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CCK2R identifies and regulates gastric antral stem cell states and carcinogenesis

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Abstract

Objective—Progastrin is the incompletely cleaved precursor of gastrin that is secreted by G-cells in the gastric antrum. Both gastrin and progastrin bind to the CCK2 receptor (*Cckbr* or CCK2R) expressed on a subset of gastric epithelial cells. Little is known about how gastrin peptides and CCK2R regulate gastric stem cells and carcinogenesis. Interconversion among progenitors in the intestine is documented, but the mechanisms by which this occurs are poorly defined.

Design—We generated CCK2R-CreERT mice and performed inducible lineage tracing experiments. CCK2R+ antral cells and Lgr5+ antral stem cells were cultured in a three-dimensional in vitro system. We crossed progastrin-overexpressing mice with Lgr5-GFP-CreERT mice and examined the role of progastrin and CCK2R in Lgr5+ stem cells during MNU-induced carcinogenesis.

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Results—Through lineage tracing experiments, we found that CCK2R defines antral stem cells at position +4, which overlapped with an $Lgr5^{neg\ or\ low}$ cell population but was distinct from typical antral $Lgr5^{high}$ stem cells. Treatment with progastrin interconverts $Lgr5^{neg\ or\ low}$ CCK2R+ cells into $Lgr5^{high}$ cells, increases CCK2R+ cell numbers and promotes gland fission and carcinogenesis in response to the chemical carcinogen MNU. Pharmacological inhibition or genetic ablation of CCK2R attenuated progastrin-dependent stem cell expansion and carcinogenesis.

Conclusions—CCK2R labels +4 antral stem cells that can be activated and expanded by progastrin, thus identifying one hormonal trigger for gastric stem cell interconversion and a potential target for gastric cancer chemoprevention and therapy.

INTRODUCTION

Gastric cancer is the second leading cause of cancer mortality worldwide with most patients dying within 5 years of their diagnosis.[12] Gastric cancer has many discrete types that may be classified by site and/or histology.[3] In this study, we focus on antral stem cells in the distal stomach and also mouse models of intestinal-type gastric cancer that predominantly, although not exclusively, arise from the distal stomach.[4–7] The events that initiate intestinal-type gastric carcinogenesis are poorly understood.

Lgr5 expression identifies long-lived, self-renewing stem cells in the gastric antrum.[8] These cells divide actively, lineage trace entire antral glands within 7–10 days and persist for the lifetime of the mouse. Activation of Wnt signalling in these Lgr5+ cells also induces gastric adenoma formation.[8] Sox2, which may overlap with Lgr5, also labels antral stem cells.[8–10] Single Lgr5+ antral stem cells can be sorted and grown into organoids or miniguts, although requiring a number of growth factors, including Wnt3A, EGF, Noggin and R-spondin1.[811] In this culture system, gastrin reportedly promotes the growth of gastric stem cells.[81213] However, the precise effects of gastrin peptides and their receptor *Cckbr* (CCK2R) signalling on antral stem cells both in vivo and in vitro have not been investigated.

Gastrin mediates its effects on the proximal and distal stomach through binding to the CCK2R, a seven-transmembrane, G protein-coupled receptor.[14] The CCK2R is highly expressed in the proximal stomach, primarily in parietal cells and enterochromaffin-like (ECL) cells in the oxyntic mucosa, where its role in acid secretion is well described, although it is also expressed in neck progenitors in the proliferative zone.[15] While the expression of CCK2R in the gastric corpus has been well characterised,[1617] its expression pattern in the distal stomach is less clear. Previous reports have shown that CCK2R knockout ($CCK2R^{-/-}$) resulted in reduced parietal, ECL and somatostatin-producing enteroendocrine cells in the stomach, but a compensatory increase in the gastrin-producing G-cells in the antrum.[1819] However, in health, $CCK2R^{-/-}$ mice, as well as hypergastrinemic (INS-GAS) or gastrin knockout (GAS-KO) mice, do not show proliferative or dramatic histological changes in the antrum.[2021] Thus, the overall role of CCK2R signalling in antral homeostasis has not been clarified.

In addition to the well-known amidated gastrins, gastrin exists in a number of molecular forms, including the longer precursor form, progastrin, an 80-amino acid peptide.[22]

Progastrin and other incompletely processed forms of gastrin typically comprise less than 10% of the total secreted peptide, but when processing is impaired, elevations in these non-amidated forms can occur. We have reported that human progastrin-overexpressing (hGAS) transgenic mice show increased colonic proliferation and enhanced tumour development, indicating a role for progastrin as a trophic growth factor for the colonic epithelium,[2324] largely through binding to the CCK2R leading to expansion of progenitors.[2526] Although progastrin binds to the identical receptor (CCK2R) as amidated gastrin, the signalling pathways are quite different,[26] accounting for the fact that progastrin stimulates colonic proliferation while amidated gastrin does not. Nevertheless, while the CCK2R is expressed at much higher levels in the gastric epithelium than in the colon, the role of progastrin on the stomach has not been elucidated.

In the current study, we used CCK2R-CreERT mice in order to clarify the location and function of CCK2R in the antrum. We found that CCK2R+ cells with low or negative expression of Lgr5 (Lgr5$^{neg\ or\ low}$) contain long-lived antral stem cells, and progastrin, but not amidated gastrin, stimulates these cell lineages in vivo and in vitro through CCK2R. Activation of CCK2R enhances antral gland fission and carcinogenesis through an expansion of antral stem cells. Targeting CCK2R significantly suppressed antral carcinogenesis in vivo. These results confirm that CCK2R signalling plays an important role in gastric carcinogenesis by regulating stem cell function and epithelial homeostasis.

MATERIALS AND METHODS

Mice and gastric cancer models

The animal protocol was reviewed and approved by the Columbia University Medical Center Institutional Animal Care and Use Committee (IACUC), and all mice were bred under specific pathogen free (SPF) conditions. INS-GAS mice[27] and hGAS mice[23] were backcrossed to C57BL6 background. Lgr5-GFP-IRES-CreER knockin mice[28] (Lgr5-GFP mice) were provided by Dr Hans Clevers, and mated with hGAS mice and B6.129S4-Gt(ROSA)26Sortm1Sor5/J, referred to as R26rLacZ mice (Jackson Laboratories). CCK2R$^{-/-}$ mice were purchased from Jackson Laboratories. CCK2R-GFP BAC transgenic mice were purchased from MMRRC (GENSAT project[29]). For generation of CCK2R-BAC-CreERT mice, BAC recombination was carried out as described at http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx using the CreTM-FrtNeoFrt cassette as described previously.[30] For lineage tracing experiments, mice were administered 1–6 mg tamoxifen in 200 μL corn oil containing 5–30 mg/mL tamoxifen by oral gavage. The CCK2R antagonist YF476 was a kind gift of Dr Keiji Miyata and Dr Hidenobu Yuki (Astellas Pharma, Tokyo, Japan). The drug was dissolved in PEG 300 at the concentration of 12 mg/mL and intraperitoneally injected twice per week at the dose of 40 mg/kg.[31]

In vitro culture system

Crypt and single-cell isolation and cultures were performed as described previously[8] with minor modifications. Two or three mice were used for each experiment, and all results were confirmed by at least two independent experiments.

