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Article

Lgr5^{+ve} Stem Cells Drive Self-Renewal in the Stomach and Build Long-Lived Gastric Units In Vitro

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SUMMARY

The study of gastric epithelial homeostasis and cancer has been hampered by the lack of stem cell markers and in vitro culture methods. The Wnt target gene *Lgr5* marks stem cells in the small intestine, colon, and hair follicle. Here, we investigated *Lgr5* expression in the stomach and assessed the stem cell potential of the Lgr5^{+ve} cells by using in vivo lineage tracing. In neonatal stomach, *Lgr5* was expressed at the base of prospective corpus and pyloric glands, whereas expression in the adult was predominantly restricted to the base of mature pyloric glands. Lineage tracing revealed these Lgr5^{+ve} cells to be self-renewing, multipotent stem cells responsible for the long-term renewal of the gastric epithelium. With an in vitro culture system, single Lgr5^{+ve} cells efficiently generated long-lived organoids resembling mature pyloric epithelium. The *Lgr5* stem cell marker and culture method described here will be invaluable tools for accelerating research into gastric epithelial renewal, inflammation/infection, and cancer.

INTRODUCTION

The stomach shares a number of features with the intestine, including a common endodermal origin and a constantly renewing epithelium. In both organs, cell renewal is fuelled from stem cell populations located in pockets within the epithelium. In the intestine, these pockets are termed crypts (Barker et al., 2008a). We have recently described a small population of cycling Lgr5^{+ve} stem cells at the base of these intestinal crypts (Barker et al., 2007). In the stomach, the epithelium is organized into multiple gastric units that are comprised of flask-shaped tubular glands, several of which feed into a single pit that opens out onto the surface epithelium. The precise structure and composition of these gastric units varies in different anatomical regions of the stomach (Lee et al., 1982).

The existence of multipotent stem cells within the glandular stomach has been inferred by elegant clonal marking studies (Bjerknes and Cheng, 2002; McDonald et al., 2008), but a paucity of specific markers has hampered their definitive identification. The gastric unit stem cell zone has been assigned to a region just above the neck of the gland, the isthmus. This is largely based on the observations that cellular proliferation is most predominant at this location and that immature, granule-free cells exist within this cycling population (Hattori and Fujita, 1976; Karam and Leblond, 1993a, 1993b, 1993c, 1993d).

In adults, each gastric unit in the pyloric and corpus region is considered to be functionally monoclonal, with all cellular progeny being derived from a single stem cell (McDonald et al., 2008; Nomura et al., 1998; Tatematsu et al., 1994). This dominant stem cell is thought to maintain a small, steady-state population of clonal, multipotent stem cells in the gastric unit through infrequent symmetric division. Daughter cells generated by much more frequent asymmetric division of these multipotent stem cells subsequently exit the stem cell niche and differentiate to generate the various epithelial cell lineages as they migrate bidirectionally toward the pit or gland (Bjerknes and Cheng, 2002).

Recently, a *villin-lacZ* transgene was found to be expressed in a very rare population of quiescent, "label-retaining" cells located at or below the isthmus in the bottom third of the pyloric glands (Qiao et al., 2007). According to in vivo lineage tracing, these villin^{+ve} cells were shown to have multilineage potential after stimulation with the proinflammatory cytokine γ-interferon. Of note, this villin^{+ve} population did not contribute to epithelial renewal in the pylorus under normal homeostatic conditions and was essentially absent from the corpus. It is therefore likely that other, more active stem cell populations exist in the glandular stomach to achieve the steady-state renewal of the stomach epithelium.

We recently identified the orphan G protein-coupled receptor Lgr5 (also known as Gpr49) as a marker of active stem cells in the

small intestine, colon, and hair follicles (Barker et al., 2007; Jaks et al., 2008). In the intestine, *Lgr5* is uniquely expressed in stem cells and is switched off in their immediate daughters, the transit amplifying cells (van der Flier et al., 2009). Independent knockin mouse models demonstrate restricted expression of *Lgr5* in a limited set of other adult tissues including the stomach, indicating that it may be a more general marker of adult stem cells (Barker et al., 2007). We therefore set out to investigate whether Lgr5 could indeed be a bona fide marker for adult stem cell populations in the stomach.

RESULTS

Restricted Expression of the Wnt Target Gene *Lgr5* at the Base of the Pyloric Glands

To document endogenous in vivo expression of the Wnt target gene *Lgr5* in the stomach, we used two independent *Lgr5* knockin mouse models in which either *lacZ* or *EGFP* reporter gene expression was driven by endogenous *Lgr5* regulatory sequences. Throughout the pyloric region of adult stomachs, both the *Lgr5-LacZ* (Figures 1A and 1B) and *Lgr5-EGFP-ires-CreERT2* (Figures 1C–1E) reporters were exclusively expressed in cells occupying the very base of the glands. On average, 3.72 ± 1.02 Lgr5-EGFP^{+ve} cells were present in isolated pyloric glands (see Figure 1E for examples). This expression pattern was independently confirmed in adult mouse pyloric glands by in situ hybridization by using an *Lgr5*-specific probe (Figure 1F; red arrows).

Robust *Lgr5* expression was maintained at the base of pylorus-type glands located at the transition zone between the pylorus and corpus regions on the lesser curvature. These glands were characterized by the presence of limited numbers of predominantly basal parietal cells (Figure S1A available online). In contrast, *Lgr5* expression was never observed in the main corpus region of adult mice (Figure S1B). However, limited numbers of corpus-type gastric units bordering the squamous forestomach (Figure S1C) and the esophagus (comparable to the gastroesophageal junction in humans) (Figure S1D) did express *Lgr5* at the gland base. No *lacZ* reporter gene activity was detected in wild-type adult stomachs, confirming the specificity of the *Lgr5*-reporter genes (Figures S1E and S1F).

Lgr5-EGFP-ires-CreERT2 mice were injected with BrdU 2 hr prior to sacrifice in order to visualize actively cycling cells within the stomach. This revealed the presence of S-phase (i.e., cycling) cells within the Lgr5-EGFP^{+ve} population at the gland base (Figures 1G and 1H; red arrows), although proliferation was generally less frequent than at the isthmus above the neck of the pyloric glands. On average, 29% ± 5% of the Lgr5 population was estimated to be actively cycling, as determined by coexpression of Lgr5-EGFP and Ki67 (Figures 1I and 1J; red arrows).

We have previously shown that *Lgr5* expression in the intestine is dependent upon active Wnt signaling (van de Wetering et al., 2002; Van der Flier et al., 2007). To investigate whether Wnt signaling was indeed active at the gland base in the pylorus, we employed an *Axin2-lacZ* knockin model as an in vivo reporter of active Wnt signaling activity (Lustig et al., 2002). *Axin2* was found to be selectively expressed at the base of the pyloric glands, indicating that cells in this region are indeed responding to an active Wnt signal (Figures S1G and S1H, black arrows). It has been reported that systemic in vivo application of the potent Wnt agonist Rspondin causes a strong Wnt response in the intestine (Kim et al., 2005). We noted highly upregulated expression of *Lgr5-lacZ* at the base of the pyloric glands 2 days after intraperitoneal R-spondin1 injection, further supporting that *Lgr5* is a Wnt-responsive target gene in the stomach (Figures S1I and S1J).

Lgr5 Marks an Active Adult Stem Cell Population at the Base of the Pyloric Glands

We have successfully used in vivo lineage tracing to prove that the Lgr5^{+ve} cells in the intestine and hair follicle represent active stem cell populations responsible for maintaining tissue renewal under physiological homeostatic conditions (Barker et al., 2007, 2008b; Jaks et al., 2008). Here, we employed the same strategy to determine whether *Lgr5* is also marking adult stem cells at the base of the pyloric glands. We crossed the *Lgr5-EGFP-ires-CreERT2* mouse strain with the *R26RlacZ* reporter strain (Soriano, 1999) and injected a single limiting dose of Tamoxifen to activate the *Lgr5*-driven Cre enzyme and consequently switch on the *Rosa-lacZ* reporter gene. LacZ activity was first observed in isolated Lgr5^{+ve} cells at the very base of the pyloric glands 2 days postinduction (PI) (Figures 2A–2C). At 4 days PI, lacZ^{+ve} progeny could already be seen populating the lower half of the gastric units, reinforcing the notion that a proportion of the Lgr5^{+ve} cells are actively proliferating (Figures 2D and 2E). At 7–10 days PI, LacZ^{+ve} progeny were distributed throughout the entire epithelium of multiple gastric units (Figures 2F–2I). This highlighted the level of active renewal occurring in the pyloric region, with an epithelial turnover rate (7–10 days) only slightly lower than that of the small intestine (5 days). At 620 day PI, the entire epithelium of multiple pyloric gastric units was LacZ^{+ve}, proving that the Lgr5^{+ve} cells at the gland base were self-renewing, multipotent stem cells responsible for maintaining epithelial renewal under normal homeostatic conditions (Figures 3A, 3C, and 3D). In contrast, no tracing was observed in the adult corpus, confirming the absence of Lgr5^{+ve} stem cell in this region (Figures 3A, 3C, and 3E).

