Conditional deletion of menin results in antral G cell hyperplasia and hypergastrinemia

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Veniaminova NA, Hayes MM, Varney JM, Merchant JL. Conditional deletion of menin results in antral G cell hyperplasia and hypergastrinemia. Am J Physiol Gastrointest Liver Physiol 303: G752–G764, 2012. First published July 5, 2012; doi:10.1152/ajpgi.00109.2012.—Antral gastrin is the hormone known to stimulate acid secretion and proliferation of the gastric corpus epithelium. Patients with mutations in the multiple endocrine neoplasia type 1 (MEN1) locus, which encodes the protein menin, develop pituitary hyperplasia, insulinomas, and gastrinomas in the duodenum. We previously hypothesized that loss of menin leads to derepression of the gastrin gene and hypergastrinemia. Indeed, we show that menin represses JunD induction of gastrin in vitro. Therefore, we examined whether conditional deletion of Men1 (Villin-Cre and Lgr5-EGFP-IRESCreERT2), with subsequent loss of menin from the gastrointestinal epithelium, increases gastrin expression. We found that epithelium-specific deletion of Men1 using Villin-Cre increased plasma gastrin, antral G cell numbers, and gastrin expression in the antrum, but not the duodenum. Moreover, the mice were hypochlorhydric by 12 mo of age, and gastric somatostatin mRNA levels were reduced. However, duodenal mRNA levels of the cyclin-dependent kinase inhibitor p27Kip1 were decreased, and cell proliferation determined by Ki67 staining was increased. About 11% of the menin-deficient mice developed antral tumors that were negative for gastrin; however, gastrinomas were not observed, even at 12 mo of age. No gastrinomas were observed with conditional deletion of Men1 in the Lgr5 stem cells 5 mo after Cre induction. In summary, epithelium-specific deletion of the Men1 locus resulted in hypergastrinemia due to antral G cell hyperplasia and a hyperproliferative epithelium, but no gastrinomas. This result suggests that additional mutations in gene targets other than the Men1 locus are required to produce gastrin-secreting tumors.

somatostatin; p27Kip1; tumor; Villin-Cre; Lgr5

MENIN IS A UBQUITOUSLY EXPRESSED tumor suppressor protein encoded by the multiple endocrine neoplasia type 1 (MEN1) gene (8). Mutations in this locus are responsible for endocrine-secreting tumors in the pituitary, parathyroid, pancreas, and duodenum (7, 9, 21). Gastrinomas are the most malignant tumor induced by autosomal-dominant mutations in the MEN1 locus, and these tumors are more common in the duodenum than the pancreas (1, 13, 17). Important features of sporadic and MEN1-driven gastrinomas include hypergastrinemia and hypersecretion of gastric acid, resulting in diarrhea and extensive duodenal peptic ulcers, collectively known as the Zollinger-Ellison syndrome (ZES) (17, 30, 37). Although not attributed to a specific genetic defect, pseudo-ZES was characterized in 1972 as hypergastrinemia due to antral G cell hyperplasia without tumors (12, 27). Described prior to the positional cloning of MEN1, antral G cell hyperplasia is considered a cause of ZES that can exist in the absence of an actual tumor (4, 27).

Since the initial cloning of MEN1, there has been heightened interest in understanding why endocrine organs are especially susceptible to hypersecretion and tumor formation. To dissect its function in vivo, the Men1 locus was deleted in mice by homologous recombination (5, 10). Complete deletion of the Men1 locus (Men1−/−) in mice causes embryonic lethality at embryonic day 11.5–13.5 due to multiple developmental defects. However, mice heterozygous for Men1 (Men1+/−) are viable, appear normal, and do not show abnormalities before 9 mo of age (5, 10). By contrast, older Men1+/− mice develop a variety of endocrine tumors consistent with those observed in the human MEN1 syndrome (5, 10). The one exception is a gastrinoma that does not develop in the Men1+/− mice, despite endocrine tumor development in the pituitary and pancreas (10). Although Bertolino et al. (5) reported an extrapancreatic gastrinoma in their Men1+/− mice, the histological evidence consisted of an adenomatous tumor with patches of G cell hyperplasia. More importantly, neither study measured the levels of plasma gastrin or determined gastrin mRNA levels in the stomach or duodenum. Thus, surprisingly, there is little evidence that deletion of Men1 alone is sufficient to induce generation of a gastrinoma as well as induce hypergastrinemia.

