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A reserve stem cell population in small intestine renders *Lgr5*-positive cells dispensable

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Abstract

The small intestine epithelium renews every 2 to 5 days, making it one of the most regenerative mammalian tissues. Genetic inducible fate mapping studies have identified two principal epithelial stem cell pools in this tissue. One pool consists of columnar *Lgr5*-expressing cells that cycle rapidly and are present predominantly at the crypt base¹. The other pool consists of *Bmi1*-expressing cells that largely reside above the crypt base². However, the relative functions of these two pools and their interrelationship are not understood. Here, we specifically ablated *Lgr5*-expressing cells using a diphtheria toxin receptor (DTR) gene knocked into the *Lgr5* locus. We found that complete loss of the *Lgr5*-expressing cells did not perturb homeostasis of the epithelium, indicating that other cell types can compensate for elimination of this population. After ablation of *Lgr5*-expressing cells, progeny production by *Bmi1*-expressing cells increased, suggesting that *Bmi1*-expressing stem cells compensate for the loss of *Lgr5*-expressing cells. Indeed, lineage tracing showed that *Bmi1*-expressing cells gave rise to *Lgr5*-expressing cells, pointing to a hierarchy of stem cells in the intestinal epithelium. Our results demonstrate that *Lgr5*-expressing cells are dispensable for normal intestinal homeostasis. In the absence of these cells, the *Bmi1*-expressing cells can serve as an alternative stem cell pool, providing the first experimental evidence for the interrelationship between these populations. The *Bmi1*-expressing stem cells may represent both a reserve stem cell pool in case of injury to the small intestine epithelium and a source for replenishment of the *Lgr5*-expressing cells under non-pathological conditions.

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AUTHOR CONTRIBUTIONS

H.T., B.B., S.W., K.G.L., L.R., O.D.K., and F.J.d.S. conceived, designed and performed experiments, and collected data. H.T., B.B., O.D.K., and F.J.d.S. analyzed the data and wrote the manuscript. All authors discussed results and edited the manuscript. O.D.K. and F.J.d.S. are joint senior authors.

Two types of stem cells have been described in the small intestine based on location and cycling dynamics¹⁻⁴. Fast-cycling stem cells express markers including *Lgr5*, *CD133* and *Sox9*^{1, 5, 6} and are present throughout the intestine. Also known as crypt base columnar cells (CBCs), these slender cells populate the crypt and villi within 3 days, and are interspersed among the Paneth cells that support them^{7, 8}. Slower-cycling stem cells, marked by enriched expression of *Bmi1* or *mTERT*, represent a rarer cell population^{2, 9}. These cells form a descending gradient from proximal to distal regions of the intestine, such that they are more prevalent in the duodenum than in the colon. Despite their rarity, *Bmi1*-expressing stem cells are crucial for crypt maintenance².

To study the function of *Lgr5*-expressing cells, we replaced the first ATG codon of *Lgr5* with two distinct cassettes. The first consisted of a dsRED-ires-creERT2 sequence to enable genetic lineage tracing studies by tamoxifen (TAM)-inducible expression of Cre in *Lgr5*-expressing cells (Supplementary Fig. 1a, *Lgr5*^{CreER} allele). The second cassette contained EGFP linked in frame to a human DTR cDNA (Supplementary Fig. 1a, *Lgr5*^{DTR} allele), producing a fusion protein. Consistent with previous reports, one injection of TAM in *Lgr5*^{CreER;R26R} mice marked *Lgr5*-expressing stem cells in a mosaic fashion and led to generation of labeled progeny for more than 60 days (Supplementary Fig. 1b). Expression of DTR-EGFP in *Lgr5*^{DTR} mice functioned as a reporter for *Lgr5* expression (Fig. 1a) and also conferred diphtheria toxin (DT) sensitivity on CBCs. Expression of EGFP in mice carrying the *Lgr5*^{DTR} allele was detected at the membrane of cycling CBCs in every crypt (Supplementary Fig. 1c-e, CBCs are marked by asterisks).

