

# Identification of alanyl aminopeptidase (CD13) as a surface marker for isolation of mature gastric zymogenic chief cells

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<sup>1</sup>Division of Gastroenterology, Departments of Medicine, Pathology, and Immunology, and Developmental Biology, Washington University, St. Louis, Missouri; <sup>2</sup>Division of Gastroenterology, Hepatology, and Nutrition, Vanderbilt University School of Medicine, Nashville, Tennessee

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**Moore BD, Jin RU, Osaki L, Romero-Gallo J, Noto J, Peek RM Jr, Mills JC.** Identification of alanyl aminopeptidase (CD13) as a surface marker for isolation of mature gastric zymogenic chief cells. *Am J Physiol Gastrointest Liver Physiol* 309: G955–G964, 2015. First published October 29, 2015; doi:10.1152/ajpgi.00261.2015.—Injury and inflammation in the gastric epithelium can cause disruption of the pathways that guide the differentiation of cell lineages, which in turn can cause persistent alterations in differentiation patterns, known as metaplasia. Metaplasia that occurs in the stomach is associated with increased risk for cancer. Methods for isolating distinct gastric epithelial cell populations would facilitate dissection of the molecular and cellular pathways that guide normal and metaplastic differentiation. Here, we identify alanyl aminopeptidase (CD13) as a specific surface marker of zymogenic chief cells (ZCs) in the gastric epithelium. We show that 1) among gastric epithelial cells alanyl aminopeptidase expression is confined to mature ZCs, and 2) its expression is lost en route to metaplasia in both mouse and human stomachs. With this new marker coupled with new techniques that we introduce for dissociating gastric epithelial cells and overcoming their constitutive autofluorescence, we are able to reliably isolate enriched populations of ZCs for both molecular analysis and for the establishment of ZC-derived ex vivo gastroid cultures.

aminopeptidase N/CD13; zymogenic chief cell; gastric epithelium; splasmolytic polypeptide expressing metaplasia; gastroid; alanyl aminopeptidase

THE DIFFERENTIATION AND MAINTENANCE of each lineage in the epithelium of the body of the mammalian stomach are orchestrated by signaling networks and molecular pathways (1, 4, 27). The three most abundant differentiated cell lineages are the mucus-secreting pit cells near the luminal surface, the acid-secreting parietal cells located mostly in the middle (neck) portion of each gastric unit, and the digestive enzyme-secreting zymogenic chief cells (ZCs) in the base of the unit. Each cell in the gastric epithelium is thought to derive from the same, undifferentiated population of stem cells and is replenished throughout adulthood (20, 30). The ZC has an unusual differentiation pattern, deriving from the stem cell by an intermediary form, the mucous neck cell, which migrates among parietal cells for ~2 wk (in mice) toward the base before terminal differentiation into ZCs (4, 19, 41).

Disruption of the molecular pathways regulating the differentiation of those cell lineages through damage or disease can cause a chronic, aberrant differentiation state known as metaplasia. In mice and humans, one of the most common such

aberrations is associated with increased risk for progression to gastric cancer and is termed, splasmolytic peptide-expressing metaplasia [SPEM; named because the progenitor marker splasmolytic peptide, aka trefoil factor 2 (TFF2), becomes reexpressed in ZCs, as they reprogram into a metaplastic lineage] (36). SPEM in humans is associated with further potential aberrations in epithelial differentiation patterns, like intestinal metaplasia, as well as progression to gastric cancer (13).

Metaplasia in the stomach, especially SPEM, is hypothesized to derive largely via the cellular reprogramming of ZCs from a postmitotic, terminally differentiated cell back into a proliferative, regenerative state (12, 29, 36). In certain injury/inflammatory states (in particular, in response to infection with the bacterium *Helicobacter pylori*, HP), ZCs can reprogram, meaning they reexpress markers of their precursor neck-cell phase and also reenter the cell cycle to become proliferative (1, 35). The molecular pathways underlying ZC reprogramming may be similar to those that govern reprogramming of other cells in other tissues during injury/repair (e.g., pancreatic acinar cells in acinar-to-ductal metaplasia) and are currently an area of intensive research (29). Isolating pure populations of ZCs to analyze the pathways that lead to reprogramming would help us understand the molecular underpinnings of this newly recognized, fundamental cellular process. Here, we identify a surface marker of ZCs that allows the isolation of an enriched population from the normal gastric epithelium, alanyl aminopeptidase (ANPEP).

ANPEP is a membrane-associated protein involved in the metabolism of peptides by diverse cell types. ANPEP-null mice have significantly impaired angiogenesis in pathological conditions (42), and ANPEP is required for endothelial cell adhesion/invasion (33, 39). ANPEP is thought to function in the final digestion of peptides in the digestive tract although its specific function in the gastric epithelium is unknown (24). Intestinal absorption of cholesterol is impaired when ANPEP is pharmacologically inhibited (23). In disease states, ANPEP expression in colonic tumors is associated with a poor prognosis for node-positive patients with colon cancer, and aberrant expression of ANPEP is a marker for multiple leukemias/lymphomas (9, 45). We show here that ANPEP is expressed exclusively in mature ZCs in the gastric epithelium, exploit this to both isolate an enriched population of ZCs for analysis and ex vivo cell culture, and document changes in the gastric epithelium in normal and metaplastic states.

## MATERIALS AND METHODS

**Bioinformatic analysis.** Affymetrix Mouse Gene 1.0ST microarrays were used to analyze gene expression in each cell lineage from

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RNA captured with laser-capture microdissection in previously performed experiments (5, 17, 41). Affymetrix Mouse Genome 430 2.0 microarrays were used to analyze gene expression in stomachs from mice treated with tamoxifen or vehicle from three pooled mice as described below (data accessible through GEO; accession no. GSE71580). Chip quality control and gene-specific ANOVA analysis were performed using Partek Flow software, version 3.0 (Partek). Zymogenic cell lineage-specific expression was determined by identifying genes that were significantly increased in ZCs (fold change >1.5) compared with other gastric epithelial cell lineages. To enrich for potential surface markers, ZC-specific genes were filtered with gene ontology terms “membrane,” “integral component of membrane,” and “external side of plasma membrane.”

**Animals.** Experiments involving animals were conducted according to protocols approved by the Washington University School of Medicine Animal Studies Committee. Mice were maintained in a specific pathogen-free barrier facility. Stomachs from germline *Mist<sup>-/-</sup>* mice and wild-type (WT) C57BL/6 mice of both sexes (Jackson Laboratory), were used at 6 wk age. Metaplasia was induced with daily intraperitoneal injection of tamoxifen (5 mg/20 g body wt) dissolved in a vehicle of 10% ethanol and 90% sunflower seed oil (Sigma) as described previously (17).