We previously showed that menin suppresses gastrin gene expression and that reducing menin expression is sufficient to induce gastrin expression (23). Therefore, we hypothesize in this study that loss of menin expression will be sufficient to induce gastrin expression. Since prior studies of heterozygous Men1 deletion did not reveal gastrinomas, we also examined whether tissue-specific homozygous Men1 deletion is sufficient for gastrinoma development. To test these hypotheses, we analyzed mice in which the Men1 locus was conditionally deleted simultaneously in the antral and intestinal epithelium (Men1FL/FL; Villin-Cre and Lgr5-CreERT2). We quantified the levels of plasma and tissue gastrin at different time points and found that deletion of Men1 was indeed sufficient to generate hypergastrinemia due to antral G cell hyperplasia but was not sufficient to induce gastrin-secreting tumors.

MATERIALS AND METHODS

Animals and genotyping. Mice with the floxed Men1 locus in which LoxP sites flank exons 1–8 of the Men1 gene (10) were purchased (stock number 005109, Jackson Laboratory) and then bred to a Villin-Cre mouse line (20) (a gift from D. Gumucio) to generate Villin-Cre × Men1FL/FL (designated VC:Men1FL/FL) mice or to a Lgr5-EGFP-IRESCreERT2 mouse line (stock number 008875, Jackson Laboratory) to generate Lgr5-EGFP-IRESCreERT2 × Men1FL/FL (designated Lgr5:Men1FL/FL) mice. In the Lgr5-EGFP-IRESCreERT2 mice, the Cre recombinase was acti-
vated by an intraperitoneal injection of tamoxifen (1 mg/mouse; Sigma-Aldrich, St. Louis, MO) once a day for 5 consecutive days. All mice were on a C57BL/6J × FVB:129S mixed background, with an average of 85.33% C57BL/6 determined by single nucleotide polymorphism analysis (Charles River Laboratories, Troy, NY). The mice were housed in a facility with a 12:12-h light-dark cycle and allowed access to food and water ad libitum. The mice were genotyped using the following primers: exon 8 [5'-ATTGATGAGACCGAAGAC-3'] (forward) and 5'-GTCCCTGAGACGAGAAAACTTGG-3' (reverse) and exon 2 [5'-CCACATCCAGCTCCTCGACT-3'] (forward) to detect tissue-specific Men1 ablation (31). PCR analysis was performed using GoTaq DNA polymerase (Promega, Madison, WI) in 20 μl of reaction mix for 35 cycles according to standard procedures. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min followed by a final single 5-min extension at 72°C. For genotyping, DNA was isolated using the HotSHOT protocol (33). For the tissue-specific Men1 deletion analysis, the DNasey Blood & Tissue Kit (Qiagen, Valencia, CA) was used. PCR products were analyzed in 1% agarose gels. Animal experiments were conducted according to protocols approved by the University of Michigan Committee on the Use and Care of Animals.

RT-quantitative PCR. After removal, fresh tissues were homogenized in TRIzol reagent (GIBCO/Invitrogen, Carlsbad, CA). RNA was isolated according to the manufacturer’s instructions using the RNeasy Mini kit (Qiagen) after DNase A treatment. The first-strand cDNA was synthesized using 1 μg of total RNA and the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCRs (qPCRs) were carried out using a thermal cycler (C1000, Bio-Rad) with SYBR Green dye (Molecular Probes, Carlsbad, CA) and Platinum Taq DNA polymerase (Invitrogen). Each reaction was performed three times in triplicate as follows: 3 min at 95°C, 40 cycles of 9 s at 95°C, 1 min at 60°C, and 1 min at 55°C. The mouse primers were as follows: hypoxanthine-guanine phosphoribosyltransferase (Hprt) [5'-AGGACGCTCTCAGAATGTGGA-TAAC-3'] (forward) and 5'-AATGTGCCTCAGTTAAGGGTTG-3' (reverse); Men1 [5'-CCAGTCCCCTCTCACTTCA-3'] (forward) and 5'-GATTGGAACATCTCTTGAGACCCA-3' (reverse); gastrin [5'-ACAACACAGCCACTTAC-3'] (forward) and 5'-CAAAGTGCATCATCAGAGATA-3' (reverse); somatostatin [5'-TCTGCACTGGCTGGCTTTAGG-3'] (forward) and 5'-TCACTTCTGTGCTTGATGATGTC-3' (reverse); p27kip1 [5'-GGGTGCTTITATTTGGGTGTCTC-3'] (forward) and 5'-GTTCCGGCTGCCCTTGGTTTGC-3' (reverse); H11003-ATPase-α [5'-TGTAATGACATGAGTTCCTCGTTG-3'] (forward) and 5'-GAGTCTTCTCTTGCTATCCACCC-3' (reverse); H11003-ATPase-β [5'-AACAGAATTGTCAAGTCTCCTC-3'] (forward) and 5'-AGACTAGGATTCCATCGG-3' (reverse); sonic hedgehog [Shh, 5'-ATGTTTTCTGTGATTCCCTGTG-3'] (forward) and 5'-ATCGTGGAGGTGTTTGTGGAT-3' (reverse); and glioma-associated oncogene homolog 1 (Gli1, 5'-TTGGAGTGAGAAGACGTG-3' (forward) and 5'-GGAGACGACGAGTCTA-3' (reverse)).

