SIDEPopulation sorting separates subfractions of intestinal stem cells

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ABSTRACT

RICHARD VON FURSTENBERG: Side Population Sorting Separates Subfractions of Intestinal Stem Cells
(Under the direction of Susan J. Henning)

The existence of intestinal stem cells (ISCs) which generate the epithelium has been recognized for over three decades. Recent work suggests there are two distinct ISC populations: active ISCs which are important for epithelial homeostasis, and quiescent ISCs that drive proliferation after injury. Although quiescent ISCs may be of greater therapeutic use, to date, isolation of this population has been achieved only with fluorescent reporter mice. My goal was to identify an isolation method for both ISC populations that can be used in wild-type mice and eventually humans. In the following experiments I use side population (SP) sorting to demonstrate that the USP: contains reporter cells of the active ISC, is highly proliferative by S phase analysis, and expresses high levels of the known active ISC transcripts. In contrast the LSP is: non-proliferative, de-enriched for active ISC transcripts, and enriched for those of quiescent ISCs.
ACKNOWLEDGEMENTS

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<td>Protein atonal homolog 1</td>
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<tr>
<td>Bmi1</td>
<td>B lymphoma Mo-MLV insertion region 1</td>
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<td>5-ethynyl-2’-deoxyuridine</td>
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<tr>
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<td>Enteroendocrine cell</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>Hopx</td>
<td>Homeobox only domain</td>
</tr>
<tr>
<td>ISC</td>
<td>Intestinal stem cell</td>
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<tr>
<td>Lgr5</td>
<td>Leucine rich repeat containing G protein coupled receptor 5</td>
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<tr>
<td>Abbreviation</td>
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<td>Lower side population</td>
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<td>Musashi RNA-binding protein 1</td>
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<td>Mouse telomerase reverse transcriptase</td>
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CHAPTER 1
INTRODUCTION

The existence of the intestinal epithelial stem cells (ISCs) that can generate the four main lineages of the small intestine (absorptive, goblet, enteroendocrine, Paneth cell) has been recognized for the last 40 years. There have been recent strides in the characterization of both ISC biology, and the niche they occupy. These advances have in large part been a product of transgenic reporter mice (Lgr5, Sox9, Bmi1, mTert, Lrig1, and Hopx). Information emerging from in vivo studies of these mice including ISC behavior in normal physiology, development, cancer, and response to damage, have shed new light on the existing notion of two distinct ISC populations or phenotypic states. The crypt base columnar cells (CBC), intercalated amongst Paneth cells in the crypt base, display high levels of Lgr5 promoter activity and are rapidly cycling (every 24h). For this reason the CBC cells are referred to as the “active ISC” and are believed to be primarily responsible for the rapid turnover of the intestinal epithelium during normal homeostasis. Located above the Paneth cells in the “+4” cell position (distributed from +2 to +7) are the long lived, slowly cycling stem cells. Validated genetic markers of the +4 cells, include Bmi1, mTert, Lrig1, Hopx.
The supra-Paneth cells labeled by these markers have demonstrated the potential to generate the four main intestinal lineages, and cycle at higher frequency following damage. The active and slow-cycling ISC populations appear to be engaged in a fluid relationship in which the +4 cell can repopulate the CBC pool, and conversely the CBC can generate the +4 cell in a bidirectional phenomenon termed interconversion. Furthermore, and in the same vein, it has been proposed that the Lgr5+ cells express transcriptional markers associated with quiescence (Bmi1, Tert, Hopx, and Lrig1) while maintaining an active status in the CBC position. Adding to this concept of plasticity, recent publications suggest that committed progenitors or subsets of both Paneth and enteroendocrine cells (EE) may re-enter the cell cycle following damage.