**Patient samples.** Examination of human gastric pathological tissue specimens was approved by the Institutional Review Board of Washington University School of Medicine, the Comité de Bioética of Nicaragua for Universidad Nacional Autónoma De Nicaragua-Facultad De Ciencias Médicas Managua, and the Research Ethics Board Manager for Health Sciences at the University of Toronto. Serial sections (4–6  $\mu$ m thick) obtained from paraffin-embedded tissue samples (hematoxylin and eosin and Alcian blue-periodic acid-Schiff stains) were reviewed by a pathologist with expertise in gastrointestinal diseases.

**Immunofluorescence.** Stomachs were prepared and stained as described previously (41). Stomachs were inflated with 10% formalin fixative and suspended in fixative for 4 h at room temperature. Tissue was rinsed with 70% EtOH multiple times, arranged in 2% agar in tissue cassettes, and paraffin processed. Sections (5  $\mu$ m) were deparaffinized and rehydrated, and antigen retrieval was performed by boiling in Trisyl Buffer (Cell Marque). Slides were blocked in 1% BSA, 0.3% Triton X-100 in PBS, then incubated in primary followed by secondary antibodies and with fluorescently labeled lectin *Griffonia simplicifolia*-II (neck cell-specific GSII; 1:1,000; Invitrogen). Finally, slides were incubated for 5 min in 1  $\mu$ g/ml bisbenzimidazole (Invitrogen) before being mounted in 1:1 PBS-glycerol. Primary antibodies used for immunostaining were mouse anti-ANPEP (1:200, Sigma), goat anti-human gastric intrinsic factor (GIF) (1:2,000; gift of Dr. David Alpers, Washington University), and sheep anti-pepsinogen (PGC) (1:10,000; Abcam). Secondary antibodies were Alexa Fluor (488, 594)-conjugated anti-mouse or anti-goat (1:500, Invitrogen).

**Single cell isolation.** Epithelial cells were isolated by methods modified from previously described work (55). Stomachs were removed and washed multiple times in PBS. Forestomach and antrum were excised, and corpus was sliced into  $\sim 1$ -mm<sup>2</sup> fragments. In some experiments, tissue was then placed in 50  $\mu$ m Medicon (Beckman, a chamber designed for efficient cutting of tissue) and mechanically dissociated with two 30-s pulses in a Medimachine (Beckman). Tissue was removed from the Medicon and incubated in 10 ml HBSS with 5 mM EDTA and 1 mM DTT for 1 h at 37° with vigorous shaking. Tissue was subsequently passed through a 50- $\mu$ m mesh filter (Partek). Sections of tissue too large to pass through the filter were placed in 10 ml RPMI 1640 with 5% BSA (Sigma) and 1.5 mg/ml Dispase II (Stem Cell Technologies) for 1.5 h at 37° with vigorous shaking and then passed through 50- $\mu$ m mesh again. The dissociated cells that passed through the 50- $\mu$ m mesh at either stage were then pooled, washed twice with cold PBS, filtered through a 50- $\mu$ m mesh filter once more, and stained for flow cytometry analysis/sorting.

**Flow cytometry cell sorting and analysis.** Single cells from the epithelial isolation were counted and suspended in PBS with 1% BSA and 5 mM EDTA at  $1 \times 10^6$  cells/ml. Cells were stained with epithelial cell adhesion molecule (EpCAM)-Alexa 647 (1:100, Cell Signaling) and ANPEP-FITC (1:200, BD Pharmingen) (20 min, 4°C). EpCAM<sup>+</sup> single cells from the ANPEP<sup>+</sup> and ANPEP<sup>-</sup> fractions were sorted using a MoFlo fluorescence-activated cell sorting (FACS) machine (Dako/Cytomation) or analyzed by FACScan (Becton Dickinson). Flow cytometry data were analyzed with FlowJo 7.6 software.

**Immunofluorescent characterization of sorted cells.** EpCAM<sup>+</sup> single cells from the ANPEP<sup>+</sup> and ANPEP<sup>-</sup> fractions were plated on slides for immunofluorescent staining as follows:  $1 \times 10^5$  cells were pipetted into a plastic chamber and centrifuged onto a slide using a Cytospin Slide Centrifuge (Cytospin) for 3 min at 800 revolution/min as previously described (40). Cells deposited on the slide were fixed with methanol (−20°C, 10 min), rinsed in PBS, and stained as described above. The number of GIF-positive cells was then quantified by an observer blinded to experimental condition in the ANPEP<sup>+</sup> and ANPEP<sup>-</sup> fractions of cells in three separate experiments.

**qRT-PCR and Western blot.** RNA was isolated using RNeasy (Qiagen) per the manufacturer's protocol. RNA was treated with DNase I (Invitrogen) and then reverse transcribed using the SuperScript III (Invitrogen) standard protocol (most cDNA syntheses started with 1  $\mu$ g of total RNA). Measurements of cDNA levels were performed by qRT-PCR using a Stratagene MX3000P detection system. Absolute QPCR SYBR green mix (Thermo Scientific) fluorescence was used to quantify relative amplicon amounts of *Gif*, *Atp4a*, and *18S*.

Cells for Western blot analysis were lysed in RIPA buffer. Proteins were quantified by DC protein assay (Bio-Rad) and then separated on NuPAGE Bis-Tris gels (Invitrogen), transferred onto Amersham Hybond ECL nitrocellulose (GE Healthcare) membranes, and detected by Immobilon chemiluminescence (Millipore). Primary antibodies used were rabbit anti-ANPEP (Sigma) and rabbit anti- $\alpha$ -tubulin and anti- $\beta$ -tubulin (Cell Signaling). Secondary antibodies were horseradish-peroxidase-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology).

**Gastroid culture.** Gastroid cultures were initiated and maintained as previously described with minor adaptations (3, 50). EPCAM<sup>+</sup>/ANPEP<sup>+</sup> single cells were isolated by flow cytometry as described above and then centrifuged at 200 g (4°C, 10 min), resuspended in Matrigel (BD Biosciences), and carefully distributed in the center of the wells. After polymerization of the Matrigel, cells were overlaid with Advanced DMEM/F12 medium (Invitrogen), 50% Wnt3a conditioned medium, 10% R-Spondin-1 and Noggin conditioned medium supplemented with penicillin/streptomycin, 10 mM HEPES,  $1 \times B27$ ,  $1 \times N-2$ ,  $1 \times$  Glutamax (Invitrogen), 2.5 mM *N*-acetylcysteine (Sigma-Aldrich), and the growth factors 50 ng/ml epidermal growth factor (Peprotech), 100 ng/ml fibroblast growth factor-10 (Peprotech), and 10 nM gastrin (Sigma-Aldrich). ROCK inhibitor (10  $\mu$ M; Y-27632, Sigma-Aldrich) was used for the first 3 days of culture. Medium was replaced every 3 days.

