Wnt/β–catenin promotes gastric fundus specification in mice and humans

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Despite the global prevalence of gastric disease, there are few adequate models in which to study the fundus epithelium of the human stomach. We differentiated human pluripotent stem cells (hPSCs) into gastric organoids containing fundic epithelium by first identifying and then recapitulating key events in embryonic fundus development. We found that disruption of Wnt/β–catenin signalling in mouse embryos led to conversion of fundic to antral epithelium, and that β–catenin activation in hPSC–derived foregut progenitors promoted the development of human fundic–type gastric organoids (hFGOs). We then used hFGOs to identify temporally distinct roles for multiple signalling pathways in epithelial morphogenesis and differentiation of fundic cell types, including chief cells and functional parietal cells. hFGOs are a powerful model for studying the development of the human fundus and the molecular bases of human gastric physiology and pathophysiology, and also represent a new platform for drug discovery.

Recently, considerable progress has been made in the development of three-dimensional in vitro organoid systems¹, ². Organoids have proven to be powerful experimental models that combine architectural complexity and cellular diversity with the tractability and scalability of traditional cell culture methods. Generation of organoids through directed differentiation of pluripotent stem cells (PSCs; comprising both embryonic stem cells and induced PSCs) offers several advantages over other approaches, including an unlimited source of starting material, the absence of a requirement for surgical acquisition of tissue, and ease of genetic manipulation. Furthermore, PSC-based methods permit direct investigation of the mechanisms that underlie normal and aberrant human development³. However, differentiating PSCs into specific organoid types depends on a robust molecular understanding of normal organ development. For some organs, such as the stomach, there are large gaps in our understanding of the molecular pathways that drive embryonic development.

The stomach is one of the most structurally diverse organs in mammals⁴. In humans, the gastric mucosa generally consists of two types of epithelial glands⁵, ⁶. Located in the more proximal anatomical domains—the corpus and fundus—of the stomach, oxyntic glands comprise acid-secreting parietal cells, protease-producing chief cells, mucus-producing cells, and endocrine cells. Antral-type glands, located in the more distal antrum and pylorus, contain mostly mucous and endocrine cells. To simplify the anatomy- and species-specific systems of nomenclature, we will use the terms ‘fundus’ and ‘antrum’ to broadly describe these two histologic types of gastric epithelia. We previously developed a method to direct the differentiation of hPSCs into three-dimensional gastric tissue (human gastric organoids; hGOs) that contained a pure antral epithelium with normal antral cell types⁷. Although these antral hGOs (hAGOs) are a robust system in which to study antral lineage allocation and host–microbe interactions in the stomach, they do not allow studies of fundic biology and disease. More recently, Noguchi et al. successfully differentiated mouse embryonic stem cells (ESCs) into organoids comprising various types of mouse gastric tissue⁸. However, this approach used mouse ESC aggregation and spontaneous differentiation, resulting in organoids that were heterogeneous, as shown by the presence of stratified epithelia. Moreover, species differences make the mouse stomach suboptimal for modelling human gastric disease⁹. Thus, a robust and efficient PSC-derived model of the human fundus epithelium would represent a significant advance in gastric biology research.

Embryonic organ development is guided by a series of instructive cues between neighbouring tissues¹⁰, ¹¹, and differentiation of hPSCs into specific lineages has relied heavily on use of these signals to direct differentiation in vitro. We previously identified a step-wise differentiation approach to generate hAGOs, whereby hPSCs were differentiated into definitive endoderm, patterned to posterior foregut, then specified into presumptive antral epithelium⁷. We hypothesized that the fundus and antrum derive from a common population of posterior foregut progenitors, which could be directed towards the fundic lineage if provided with the appropriate signals. However, given that the mechanisms that drive fundus development in vivo were not previously known, we first had to identify signalling pathways that pattern the embryonic stomach along the proximal–distal axis.

Embryonic stomach pattern formation

To investigate the pathways that regulate fundus specification during embryonic development, we analysed mouse embryos to identify molecular markers that could distinguish between presumptive fundus, antrum and forestomach. At embryonic day (E)14.5, Sox2 was expressed in all foregut organ lineages whereas Gata4 was restricted to the glandular stomach epithelium. Within the Gata4+ domain, Pdx1 was specific to the presumptive antral region (Extended Data Fig. 1a); thus, the embryonic fundus domain is Sox2+ Gata4− Pdx1−. We also analysed published microarray data sets (GSM326648–GSM326650, 12 and GSM80809–GMS80816) and dissected regions of the E14.5 foregut to demonstrate that expression of the transcription factors Irx2, Irx3 and Irx5 was more than tenfold higher in the embryonic fundus than in the antrum (Extended Data Fig. 1b, c), indicating that expression of these transcription factors can further distinguish between regions of the glandular gastric epithelium.

