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Progastrin Stimulates Colonic Cell Proliferation via CCK2R- and β-Arrestin–Dependent Suppression of BMP2

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Abstract

Background & Aims—Progastrin stimulates colonic mucosal proliferation and carcinogenesis through the cholecystokinin 2 receptor (CCK2R)—partly by increasing numbers of colonic progenitor cells. However, little is known about the mechanisms by which progastrin stimulates colonic cell proliferation. We investigated the role of bone morphogenetic proteins (BMPs) in progastrin induction of colonic cell proliferation via CCK2R.

Methods—We performed microarray analysis to compare changes in gene expression in the colonic mucosa of mice that express a human progastrin transgene (hGAS), gastrin knockout

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(GAS−/−) mice, and C57BL/6 mice (controls); the effects of progastrin were also determined on in vitro colonic crypt cultures from cholecystokinin 2 receptor knockout (CCK2R−/−) and wild-type mice. Human colorectal and gastric cancer cells that expressed CCK2R were incubated with progastrin or Bmp2 protein; levels of β-arrestin-1 and -2 (ARRB1 and ARRB2) were knocked down using small interfering RNAs. Cells were analyzed for progastrin binding, proliferation, changes in gene expression, and symmetric cell division.

Results—The BMP pathway was downregulated in the colons of hGAS mice, compared with controls. Progastrin suppressed transcription of Bmp2 through a pathway that required CCK2R and was mediated by ARRB1 and ARRB2. In mouse colonic epithelial cells, downregulation of Bmp2 led to decreased phosphorylation of Smads1/5/8 and suppression of Id4. In human gastric and colorectal cancer cell lines, CCK2R was necessary and sufficient for progastrin binding and induction of proliferation; these effects were blocked when cells were incubated with recombinant Bmp2. Incubation with progastrin increased the number of $CD44^+$, bromodeoxyuridine+, and $NUMB^+$ cells, indicating an increase in symmetric divisions of putative cancer stem cells.

Conclusions—Progastrin stimulates proliferation in colons of mice and cultured human cells via CCK2R- and ARRB1- and 2-dependent suppression of Bmp2 signaling. This process promotes symmetric cell division.

Keywords

Progastrin; CCK2R; BMP

Introduction

Progastrin, the incompletely processed form of gastrin, is an important colonic growth factor.[1] In mice, overexpression of human progastrin (hGAS) leads to increased colonic Lgr5 stem cells, mucosal thickness, and susceptibility to AOM-dependent carcinogenesis.[2-4] Furthermore, overexpression or exogenous administration of progastrin is sufficient to drive colonic epithelial regeneration after DNA damage, suggesting effects on colonic stem or progenitor cells.[5] While these biological effects have been reproducible *in vivo*, the downstream mechanisms have yet to be defined.[6]

The CCK2R is a G-protein coupled receptor that binds both amidated gastrin and cholecystokinin (CCK), but was initially thought not to bind incompletely processed forms of gastrin such as progastrin.[7] Our group demonstrated that CCK2R is expressed in colonic crypt, and that progastrin stimulates proliferation, increases colonic stem cell numbers[2] and promotes colonic tumorigenesis in a CCK2R-dependent manner.[8] Accordingly, knockout of the CCK2R or treatment with a small molecule receptor antagonist (YM022) strongly inhibited AOM-dependent aberrant crypt foci (ACF) and tumor formation.[8] This study suggested that progastrin interacts with the CCK2R, and modulates the colonic stem cell compartment, but fell short of demonstrating a direct interaction between progastrin and epithelial stem cells. Recently, Duckworth et al demonstrated that progastrin could potentially increase colonic proliferation in a CCK2R-independent manner by stimulating IGF-2 secretion from colonic myofibroblasts.[9] Furthermore, other candidate receptors, such as Annexin II,[10] also appear to play a role in mediating some of progastrin's effects. Nevertheless, the precise mechanisms by which the progastrin/CCK2R axis promotes colonic proliferation and carcinogenesis have not been elucidated.

Hyperproliferation of the colonic epithelium is a risk factor for colorectal cancer, and typically involves an expansion of stem cells resulting in crypt fission, whereby a new colonic crypt is formed and branches off from the original crypt.[11] Stem cell expansion requires symmetric stem cell division,[12] and the assessment of symmetric versus asymmetric

cell division has been analyzed by the segregation of cell fate determinants such as Numb[13] or through assessment of pulse-chase 5-bromo-2-deoxyuridine (BrdU)-labeled DNA strands.[14]

One signaling cascade of the colonic epithelium that has been linked to stem cell expansion, crypt fission and colon cancer, is the bone morphogenetic protein (BMP) pathway. BMP proteins are members of the transforming growth factor-β (TGF-β) superfamily, and play a key role in gastrointestinal development and adult tissue homeostasis. The main BMP ligands in the intestine, Bmp2 and Bmp4, function by binding to the type II receptor (Bmpr2) and recruitment of type 1 receptors (Bmpr1a or Bmpr1b), leading to phosphorylation of Bmpr1 and signal transduction through SMAD transcription factors. Colonic epithelial cells produce Bmp2, which acts in an autocrine fashion to inhibit colonic proliferation and induce apoptosis.[15] The BMP pathway itself is inactivated in the majority of sporadic colorectal cancer during tumor progression.[16] In addition, inactivation of BMP signaling in the intestinal epithelium results in the expansion of intestinal stem cells, crypt fission, and the development of intestinal tumors.[17] Finally, aberrant expression of the BMP antagonist Gremlin1 underlies human hereditary mixed polyposis syndrome.[18] In the current study, we sought to understand the CCK2R-dependent mechanisms by which progastrin stimulates proliferation and cancer susceptibility in the murine colon. These studies reveal a novel connection to BMP suppression and expansion of progenitor cells through symmetric division.