**Graphing and statistics.** Experiments were performed at least three times independently. Values represent means  $\pm$  SD or SE as indicated. All statistics and graphs were determined using GraphPad Prism and visualized with Adobe Illustrator. Statistical analysis was by one- or two-tailed Student's *t*-test, depending on the hypothesis before commencing the experiment.

## RESULTS

**ANPEP is a membrane-associated protein expressed in fully differentiated ZCs.** Given that ZCs have previously been purified by differential centrifugation and not by specific markers in published reports, and given our aim to develop flow cytometric techniques to isolate them, we sought to identify a surface marker exclusively expressed in ZCs. We identified

potential ZC-specific markers with a screen using previously obtained microarray data coupled with gene ontology analysis (5). We compared RNA isolated from pit, parietal, neck, and ZCs previously obtained using laser-capture microdissection and measured gene expression changes among these cell populations (Fig. 1A). We first looked for targets enriched in ZCs vs. other cell types. We then sorted for targets associated with the plasma membrane, determined by gene ontology terms (Fig. 1B). Using this method, we found a short list of targets potentially expressed on the surface of the ZCs that would allow isolation via flow cytometry.

The ZC in a normal stomach is a terminally differentiated, professional secretory cell residing in the base of the gastric unit. The ZC may act as a bellwether of damage or disease with the capacity to undergo SPEM (i.e., to reprogram to reexpress progenitor markers and proliferate to repair damage to the gastric unit). To identify potential surface markers to enable the isolation of mature, fully differentiated ZCs, we looked for those surface markers that were lost during SPEM induced by gastrototoxic doses of intraperitoneal tamoxifen. High-dose tamoxifen causes rapid parietal cell death, spurring ZC reprogramming to SPEM (17, 46). We performed Affymetrix GeneChip microarrays of the body of the stomach 12 h after injection of tamoxifen (5 mg/20 g mouse weight) or vehicle control. As expected, markers of terminally differentiated parietal cells were rapidly lost upon treatment with tamoxifen, consistent with parietal cells beginning to die, e.g., ATPase,  $H^+/K^+$  exchanging,  $\alpha$ -polypeptide (*Atp4a*). A feature of ZCs undergoing SPEM is that, although they begin to reexpress progenitor markers, they maintain expression of some ZC genes, while turning off expression of others. One gene whose expression is known to be rapidly extinguished is basic helix-

loop-helix family member a15 (*Mist1*). (26) We used this GeneChip screen to determine which of the potential ZC surface markers followed the *Mist1* pattern, indicating loss of expression in concert with ZC reprogramming. The only ZC-specific surface marker (predicted from the screen in Fig. 1B) that followed that pattern was *Anpep* (aka CD13, Fig. 1C), whereas the other markers were not changed significantly or actually increased during SPEM. Western blot of the mouse corpus gastric epithelium showed that ANPEP protein lingered at the 12-h time point after tamoxifen, but the loss of *Anpep* mRNA did eventually correlate with decreased protein also by 3 days of daily injections (Fig. 1D). Thus ANPEP is a surface marker, specific to ZCs in the normal stomach and lost upon ZC reprogramming in metaplasia.

*ANPEP is expressed exclusively in mature mouse and human ZCs.* To confirm that ANPEP expression is specific to ZCs, we used immunofluorescent staining in mouse and human stomachs. With confocal microscopy, we observe that ANPEP was expressed in ZCs but not in ZC-precursor cells (Fig. 2, A–C). As neck cells move toward the base of the gastric epithelium, they transition into ZCs, rapidly losing expression of neck cell markers like TFF2 and GSII, and begin to express ZC markers, e.g., PGC and, in mice, GIF (19, 41). ANPEP expression distinctly marks cells expressing mature ZC markers and does not overlap with cells expressing ZC-precursor neck cell markers (Fig. 2C). Thus ANPEP specifically identifies mature ZCs in a pattern that indicates expression on both intracellular and plasma membranes, supporting the possibility that it might be used as a surface marker to isolate pure ZCs from the gastric epithelium.

MIST1 is a transcription factor responsible for scaling up the ZC secretory apparatus during terminal differentiation of these

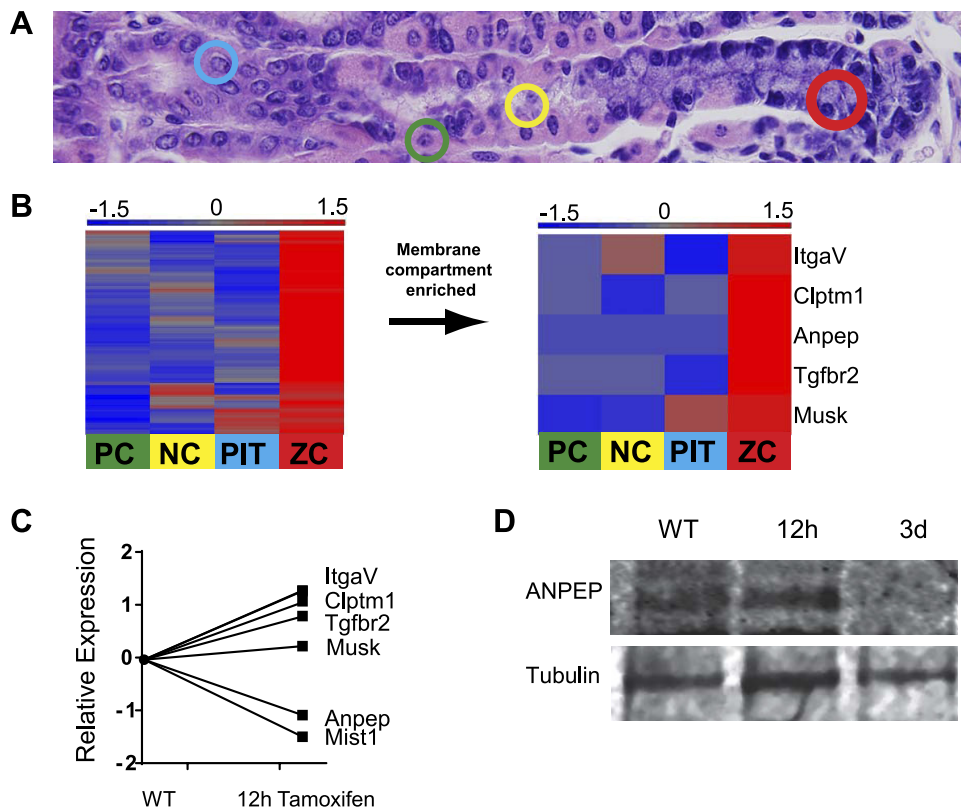


Fig. 1. Alanyl aminopeptidase (ANPEP) is expressed in mature gastric zymogenic cells (ZCs). **A**: hematoxylin and eosin-stained gastric unit. Each circle represents a distinct cell lineage isolated for microarray analysis. **B**: heat map representing enrichment of surface markers of ZCs compared with other cell populations were sorted by gene ontology terms for plasma membrane markers. 5 potential candidates were identified. **C**: change in candidate gene expression in response to tamoxifen-induced metaplasia, measured by microarray of RNA isolated from corpus portion of mouse stomach. MIST1 is known to be lost in metaplasia. ANPEP was the only candidate from B with decreased expression. **D**: Western blot of mouse stomach. ANPEP expression is lost when metaplasia is induced with tamoxifen although loss of protein trails loss of mRNA expression in C. PC, parietal cells; NCs, neck cells; WT, wild-type.



