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BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

Mouse Label-Retaining Cells Are Molecularly and Functionally Distinct From Reserve Intestinal Stem Cells

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BACKGROUND & AIMS: Intestinal homeostasis and regeneration after injury are controlled by 2 different types of cells: slow cycling, injury-resistant reserve intestinal stem cells (ISCs) and actively proliferative ISCs. Putative reserve ISCs have been identified using a variety of methods, including CreER insertions at *Hopx* or *Bmi1* loci in mice and DNA label retention. Label-retaining cells (LRCs) include dormant stem cells in several tissues; in the intestine, LRCs appear to share some properties with reserve ISCs, which can be marked by reporter alleles. We investigated the relationships between these populations. **METHODS:** Studies were performed in *Lgr5–EGFP-IRESCreERT2*, *Bmi1-CreERT2*, *Hopx-CreERT2*, and *TRE-H2BGFP::Hopx-CreERT2::lox-stop-lox-tdTomato mice*. Intestinal epithelial cell populations were purified; we compared reporter allele-marked reserve ISCs and several LRC populations (marked by H2B-GFP retention) using histologic flow cytometry and functional and single-cell gene expression assays. **RESULTS:** LRCs were dynamic and their cellular composition changed with time. Short-term LRCs had properties of secretory progenitor cells undergoing commitment to the Paneth or enteroendocrine lineages, while retaining some stem cell activity. Long-term LRCs lost stem cell activity and were a homogenous population of terminally differentiated Paneth cells. Reserve ISCs marked with HopxCreER were primarily quiescent (in G0), with inactive Wnt signaling and robust stem cell activity. In contrast, most LRCs were in G1 arrest and expressed genes that are regulated by the Wnt pathway or are in the secretory lineage. **CONCLUSIONS:** LRCs are molecularly and functionally distinct from reporter-marked reserve ISCs. This information provides an important basis for future studies of relationships among ISC populations.

Keywords: Quiescence; Epithelium; Differentiation; Crypt.

Emerging data provide compelling evidence of the existence of reserve stem cells in the intestinal epithelium. Functionally, we define the reserve intestinal stem cell (ISC) as an indispensible stem cell capable of giving rise to all intestinal cell types and is injury-resistant and indispensible for tissue maintenance, although this definition is arbitrary and a potential source of contention in the literature. Recently, a burgeoning body of literature using a plethora of proxy markers has identified cells with all of these functional qualities, or subsets thereof. These studies primarily employ 2 technical approaches: the use of proxy-reporter alleles (either targeted or randomly integrated transgenes) for cell isolation, ablation, and lineage tracing (via CreER), and the use of label-retention assays employed under the assumption that reserve ISCs should be slow cycling and retain DNA label (although methods for how this label is initially introduced vary widely).

The most extensively studied proxy knockin reporter alleles used to mark reserve ISCs include CreER insertions into the endogenous *Hopx* and *Bmi1* loci.[1–6] Lineage tracing from these alleles verified the presence of bona fide stem cells within marked populations capable of giving rise to all cell types of the intestinal epithelium, including the active crypt base columnar (CBC) ISC[2,4–6] In addition, single-cell gene expression analysis of *Hopx-* and *Bmi1-CreER*–marked cells revealed these populations to be largely overlapping with one another, but distinct from CBCs marked by *Lgr5-eGFP-IRES-CreER*.[2,7] These reserve ISCs are also resistant to high doses of gamma irradiation (IR) that ablate actively cycling CBCs (12 Gy and higher), although recent evidence indicates that a rare population of $Lgr5^{low}Wnt^{low}$ cells above the crypt base but still marked by the *Lgr5-eGFP-IRES-CreER* reporter are also radioresistant.[6,8] Further functional evidence illustrating the critical importance of *Bmi1-/Hopx-CreER*–marked ISCs within the ISC niche come from diphtheria toxin cell ablation experiments driven by *Bmi1-CreER* that demonstrate that these reserve ISCs are required for tissue fidelity and maintenance of normal crypt-villus architecture; in contrast, Lgr5-expressing cells are completely dispensable.[3,5] The de novo generation of Lgr5-expressing cells is, however,

Abbreviations used in this paper: CBC, crypt base columnar; Dox, doxycycline; IR, irradiation; ISC, intestinal stem cell; LRC, label-retaining cell; LT, long-term; mRNA, messenger RNA; ST, short-term.

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required for regeneration after radiation injury.[9] These studies collectively demonstrate the existence of an indispensable, Wnt-negative, radioresistant reserve ISC that gives rise to active Wnt^{High} CBCs. It is important to point out here that these functional assays were all performed using CreER knockin reporter alleles, and that the populations marked by these alleles are not equivalent to those containing endogenous *Bmi1* or *Hopx* messenger RNAs (mRNAs), both of which can be found nonspecifically throughout the crypt base and cannot serve as proxies for specific stem cell identity.[2,10,11] Numerous other proxy alleles have been described that almost certainly mark populations overlapping to various degrees with the *Hopx-/Bmi1-CreER*–marked reserve ISC population; however, the rigorous molecular and functional assays applied to the *Hopx-/Bmi1-CreER*-marked cells are incomplete for many of these other populations (specifically lineage tracing to verify long-term self-renewal and differentiation capacity, cell ablation to determine requirement for tissue fidelity, and single-cell profiling to address heterogeneity) and we do not describe this literature here in detail.[12–16] To date, no nongenetic means exist for the prospective identification and isolation of these reserve ISCs.