Detailed information is described in online supplementary methods.

RESULTS

CCK2R marks long-lived gastric stem cells in the antrum

To analyse the localisation of CCK2R expression within the stomach, we generated CCK2R-CreERT-BAC transgenic mice, crossed them to reporter mice and induced recombination (see online supplementary figure S1A). Twenty-four hours after tamoxifen induction, reporter gene expression was observed in CCK2R+ H/K-ATPase+ parietal cells and chromogranin A+ (ChgA+) ECL cells in the corpus (see online supplementary figure S1, B, C). Gastric intrinsic factor+ (GIF+) chief cells were not recombined (see online supplementary figure S1D). Thus, this BAC-transgenic line fairly reflected the known distribution of CCK2R. Interestingly, CCK2R+ recombined cells were detected most frequently as single cells in the gastric antral epithelium at a position just above the Lgr5+ cells at the base of the gland (figure 1A).[8] These CCK2R+ cells were confirmed as epithelial cell types by E-cadherin and 3,3'-diaminobenzidine (DAB) staining (figure 1A). The majority of these antral CCK2R+ cells were ChgA-negative (9.1% of recombined cells in the antrum were ChgA+, figure 1B and online supplementary figure S1I), and also negative for other differentiated markers including TFF1, Dclk1, gastrin and somatostatin (see online supplementary figure S1E–H). Interestingly, analysis of a separate CCK2R-GFP transgenic mouse line[29] confirmed the presence of CCK2R+ cells in the antrum near the base and also established that CCK2R-GFP+ cells were predominantly ChgA-negative cells (11.1% of antral GFP+ cells were ChgA+, figure 1C and see online supplementary figure S1I). Thus, the vast majority of CCK2R+ cells in the antrum were undifferentiated. The expression of CCK2R in the gastric antrum was also validated by immunofluorescence (figure 1D). In CCK2R-CreERT mice, the recombined cells expanded rapidly to trace whole antral glands within 10 days after tamoxifen induction and the traced glands persisted beyond 12 months (figure 1E, F). Although we observed occasional apoptosis in the corpus after giving 6 mg tamoxifen, we did not find any such histological changes or apoptotic cells in the antrum (see online supplementary figure S1J, K). Furthermore, lineage tracing from CCK2R+ cells occurred with 1 mg tamoxifen administration (see online supplementary figure S1L), suggesting that CCK2R+ lineage tracing in the antrum is independent of the tamoxifen dose. The CCK2R+ cell progeny differentiated into all mature antral cell types, including G-cells (gastrin+), pit cells (TFF1+), tuft cells (Dclk1+) and D-cells (somatostatin+) (figure 1G).

Next, we performed in vitro culture of CCK2R+ antral cells. Recombined CCK2R+ cells were sorted 1 day after tamoxifen induction by fluorescence-activated cell sorting (FACS) and placed in our three-dimensional culture system (figure 1H).[8] Approximately 0.8±0.23% antral epithelial cells were labelled by CCK2R, and these CCK2R+ cells robustly formed gastric organoids in culture (colony formation efficiency 2.1±0.56 (average±SE) %), which could be maintained for more than 2 months with the growth of budding events (figure 1I). In contrast, CCK2R-negative antral cells were relatively inefficient at organoid formation in vitro (colony formation efficiency 0.2±0.36%, p=0.004 compared with CCK2R+ cells, figure 1J). These data suggest that CCK2R marks long-lived antral stem cells in vivo and in vitro.

Actively cycling CCK2R+ stem cells are higher up in the antral gland than Lgr5+ stem cells

Next, we investigated the characteristics of CCK2R+ stem cells in comparison to Lgr5+ stem cells in the antrum. As reported previously,[8] Lgr5+ cells were located at the base of antral glands in Lgr5-GFP-CreERT mice, predominantly below position +4 (figure 2A, B). In contrast, analysis of CCK2R-CreERT mice crossed to reporter mice showed that at day 1 following tamoxifen, CCK2R+ cells were quite rare (average 1–2 cells/gland) and were distributed in the lower half of glands from position +1 to +13, with the peak at position +4 (figure 2B) and the vast majority between +3 and +7, indicating that most CCK2R+ stem cells reside above the basally located Lgr5+ cells. Lgr5+ cells in the antrum are reported to be actively cycling.[8] We found that approximately 10% of Lgr5+ cells are Ki67+ (figure 2C, D), although the proliferation rate appears slightly lower than that previously reported.[8] In contrast, approximately 30% of CCK2R+ antral cells were Ki67+ (figure 2C, D), suggesting that CCK2R+ cells are in general more rapidly cycling than Lgr5+ cells. Double staining of ChgA and Ki67 revealed that there were no proliferating cells among the ChgA+ cells, indicating that the CCK2R+ stem cells likely reside among the ChgA-negative population (see online supplementary figure S2A). Thus, these data demonstrate that CCK2R+ cells and Lgr5+ cells are distinct stem cells in the gastric antrum. Indeed, immunofluorescence of CCK2R in Lgr5-GFP mice confirmed that CCK2R are predominantly expressed in GFP-negative cells (figure 2E).

CCK2R is expressed in Lgr5$^{neg\ or\ low}$ cells, which could convert to Lgr5^{high} cells in response to progastrin

We have previously reported that progastrin binds to and activates CCK2R, leading to the expansion of CCK2R-expressing cells.[2532] To test the effects of progastrin on antral stem cells in vivo, we generated Lgr5-GFP;hGAS mice and analysed CCK2R expression. Interestingly, in the Lgr5-GFP;hGAS double transgenic mice, the expression of CCK2R in the Lgr5-GFP+ population is markedly increased (figure 3A, B), suggesting the possibility of interconversion of Lgr5$^{neg\ or\ low}$CCK2R+ cells to Lgr5-high expressing (Lgr5^{high}) cells in response to progastrin. In contrast, ChgA+ endocrine cells did not become Lgr5-positive even with progastrin overexpression (see online supplementary figure S2B), indicating that progastrin does not activate in this manner the ChgA+ cell population, but instead interconverts undifferentiated CCK2R+ stem cells. We observed by immunofluorescence that each normal antral glands only contained one or two of CCK2R+ cells, but progastrin overexpression expanded the CCK2R+ population (figure 3C). However, the Ki67+ proliferation index in CCK2R+ cells was almost identical in wild-type (WT) and hGAS mice mated with CCK2RCreERT mice (see online supplementary figure S2C, D), suggesting that progastrin-dependent activation of CCK2R leads to increased CCK2R +Lgr5+ cells (figure 3A) through interconversion or symmetric cell division rather than simply enhancing proliferation.