While the various ISC transgenic mice have allowed for elegant experiments and new insight, this approach is limited to these specifically-engineered animals and thus has experimental and therapeutic limitations. In contrast, fluorescence-activated cell sorting (FACS) approaches using antibodies against surface markers such as DCLK1, CD24, and Lrig1 offer the potential to sort ISC from any mouse or human. However, to date the published antibody-based methods each have caveats of their own: DCLK1 is expressed on slow-cycling ISC and differentiated tuft cells, CD24 is expressed on actively-cycling ISC and Paneth cells, and while Lrig1 is expressed on slow-cycling ISC, high levels of Lrig1 are expressed in the CBC. In light of these caveats a selection method based on a conserved stem cell property of dye efflux and DNA content offers a parallel approach to surface marker strategies.

The side population (SP) phenotype is due to the presence of xenobiotic efflux transporters found on the membrane of stem cells. The technique, originally developed
utilizing bone marrow cells, has now been used to successfully isolate stem cells from many solid tissues including skeletal muscle, lung, liver, testis, kidney, skin, mammary gland, heart, and brain.\textsuperscript{23} Our laboratory reported in 2005 the use of SP sorting to enrich for ISC from WT mouse intestine.\textsuperscript{24} A microarray comparing the intestinal SP to intact jejunum, and consequent Gene Ontology analyses, revealed enrichment in \textit{Msi1} and interestingly a de-enrichment of markers associated with cell cycle.\textsuperscript{25} Gulati et al. also validated the SP as originating primarily from the crypt base by performing in situ for 36 transcripts enriched in the aforementioned SP microarray, of which 32 were found to be restricted to the crypt base.

I hypothesized that the SP we have previously reported on represents the slowly-cycling stem cell population and that an effluxing population exists above the traditional intestinal SP which represents the actively cycling CBC.\textsuperscript{24,25} For the purpose of this paper the traditional SP will be referred to as the lower SP (LSP) and the newly described upper SP as (USP) from here forward as I report a series of experiments to test this hypothesis. I conclude from these studies that this one method of SP sorting will allow for the simultaneous isolation of a fraction enriched for actively cycling and slowly cycling ISC from WT mice.


CHAPTER 2
METHODS AND MATERIALS

MICE

WT adult male C57BL/6J mice and heterozygote breeder pairs of Lgr5-EGFP-IRES-creERT2 (called Lgr5-EGFP from here on) mice were obtained from The Jackson Laboratories (Bar Harbor, ME), and housed under a 12:12-h light-dark cycle in American Association for Accreditation of Laboratory Animal Care-approved facilities. All animals were used within the age range of 6-8 weeks. All WT and Lgr5-EGFP animal usage had Institutional Animal Care and Use Committee approval.

CELL PREPARATION AND SP STAINING AND ANALYSIS

Epithelial cells were isolated from the jejunum of WT and Lgr5-EGFP mice using our previous published EDTA method. For SP analysis, cells from the preparation were incubated in “SP buffer” made with 2% FBS, and 10mM HEPES in HBSS and either Hoechst 33342 (10ug/ml equal to 17µM) (Sigma) or Vybrant DyeCycle Violet (DCV) 10µM (Invitrogen) for 90min at 37°C. Following the incubation the cells were washed with ice cold HBSS and labeled with CD45-FITC (Biolegend) at 0.5ug/10^6 cells and propidium iodide (PI) at 1µg/ml for removal of cells that were dead and/or of hematopoietic origin. For validation purposes, in order to block vital dye efflux, cells
were pre-incubated with 100µM verapamil for 20min at 37°C and incubated with the above SP solution for 90min at 37°C with the addition of 100µM verapamil. To generate the SP fluorescent phenotype Hoechst 33342 samples were excited using a UV laser (Dako MoFlo) while DCV samples were excited using a 405nm laser (Dako CyAn and BD LSRll). Corresponding band-pass filter sets were: Dako MoFlo (blue 450-50, red 670-30), Dako CyAn (blue 450-50, red 680-20), and BD LSRll (blue 450-50, red 660-20). On each machine, the SP was defined on the red<sup>lo</sup> population which was eliminated by verapamil (Figure 1). For analyses and cell collections, the SP was subdivided into two regions: upper SP (USP), and lower SP (LSP).