To formally demonstrate the multilineage potential of the Lgr5^{+ve} stem cells, we performed serial stains for the most common epithelial cell types present on the pyloric epithelium. Muc5AC^{+ve} mucus cells (Figure 3F) and gastrin G^{+ve} enteroendocrine cells (Figure 3G) were readily visible within the lacZ^{+ve} clones derived from the Lgr5^{+ve} cells in the true pylorus. In addition, H-K-ATPase^{+ve} parietal cells were observed toward the base of lacZ^{+ve} pyloric-type glands located at the transition zone between the pylorus and true corpus on the lesser curvature (Figure 3H).

Importantly, the number of tracing units in the pyloric region did not significantly change over the 620 day period (24% at 620 day PI versus 31% at 4 day PI), strongly indicating that all the pyloric Lgr5^{+ve} cells are long-term, self-renewing adult stem cells (Figures S2A and S2B). Corpus-type LacZ^{+ve} tracing gastric units were also observed at the border of the squamous forestomach (not shown) and the border of the esophagus, indicating that stemness is probably a more general feature of the Lgr5^{+ve} cells in the stomach (Figures S2C–S2F). In contrast, no lacZ^{+ve} tracing units were observed in the stomach of a 752-day-old, noninduced *Lgr5-EGFP-ires-CreERT2/Rosa26RlacZ* mouse, confirming the tight regulation of Lgr5-Cre activity in the stem cells (Figure 3B).

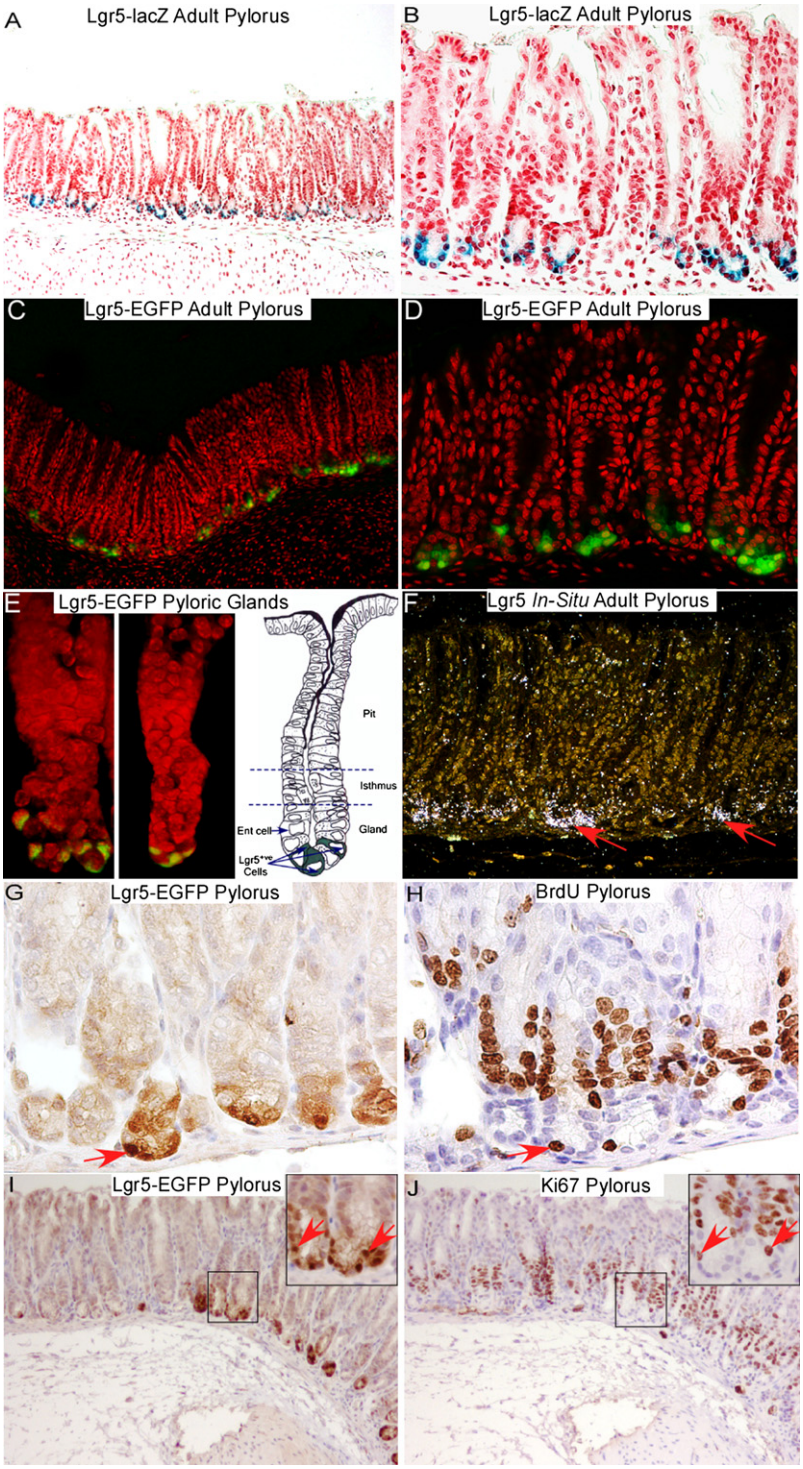

Figure 1. Restricted Expression of *Lgr5* at the Base of Adult Pyloric Glands

(A and B) Lgr5 expression is restricted to the base of the pyloric glands in adult *Lgr5-lacZ* KI mice (A, 10× magnification; B, 20× magnification).

(C and D) Confocal images showing *Lgr5-EGFP* expression at the base of the pyloric glands in adult *Lgr5-EGFP-ires-CreERT2* mice (C, 10× magnification; D, 20× magnification).

(E) Confocal images of two independent isolated Lgr5-EGFP pyloric glands (63× magnification). Right: A cartoon of a pure pyloric gastric unit depicting the location of the Lgr5^{+ve} cells (green). Ent, enteroendocrine cell.

(F) In situ hybridization for *Lgr5* confirms restricted expression at the base of the pyloric glands in adult mouse stomach (red arrows; 20× magnification).

(G and H) Serial sections of adult pyloric epithelium stained for Lgr5-EGFP (G, 40× magnification) and BrdU (H, 40× magnification) after a 2 hr pulse of BrdU. Costaining of these markers (red arrows) demonstrates the presence of cycling cells within the Lgr5^{+ve} population.

(I and J) Serial sections of adult pyloric epithelium stained for Lgr5-EGFP (I, 10× magnification, inset 20× magnification) and KI67 (J, 10× magnification, inset 20× magnification). Quantification of EGFP/KI67 costaining (red arrows) estimates on average 29% (±5% SEM, n = 3) of the Lgr5^{+ve} cells to be actively proliferating.

See also Figure S1.

Single Adult Lgr5^{+ve} Stem Cells Build Long-Lived Gastric Organoids Closely Resembling Pyloric Gastric Units In Vitro

Recently, we have established a long-term culture system that facilitates the generation and maintenance of basic intestinal crypt-villus architecture in vitro from single intestinal Lgr5^{+ve} stem cells (Sato et al., 2009). To determine whether gastric Lgr5^{+ve} cells were also capable of generating and maintaining pyloric gastric units in vitro, isolated gastric gland units from *Lgr5-EGFP-ires-CreERT2* mice (Figure 4A) were suspended in Matrigel and cultured under different conditions. Gastric culture growth conditions were similar to those of the small intestine cultures (including EGF, Noggin, and R-spondin1), except for a strict dependence on Wnt3A in the form of conditioned media. This requirement was confirmed with purified Wnt3a protein (Figure S3A). Additionally, FGF10 was essential for driving budding events and for the expansion of the cultures into multiunit organoids (Figure S3B). The newly formed gastric organoids underwent continuous budding events, while maintaining their polarity, with gastric gland-domain buds distributed around a central lumen (Figure S3C). In the absence of Wnt3A conditioned medium, the gastric organoids rapidly deteriorated (Figure S3D). Each week, organoids were mechanically dissociated and split to one-fifth of their preplating density.

