Defining hierarchies of stemness in the intestine: evidence from biomarkers and regulatory pathways

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Gracz AD, Magness ST. Defining hierarchies of stemness in the intestine: evidence from biomarkers and regulatory pathways. Am J Physiol Gastrointest Liver Physiol 307: G260–G273, 2014. First published June 12, 2014; doi:10.1152/ajpgi.00066.2014.—For decades, the rapid proliferation and well-defined cellular lineages of the small intestinal epithelium have driven an interest in the biology of the intestinal stem cells (ISCs) and progenitors that produce the functional cells of the epithelium. Recent and significant advances in ISC biomarker discovery have established the small intestinal epithelium as a powerful model system for studying general paradigms in somatic stem cell biology and facilitated elegant genetic and functional studies of stemness in the intestine. However, this newfound wealth of ISC biomarkers raises important questions of marker specificity. Furthermore, the ISC field must now begin to reconcile biomarker status with functional stemness, a challenge that is made more complex by emerging evidence that cellular hierarchies in the intestinal epithelium are more plastic than previously imagined, with some progenitor populations capable of dedifferentiating and functioning as ISCs following damage. In this review, we discuss the state of the ISC field in terms of biomarkers, tissue dynamics, and cellular hierarchies, and how these processes might be informed by earlier studies into signaling networks in the small intestine.

Bmp signaling; cell fate; differentiation; intestinal stem cells; Wnt signaling

INTESTINAL EPITHELIAL STRUCTURE AND FUNCTION

The small intestine is a complex, multilayered tissue that is responsible for the processes of nutrient absorption and digestion critical for organismal survival. These layers consist of the intestinal mucosa, submucosa, muscularis, and serosa, each with a distinct cellular composition and function (Fig. 1, A and B). The intestinal epithelium is a monolayer of columnar epithelium that lines the luminal surface of the small intestine and is responsible for the bulk of digestive processes. Since the intestinal lumen is constantly subjected to mechanical stress and ingested matter that may contain potentially toxic and infectious agents, the epithelium provides an important barrier between the lumen and the body, and its integrity must be maintained in order to preserve barrier function. In addition to protecting the systemic circulation from ingested pathogens, this barrier function is also critical in preventing the commensal microflora from accessing the bloodstream. The lamina propria lies immediately beneath the epithelium and consists mainly of fibroblasts, myofibroblasts, capillaries, and lymphocytes, which support the digestive and barrier functions of the epithelium. Collectively, the epithelium and lamina propria form the intestinal mucosa. The outer layers of the small intestine, which include the submucosa, muscularis, and serosa, support the mucosa and contain the complex network of vasculature and nerves that regulate the movement of ingested matter down the gastrointestinal tract, and will not be further discussed here (8).

The intestinal epithelium is arranged along a crypt-villus axis (Fig. 1). The crypts, which exist as regularly dispersed invaginations in the epithelial monolayer, have long been appreciated as the proliferative compartment of the epithelium (105). A multipotent stem cell population, located at the base of the crypts, maintains the epithelium and drives near-total tissue renewal approximately every 7–10 days, making it one of the most proliferative tissues in the body (105). In addition to the intestinal stem cell (ISC) population, the crypts contain transit-amplifying progenitor cells (TA), which proliferate at a higher rate than ISCs, but exhibit a more restricted degree of potency and lack the ability to self-renew under physiological conditions (15). The villi, which serve as the main site of digestive function in the intestine, are made up mostly of absorptive enterocytes, with some secretory lineage cells (enteroendocrine and goblet) dispersed throughout, but at much lower numbers (11, 13, 14). The crypts are largely devoid of postmitotic, differentiated cells, with the important exception of Paneth cells, which are intercalated between ISCs at the crypt base (12, 75). Therefore, the hierarchy of proliferation in the intestinal epithelium flows from the ISCs, which divide...
The function of Paneth cells is to secrete antimicrobial peptides has not been tested experimentally (12). The canonical zone and migrate downward to the crypt base, although this been hypothesized that Paneth cells differentiate in the TA where they reside exclusively at the base of the crypts. It has long been hypothesized that Paneth cells differentiate in the TA zone and migrate downward to the crypt base, although this has not been tested experimentally (12). The canonical function of Paneth cells is to secrete antimicrobial peptides into the intestinal lumen, to prevent overgrowth of any commensal microbes present in the small intestine, as well as eliminate any ingested pathogens (18). In addition to their antimicrobial function, there is intense and growing interest in the function of Paneth cells as “niche,” or supporting, cells to the ISCs, which will be discussed in greater detail later in this review (75).

