CCK2R appears early in the adenoma-carcinoma sequence and is often upregulated early at the adenoma stage, consistent with its localization in crypt progenitors (20). Thus, our findings are likely to be of physiological relevance, given the decreased tumors in *Cck2r*$^{-/-}$ mice compared with those in WT mice. Enhanced expression of CCK2R has been shown to correlate with more rapid progression of colorectal cancer (53), and gastrinomas have been linked in case reports to increased colon polyposis (54). In addition, overexpression of CCK2R has been shown to promote the development of pancreatic tumors in animal models (34).

With respect to the potential mechanism for the progastrin's carcinogenic effects, recent reports have linked progastrin stimulation to activation of β-catenin/TCF-4 activity and inhibition of apoptosis (38). Here we show that hGAS$^{/+}$ mice treated with AOM show strong accumulation of nuclear β-catenin, along with increased expression of CD44 and cyclin D1, 2 downstream targets of Wnt signaling. β-Catenin activation was not observed in hGAS$^{/+}$ mice not treated with AOM, and since AOM is known to induce Catnb mutations in colonic crypts, this suggests that progastrin acts primarily to stabilize β-catenin activity in mutated progenitor cells. Inactivation of CCK2R inhibited β-catenin activation and upregulation of expression of CD44, which has also been shown to be a marker of cancer stem cells (55), and this suggests that CCK2R may either act to amplify Wnt signaling or perhaps help expand colon progenitors that carry these mutations.