When antral glands were cultured with standard growth factors, including Wnt3A, R-spondin1, EGF and Noggin, treatment with progastrin did not affect organoid numbers (data not shown). Since progastrin induces expansion of colonic progenitors through downregulation of bone morphogenetic protein (BMP) 2 expression,[32] we reasoned that removal of Noggin (a BMP antagonist) from the culture media might enhance progastrin-

mediated effects. We observed that progastrin treatment increased organoid number following the first passage in Noggin-free conditions compared with the vehicle-treated group (figure 3D). Abrogating the effects of progastrin by either YF476, a CCK2R antagonist, or genetic knockout of CCK2R ($CCK2R^{-/-}$), abolished these changes (figure 3D). However, the size of the organoids was not affected by progastrin or YF476, suggesting less of an effect on proliferation and more of an effect on stem cell self-renewal (figure 3E). Gene expression of CCK2R was significantly upregulated by progastrin treatment (figure 3F), which supports the in vivo CCK2R+ cell expansion in hGAS mice (figure 3C).

We then sorted single gastric epithelial cells from the antral crypts of Lgr5-GFP mice and analysed both $Lgr5^{low}$ cells and $Lgr5^{high}$ cells separately (figure 3G). When we cultured sorted single $Lgr5^{high}$ cells with standard culture medium, the colony formation efficiency was 5%, while $Lgr5^{low}$ cells had a much lower efficiency (about 0.3%, p=0.01, compared with $Lgr5^{high}$, figure 3H, I), consistent with previous reports.[81133] Amidated gastrin treatment did not significantly enhance colony formation in either $Lgr5^{high}$ or $Lgr5^{low}$ cell populations (figure 3I). In contrast, progastrin significantly increased colony formation, but only in $Lgr5^{low}$ cells (some of which express CCK2R), while there was no effect of progastrin in $Lgr5^{high}$ cells (mostly CCK2R-negative) (figure 3I). Quantitative RT-PCR indicated that Lgr5 expression was significantly upregulated in cultured $Lgr5^{low}$ populations by progastrin treatment, and this upregulation and colony formation were inhibited by YF476 (figure 3J and see online supplementary figure S2E). Furthermore, CCK2R gene expression in $Lgr5^{high}$ cells was markedly increased in sorted cells from Lgr5-GFP;hGAS mice compared with Lgr5-GFP mice, and this increase was blocked by 2-week YF476 administration (figure 3K). Thus, $Lgr5^{neg\ or\ low}$CCK2R+ cells respond to progastrin by rapid interconversion into $Lgr5^{high}$ cells, thus expanding an active antral stem cell pool.

Progastrin increases gastric stem cell number and crypt fission through CCK2R

To test whether progastrin also promoted interconversion of $Lgr5^{neg\ or\ low}$ to $Lgr5^{high}$ antral stem cells to initiate gastric carcinogenesis, we treated Lgr5-GFP mice and Lgr5-GFP;hGAS mice with N-nitroso-N-methylurea (MNU), an antral carcinogen.[34] Without MNU treatment, gastric histology, acid secretion and the number of Lgr5+ cells appeared to be similar between Lgr5-GFP mice and Lgr5-GFP;hGAS mice at baseline (see online supplementary figure S3A–C). The serum progastrin level is consistently high in the hGAS mice during all experiments (see online supplementary figure S3D). However, 3 months following treatment with MNU, Lgr5-GFP;hGAS mice showed a greater increase in the number of GFP+ cells compared with MNU-treated Lgr5-GFP mice (figure 4A, B). Lgr5 expression was upregulated in the MNU-treated WT and hGAS mice, particularly in the hGAS group (figure 4C). At this 3-month time point, hGAS mice displayed more severe hyperplasia and gland irregularity compared with WT mice. The expression of Ki67 and CD44 in hGAS mice was also significantly greater than in WT mice (see online supplementary figure S4A, B). These results suggest that progastrin overexpression promotes the expansion of Lgr5+ stem cells following exposure to a gastric carcinogen.

In the setting of carcinogenic exposure, the expansion of gastrointestinal stem cells leads to crypt fission.[35] Recent studies have shown that progastrin increases crypt fission and symmetric cell division in the colon.[32] We observed that in the MNU-induced antral cancer model, progastrin also increased antral gland fission (figure 4D, E). To test the relationship between antral gland fission and stem cell expansion, we lineage-traced antral glands following MNU treatment in Lgr5-GFP-CreERT mice mated with Rosa26rLacZ reporter mice (figure 4F). Twelve weeks after the start of MNU treatment, we observed that the number of contiguously labelled glands was increased by progastrin overexpression, consistent with the observed increases in antral gland fission (figure 4G, H). Similarly, in vitro lineage tracing experiments of CCK2R also revealed that CCK2R-lineage expansion was enhanced by progastrin treatment compared with the untreated group (see online supplementary figure S5A, B). Taken together, progastrin expands CCK2R+ stem cells through interconversion and gland fission (figure 4I).

CCK2R inhibition prevents antral crypt fission

Next, we examined the effects of CCK2R gene ablation and inhibition of CCK2R with small molecule antagonists during MNU-induced carcinogenesis. After five cycles of MNU treatment, $CCK2R^{-/-}$ mice showed significantly fewer gland fission events (figure 5A, B). The initiation of CCK2R antagonism by YF476 at an early stage (from the beginning of MNU treatment) (see online supplementary figure S6A) also successfully suppressed crypt fission in the antrum in WT and hGAS mice (figure 5C). Furthermore, YF476 treatment prevented Lgr5+ cell expansion in hGAS mice (figure 5D, E), suggesting that the CCK2R signal is important for the gland fission and stem cell expansion following exposure to MNU.

To confirm that the effects of progastrin were CCK2R-dependent in vivo, we crossed Lgr5-GFP;hGAS mice to $CCK2R^{-/-}$ mice. The number of Lgr5+ cells was similar between hGAS;$CCK2R^{-/-}$ mice and hGAS;$CCK2R^{+/-}$ mice without MNU treatment (see online supplementary figure S7). After five cycles of MNU treatment, however, $CCK2R^{-/-}$ mice failed to expand their Lgr5+ stem cell pool to the same extent (figure 5F, G). Thus, progastrin overexpression expanded Lgr5+ cells in a CCK2R-dependent fashion.