Two additional cell lineages of the small intestine epithelium are understood in less detail, and their classification into traditional “absorptive” and “secretory” lineages is controversial. The first, tuft cells, are identified by expression of COX1, COX2, and DCAMKL1, and their precise function remains unknown (28, 29). Recent studies have argued for and against classification of tuft cells as “secretory” in nature, based on conflicting results demonstrating dependence or independence of tuft cell differentiation on the transcription factor Atoh1 (Fig. 2) (7, 29). Microfold (M) cells are also poorly understood, but are known to facilitate proper immune function in the intestine by delivering ingested antigens to submucosal immune cells (50). While this function is technically “absorptive” in nature, M cells differentiate independently of the genetic programs associated with absorptive enterocytes, and recent studies demonstrate that M cell differentiation requires the hematopoietic-associated transcription factor, Spib (Fig. 2) (42). Despite the further work needed to characterize the exact functional and genetic nature of tuft and M cells, they are both known to derive from ISCs (4).

**Identification of Intestinal Stem and Progenitor Cells**

Somatic stem cells are defined by their ability to meet the dual functional criteria of “stemness”: self-renewal, or the ability to produce a daughter stem cell, and multipotency, or the ability to produce all postmitotic lineages in a given tissue. Its high rate of physiological renewal, coupled with well-defined postmitotic lineages, makes the intestinal epithelium an ideal system to study stem cell biology. Somatic stem cells are defined by their ability to meet the dual functional criteria of “stemness”: self-renewal, or the ability to produce a daughter stem cell, and multipotency, or the ability to produce all postmitotic lineages in a given tissue. Its high rate of physiological renewal, coupled with well-defined postmitotic lineages, makes the intestinal epithelium an ideal system to study stem cell biology.
attractive tissue for the study of somatic stem cell maintenance and differentiation. While the dynamics of intestinal epithelial turnover have been appreciated for decades, direct studies on ISCs were long hampered by a lack of specific genetic biomarkers. Until the recent identification of ISC biomarkers, two predominant theories existed concerning the location and properties of ISCs. Cheng and Leblond hypothesized that the crypt-base columnar cell (CBC), intercalated between Paneth cells, represented the ISC population, based on observations made by light and electron microscopy and evidence of proliferation (13, 15). An alternative hypothesis was generated based on early label-retention studies, which relied on \( [3H] \) thymidine-labeling followed by long "wash-out" periods to identify relatively slowly dividing, or label retaining cells (LRCs, hypothesized to be stem cells), that were localized mainly to the \(+4\) position, relative to the base of the crypt (65, 66). It remains somewhat controversial if label retention is driven by low rates of proliferation, or by retention of a single DNA template strand in the putative ISC, as conflicting studies have been published (22, 68). Unification of the two theories on ISC position has resulted in the general acceptance of two potential ISC populations: an "active", rapidly-cycling ISC population of CBCs, and a "quiescent" LRC or reserve ISC population located at the \(+4\) position (Fig. 3) (52). However, proving, disproving, or reconciling these theories remained a significant hurdle for the ISC field for decades, as technological limitations prevented the functional testing of stemness.

In a seminal study, the G protein-coupled receptor Lgr5 (Gpr49) became the first ISC biomarker to be validated by in vivo lineage-tracing (5). Using a targeted knock-in of an enhanced green fluorescent protein (EGFP) reporter gene linked to tamoxifen-inducible Cre recombinase by an internal ribosomal entry site (IRES), Barker et al. (5) demonstrated that Lgr5 is expressed at high levels in CBCs with low but present expression in the TA region immediately above the Paneth cells. Importantly, lineage tracing using the \( Lgr^{5EGFP-CreERT2} \) allele demonstrated that derivatives of these cells were capable of forming long-lived clonal units that contained all postmitotic lineages of the intestinal epithelium and persisted for 60 days or longer. These landmark studies established Lgr5 as a specific marker of CBC ISCs through the gold standard of in vivo lineage tracing and resulted in the rapid identification and characterization of other CBC ISC biomarkers, including Olfm4, Ascl2, and Sox9 (Table 1) (27, 31, 89, 91). Further studies demonstrated that isolated Lgr5\(^{High}\) cells were capable of forming structures containing all postmitotic lineages of the small intestine, termed enteroids, in vitro (76, 83). In addition to the obvious biological implications of these studies, the development of the \( Lgr^{5EGFP-CreERT2} \) allele was an important technological contribution to the field, as it provided the first methodology for the isolation and downstream analysis of actively cycling ISCs. One important caveat of this mouse model is that the reporter gene is expressed in a mosaic manner, with only some crypts exhibiting GFP expression, despite the fact that Lgr5 is expressed in all intestinal crypts (5).
The characterization of Lgr5 as an ISC marker was quickly followed with a “boom” in ISC biomarker discovery. In addition to the growing number of CBC ISC markers, a number of studies were published characterizing putative markers of +4 ISC4, the first of which was Bmi1. Like Lgr5, lineage-tracing experiments in Bmi1CreER4T2 mice indicated that Bmi1+ cells are capable of forming long-lived clonal units that contain all postmitotic intestinal epithelial lineages in vivo (73). However, examination of Bmi1 expression by Cre induction in a Cre reporter mouse revealed that cells expressing Bmi1 are located primarily at the +4 position, which provided the first direct evidence for a secondary, +4 ISC population in addition to the Lgr5+ CBC ISC (Fig. 3) (73, 106). Subsequent studies identified other genes associated with the +4 position that also functioned as ISC biomarkers in vivo, including mTert and Hopx (57, 85). The ErbB inhibitor Lrig1 was shown to be an ISC marker in vivo, but appears to be expressed at both the CBC and +4 positions, with conflicting reports highlighting the need for further study into the specificity of this marker (69, 104). Interestingly, while all of the +4 ISC markers were shown to mark populations with some degree of stemness under normal physiological conditions, a majority of these studies also demonstrated increased lineage tracing from these markers following irradiation damage, resulting in a proposed model in which CBC ISC4 represent an active stem cell population, and +4 ISC populations as a reserve or facultative population that activate to initiate repair following damage to the CBCs (Fig. 3) (57, 85, 106). This model is currently under intense scrutiny, due to a recent report that Lgr5High ISC4 are also radioresistant and capable of initiating epithelial repair following irradiation damage (37).