We next set out to test the effects of eliminating *Lgr5*-expressing cells by administering DT to *Lgr5*^{DTR} mice. Twenty-four hours after DT administration, all EGFP-positive cells were depleted, including CBCs (Fig. 1a, 1b, 1j, 1k, 1p, 1q). Loss of *Lgr5*-expressing cells was further confirmed by the absence of *Lgr5* mRNA (Fig. 1d, 1e) and was accompanied by extensive apoptosis at the base of the crypts, with shedding of dead cells into the lumen (Fig. 1n).

After sustained DT exposure for 10 days, both the EGFP reporter and *Lgr5* mRNA were completely absent from the base of the crypts (Fig. 1c, 1f, and Supplementary Fig. 2), but strikingly, crypt architecture was comparable to controls (Fig. 1g, 1i, 1j, 1l). Proliferating CBCs were absent from the crypt (Fig. 1l, 1r), such that the crypt base was occupied mostly or entirely by Paneth cells (Supplementary Fig. 3a, 3b). The extensive apoptosis detected 24 hours after DT treatment had significantly decreased by day 10 (compare Fig. 1n with Fig. 1o) but was still detectable. No increase in crypt fission after DT treatment was observed by H&E staining at any time point (Fig 1g-i).

Because *Lgr5*-expressing cells have been proposed to play a critical role in renewal of the intestine, it was surprising that the architecture of the intestinal epithelium was essentially intact after ablation of *Lgr5*-expressing CBCs (Fig 1g-i). Within the villi, very little change in the total number of endocrine cells was observed (Supplementary Fig. 3c, 3d), and goblet cells were abundant in the crypts and villi (Supplementary Fig. 3g, 3h, 3j). Upon CBC ablation, Paneth cells were found at the bottom of the crypts and in some cases were mislocalized to the villi (Supplementary Fig. 3a, 3b and data not shown); additionally,

migration of cells as assessed by BrdU pulse-chase labeling was normal (Supplementary Fig. 4). The only major difference from controls that we observed was in the secretory cell lineage; the number of chromogranin A positive enteroendocrine cells in the crypts doubled 10 days post DT (Supplementary Fig. 3e, 3f, 3i).

We did not detect any *Lgr5*-expressing CBCs using either the EGFP reporter or *in situ* hybridization after 10 days of DT (Fig. 1c, 1f and Supplementary Fig. 2), but it was still possible that a few CBCs could have escaped ablation and repopulated the epithelium, as a similar scenario was reported in *c-Myc* and *Ascl2* conditional null animals^{10, 11}. To directly address this possibility, we visualized *Lgr5*-expressing cell activity during DT selection by producing *Lgr5*^{DTR/CreER}; *R26R* mice. These mutant mice carried two null alleles at the *Lgr5* locus, one of which enabled ablation of *Lgr5*-expressing cells and the other enabled lineage tracing of any possibly remaining *Lgr5*-expressing cells. These mice died at postnatal day (P) 1, consistent with previous reports that *Lgr5* null mice are not viable¹². To analyze the postnatal gut, we grew pieces of small intestine from embryonic day (E) 15 *Lgr5*^{DTR/CreER}; *R26R* embryos under the kidney capsule of immunocompromised mice for three weeks, at which point they formed crypts comparable to P17 intestine (Fig. 2a-e)¹³. Following 10 days of TAM treatment, columns of blue cells emanated from the crypt base, and progeny of *Lgr5*-expressing cells differentiated into all four major cell types of the intestinal epithelium (Fig. 2a-e). Concomitant administration of DT and TAM for 10 days eradicated all EGFP-positive CBCs (Fig. 2g), and no cells descended from *Lgr5*-expressing cells were observed (Fig. 2f), confirming that the *Lgr5*^{DTR} allele leads to complete elimination of these cells. Importantly, no abnormalities in graft morphology, differentiation or proliferation were observed in these mice compared to controls (Fig. 2a-j).