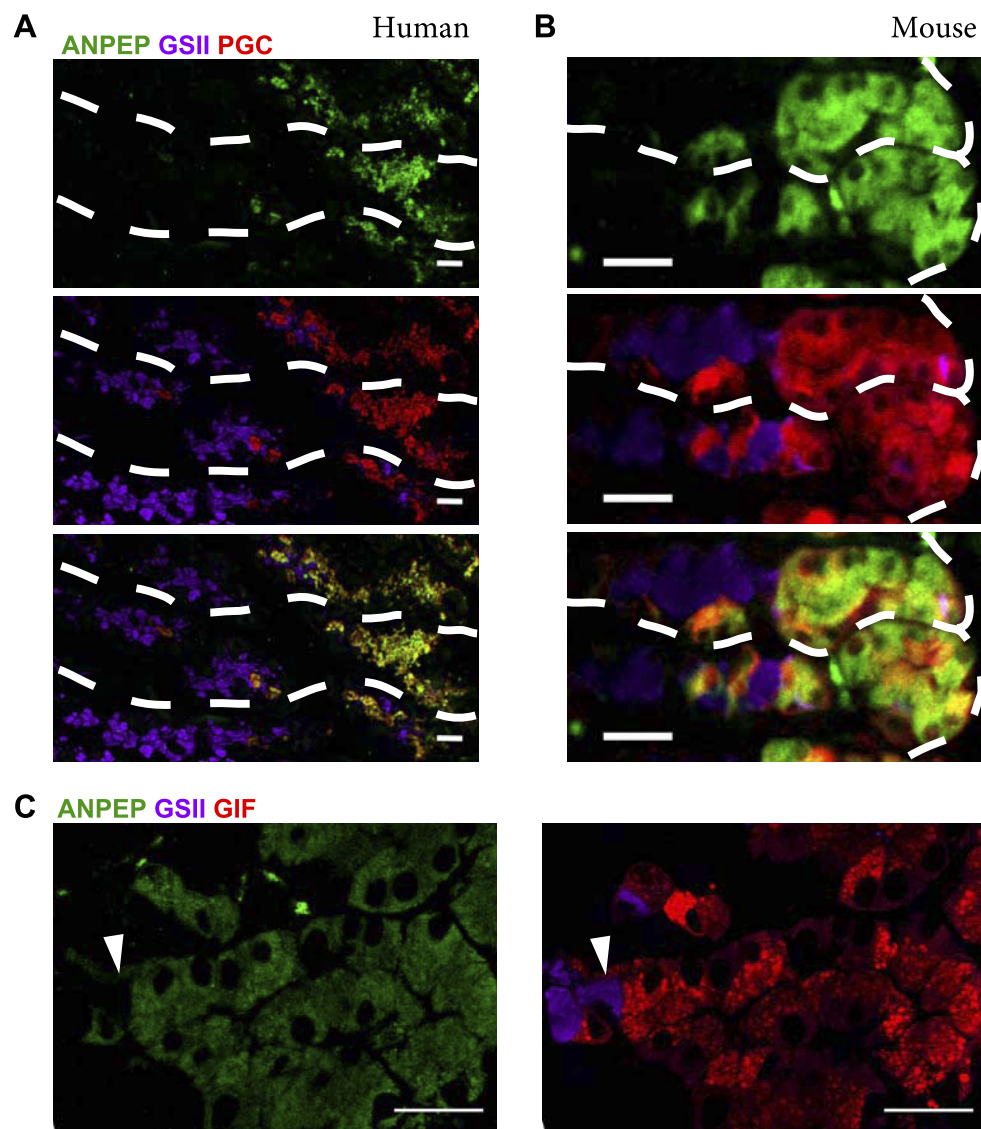


Fig. 2. ANPEP is expressed exclusively in ZCs. *A* and *B*: human and mouse gastric epithelium stained with ANPEP (green), neck cell-specific *Griffonia simplicifolia*-II (GSII) (purple), and ZC-specific pepsinogen (PGC) (red). In every mature ZC, PGC and ANPEP are expressed but not GSII. *C*: confocal image of ZC region of mouse gastric epithelium stained as described above. ANPEP expression is absent in cells that express GSII. Arrowhead marks a cell in transition between neck and ZC zones. ANPEP is not expressed where GSII epitope is maintained and marks only cells that express the ZC marker gastric intrinsic factor (GIF, red). All scale bars represent 20 μm.

cells (32). Like ANPEP, in the stomach, MIST1 is expressed exclusively in mature ZCs, and its expression is rapidly lost as ZCs scale down their secretory apparatus en route to SPEM (26, 35). To determine whether *Anpep* expression might be dependent on MIST1, we analyzed *Anpep* expression in RNA isolated from ZCs and their mucous neck cell precursors, laser-capture microdissected from WT and *Mist1*<sup>-/-</sup> stomach bodies (5). As expected, *Anpep* expression increased ~10-fold in mature ZCs compared with mucous neck cell precursors; however, this increase occurred in both WT and *Mist1*<sup>-/-</sup> mice. Thus *Anpep* expression in ZCs is clearly not dependent on MIST1. Additionally, ANPEP was clearly expressed at substantial levels in *Mist1*<sup>-/-</sup> ZCs and not mucous neck cells, just as occurs in WT (data available upon request from corresponding author).