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At the molecular level, the presumptive fundic and antral domains of the stomach were already established by E10.5 (Fig. 1a). At that point in development, the canonical Wnt signalling pathway was active in the proximal stomach but exhibited little or no activity in the distal stomach, as shown using the Wnt reporter mouse strain Axin2: LacZ (Fig. 1b). Although the regulation of Wnt/β-catenin signalling is known to play a role in establishing the pyloric–duodenal boundary, its role in gastric epithelial patterning has not been investigated. To determine whether Wnt/β-catenin signalling was functionally required for establishing the fundus in vivo, we deleted β-catenin (also known as Ctnnb1) in the foregut epithelium using Shh-cre (Shh-cre;β-cateninfl/fl) (cKO) embryos. Disruption of Wnt/β-catenin signalling resulted in the loss of fundic identity, demonstrated by ectopic Pdx1 expression in the fundus at E10.5 (Fig. 1c). Ectopic Pdx1 was initially restricted to the ventral half of the fundic epithelium, consistent with previously reported recombination activity using this Shh-cre line, but it then expanded over time to include most of the proximal stomach and greater curvature by E14.5 (Extended Data Fig. 2a). In addition, expression of the fundus markers Irx2, Irx3 and Irx5 was greatly reduced in the cKO embryos (Extended Data Fig. 2b). Collectively, these data support the conclusion that epithelial Wnt/β-catenin signalling regulates gastric pattern formation, as it is required for the initial specification of fundus identity while repressing antral fate in the embryonic mouse stomach.

To understand the effect of early Wnt/β-catenin-mediated patterning abnormalities on subsequent cytodifferentiation, we analysed cKO embryos at E18.5. The stomach in cKO embryos was malformed and reduced in size at E18.5 (Fig. 1d and Extended Data Fig. 2c–d), which suggests that Wnt/β-catenin is involved in promoting stomach growth during later stages of development. Moreover, the stomach of cKO embryos was completely mis-patterned, with ectopic Pdx1 expression throughout the proximal-most regions of the epithelium (Fig. 1d). Parietal cells, a fundic cell type marked by expression of Atp4b, were reduced in the cKO stomach (Fig. 1d) and completely absent in β-catenin-deficient epithelium (Fig. 1e). Those parietal cells that did develop were observed only in β-catenin-expressing epithelium that did not undergo Cre-mediated recombination (Fig. 1e and Extended Data Fig. 2d, e). Together, these in vivo data support a model in which Wnt/β-catenin signalling induces fundus specification and inhibits antral identity. Disruption of this early patterning coincides with subsequent cell-autonomous loss of parietal cells, suggesting that cytodifferentiation is impaired secondary to developmental patterning defects.

**Differentiation of fundic hGOs from hPSCs**

We next investigated the role of Wnt/β-catenin signalling in establishing the fundic–antral pattern of the developing human stomach. To model the early stages of stomach differentiation, we started with a previously described protocol for differentiating hPSCs into antrum-like gastric organoids, which recapitulates the normal stages of early gastric development with high fidelity. Starting with three-dimensional posterior foregut spheroids (SOX2+ HNF1β+), we tested whether stimulation of Wnt/β-catenin signalling would direct posterior foregut epithelium into the fundic (SOX2+ GATA4+ PDX1+) rather than antrum (SOX2+ GATA4+ PDX1+) lineage during the gastric specification stage (Fig. 2a). Indeed, activation of β-catenin with the GSK3β inhibitor CHIR99021 (CHIR) for three days resulted in nearly complete repression of PDX1 expression at day 9, accompanied by significantly increased expression of IRX2, IRX3 and IRX5 (Fig. 2b, c; P < 0.05). Importantly, levels of SOX2 and GATA4 were unaffected by CHIR treatment, confirming that spheroids retained their gastric identity. Thus, CHIR exposure resulted in the formation of SOX2+ GATA4+ PDX1– epithelium with increased IRX gene family expression, a signature consistent with the presumptive fundic epithelium.

We then sought to determine whether CHIR-treated spheroids would further develop into more mature hGOs containing a fundus-like epithelium. Notably, a three-day pulse of CHIR from days 6–9 was not sufficient to irreversibly specify a fundic identity, as the hGOs ultimately reverted to a PDX1+ antral phenotype at later stages. However, continued Wnt stimulation via CHIR treatment through at least day 29 led to stable induction of fundic gene expression (Extended Data Fig. 3a). This was consistent with the prolonged activity of Wnt/β-catenin signalling during embryonic stomach development in vivo. Although previous studies indicated that ectopic Wnt activation in the embryonic stomach promoted an intestinal fate, CHIR-treated hGOs did not exhibit a significant increase in the intestinal markers CDX2, MUC2, CCK and SCT (Extended Data Figs 3e, 4a, b). We further demonstrated that CDX2 remained suppressed despite Wnt/β-catenin activation owing to concomitant inhibition of BMP signalling, as replacing Noggin with BMP4 led to robust expression of the intestinal transcription factor (Extended Data Fig. 4c).

Once regional domains have been established in early development, the primitive gastric epithelium undergoes periods of growth, glandular morphogenesis, and differentiation of definitive cell types. We have previously shown that hAGOs undergo a similar progression of morphologic and cellular development. CHIR-treated hFGOs grew at a similar rate and efficiency compared to hAGOs, as 75–90% of all spheroids plated grew into organoids (Extended Data Fig. 3d). At day 20, both types of hGOs contained epithelia that expressed the gastric SOX2–GATA4 signature in more than 90% of cells, while PDX1 was restricted to hAGOs (87.1 ± 8.4% in hAGOs and 3.9 ± 2.0% in hFGOs, P = 3.07 × 10−4; Extended Data Fig. 3e). The organoids maintained their respective gastric identities throughout their development (Extended Data Fig. 3b, c). By day 34, hFGOs and hAGOs comprised CDH1 CTNNB1 KRT8–polarized, columnar epithelia