**SP AND EdU S PHASE ANALYSIS**

*WT* mice were injected intraperitoneally with 100µg EdU in PBS, and sacrificed 1h post-injection. The jejunum was harvested and epithelial cells were isolated, labeled for SP analysis (Hoechst) as mentioned above, and the sub-populations of the SP phenotype (LSP and USP) were collected using a Dako MoFlo cell sorter. The LSP and USP cell collections were then processed for EdU detection using the Click-it kit (Invitrogen) with an Alexa-647 fluor. The LSP and USP cell collections were subsequently analyzed for EdU positivity using a Dako CyAn flow cytometer.
SP AND LGR5-EGFP TRACKING

Lgr5-EGFP^{hi} mice were sacrificed, and jejunal epithelial cells isolated. The cells were incubated and labeled for SP analysis (DCV) as described above. The cells were then analyzed using the Dako CyAn to detect both SP fluorescence as well Lgr5-EGFP signal from the same sample. Summit 4.2 software was used to track the Lgr5-EGFP^{hi} cells with respect to their SP phenotype.

qRT-PCR OF SP SUBPOPULATIONS

LSP and USP were collected into lysis buffer and RNA isolated using the RNAqueous-Micro kit (Ambion). cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative reverse transcriptase PCR (qRT-PCR) was conducted for each sample in triplicate. Taqman probes (Actb, M m00607939_s1; Dclk1, M m00444950_m1; Lgr5, M m00438890_m1; Lrig1, M m00456116_m1; mTert, M m00436931_m1; Ascl2, M m01268891_g1; Olfm4, M m01320260_1; Bmi1 M m03053308_g1; Hopx, M m00558630_m1; SI, M m01210305_m1; Lyz, M m00727183_s1; ChgA M m00514341_m1; Muc2, M m00458299_m1; Syp, M m00436850_m1) were obtained from Applied Biosystems and used according to manufacturer’s protocol.
B-Actin RNA was used as an internal control due to its similar Ct threshold values between USP and LSP when the same number of cells were collected: difference in $^2$-Actin threshold Ct's of USP and LSP for 100,000 cells each = 0.18±0.48 (expressed as mean ± SEM), n=3. ""'Ct (cycle threshold) values were calculated to obtain fold changes vs. pooled intact jejunum (WT C57BL6J male 7wk old mice).

**QUANTIFICATION OF ENTEROENDOCRINE CELLS IN THE LOWER SP**

The jejunum was harvested and epithelial cells were isolated, labeled for SP analysis (Hoechst) as mentioned above, and the sub-populations of the SP phenotype (LSP and USP) were collected using a Dako MoFlo cell sorter. The LSP and USP cell collections were then labeled with synaptophysin antibody (Epitomics) as described by Bjerknes and Cheng, with the only exception being our use of the synaptophysin antibody at an increased 1:500 concentration.²⁶ To validate the synaptophysin antibody in our hands it was tested on intestinal cells isolated from peptide YY-GFP mouse, a reporter mouse in which the subset of peptide-Y Y EE cells express GFP.
REFERENCES