Cultured pyloric units were single-layered epithelial structures, as evidenced by E-Cad staining, and resembled the intestinal crypt organoids, displaying a sealed glandular lumen filled with apoptotic cells (Figures S3E and S3F). We have successfully cultured gastric organoids for at least 9 months without any detectable loss of the properties described above.

When single Lgr5-EGFP^{hi} cells were sorted (Figure 4B), an average of 9% of the cells grew into organoids, whereas the

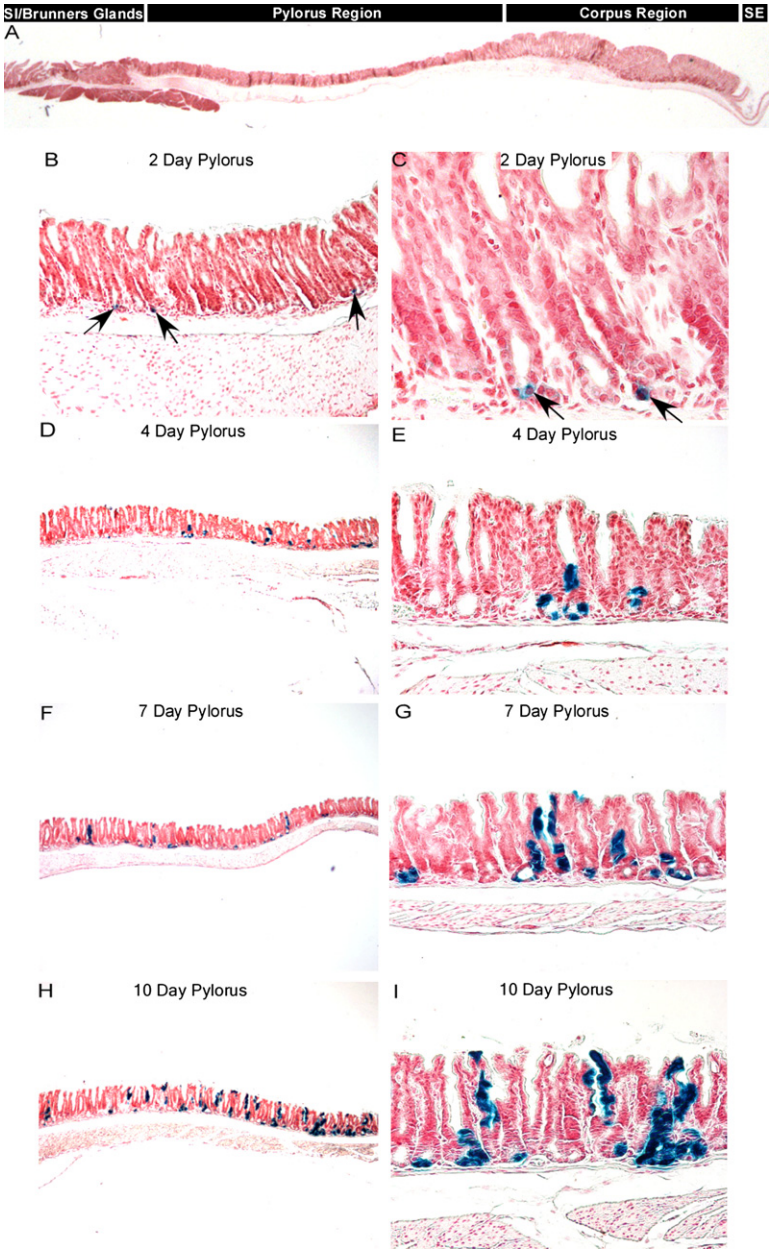

Figure 2. Lgr5^{+ve} Cells at the Pylorus Gland Base Contribute to the Rapid Self-Renewal of the Adult Pyloric Epithelium

(A) Low-power image of a section through the small intestine, Brunners glands, pylorus, corpus, and the boundary between the glandular stomach and squamous epithelium (SE) of an adult mouse stomach.

(B and C) Stochastic expression of the *ROSA-LacZ* reporter gene in Lgr5^{+ve} cells at the base of the pyloric glands (black arrows) 2 days postinduction (B, 10× magnification; C, 40× magnification).

(D and E) LacZ-positive progeny of the Lgr5^{+ve} cells rapidly populate the glands within 4 days (D, 4× magnification; E, 20× magnification).

(F–I) LacZ-positive progeny of the Lgr5^{+ve} cells are already visible throughout the gastric units in the pylorus 7 days (F, 4× magnification; G, 20× magnification) to 10 days (H, 4× magnification; I, 20× magnification) postinduction.

remaining cells died within 24 hr. The sorted Lgr5-EGFP^{hi} cells rapidly began dividing and small cyst-like structures were already visible after 5 days. During the following days, the newly formed (cyst-like) structures started to generate gland-like domains (Figure 4C). After 9–11 days in culture, gastric organoids were dissociated manually and split to generate new organoids. Gastric organoids derived from single cells have been successfully replated on a weekly basis for at least 3 months, without losing the properties described (Figure 4D). From day 7 onward, *Lgr5-EGFP* expression was restricted to the base of the gland-like domains (Figure 4E). As evidenced by EdU staining, proliferating cells were located at the base of these gland-like domains (Figure 4F), whereas apoptotic caspase 3-positive cells were found extruded into the lumen (data not shown). Importantly, only the *Lgr5*-expressing cells were capable of generating gastric organoids in vitro (Figure 4G).

To formally prove the constant self-renewal capacity of the Lgr5^{+ve} cells in vitro, lineage tracing was studied in established organoids derived from single Lgr5^{+ve} cells isolated from an *Lgr5-EGFP-ires-CreERT2/Rosa26-YFP* reporter mouse. After tamoxifen induction, the YFP^{+ve} reporter gene was rapidly activated in single Lgr5^{+ve} cells within the gland-like domains. Over the next few days, the YFP expression domain expanded considerably within the growing organoids, confirming the contribution of the Lgr5^{+ve} stem cells to organoid growth in vitro (Figure 4H).

The organoids derived from single-cell cultures were single-layered epithelial structures, as evidenced by E-cadherin staining (Figure 4J). In addition to *Lgr5*, the cultures expressed the gastric epithelial markers Gastric intrinsic Factor, Mucin 6, and Pepsinogen C. No differentiation to the pit or enteroendocrine lineages was observed under these culture conditions. Reduction of the Wnt3A concentration in the culture media resulted in the formation of comparable gastric structures harboring polarized pit cells, as evidenced by the expression of gastric mucin 5AC (MUC5AC) and Periodic acid-Schiff (PAS), Tff2-positive mucus neck cells, and scattered immature Chromogranin A-positive enteroendocrine cells (Figures 4I and 4J).

The Ultrastructure of Lgr5^{+ve} Adult Stem Cells in the Pylorus

We next employed transmission electron microscopy (EM) to investigate the ultrastructure of the Lgr5^{+ve} adult stem cells at the pyloric gland base in *Lgr5-EGFP-ires-CreERT2* mice. We performed quantitative cryo-immunogold labeling (Peters et al., 2006) for EGFP to identify the Lgr5^{+ve} EGFP^{hi} stem cells and their Lgr5-EGFP^{lo} immediate progeny at the base of the pyloric glands. Typically, 2–3 Lgr5^{+ve} EGFP^{hi} stem cells (Figures S4A and S4E; cell nos 2 & 4 with relative labeling density > 0.5) were found