Materials and methods

Animals

Human progastrin (hGAS) transgenic mice, gastrin knockout mice ($GAS^{-/-}$), Lgr5-GFP and UBC-GFP mice, cholecystokinin receptor knockout mice ($CCK2R^{-/-}$), and wild type mice on an C57BL/6 background 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.

For detailed methods, see Supplemental information.

Results

Progastrin overexpression suppresses Bmp2 pathway in colonic mucosa

To investigate differences in gene expression induced by progastrin which might be responsible for increased proliferation, we carried out a microarray analysis on three groups of mice colonic mucosa. These measurements revealed a number of genes that are differentially expressed in hGAS vs WT hGAS vs $GAS^{-/-}$ and $GAS^{-/-}$ vs WT (Supplemental Table 1). Pathway Express[19] analysis showed that many of these genes are associated with proliferative pathways, as expected (Supplemental Table 2). These include WNT pathway genes, which have been reported to be involved in progastrin signaling.[20] However, Tcf3, Tcf4, and Tcf7, key genes in the canonical WNT pathway (Supplemental Table 3), and WNT target genes (Supplemental Table 4) were downregulated or without significant difference in hGAS vs. WT mice, the comparison most indicative of the action of progastrin alone, suggesting a lesser role for WNT in progastrin's proliferative effects. In contrast, the BMP pathway was significantly dysregulated in the hGAS vs. WT comparison (Supplemental Table 5), particularly Bmp2, a known tumor suppressor in colon cancer.[15, 21] We analyzed the relative expression of BMP genes by RT-PCR (Figures 1B, 1C) and found that Bmp2 and Bmp7 were significantly decreased in hGAS vs. WT mice. Furthermore, immunostaining, ELISA, and Western blot showed downregulation of Bmp2 protein in the

colon of hGAS mice when compared with WT controls (Figures 1A, 1E, 1F, Supplemental Figure 1A).

A cluster diagram of the 3 comparisons for select BMP pathway genes is shown in Supplemental Figure 2A. Bmp2 indirectly induces the transcription of inhibitor of DNA binding 4 (Id4),[22] a postulated tumor suppressor,[21, 23] such that downregulation of Bmp2 would be expected to decrease Id4. Indeed, Id4 was downregulated in hGAS mice in our microarray study confirmed by quantitative RT-PCR (Figure 1D) and by immunohistochemistry (Figure 1A). Thus, downregulation of Bmp2 leads to decreased phosphorylated Smads1/5/8 (pSmads1/5/8) and downregulation of Id4 (Supplemental Figure 2B), changes which could enhance tumorigenesis. Taken together, microarray and PCR analyses strongly suggest that progastrin stimulates colonic proliferation and progenitor expansion at least in part by suppression of the Bmp2 pathway.

Progastrin downregulates phosphorylated Smad1/5/8 protein expression in the mouse colon

Smads1/5/8 transduce the majority of intracellular signaling by bone morphogenetic proteins.[24] Western blot analysis of colonic mucosa revealed significant downregulation of pSmad1/5/8 upon progastrin overexpression (Figure 1F). Immunoreactivity for pSmads1/5/8 was easily detectable in the nuclei of colonic crypt epithelial cells from WT mice, but was markedly lower in hGAS mice (Figure 1A). ID proteins, which have also been linked to the BMP pathway,[25] are key regulators in embryonic development where they inhibit premature differentiation of stem cells. In contrast, inappropriate suppression of ID proteins in differentiated cells can contribute to tumorigenesis.[26] Consistent with our findings of decreased Id4 mRNA, we found significantly downregulated Id4 protein level in the hGAS colon (Figure 1A, 1F). Thus, our findings strongly suggest that progastrin stimulates colonic growth in part by suppression of the BMPs, Smads1/5/8 and Id4.

Progastrin increases colonic epithelial proliferation through Bmp2

In order to investigate the possibility that progastrin regulates Bmp2 expression through the CCK2R, we isolated colonic crypts[27] from WT and CCK2R$^{-/-}$ mice. Noggin, a Bmp2 antagonist, is required for successful colonic crypt culture.[27] Progastrin had no effect in noggin-containing media (Supplemental Figure 3A); however, following three to seven days in culture under noggin-free media conditions, progastrin treatment led to increased colonic organoid survival (Figure 2A, 2B, Supplemental Figure 3A) and suppressed Bmp2 mRNA expression in cultured organoids (Figure 2C), suggesting that progastrin restores noggin function through Bmp2 suppression. These effects were clearly mediated through CCK2R, since they were absent in CCK2R$^{-/-}$ organoids. To assess the effects of progastrin on colonic organoid budding, the *in vitro* correlate of crypt fission[28], we cultured colonic organoids from UBC-GFP mice and indeed observed increased budding after seven days of progastrin treatment (Supplemental Figure 3B, 3C). Finally, to determine if dysregulation of BMP signaling is critical for progastrin-mediated proliferation *in vivo*, we injected recombinant Bmp2 protein (0.33ug/g) once a day for 7 days into both hGAS and WT mice, and found that systemic Bmp2 treatment significantly reduced colonic mucosa proliferation in hGAS mice but had no effect in controls (Fig 2D, 2E). These findings support the conclusion that CCK2R-mediated downregulation of Bmp2 is largely responsible for the increase in colonic proliferation in hGAS mice.

