Limitations to Understanding Intestinal Stem Cell Activity via Cre-Lox-Based Lineage Tracing

The intestinal epithelium is a highly proliferative tissue with robust regenerative potential, governed by the intestinal stem cell (ISC) compartment. Our understanding of the cellular hierarchy within this compartment is drawn from Cre-lox-based lineage tracing studies, initially via the identification of crypt base columnar stem cells (CBCs) using a Lgr5-CreER reporter,3 showing that CBCs reconstitute all epithelial cell types during homeostasis.4 Despite their proliferative capacity, CBCs are exquisitely sensitive to injury, requiring facultative stem cells for postinjury regeneration.3,4 Many groups have shown facultative ISC activity within populations marked by a Cre-estrogen receptor fusion (CreER) targeted to Hopx, Bmi1, Lrig1, and additional loci.5-7 These Cre drivers mark heterogeneous yet overlapping populations that give rise to all epithelial cell types in response to injury.8 More recently, plasticity and facultative ISC activity have been described in populations marked by CreER reporters in loci purportedly specific to terminally differentiated cells, particularly in the secretory lineages. Facultative ISCs are lineage-committed cells that reacquire stem cell function in response to injury to facilitate tissue regeneration and are defined by in vivo lineage tracing, usually using tamoxifen (Tam)-inducible CreER recombinase, expressed via a putative cell type–specific promoter, to excise a locus of X-over P1 (loxP)-flanked stop cassette (lox-stop-lox, or LSL) proceeding a ubiquitous promoter and preceding a reporter protein (usually a fluorophore or β-galactosidase). Tam treatment results in irreversible reporter activation, enabling lineage tracing, and thus functionally defining a stem cell. Lox-stop-lox reporters typically are inserted into the ROSA26 locus, and different reporter alleles often are used with the same CreER driver interchangeably under the assumption that they mark analogous populations. However, clear differences in recombination efficiencies exist across lox-stop-lox reporters.

We aimed to address these differences by directly comparing recombination efficiencies between two commonly used reporter alleles within the same cell. We generated mice harboring a Hopx CreER allele (among the most broadly used reporters of facultative ISC activity9), and heterozygous for each of two ROSA26 reporters: R26SL-tdTomato1,3 and R26SL-eYFP14 (Figure 1A and B). This allowed us to directly compare recombination efficiency within the same cell, eliminating potential discrepancies from differences in Cre activity or expression levels. We induced recombination in Hopx CreER::R26SL-tdTomato/LSL-eYFP mice and probed via flow cytometry and immunofluorescence for the relative number of tandem-dimer Tomato (tdTomato)+, enhanced yellow fluorescent protein (eYFP)+, and double-positive cells (Figure 1C–E).

The shortest Tam regimen (1 × 24 hours) often is used to characterize putative parental stem cells before cell division and tracing into progeny. In response to this Tam regimen, approximately 1.5% of cells were tdTomato+, while eYFP+ (and double-positive) cells were nearly undetectable. Histologically, tdTomato+ cells were observed around the +4 position near the crypt base, a location and frequency consistent with previous studies.5,6 (Figure 1C). Mice receiving a 5 × 24-hour Tam regimen had frequent tdTomato+ cells in the crypt base and scattered throughout the villi. We found only rare instances of eYFP+/tdTomato+ cells (Figure 1C). Consistent with the histology, approximately 15% of epithelial cells were tdTomato+ at this time point, and <0.2% were eYFP+ or eYFP+/tdTomato+ (Figure 1D and E). After the longest Tam chase period, we found frequent ribbons of tdTomato+ cells, but only rare eYFP+/tdTomato+ ribbons. Here, tdTomato+ cells represented approximately 13% of the epithelium, while eYFP+ and eYFP+/tdTomato+ cells made up <0.1% (Figure 1C–E). Thus, the recombination efficiency of the R26SL-eYFP allele is markedly less efficient than that of the R26SL-tdTomato allele.