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**Figure 1 | Wnt/β-catenin signalling is required for specification of the embryonic fundus in mice.** a, Pdx1 and Sox2 were expressed in the antrum (A), whereas Pdx1 was absent from the fundus (F), as shown in Atp4b-expressing parietal cells at E18.5. b, X-gal staining of an E10.5 foregut from an Axin2: LacZ reporter embryo shows that Wnt activity was restricted to the anterior domain of the stomach (Ant) but excluded from the posterior stomach (Post). c, Deletion of β-catenin from the gastric epithelium caused anterior expansion of Pdx1 into the fundic region of the stomach. The dorsal pancreas (DP) and ventral pancreas (VP) mark the boundary between the stomach and duodenum. d, In E18.5 Shh-cre;β-cateninfl/fl (cKO) embryos, Pdx1 was expressed throughout the stomach, except in some remaining patches of parietal cell–containing epithelium. Insets 1–3 and 4–6 show boxed regions in control and cKO stomachs, respectively. e, In the cKO stomach, Ctnnb1 exhibited mosaic deletion, and parietal cells differentiated only in Ctnnb1–sufficient epithelium. Scale bars, 250 μm (a), 200 μm (c) and 500 μm (d, e).
that ubiquitously expressed the gastric-specific claudin CLDN18 (Fig. 2e and Extended Data Fig. 4d), as well as comparable undifferentiated mesenchymal cells (Extended Data Fig. 5b). One notable difference was that hFGOs had a distinctive architecture with organized glands that budded from the organoid epithelium (Fig. 2d, e and Extended Data Fig. 5a), whereas hAGOs had complex folding and glandular buds. Thus, the novel Wnt/β-catenin-dependent mechanism that specifies the fundus is conserved in humans and can be manipulated to generate three-dimensional hFGOs with a glandular epithelium that molecularly resembles the developing fundus.

**Region-specific gastric cytodifferentiation**
Differentially expressed cell types (Fig. 3c), but expression of the hormone GAST was specific to hAGOs and GHRL expression was enriched tenfold in hFGOs (Fig. 3d), consistent with the normal gastroendocrine pattern.

To functionally define the region-specific competence of hOGs, we used an inducible system to overexpress the proendocrine transcription factor NEUROG3 in a NEUROG3-deficient PSC line. Expression of NEUROG3 in both hGO subtypes resulted in robust expression of the pan-endocrine marker SYP. Diverse hormone cell types were identified in hFGOs, including GHRL-, SST-, and histamine-expressing endocrine cells. The antral-specific G-cell marker GAST was expressed in hAGOs but not hFGOs; conversely, GHRL was enriched in hFGOs. hAGOs and hFGOs respectively, data representative of six independent experiments. hAGOs, but not hFGOs, were competent to give rise to antral-specific GAST-expressing endocrine cells in response to expression of the proendocrine transcription factor NEUROG3 (+doxycycline). *P < 0.01; two-tailed Student’s t-test; n = 4 biological replicates, data representative of three independent experiments. Error bars represent s.e.m. black dots show individual data points.
Figure 4 | Formation of chief cells in hFGOs. a, hFGOs had both MIST1- and pepsinogen C (PGC)-positive cells. b, High magnification of boxed region in a showing a gland with a cluster of cells with apical PGC staining. c, hFGOs had significantly increased expression of the chief cell markers PGA5 (1,000-fold), PGC (100-fold), and MIST1 (>10-fold) as compared to hAGOs. **P < 0.05; two-tailed Student’s t-test. n = 3 biological replicates, data representative of four independent experiments. d, Transmission electron micrograph of an hFGO cell containing dense zymogen granules, indicative of a chief cell. e, Pepsinogen protein content in hFGOs as compared to hAGOs in the presence or absence of the MEK inhibitor (PD03). There was no significant difference in the production of pepsinogen in hFGOs with or without PD03. **P < 0.0001 compared to hAGOs, two-tailed Student’s t-test, n = 8, 12, and 11 biological replicates in hAGOs, control hFGOs and hFGOs (no PD03), respectively. Scale bars, 200 μm (a), 25 μm (b) and 10 μm (d). Error bars represent s.e.m.

Pathways controlling parietal cell differentiation

At baseline, hFGOs contained only a small number of parietal cells (Fig. 5a, b), the defining cell type of fundic glands that acidify the gastric lumen via the proton pump (which consists of ATP4A and ATP4B subunits). Identification of efficient methods to increase parietal cell populations has remained elusive owing to a lack of understanding of the signalling mechanisms that drive their development. We therefore used PSC-derived hFGOs as a platform to functionally screen candidate signalling pathways for a role in regulating parietal cell differentiation. We exposed day 30 hFGOs to signalling agonists or antagonists for 2 days and analysed parietal cell differentiation on day 34.