Western blots. Tissues were homogenized in RIPA lysis buffer (Sigma-Aldrich) supplemented with Complete protease inhibitor cocktail (Roche, Indianapolis, IN). Protein concentration in the lysates was measured using the Bradford colorimetric assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Protein samples were prepared in 2× Laemmli buffer (Bio-Rad, Hercules, CA) and resolved by 10.2% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Temecula, CA). The membranes were blocked with 5% nonfat dry milk dissolved in 1× Tris-buffered saline and 0.1% Triton X-100 and then for 1 h at room temperature with a 1:2,000 dilution of mouse monoclonal anti-GAPDH (Millipore, Temecula, CA) antibody. After the sample was washed, horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) were used to visualize the proteins.

Immunohistochemical staining. After the animals were euthanized, the stomach and duodenum were removed, opened along the greater curvature, immobilized on bite wax, and fixed in place overnight at 4°C in 4% formaldehyde solution (Sigma-Aldrich) diluted in 1× PBS. Paraffin sections (5 μm) were coimmunostained with goat anti-gastrin antibody (1:200 dilution; Santa Cruz Biotechnology) and then incubated with rabbit anti-menin antibody (1:2,000 dilution; Bethyl Labs). In separate experiments, specific gastric cell types were identified by immunostaining for rabbit anti-gastrin (1:1,000 dilution; Dako, Carpinteria, CA), rabbit anti-Ki67 (1:200 dilution; Thermo Scientific), rabbit anti-phosphorylated (Ser10) histone H3 (Ser10) histone H3 (1:500 dilution; Millipore) and mouse anti-proton pump/H+-K+-ATPase-α (1:500 dilution; MLB, Woburn, MA). A rabbit anti-somatostatin antibody (1:200 dilution; Sigma-Aldrich) was used to stain D cells. Griffonia simplici-
results

Gastrointestinal-specific deletion of Men1. We previously showed that menin inhibits gastrin gene expression in vitro and that reduced levels of menin increase gastrin expression (23). Therefore, to test whether deletion of the menin locus increased gastrin expression in vivo, mice expressing Cre recombinase from a modified villin transgene (Villin-Cre) were bred to the Men1FL/FL line (VC:Men1FL/FL). Prior expression analysis using lineage-tracing studies showed that Villin-Cre is expressed in all epithelial cell lineages of the small and large intestines, as well as all gastric epithelial cell lineages of the antral glands (20, 28). To demonstrate the efficiency of Villin-Cre recombinase at the Men1 locus, we isolated DNA from tissues expected to express the transgene (duodenum, jejunum, ileum, colon, and stomach antrum) and compared these tissues with those not expected to express Cre recombinase on the basis of the expression pattern of the modified villin promoter used to generate the transgene (tail, stomach corpus, and liver). PCR analysis using primers that distinguish wild-type (WT), floxed, and Cre-mediated deletion of the Men1 gene was performed (31). The location of the LoxP sites within the Men1 gene and the primer locations are shown in Fig. 1A. As expected, a single band, corresponding to the WT Men1 allele, was detected in all tissues examined in the VC:Men1FL/FL (WT) mice (Fig. 1B, top). PCR using tissues from mice carrying floxed alleles resulted in amplification of additional PCR products in the antrum, duodenum, jejunum, ileum, and colon, corresponding to the deleted Men1 allele (Fig. 1B, bottom). In tissues where knockout of the Men1 gene was detected, we analyzed the ratio of the knockout to the floxed bands to compare the efficiency of Men1 recombination (Fig. 1C). We