Although *Lgr5*-expressing cells were completely depleted within 24 hours of DT treatment, persistence of apoptotic bodies at the crypt base throughout the 10 day DT treatment suggested that *Lgr5*-expressing CBCs were continuously generated and eliminated during the treatment (Fig. 1n, 1o). This notion was supported by the quick recovery of *Lgr5*-expressing cells between 48 to 96 hours after the final dose of DT (Fig. 1s-v). To follow the fate of the newly generated *Lgr5*-expressing cells, mice implanted with *Lgr5*^{DTR/CreER}; *R26R* embryonic intestine fragments in the kidney capsule were allowed to recover for 6 days following 6 days of DT treatment. A row of blue cells emanated from the crypt base (Supplementary Fig. 5a), indicating that the newly formed *Lgr5*-expressing stem cells (Supplementary Fig. 5b, GFP-positive cells) gave rise to progeny that migrated out of the crypt. When the converse experiment was performed by injecting TAM for 6 days and then dosing with DT from days 6 to 12, blue cells were only present in the upper region of the villi (Supplementary Fig. 5c), indicating that progeny of *Lgr5*-expressing cells marked between day 1 and 6 migrated out of the crypts into the villus, but that during DT treatment between days 6 and 12, *Lgr5*-expressing stem cells were no longer available (Supplementary Fig. 5d, absence of GFP signal) to supply labeled (blue) progeny to replenish the epithelium.

To study the long-term effects of CBC ablation, we isolated crypts from *Lgr5*^{DTR/+} mice to perform *in vitro* crypt organoid zcultures¹⁴. Crypts depleted of *Lgr5*-expressing CBCs by treatment for 10 days with DT, as indicated by absence of GFP expression, gave rise to organoids with similar efficiency as wild-type controls (Supplementary Fig. 6a, 6b). These

could be passaged *in vitro* in DT for up to 2 months without losing their ability to expand and proliferate. No *Lgr5*-expressing (GFP-positive) cells were detected in organoid epithelium as long as the organoids were maintained in medium containing DT (Supplementary Fig. 6d). However, when DT was removed from the culture medium, *Lgr5*-expressing cells reappeared at the bottom of crypt-like structures within 3 days (Supplementary Fig. 6c, GFP-positive cells).

Because we found that *Lgr5*-expressing CBCs were dispensable for crypt maintenance, we next asked whether *Bmi1*-expressing stem cells were mobilized to compensate for the loss of the *Lgr5*-expressing stem cells. BMI1 regulates self-renewal of hematopoietic and neuronal stem cells¹⁵. We employed a GFP knock-in allele (*Bmi1*^{GFP/+}) to monitor *Bmi1* gene expression¹⁶. *Bmi1* expressing GFP-positive cells were most commonly observed at positions 3 to 6 from the crypt base (Fig. 3a), consistent with the *Bmi1* mRNA expression pattern in the small intestine². Upon depletion of *Lgr5*-expressing CBCs in *Lgr5*^{DTR/+};*Bmi1*^{GFP/+} animals after 9 days of DT treatment, the number of GFP-positive cells per crypt increased three fold (Fig. 3a-d, and Supplementary Fig. 7a), and the proportion of crypts containing either single or multiple GFP-expressing cells increased by 40 percent compared to control animals (Supplementary Fig. 7b). Of note, 55 percent of the total number of GFP-positive crypts in *Lgr5*^{DTR/+};*Bmi1*^{GFP/+} animals now contained multiple GFP-positive cells (Fig. 3d and Supplementary Fig. 7b), compared with only 22 percent in *Bmi1*^{GFP/+} control animals.