*ANPEP expression is lost in SPEM in human and mouse gastric tissue.* Recent studies have shown that expression of ANPEP is altered during tumorigenesis (43, 53). For example, loss of ANPEP is an adverse prognostic factor in prostate cancer (49). Because of our data showing loss of ANPEP in ZCs upon induction of gastric metaplasia, we sought to deter-

mine whether ANPEP expression was altered in other models of gastric disease. Infection by the bacterium HP can cause metaplasia in many people and thereby greatly increase risk for progression to gastric cancer (7). Indeed, the high-dose tamoxifen protocol discussed earlier is a rapid, reversible model for the effects of HP infection (17). In animal models, HP causes, over the course of 2–3 mo, parietal cell atrophy and ZC reprogramming into SPEM (21, 54). In other words, in mice colonized by HP, ZCs reexpress precursor neck cell markers and become proliferative. Eventually, HP infection causes hyperplasias and dysplasias (14, 16). We analyzed the base of gastric corpus units in 3 mice infected with a CagA<sup>+</sup> strain of *Helicobacter* PMSS1 8 wk after infection. As expected, infection caused parietal cell atrophy and SPEM in multiple regions throughout most of the corpus, by this time point (data not shown, but see Refs. 5, 21 for previous characterization of infections with this HP strain in these mice). Using immunofluorescent microscopy, we observed that mice colonized by HP labeled with both the neck cell marker GSII and the ZC marker GIF in gastric ZCs had reprogrammed into a SPEM differentiation state (Fig. 4A). Only cells that had not yet

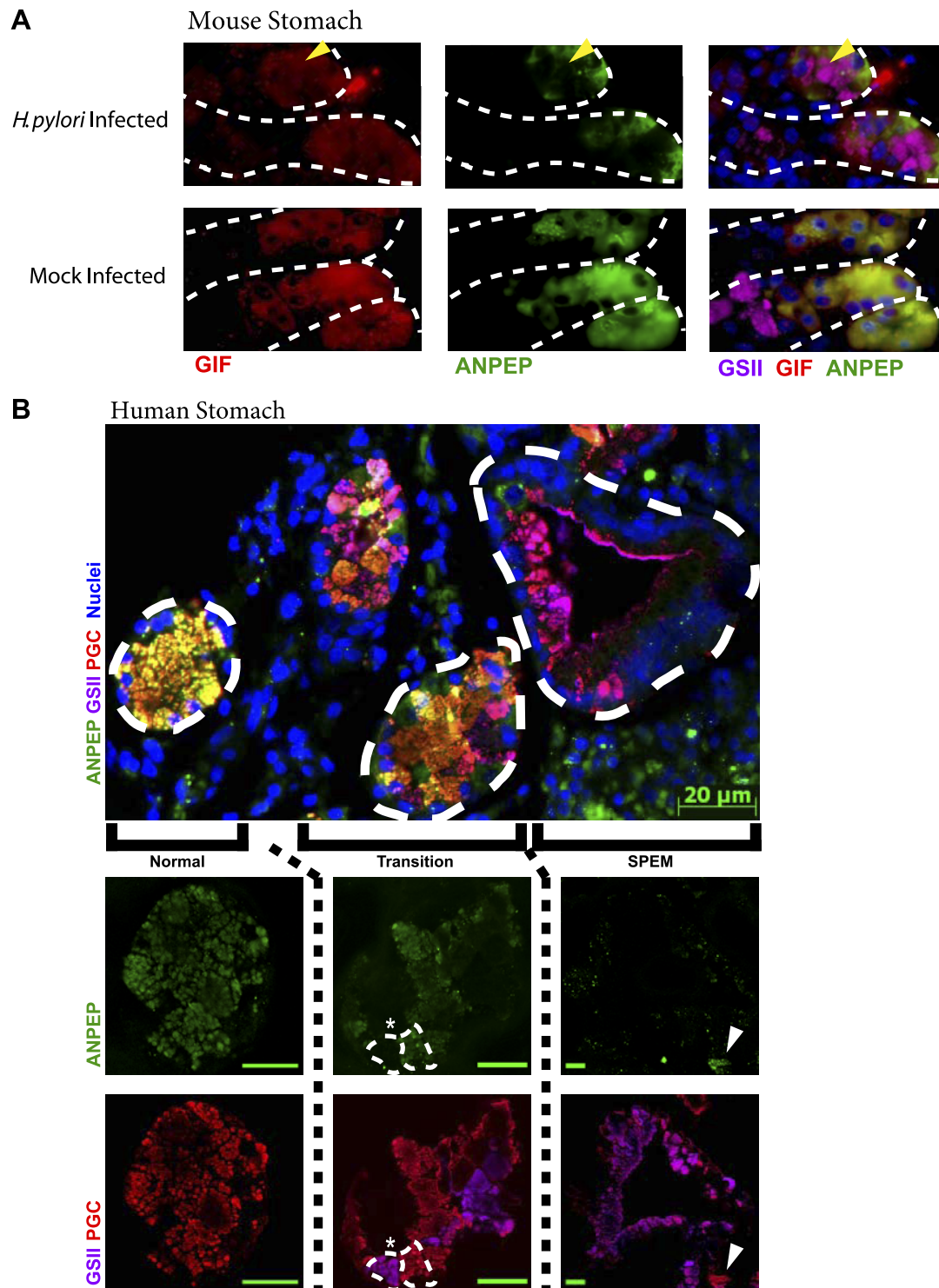


Fig. 3. ANPEP is lost during ZC metaplasia. *A*: ZCs in mouse stomachs infected with *Helicobacter pylori* (*H. pylori*) undergo splasmodic peptide-expressing metaplasia (SPEM), evidenced by overlapping PGC and GSII expression in the base of units. SPEM cells that express both GSII and PGC do not express ANPEP (yellow arrowhead). *B*: tissue from a patient infected with *H. pylori* in a region of transition between normal basal gastric gland architecture and SPEM-type metaplastic differentiation pattern. ANPEP is expressed only in mature ZCs; i.e., only in cells expressing the digestive enzyme PGC and not the SPEM/mucous neck cell marker GSII. Normal tissue, tissue transitioning to SPEM, and SPEM tissue are highlighted from left to right, respectively. Asterisk indicates a cell that expresses both neck and ZC markers (i.e., a SPEM cell), but not ANPEP, adjacent to a cell that expresses only ZC markers and has not lost ANPEP expression (i.e., a normal ZC). White arrowhead denotes the only cell in the SPEM unit that expresses only ZC markers and retains ANPEP expression.

reprogrammed at this time point (i.e., did not label with GSII) retained their ANPEP expression.

To determine whether ANPEP expression was also lost in human gastric metaplasia, we looked at ANPEP expression in tissue sections from a database of tissues infected with HP that show regions of transition between normal and SPEM-type metaplasia that we have previously described (5, 21, 26). Figure 3B shows a representative region of transition to SPEM. As in completely normal human stomach, ZCs labeled with antibodies against the ZC digestive enzyme PGC also labeled with antibodies against ANPEP (Fig. 3B). However, ZCs transitioning to SPEM, identified by labeling with both PGC and the mucous neck/precursor marker GSII, lose ANPEP expression (Fig. 3B). In glands showing complete SPEM, nearly every cell expresses both neck and zymogenic markers and has undetectable ANPEP. Even in units nearly completely transformed to SPEM, a rare PGC-positive/GSII-negative ZC (e.g., cell marked by arrowhead, Fig. 3B) will still be identifiable by its maintained expression of ANPEP.