Progastrin promotes antral gastric cancer in mice

Thirty-six weeks after *Helicobacter felis* inoculation and five cycles of MNU treatment, WT and hGAS mice developed antral cancer. The tumour number and tumour size, however, were significantly increased in hGAS mice compared with WT mice (figure 6A, B), with hGAS mice also showing more advanced grade tumours along with increased Lgr5 expression (figure 6C). In the *H. felis* gastric cancer model (without MNU), infected hGAS mice at 18 months exhibited polypoid lesions at the gastric pylorus (see online supplementary figure S8A), a phenotype not typically observed in *H. felis*-infected WT mice.[36] Histologically, *H. felis*-infected hGAS mice had moderate-to-severe hyperplasia with dysplastic changes in the gastric antropyloric region, while *H. felis*-infected WT mice showed minimal changes in the corresponding distal stomach (see online supplementary figure S8B). Accordingly, immunohistochemistry of Ki67 and CD44 revealed that progastrin overexpression enhanced cell proliferation and dysplastic changes in the antrum

(see online supplementary figure S8C, D). Moreover, when we treated MNU-treated mice with YF476 at a later stage (starting at 30 weeks) (see online supplementary figure S6B), we observed a significant decrease in tumour number and tumour size in hGAS mice (figure 6D–F), but not in WT mice (data not shown). These data suggest that progastrin/CCK2R axis, and following stem cell expansion with gland fission, contribute to antral tumour initiation and progression.

DISCUSSION

We investigated the role of CCK2R signalling in antral homeostasis and carcinogenesis. Using inducible Cre-dependent lineage tracing, we established that CCK2R is expressed in $Lgr5^{neg\ or\ low}$ cells located above $Lgr5^{high}$ cells, at position +4 in the antral gland. These CCK2R+ cells are antral stem cells that can lineage trace Lgr5+ antral stem cells and all mature antral lineages, with recombination persisting more than 12 months following adult induction. Progastrin accelerated antral carcinogenesis through an expansion of antral gastric stem cells as a result of progastrin-mediated interconversion of CCK2R+$Lgr5^{neg\ or\ low}$ stem cells into $Lgr5^{high}$ cells. In turn this increased crypt fission and predis-posed to antral carcinogenesis. These effects were blocked by inhibition of CCK2R signalling either by YF476 or genetic knockout of CCK2R.

Given that Lgr5+ cells are a well-validated antral stem cell population, we examined possible overlap of CCK2R with Lgr5. We found that CCK2R was primarily expressed in $Lgr5^{neg\ or\ low}$ cells at peak position +4, rather than in $Lgr5^{high}$ cells located at the base. Gastrointestinal stem cells are thought to be heterogeneous.[37] In the small intestine, Potten *et al* suggested that the intestinal +4 label retaining stem cells by nucleotide labelling assay.[38] Lgr5+ cells at the base of glands were later identified as rapidly cycling stem cells,[39] while the relatively quiescent stem cells at position +4 have been fate mapped using the expression of Bmi1, Hopx and mTERT.[40–42] In the gastric antrum, it has been suggested that the anatomical narrowing at the lower third of the gland, known as the isthmus, is the site of cellular renewal, and that immature cells migrate and differentiate bidirectionally from this point.[43–45] Proliferation rate of antral isthmal cells are reported to be approximately 37%,[44] which seems equivalent to antral CCK2R+ cell population (30%). The distribution and lineage tracing that we described confirms, for the first time, that CCK2R+ marks this isthmus (or +4) stem cell in the gastric antrum. The CCK2R+ cell population in the gastric antrum may also be heterogeneous, but includes a bona fide actively proliferating stem cell population that does not overlap with Sox2 cells (see online supplementary figure S9). Although TFF2+ cells also exist at the base of antral glands, our previous report showed that no lineage tracing was observed in the antrum.[30] Thus, CCK2R + antral stem cells do not express TFF2. The presence of rare CCK2R+ antral stem cells at the +4 position, along with multiple Lgr5+ cells at the antral base, raises questions about whether there is an antral stem cell hierarchy. Previous radioautographic and electron microscopic analysis revealed that the antral isthmal cells contain five different cell types that can be labelled by ^{3}H-thymidine in 1 h.[4445] Further work is needed to identify other isthmal stem/progenitor cell markers and define relationships among these multiple stem cell populations.

Previous reports suggest that intestinal +4 stem cells and Lgr5+ crypt base columnar cells (CBCs) may be able to dynamically interconvert especially in response to acute epithelial injury,[414647] although it is unclear how this interconversion is regulated. We observed that progastrin increased CCK2R/Lgr5 double-positive cell numbers in mice and expanded Lgr5+ cells after MNU treatment. Also, our single-cell cultures showed that progastrin enhanced both colony formation efficiency and Lgr5 expression in CCK2R+ cells. Thus, we conclude that progastrin promotes expansion and renewal of CCK2R+ stem cells by converting CCK2R+Lgr5$^{\text{neg or low}}$ cells into Lgr5$^{\text{high}}$ stem cells. Although the colony-forming efficiency of CCK2R+ cells are lower than Lgr5+ cells (2% vs 5% in our data), the highly Wnt-dependent culture condition seems to be optimised for Lgr5+ or Wnt-responsive cell types. The culture method is likely to be critical for specific stem cell propagation in vitro, and as would be expected from our in vivo findings, different stem cells are best supported by unique conditions and microenvironments. The very premise of which underpins the entire concept of stem cell interconversion. We have recently shown that CCK2R signalling downregulated BMP2 expression, which may also contribute to interconversion.[32] Hormone-mediated stem cell interconversion may be a common mechanism by which trophic hormones expand stem cells in development and repair.

We have reported that progastrin promotes colonic cell proliferation and cell division through CCK2R in vivo and in vitro.[253248] Given that CCK2R is expressed at higher levels in the stomach,[25] where a physiological role is well established, and that progastrin is overexpressed in some gastric cancers,[49] antagonising progastrin signalling could theoretically be effective as therapy in some distal gastric cancers. Nevertheless, we have also shown that amidated gastrin, the most abundant form of gastrin that signals through CCK2R, inhibits antral gastric cancer.[50] Thus, progastrin and amidated gastrin induce opposite effects in the distal stomach, possibly through ligand competition, with each serving as a partial antagonist for the other. The opposite effects of amidated gastrin and progastrin on the distal stomach suggest important signalling differences between these two gastrin isoforms. We did not find any promoting effect of amidated gastrin on colony formation ability in Lgr5+ cells. The effect of amidated gastrin in gastric stem cell regulation needs to be further clarified.

The CCK2R antagonist YF476 reduced crypt fission and tumourigenesis in hGAS mice, suggesting that YF476 blocks progastrin-mediated cancer promoting effects. While YF476-treated WT mice treated early showed decreased crypt fission, mice that were only treated at a later stage failed to show any tumour regression. Consistently, CCK2R$^{-/-}$ mice also had reduced crypt fission at the beginning but ultimately developed comparable tumour burden (data not shown). Thus, CCK2R inhibition would only likely to be therapeutically useful for a subset of patients with distal gastric cancer, particularly in those with an increased progastrin to gastrin ratio. The balance of these hormones may influence an individual's susceptibility to gastric cancer as well as the effectiveness of any CCK2R-directed therapy.

In summary, these data reveal that CCK2R marks a new stem cell population in the gastric antrum position +4, and that progastrin, but not amidated gastrin, is a critical modulator of antral stem cell states. These findings have implications for hormonal regulation of other

stem cell niches and highlight a novel stem cell target that may have therapeutic potential in patients with, or at risk of, antral cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance of this study

What is already known on this subject?