Figure 5 | Identification of pathways that drive differentiation of functional parietal cells in hFGOs. a, Expression of parietal cell genes ATP4, ATP4B and GIF exhibited 10–100-fold increase in hFGOs compared to antral tissue at baseline, but was greatly increased by exposing hFGOs to a two-day pulse of PD03/BMP4. **P < 0.05 compared to hAGOs; *P < 0.05 compared to control hFGOs, two-tailed Student’s t-test, n = 4 biological replicates, data representative of 15 independent experiments. b, Stimulated differentiation of ATP4B-expressing parietal cells following treatment with PD03/BMP4. c, hFGO-derived parietal cells resembled those found in the maturing mouse fundic epithelium in vivo. d, Transmission electron micrograph of an hFGO cell with canalicular structure reminiscent of parietal cells. e, The epithelium of human fundic glands and hFGO epithelium were organized into MUC5AC-expressing cells in the surface epithelium and ATP4B-expressing parietal cells in the glandular units. f, Analysis of luminal pH in organoids in response to histamine by luminal injection of SNARF-5F. The luminal pH in hFGOs dropped rapidly, whereas hAGOs exhibited no response. The acidification was blocked by pretreating the organoids with famotidine or omeprazole. n = 9, 9, 7 and 4 biological replicates in hFGOs (histamine), hFGOs (histamine and famotidine), hFGOs (histamine and omeprazole) and hAGOs (histamine), respectively; data representative of three independent experiments. g, Histamine-induced acridine orange (AO) dye accumulation in a canalicular-type pattern in isolated mouse gastric glands and in hFGOs after 60 min. Scale bars, 100 μm (b, e (human fundus)), 10 μm (c, d, g) and 20 μm (e (hFGO)). Error bars represent s.e.m.
Whereas the majority of signalling manipulations had no appreciable effect, transient inhibition of the MEK pathway with PD0325901 (PD03) resulted in substantial upregulation of both ATP4A and ATP4B (Extended Data Fig. 8a). Furthermore, while BMP4 alone did not affect parietal cell gene expression, it could enhance the effect of PD03 (data not shown). Thus, a two-day pulse of PD03 and BMP4 was sufficient to induce rapid and robust expression of the parietal cell markers ATP4A, ATP4B and GIF (Fig. 5a, b and Extended Data Fig. 8d). Interestingly, this effect was not induced by simply removing EGF or FGF from the culture medium (Extended Data Fig. 8b), suggesting that there are likely to be endogenous signalling interactions upstream of MEK/ERK that are responsible for limiting parietal cell differentiation in hFGO cultures. Furthermore, treatment with PD03 and BMP4 affected only the parietal cell lineage (Extended Data Fig. 8e), and was unable to induce parietal cells in hAGOs (Extended Data Fig. 8c), further emphasizing that early patterning of the gastric epithelium defines its ultimate differentiation potential.

At day 34, hFGO epithelium showed organization similar to that of the human stomach, with mucous cells lining the surface domain and parietal cells concentrated in the glandular portion (Fig. 5c). Moreover, parietal cell morphology closely resembled that of maturing parietal cells in vivo (Fig. 5c). Given their resemblance to parietal cells in vivo and their tubulovesicular ultrastructure as seen on TEM (Fig. 5d), we hypothesized that the parietal cells in hFGOs would be able to secrete acid in response to appropriate stimuli. Measured using a pH-sensitive dye (SNARF5F) with real-time confocal microscopy (Extended Data Fig. 5a), hFGOs produced a swift and marked decrease in luminal pH in response to histamine that was blocked by either the H+ antagonist famotidine or the H+K+ -ATPase antagonist omeprazole (Fig. 5f and Extended Data Fig. 9b). To visualize the cellular response to histamine, hFGOs were cultured with the fluorescent dye acridine orange, which shifts to an orange colour when sequestered in acidic compartments.23. Similar to isolated mouse gastric glands, acridine orange accumulated in acidified cellular vesicles in hFGO glands in response to histamine (Fig. 5g and Extended Data Fig. 9c, d). These data indicate that the parietal cells underwent appropriate changes in secretory canalicular structure in response to acid-inducing stimuli.

In vivo, differentiated gastric cell lineages are thought to derive from a common pool of undifferentiated stem or progenitor cells. Here we have shown that the relative proportions of cell types in hFGOs can be altered either through genetic means (NEUROG3-mediated regulation of endocrine cells) or by manipulation of extrinsic signalling pathways (PD03 and BMP4 for parietal cells). These observations led to the hypothesis that hFGOs might contain a population of gastric stem cells analogous to those that have been isolated from the adult stomach. Indeed, we found that dissociated day 34 hFGOs could be passaged serially to give rise to new organoids (Extended Data Fig. 10a, b). Re-growth of organoids from passaged hFGOs was dependent on high-Wnt and high-FGF culture medium, similar to what is used to grow primary gastric tissue organoids.24,25. Following two rounds of passaging, hFGOs maintained expression of the lineage markers MUC5AC, MUC6, PGC, and GHRL; however, they did not contain parietal cells and were refractory to PD03-BMP4-mediated induction of the parietal lineage (Extended Data Fig. 10c, d). This finding was similar to what has been observed in adult stem cell-derived gastric organoids, which do not robustly produce parietal cells despite being derived from the bona fide oxyntic mucosa.26,27. Thus it will be important to identify conditions that preserve parietal cell competence in long-term cultures of hFGOs and adult gastric organoids.

Summary

To our knowledge, this is one of the first studies in which combined in vivo and in vitro methods have been directly applied to the differentiation of hPSCs into a new tissue type. We have shown that Wnt/β-catenin signalling is involved in specifying the fundic domain during stomach development in mice, and used Wnt modulation as the mechanistic basis to direct differentiation of hPSCs into three-dimensional human fundic organoids. In both mouse and human, Wnt-mediated fuscin specification was required for the subsequent formation of parietal cells. The fundus-specific manipulations at each stage of this directed differentiation protocol were essential for robust parietal cell induction (Extended Data Fig. 5f), emphasizing the importance of methodically reproducing the embryonic signalling environment. Previous reports have shown that the mesenchymal factor Barx1 acts indirectly to repress Wnt signalling and thereby helps to prevent intestinal gene expression in the stomach.14,15. Given that we have identified an epithelial Wnt/β-catenin function, and the previous work identified a mesenchymal pathway, it seems likely that Wnt/β-catenin may have distinct roles in the epithelium and mesenchyme. For example, the mesenchymal role for Wnt/β-catenin could modulate other signalling pathways such as BMP27, which our data show synergizes with Wnt to promote intestinal specification from early endoderm7 (Extended Data Fig. 4c). The human gastric organoid systems might be useful, in combination with animal models, to dissect how these signalling pathways interact in the mesenchyme and epithelium to coordinate early embryonic gastrointestinal development.