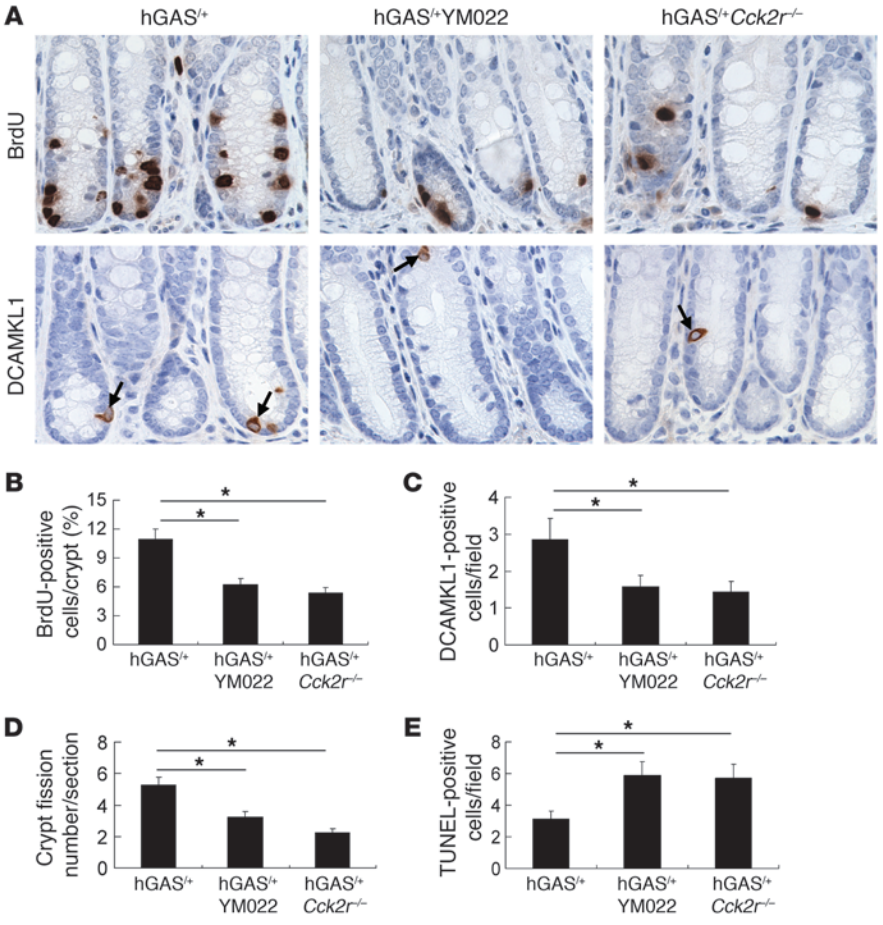

Figure 8
The CCK2R antagonist YM022 blocks progastrin-dependent colonic mucosal proliferation and increases colonic apoptosis. (**A**) Immunohistochemical staining of BrdU-positive cells (upper panels), and DCAMKL1-positive cells (lower panels) after YM022 treatment in 3 groups of mice (original magnification, ×600). The arrows indicate the locations of DCAMKL1-expressing cells. (**B** and **C**) Percentage of BrdU-positive cells (**B**) and DCAMKL1-positive cells (**C**) were measured under a high-power light microscope in the 3 groups of mice (n = 4/group). (**D**) Relative numbers of mouse colonic mucosa crypt fissions in each slide section (n = 4/group). (**E**) Average percentage of TUNEL-positive cells at different locations in the colon in the 3 groups of mice (n = 4/group). All values represent the mean ± SD. *P < 0.05.

The important role of CCK2R in colonic proliferation and colonic carcinogenesis appears to be related to its location in the colon crypts and on colon stem cells. A number of potential intestinal stem cell markers have been described, some of which appear to characterize active stem cells and others that characterize quiescent (position 4) stem cells (56, 57). Previous studies have shown that progastrin-derived peptides bind preferentially to the colonic crypts (58). In the current study, we were able to localize CCK2R to the colonic crypts of WT mice, and showed that CCK2R cells were expanded in mice overexpressing progastrin. In addition, elevations in progastrin led to an expansion of DCAMKL1- and LgR5-positive cells in the colonic crypts. DCAMKL1, a microtubule-associated kinase expressed in postmitotic neurons (59), was first identified as a gene ontogeny–enriched transcript expressed in comparison with gastric epithelial progenitor and whole stomach libraries (42) and has since been suggested to be a putative intestinal and colonic stem cell marker (43). LgR5/GPR49, a leucine-rich orphan G protein–coupled receptor, was elegantly shown to specifically label stem cells in the mouse small intestine in the crypt base columnar (CBC) cells between Paneth cells (44). In our current study, we found that inactivation of CCK2R reduced both proliferation and the number of DCAMKL1- or LgR5-positive cells in hGAS$^{/+}$ mice. These results strongly suggest that CCK2R expression is likely present on, but perhaps not specific for, colonic stem/progenitor cells.