CHAPTER 3
S PHASE ANALYSIS OF SIDE POPULATION

To investigate the proliferative activity of the SP sub-populations LSP and USP were analyzed for EdU positivity 1h after injection of WT mice with the thymidine analog. Because SP phenotype is reliant on the extrusion of vital dyes by membrane efflux pump of the ATP-binding cassette (ABC) transporter superfamily, the collection of the SP sub-populations had to be performed before fixation of the cells for EdU detection (Figure 2A). After collection and reanalysis of the SP sub-populations, the EdU positive percentages showed marked differences. The USP was 36 ± 4% EdU positive upon reanalysis (Figure 2B) while the LSP was only 0.4 ± 0.04% EdU positive (Figure 2C). These data suggest the actively cycling ISC are likely to reside in the USP. Conversely the paucity of S phase cells in the LSP, taken along with our group’s previous SP publications, suggests it may harbor ISC that cycle very infrequently. This approximate 100 fold difference in the percentage of cells undergoing S-phase between the two SP subpopulations dramatically illustrates the disparity in proliferative activity between USP and LSP.
Since Lgr5 is a commonly used marker of actively cycling ISCs, Lgr5-EGFP mice were used to assess the distribution of Lgr5+ cells between the USP and the LSP. To this end, following the SP analysis of intestinal epithelial cells from Lgr5-EGFP mice (Figure 3A), the Lgr5-EGFP\textsuperscript{hi} fraction was gated forward onto an SP plot (Figure 3B), with SP sub-population gates based on a standard SP sample of all cells from the same mouse. Supporting the S phase data from the EdU experiment, 96 ± 2% of the Lgr5-EGFP\textsuperscript{hi} cells tracked to the USP gate (Figure 3C). Quantitation of the actual Lgr5-EGFP\textsuperscript{hi} contribution to the overall makeup of the USP was not possible due to the mosaic expression of Lgr5-EGFP in the intestine.\textsuperscript{21}
CHAPTER 5
TRANScripT ANALYSIS OF UPPER AND LOWER SP

The experiments to this point suggest that the actively cycling ISC are restricted to the USP. To explore the transcriptional profile of the SP sub-populations, I utilized qRT-PCR and primer/probes for active and quiescent ISC markers. Additionally I used primer/probes for the four intestinal lineages to give some insight into the non-stem cell makeup of the SP sub-populations.

qRT-PCR OF INTESTINAL STEM CELL mRNA MARKERS

As expected from the EdU analysis and Lgr5-EGFP hi cell tracking experiments, the USP was enriched in all active ISC markers examined, specifically: Lgr5 (25 ± 3), Ascl2 (47 ± 16), and Olfm4 (10 ± 2), (Figure 4). The quiescent ISC associated transcripts were enriched as well: Lrig1 (37 ± 15), Bmi1 (84 ± 15), mTert (39 ± 15), Hopx (13 ± 4), and Dclk1 (5 ± 2).

In contrast the LSP was de-enriched for active ISC markers relative to intact jejunum, Lgr5 (0.7 ± 0.5), Ascl2 (0.9 ± 0.6), and Olfm4 (0.6 ± 0.5). mTert mRNA was significantly enriched while other q-ISC markers were increased but did not achieve
statistical significance, Lrig1 (4.8 ± 2.2), Bmi1 (9.2 ± 3.9), Dclk1 (0.5 ± 0.3), the q-ISC/tuft cell marker was de-enriched.

Interestingly, markers associated with quiescence (Lrig1, Bmi1, mTert, HopX, and Dclk1) were also enriched in the USP compared to intact jejunum. This is in agreement with the finding by Munoz et al. that the Lgr5+ cells contain both transcriptional profiles (active/quiescent). Notably the LSP was void of active ISC markers and enriched for quiescent ISC markers.

qRT-PCR OF LINEAGE mRNA MARKERS

In regard to lineage positive cells (Figure 5) the USP was enriched in Muc2 (4.0 ± 1.0), Lyz (23 ± 8), ChgA (6.3 ± 1.7), and Syp (3.3 ± 1.5) transcripts while de-enriched for SI (0.4 ± 0.1). The LSP appeared to have minimal amounts of absorptive, goblet, and Paneth cell transcripts: SI (1.2 ± 0.1), Muc2 (2.7 ± 1.9), Lyz (1.0 ± 0.9) respectively. The EE markers ChgA (17.3 ± 6.6) and Syp (4.1 ± 2.0) were elevated in the LSP. With regard to lineage contamination of the USP, the levels of Lyz, Muc2, and EE transcripts were increased over intact jejunum. LSP however was relatively free from contaminating lineage markers with the exception of the EE marker ChgA.
CHAPTER 6
ENTEROENDOCRINE CELL QUANTIFICATION