Our understanding of pathways that control the differentiation of gastric progenitor cells into distinct lineages is also incomplete. Our hGO platform has shown that MEK/ERK signalling potently represses parietal cell specification. Consistent with these findings, transgenic activation of MEK/MAPK-dependent pathways led to loss of parietal cells in vivo.28,29. Therefore, hFGOs are a tractable human model system in which to identify and study signalling mechanisms involved in normal cellular homeostasis in the fundus and antrum. Furthermore, aberrant regulation of developmental programs may also contribute to gastric disease, as corpus and/or fundus pathology is often associated with parietal cell atrophy30–32, antral-type histology33, and even misexpression of Pdx1.34. Thus, targeting of these pathways could have clinical applications, as Choi et al. recently demonstrated that pharmacological inhibition of MEK was sufficient to restore normal parietal cell differentiation in a mouse model of metaplasia35. Additionally, having now established both antral- and fundic-type hFGOs, it is possible to study how these human gastric tissues interact physiologically, react differentially to infection and injury, and respond to pharmacological treatments.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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**METHODS**

Mouse experiments. The following genetic mouse strains were obtained from The Jackson Laboratory, housed at Cincinnati Children's Hospital Research Foundation animal facility, and maintained according to IACUC protocol (0809074): Axin2:LacZ (stock no. 009120), Shh:Cre (stock no. 005622), and β-cateninfluorescent (stock no. 004152). Timed matings, with the morning the vaginal plug was observed being denoted as E0.5, were used to generate embryos at various stages that were used for either wholemount staining or tissue dissection. At least two litters of embryos were analyzed at each developmental stage examined. Both male and female embryos were analyzed.

Pluripotent stem cell culture. Human embryonic stem cell line WA01 (H1; obtained from WiCell) was supplied by the Pluripotent Stem Cell Facility at Cincinnati Children’s Hospital Medical Center. Cell identity was confirmed by short tandem repeat analysis (Microsatellite STR Analysis; Applied Biosystems), and cells were routinely tested for mycoplasma contamination (MycoAlert Mycoplasma Detection Kit; Lonza). Pluripotent cells were maintained in feeder-free conditions on HESC-qualified Matrigel (BD Biosciences) in mTeSR1 media (Stem Cell Technologies). Colonies were passaged every four days using dispase (Invitrogen).

Differentiation of posterior foregut spheroids. The protocol for directed differentiation of gastric organoids was adapted from our previous protocol2, and Supplementary Table 1 contains the complete list of media and growth factors for each stage. For differentiation, hPSCs were dissociated into single cells using Accutase (Stem Cell Technologies) and plated into 24-well plates at a density of roughly 200,000 cells per well in mTeSR1 with Y-27632 (10 μM; Stemgent). The following day, cells were differentiated into definitive endoderm (DE) by adding Activin A (100 ng/ml; Gibco) and defined FBS (dFBS; Invitrogen) at 0%, 20%, and 2.0% on days 1, 2, and 3, respectively. Subsequently, BMP4 (50 ng/ml; R&D Systems) was added to fundic hGOs from day 20 to day 30 as it was shown to promote differentiation of gastric organoids. hGOs were processed as previously described7. Briefly, organoids were fixed in 3% formalin in PBS, incubated in secondary antibody overnight at 4 °C, then washed thoroughly in PBS. For wholemount immunofluorescence, slides were deparaffinized with xylene, then embedded in paraffin. For staining, slides were deparaffinized, rehydrated in methanol and washed in PBS, incubated in secondary antibody overnight at 4 °C, and thoroughly washed. For confocal imaging, sections were blocked for 1 h, incubated in primary antibody overnight at 4 °C, then washed thoroughly in PBS. For wholemount staining, sections were stained with primary antibody (at dilution of 1:500) for 1 h at room temperature. Secondary antibodies (Jackson ImmunoResearch Laboratories) were made in donkey and conjugated to Alexa Fluor 488, 594, or 647.

Primary antibodies. Antibodies used for immunofluorescence staining are listed with antigen, host species, manufacturer and catalogue number, and dilution used for staining. Atpflb, rabbit, Santa Cruz sc48304, 1:300; Cdh1, goat, R&D Systems AF648, 1:500; Cdh1, mouse, BD Biosciences 610182, 1:500; Cdx2, mouse, Biogenex MUI392A, 1:500; Cldn18, rabbit, Sigma HPA018446, 1:200; Cmnb1, rabbit, Santa Cruz sc7190, 1:100; Foxf1, goat, R&D Systems F4798, 1:500; Gastrin, rabbit, Dako, clone G17G1, 1:1000. Cuxl1, goat, Santa Cruz sc13271, 1:200; Ghrl, rabbit, Sigma HPA04774, 1:100; Glut, goat, Santa Cruz sc10368, 1:200; Histamine, rabbit, Immunostar 22939, 1:1000; Krt8, rat, DSHB troma-1-s, 1:100; Mist1, rabbit, Sigma MUC5AC; hGOs were processed as previously described7. Briefly, they were permeabilized in Dent's bleach (4:1:100:20% H2O2) for 2 h at room temperature and rehydrated through series of methanol washes. Embryos were then blocked for 1 h, incubated in primary antibody overnight at 4 °C, washed in PBS, incubated in secondary antibody overnight at 4 °C, and thoroughly washed. For confocal embedding, tissues were dehydrated through series of ethanol washes, washed in xylene, then embedded in paraffin. For staining, slides were deparaffinized and rehydrated. Antibody retrieval was performed in citrate buffer for 45 min in steamer. Primary antibodies were incubated overnight at 4 °C. Following primary antibody, slides were washed in PBS then incubated with secondary antibody (at dilution of 1:500) for 1 h at room temperature. Secondary antibodies (Jackson ImmunoResearch Laboratories) were made in donkey and conjugated to Alexa Fluor 488, 594, or 647.