fig. 2. epithelium-specific Men1 deletion in the duodenum. A: Men1 mRNA determined by RT-qPCR analysis of duodenal mucosa from 3-, 6-, and 12-mo-old VC:Men1FL/FL and WT mice. Values (means ± SE; n = 3–4) are shown relative to Hprt mRNA expression measured in the same samples. *P < 0.05. B: Western blot analysis of menin protein levels performed with duodenal mucosal scrapings from 2 WT (lanes 1 and 2) and 2 VC:Men1FL/FL (lanes 3 and 4) mice at 12 mo of age. C and D: representative images of menin (red) immunofluorescent staining using duodenal tissues from 12-mo-old WT and VC:Men1FL/FL mice. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Original magnification: ×200.
found ratios of 0.23–0.33 in the antrum and 0.6–0.83 in the duodenum, jejunum, ileum, and colon of VC:Men1\textsuperscript{FL/FL} mice. Our analysis confirmed that the Cre recombinase was \textasciitilde50\% less active in the antrum than in the remainder of the GI tract, consistent with the Villin-Cre promoter expression. Specifically, the expression of Cre in the antrum was as previously reported (19, 28). In addition, we performed RT-qPCR analysis of Men1 mRNA expression in the same tissues of the luminal GI tract and found that Men1 mRNA levels were significantly (up to 95\%) reduced in the antrum, duodenum, jejunum, ileum, and colon, but not the corpus, of VC:Men1\textsuperscript{FL/FL} compared with WT mice (Fig. 1D). Thus the PCR analysis confirmed that Cre-mediated Men1 deletion occurred in the antrum, duodenum, jejunum, ileum, and colon, but not in other tissues, including the stomach corpus. Interestingly, even a low level of Cre was sufficient to significantly reduce Men1 RNA levels in the antrum.

**Effect of Men1 deletion in the duodenum.** Since Men1-related gastrinomas frequently arise in the duodenum (1), we first analyzed the effect of menin deletion on the duodenal epithelium. Men1 mRNA expression was significantly reduced (up to 90\%) in the duodenal mucosa from VC:Men1\textsuperscript{FL/FL} mice compared with WT (control) mice as early as 3 mo of age (Fig. 2A). In addition, reduced levels of menin protein at 12 mo of age were detected by Western blot (Fig. 2B). Menin is ubiquitously expressed and is primarily a nuclear protein (8). Indeed, in the duodenum of a WT mouse, nuclear localization of menin was confirmed by immunofluorescence (Fig. 2C). In contrast, we observed reduced menin protein levels in the epithelial cells of VC:Men1\textsuperscript{FL/FL} duodenum, but not lamina propria (Fig. 2D). This result confirms faithful epithelium-specific expression of the Villin-Cre transgene in the duodenum and correlates with the genotyping in Fig. 1B (20).

Men1 deletion in the duodenum did not affect gastrin expression but increased proliferation. Menin is a negative regulator of gastrin gene expression (23), prompting us to examine the effect of epithelium-specific menin deletion on gastrin expression in the duodenum. Since the levels of gastrin

![Figure 3](http://ajpgi.physiology.org/)

**Fig. 3.** Conditional Men1 deletion in the duodenum does not alter G cell number. A and B: gastrin immunostaining in duodenum of 12-mo-old WT and VC:Men1\textsuperscript{FL/FL} mice. Original magnification: \texttimes200. C: G cell quantification in duodenum of 12-mo-old VC:Men1\textsuperscript{FL/FL} and WT mice. Values are numbers of gastrin-immunostained cells per high-power field (HPF). D–G: coimmunostaining of paraffin sections from duodenum for gastrin (green) and menin (red) with DAPI nuclear stain (blue) to identify double-positive cells in WT and VC:Men1\textsuperscript{FL/FL} mice within crypt (D and F) and villi (E and G). Original magnification: \texttimes600.
mRNA were not detectable in the duodenum by RT-qPCR, we performed immunohistochemical staining for gastrin. There was no difference in the number of gastrin-expressing (G) cells in the duodenums of the VC:Men1FL/FL compared with WT mice (Fig. 3, A–C). We observed an average of one to two G cells per approximately three crypts or villi of the duodenum. Double immunofluorescent staining revealed that menin is highly expressed in duodenal gastrin-producing cells of the crypts (Fig. 3D) and villi (Fig. 3E) of the WT mice. However, in the VC:Men1FL/FL mice, menin protein was immunodetected in duodenal G cells, but the levels appeared significantly reduced (Fig. 3, F and G).