To trace the fate of cells descended from *Bmi1*-expressing cells after elimination of *Lgr5*-expressing CBCs, we generated a *Bmi1**CreER* BAC transgenic allele (Supplementary Fig. 8). Labeling kinetics using the *Bmi1**CreER* transgenic line crossed with the *R26R* reporter were identical to previously reported results using the *Bmi1*^{CreER} knock-in allele² (Fig. 3f). *Bmi1**CreER*;*R26R*;*Lgr5*^{DTR/+} animals were treated with alternating doses of DT and TAM per day for 7 days (Fig. 3e). Because *Bmi1*-expressing cells are most abundant in the first 5 cm of the duodenum, we focused our analysis on this region. Consistent with the increased number of *Bmi1*-expressing cells (Supplementary Fig. 7a), the proportion of *LacZ*-positive crypts (either partially or fully labeled) also increased 34 percent upon loss of *Lgr5*-expressing CBCs (Supplementary Fig. 7c). The most dramatic difference was in the number of fully labeled crypts. Only 2.3 percent of crypts were fully labeled in *Bmi1**CreER*;*R26R* control animals during a 6 day lineage tracing period, which was comparable with previous studies using a *Bmi1*^{CreER} knock-in allele². Upon loss of *Lgr5*-expressing CBCs, the number of fully labeled crypts increased approximately 15-fold (Fig. 3h, 3i and Supplementary Fig. 7c.). These results indicate that in the absence of *Lgr5*-expressing cells, *Bmi1*-expressing cells are capable of directly giving rise to all intestinal cell types without going through *Lgr5*-positive intermediate cells. However, *Bmi1*-expressing stem cells did not give rise to an increased number of labeled crypts in more distal regions of small intestine and colon upon loss of *Lgr5*-expressing CBCs (Fig 3f, 3g), indicating that different stem cell pools must compensate for the loss of *Lgr5*-expressing stem cells in distal regions of the gut.

Lastly, we tested whether *Bmi1*-expressing cells give rise to *Lgr5*-expressing cells under normal conditions. Since *Bmi1*- and *Lgr5*-expressing cells represent distinct though overlapping cell populations, we carried out a series of short term pulse-chase experiments

using *Bmi1**CreER*; *R26R*; *Lgr5*^{DTR/+} animals. Twenty-four hours after TAM administration, most of the β -galactosidase (β -gal)-positive cells appeared as individuals, reflecting the normal pattern of *Bmi1* expression (Fig. 4a) in the initially labeled cells. *Bmi1*-expressing cells (β -gal positive) overlapped with *Lgr5*-expressing cells (GFP positive) between positions 1 to 6 at the crypt base; the double-positive cells peaked at positions 3 and 4 (Fig. 4a-c). This observation is consistent with a previous report stating that *Bmi1* mRNA expression (via qPCR analysis) was readily detectable in *Lgr5*-positive cells¹¹. Later, between 48–72 hours, clonal expansion from *Bmi1*-expressing cells was evident, as β -gal/GFP double-positive cells now appeared as doublets or triplets (Fig. 4d-i). We scored a total of 500 crypts at each time point and found that while a few cells were β -gal/GFP double positive (i.e., expressing both *Bmi1* and *Lgr5*) at 24 hours after TAM induction, this number doubled at 48 hours (Fig. 4j,k). Similarly, lineage tracing from *Bmi1*-expressing cells carried out in mice treated for 6 days with DT and allowed to recover for 72 hours demonstrated that newly formed *Lgr5*-positive cells at the bottom of the crypts arose from *Bmi1*-expressing cells (Fig 4m-o). Together, these data show that *Bmi1*-expressing cells can give rise to *Lgr5*-expressing cells both under normal physiological conditions and following insults that deplete CBCs. Similar to our observation, mTERT-expressing stem cells could also give rise to *Lgr5*-positive cells over a 5 day lineage tracing period⁹.