Next, we compared recombination efficiency using CreER alleles that mark mature cells within the secretory lineage, which recently has garnered attention as a source of facultative ISC activity.10,11 First, to mark enteroendocrine cells, we used a ChgaCreER-2A-tdTomato allele coupled with either the R26SL-tdTomato or R26SL-eYFP reporter.1 Comparing the number of labeled cells in ChgaCreER::R26SL-tdTomato and ChgaCreER::R26SL-eYFP mice, we observed significantly more LacZ-marked cells than eYFP-marked cells (Figure 2A and B). Next, to mark goblet cells, we used a novel Muc2CreER allele combined with either the R26SL-tdTomato or R26SL-eYFP reporter. We found significantly more tdTomato-marked cells than LacZ-marked cells (Figure 2C and D). Taken together, these results indicate that the R26SL-tdTomato reporter is the most sensitive to Cre-mediated recombination, followed by the R26SL-eYFP, then R26SL-LacZ.

We postulate that discordance among reporters may result from differences in the size of the floxed stop cassette (because distance between loxP sites correlates inversely with recombination efficiency15), and/or variation in the sequences of the loxP sites. Indeed, the R26SL-tdTomato reporter has a much smaller distance between the loxP sites (~900 bp) compared with the R26SL-LacZ and R26SL-eYFP alleles (~2.7 kb) (Figure 2E). However, neither distance nor loxP sequence can explain the difference in recombination efficiency between R26SL-LacZ and R26SL-eYFP alleles. It is possible that differences in detection methods...
Figure 1. R26LSL-tdTomato and R26LSL-eYFP recombination efficiencies within single HopxCreER+ cells. (A) Tamoxifen schematic. (B) Schematic of R26LSL-tdTomato and R26LSL-eYFP reporter alleles. (C) Representative images of tdTomato and eYFP immunofluorescence staining of jejunum after different TAM regimens. Scale bar: 50 um. (D) Percentage of tdTomato+ and eYFP+ cells in small intestinal epithelium after different Tam regimens, measured by fluorescence-activated cell sorter. N = 3 mice/group. (E) Representative flow plots from mice quantified in panel D. CAG, (C) the cytomegalovirus (CMV) early enhancer element, (A) the promoter, the first exon and the first intron of chicken beta-actin gene, (G) the splice acceptor of the rabbit beta-globin gene; DAPI, 4',6-diamidino-2-phenylindole.
Figure 2. Relative efficiencies of R26LSL-tdTomato, R26LSL-LacZ, and R26LSL-eYFP reporters in goblet and enteroendocrine cells. (A) Histology and immunofluorescence of ChgaCreER::R26LSL-LacZ and ChgaCreER::R26LSL-eYFP small intestine 48 hours after 5 daily TAM doses. Scale bar: 100 μm. (B) Quantification of marked cells from histology in panel A, N = 3 mice/group, n = 4 fields of view/mouse. **P < 10⁻⁶. (C) Histology and immunofluorescence of Muc2CreER::R26LSL-LacZ and Muc2CreER::R26LSL-tdTomato small intestine 24 hours after 5 daily TAM doses. Scale bar: 100 μm. (D) Quantification of marked cells from histology in panel C, N = 3 mice/group, n = 4 fields of view/mouse. S1, S2, and S3 represent proximal, middle, and distal small intestine segments, respectively. S1, **P < 10⁻⁶; S2, **P < 10⁻⁹; and S3, **P < 10⁻⁶. (E) Schematic of R26LSL-tdTomato, R26LSL-LacZ, and R26LSL-eYFP alleles. CAG, (G) the cytomegalovirus (CMV) early enhancer element, (A) the promoter, the first exon and the first intron of chicken beta-actin gene, (G) the splice acceptor of the rabbit beta-globin gene.
could explain the greater proportion of LacZ+ cells (enzymatic detection, more sensitive) vs eYFP+ cells (immunoﬂuorescence, less sensitive). We did not, however, directly compare all 3 reporter alleles with the same Cre driver, and thus these data should be interpreted with that limitation in mind.

Reporter choice is particularly important in the study of intestinal stem cell biology because quantifying the degree to which different cell populations contribute to regeneration is greatly inﬂuenced by reporter efﬁciency. To date, many Cre drivers have been reported to mark facultative ISCs (eg, Dll1, Mex3a, Hopx, Bmi1, Lyz1, Chu, Atoh1, Krt19, Alpi, Lrig1, Sox9, mTert, Dclk1, Prox1, and H2B-splot-Cre), with postinjury lineage tracing events occurring at varying frequencies, from robust (>40% with HopxCreER) to exceedingly rare (<1% with Dll1CreER).17 Our ﬁndings highlight the importance of understanding recombination efﬁciencies when interpreting the literature describing these various proxy markers of facultative ISC activity. Our studies suggest that R26LSL-eYFP is too inefficient to reliably gauge stem cell frequency, and although R26LSL-tdTomato readily recombines in target cell types, it may suffer from excess sensitivity because spurious recombination or recombination in progenitors upstream of the target cell type can be observed. The R26LSL-LacZ reporter is perhaps the best alternative, offering robust recombination with the added beneﬁt of being amenable to whole-mount imaging, enabling the quantiﬁcation of relatively rare events across large regions of tissue. Ultimately, interpretation of the literature describing facultative intestinal stem cell activity should be performed with caution and respect to reporter allele choice.