Immunofluorescent staining. Tissues were fixed in 4% paraformaldehyde overnight at 4 °C, then washed thoroughly in PBS. For wholemount immunofluorescent staining, embryos were processed as previously described7. Briefly, they were permeabilized in Dent’s bleach (4:1:100:20% H2O2) for 2 h at room temperature and rehydrated through series of methanol washes. Embryos were then blocked for 1 h, incubated in primary antibody overnight at 4 °C, washed in PBS, incubated in secondary antibody overnight at 4 °C, and thoroughly washed. For paraffin embedding, tissues were dehydrated through series of ethanol washes, washed in xylene, then embedded in paraffin. For staining, slides were deparaffinized and rehydrated. Antibody retrieval was performed in citrate buffer for 45 min in steamer. Primary antibodies were incubated overnight at 4 °C. Following primary antibody, slides were washed in PBS then incubated with secondary antibody (at dilution of 1:500) for 1 h at room temperature. Secondary antibodies (Jackson ImmunoResearch Laboratories) were made in donkey and conjugated to Alexa Fluor 488, 594, or 647.

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Imaging. Confocal imaging was performed on a Nikon A1r inverted confocal microscope. For whole-mount imaging, embryos were dehydrated in methanol and cleared in Murray’s clear (2:1 benzyl benzoate/benzyl alcohol) just before imaging. For wholemount staining imaging, embryos were fixed in 4% paraformaldehyde in Fluoromount G (SouthernBiotech), and air-dried overnight at room temperature.

Transmission electron microscopy. For transmission electron microscopy, hGOs were processed as previously described7. Briefly, organoids were fixed in 3% glutaraldehyde, washed in 0.1 M sodium cacodylate buffer, and incubated for 1 h in
4% osmium tetroxide. They were subsequently washed then dehydrated in ethanol series, and finally embedded in propylene oxide/LX112. Tissue was then sectioned and stained with 2% uranyl acetate followed by lead citrate. Images were visualized on Hitachi transmission electron microscope.

**Pepsinogen ELISA.** ELISA was performed using the Human Pepsinogen I (PGI) ELISA Kit (Thermo Scientific, EHPGI) according to the manufacturer's instructions. Briefly, day 34 hGOs were collected and incubated in Cell Recovery Solution (Corning) for 1 h at 4 °C then washed in PBS. Organoids were lysed with RIPA buffer followed by vigorous vortexing at high velocity for 30 min at room temperature. Lysates were pelleted and supernatant was collected and stored at −80 °C. For ELISA, the samples and standards were performed in technical replicates. The reactions were measured on a μQuant microplate plate reader (Bio Tek). Absorbance at 450 nm was measured, and the 570 nm absorbance was subtracted.

**Acid secretion assays.** Acid secretion assays were performed as previously described. hGOs were grown in the chambered coverglass (Thermo Scientific) and the chamber was placed on an inverted confocal microscope (Zeiss LSM 710), and experiments were performed under 5% CO2 at 37 °C (incubation chamber, PeCon).

Freshly isolated mouse gastric fundic glands or cultured hGO were incubated with acridine orange (10 mM), then acridine orange fluorescence was excited at 458 nm or 488 nm and images were collected at 600–650 nm (red) or 500–550 nm (green), respectively. On the other hand, to monitor the luminal pH of hGOs, the ratiometric pH-sensitive dye, 5-(and-6)-carboxy SNARF-5F (5 mM stock: EX 560 nm, EM 565–605 (green) and 620–680 (red) nm: Invitrogen) was microinjected (46–92 nl) into the lumen and monitored. Fluorescent dye was also added to the medium. Histamine (100 μM; Sigma) was added to the medium, while famotidine (100 μM; Sigma) or omeprazole (100 μM; Sigma) were pre-incubated for at least 30 min before histamine. Images were analyzed using MetaMorph software (Molecular Devices). Background corrected 620–680/565–605 nm ratio values were converted to pH using a standard curve.

**Statistical analysis.** Statistical significance was determined using unpaired Student’s t-test or one-way ANOVA with Dunnett’s multiple comparison post-hoc test. P<0.05 was considered significant.

**Statistics and experimental reproducibility.** No statistical analysis was used to determine experimental sample size, no specific method of randomization was used, and the investigators were not blinded during experiments. Statistical methods and measures are described in the figure legends. The protocol for differentiation of fundic hGOs was successfully completed more than 20 times by seven independent users in the laboratory. In all cases, data shown are derived from a single experiment that is representative of multiple experiments.

**Data availability statement.** Source data are available for Figs 2, 3, 4 and 5 and Extended Data Figs 2, 4, 5, 6, 8, 9 and 10.