Besides the use of genetic proxy reporter alleles for reserve ISC identification, it has been proposed that such a stem cell should cycle slowly, and thus retain DNA label if the label is introduced during a period before the induction of dormancy (ie, in the newborn or after injury). The presence of label-retaining cells (LRCs) was first proposed by Potten et al to be evidence of the immortal strand hypothesis, although support for this idea lacks consensus in the face of conflicting data.[17–21] Nonetheless, DNA labeling in young mice or in mature animals after injury revealed the presence of long-term (LT) LRCs in the intestine (up to 1 month chase), which consisted of Paneth cells (long-lived cells intercalated between CBCs) as well as non-Paneth cells residing around the +4 to +15 position from the crypt base.[22] These non-Paneth LRCs were posited to be ISCs, but no functional evidence for this existed at the time. Over a decade later, these findings were reproduced using inducible H2B fusion proteins rather than the DNA radiolabeling used in the original studies.[13,23,24] Interestingly, these studies incorporated label not in newborn mice or after injury, but in mature mice under basal conditions, suggesting that the LRCs may not be dormant stem cells upstream of CBCs, but rather a downstream cell that incorporates label while undergoing cell cycle exit and differentiation (ie, the label should not incorporate into dormant stem cells in the absence of DNA replication, either induced post-injury or during development).

The use of H2B-eGFP fusion proteins to identify these LRCs enabled for the first time their prospective isolation and molecular profiling. These studies, performed on shorter-term LRCs (usually around 8–12 days), revealed that the LRC population expresses direct target genes of the canonical Wnt/β-catenin pathway, such as *Sox9* and *Lgr5* and markers of the secretory lineages.[13,23,24] Single-cell profiling of 10-day LRCs, however, found them to be a highly heterogeneous population.[24] Remarkably, the use of an H2B-split-Cre reporter allele that enables lineage tracing from LRCs revealed stem cell activity from at least some cells contained within this population.[24] Further, these 10-day LRCs could give rise to clonal lineage tracing events after exposure to mid-dose gamma IR (6 Gy), although the frequency of these events was vanishingly small, with <10 tracing events observed along the entire length of the intestine.[24] Taken together, these studies suggested that non-Paneth LRCs are a secretory progenitor cell population that can serve as a reserve ISC. These observations, coupled with their location above the crypt base and slow cycling nature, have led researchers to posit that the short-term (ST) LRCs and reserve ISCs marked by the *Bmi1*- and *Hopx-CreER* proxy alleles are one and the same,[1] although no cell ablation evidence exists demonstrating a functional importance for LRCs, as it does for the proxy allele-marked reserve ISCs.

In order to understand the relationship between intestinal LRCs and proxy-reporter allele-marked ISCs, the current study undertakes a comprehensive and direct comparison of single cells within these 2 populations, including both ST and LT LRCs (10 days, 1 month, 3 months), and reserve ISCs marked by *Hopx-/Bmi1-CreER.*

Methods

Mouse Strains

Lgr5–EGFP-IRESCreERT2 (JAX strain 008875) and *Bmi1-CreERT2* (JAX strain, 010531) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). *Hopx-CreERT2* (JAX strain 017606) mice were a kind gift from Dr Jon Epstein. *TRE-H2BGFP* mice were obtained from Jackson Laboratory (JAX strain 016836). Mice were maintained on a C57/BL6N background. Mice (including the *TRE-H2BGFP*) received a single intraperitoneal injection of 100 μL tamoxifen (Sigma, St Louis, MO; T5648, 10 mg/mL in corn oil). All mouse protocols were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania under protocol 803415 to Dr Lengner.

H2B-GFP Labeling

TRE-H2BGFP::Hopx-CreERT2::Lox-Stop-Lox-tdTomato mice were maintained on doxycycline (Dox) (Sigma; D9891, 1 mg/mL in 1% sucrose) for 6 weeks starting at postnatal day 14 in order to fully label nuclei with GFP. Dox was withdrawn when mice reached 8 weeks of age and mice were sacrificed 10 days, 1 month, or 3 months after Dox withdrawal and initiation of tracing. *Hopx-CreERT2::Lox-Stop-Lox-tdTomato* activity was initiated with 1 dose of tamoxifen 18 hours before sacrifice.

EdU Labeling, RNA Content Staining, Flow Cytometry, and Single-Cell Fluorescence-Activated Cell Sorting

The intestine was cut open longitudinally and incubated with 5 mM EDTA and Hank's balanced salt solution solution at 4°C for 30 minutes to isolate epithelial cells. To generate a single-cell suspension, cells were incubated with Accutase (BD Biosciences, San Jose, CA) at 37°C for 10 minutes. Flow cytometry analysis was performed with BD LSRFortessa cell analyzer (BD Biosciences). 4′,6-Diamidino-2-