However, in contrast to other stem/progenitor markers that have been reported, the current data suggest an important functional role for CCK2R in regulating colonic stem cell function. Elevated levels of progastrin led to a CCK2R-dependent expansion of crypt progenitors both in the basal state as well as after whole-body radiation. Previous studies have demonstrated that intestinal stem cells are sensitive to low-dose radiation, and DCAMKL1-positive cells undergo cell cycle arrest with apoptosis 24 hours after radiation (43). In addition, we have shown the progastrin stimulation prevents crypt apoptosis and maintains crypt mitosis after γ irradiation (12). Anti-apoptotic effects for progastrin have been reported by others (60, 61). Here, we show that progastrin's effects on inhibition of apoptosis and stimulation of mitosis are CCK2R dependent and that progastrin prevents loss of DCAMKL1 cells after radiation. Furthermore, progastrin appeared to stimulate crypt fission in a CCK2R-dependent fashion. Crypt fission is a physiologic mechanism of crypt reproduction that is most commonly seen in neonatal animals and humans in situations requiring rapid mucosal growth (62). Crypt fission involves the reduplication of crypts from colonic progenitor cells and is a rare event in adults but increases in pathophysiologic situations in which intestinal regeneration is required (e.g., radiation injury) (63). In our study, elevated levels of circulating progastrin in hGAS$^{/+}$ mice significantly increased mice colonic crypt fission, but these increases were inhibited when mice were treated with a CCK2R antagonist or bred into a *Cck2r*$^{-/-}$ background. The increase in crypt fission correlated with an increase in DCAMKL1 progenitors and an expansion of crypt stem cells, likely resulting from symmetrical stem cell division that has been postulated to contribute to crypt fission and to cancer (64). Previous studies have suggested that APC mutation, which activates β-catenin, also stimulates the expansion of the crypt stem cells, leading to the initiation of crypt fission (62, 65).