▶ The gastrin family of peptides has been reported to play an important role in gastric homeostasis and carcinogenesis.

▶ The CCK2R is highly expressed in the proximal stomach, primarily in parietal cells and enterochromaffin-like (ECL) cells, although the expression of CCK2R in the gastric antrum is less clear.

▶ Lgr5+ cells at the base of antral glands label long-lived, self-renewing stem cells; however, the heterogeneity of antral stem cells and their regulation has not been investigated.

What are the new findings?

▶ CCK2R marks $Lgr5^{neg\ or\ low}$ antral stem cells located at position +4, slightly above typical $Lgr5^{high}$ stem cells.

▶ CCK2R regulates the interconversion of CCK2R + cells into $Lgr5^{high}$ cells, and induces clonal expansion.

▶ CCK2R modulates antral carcinogenesis following progastrin stimulation.

How might it impact on clinical practice in the foreseeable future?

▶ These results highlight a novel mechanism for hormonal regulation of gastric stem cell interconversion.

▶ We identify CCK2R as a potential target for gastric cancer chemoprevention and therapy.

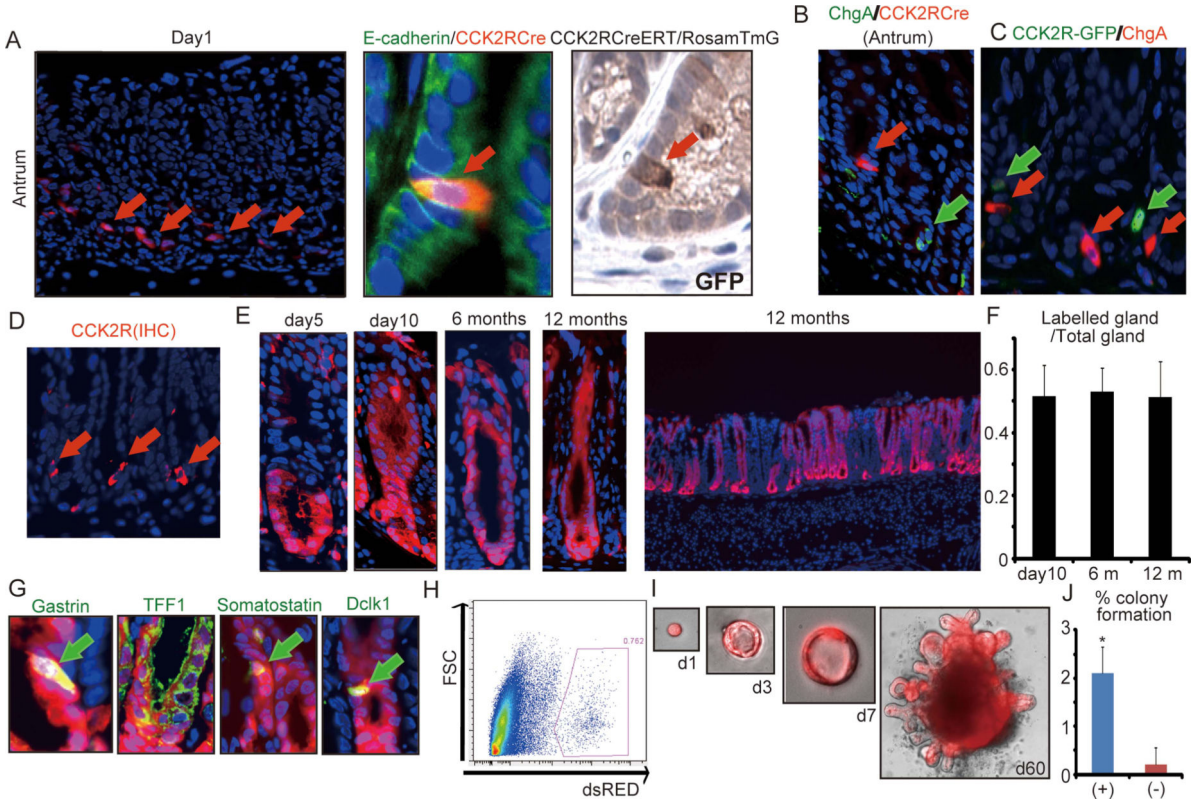

Figure 1.

CCK2R marks long-lived gastric stem cells in the antrum. (A) CCK2R-BAC-CreERT2;Rosa26rTdTomato mice were given 6 mg tamoxifen and sacrificed after 24 h. Recombined red cells (allows) are observed at near the gland base. Middle panel was stained by E-cadherin antibody. Right panel is GFP staining of CCK2R-BAC-CreERT2;Rosa26mTmG mouse antrum. (B) Immunostaining of ChgA (green) with CCK2RCreERT/TdTomato mice antrum 24 h after tamoxifen. (C) Immunostaining of ChgA (red) with CCK2R-GFP (green) transgenic mice antrum. (D) Immunofluorescence of CCK2R (red) in mouse antrum. (E) Representative lineage-tracing pictures after 5 days, 10 days, 6 months and 12 months. (F) Ratio of the number of lineage-traced glands per total glands at day 10, 6 months and 12 months. (G) Double staining of gastrin, TFF1, somatostatin and Dclk1 (green) with CCK2RCreERT/TdTomato mice 6 months after tamoxifen. (H, I) Single-cell sorting (H) and time course (days 1, 3, 7 and 60) of CCK2R+ red single-cell culture (I) isolated 24 h after tamoxifen induction. (J) The percentage of single-cell colony formation of CCK2R+ and CCK2R- cells. Data are plotted as the means ±SE (*$p<0.05$).

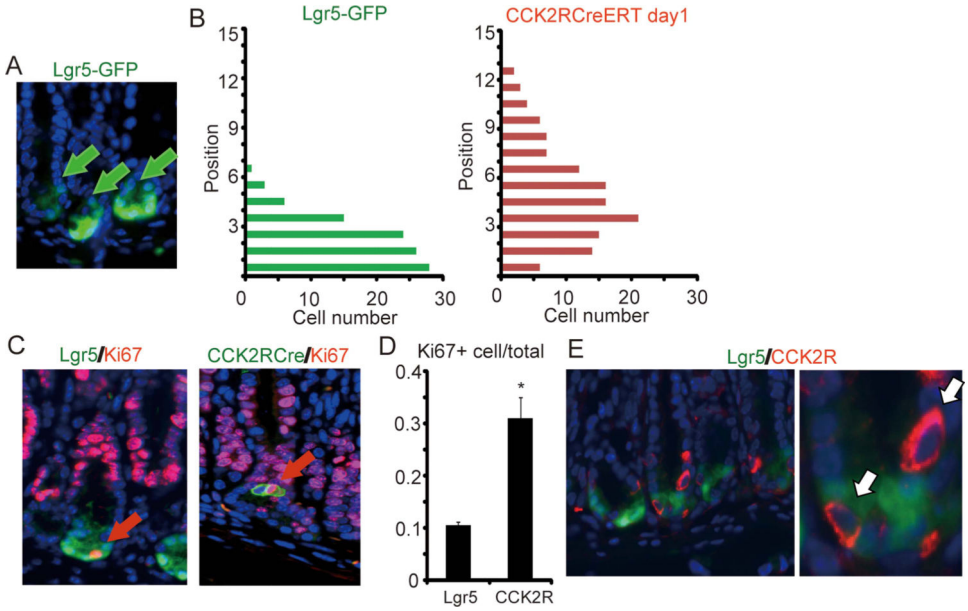

Figure 2.