Progastrin increases cell proliferation through CCK2R

CCK2R is expressed in colonic crypts[8] and CCK2R inactivation inhibits gastrointestinal proliferation and tumorigenesis.[8, 29] To investigate whether progastrin binds to CCK2R, we studied the gastric cancer cell line AGSE and the colorectal cancer cell line Colo320(+) that

stably express CCK2R. *In vitro*, we observed an increase of intracellular CCK2R staining upon progastrin treatment, arguing for internalization of the receptor following ligand binding (Figure 3A). Next, we co-cultured AGSE and AGS cells in a single plate, and following treatment with progastrin, found that progastrin bound to the surface of AGSE cells but not to AGS cells (white arrows) (Supplemental Figure 4A). These findings were confirmed when AGS and AGSE cells were mixed in solution and incubated with progastrin (Supplemental Figure 5). To confirm the specificity of progastrin binding, we took advantage of the fact that ligand binding to GPCRs is temperature sensitive.[30] As expected, lowering the temperature of the culture medium to 4°C resulted in a marked decrease of progastrin binding to Colo320(+) cells (Supplemental Figure 6). Taken together, these results demonstrate that progastrin binds to the CCK2R, and that expression of CCK2R is both necessary and sufficient for progastrin binding.

To determine whether progastrin induces cell proliferation in part through the CCK2R, AGSE and Colo320(+) cells were treated with progastrin in serum free medium. Using the MTT assay, we found a significant stimulatory effect of progastrin on proliferation of cells that expressed CCK2R (Figure 3B, Supplemental Figure 7A). This effect was absent in AGS and Colo320(−) cells (both lacking CCK2R expression) (Figure 3B, Supplemental Figure 7B). Additionally, progastrin treatment significantly increased BrdU uptake in Colo320(+) cells but not in Colo320(−) cells (Figure, 4A, 4B). Interestingly, in addition to more rapid proliferation, progastrin-treated AGSE cells also showed morphological changes suggestive of increased mitotic activity (arrows) (Figure, 4C, 4D). YF476, a highly selective gastrin/CCK2 receptor antagonist[31] previously been shown to specifically inhibit gastrin-dependent proliferation and gastric carcinogenesis,[29] effectively inhibited progastrin binding to AGSE cells (Supplemental Figure 4B). Furthermore, YF476 had no effect on Colo320(−) cells (Figure 3D), but abolished the growth stimulatory effect of progastrin in Colo320(+) cells in a dose-dependent manner (Figure 3C), as well as all morphological changes seen upon progastrin treatment (Figure 4C). Taken together, these findings demonstrate that progastrin stimulates cell proliferation, at least in part, through direct interactions with the CCK2R.

While both progastrin and gastrin bind to the G-protein coupled receptor CCK2R, they appear to have distinct effects, with only amidated gastrin causing gastric acid secretion in the stomach.[32] In contrast, while progastrin stimulated proliferation of CCK2R-expressing epithelial cells, amidated gastrin (G-17) could not. G-17 at a concentration of 8×10^{-8} mol/L was not able to stimulate proliferation of AGSE cells, nor was it able to affect Bmp2 gene expression (data not shown). To investigate possible differential signaling by these two ligands, we treated AGSE cells with either G-17 or progastrin and analyzed changes in intracellular calcium as a measure of classical GPCR activation. We found that G-17 induced a significant increase in cytosolic calcium, while progastrin treatment had no effect (Figure 5A). One possible explanation for these distinct effects is different receptor coupling, since G protein-coupled receptors have been shown to signal through beta-arrestin as well as through G proteins.[33] To determine whether beta-arrestin plays a role downstream of the CCK2R, we treated Colo320 cells with siRNA against beta-arrestin 1 and 2, and found a significant decrease in beta-arrestin 1 (Supplemental Figure 8A) and beta-arrestin 2 (Supplemental Figure 8B) mRNA levels in Colo320(+) cells. Importantly, progastrin failed to induce proliferation after knockdown of either beta-arrestin 1 or beta-arrestin 2 (Figure 5B). Furthermore, beta-arrestin knockdown blocked progastrin-induced Bmp2 downregulation (Figure 5D). Interference with the beta-arrestins had no effect on Colo320(−) cells (Figure 5C&E). We also assessed symmetric division after tranfection of beta-arrestin 1 and 2 siRNA, and found a significant decrease in symmetric cell division in Colo320(+) cells (Figure 5F). This effect was not seen in CCK2R negative Colo320(−) cells (Figure 5F). These findings suggest that, in contrast to activation of classical G protein-

coupled pathways seen with amidated gastrin/CCK2R interactions, progastrin binding to CCK2R signals through a beta-arrestin mediated pathway.