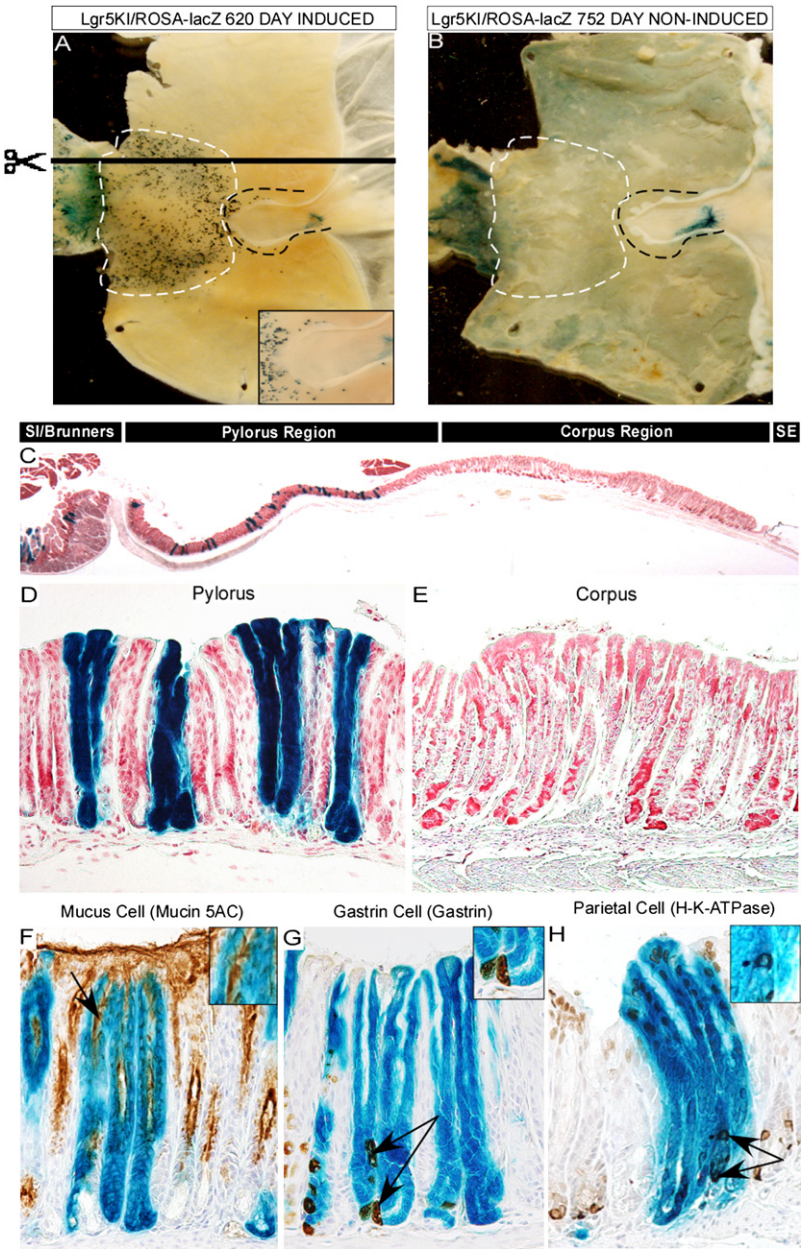

Figure 3. Lgr5^{+ve} Cells at the Pylorus Gland Base Are Multipotent, Self-Renewing Adult Stem Cells

(A) Whole-mount of a 620-day induced *Lgr5KI/ROSA-lacZ* stomach stained for lacZ. Lineage tracing is still evident throughout the pylorus (white, dashed outline) and a limited region adjacent to the esophagus (black, dashed outline + inset). Filled black line corresponds to the section in (C).

(B) Whole-mount of a 752-day noninduced *Lgr5KI/ROSA-lacZ* stomach stained for lacZ demonstrating that lineage tracing is never initiated in the absence of Tamoxifen induction.

(C) Low-power image of a section (black line in A) through the stomach of the 620-day induced *Lgr5KI/ROSA-lacZ* stomach. Long-term lineage tracing is evident in the small intestine and throughout the pyloric region.

(D) Multiple lacZ-positive gastric units are still visible in the pylorus 620-days postinduction (20× magnification).

(E) No lacZ-positive gastric units are visible in the corpus region 620 days postinduction (20× magnification).

(F) Clonal lacZ-positive gastric units contain mucin5-secreting cells (black arrow; 40× magnification).

(G) Clonal lacZ-positive gastric units contain gastrin-positive cells at their base (black arrows; 40× magnification).

(H) Clonal lacZ-positive gastric units at the pyloric/corpus transition zone contain H-K-ATPase positive Parietal cells close to the gland base (black arrows; 40× magnification).

See also Figure S2.

intermingled with 3–4 of their predicted EGFP^{lo} progeny (Figures S4A and S4E; cell numbers 3, 5 & 6 with relative labeling density < 0.4) at the gland base. Both cell types were characterized by the presence of limited basal rER, a large, centrally located nucleus, and apical microvilli (Figures S4C and S4D). More mature cells (EGFP^{-ve}) with abundant apical granules occupied the positions just above the Lgr5-EGFP^{hi} stem cell zone (Figure S4A; cell no. 1). No EGFP^{+ve} cells were observed in the predicted isthmus region of these pyloric glands (Figure S4E).

The Transcriptome of the Adult Lgr5^{+ve} Stem Cells in the Pylorus

Having established that the Lgr5^{+ve} cells at the base of the pyloric glands in adult stomachs are bona fide stem cells, we set out to determine their individual gene signature by using a microarray approach similar to that employed previously for profiling the Lgr5^{+ve} intestinal stem cells (van der Flier et al., 2009). Distinct EGFP^{hi} and EGFP^{lo} populations, corresponding to the Lgr5^{+ve} stem cells and their presumed immediate daughter cells, respectively, were FACS sorted from isolated pyloric gastric units of adult *Lgr5-EGFP-ires-CreERT2* mice (see Figure 4B for a typical EGFP FACS profile). The Lgr5^{+ve} gene signature was subsequently derived via comparative gene expression profiling of mRNA samples from these two populations. Lgr5 was one of the most highly enriched genes in this signature, confirming the separate identities of the Lgr5-EGFP^{hi} stem cell and Lgr5-EGFP^{lo} daughter cell populations (Table S1). Multiple additional Wnt target genes were also selectively expressed in the Lgr5^{+ve} stem cells, including Cd44, Sox9, Sord, Prss23, and Sp5 (Table 1 and Figure S5). Collectively, 48% (153 genes in total) of the intestinal Wnt target gene signature (Van der Flier et al., 2007) was expressed in the Lgr5^{+ve} stem cell/daughter cell population, providing strong evidence of robust canonical Wnt signaling activity at the base of the pyloric glands (data not shown). Several enteroendocrine-specific genes, including Chromogranin A&B, Somatostatin, and Gastrin G, were highly upregulated in the Lgr5-EGFP^{lo} daughter cell population, implying a rapid commitment toward the enteroendocrine lineage.