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37. Ahnfelt-Ronne, J. et al. An improved method for three-dimensional reconstruction of protein expression patterns in intact mouse and chicken embryos and organs. **J. Histochem. Cytochem.** 55, 925–930 (2007).
Extended Data Figure 1 | Defining molecular domains in the developing stomach in vivo. 

a. Analysis of Sox2, Pdx1, and Gata4 in the embryonic mouse stomach (E14.5) showed that the fundus (f) was Sox2⁺Gata4⁺Pdx1⁻, whereas the antrum (a) was Sox2⁺Gata4⁺Pdx1⁺. The forestomach (fs) expressed Sox2 but neither Gata4 nor Pdx1.

b. Brightfield stereomicrograph showing dissected regions of the E14.5 mouse stomach that were analysed by qPCR. fs, forestomach; f, fundus; a, antrum; d, duodenum.

c. Dissected regions in b were analysed by qPCR for known regionally expressed markers (Sox2, P63, Gata4, Pdx1, and Cdx2) to validate the accuracy of micro-dissection. qPCR analysis of the dissected E14.5 stomach regions showed that putative fundus markers Irx1, Irx2, Irx3, Irx5, and Pitx1 were enriched in the fundus compared to the antrum. n = 4 biological replicates per dissected region. Scale bar, 500 μm. Error bars represent s.d.
Extended Data Figure 2 | Analysis of β-catenin cKO embryos. 
a. By E12.4 and E14.5, ectopic Pdx1 expression was observed throughout the
dorsal gastric epithelium and in the most proximal gastric epithelium
of cKO embryos. 
b. qPCR analysis of dissected regions (as shown in Extended Data Fig. 1b) of E14.5 cKO foregut showed significant
upregulation of Pdx1 in the fundus and forestomach domains. Conversely,
expression of Irx2, Irx3, and Irx5 was markedly reduced in these proximal
regions. *P < 0.05; two-tailed Student's t-test, n = 3 biological replicates
per dissected region for each genotype. 
c. Stereomicrographs of E18.5
dissected viscera demonstrated that cKO embryos exhibited lung agenesis
as previously reported. The GI tract, particularly the stomach, was greatly
reduced in size. 
d. Immunofluorescent staining at E18.5 revealed mosaic
deletion pattern of Ctnnb1. Boxed regions are shown in Fig. 1e. e. In the
E18.5 cKO stomach, recombined glands lacking Ctnnb1 staining did
not contain parietal cells whereas robust parietal cell differentiation was
observed in Ctnnb1-positive glands. Scale bars, 200 μm (a), 500 μm (d)
and 50 μm (e). Error bars represent s.d.
Extended Data Figure 3 | Stable induction of fundic fate in hGOs and efficiency of protocol. a, We investigated how long CHIR treatment was necessary to establish fundus identity. Brief CHIR treatment (days 6–9) and subsequent growth of organoids in control growth medium until day 34 resulted in fundic organoids expressing the antral marker PDX1, suggesting that short CHIR treatment did not produce a stable fundic fate. We then tested whether longer exposures to CHIR were required to retain fundic fate and found that only continuous treatment through at least day 29 could maintain low expression of the antral marker PDX1. *P < 0.05 compared to control antral hGOs; two-tailed Student’s t-test; n = 3 biological replicates, data representative of two independent experiments. b, c, Over the course of the protocol, PDX1 remained low in CHIR-treated organoids, whereas IRX5 expression was persistently elevated. *P < 0.05; two-tailed Student’s t-test; n = 3 biological replicates per time point. d, Conversion of day 6 posterior foregut spheroids to early stage gastric organoids (day 20) is greater than 80% efficient in both the hAGO and hFGO protocols. e, At d20, hFGO epithelium is ~90% GATA4^+ SOX2^+ PDX1^- whereas hAGO epithelium is ~90% GATA4^+ SOX2^- PDX1^+. **P < 0.001, two-tailed Student’s t-test, n = 4 biological replicates per experiment, two individual experiments shown. Scale bars, 100 μm (c) and 200 μm (d).
Extended Data Figure 4 | BMP-dependence of induction of intestinal fate from foregut progenitors by Wnt/β-catenin activation. a, The intestine-specific transcription factor CDX2 was not significantly induced in CHIR-treated hGOs at either day 9 or day 20. b, Neither fundic nor antral hGOs expressed genes associated with intestinal cell types, including MUC2, CCK, and SCT, when compared to human intestinal organoids (hIOs). *P < 0.05 compared to hIO; two-tailed Student’s t-test. n = 3 biological replicates. c, Anterior–posterior fate is coordinately controlled by WNT and BMP activity. In the presence of the BMP inhibitor Noggin, all organoids maintained foregut (SOX2⁺) regardless of Wnt/β-catenin pathway activity; however in the presence of BMP4, all organoids were posteriorized (CDX2⁺). Activation of Wnt (CHIR) in a BMP inhibited state resulted in fundus pattern (SOX2⁺, PDX1⁻, CDX2⁻) whereas activation of WNT (CHIR) and addition of BMP4 resulted in an intestinal fate (CDX2⁺). *P < 0.05 compared to analogous Noggin-treated condition; two-tailed Student’s t-test. n = 3 biological replicates. d, Immunofluorescent staining of human tissues revealed that CLDN18 was a gastric-specific epithelial marker that is not found in the intestine. Scale bar, 200 μm. Error bars represent s.e.m.
Extended Data Figure 5 | hFGOs contain organized glands supported by associated mesenchymal layer. a, Transmission electron micrographs show that hFGO glands exhibited organized architecture with narrow apical membranes. b, Both hFGOs and hAGOs contained a supporting layer of FOXF1⁺ VIM⁺ undifferentiated fibroblasts. Scale bars, 5 μm (a) and 100 μm (b).
Extended Data Figure 6  |  Region-specific cytodifferentiation in human gastric organoids. 