Finally, our data showing reduced proliferation (DCAMKL1 progenitor number), crypt fission, and ACFs in mice treated with a CCK2R inhibitor suggest a possible role for CCK2R antagonism in colorectal cancer chemoprevention. We have previously shown the efficacy of another CCK2R antagonist, YF476, in the chemoprevention of *Helicobacter*-dependent gastric cancer (66). Other groups have demonstrated in preclinical studies the possible efficacy of CCK2R antagonists in carcinogenesis (67, 68). A number of CCK2/gastrin receptor antagonists have been developed, but only a few have been tested in clinical trials in human patients, and mostly in patients with advanced cancer (69). Thus, future studies should explore further the utility of targeting CCK2R in cancer prevention.

Methods

Mice experiments. hGAS transgenic mice on an FVB/N background were crossed with *Cck2r*$^{-/-}$ mice (F9 on FVB/N). Breeding resulted in littermates that included mice of hGAS$^{/+}$, hGAS$^{/+}$*Cck2r*$^{-/-}$, *Cck2r*$^{-/-}$, and WT (FVB/N). *Cck2r*$^{-/-}$ mice were provided by Alan S. Kopin (Tufts New England Medical Center, Boston, Massachusetts, USA). Mice were bred and maintained under specific pathogen–free conditions at the animal facility of Columbia University Medical Center (CUMC). All experiments were approved by the Subcommittee on the Research and Animal Care in the Irving Cancer Research Center at CUMC.

In the study of mouse colonic mucosa proliferation, four 6-week-old male mice in each group were injected intraperitoneally with 50 mg/kg BrdU (Sigma-Aldrich) 1 hour before being sacrificed. To further investigate the role of CCK2R in colonic mucosa proliferation, hGAS$^{/+}$ mice (n = 4) were injected intraperitoneally with 1 mg/kg CCK2R inhibitor YM022 (Tocris Bioscience) on days 1, 3, and 5 and sacrificed on day 7. To investigate the DCAMKL1 expression after whole-body irradiation (8 Gy), hGAS$^{/+}$ and WT mice (n = 3/group) were treated with γ irradiation with a ^{137}Cs source and sacrificed after 4.5 hours. To induce ACFs, 4 groups of 6-week-old sex-matched mice (n = 12/group) were given weekly intraperitoneal injections of 10 mg/kg AOM (Sigma-Aldrich; molecular weight, 36,000–50,000) in PBS for 2 weeks. Two weeks after the last injection, the mice were sacrificed and full-length colons were removed. Longitudinally dissected colons were spread on Whatman filter paper, followed by fixing with 70% ethanol for 24 hours. After staining with 0.3% methyl-blue for 1 minute, the total number of ACFs per colon was counted under a light microscope. To induce colorectal cancer, 4 groups of 6-week-old mice (n = 12/group) were injected weekly with AOM (10 mg/kg) for 6 weeks. Eleven weeks after the last injection, the mice were sacrificed, the colons were removed, and the total number and size (diameter in millimeters) of tumors per colon were analyzed. For animal mortality studies, 2 groups of 6-week-old mice (hGAS$^{/+}$, n = 25; hGAS$^{/+}$*Cck2r*$^{-/-}$, n = 23) were injected with AOM (10 mg/kg) once a week for 6 weeks and housed in separate cages under specific pathogen–free conditions at the animal facility of CUMC.