Bmp2 suppresses progastrin-dependent colon cancer cells proliferation *in vitro*

To determine the functional significance of progastrin-dependent Bmp2 downregulation, we studied the effects of Bmp2 reconstitution. First, we confirmed that progastrin stimulation of Colo320(+) and AGSE cells resulted in a significant downregulation of endogenous Bmp2 mRNA compared to untreated cells (Figure 6A, Supplemental Figure 7C). Parental Colo320(−) and AGS cells showed no change in Bmp2 mRNA levels after progastrin treatment (Figure 6A, Supplemental Figure 7D), confirming that downregulation of Bmp2 gene expression was indeed CCK2R-dependent. Progastrin stimulation also suppressed Bmp2 protein levels, as demonstrated by ELISA (Figure 6B) and western blot (Figure 6C, Supplemental Figure 1B, 1C). Notably, Bmp2 (50ng/ml) treatment completely abrogated progastrin-induced proliferation in Colo320(+) cells (Figure 6D). As expected, treatment of Colo320(+) and Colo320(−) with the BMP antagonist Noggin increased cell proliferation in both cell lines (Figure 6E). Furthermore, YF476 treatment efficiently blocked progastrin induced suppression of Bmp2 expression in Colo320(+) cells (Supplemental Figure 8C) but had no effect on Bmp2 expression in Colo320(−) cells (Figure Supplemental Figure 8D). Additionally, progastrin treatment of Colo320(+) cells caused a significant downregulation of Id4 mRNA (Figure 6F). In summary, progastrin stimulated cellular proliferation through suppression of the Bmp2 pathway in a CCK2R-dependent manner.

To determine whether the suppression of Bmp2 mRNA expression by progastrin was a transcriptional response; we analyzed the effects of progastrin on Bmp2 promoter activity using previously generated Bmp2 promoter luciferase constructs.[34] In Colo320(+) cells, progastrin treatment markedly suppressed the relative luciferase activities of −2712/165 and −1997/165 Bmp2 promoters constructs, while it had no effect on the short −212/165 construct (Supplemental Figure 8E), indicating that transcription factor binding occurs upstream of −212. In Colo320(−) cells, robust Bmp2 promoter activity was found with all constructs transfected (Supplemental Figure 8E). Afterwards, we carried out an in *silico* analysis for potential transcription factor binding sites within the Bmp2 promoter region. Not surprisingly, we identified multiple different potential binding sites (Supplemental Table 6). To specify the transcription factors involved further, we compared the human and murine promoter sequence for conserved regions. Indeed, we identified a conserved region, which included eleven transcription factor binding sites. We reason that transcription factors binding within this conserved region are the most likely candidates regulating Bmp2 expression through CCK2R (Supplemental Table 6 - bold).

Progastrin increases stem or progenitor cell proliferation

The Bmp2 pathway, which is suppressed by progastrin, has previously been linked to the maintenance of stem cells in the gastrointestinal tract.[17] The BMP inhibition increases Wnt and AKT signaling.[17, 35] In line with this observation, hGAS mice crossed to Lgr5-GFP mice showed a significant increase in Lgr5 positive colonic stem cells (Supplemental Figure 9A, 9B), which is regulated by suppression of BMP signaling. Given its effect on normal colonic stem cells, we hypothesized that progastrin has a similar effect on putative cancer stem cells. CD44 appears to be a useful marker of both colorectal and gastric cancer stem cells.[36] Therefore, we treated AGSE cells with progastrin and analyzed the proportion of CD44+ cells by FACS. At baseline, in the absence of progastrin, 11% of cells (both AGSE and AGS cells) were positive for CD44. However, following progastrin treatment, the proportion of CD44+ cells increased to 16% in the AGSE cells, but remained unchanged in AGS cells (Figure 7A, 7B).

Numb is a cell fate determinant which normally controls cell fate through asymmetric partitioning at mitosis,[13] and is used as a standard measure of symmetric or asymmetric cell division.[13, 37] To investigate the possible modulation of symmetric versus asymmetric cell division by progastrin, we treated Colo320(+) cells with progastrin and analyzed Numb expression in mitotic cells by immunohistochemistry. Cells undergoing symmetric cell division were characterized by an equal expression of Numb in both daughter cells, while cells undergoing asymmetric division were characterized by Numb expression in only one daughter cell (Supplemental Figure 10A). At baseline, there were 1.4% and 4.9% cells that were Numb positive in Colo320(−) and Colo320(+) cells, respectively. Following progastrin treatment, the percentage of numb positive cells increased significantly to 17.9% in Colo320(+) cells, compared with untreated Colo320(+) cells (Supplemental Figure 10B, 10C). Progastrin treatment of Colo320(−) cells did not have any effect on Numb positive cells (Supplemental Figure 10B, 10C). These results argue for a progastrin-mediated increase in symmetric cell division.

BrdU has been used to label DNA and track label-retaining cells that are proliferating slowly or dividing asymmetrically.[38] Thus, we grew AGSE cells for a number of passages in the presence of 1μM BrdU then, removed the BrdU and confirmed our ability to address symmetric versus asymmetric cell divisions (Figure. 7C). In separate experiments, this was repeated in the presence or absence of progastrin. As expected, progastrin increased symmetric divisions and decreased asymmetric divisions in CCK2R-expressing AGSE cells (Figure. 7D). Moreover, Bmp2 treatment suppressed progastrin-dependent AGSE cell symmetric division (Figure. 7E), supporting the notion that progastrin increased symmetric division through the CCK2R-dependent Bmp2 pathway.