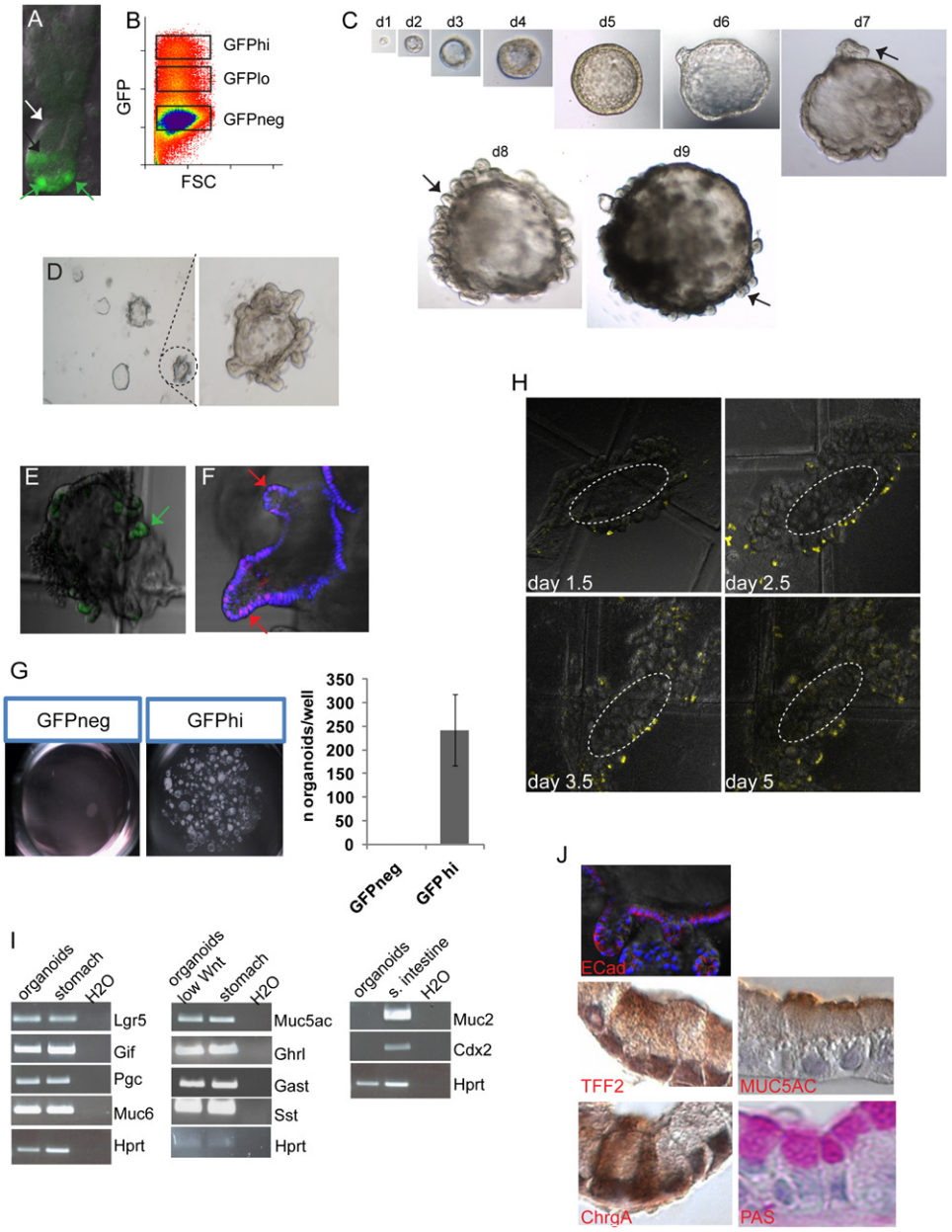

Figure 4. Single Lgr5^{+ve} Cells Build Long-Lived Gastric Organoids In Vitro

(A) Confocal analysis of a freshly isolated pyloric gastric unit from an *Lgr5-EGFP-ires-CreERT2* mouse stomach. Arrows showing EGFP^{hi} (green), EGFP^{lo} (black), and EGFP^{NEG} (white) distinct populations.

(B) Lgr5-EGFP^{+ve} cells are discriminated from the EGFP^{lo} and EGFP^{NEG} populations according to their EGFP expression level. FSC, forward scatter.

(C) Representative example of a growing organoid originating from a single Lgr5^{+ve} cell. Arrows showing the formation of gland-like domain buds at day 7. Original magnifications: days 1–4, 40× magnification; days 5–6, 20× magnification; days 7–8, 10× magnification; and day 9, 5× magnification.

(D) Organoids derived from single Lgr5^{+ve} cells have been dissociated and split every 5–7 days. Representative images of a 3-month-old culture. Original magnifications: left, 4× magnification; right, 10× magnification.

(E) Confocal analysis of Lgr5 EGFP-expressing cells in a 14-day-old gastric culture grown from a single EGFP^{hi} cell. Note that Lgr5-EGFP^{+ve} cells are located at the bottom of the gland domains (green arrow; 10× magnification).

(F) Organoids cultured with the thymidine analog EdU (red) for 1.5 hr. Only gland domains incorporate EdU (red arrows; 20× magnification). Counterstain, 4,6-diamidino-2-phenylindole (DAPI; blue).

(G) Colony-forming efficiency of Lgr5-EGFP^{hi} and Lgr5-EGFP^{NEG} populations. The number of organoids/well was counted after the first passage of the culture (14 days after seeding). Representative images are shown. Data are expressed as the mean ± SEM of three independent experiments.

(H) A 2-week-old culture from a single-cell culture of *Lgr5-EGFP-ires-CreERT2/Rosa26-YFP* reporter mouse was stimulated with tamoxifen in vitro for 20 hr and imaged on the indicated days. YFP fluorescence (yellow) shows that scattered single yellow cells (day 1.5) generate multiple offspring in vitro. Note that YFP^{+ve} cells migrate toward the central lumen (white dotted circle).

Table 1. Wnt Target Genes Enriched in Adult Lgr5^{+ve} Pyloric Stem Cells

Gene Name	Systematic Name	Average log2 Ratio GFP High/Low	Description
Lgr5	NM_010195	3.13	leucine-rich repeat containing G protein-coupled receptor 5
Sp5	NM_022435	2.36	*trans*-acting transcription factor 5
Sord	NM_146126	2.03	sorbitol dehydrogenase
Cd44	NM_009851	1.84	CD44 antigen, transcript variant 1
Prss23	NM_029614	1.75	protease serine 23
Sox9	NM_011448	1.72	SRY-box containing gene 9
Vegfa	NM_001025250	1.67	vascular endothelial growth factor A (Vegfa), transcript variant 1
Rnf43	NM_172448	1.51	ring finger protein 43
Cldn2	NM_016675	1.48	claudin 2
Bambi	NM_026505	1.29	BMP and activin membrane-bound inhibitor homolog
Nelf	NM_001039386	1.17	nasal embryonic LHRH factor (Nelf), transcript variant 1
Enc1	NM_007930	1.06	ectodermal-neural cortex 1

Wnt target genes specifically expressed in the Lgr5^{+ve} pyloric stem cells were identified by comparing the pyloric stem cell signature (Table S1) to the intestinal Wnt target gene program as defined in Van der Flier et al. (2007). Arbitrarily selected genes were validated by q-PCR comparison of Lgr5-EGFP^{hi}, Lgr5-EGFP^{lo}, and Lgr5-EGFP^{-ve} expression levels, or immunohistochemical staining of adult pyloric tissue to confirm restricted expression at the gland base (Figure S5).

Neonate Lgr5^{+ve} Cells Contribute to the Development of Mature Gastric Epithelium in Both Pylorus and Corpus Regions

Analysis of Lgr5 expression in both human (Figures S6A and S6B) and mouse (Figures S6C and S6D) embryonic stomachs revealed robust expression within shallow indentations, the prospective glands, throughout the developing glandular epithelium.

A more detailed analysis of newborn (P1) *Lgr5-EGFP-ires-CreERT2* animals confirmed expression of Lgr5-EGFP in the developing glands of both the pylorus and corpus regions (Figures 5A–5C). The stomach, like much of the gastrointestinal tract, continues to undergo a process of morphogenetic rearrangement during the first few weeks of birth, resulting in the formation of the mature, functional glandular epithelium. In vivo lineage tracing initiated in neonate (P1) *Lgr5-EGFP-ires-CreERT2/Rosa26RlacZ* mice revealed the presence of multiple lacZ^{+ve} units (Figures 5D and 5E) throughout the Brunner's glands (Figures 5F and 5G), pylorus (Figures 5F and 5H), and corpus regions (Figures 5F and 5I) 8 months after induction.

These results identify the neonatal Lgr5^{+ve} cell populations of both the developing pylorus and corpus regions as early stem/progenitor cells contributing to the generation of the mature glandular epithelium. The observed long-term maintenance of this tracing implies that both the Lgr5^{+ve} stem cell population of the adult pylorus and the Lgr5^{-ve} stem cell population of the adult corpus are derived from these neonatal Lgr5^{+ve} cell stem/progenitor populations.

Wnt-Driven Transformation of Adult Lgr5^{+ve} Stem Cells Initiates Tumor Formation in the Distal Stomach

Inappropriate activation of the Wnt signaling pathway via mutations in core pathway components such as *APC* is firmly established as the initiating event in colon cancer (Korinek et al., 1997). By using our *Lgr5-EGFP-CreERT2* mice as a stem cell-specific Cre line, we recently demonstrated that conditional deletion of *APC* from Lgr5^{+ve} intestinal stem cells efficiently drives intestinal cancer formation (Barker et al., 2009). Conditional expression of mutant β-catenin in the intestinal stem cells achieved similar results (Zhu et al., 2009). In contrast, transformation of non-stem cell populations fails to initiate tumor formation.

Aberrant Wnt signaling pathway activity has also been implicated in a subset of human and mouse gastric cancers, particularly intestinal (well-differentiated)-type cancers arising predominantly in the pyloric region of the stomach (Clements et al., 2002; Nakatsuru et al., 1992; Park et al., 1999). Furthermore, spontaneous inactivation of *Apc* in the *APC^{min}* mouse model was found to promote the formation of gastric adenomas within the pyloric region (Gravaghi et al., 2008; Tomita et al., 2007).

To assess the tumorigenic potential of the Lgr5^{+ve} pyloric stem cells, we injected *Lgr5-EGFP-CreERT2 /APC$^{flox/flox}$* mice with a single i.p. dose of Tamoxifen to activate the *Lgr5*-driven Cre in the pyloric stem cells. The resulting deletion of *APC* in the Lgr5^{+ve} stem cells first became apparent 3 days after induction, when single cells expressing elevated levels of the Wnt-effector protein β-catenin (referred to as β-catenin^{hi}) were visualized at the base of the pyloric glands (Figure 6A). These β-catenin^{hi} stem cells began to rapidly divide (Figure 6B), generating ribbons of transformed progeny emanating from the gland base (Figure 6C). Over the course of the next 2–3 weeks, the transformed Lgr5-EGFP^{+ve} stem cells persisted (black arrow), while fuelling the growth of highly proliferative, β-catenin^{hi} adenomas (Figures 6D–6H). The Lgr5-EGFP stem cell marker was expressed throughout these small adenomas, indicating an expansion of the Lgr5 stem cell compartment (Figures 6G and 6I). At this stage, the tumor load in the intestine necessitated the sacrifice of the mice, preventing further analysis of tumor progression in the pylorus of the stomach. Adenomas were never detected in the corpus, confirming the absence of Lgr5 in this region.