Histopathological and immunohistochemical analysis. Removed mouse colon tissues were rolled on a plastic bar using the Swiss roll technique (70), fixed in 10% neutral buffer formalin (VWR International) for paraffin embedding or fixed in 4% paraformaldehyde for freezing in Tissue-Tek O.C.T. compound (Sakura). Paraffin-embedded tissues were cut in 4-μm slices and deparaffinized for hematoxylin and eosin staining or further immunohistochemical analysis. β-Catenin staining was performed with the Animal Research Kit (Dako). The deparaffinized colon tissues were blocked with peroxidase blocking solution for 5 minutes, washed, and incubated for 15 minutes in prepared anti–β-catenin antibody (BD Biosciences), followed by 15 minutes of incubation with streptavidin-peroxidase. The staining was completed with 8 minutes of incubation with diaminobenzidine (Dako). For BrdU, CD44, DCAMKL1, and LgR5 immunohistochemistry, the deparaffinized tissues were incubated in hydrogen peroxide (3%) for 5 minutes and blocked with 2% BSA (Sigma-Aldrich) for 1 hour, followed

by incubation in anti-BrdU (Abcam Inc), anti-CD44 (BD Biosciences), anti-DCAMKL1 (Abgent), and anti-LgR5 (Novus Biologicals) antibodies for 1 hour. Samples were washed and incubated with HRP-conjugated anti-rat or anti-rabbit IgG (Dako). The epithelial cell proliferation rate was expressed as the number of BrdU-positive cells divided by the total number of cells in each crypt. Twenty crypts in different sites of each colon were randomly chosen. For immunofluorescence analysis of CCK2R expression in the colonic crypt, frozen sections were blocked with 2% BSA for 1 hour, followed by incubation in anti-CCK2R antibody (Santa Cruz Biotechnology Inc.) and anti–E-cadherin antibody (Cell Signaling Technology) at 4°C overnight. The samples were then washed and incubated with Texas Red–conjugated anti-goat IgG (Jackson ImmunoResearch Laboratories Inc.) and FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc.) and analyzed under a Nikon TE2000 microscope.

In situ hybridization. The removed colons of 6-week-old hGAS$^{/+}$ and WT mice were fixed in RNase-free 4% paraformaldehyde (VWR International) at 4°C overnight, dehydrated in RNase-free 30% sucrose (VWR International) for 24 hours, imbedded in O.C.T. compound, frozen cut into 8-μm sections, and stored at –80°C. Murine *Cck2r* gene cDNA (338 base pairs) was cloned into pCRII-TOPO vector (Invitrogen), and the oligonucleotide probes were synthesized with a Digoxin RNA labeling kit (Roche Applied Science). cRNA was used at a concentration of 500 ng/ml in hybridization buffer (50% formamide, 2× standard saline citrate [SSC], 5× Denhardt's solution, 1.0 mg/ml transfer RNA, 1.0 mg/ml salmon sperm DNA, 5 mM EDTA, 1 M DTT). Hybridization was performed at 55°C water bath overnight. Sections were washed in 5× SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) at room temperature (15 minutes), 2× SSC at 50°C (15 minutes), and 1× SSC at 50°C (15 minutes). To remove nonspecific bound DIG-labeled RNA probes, slides were incubated with RNase A (10 μg/ml in 500 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) for 15 minutes at 37°C, then washed once more with 2× SSC at 50°C (15 minutes) and 1× SSC at 50°C (15 minutes). Blocking was performed with 1% blocking solution (Roche Applied Science). Antibody incubation and detection with anti-digoxigenin–alkaline phosphatase were performed according to the manufacturer's instructions (Roche Applied Science). Sections were dehydrated, mounted with coverslips, and analyzed using light microscopy.

Analysis of apoptosis by TUNEL assay. Deparaffinized colonic tissues slides were stained with an In Situ Apoptosis Detection Kit (Chemicon) according to the manufacturer's instructions. The apoptotic index of TUNEL-positive cells was calculated using the total number of positive cells per field of sight at 10 different locations in each colon under light microscopy (×400).