In light of recent publications from Sei et al. and Van Landeghem et al., that a subset of EE cells have ISC potential, I felt it was important to investigate the possibility that the LSP was selecting for this EE subset. To directly assess the number of EE cells in the SP regions I opted to collect USP and LSP cells from WT mice and reanalyze by permeabilizing the cells and labeling with an antibody against the EE marker Syp. The resulting analysis showed less than 2% of the LSP to be Syp positive indicating the LSP is likely a different population (Figure 6). To validate the pan-EE specificity of the Syp antibody I tested it against intestinal epithelial cells isolated from the PYY-GFP mouse, developed by Bohorquez et al., in which the peptide-YY producing subset of EE cells express GFP. Our resulting flow analysis demonstrated that almost all PYY-GFP cells were also Syp positive (Figure 7). The ability of a committed secretory progenitor to revert back to a stem cell phenotype following damage has been recently demonstrated by van Es et al. in the case of the Dll1hi cell and the characterization of the H2B-LRC secretory progenitor by Buczacki et al. However I did not find significant expression of Dll1 or Atoh1 mRNA in the LSP (data not shown). Thus, under homeostatic conditions these progenitors do not exhibit the stem cell phenotype of dye efflux.


CONCLUSION

This paper reports the use of SP sorting to successfully isolate two phenotypically different putative ISC populations from WT mice. Most notable is the near 100 fold difference in the percentage of cells undergoing S phase when comparing the USP to the LSP. I demonstrated that Lgr5-EGFP$^{hi}$ cells, which represent the active ISC, were found to reside in the USP when tracked on the SP plot. Upon sorting and processing the USP and LSP for mRNA analysis, I found differing transcriptional profiles via qRT-PCR. In support of the notion that the LSP is characteristic of the quiescent ISC pool, mRNA markers associated with quiescent ISC were enriched, while those of the active ISC were de-enriched. The LSP, otherwise free from lineage transcripts, displayed a high level of ChgA mRNA. To evaluate the possibility that the LSP contained a significant proportion of EE cells, I collected the LSP along with the USP and labeled the cells with anti-Syp antibody. Less than 2% of the LSP was positive for the widely reported pan-EE marker Syp.$^{18,16}$ This finding exemplifies the caution by that transcript levels, especially from secretory cells, are not necessarily indicative of cell number.$^{29}$

The SP phenotype is conferred when a cell expresses a combination of effluxing membrane proteins belonging to the ATP-binding cassette (ABC) superfamily including multi-drug resistance 1 (Mdr1 human, Mdr1a/1b mouse) and ATP-binding cassette, subfamily G (WHITE), member 2 (Abcg2) also referred to as breast cancer resistance
protein1 (Bcrp1) which work in concert to eliminate a DNA binding dye such as Hoechst 33342 or DyeCycle Violet.\textsuperscript{23,30} Despite this efflux some amount of dye does bind to the DNA and resulting fluorescence is an index of DNA content and allows for discrimination of cell cycle activity.\textsuperscript{23} The interpretation of cell cycle activity/ DNA content from SP has been demonstrated by both Goodell et al. and Petritz et al. with bone marrow cells and yielded plots analogous to the patterning seen in intestine when visualized using lower signal amplification of the vital dye emission than we had in 2005.\textsuperscript{31,32} A adapting these instrument settings I could now interpret an additional effluxing population (USP) above the traditional SP (now LSP) illustrated in the 2005 publication from our group.\textsuperscript{24} This “upper SP” region was in an orientation much like the published S-G2M phase cells which exist, spatially, above the SP along the Hoechst blue axis. Confirming this, I found the USP to be highly enriched in cells which incorporated EdU following a 1h pulse when compared to the LSP.