**a.** Antral and fundic hGOs exhibited comparable expression of the mucous cell markers MUC5AC and MUC6. 

**b.** As shown in a transmission electron micrograph, hFGOs contained abundant cells exhibiting granule pattern consistent with mucous neck cells, the precursors to differentiated chief cells.

**c.** Exogenous expression of NEUROG3 in hGOs derived from a NEUROG3-deficient hESC line induced robust differentiation of SYP-positive endocrine cells. While both hAGOs and hFGOs formed GHRL⁺ and SST⁺ endocrine cells, specification of GAST⁺ G-cells was observed only in hAGOs.

**d.** Expression comparison of cell lineage markers in hGOs and human gastric biopsy tissue. qPCR analyses demonstrated that hGOs exhibited comparable expression levels of several lineage markers (MUC5AC, ATP4B), while other markers were expressed at much lower levels (ATP4A, PGA5, and PGC) than found in the fully differentiated, mature human stomach. Scale bars, 5 μm (b) and 100 μm (c). Error bars represent s.d. (a) and s.e.m. (b).
Extended Data Figure 7 | Analysis of murine chief cell development.

a. Unlike parietal cells, which expressed functional markers (Atp4b) as early as late embryonic stages, chief cell gene products were not detectable until much later stages of development. In the embryonic (E18.5) and juvenile (P12) stomach, Gif and Pgc were not yet expressed, indicating that chief cells mature much later in development than other lineages in the gastric epithelium. b. Despite the absence of Pgc, the P12 mouse stomach did contain abundant glandular cells expressing nuclear Mist1, a chief cell-specific marker. Thus, chief cells were specified earlier but took several weeks to develop robust expression of terminal differentiation markers. Scale bars, 100 μm (a) and 200 μm (b).
Extended Data Figure 8 | Screen for pathways that promote differentiation of parietal cells in fundic hGOs. a, To test for growth factors or small molecules capable of inducing parietal cell differentiation, hFGOs were exposed for two days (30–32) to the indicated agonist or antagonist and then analysed at day 34. In a screening experiment of different pathways, only MEK inhibition with PD03 was found to robustly induce expression of \(ATP4A \) and \(ATP4B\). b, Reduction or removal of EGF from the culture medium was not sufficient to reproduce the effect of MEK inhibition. c, The ability of PD03/BMP4 to induce parietal cell development was exclusive to fundic hGOs, as antral hGOs did not express fundic markers in response to PD03/BMP4. d, Exposure to PD03/BMP4 rapidly increased expression of \(ATP4A\) and \(ATP4B\) in fundic hGOs. e, Induction of parietal cell generation with PD03/BMP4 did not significantly alter the differentiation of chief cells (PGA5 and PGC) and endocrine cells (CHGA). f, The manipulations at each stage of the hFGO differentiation protocol were required for robust parietal cell differentiation, as removal of any single step led to loss of \(ATP4A/B\) expression. Error bars represent s.d. (a–c) and s.e.m. (d–f).
Extended Data Figure 9 | Live in vitro pH monitoring in gastric organoids. a, The dye SNAFR5F exhibits responsiveness over pH range of 5–8, which makes it well suited to detect physiological changes in response to parietal cell-mediated acid secretion. b, Media and luminal pH measurements recorded before (closed circles) and 60 min after addition of histamine (open circles). Antral hGOs did not respond, whereas the fundic hGO luminal pH decreased in response to histamine. The acidification was inhibited by pre-treatment of organoids with famotidine or omeprazole. Furthermore, omeprazole was sufficient to raise the pH in fundic organoids before histamine exposure, suggesting baseline acid secretion in the fundic organoids. Media pH did not change in any organoids. ***P < 0.001 compared to before histamine; ###P < 0.001 compared to luminal pH without histamine; $$$P < 0.001 compared to luminal pH with histamine; 2-tailed Student’s t-test. c, hFGOs contained parietal cell-dense glands in which acridine orange (AO) accumulated in nearly all of the cells lining the lumen of the gland. AO accumulation was observed in a canalicular-type pattern in parietal cells in hFGOs. Scale bars, 10 μm. Error bars represent s.d.
Extended Data Figure 10 | Serial passaging of human gastric organoids.

a, Schematic representation of experiments to determine the presence of gastric stem cells in hGOs. b, When fragments were grown in culture medium containing only EGF, they did not grow or expand to form new organoids. However, addition of CHIR and FGF10 to the culture medium was sufficient to support the growth of individual fragments into newly formed organoids. c, Following two passages, hFGOs still expressed genes consistent with a gastric phenotype, including *PGC*, *MUC6*, *MUC5AC*, and *GHRL*. This ability to undergo serial passaging with maintenance of gastric identity supports the conclusion that hFGOs contain cells with properties analogous to those of adult gastric stem cells. d, Although passaged hFGOs expressed markers associated with several differentiated gastric cell types, they did not express genes associated with parietal cells, such as *ATP4B*. Furthermore, differentiation of parietal cells could not be induced through MEK inhibition as they could before passaging. Error bars represent s.d.
Erratum: Wnt/β–catenin promotes gastric fundus specification in mice and humans

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In Fig. 1 of this Article, owing to an error during the production process, the bottom image was incorrectly labelled ‘ShhCre+/β–cateninfl/fl’ instead of ‘ShhCre+/β–cateninfl/fl’; this has been corrected online.