Actively cycling CCK2R+ stem cells reside at higher position than Lgr5+ stem cells. (A) Representative picture of Lgr5-GFP mouse antrum. (B) Position of Lgr5-GFP+ cells and CCK2R+ red recombined cells in the antrum 24 h after tamoxifen induction. Total 60 glands from three mice were counted. (C) Representative images of Ki67 staining (red) in Lgr5-GFP and CCK2RCreERT; Rosa26mTmG mouse. Red arrows indicate double-positive cells. (D) The percentage of Ki67+ cells in total Lgr5-GFP or CCK2R+ cells. Total 50 glands from three mice were counted (*$p<0.05$). (E) Immunofluorescence of CCK2R (red, arrow) in Lgr5-GFP mice antrum.

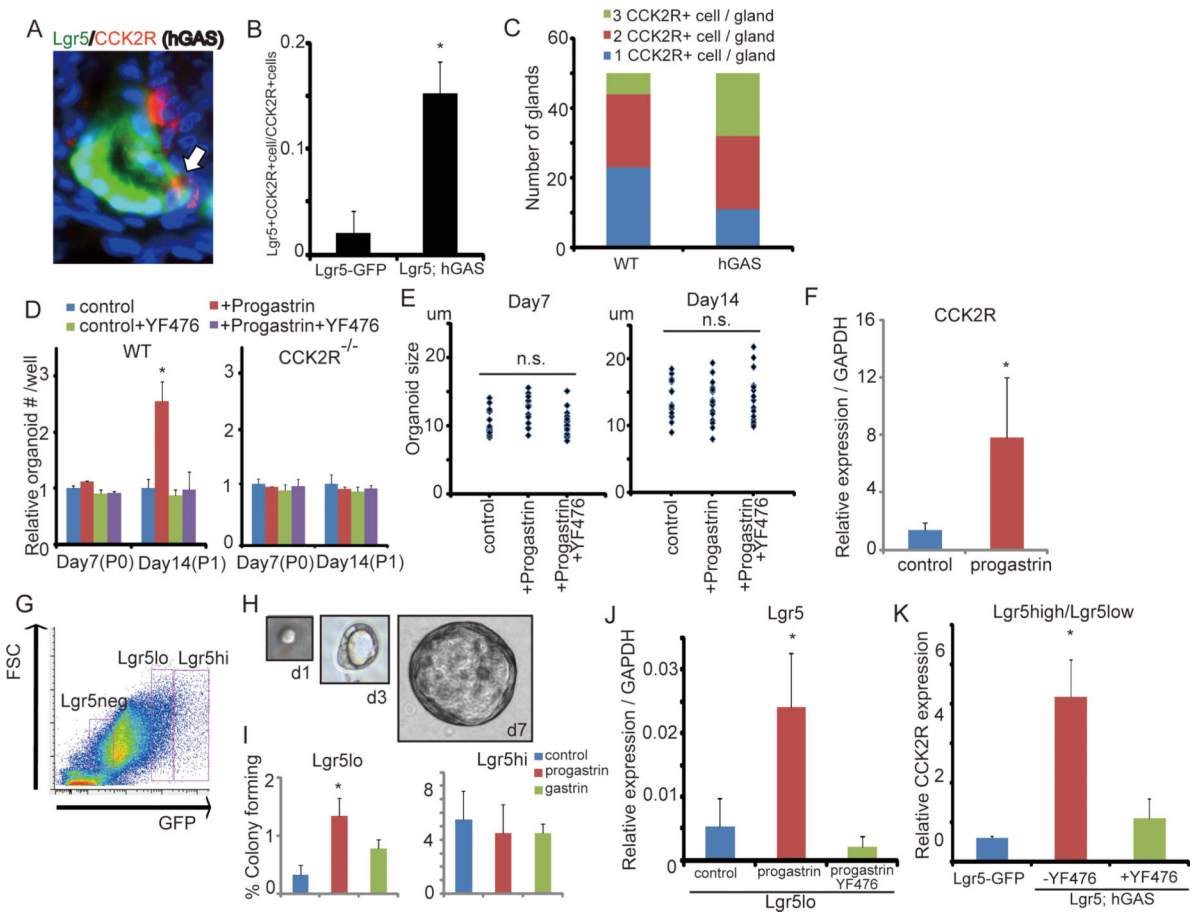

Figure 3.

CCK2R is expressed in Lgr5^{low} cells, which could convert to Lgr5^{high} cells in response to progastrin. (A) Immunofluorescence of CCK2R (red, arrow) in Lgr5-GFP;hGAS mice antrum. (B) Average numbers of Lgr5+CCK2R+ double-positive cells in total CCK2R+ cells in Lgr5-GFP mice (n=3) and Lgr5-GFP;hGAS mice (n=3). Data are plotted as the means±SE (*p<0.05). (C) The numbers of CCK2R+ cells in WT and hGAS mouse antrum by immunofluorescence. Data are shown as numbers of glands with one, two or three cells in each glands. (D, E) The numbers (D) and sizes (E) of gastric organoids from WT and CCK2R$^{-/-}$ mice (n=4 well/group) after 14 days (passage 1) in culture in Wnt3A, EGF, R-spondin1. Organoids were treated with progastrin (1 μg/mL) and/or 1 uM YF476, *p<0.05. (F) GAPDH-normalised gene expression of CCK2R in cultured organoids with or without progastrin (n=4). Data are plotted as the means±SE (*p<0.05). (G) Fluorescence-activated cell sorting (FACS) plot of digested single cells from Lgr5-GFP mouse stomach. Boxes indicate Lgr5^{high}, Lgr5^{low} and Lgr5^{neg} groups. (H) Representative time course of Lgr5+ gastric single-cell culture. (I) Average colony-forming efficiency of Lgr5^{low} and Lgr5^{high} cell culture after 7 days (n=5). Cells were treated with progastrin (1 μg/mL) or amidated gastrin (G-17, 100nM) as indicated. Data are plotted as the means±SE (*p<0.05). (J) GAPDH-normalised Lgr5 gene expression in sorted Lgr5^{low} cells with or without progastrin treatment or YF476 after 5 days. Data are plotted as the means±SE (n=5, *p<0.05). (K) Relative CCK2R expression in Lgr5^{high} population compared with Lgr5^{low} population in

Lgr5-GFP mice and Lgr5-GFP;hGAS mice. Mice were treated with YF476 or vehicle for 2 weeks prior to sorting. Data are plotted as the means±SE (n=5, *p<0.05).