*Disaggregation and flow cytometric analysis of gastric epithelial cells.* Because of strong cell-cell junctions and the high mucus environment of the stomach, gastric epithelial cells are challenging to dissociate into single cell populations for isolation by differential centrifugation or flow/magnet-based sorting. In the past, we have used laser-capture microdissection to isolate cells from tissue, which has been useful for isolating smaller numbers of cells to generate RNA that can be amplified for qRT-PCR or microarray analysis (e.g., Fig. 1, A and B). Laser-capture purification of cells is restricted to relatively small numbers of cells and is not the ideal technique for isolating pure populations of cell populations that are intermingled in tissues with complex organization like the gastric epithelium (4). However, to isolate larger numbers of cells for biochemistry or for culture *ex vivo* in organoids, we experimented with multiple published protocols and with previous methods for dissociation used in our laboratory (28, 31, 55). We found that, by mechanically disaggregating epithelial tissue before enzymatic digestion using a Medimachine (37), we were able to reduce the fraction of cell aggregates and doublets approximately fivefold compared with tissue cut into small pieces with a razor blade before subsequent enzymatic digestion (Fig. 4A). Mechanical disaggregation did not affect cell viability as measured by propidium iodide incorporation (Fig. 4B). We have also found that, in addition to difficulty in achieving single cell dissociation, analysis of gastric epithelial cells with flow cytometry is complicated by high levels of autofluorescence of gastric epithelial cells. Dissociated single cells from normal mouse stomach fluoresce across the detectable spectrum in response to excitation at multiple wavelengths in the absence of any exogenous label. Indeed, when light emitted from an unstained stomach is compared with that of an unstained spleen (Fig. 4C), it is evident that any positive signal from staining with antibody-conjugated fluorophores could be potentially obscured by nonspecific signal from unstained cells.

When we sorted gastric epithelial cells based on their intrinsic autofluorescence and characterized them thereafter by lineage marker expression, we observed no consistent pattern in autofluorescence intensity relative to cell lineage or viability (not shown). We established a method that reliably detects true positive cells with specific staining by first analyzing emission

of a gastric epithelial cell population in two close wavelengths on the same plot as shown (Fig. 4D). The autofluorescence in both channels is similar, causing a linear plot of approximately  $x = y$  light emission in each channel. When these cells are stained with an antibody conjugated with a fluorophore that emits at one of the wavelengths, the additional signal is detectable, so positive cells can be identified as a population distinct from the autofluorescent cells on the diagonal of the plot. By looking simultaneously at the detectors for allophycocyanin (APC) (positive channel, stained with APC-conjugated EpCAM antibody) and APC-Cy7 (negative control channel, used to adjust for autofluorescence) (Fig. 4D), we can see that a population of EpCAM-positive cells is distinguishable. This distinct population would not be readily observable by analyzing APC signal alone. Mechanical disaggregation and FACS analysis, carefully controlled for autofluorescence, allowed for accurate and repeatable analysis of individual populations of gastric epithelial cells.

*ANPEP can be used to isolate mature ZCs for FACS analysis and organoid culture.* We used the above method with ANPEP as a surface marker to isolate a purified population of ZCs (Fig. 5A). We dissociated and then stained gastric epithelial cells with an APC-Cy7-conjugated anti-ANPEP antibody. We found ~14% of EPCAM-positive epithelial stomach cells were also ANPEP positive, consistent with the fraction of gastric epithelial cells that are differentiated ZCs (28). Additionally, flow-sorted ANPEP-positive cells expressed the ZC-specific marker GIF (Fig. 5, B and C) but not the parietal cell-specific marker *Atp4a*, whereas the ANPEP-negative fraction expressed *Atp4a* but not GIF (Fig. 5C). We next induced SPEM using tamoxifen, which led, as expected, to a decreased fraction of ANPEP-positive epithelial cells, consistent with earlier findings in tissue (Fig. 5D).

We next cultured isolated ZCs in conditions that allow formation of three-dimensional organ-like gastroid structures, which we have shown previously can be formed from ZCs isolated by expression of fluorescent protein driven by ZC-specific promoter (*Mist1* or *Troy*) (50). As expected, ZCs isolated solely by EpCAM/ANPEP positivity were similarly able to form large, proliferating gastroids (Fig. 6A). On average, ~0.1% of isolated ZCs formed gastroids. The cells constituting the gastroids were strongly enriched for cells of the ZC lineage (Fig. 6, B–D).

## DISCUSSION

Elucidation of the molecular mechanisms regulating normal homeostasis in the stomach as well as aberrant differentiation patterns like SPEM has been hindered by a limited toolkit for isolating and studying individual cell populations. There are few markers of mature gastric ZCs, and to our knowledge no ZC-specific surface marker has ever been characterized. Our data show that ANPEP is expressed exclusively in mature ZCs and that its expression is lost when they reprogram to SPEM-type metaplastic cells in both mice and humans. The only other molecular marker reported to be lost as rapidly and completely during ZC to SPEM reprogramming is MIST1. However, our results indicate that ANPEP expression is not a target of MIST1 (35). Here, we have also presented what has proven to be a useful protocol for cell dissociation and flow cytometric sorting using ANPEP as a marker to purify ZCs. ANPEP-