Analysis of serum progastrin and amidated gastrin levels. Mice serums were collected by heart puncture on anesthetized mice, followed by centrifugation of the blood (10 minutes at 4,500 *g*) and separation of the serum. Serum progastrin was extracted with ethanol extraction in a 1:2 ratio of serum to ethanol and measured using antiserum 1137 (19). Amidated gastrin levels were measured in unextracted serum with antiserum 1296.

Gene expression analyses. For qRT-PCR analysis of CCK2R, CD44, caspase-3, FAS, and cyclin D1, 6-week-old male WT FVB/N mouse stomach, small intestine, colon, and colon tumor tissues were homogenized with IKA ULTRA-TURRAX Dispersers (IKA Works Inc.). The RNA was isolated using the TRIzol (Invitrogen) reagent, and reverse transcription was performed using the SuperScript III First-Strand Synthesis System (Invitrogen). qRT-PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems) using the comparative Ct quantitation method with QuantiTect SYBR Green PCR kit. Reactions were done at 95°C for 15 minutes, followed by 40 cycles of 94°C for 15 seconds, 55°C for 20 seconds, and 72°C 30 seconds.

Statistics. Group measures are expressed as mean ± SD for all parameters determined, unless otherwise indicated. Statistical analysis was performed using 1-way ANOVA followed by a Tukey or Dunnet's test. *P* values less than 0.05 were considered statistically significant. The mouse mortality analysis used the Mantel-Haenszel/log-rank test.

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1. Jemal, A., et al. 2003. Cancer statistics, 2003. *CA Cancer J. Clin.* **53**:5–26.
2. Bos, J.L., et al. 1987. Prevalence of ras gene mutations in human colorectal cancers. *Nature.* **327**:293–297.
3. Fearon, E.R., Hamilton, S.R., and Vogelstein, B. 1987. Clonal analysis of human colorectal tumors. *Science.* **238**:193–197.
4. Groden, J., et al. 1991. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell.* **66**:589–600.
5. Lipkin, M., and Newmark, H. 1988. Application of intermediate biomarkers and the prevention of cancer of the large intestine. *Prog. Clin. Biol. Res.* **279**:135–150.
6. Dockray, G.J. 2001. Gastrin-releasing peptide: an identity crisis at 30? *Lancet.* **358**:778–779.
7. Wang, T.C., et al. 1996. Processing and proliferative effects of human progastrin in transgenic mice. *J. Clin. Invest.* **98**:1918–1929.
8. Cobb, S., et al. 2004. Intestinal expression of mutant and wild-type progastrin significantly increases colon carcinogenesis in response to azoxymethane in transgenic mice. *Cancer.* **100**:1311–1323.
9. Baldwin, G.S., et al. 2001. Biologically active recombinant human progastrin(6-80) contains a tightly bound calcium ion. *J. Biol. Chem.* **276**:7791–7796.
10. Brown, D., Yallampalli, U., Owlia, A., and Singh, P. 2003. pp60c-Src Kinase mediates growth effects of the full-length precursor progastrin1-80 peptide on rat intestinal epithelial cells, in vitro. *Endocrinology.* **144**:201–211.
11. Ottewell, P.D., et al. 2003. Progastrin stimulates murine colonic epithelial mitosis after DNA damage. *Gastroenterology.* **124**:1348–1357.
12. Ottewell, P.D., et al. 2005. COOH-terminal 26-amino acid residues of progastrin are sufficient for stimulation of mitosis in murine colonic epithelium in vivo. *Am. J. Physiol. Gastrointest. Liver Physiol.* **288**:G541–G549.
13. Cobb, S., et al. 2002. Deletion of functional gastrin gene markedly increases colon carcinogenesis in response to azoxymethane in mice. *Gastroenterology.* **123**:516–530.
14. Koh, T.J., et al. 1997. Gastrin deficiency results in altered gastric differentiation and decreased colonic proliferation in mice. *Gastroenterology.* **113**:1015–1025.
15. Koh, T.J., et al. 2000. Gastrin is a target of the beta-catenin/TCF-4 growth-signaling pathway in a model of intestinal polyposis. *J. Clin. Invest.* **106**:533–539.
16. Singh, P., Velasco, M., Given, R., Varro, A., and Wang, T.C. 2000. Progastrin expression predisposes mice to colon carcinomas and adenomas in response to a chemical carcinogen. *Gastroenterology.* **119**:162–171.
17. Singh, P., et al. 2000. Mice overexpressing progastrin are predisposed for developing aberrant colonic crypt foci in response to AOM. *Am. J. Physiol. Gastrointest. Liver Physiol.* **278**:G390–G399.
18. Siddheshwar, R.K., Gray, J.C., and Kelly, S.B. 2001. Plasma levels of progastrin but not amidated gastrin or glycine extended gastrin are elevated in patients with colorectal carcinoma. *Gut.* **48**:47–52.
19. Ciccotosto, G.D., McLeish, A., Hardy, K.J., and Shulkes, A. 1995. Expression, processing, and secretion of gastrin in patients with colorectal carcinoma. *Gastroenterology.* **109**:1142–1153.
20. Smith, A.M., and Watson, S.A. 2000. Gastrin and gastrin receptor activation: an early event in the adenoma-carcinoma sequence. *Gut.* **47**:820–824.
21. Van Solinge, W.W., Nielsen, F.C., Friis-Hansen, L., Falkmer, U.G., and Rehfeld, J.F. 1993. Expression but incomplete maturation of progastrin in colorectal carcinomas. *Gastroenterology.* **104**:1099–1107.
22. Rehfeld, J.F., and van Solinge, W.W. 1994. The tumor biology of gastrin and cholecystokinin. *Adv. Cancer Res.* **63**:295–347.
23. Koh, T.J., and Chen, D. 2000. Gastrin as a growth