Discussion

In this study, we show that progastrin stimulates colonic proliferation through a CCK2R-dependent pathway that involves suppression of Bmp2 expression. Down-regulation of Bmp2 transcription by progastrin was confirmed through unbiased gene expression arrays, quantitative RT-PCR and reporter gene assays. Downregulation of Bmp2 strongly contributes to the proliferative effects of progastrin, since reconstitution with exogenous Bmp2 blocked the stimulatory effects of progastrin on epithelial growth. The effects of progastrin on proliferation and Bmp2 expression were clearly mediated through binding to the CCK2R, as they were blocked with CCK2R antagonists. In contrast to signaling with amidated gastrin, progastrin stimulation of the CCK2R appears to be mediated through the beta-arrestin pathway. Finally, we link progastrin stimulation and Bmp2 suppression to increased symmetric cell division of cancer stem cells, consistent with the proposed role for Bmp2 in suppressing the expansion of intestinal stem cells.

We recently proposed that the CCK2R is a critical component of the receptor complex that binds progastrin in gastrointestinal epithelial cells, since progastrin-dependent stimulation of colonic proliferation was ablated by knockout of the CCK2R gene or antagonism with YF476.[8] In addition, CCK2R expression was localized to the progenitor region at the base of the colonic crypts.[8] We now show more directly that progastrin binds to the CCK2R. *In vitro*, progastrin bound only to cells that expressed CCK2R, but not to CCK2R negative cells. Progastrin binding occurred within 1 minute of stimulation, and the ligand could be found in both membranous and cytoplasmic locations, suggesting CCK2R internalization after ligand binding. While other proteins, such as Annexin II, have been shown to interact with progastrin and may be essential for the proliferative response,[10] our findings confirm a critical role for CCK2R mediating progastrin interactions with epithelial cells. Additionally, although progastrin-mediated IGF-2 release from colonic myofibroblasts may also influence colonic proliferation,[9] the findings in the current study clearly demonstrate that progastrin

exerts direct effects on colonic epithelial proliferation. Moreover, despite the fact that the myofibroblasts effects are CCK2R-independent, previous studies showed that both CCK2R knockout and CCK2R antagonism inhibit progastrin-dependent proliferation and colonic carcinogenesis *in vivo*.[8]

Numerous reports have demonstrated that progastrin stimulates colonic proliferation, while amidated gastrin does not.[39] Our finding that progastrin also binds to the CCK2R was initially somewhat puzzling, since studies to date have not shown the activation of classical GPCR signaling pathways by progastrin stimulation. Thus, while numerous G protein-coupled pathways have been linked to signaling by amidated gastrin through the CCK2R, including Ca^{2+} and ERK signaling,[40] we show for the first time that progastrin signals not through classical G protein-coupling, but rather through a beta-arrestin pathway. In contrast to amidated gastrin, progastrin failed to activate intracellular calcium fluxes, a classical measure of Gq-mediated activation of protein kinase C. Furthermore, progastrin-dependent proliferation was blocked by siRNAs directed against beta-arrestin 1 and 2. It is now well accepted that, depending on the ligand, multiple GPCRs can signal either through G-proteins or beta-arrestins.[41, 42] If a ligand preferentially signals through either of the two pathways, it is termed a *biased* agonist.[43] While biased agonism has been described for the CCK1R,[43] such a mechanism has not been demonstrated for the CCK2R. The finding that CCK2R can also signal through beta-arrestin could therefore, explain the differential effects of gastrin and progastrin on the gastrointestinal tract.

Regulation of the BMP pathway through beta-arrestins has previously been demonstrated.[44] We confirmed in this study that Bmp2 is expressed primarily in the colonic epithelium,[15] and gene expression arrays and pathway expression analysis comparing hGAS and WT mice showed the consistent finding that the BMP pathways were strongly suppressed in colonic tissue from hGAS mice. Our gene expression arrays revealed less of a change in WNT pathways, despite previous reports linking progastrin signaling and the WNT pathway.[20] In addition, we show the downregulation of BMP downstream targets Id4 and Smads1/5/8. Our finding that Id4 is downregulated in hGAS mice is in line with the important tumor suppressor function of Id4.[45] While the regulation of Id4 through Bmp2 under physiological conditions had previously been described,[46] our findings have important implications as we demonstrate that this axis is also functional and of relevance under pathological conditions.

Given the strong effect of progastrin on colonic tumorigenesis, downregulation of BMP signaling appears to be a plausible mechanism for progastrin's carcinogenic effects. BMP signaling is inactivated in the majority of sporadic colon cancer,[16] and in individuals with juvenile polyposis approximately 50% of patients carry germline mutations in either the BMPR1A or Smad4 gene.[47] Thus, Bmp2 appears to represent an important intestinal tumor suppressor, with multiple lines of evidence suggesting that disturbances in BMP signaling may contribute to tumorigenesis.[15]