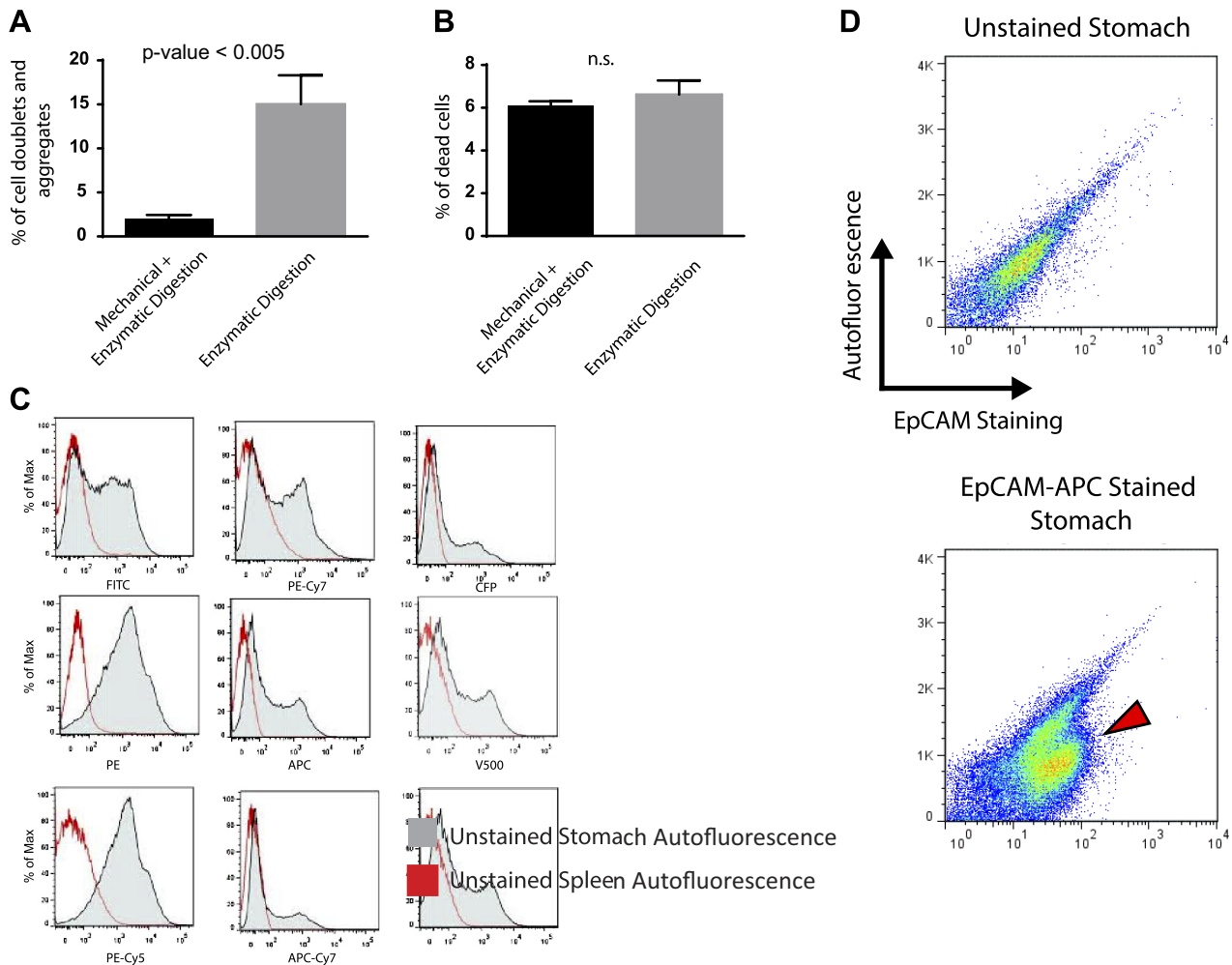


Fig. 4. Optimization of fluorescence-activated cell sorting (FACS) to sort and analyze gastric epithelium. **A**: doublet discrimination analysis of mouse gastric epithelium shows that mechanical disaggregation is necessary to achieve optimal single cell isolation (means  $\pm$  SE,  $n = 3$  biological replicates). **B**: mechanical disaggregation does not increase cell death in mouse gastric epithelial cells (means  $\pm$  SE,  $n = 3$  biological replicates). **C**: autofluorescent histograms of unstained spleen (red) and stomach (gray) throughout detectable fluorescent spectrum. High levels of autofluorescence are present throughout the spectrum in gastric epithelial cells. **D**: epithelial cell adhesion molecule (EpCAM) staining of single gastric cells. APC, allophycocyanin.

mediated isolation of ZCs can be used in “Omics” studies in the future to determine, for example, ZC-specific gene expression, chromatin modifications, and transcription factor binding sites under different conditions. Alternatively, we have shown that ZCs expressing specific promoters can serve as stem/progenitor cells *ex vivo* in organoid systems (50). A method for isolating all ZCs that is not genetically based (i.e., not based on transgenic or knockin expression of inducible Cre recombinases) would be useful in determining the progenitor properties of ZCs in a parallel, complimentary manner. We show here that we can form organoids from ZCs using ANPEP without using fluorescent promoters driven by ZC-specific promoters. It will be interesting to compare the two approaches in terms of their relative clonogenic potential and relative propensity for giving rise to other lineages because it is not clear whether the inducible stem cell activity inherent to ZCs is inherent to all ZCs or only specific subpopulations. For example, the *Troy*-expressing ZCs could be purified from the ANPEP-positive general population to examine whether, as predicted (50), they have increased stem cell potential.

ANPEP is an integral membrane protein that hydrolyzes peptides in multiple tissues and has varying functions that depend on the cells and tissues where it is expressed. In the small intestine, ANPEP hydrolyzes peptides from proteins partially digested by gastric and pancreatic proteases (24); it is also required for normal cholesterol absorption. In endothelial cells, ANPEP is required for cell motility and adhesion, and disruption prevents endothelial cell invasion in Matrigel assays (11, 33, 39). ANPEP-null mice are phenotypically normal in unstressed conditions but have exhibited impaired angiogenesis in hypoxic settings (42). We used several small-molecule inhibitors of ANPEP function (ezetimibe, bestatin, tosedostat, curcumin) (23, 44, 48, 52) but found no altered morphology in the normal gastric epithelium (data not shown). ANPEP expression correlates negatively with progression to prostate cancer and with aggressiveness of disease (49). Expression of ANPEP is also known as myeloid antigen because it is expressed in myeloid lineages of the bone marrow and has been proposed to correlate with worse prognosis in lymphoblastic leukemias (8, 9, 47). Despite a large and somewhat