factor in the gastrointestinal tract. *Regul. Pept.* **93**:37–44.
24. Chakladar, A., et al. 2005. Synergistic activation of the murine gastrin promoter by oncogenic Ras and beta-catenin involves SMAD recruitment. *Biochem. Biophys. Res. Commun.* **336**:190–196.
25. Baldwin, G.S. 1995. The role of gastrin and cholecystokinin in normal and neoplastic gastrointestinal growth. *J. Gastroenterol. Hepatol.* **10**:215–232.
26. Singh, P., et al. 2003. Progastrin1-80 stimulates growth of intestinal epithelial cells in vitro via high-affinity binding sites. *Am. J. Physiol. Gastrointest. Liver Physiol.* **284**:G328–G339.
27. Singh, P., Wu, H., Clark, C., and Owlia, A. 2007. Annexin II binds progastrin and gastrin-like peptides, and mediates growth factor effects of autocrine and exogenous gastrins on colon cancer and intestinal epithelial cells. *Oncogene.* **26**:425–440.
28. Dubeykovskiy, A., Nguyen, T., Dubeykovskaya, Z., Lei, S., and Wang, T.C. 2008. Flow cytometric detection of progastrin interaction with gastrointestinal cells. *Regul. Pept.* **151**:106–114.
29. Kopin, A.S., et al. 1992. Expression cloning and characterization of the canine parietal cell gastrin receptor. *Proc. Natl. Acad. Sci. U. S. A.* **89**:3605–3609.
30. Lay, J.M., Jenkins, C., Friis-Hansen, L., and Samuelson, L.C. 2000. Structure and developmental expression of the mouse CCK-B receptor gene. *Biochem. Biophys. Res. Commun.* **272**:837–842.
31. de Weerth, A., et al. 1998. Gastrin/cholecystokinin type B receptors in the kidney: molecular, pharmacological, functional characterization, and localization. *Eur. J. Clin. Invest.* **28**:592–601.
32. Noble, F., and Roques, B.P. 1999. CCK-B receptor: chemistry, molecular biology, biochemistry and pharmacology. *Prog. Neurobiol.* **58**:349–379.
33. Todisco, A., et al. 2001. Molecular mechanisms for the antiapoptotic action of gastrin. *Am. J. Physiol. Gastrointest. Liver Physiol.* **280**:G298–G307.
34. Clerc, P., et al. 2002. Expression of CCK2 receptors in the murine pancreas: proliferation, transdifferentiation of acinar cells, and neoplasia. *Gastroenterology.* **122**:428–437.
35. McLellan, E.A., Medline, A., and Bird, R.P. 1991. Dose response and proliferative characteristics of aberrant crypt foci: putative preneoplastic lesions in rat colon. *Carcinogenesis.* **12**:2093–2098.
36. Langhans, N., et al. 1997. Abnormal gastric histology and decreased acid production in cholecystokinin-B/gastrin receptor-deficient mice. *Gastroenterology.* **112**:280–286.
37. van Es, J.H., Barker, N., and Clevers, H. 2003. You Wnt some, you lose some: oncogenes in the Wnt signaling pathway. *Curr. Opin. Genet. Dev.* **13**:28–33.
38. Pannequin, J., et al. 2007. Beta-catenin/Tcf-4 inhibition after progastrin targeting reduces growth and drives differentiation of intestinal tumors. *Gastroenterology.* **133**:1554–1568.
39. Germann, A., Dihlmann, S., Hergenhahn, M., Doeberitz, M.K., and Koesters, R. 2003. Expression profiling of CC531 colon carcinoma cells reveals similar regulation of beta-catenin target genes by both butyrate and aspirin. *Int. J. Cancer.* **106**:187–197.
40. Liu, J., and Jiang, G. 2006. CD44 and hematologic malignancies. *Cell. Mol. Immunol.* **3**:359–365.
41. Alao, J.P., et al. 2006. The cyclin D1 proto-oncogene is sequestered in the cytoplasm of mammalian cancer cell lines. *Mol. Cancer.* **5**:7.
42. Giannakis, M., et al. 2006. Molecular properties of adult mouse gastric and intestinal epithelial progenitors in their niches. *J. Biol. Chem.* **281**:11292–11300.
43. May, R., et al. 2008. Identification of a novel putative gastrointestinal stem cell and adenoma stem cell marker, doublecortin and CaM kinase-like-1, following radiation injury and in adenomatous polyposis coli/multiple intestinal neoplasia mice. *Stem Cells.* **26**:630–637.
44. Barker, N., et al. 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature.* **449**:1003–1007.
45. He, X.C., et al. 2007. PTEN-deficient intestinal stem cells initiate intestinal polyposis. *Nat. Genet.* **39**:189–198.
46. Przemeck, S.M., et al. 2008. Hypergastrinemia increases gastric epithelial susceptibility to apoptosis. *Regul. Pept.* **146**:147–156.