(I) Expression analysis of gastric-specific genes from 2-month-old cultures derived from Lgr5^{+ve} single cells. Cultures maintained in high (left) or low (middle) Wnt3A medium. Note that gastric-derived cultures are negative for intestine-specific genes (right).
(J) Cultures maintained in low Wnt3A media for at least 10 days. Top: confocal image of ECad staining (red; organoid epithelium). Counterstain, Hoescht 33345 (blue). Bottom: paraffin sections stained for Tff2 (brown, mucus neck cells), periodic acid-Schiff (red, pit cells), MUC5AC (brown, pit cells), and chromogranin A (brown, enteroendocrine cells).
See also Figure S3.

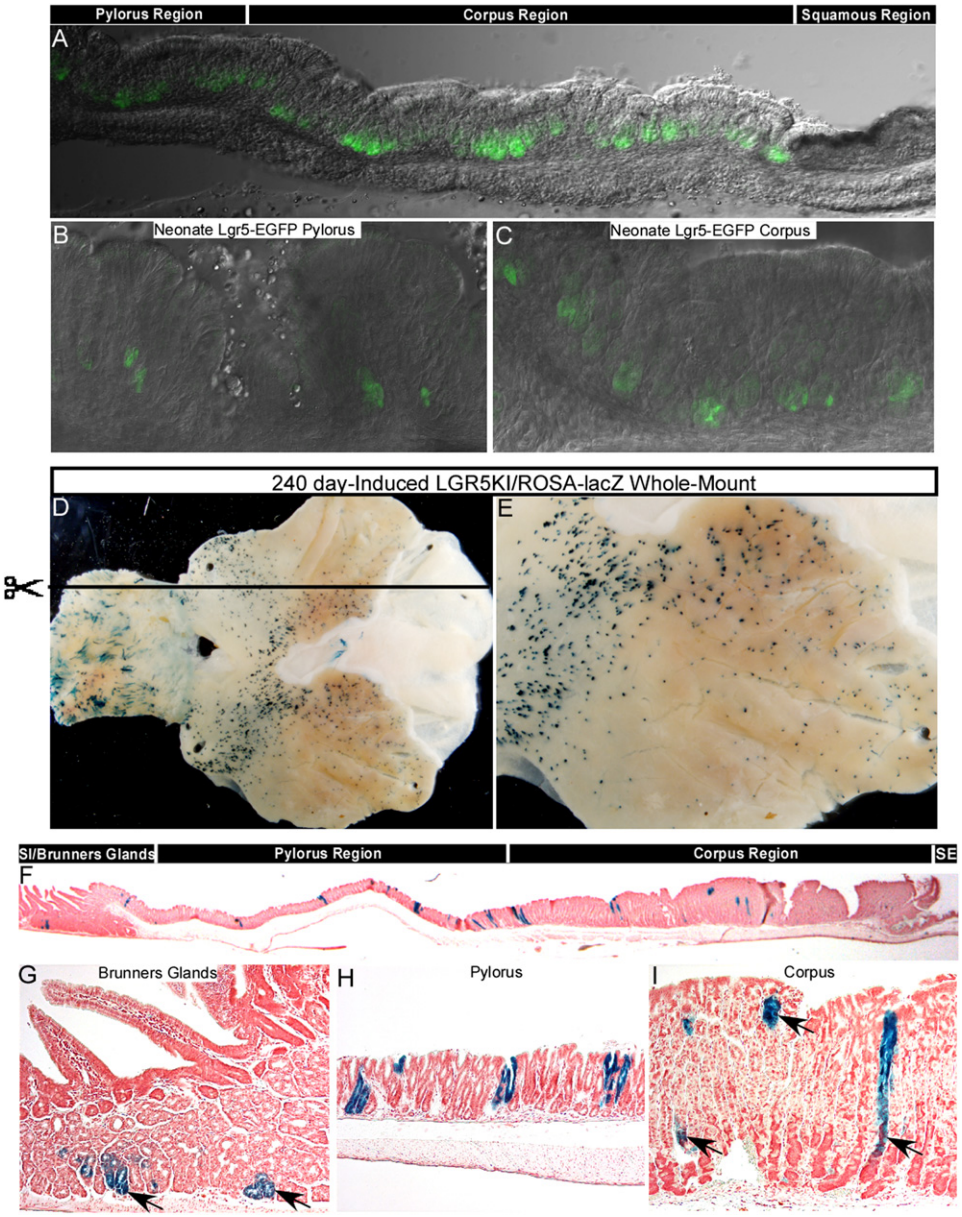

Figure 5. Neonate Lgr5^{+ve} Cells Contribute to Formation of the Mature Glandular Epithelium in Both the Pylorus and Corpus

(A) Low-power confocal image showing *Lgr5-EGFP* expression at the base of the glands throughout the pylorus and corpus regions in neonate *Lgr5-EGFP-ires-CreERT2* mice (2× magnification). Expression is absent from the nonglandular squamous forestomach.

(B and C) Confocal images showing *Lgr5-EGFP* expression at the base of the pyloric glands (B) and corpus glands (C) in neonate *Lgr5-EGFP-ires-CreERT2* mice (20× magnification).

(D and E) Whole-mount of a lacZ-stained stomach from a 240-day-old *Lgr5KI/ROSA-lacZ* mouse induced 1 day after birth. Filled black line corresponds to the section in (F). Lineage tracing is evident throughout the pylorus and corpus regions (higher magnification in E).

(F) Low-power image of a section (black line in D) through the stomach of the 240-day-old *Lgr5KI/ROSA-lacZ* mouse induced 1 day after birth. Long-term lineage tracing is evident in the Brunners glands, pylorus, and corpus regions.

(G–I) Higher-power images of lineage tracing evident in Brunners glands (G: black arrows), pylorus (H), and corpus (I: black arrows) (10× magnifications).

See also Figure S6.

DISCUSSION

Here, we report the identification of a previously unappreciated population of adult stem cells located at the base of the glandular units throughout the pylorus by using the orphan G protein-coupled receptor Lgr5 (also known as GPR49) as a specific marker. We demonstrate that 3–4 Lgr5^{+ve} cells are typically present intermingled with their presumptive immediate progeny at the base of each glandular unit in the adult pyloric region and limited numbers of glandular units adjacent to the esophagus