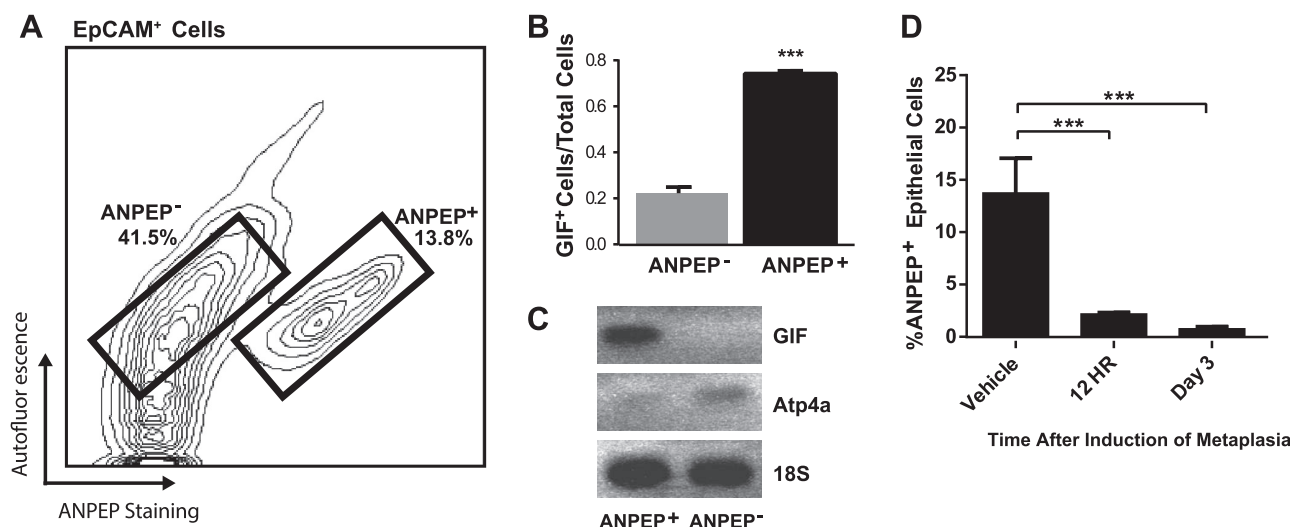


Fig. 5. ANPEP can be used to isolate ZCs. **A:** FACS plot of ANPEP-stained gastric epithelium. EpCAM-positive cells were stained with an APC-Cy7-conjugated ANPEP antibody (x-axis) and plotted against autofluorescence in an unstained channel (APC). **B:** quantification of ZC marker-positive cells in sorted fractions. ANPEP-positive mouse gastric epithelial cells were isolated using flow cytometry and attached to a slide using cytopsin. Cells were then stained for the ZC marker GIF and quantified (means  $\pm$  SE,  $n = 3$  biological replicates). **C:** semi-qPCR of ANPEP-positive and -negative mouse gastric epithelial cell fractions for ZC (*Gif*)- and parietal cell (*Atp4a*)-specific genes. **D:** FACS quantification of ANPEP expression in EpCAM-positive gastric epithelial cells treated with vehicle or high-dose tamoxifen for 12 h or 3 days to induce SPEM (means  $\pm$  SE,  $n = 3$  biological replicates; \*\*\* $P$  value of  $<0.0005$  as determined by 1-tailed Student's  $t$ -test).

controversial literature on the topic, it is not clear that there is functional significance for ANPEP expression in lymphoid leukemias; it may simply be a reflection of abnormal gene expression as a whole, in which genes normally restricted to the myeloid lineage are misexpressed in lymphoid neoplasms (2, 38, 45). ANPEP expression has been shown to be increased in gastric cancers with poor prognosis although we are unaware

of previous reports on its expression in normal fundic-type mucosal cells like ZCs (6). Given that ANPEP is a known intestinal protein and that gastric cancers frequently have intestinal differentiation (10, 15), it is possible that ANPEP expression is correlated with progression from SPEM (thought to be the earliest lesion in gastric cancer tumorigenesis, as it correlates with loss of parietal cells in the stage known as atrophic gastritis) (36) to later-stage disease like intestinal metaplasia, dysplasia, and cancer, where it returns.

The role of ANPEP in ZCs is not clear. Its rapid loss during SPEM in multiple mouse models and human tissue indicates that it may play a role in their secretory function, as one of the first events in ZC reprogramming during injury is scaling down MIST1 and the secretory apparatus (26). ANPEP expression is on the plasma membrane and also seems to be distributed around secretory granules in ZCs. It has been shown to help regulate phagocytosis (51), being required for development of secretory tissue like mammary glands (22) and for secretion of cytokines (25). An interesting function of ANPEP seems to be to specifically degrade the cytokine-like IL-8 (18, 34). Perhaps ZC ANPEP helps reduce IL-8 abundance under homeostatic conditions. More severe damage that can lead to reprogramming of ZCs and loss of ANPEP would allow increased abundance of this cytokine to the gastric epithelium.

In summary, we have refined protocols for the isolation and analysis of gastric epithelial cells, have identified the first surface marker of mature ZCs useful for flow cytometry, and have described the pattern of this protein ANPEP in normal and metaplastic gastric epithelium. The tools should allow for additional characterization of other isolated gastric epithelial cells with other markers and help us better understand the biology of the elaborate but highly plastic digestive enzyme-secreting cells of the stomach.

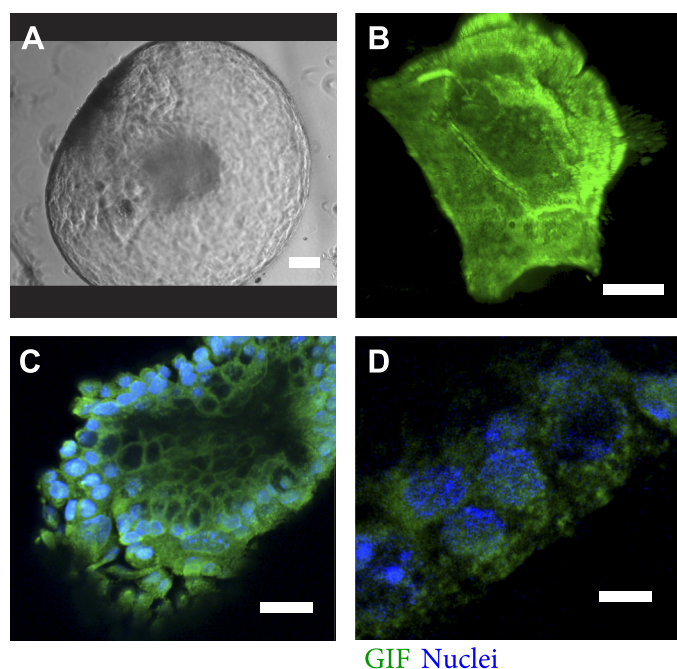


Fig. 6. ZC-derived gastroid formation. **A:** representative example of growing gastroid derived from single ZC (scale bar = 100  $\mu$ m). **B:** confocal stack of GIF staining in entire gastroid (scale bar = 100  $\mu$ m). **C** and **D:** representative image of vesicular GIF staining in cells throughout the gastroid. (scale bars = 20  $\mu$ m and 5  $\mu$ m, respectively).



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## DISCLOSURES

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

## AUTHOR CONTRIBUTIONS

Author contributions: B.D.M., R.U.J., L.O., R.M.P., and J.C.M. conception and design of research; B.D.M., L.O., J.R.-G., and J.M.N. performed experiments; B.D.M., R.U.J., and L.O. analyzed data; B.D.M., L.O., and J.C.M. interpreted results of experiments; B.D.M. and J.C.M. prepared figures; B.D.M. and J.C.M. drafted manuscript; B.D.M., R.U.J., L.O., and J.C.M. edited and revised manuscript; B.D.M., R.U.J., and J.C.M. approved final version of manuscript.

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