The cyclin-dependent kinase inhibitor p27Kip1 regulates the transition of cells from the G1 to the S phase and is an important readout of the menin signaling pathway (15, 16, 24). Therefore, we quantified the levels of p27Kip1 mRNA in the duodenum of VC:Men1FL/FL mice at 3, 6, and 12 mo of age. A decrease in p27Kip1 mRNA in the duodenal mucosa of VC:Men1FL/FL mice was detected at 3 mo of age and achieved significance by 12 mo (Fig. 4A). The reduced levels of p27Kip1 correlated with an increase in the proliferation marker Ki67 (Fig. 4C vs. 4B), as well as the decrease in menin expression (Fig. 2A). Additionally, we observed a significant increase in the mitosis marker phosphorylated histone H3 (pH3, red) in the duodenal crypts (Fig. 4, E and F). Thus, in the duodenum, reduced Men1 mRNA and protein levels correlated with a decrease in p27Kip1 mRNA expression and an increase in epithelial proliferation.

Antral Men1 deletion leads to G cell hyperplasia. We examined the levels of Men1 mRNA in the stomach of the VC:Men1FL/FL and WT mice at 3, 6, and 12 mo of age. We observed a reduction in Men1 mRNA in 6- and 12-mo-old VC:Men1FL/FL mice (68% and 90%, respectively; Fig. 5A). Consistent with reduced Men1 mRNA, the levels of menin protein, determined by Western blotting (Fig. 5B, lanes 3 and 4 vs. lanes 1 and 2) or immunofluorescent staining (Fig. 5D vs. 5C), were also reduced in the antrum of 12-mo-old VC:Men1FL/FL mice.

The major source of circulating gastrin is the G cells in the stomach antrum (6, 34). Gastrin mRNA levels in the antrum were significantly increased in 12-mo-old VC:Men1FL/FL compared with WT mice. In addition, one of four (25%) and two of four (50%) mutant mice at 3 and 6 mo of age, respectively, showed elevated (in some cases up to 7-fold) gastrin mRNA levels (Fig. 6A). RIA revealed a significant 1.7-fold increase in the plasma gastrin levels of VC:Men1FL/FL mice by 12 mo of age (Fig. 6B). This increase in gastrin mRNA and protein correlated with a twofold increase in the number of antral G cells (Fig. 6, C–E), suggesting that these cells are the main source of circulating gastrin in the VC:Men1FL/FL mice. We previously reported that menin colocalizes with gastrin in G cells of the mouse antrum (23). Surprisingly, double immunostaining of menin and gastrin revealed residual menin protein expression in some antral G cells (Fig. 6, F and G). Nevertheless, the significant increase in gastrin mRNA, plasma gastrin,
and G cell numbers demonstrated that a sufficient number of G cells exhibited reduced menin levels to permit derepression of gastrin gene expression.

Men1 deletion in the antrum correlates with decreased somatostatin. As observed in the duodenum, p27Kip1 mRNA, an important menin target, was depressed in the antrum of ~90% of the VC:Men1FL/FL mice by 12 mo of age (Fig. 7A). Interestingly, somatostatin, a paracrine inhibitor of gastrin, was also downregulated (Fig. 7B). Somatostatin mRNA levels were reduced ~80% in the VC:Men1FL/FL mice at 12 mo of age, consistent with reciprocal changes in the levels of this paracrine inhibitor of gastrin (Fig. 7B). Double staining of menin and somatostatin in the corpus and antrum of WT mice revealed colocalization of menin to somatostatin-producing D cells (Fig. 7, C and E); even in the VC:Men1FL/FL mice, menin was detected in the D cells, suggesting an indirect effect of menin ablation on the regulation of somatostatin mRNA levels (Fig. 7, D and F). Despite changes in somatostatin mRNA, there was no effect on D cell number in the corpus and antrum (Fig. 7, G and H), indicating that menin regulates a change in somatostatin mRNA expression per cell.