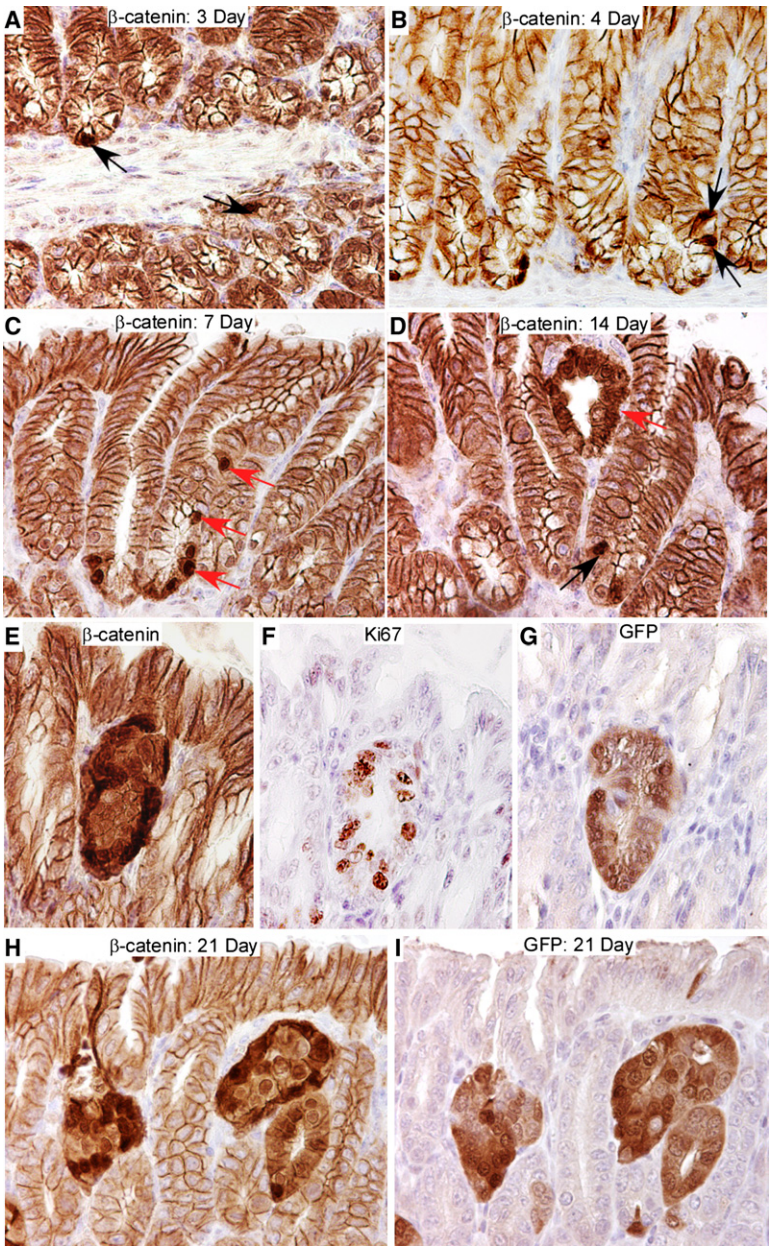

Figure 6. Wnt-Driven Transformation of Adult Lgr5^{+ve} Stem Cells Efficiently Drives Gastric Adenoma Formation in the Pylorus

Adult *Lgr5-EGFP-ires-CreERT2/APC$^{flox/flox}$* mice were treated with a single i.p. injection of tamoxifen to activate the *Lgr5*-driven Cre and facilitate loss of APC in the pyloric stem cells.

(A) Accumulation of the Wnt-effector protein β-catenin is first observed in isolated Lgr5^{+ve} stem cells at the gland base after 3 days (black arrows).

(B) Initial expansion of the transformed, β-catenin^{hi} population is observed at the gland base after 4 days (black arrows).

(C) β-catenin^{hi} progeny begin populating the gastric units within 7 days (red arrows).

(D) After 14 days, transformed stem cells persist (black arrow) and fuel the growth of β-catenin^{hi} gastric adenomas (red arrow).

(E–G) β-catenin^{hi} gastric adenomas (E) express high levels of the proliferation marker Ki67 (F) and the Lgr5-EGFP stem cell marker (G).

(H and I) Larger β-catenin^{hi} gastric adenomas seen after 21 days (H) demonstrate uniform expression of the *Lgr5-EGFP* stem cell marker (I).

(comparable to the gastroesophageal junction in humans) and squamous forestomach boundaries. In contrast, Lgr5^{+ve} cells were absent from the main corpus region of the adult stomach. A similar expression pattern was confirmed in adult human pyloric stomach via in situ hybridization. As published, cell proliferation was most evident in the isthmus region above the neck of the glands, but a proportion of the Lgr5^{+ve} population at the gland base was also actively proliferating.

We then took advantage of our *Lgr5-EGFP-ires-CreERT2* in vivo lineage-tracing model to demonstrate that the Lgr5^{+ve} cells at the gland base are active, multipotent stem cells contributing to the regular self-renewal of the glandular units in the pyloric region. After stochastic activation of the *Rosa-LacZ* reporter gene in isolated Lgr5^{+ve} cells at the pyloric gland base, we observed the rapid appearance of Lgr5-derived lacZ^{+ve} progeny, confirming the active proliferative status of a proportion of the Lgr5^{+ve} cells. Within 7–10 days, lacZ^{+ve} progeny could be found distributed throughout the glands and pits of the pyloric units, providing an estimate of the rate of self-renewal occurring in this region of the stomach. We speculate that the immediate Lgr5 progeny generated at the gland base rapidly migrate to the isthmus, where they undergo transient amplification via rapid proliferation before committing to the various functional lineages during bidirectional migration toward the pit or the gland base.

Multiple tracing units populated exclusively by lacZ^{+ve} cells of all pyloric lineages were evident throughout the pyloric region of a mouse induced nearly 21 months earlier. Moreover, the frequency of lineage tracing was essentially maintained over this extensive period, demonstrating the long-term self-renewal capacity and multipotent nature of the Lgr5^{+ve} cells. Long-term tracing was also observed in the corpus-type units adjacent to the esophagus and the border of the squamous forestomach, indicating that the Lgr5^{+ve} cells at the base of these glands are also adult stem cells contributing to epithelial self-renewal at these locations.

The absence of Lgr5^{+ve} stem cell populations in the main corpus region of the adult stomach indicates the existence of other, Lgr5^{-ve} stem cell pools responsible for maintaining epithelial self-renewal in this region. However, our analysis of Lgr5^{+ve} stem/progenitor activity in newborn stomachs has revealed that Lgr5^{+ve} cells contribute to the rapid postnatal development of the mature glandular epithelium in both the pyloric and corpus regions. The appearance and long-term maintenance of lacZ^{+ve} glandular units in the corpus supports a model in which the Lgr5^{-ve} adult stem cell population originates from the early Lgr5^{+ve} stem/progenitor cells in the developing corpus. Once the glandular epithelium is fully developed, the

early corpus Lgr5^{+ve} stem cells are lost (or simply lose Lgr5 expression), whereas the early Lgr5^{+ve} stem cells of the developing pyloric region are maintained throughout adulthood. We speculate that the pylorus, like the intestine, colon, and hair follicle, maintains an active Lgr5^{+ve} stem cell population to drive the rapid epithelial turnover that is so characteristic of these tissues. In contrast, the lower rate of epithelial turnover present in the adult corpus negates the requirement for such an active Lgr5^{+ve} stem cell population.

With a similar in vivo lineage tracing approach, Qiao and colleagues elegantly demonstrated the presence of rare villin^{+ve} stem/progenitor cells in the pyloric region of a *villin* transgenic mouse (Qiao et al., 2007). These villin^{+ve} stem/progenitor cells differ markedly from the Lgr5^{+ve} stem cells described here in that they are predominantly located at or just below the isthmus and they are a largely quiescent population that requires cytokine stimulation to activate their self-renewal capacity. Although *villin* is not normally expressed in the adult stomach and, consequently, cannot be considered an endogenous stem/progenitor cell marker, this study does imply the existence of a quiescent population of adult stem cells in the pyloric stomach. We speculate that the active Lgr5^{+ve} stem cells effect the daily self-renewal of the pyloric epithelium, whereas the more quiescent villin^{+ve} pool serves as a reserve stem cell population that is activated only in response to damage to the Lgr5^{+ve} stem cell compartment. Similar models have been proposed to account for the extreme regenerative capacity of the small intestine after injury, although there is currently no functional evidence for the existence of a quiescent stem cell population in this tissue.

Current studies of gastric epithelial homeostasis and the underlying molecular causes of gastric cancer rely heavily on the use of multipassage cell lines harboring an array of genetic mutations, or complex in vivo mouse models that are expensive and time-consuming to generate. This has led to a concerted effort in recent years to generate in vitro systems supporting the efficient, long-term culture of primary gastric epithelium. Progress has been made in this endeavor, most notably the development of a 2D culture method by Ootani and colleagues, which facilitates the generation of a gastric monolayer comprising highly differentiated gastric surface mucous cells (Ootani et al., 2003). However, this system was unable to sustain growth for longer than a week, consistent with the rapid loss of stem cell/progenitor self-renewal function. We now report the development of a culture system for generating and expanding long-lived gastric organoids in vitro. In this system, single Lgr5-EGFP^{+ve} pyloric stem cells efficiently generate three-dimensional gastric organoids that closely resemble the pyloric gastric units found in vivo.

Taken together with the in vivo lineage tracing data, we believe this conclusively establishes the Lgr5^{+ve} stem cells at the base of the pyloric glands as a previously unidentified population of active adult stem cells contributing to epithelial self-renewal in the distal stomach.

Comparative gene profiling of the sorted Lgr5^{+ve} adult stem cells and their presumed direct descendents from the pylorus has revealed robust expression of almost 50% of the 318 Wnt target gene signature of the intestine (Van der Flier et al., 2007). In addition to *Lgr5* itself, expression of a subset of the Wnt target genes including *Cd44*, *Sox9*, and *Sord* is highly enriched in the adult stem cells. This probably reflects a central role for the canonical Wnt pathway in maintaining adult Lgr5^{+ve} stem cell function at the gland base in vivo. The strict requirement for Wnt activation that we observe for adult Lgr5^{+ve} stem cell-driven gastric organoid growth in vitro, together with the expansion of the Lgr5 population in vivo, upon RSpondin 1 injection, strongly supports this notion. Whether active Wnt signaling is influencing other regions of the gastric units, such as the proliferative isthmus, remains to be established.