The link between progastrin, BMP signaling and carcinogenesis may well be related to effects of BMP signaling on stem and progenitor cells. Several groups have postulated that the early steps in colonic carcinogenesis involve an expansion of stem and progenitor cells.[48] While many stem cells favor asymmetric cell division under homeostatic conditions, in the setting of regeneration or neoplasia, the number of stem cells is increased by symmetric cell division, whereby two stem cells are produced following mitosis.[12] Progastrin stimulation, through downregulation of BMP2, appears to stimulate symmetric cell division, as revealed by increased murine colonic organoids budding *in vitro*, percentages of CD44+ cancer stem cells, as well as through BrdU pulse labeling and changes in Numb expression. Interestingly, conditional inactivation of Bmpr1a causes a disruption of epithelial BMP signaling in mice and disturbs intestinal homeostasis to result

in intestinal polyposis through an expansion of the stem and progenitor cell populations.[17] These findings are in line with our observation that progastrin leads to an expansion of colonic Lgr5 cells.[2] Previous studies have suggested that many tumor suppressor genes (such as Apc) may modulate tumor risk through promotion of symmetric stem cell division.[49] This is the first demonstration that symmetric stem cell division can be regulated through biased signaling through a GPCR, and may represent an attractive target for chemoprevention and modulation of cancer risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard abbreviations used

(hGAS)	Human progastrin
(Bmp2)	Bone morphogenetic protein 2
(CCK2-R)	Cholecystokinin-2 receptor
(Id4)	Inhibitor of DNA binding 4
($GAS^{-/-}$)	Gastrin knockout
(RT-PCR)	Reverse-transcription polymerase chain reaction
(pSmad1/5/8)	Phosphorylated Smads1/5/8
(BrdU)	5-bromo-2′-deoxyuridine
(UBC)	Human ubiquitin C
(GFP)	Green Fluorescent Protein
(G-17)	Amidated gastrin 17

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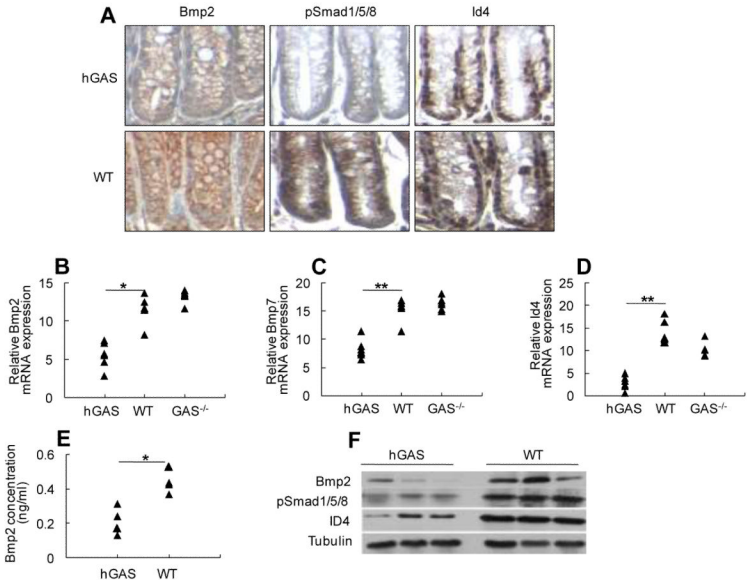

Figure 1. Progastrin downregulates BMP pathway and Id4 genes in the colonic mucosa of hGAS mice

(A) Immunohistochemistry for Bmp2, pSmad1/5/8, and Id4 protein expression in the colonic mucosa of hGAS and WT mice (original magnification, −800). Quantitative RT-PCR analysis of (B) Bmp2 (C) Bmp7 and (D) Id4 mRNA levels in the colonic mucosa of hGAS, WT, and $GAS^{-/-}$ mice ($n = 6$ mice/group). Expression levels were normalized to GAPDH mRNA. (E) ELISA for Bmp2 protein in the colonic mucosa of hGAS and WT mice ($n = 4$ mice/group). All values represent the mean ± SD. $*P < 0.05$, $**P < 0.01$. (F) Western blot analysis of Bmp2, pSmad1/5/8, Id4 protein levels from murine colonic mucosa ($n = 3$ mice/group).

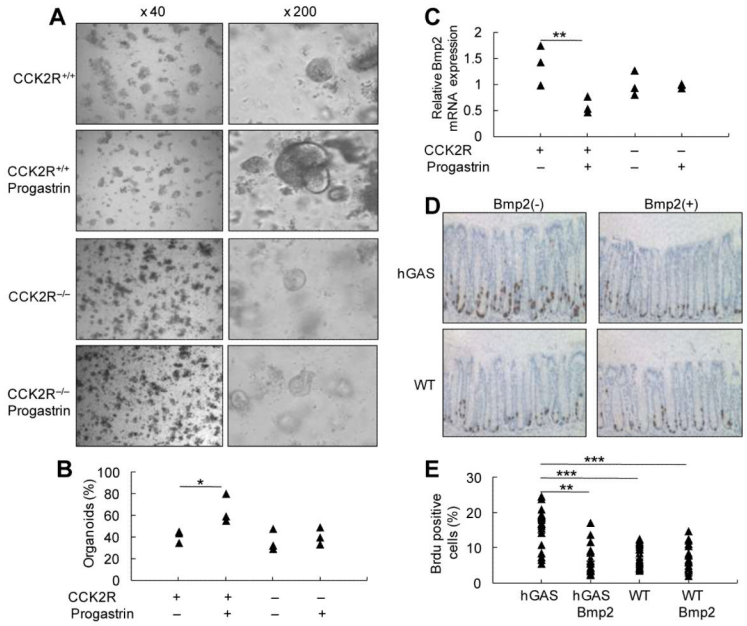

Figure 2. Progastrin increases mucosal proliferation through CCK2R dependent suppression of Bmp2 pathway