The Lgr5-EGFP\textsuperscript{hi} cells from the Lgr5-EGFP-ires-CreERT2 mice have been previously used as a surrogate for the mitotically active ISC.\textsuperscript{13} To examine the relationship between active ISC and SP phenotype I performed SP analysis on jejunal epithelium from these mice, and found that almost all Lgr5-EGFP\textsuperscript{hi} active ISC reside in the USP. Unfortunately, as referenced in our results, due to the mosaicism of Lgr5-EGFP expression in this mouse line I could not estimate the contribution of Lgr5\textsuperscript{hi} cells to the USP. As mentioned in the introduction there no published ISC membrane markers which are exclusive to the active ISC so I was precluded from further investigation as to the exact ISC percentage of the USP.
Next I evaluated the transcriptional profiles of the USP and LSP in terms of ISC genes (quiescent and active) as well as genetic markers of the committed lineages. As expected from the Lgr5-EGFP<sup>hi</sup> tracking experiment I found an enrichment of Lgr5, Ascl2, and Olfm4 in the USP. Interestingly, markers associated with quiescence (Lrig1, Bmi1, mTert, HopX, and Dclk1) were also enriched in the USP compared to intact jejunum. This is in agreement with the findings of that the Lgr5+ cells contain both transcriptional profiles (active/quiescent).<sup>15</sup> The LSP in contrast expressed only the quiescent ISC markers (Lrig1, Bmi1, mTert, and HopX) at levels above intact jejunum, and were de-enriched for a-ISC transcripts (Lgr5, Ascl2, and Olfm4). With regard to lineage contamination of the USP, the levels of Lyz, Muc2, and EE transcripts were increased over intact jejunum. LSP however was relatively free from contaminating lineage markers with the exception of the EE marker ChgA.

In light of recent publications from Sei et al. and Van Landeghem et al. that a subset of EE cells have ISC potential, I felt it was important to investigate the possibility that the LSP was selecting for this EE subset.<sup>27,19</sup> To directly assess the number of EE cells in the SP regions I opted to collect USP and LSP cells from WT mice and reanalyze by permeabilizing the cells and labeling with an antibody against the EE marker Syp. The resulting analysis showed less than 2% of the LSP to be Syp positive indicating the LSP is likely a different population. To validate the pan-EE specificity of the Syp Ab I tested it against intestinal epithelial cells isolated from the peptide YY-GFP mouse in which the peptide YY producing subset of EE cells express GFP. Our resulting flow analysis demonstrated that almost all peptide YY-GFP cells were also Syp positive. The ability of a committed secretory progenitor to revert back to a stem cell phenotype following
damage has been recently demonstrated as in the case of the Dll1^hi cell and the H2B-LRC secretory progenitor.\textsuperscript{16,17} However I did not find significant expression of Dll1 or Atoh1 mRNA in the LSP.

A recent study that has strengthened the link between intestinal SP and ISC is an inducible A bgc2-LacZ mouse model allowing for lineage tracing.\textsuperscript{33} Previously mentioned as one of the vital dye effluxers, A bgc2 has been demonstrated to be the most critical to the SP phenotype in bone marrow, essentially eliminating the SP population when the breast cancer resistance protein 1 (B crp1) the mouse homolog of A bgc2 was knocked out.\textsuperscript{34} Fatima et al. tamoxifen treated adult A bgc2\textsuperscript{CreERT2/+} RosaLacZ/+ and found 50% of the crypts and associated villi stained positive for LacZ one month afterward, with some crypts/villi remaining blue out to 21 months. This suggests that intestinal ISC of varying lifespan express A bgc2. The division of the SP into an upper and lower portion with respective active and quiescent phenotypes is a concept already applied in the hematopoietic stem field identified a USP and LSP among HSC’s, with the USP representing a more proliferative HSC with a shorter lifespan and the LSP exhibiting quiescence, longer life span, and responsive to TGF-\textsuperscript{-2} stimulation. In their study the LSP exhibited a higher engraftment rate and contribution to peripheral blood regeneration than the USP when transplanted into lethally irradiated mice.\textsuperscript{35} While these studies from the HSC field are encouraging, there is still a significant gap to close in our understanding of the intestinal SP with regard to both therapeutic potential and its relationship to ISC physiology. I have made progress in this direction by demonstrating that SP sorting can be used to identify and collect two populations, LSP and USP, from WT mice which are enriched for quiescent and active cycling ISC subsets respectively.
Currently, the only way to identify the quiescent or reserve ISC population is with genetically engineered mice. This method may offer an alternative that should be applicable to WT mice or human tissue because of the evolutionary conservation of the biology central to the SP phenotype.