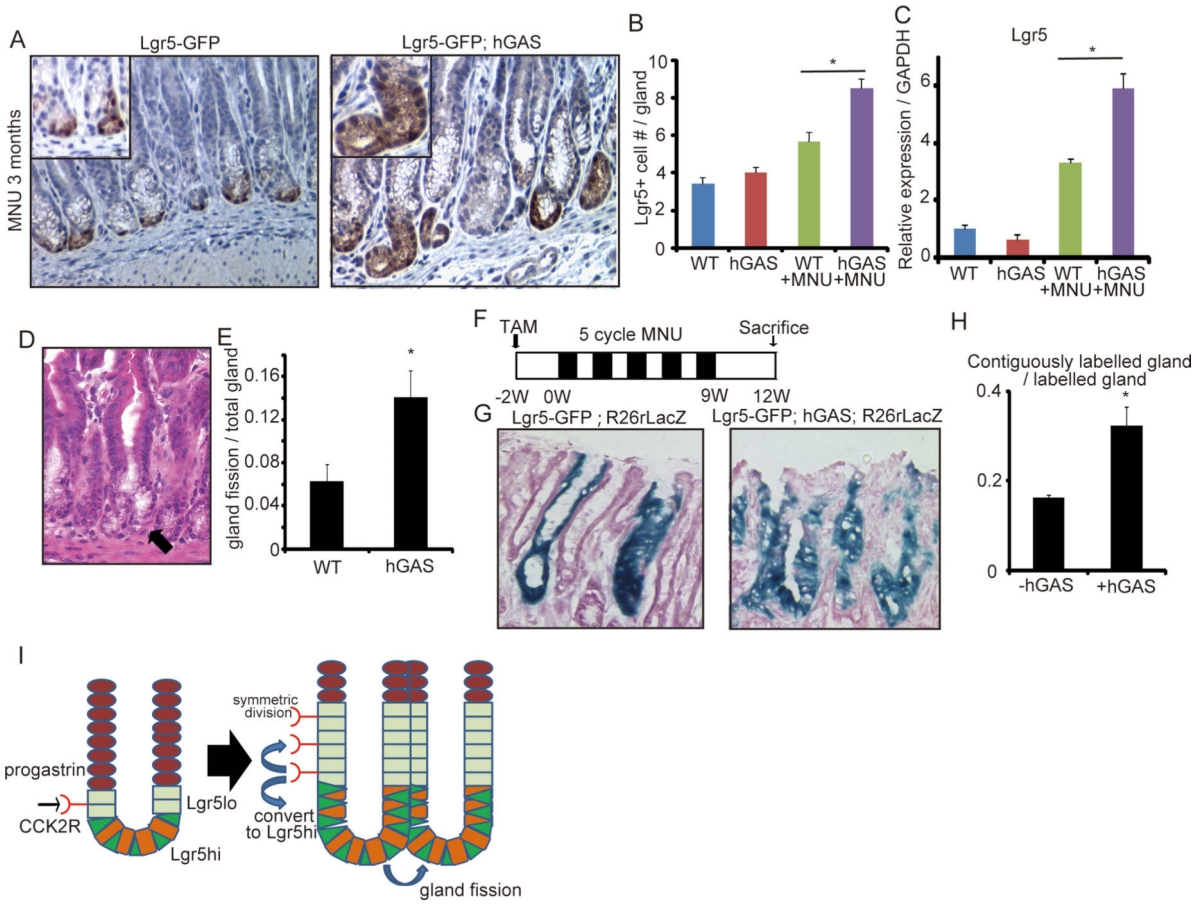

Figure 4.

Progastrin increased gastric stem cell number and crypt fission through CCK2R. (A) GFP staining of Lgr5-GFP and Lgr5-GFP;hGAS mice 3 months after MNU treatment. (B) The numbers of GFP+ cells in Lgr5-GFP and Lgr5-GFP;hGAS mice with or without MNU. Cells were counted at 20 different glands in the each group mice (n=3). All values were expressed as mean±SE (*p<0.05). (C) GAPDH-normalised expression of the Lgr5 gene in the stomachs of WT and hGAS with or without MNU treatment (n=3 for each group). Relative expression in untreated WT mice is represented as 1.0. Data are plotted as the means±SE (*p<0.05). (D) Representative crypt fission picture (arrow). (E) The numbers of crypt fission per total glands in WT and hGAS mice (three mice for each group, and three sections per mouse were counted) after MNU treatment. Data are plotted as the means±SE (*p<0.05). (F) Protocol of tamoxifen induction and MNU treatment. (G) Representative sections of β-gal+ labelled antral crypts in Lgr5-CreER;R26rLacZ and Lgr5-CreER;hGAS;R26rLacZ mice exposed to five cycles of MNU. (H) Average ratio of β-gal+ labelled contiguous labelled glands to total β-gal+ labelled antral crypts. Each group has three mice and 20 labelled crypts per mouse were measured. Data are plotted as the means ±SE (*p<0.05). (I) Models of the role of progastrin in the antrum during carcinogenesis.