47. Stepan, V.M., et al. 1999. Glycine-extended gastrin regulates HEK cell growth. *Am. J. Physiol.* **277**:R572–R581.
48. Sandvik, A.K., and Dockray, G.J. 1999. Biological activity of carboxy-terminal gastrin analogs. *Eur. J. Pharmacol.* **364**:199–203.
49. Cho, N.L., et al. 2008. Aberrant crypt foci in the adenoma prevention with celecoxib trial. *Cancer Prev. Res. (Phila. Pa.).* **1**:21–31.
50. Upp, J.R., Jr., Singh, P., Townsend, C.M., Jr., and Thompson, J.C. 1989. Clinical significance of gastrin receptors in human colon cancers. *Cancer Res.* **49**:488–492.
51. Smith, J.P., Stock, E.A., Wotring, M.G., McLaughlin, P.J., and Zagon, I.S. 1996. Characterization of the CCK-B/gastrin-like receptor in human colon cancer. *Am. J. Physiol.* **271**:R797–R805.
52. Baldwin, G.S., and Shulkes, A. 2007. CCK receptors and cancer. *Curr. Top. Med. Chem.* **7**:1232–1238.
53. Yu, H.G., et al. 2006. Enhanced expression of cholecystokinin-2 receptor promotes the progression of colon cancer through activation of focal adhesion kinase. *Int. J. Cancer.* **119**:2724–2732.
54. Goswami, R.S., et al. 2006. Hyperplastic polyposis and cancer of the colon with gastrinoma of the duodenum. *Nat. Clin. Pract. Oncol.* **3**:281–284; quiz 285.
55. Dalerba, P., et al. 2007. Phenotypic characterization of human colorectal cancer stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **104**:10158–10163.
56. Scoville, D.H., Sato, T., He, X.C., and Li, L. 2008. Current view: intestinal stem cells and signaling. *Gastroenterology.* **134**:849–864.
57. Quante, M., and Wang, T.C. 2008. Inflammation and stem cells in gastrointestinal carcinogenesis. *Physiology (Bethesda).* **23**:350–359.
58. Karelina, Y., and Baldwin, G.S. 2001. Binding sites for progastrin-derived peptides in colonic crypts. *J. Gastroenterol. Hepatol.* **16**:169–175.
59. Lin, P.T., Gleeson, J.G., Corbo, J.C., Flanagan, L., and Walsh, C.A. 2000. DCAMKL1 encodes a protein kinase with homology to doublecortin that regulates microtubule polymerization. *J. Neurosci.* **20**:9152–9161.
60. Wu, H., Owlia, A., and Singh, P. 2003. Precursor peptide progastrin(1-80) reduces apoptosis of intestinal epithelial cells and upregulates cytochrome c oxidase Vb levels and synthesis of ATP. *Am. J. Physiol. Gastrointest. Liver Physiol.* **285**:G1097–G1110.
61. Rengifo-Cam, W., Umar, S., Sarkar, S., and Singh, P. 2007. Antiapoptotic effects of progastrin on pancreatic cancer cells are mediated by sustained activation of nuclear factor-{kappa}B. *Cancer Res.* **67**:7266–7274.
62. Humphries, A., and Wright, N.A. 2008. Colonic crypt organization and tumorigenesis. *Nat. Rev. Cancer.* **8**:415–424.
63. Thompson, J.S., Saxena, S.K., and Sharp, J.G. 1999. Difluoromethylornithine inhibits crypt fission. *J. Gastrointest. Surg.* **3**:662–667.
64. Morrison, S.J., and Kimble, J. 2006. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature.* **441**:1068–1074.
65. Boman, B.M., et al. 2004. Colonic crypt changes during adenoma development in familial adenomatous polyposis: immunohistochemical evidence for expansion of the crypt base cell population. *Am. J. Pathol.* **165**:1489–1498.
66. Takaishi, S., et al. 2005. Synergistic inhibitory effects of gastrin and histamine receptor antagonists on Helicobacter-induced gastric cancer. *Gastroenterology.* **128**:1965–1983.
67. Fontana, M.G., et al. 2001. Distribution of 1,2 DMH-induced colonic aberrant crypt foci after administration of a gastrin receptor antagonist (CR2945), in the murine model. *Ann. Ital. Chir.* **72**:221–225.
68. Grabowska, A.M., et al. 2008. Pre-clinical evaluation of a new orally-active CCK-2R antagonist, Z-360, in gastrointestinal cancer models. *Regul. Pept.* **146**:46–57.
69. Chau, I., et al. 2006. Gastrazole (JB95008), a novel CCK2/gastrin receptor antagonist, in the treatment of advanced pancreatic cancer: results from two randomised controlled trials. *Br. J. Cancer.* **94**:1107–1115.
70. Weiner, M.L., Nuber, D., Blakemore, W.R., Harriman, J.F., and Cohen, S.M. 2007. A 90-day dietary study on kappy carrageenan with emphasis on the gastrointestinal tract. *Food Chem. Toxicol.* **45**:98–106.