Our data support the existence of two stem cell pools in the epithelium of the small intestine: an actively proliferating stem cell compartment responsible for the daily maintenance of the intestine epithelium that is characterized by the expression of *Lgr5*, *Ascl2*, and *Olfm4*^{1, 11, 17} and a distinct pool of stem cells expressing *Bmi1*. Our results lend support to the two-stem-cell pool model that is based on computational approaches¹⁸, and provide experimental evidence for recent models predicting that the intestine could fully recover after complete elimination of cellular subpopulations deemed to be functional stem cells¹⁹. Our data do not support the recent proposal that *Bmi1*-expressing cells are exclusively a subset of *Lgr5*-expressing cells¹¹; rather they indicate that under normal circumstances, *Bmi1*-positive stem cells are upstream of rapidly cycling, *Lgr5*-expressing stem cells and replenish the pool of active stem cells, either to avoid exhaustion of actively cycling stem cells or to prevent the accumulation of damaged cells that may lead to the development of tumors. Importantly, we also demonstrate that when the *Lgr5*-expressing cell compartment is eliminated by DT treatment, the *Bmi1*-expressing cells can increase in number, presumably as a compensatory mechanism. Under these conditions, *Bmi1*-expressing cells contribute directly to the generation of all cell types of the intestinal epithelium to produce a functional organ until the rapidly-cycling stem cell compartment is able to recover. While it has been suggested that *Bmi1*-expressing stem cells are quiescent², this remains to be conclusively demonstrated.

Distinct stem cell pools with differing cycling dynamics have previously been observed in the hair follicle and in blood, organs that, like the intestine, undergo regular bouts of proliferation and regeneration^{20–22}. The factors that regulate the interplay between discrete populations of stem cells, and the precise hierarchical relationships among such populations, remain to be characterized. While we have found that loss of *Lgr5*-positive cells is sustainable under short-term conditions *in vivo*, it remains to be determined if such a scenario can persist for longer periods of time in the animal. Interestingly, depletion of

Paneth cells, which are thought to be important for the maintenance of CBCs⁷, can be tolerated by mice for over 6 months without significant structural defects of the epithelium^{23, 24}, supporting the idea that the intestine can function normally in the absence of CBCs. It will be important to determine how different stem cell populations sense the activity of other populations, whether rapidly cycling cells can repopulate more quiescent stem cell populations, and whether additional sub-populations of stem cells exist.

Methods

Mice

Lgr5^{DTR/+}, *Lgr5*^{CreER/+} and *Bmi1*^{CreER} alleles were generated as described in supplementary methods online. *Bmi1*^{GFP/+} mice were kindly provided by Irving Weissman¹⁶. All studies and procedures involving animal subjects were approved by Institutional Animal Care and Use Committees of Genentech and UCSF, and were conducted strictly in accordance with the approved animal handling protocol.

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/nature>

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of diphtheria toxin-mediated crypt base columnar cell (CBC) ablation

(a) EGFP is detected on the membrane of Ki67⁺ proliferating CBCs in saline treated *Lgr5*^{DTR/+} animals. (b) One dose of diphtheria toxin (DT) eliminates all DTR-EGFP-positive cells at 24 hours. (c) DT treatment for up to 10 days prevents reappearance of *Lgr5*-expressing cells.

(d) *Lgr5* mRNA is normally present at the bottom of the crypts and (e) is not detected after 24 hours or (f) 10 days of DT treatment.

- (g-i) Crypt architecture is intact after ablation of *Lgr5*-expressing CBCs.
- (j-l) Proliferation above the crypt base is normal after ablation of *Lgr5*-expressing CBCs.
- (m-o) Extensive apoptosis is observed at the crypt base 24 hours after DT and tapers off by 10 days, but is still higher than controls.
- (p-r) Electron microscopy shows that CBCs in controls are characterized by slender nuclei and scant cytoplasm. No CBCs remain at the crypt base after one dose or 10 days of DT treatment. The crypt base is filled with granule-rich Paneth cells.
- (s) Dosing regimen for study of the recovery of *Lgr5*-expressing CBCs
- (t) No CBCs are detected 24 hours after DT. (u) A few *Lgr5*⁺/*Ki67*⁺ CBCs (arrow) were detected 48 hours after the last dose of DT. (v) More *Lgr5*⁺/*Ki67*⁺ CBCs (arrow) recovered after 96 hours.

Figure 2. Maintenance of normal crypt architecture is not mediated by *Lgr5*-positive cells that have escaped ablation

(a) 10 day lineage tracing of descendants of *Lgr5*-expressing stem cells shows a blue ribbon emanating from the base of the crypt in a grafted intestine piece from E15 *Lgr5*^{DTR/CreER} embryos.