Figure 1. Representative SP histograms and verapamil validation using DyeCycle Violet (DCV) or Hoechst 33342 (Ho) vital dyes on the LSRII (DCV), CyAn (DCV), and MoFlo (Ho).
Figure 2. Locating the actively cycling cells with regard to the SP subpopulations. Adult C57/BL6 mice were injected IP with 100µg EdU. 1h post injection, jejunal epithelial cells were prepped for SP sorting and the upper SP (USP) and lower SP (LSP) were collected by FACS for reanalysis of EdU positive cells on a second pass through a flow cytometer. Whole epithelium, shown for reference, is 6 ± 1% EdU positive. The USP is 36 ± 4% EdU positive, roughly 100 fold increase over the LSP at 0.4 ± 0.04% EdU positive, n=3.
Figure 3. Tracking the Lgr5-EGFP\textsuperscript{hi} cells to the SP. (A) All jejunal epithelial cells from Lgr5-EGFP mouse are analyzed based on SP phenotype and gates are drawn. Using Summit 4.3 software, only the EGFP\textsuperscript{hi} cells (B) were gated forward onto the previously established SP gates (C), 96±2% Lgr5-EGFP\textsuperscript{hi} cells track to USP (n=3).
Figure 4. RT-PCR analysis of active and quiescent ISC mRNA markers from USP and LSP. Data are shown as fold change vs. intact jejunum. LSP (gray) fold changes are as follows: Lgr5 (0.7 ± 0.5), Ascl2 (0.9 ± 0.6), and Olfm4 (0.6 ± 0.5). Several quiescent ISC markers were analyzed: Lrig1 (4.8 ± 2.2), Bmi1 (9.2 ± 3.9), mTert (4.8 ± 1.6), Hopx (2.3 ± 1.3); while Dclk1 (0.5 ± 0.3) was de-enriched. The USP (black) was analyzed for the following active ISC markers: Lgr5 (25 ± 3), Ascl2 (47 ± 16), and Olfm4 (10 ± 2). The quiescent ISC associated transcripts examined were: Lrig1 (37 ± 15), Bmi1 (84 ± 48), mTert (39 ± 15), Hopx (13 ± 4), and Dclk1 (5.1 ± 1.7), n=3.
Figure 5. RT-PCR analysis of intestinal lineage mRNA markers from USP and LSP. Data are shown as fold change vs. intact jejunum. LSP (gray) was analyzed for the following markers SI (1.2 ± 0.1), Lyz (1.0 ± 0.9), Muc2 (2.7 ± 1.9), ChgA (17 ± 7) and Syp (4.1 ± 2.0). The same marker examination of USP (black) yielded: SI (0.4 ± 0.1), Lyz (23 ± 8.0), Muc2 (4.0 ± 1.0), ChgA (6.3 ± 1.7), and Syp (3.3 ± 1.5), n=3.
Figure 6. Quantifying the percent of enteroendocrine cells in SP subpopulations using flow cytometric analysis of synaptophysin. Percent synaptophysin positive of SP subpopulations as follows: Upper SP (0.3 ± 0.08%), Lower SP (1.8 ± 0.5%), and Non SP (0.02 ± 0.01%), n=3.
Figure 7. Validation of the synaptophysin antibody against small intestinal cells from a PYY-GFP mouse. Panel (A) is a plot of PYY-GFP cells labeled with an isotype control. Panel (B) are PYY-GFP cells labeled with an antibody against synaptophysin. Analysis demonstrated that 93.5 ± 1.5% PYY-GFP cells are synaptophysin positive, n=4.
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