Menin deficiency rarely leads to adenoma development in the antrum. Although gastrin-expressing tumors were not observed in any of the mice, 2 of 18 (11.1%) 12-mo-old VC:Men1FL/FL mice developed adenomas in the antrum, but not the duodenum. The histology of the adenomas is shown in Fig. 8A, and staining for gastrin peptide confirmed that G cells were essentially absent in these hyperplastic regions (Fig. 8B). By contrast, most of the cells in the hyperplastic antrums were essentially absent in these hyperplastic regions (Fig. 8C). Costaining for menin and Ki67 revealed that menin expression was dramatically reduced in the proliferating cells (Fig. 8E), with patches of cells completely negative for menin (Fig. 8, D–F). Since previous studies showed that the Shh pathway is altered in the hypergastrinemic mice (11), we investigated the effects of menin loss on the Shh pathway. We found that mRNA levels of Shh ligand and the Gli1 transcription factor trended downward, but the change was not statistically significant (Figs. 8, G and H). Therefore, canonical Shh signaling does not appear to be playing a role in the hyperplastic changes of the antrum after Men1 deletion.

Menin expression in the corpus of VC:Men1FL/FL mice is not affected. Western blotting revealed no difference in menin expression in the corpus of 12-mo-old VC:Men1FL/FL and WT mice (Fig. 9A). We measured the basal acid content and mRNA expression of H⁺-K⁺-ATPase-α- and -β from 3-, 6-, and 12-mo-old VC:Men1FL/FL and WT mice (Fig. 9, B–D). There were no changes in gastric acid level at 3 mo of age in the menin-deficient mice (Fig. 9B), despite some increase in gastrin mRNA and plasma levels (Fig. 6, A and B). Therefore, gastrin expression and peptide increased before changes in gastric acid levels were detected in the menin-deficient mice. By 6 mo of age, gastric acid levels peaked, but by 12 mo of age they were suppressed, in menin-deficient mice (Fig. 9B). These changes in gastric acid levels were mirrored by changes in H⁺-K⁺-ATPase-α- and -β mRNA expression (Fig. 9, C and D). By contrast, the menin-deficient mice remained hypergastrinemic (Figs. 6, A and B). Therefore, our data suggest that conditional deletion of menin in the antral epithelial cells increases gastrin expression in the antrum first, then as a secondary effect, the stomach acidity changes. Hypergastrinemic VC:Men1FL/FL mice showed initial increases followed by sustained decreases in acid secretion, which is consistent with the hypergastrinemic insulin-gastrin (InsGas) mouse model (35). Analysis of parietal cells by immunostaining for H⁺-K⁺-ATPase-α revealed no significant changes in the number of acid-secreting cells at 12 mo of age (Fig. 9, E and F). In
addition, VC:Men1<sup>FL/FL</sup> mice rarely developed hyperplastic lesions in the corpus (Fig. 10, A–C). In the corpus of WT, as well as VC:Men1<sup>FL/FL</sup>, mice, menin was ubiquitously expressed as a nuclear protein (Fig. 10, D and E). The hyperplastic lesions in the corpus did not show a decrease in menin expression (Fig. 10 F), were negative for an increase in Ki67 (Fig. 10 I), and primarily showed expansion of cells staining for the mucous neck cell marker GSII (Fig. 10 L).

We attributed the changes in the corpus to the indirect effects of hypergastrinemia as observed in the InsGas mouse model (35).