(A) Representative pictures (original magnification, −40 and −200) and (B) the numbers of colonic crypt organoids from WT and CCK2R$^{-/-}$ mice (n = 3 plates/group) after three days in culture in Wnt3A, EGF, and R-spondin1 containing media with or without progastrin (1ug/ml). (C) Quantitative RT-PCR analysis of Bmp2 mRNA expression in colonic organoids (n =3 plates/group). Expression levels were normalized to GAPDH mRNA. (D) Immunohistochemistry for BrdU in the colonic mucosa after Bmp2 protein injection (original magnification, −300). (E) The percentage of BrdU positive cells in colonic crypts (n = 23 crypts/group). All values represent the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

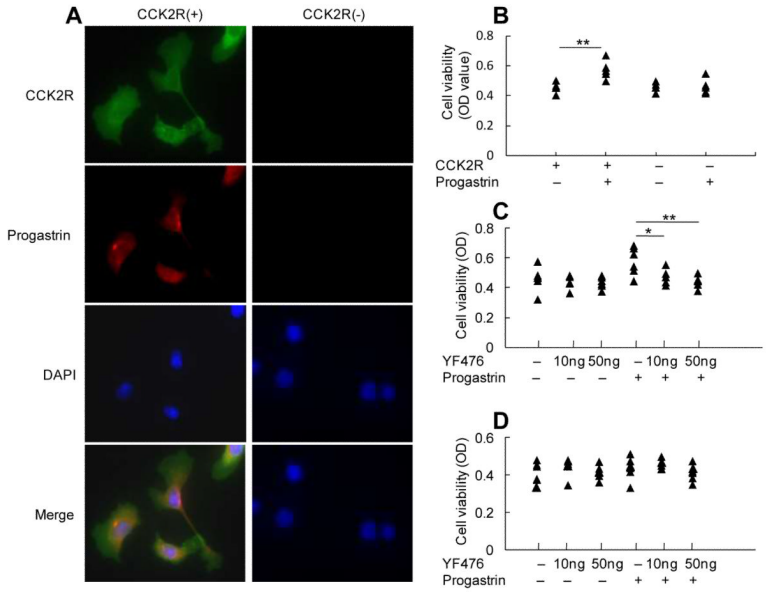

Figure 3. Progastrin binds to CKK2R to increase cell proliferation

(A) Immunofluorescence staining of progastrin binding on CCK2R expressing cells after 30 minutes of progastrin treatment (original magnification, −800). MTT assay after progastrin treatment on Colo320(+) and Colo320(−) cells (B), or pretreatment with CCK2R antagonist YF476 on Colo320(+) (C) and Colo320(−) cells (D) (n=6 plates/group). All values represent the mean ± SD. $*P < 0.05$, $**P < 0.01$.

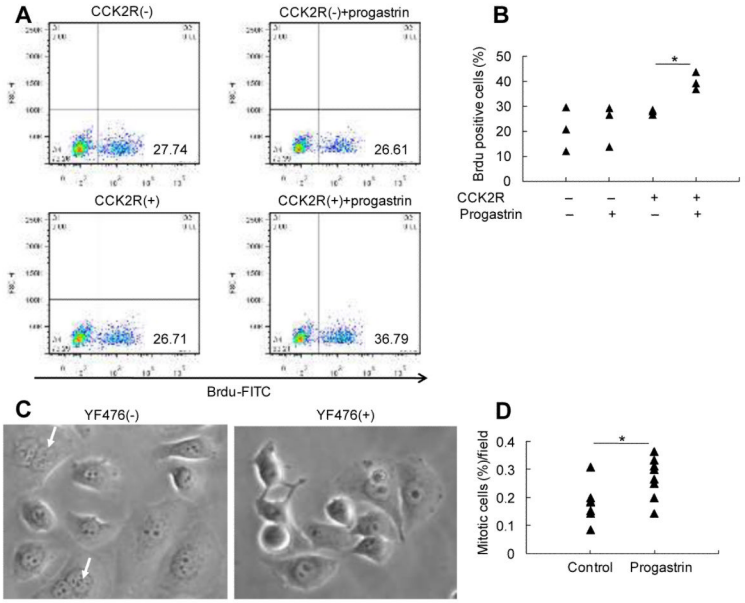

Figure 4. Progastrin stimulates proliferation through CCK2R dependent mechanism

(A) FACS analysis of BrdU incorporation after progastrin treatment to the Colo320(+) and Colo320(−) cells. Horizontal axis: BrdU-FITC. (B) Comparison of BrdU positive cell populations after progastrin treatment of Colo320(+) and Colo320(−) cells (n=3 plates/group). (C) AGSE cell morphology in serum free medium with or without YF476 treatment (white arrow indicate mitotic cells) (original magnification, −600). (D) Percentage of mitotic events in AGSE cells with or without progastrin treatment (n=8 yields/group). All values represent the mean ± SD. *$P < 0.05$.