The pathological cascade that characterizes the onset of stomach cancer is well defined, with *Helicobacter pylori* infection driving chronic inflammation (Correa, 1996) and the consequent loss of parietal cells (oxyntic atrophy). The resulting intestinal metaplasia (predominantly in the pylorus) or spasmolytic polypeptide-expressing metaplasia (SPEM; predominantly in corpus) then progresses to dysplasia and ultimately cancer. The underlying molecular changes in the stomach epithelium accompanying this sequence are less well defined, although a growing body of evidence supports a contribution of aberrant Wnt signaling activity to predominantly intestinal-type stomach cancers arising in the distal stomach (Clements et al., 2002; Gravaghi et al., 2008; Nakatsuru et al., 1992; Offerhaus et al., 1992; Park et al., 1999; Tomita et al., 2007). Our observation that loss of APC in the Lgr5^{+ve} stem cells efficiently drives the rapid appearance of proliferating adenomas in the pylorus strongly indicates that stomach cancer can be readily initiated by deregulation of Wnt signaling activity. Future dedicated studies are needed to investigate the possibility that both intestinal cancer and a subset of stomach cancers indeed share a common origin, namely the Lgr5^{+ve} stem cells.

Collectively, this work establishes Lgr5 as a unique marker of adult stem cells in the distal stomach. The novel gastric culture system we describe here should facilitate the routine growth and in vitro manipulation of primary gastric epithelium, thereby accelerating the pace of research into gastric homeostasis and the molecular causes of gastric cancer.

EXPERIMENTAL PROCEDURES

Mice

The generation of the *Lgr5-EGFP-Ires-CreERT2* and *Lgr5-lacZ* mice was described earlier (Barker et al., 2007). *Lgr5-EGFP-Ires-CreERT2/APC$^{flox/flox}$* mice were generated by interbreeding mice carrying a floxed *APC* allele (*Apc$^{580S/flox}$*) (Shibata et al., 1997) and the *Lgr5-EGFP-Ires-CreERT2* allele (Barker et al., 2007). All animal experiments were approved by the Animal Experimentation Committee of the Royal Dutch Academy of Science.

Tamoxifen Induction

Mice aged 6–8 weeks were injected intraperitoneally with a single 200 μl dose of Tamoxifen in sunflower oil at 10 mg/ml. Newborn (neonate) mice (P1) were injected intraperitoneally with a single 20 μl dose of Tamoxifen in sunflower oil at 5 mg/ml.

β-Galactosidase Staining

To determine the pattern of Lgr5 Cre-mediated recombination at the *Rosa26R* lacZ reporter locus, stomachs were isolated and immediately incubated for 2 hr in a 20-fold volume of ice-cold fixative (1% formaldehyde; 0.2 % gluteraldehyde; 0.02% NP40 in PBS0) at 4°C on a rolling platform. Staining for the presence of β-galactosidase (lacZ) activity was performed exactly as described in Barker et al. (2007). Details are included in the Supplemental Experimental Procedures.

The stained tissues were transferred to tissue cassettes and paraffin blocks prepared with standard methods. Tissue sections (4 μM) were prepared and counterstained with neutral red.

BrdU Injection

Adult mice were injected intraperitoneally 2 hr prior to sacrifice with 200 μl BrdU solution in PBS at 5 mg ml^{-1}.

Immunohistochemistry

The immunostaining procedure used here was described earlier (Batlle et al., 2002). The primary antibodies were mouse anti-KI67 (1:250; Monosan), mouse anti-Sox9 (1:500), rabbit anti-GFP (1:6000, gift from E. Cuppen), mouse anti β-catenin (1:100; BD Transduction Labs), mouse anti-BrdU (1:250; DAKO), mouse anti-Muc5AC (1:200; Novocastra), rabbit anti-Gastrin (1:500; Novocastra), and mouse anti-H-K-ATPase (1:1000; MBL). The peroxidase conjugated secondary antibodies used were Mouse or Rabbit EnVision+ (DAKO).

Gastric unit/organoid immunohistochemistry is described in detail in the Supplemental Experimental Procedures.

In Situ Hybridization

ISH was carried out with ^{3}H or ^{35}S antisense riboprobes essentially as described (Poulsom et al., 1998) with SP6 RNA polymerase and EcoRI linearized sequence verified templates prepared in pGEM3Z by Dr. Stefania Segditsas: mouse *Lgr5* 562 bp from 5′ UTR to exon5 (UCSC chr10:114,915,553-115,024,577, introns excluded); human *Lgr5* 566 bp from 5′UTR to exon5 (UCSC chr12:70,120,102-70,233,231, introns excluded).

Immunoelectron Microscopy

Stomachs were dissected and immersion fixed in 2% paraformaldehyde (PFA) in 0.2 M PHEM buffer (240 mM PIPES, 40 mM EGTA, 100 mM HEPES, 8 mM $MgCl_2$ [pH 6.9]), embedded in gelatine, and cryosectioned with a Leica FCS cryoultratome. Cryo-immuno gold staining (Peters et al., 2006) for GFP expression was performed as previously described (Barker et al., 2007).

Gastric Unit Isolation, Single-Cell Dissociation, and EGFP^{+ve} Cell Sorting

Gastric glands units were isolated from mouse pyloric stomach as previously described by Bjerknes and Cheng with some modifications, depending on whether pyloric units were intended for immunohistochemistry or single-cell dissociation (Bjerknes and Cheng, 2002). Detailed protocols are described in Supplemental Experimental Procedures.

Gastric Culture Conditions

Isolated gastric glands were counted and a total of 100 glands mixed with 50 μl of Matrigel (BD Bioscience) and plated in 24-well plates. After polymerization of Matrigel, gastric culture medium (Advanced DMEM/F12 supplemented with B27, N2, nAcetylcysteine (Invitrogen), and Gastrin (10 nM [Sigma-Aldrich]) containing growth factors (50 ng/ml EGF [Peprotech], 1 μg/ml R-spondin1, 100 ng/ml Noggin [Peprotech], 100 ng/ml FGF10 [Preprotech] and Wnt3A-conditioned media) was overlaid. To differentiate to enteroendocrine lineage, Exendin 4 (50 nM) was added.

For the single-cell culture, a total of 50 sorted EGFP^{hi} cells/well were collected in gastric culture medium and embedded in Matrigel (BD Bioscience). After polymerization of Matrigel, gastric culture media was overlaid. For the first 2 days after seeding, the media was also supplemented with 10 μM ROCK inhibitor Y-27632 (Sigma Aldrich) to avoid anoikis. Growth factors were added every second day and the entire medium was changed every 4 days. For passage, gastric organoids were removed from Matrigel, mechanically dissociated, and transferred to fresh Matrigel. Passage was performed every week with a 1:5–1:8 split ratio.

To confirm the Wnt3A requirement, mouse Wnt3A recombinant protein (Stem Cell Technologies) was supplemented instead of the Wnt3A-conditioned media. In vitro tracing experiments were performed as described in Supplemental Information. Organoid immunohistochemistry was performed as described with some modifications (Huch et al., 2009). Detailed protocols are found in Supplemental Experimental Procedures.

Microarray Analysis

For the expression analysis of pyloric Lgr5^{+ve} cells, RNA was isolated from sorted EGFP^{hi} and EGFP^{lo} cell fractions of the pyloric region of the stomach from 20 adult (~8-week-old) *Lgr5-EGFP-ires-CreERT2* mice. 150 ng of total RNA was labeled with low RNA Input Linear Amp kit (Agilent Technologies, Palo Alto, CA). Differentially labeled cRNA from EGFP^{hi} and EGFP^{lo} cells were hybridized on 4X44K Agilent Whole Mouse Genome dual color Microarrays (G4122F) in a dye swap experiment. Labeling, hybridization, and washing were performed according to Agilent guidelines (see Supplemental Experimental Procedures for more details).

Statistical Analysis

The descriptive statistical analysis was performed on SPSS software (SYSTAT software, Inc., Chicago, IL). Results are expressed as mean ± SEM. A Mann-Whitney nonparametric test was used for the statistical analysis (2-tailed). $p < 0.05$ was taken as the level of significance.

ACCESSION NUMBERS

Array data can be retrieved via Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE17485.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at doi:10.1016/j.stem.2009.11.013.

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