Men1 deletion in Lgr5-positive stomach antral stem cells does not affect menin expression in G cells. An orphan G protein-coupled receptor, Lgr5, has been shown to mark crypt base columnar stem cells in the intestine and stomach antrum (2, 3). To further characterize the role of menin in the GI epithelium, we bred Men1<sup>FL/FL</sup> mice to the Lgr5-EGFP-IRES-CreERT2 mouse line to generate Lgr5:Men1<sup>FL/FL</sup> mice. At 5 mo after tamoxifen-induced Cre activation, we observed loss of menin expression cells in stem cell progeny in the duodenum (Fig. 11B) and whole glands in the antrum (Fig. 11D). RT-qPCR revealed a significant decrease in Men1 mRNA expression in the antrum, but not the corpus and duodenum, of the Lgr5:Men1<sup>FL/FL</sup> vs. WT mice (Fig. 11E). The low efficiency of Men1 deletion in the duodenum is consistent with variegated expression of the Lgr5 promoter in the small intestine, which limits the use of these mice in Lgr5-Cre-driven knockout strategies. Interestingly, double immunostaining of menin and gastrin revealed residual menin protein expression in some antral G cells, even in the glands that had undergone successful menin deletion (Fig. 11F). Surprisingly, we observed a pattern of deletion in the epithelial vs. gastrin-expressing cells that was the same as that observed with Villin-Cre (Fig. 6G). In addition, G cell numbers (Fig. 11G), gastrin mRNA expression (Fig. 11H), and circulating plasma gastrin levels (Fig. 11I) tended to be higher in these Lgr5:Men1<sup>FL/FL</sup> mice than in control mice, although the numbers did not reach statistical significance. These results were consistent with data we obtained using the epithelium-specific Cre driver Villin-Cre (Fig. 6, A, B, and E).

Fig. 6. Menin deficiency in the antrum leads to G cell hyperplasia and hypergastrinemia. A: RT-qPCR measurement of gastrin mRNA normalized to Hprt mRNA levels in stomach of VC:Men1<sup>FL/FL</sup> and age-matched WT mice. Values are means ± SE; n = 3–4. *P < 0.05. B: RIA and enzyme immunoassay measurement of gastrin concentration in circulating plasma from mice fasted for 16 h. Values are means ± SE; n = 4–13. *P < 0.05. C and D: paraffin sections of stomach antrum from 12-mo-old WT and VC:Men1<sup>FL/FL</sup> immunostained for gastrin. Original magnification: ×400. E: morphometric analysis of G cell number in 12-mo-old WT and VC:Men1<sup>FL/FL</sup> mice. Values are numbers of gastrin-immunostained cells per HPF (×400 magnification). *P < 0.05. F and G: coimmunostaining of paraffin sections for menin (red) and gastrin (green) revealing colocalization of these proteins to antral G cells of WT and VC:Men1<sup>FL/FL</sup> mice. Nuclei were stained with DAPI (blue). Original magnification: ×400. Open arrowheads indicate menin-expressing G cells; closed arrowhead indicates a menin-negative G cell.
DISCUSSION

It is well established that MEN1-driven tumor development follows Knudson’s “two-hit” gene model for transformation (18). Because complete Men1-null mice are not viable, analysis of the Men1 locus requires analysis of heterozygous mice or a conditional null allele. All prior studies describing the heterozygous Men1-related phenotype reported tumors in a variety of endocrine organs except the GI tract (5, 10). We used the Villin-Cre mouse line to perform tissue-specific Men1 deletion in the epithelial cells of the duodenum and antrum (19, 20, 28). Deletion of Men1 in the antral epithelial cells induced a nearly twofold increase in gastrin mRNA expression in antral G cell numbers and levels of plasma gastrin. Nearly 50% (6 of 13) of the 12-mo-old VC:Men1FL/FL mice were hypergastrinemic without evidence of gastrin-secreting tumors. These changes in the antrum, in ~10% of the mice, affected the corpus by inducing the expansion of the GSII-positive mucous neck cell lineage, typically called spasmyloytic polypeptide-expressing metaplasia (26). However, in the absence of a robust increase in corpus proliferation and no changes in menin expression, the expansion of these mucous neck cells likely represents a compensatory change in cellular differentiation due to increased gastrin (25).