(b-e) Normal proliferation and differentiation of intestinal epithelium after loss of *Lgr5* gene function. *Lgr5*-expressing stem cells can give rise to all four major differentiated cell types. X-GAL-positive cells mark *Lgr5*-positive stem cell progeny, which overlap with differentiated cell markers for goblet (c), Paneth (d) and endocrine cell (e) lineages.

(f) Concurrent TAM and DT treatment kills all *Lgr5*-expressing cells. No progeny of *Lgr5*-expressing cells (blue) are detected in the grafted intestine.

(g-j) No GFP-positive cells are detected but proliferation and differentiation are normal after DT-mediated ablation of *Lgr5*-expressing CBCs

Figure 3. *Bmi1*-expressing stem cells are mobilized to compensate for the loss of *Lgr5*-expressing CBCs

- (a) Rare *Bmi1*-expressing cells are detected at positions 3 to 6 of the crypt base in the duodenum of *Bmi1*^{GFP/+} reporter mice.
- (b) Increased *Bmi1*-expressing cells appear at the crypt base upon ablation of *Lgr5*-expressing CBCs.
- (c) Higher magnification showing a *Bmi1*-expressing cell at position 4 of crypt base in *Bmi1*^{GFP/+} reporter mice.

- (d) Close up view of a crypt with multiple *Bmi1*-expressing cells after ablation of *Lgr5*-expressing cells.
- (e) Dosing regimen for lineage tracing of *Bmi1*-expressing cell progeny after ablation of *Lgr5*-expressing CBCs.
- (f-g) Whole-mount X-GAL staining of the gastrointestinal tract. Proximal to distal gradient of decreasing progeny production by *Bmi1*-expressing cells is maintained after ablation of *Lgr5*-expressing CBCs.
- (h-i) Close up view of X-GAL positive crypts in duodenum. Most of the labeled crypts have less than five X-GAL positive cells in *Bmi1**CreER*;*R26R* control animals. Ablation of *Lgr5*-expressing CBCs stimulates production of progeny by *Bmi1*-expressing cells. 36% of the crypts in the first 5 cm of duodenum now become fully labeled (marked by arrows).

Figure 4. *Bmi1*-expressing cells give rise to *Lgr5*-expressing CBCs under normal and injury conditions

(a-c) *Bmi1*^{CreER};*R26R*;*Lgr5*^{DTR/+} animals were dosed with 5 mg TAM and harvested 24 hours later. β-gal-positive cell (red) derived from *Bmi1*-expressing cells overlap with *Lgr5*-expressing CBCs (GFP-positive, green) at position 4 (arrow). A non-overlapping β-gal-positive cell was detected at position 7 in the same crypt (asterisk).

(d-f) More β-gal-positive cells (red) display overlapping expression (marked by arrow) with *Lgr5*-expressing CBCs (GFP⁺, green) at 48 hours.

(g-i) At 72 hours, clonal expansion from *Bmi1*-positive stem cells is now evident by a streak of β -gal positive cells migrating upward (red). β -gal-positive clones at lower crypt positions overlap with *Lgr5*-expressing CBCs (arrow).

(j-k) Distribution of the *Bmi1* positive stem cell progeny (β -gal⁺ cells) within the crypt at 24 and 48 hours post TAM induction. *Bmi1*-expressing cells appear as singles throughout the crypt base between positions 1 to 15 (j). More cells are derived from *Bmi1*-expressing stem cells at 48 hours (k). A significant portion of β gal⁺ cells also express *Lgr5* (GFP⁺, green column), at positions 1 to 6. Overlapping cells (green) peak around positions 3, 4, or 5.

(l) Dosing regimen used to study the recovery of *Lgr5*-expressing CBCs from *Bmi1*-positive cells.

(m-o) *Bmi1*-positive cells give rise to a fully labeled crypt (red), including newly formed *Lgr5*-expressing CBCs (GFP positive, arrows).