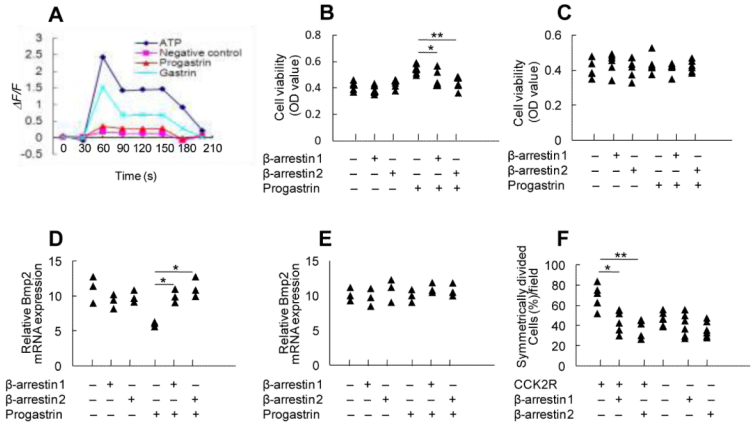

Figure 5. Progastrin increases cell proliferation through CCK2R-dependent, beta-arrestin pathway

(A) Calcium influx in AGSE cells after progastrin and gastrin 17 treatment. MTT assay of Colo320(+) (B) and Colo320(−) (C) cells (n=6 plates/group), and quantitative RT-PCR analysis of Bmp2 mRNA levels in Colo320(+) (D) and Colo320(−) (E) cells (n=3 plates/group) after transfection with 200ng/140ul of beta-arrestin 1 and 2 siRNA. (F) Percentage of symmetric divisions of BrdU positive cells after transfection with beta-arrestin 1 and 2 siRNA in Colo320(+) and Colo320(−) cells (n=6 plates/group). All values represent the mean ± SD. $*P < 0.05$, $**P < 0.01$.

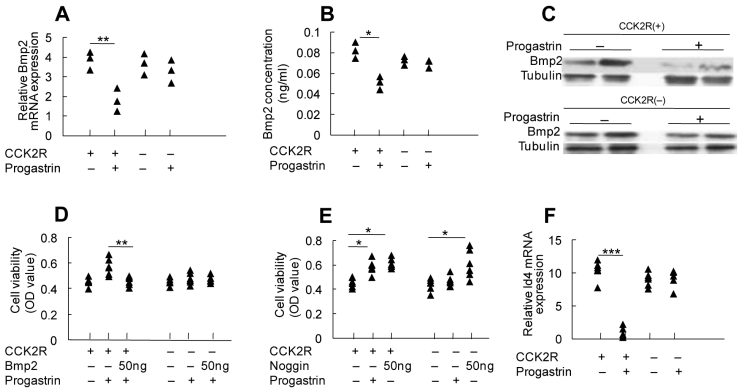

Figure 6. Progastrin increases cell proliferation through Bmp2 suppression

Quantitative RT-PCR analysis of Bmp2 mRNA expression (n=3 plates/group), the expression levels were normalized to GAPDH (A), protein levels with ELISA (n=3 plates/group) (B) and western blot (n=2 plates/group) (C) in Colo320(+) and Colo320(−) cells after progastrin treatment. MTT assay of Colo320(+) and Colo320(−) cells after Bmp2 protein (n=6 plates/group) (D) and Noggin treatment (n=6 plates/group) (E). (F) Quantitative RT-PCR analysis of Id4 mRNA expression levels after progastrin treatment of Colo320(+) and Colo320(−) cells (n=6 plates/group). All values represent the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

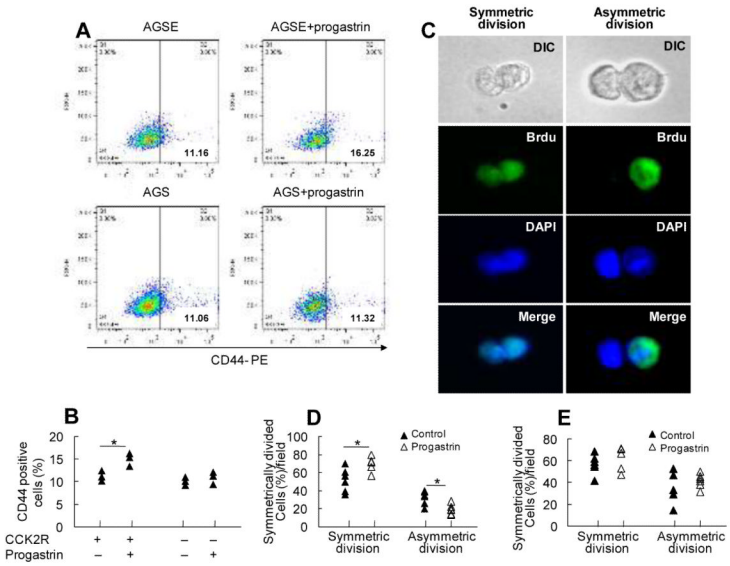

Figure 7. Progastrin increases stem or progenitor cell proliferation in AGSE cells

(A) FACS analysis of CD44 positive cells in AGSE and AGS cells after progastrin treatment. Horizontal axis: CD44-PE. (B) CD44 positive cell populations in AGSE and AGS cells after progastrin treatment (n=3 plates/group). (C) Immunofluorescence analysis of BrdU positive cells. Representative examples for symmetric or asymmetric cell divisions are shown (original magnification, −600). (D) Symmetric and asymmetric cell populations after progastrin treatment to the AGSE cells (n=6 plates/group). (E) Symmetric and asymmetric cell populations after Bmp2 protein treatment to the AGSE cells (n=6 plates/group). All values represent the mean ± SD. *$P < 0.05$.