The effect of Men1 deletion on somatostatin gene expression and subsequent hypergastrinemia was less clear. Although somatostatin mRNA expression was downregulated in the stomach of menin-deficient mice, we did not observe changes in the number of D cells and, specifically, the number of D cells that express menin. We previously showed that somatostatin increases menin protein levels in the gastrin-expressing human gastric adenocarcinoma (AGS) cell line and that the induction requires inhibition of protein kinase A (22). Thus we expected that a deletion of Men1 would prevent somatostatin-mediated induction of menin protein and subsequent suppression of gastrin gene expression by menin in the G cell. Indeed, our results are consistent with this mechanism in the G cell but...
do not explain why somatostatin levels were also suppressed by 12 mo of age. We considered the possibility that menin might also regulate somatostatin gene expression, but this has not been directly tested. In fact, little is known regarding the upstream regulators of somatostatin gene expression, with the exception that hypergastrinemia correlates with reduced somatostatin levels (36). Since menin levels in the corpus did not change, our results support the notion that reduced menin protein levels prevent suppression of gastrin gene expression by somatostatin, at least in some G cells. Moreover, it raises the possibility that chronic hypergastrinemia, which apparently is an early change after menin deletion, is sufficient to suppress somatostatin.

Consistent with a prior study (29), we observed an increase in proliferation in the duodenal mucosa of 12-mo-old VC:Men1FL/FL mice that correlated with epithelium-specific Men1 deletion. p27Kip1 is a well-known target of somatostatin and menin signaling (14–16, 24, 32). Therefore, the mRNA levels of this cyclin-dependent kinase inhibitor were suppressed in the VC:Men1FL/FL mice as expected and were sufficient to explain the hyperplastic changes in the antral and duodenal mucosa. However, adenomas were observed only in antrums of VC:Men1FL/FL mice, albeit rarely. The duodenal hyperplasia was restricted to the crypt proliferative zone and, surprisingly, did not develop into tumors. More interestingly, no hyperplastic changes occurred in gastrin-expressing cell types in the stomach or duodenum, suggesting that epithelial deletion of menin alone was not a sufficient catalysis for transformation, despite a hyperplastic epithelium. A corollary to this observation was the fact that several months were required before phenotypic changes, such as hypergastrinemia, emerged in the VC:Men1FL/FL mice, suggesting that other genetic defects are likely necessary for development of gastrinomas.

Despite one report suggesting that Men1 deletion alone is sufficient to produce gastrinomas (5), our study and the original report on the conditional deletion of this locus (10) agree that loss of menin alone is insufficient to produce gastrinomas. Moreover, Crabtree et al. (10) examined Men1+/− mice that developed loss of heterozygosity in the pituitary and adrenal glands and pancreatic islets, where hormone-producing tumors eventually developed. More importantly, neither study measured plasma gastrin levels to demonstrate that loss of menin...
derepresses gastrin gene expression. While the number of antral G cells that remained positive for menin was surprising, we concluded that a sufficient number of these neuroendocrine cells exhibited reduced menin levels, permitting derepression of the gastrin gene. Even deletion of the Men1 gene in the Lgr5-positive stem cells mimicked our findings with respect to gastrin in 6-mo-old VC:Men1FL/FL mice.

In conclusion, tissue-specific deletion of Men1 in the mucosa of the luminal GI tract is sufficient to induce hypergastrinemia and epithelial hyperplasia, but not gastrinomas. This result raises the possibility that gastrin-expressing tumors exhibit differential sensitivity to regulation by menin. In particular, transformation of the gastrin-expressing cell type to a bona fide malignancy appears to require additional gene mutations that have yet to be determined.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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Fig. 10. Characteristics of corpus hyperplasia in 12-mo-old VC:Men1 FL/FL mice. A–C: representative hematoxylin-eosin-stained sections of the corpus from a WT and a VC:Men1 FL/FL mouse and hyperplastic corpus from a VC:Men1 FL/FL mouse. D and E: immunofluorescent staining of sections of corpus from a WT and a VC:Men1 FL/FL mouse and hyperplastic corpus from a VC:Men1 FL/FL mouse. Nuclear staining of menin (red) and DAPI (blue). G–I: proliferative cells in sections of corpus from a WT and a VC:Men1 FL/FL mouse and hyperplastic corpus from a VC:Men1 FL/FL mouse visualized by immunostaining for Ki67. J–L: mucous neck cells visualized by staining with *Griffonia simplicifolia* II (GSII) lectin in sections of corpus from a WT and a VC:Men1 FL/FL mouse and a hyperplastic corpus from a VC:Men1 FL/FL mouse. Original magnification: ×200.


Disruption of Klf4 in villin-positive gastric progenitor cells promotes formation and progression of tumors of the antrum in mice. Gastroenterology 139: 2028 –2037; e2029, 2010.