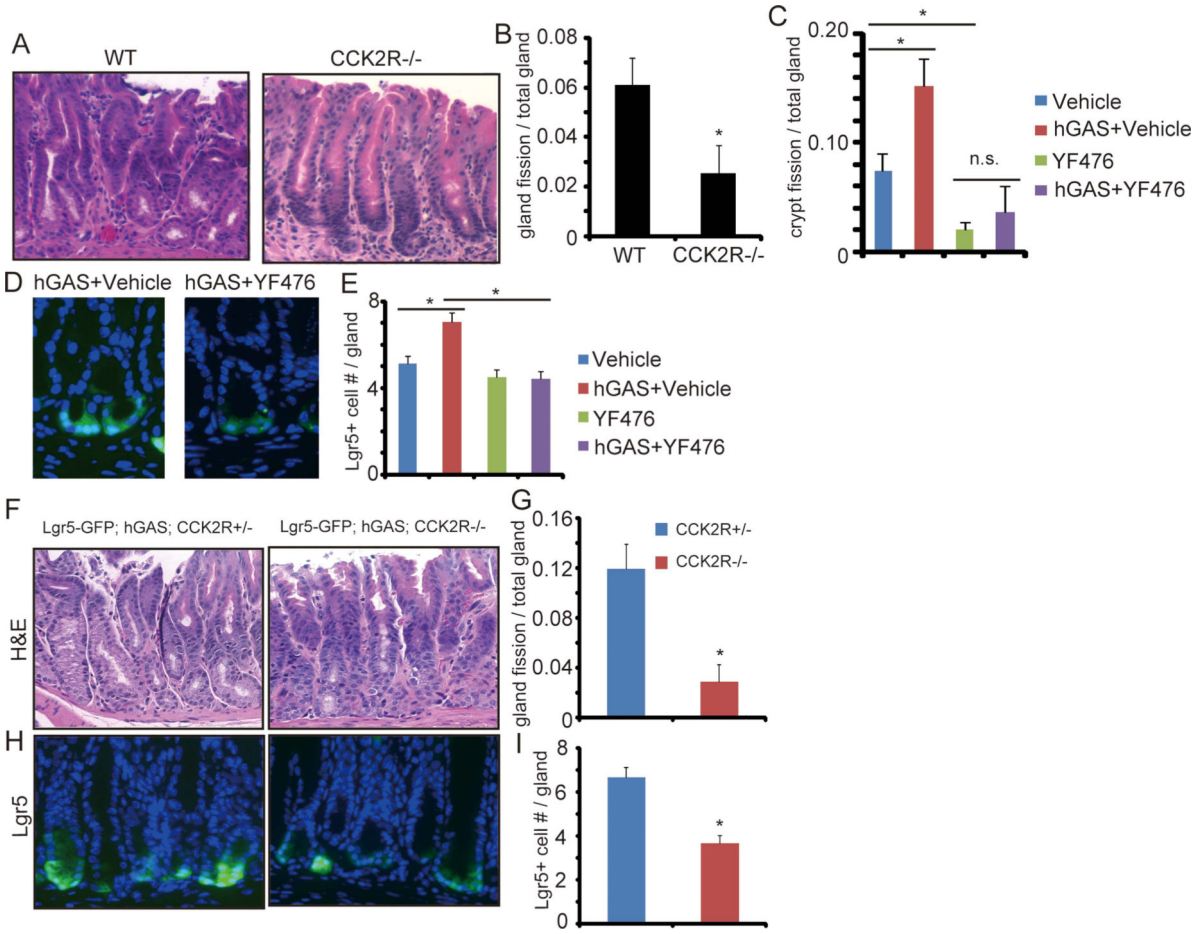

Figure 5.

CCK2R inhibition prevents antral crypt fission. (A) H&E staining of five-cycle MNU-treated antrum of WT and CCK2R$^{-/-}$ mice. (B) The numbers of crypt fission per total glands in WT and CCK2R mice (n=3, each group) after MNU treatment. Data are plotted as the means±SE (*p<0.05). (C–E) The numbers of crypt fission per total glands (C) and Lgr5+ cell number (E) in Lgr5-GFP and Lgr5-GFP;hGAS mice (n=3, each group) treated with YF476 or vehicle during MNU treatment. Representative Lgr5-GFP pictures of Lgr5-GFP;hGAS mice with or without progastrin are shown in (D). Data are plotted as the means ±SE (*p<0.05). (F) H&E staining (top) and GFP expression (bottom) in MNU-treated Lgr5-GFP;hGAS; CCK2R$^{+/-}$ mice and Lgr5-GFP;hGAS;CCK2R$^{-/-}$ mice (n=4, each group). (G) Quantification of crypt fission and GFP+ cell number. Data are plotted as the means±SE (*p<0.05).

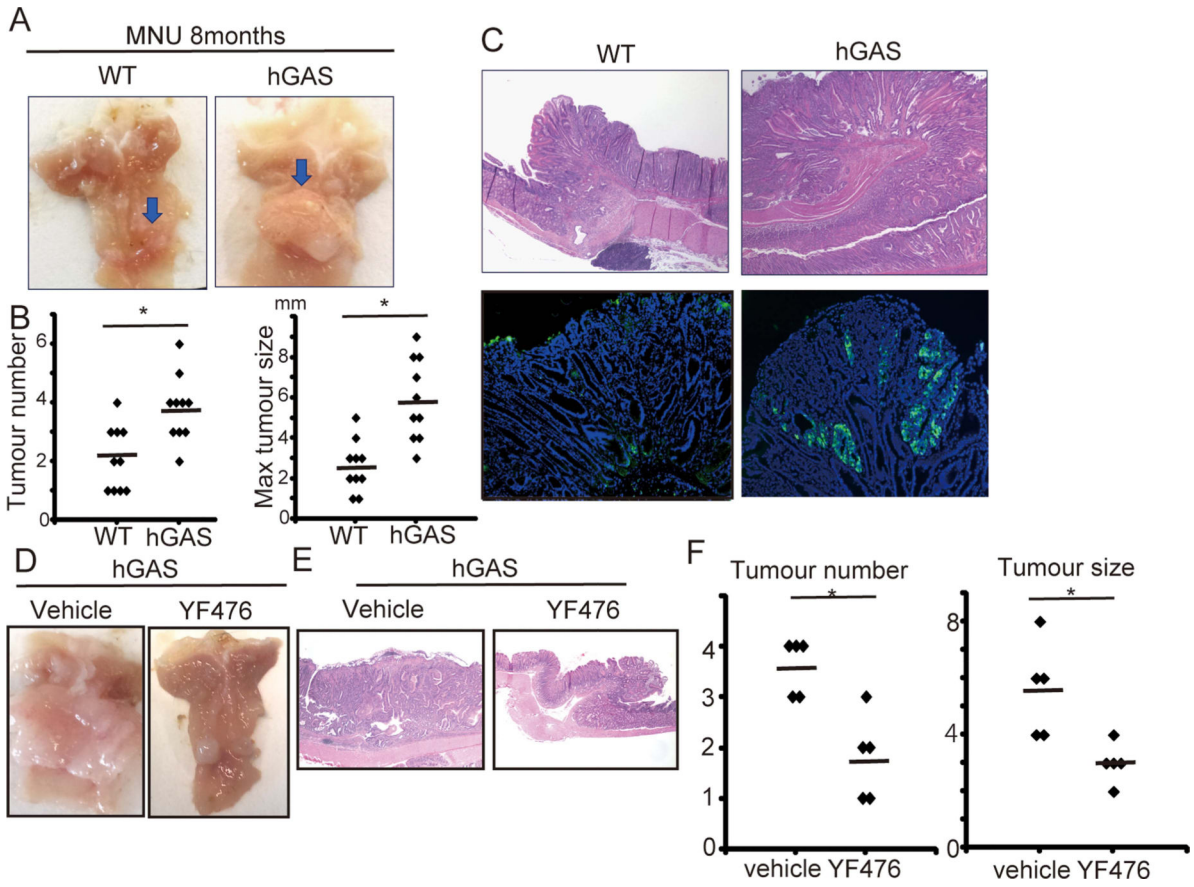

Figure 6.

Progastrin promotes antral carcinogenesis in mice. (A–C) WT and hGAS mice with Lgr5-GFP were treated with MNU and *H. felis*, and sacrificed at 36 weeks after the beginning of MNU (n=10). Macroscopic images (A), dot plots of tumour number and max tumour size (B), H&E staining (C, top) and Lgr5-GFP expression (C, bottom) are shown. Arrows indicates tumour area (*p<0.05). (D–F) Macroscopic images (D), H&E staining (E) and dot plots of tumour numbers and sizes (F) of YF476-treated or vehicle-treated hGAS mouse stomachs are shown (n=5, each group). *p<0.05.