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**Supplementary Materials and Methods**

**Animals**

All mice used for these studies were between 20 and 30 weeks of age, fed ad libitum, and housed under standard University Laboratory Animal Resources conditions. The following mice were obtained from Jackson Laboratories (Bar Harbor, ME): Hopx-CreER (017606), R26-eYFP (006148), R26-tdTomato (007909), and R26-LacZ (003474); Chga-CreER mice were generated as described later. Muc2-CreER mice were generated in house as previously described. Muc2-CreER mice were described as generation 2.

The Muc2-CreERT2 targeted allele was generated by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-assisted homologous recombination in mouse embryonic stem cells. Briefly, a targeting construct was synthesized by GenScript (Piscataway, NJ) to insert a mouse codon optimized 2xV5 epitope tag-T2A sequence against the immediate downstream untranslated region 5'-GACCTTCTCCACTCCTGGCT-3'. This construct was cloned into the eSpCas9(1.1) plasmid (71814; Addgene) and co-transfected into V6.5 mouse embryonic stem (ES) cells with subsequent neomycin selection and propagation of appropriately targeted ES cells initially screened by polymerase chain reaction, and then verified by full-length sequencing of the insertion using primers flanking the arms of homology.

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**Isolation of Small Intestinal Crypts and Fluorescence-Activated Cell Sorter Analysis**

After the mice were killed, the gastrointestinal tract of the mice was dissected and the first 2 cm of duodenum was removed, the next 5 cm of jejunum was taken for histology, and the following 10 cm was isolated in phosphate-buffered saline. The tissue was briefly washed in fresh phosphate-buffered saline and subsequently was splayed open and transferred to a tube containing 10 mL 1 × Hank's balanced salt solution with 1 mmol/L N-acetyl cysteine. After collection, the tissue was vortexed for 15 seconds followed by a 15-second rest on ice; this was performed repeatedly during a 2-minute period. The tissue was then transferred to a tube containing 10 mL 1 × Hank's balanced salt solution with 1 mmol/L NAC and 10 mmol/L EDTA and was placed on a rotator in 4°C for 45 minutes. After the incubation period, the tissue was vortexed for 30 seconds followed by a 30-second rest period on ice; this was performed repeatedly during a 3-minute period. After vortexing, the tissue digestion was filtered through a 70-umol/L filter and the flow-through was centrifuged at 300 × g for 3 minutes. To generate a single-cell suspension, the cell pellet was resuspended in a single-cell suspension buffer containing DNase (35 μg/mL) and Liberase (20 μg/mL) (Sigma-Aldrich, St. Louis, MO) and was incubated at 37°C for 20 minutes. After digestion, the cells were washed in phosphate-buffered saline and resuspended in fluorescence-activated cell sorter buffer (phosphate-buffered saline with 4% fetal bovine serum) before fluorescence-activated cell sorter analysis. The viability dye 4',6-diamidino-2-phenylindole was used to exclude dead cells. Cells were analyzed on a FACSCalibur (BD Biosciences, Franklin Lakes, NJ) using FlowJo software (BD Biosciences, Franklin Lakes, NJ). All secondary antibodies were used at a 1:600 dilution.

LacZ staining was performed as previously described. The entire length of the small intestine was divided into 4 segments, labeled as S1, S2, S3, and S4, with S1 being the most proximal and S4 being the most distal. Each segment was flushed with fixative and stained with X-Gal (1070372901; Sigma-Aldrich), Swiss rolled, embedded in paraffin, sectioned, and stained with neutral red.

**Statistical Methods**

Data were analyzed using unpaired and paired 2-tailed Student t tests, and P values are indicated in individual Figures. Specific experimental replicates are described in each Figure legend.

**Supplementary References**

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