The extent to which CBC and +4 ISC populations are distinct from one another remains controversial. A recent study that analyzed transcriptomic and proteomic data from intestinal epithelial cell populations expressing variable levels of Lgr5 reported that all of the previously described +4 ISC markers were most significantly upregulated in the Lgr5High population, which was initially characterized as a CBC-specific marker (5, 60). Additionally, the study demonstrated that Bmi1 is not exclusively expressed in +4 cells, but is also present in CBCs, as well as the TA zone of the crypts (60). However, these data are subject to important caveats. The principal assumption of the study by Munoz et al. is that Lgr5High expression is restricted to CBCs, and it is upon this assumption that the authors conclude that previously characterized +4 markers are most strongly expressed in CBC ISC4. It is possible that, depending on their functional status, some +4 ISC4 may express upregulated levels of Lgr5 that are consistent with those found in CBCs. Examination of Lgr5EGFP expression reveals that some crypts do express high levels of GFP in the supra-Paneth cell position (5, 9). This leaves open the possibility that heterogeneity within the Lgr5High population could bias gene expression data aimed at determining the existence of two distinct ISC populations. Since most studies to date focus on a single ISC biomarker, future work comparing the transcriptome of putative CBC and +4 cells isolated independently of Lgr5 (e.g., Olfm4, Ascl2, Bmi1, Hopx) could provide a more detailed understanding of the distinct and overlapping genetic characteristics of these populations.

Our group previously demonstrated that low levels of Sox9 expression are associated with actively cycling CBCs (25, 31). A Sox9EGFP BAC transgenic mouse was used to isolate cellular populations based on levels of EGFP expression, which faithfully recapitulated expression of the endogenous mRNA and protein (25). Based on gene expression studies, we demonstrated that the Sox9abeled population correlated with markers of TA cells; the Sox9low population correlated with markers of actively cycling CBCs; and the Sox9High population correlated with markers of enteroendocrine cells, as well as previously described +4 ISC markers, including Bmi1, Hopx, and mTert.

### Table 1. Intestinal stem cell markers and their characteristics

<table>
<thead>
<tr>
<th>Marker</th>
<th>Type</th>
<th>Associated ISC Population</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Lgr5</td>
<td>genetic</td>
<td>CBC</td>
<td>5</td>
</tr>
<tr>
<td>Olfm4</td>
<td>genetic</td>
<td>CBC</td>
<td>91</td>
</tr>
<tr>
<td>Ascl2</td>
<td>genetic</td>
<td>CBC</td>
<td>91</td>
</tr>
<tr>
<td>Smoc2</td>
<td>genetic</td>
<td>CBC</td>
<td>60</td>
</tr>
<tr>
<td>Troy</td>
<td>genetic</td>
<td>CBC</td>
<td>23</td>
</tr>
<tr>
<td>Sox9</td>
<td>genetic</td>
<td>CBC (Sox9high), +4/reserve</td>
<td>1, 25, 27, 31, 96</td>
</tr>
<tr>
<td>Bmi1</td>
<td>+4/reserve</td>
<td>60, 73, 106</td>
<td></td>
</tr>
<tr>
<td>Lrig1</td>
<td>+4/reserve</td>
<td>60, 69, 104</td>
<td></td>
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<tr>
<td>Hopx</td>
<td>+4/reserve</td>
<td>60, 85, 96</td>
<td></td>
</tr>
<tr>
<td>Tert</td>
<td>+4/reserve</td>
<td>57, 60</td>
<td></td>
</tr>
<tr>
<td>Label</td>
<td>functional</td>
<td>+4/reserve</td>
<td>9</td>
</tr>
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ISC, intestinal stem cell.
(unpublished data and Refs. 25, 31). Furthermore, we demonstrated that enteroid-forming capacity in vitro was restricted to the Sox9<sup>low</sup> population, consistent with the initial reports for Lgr5<sup>High</sup> cells (31). Compellingly, our gene expression data support a model in which CBC ISCs are distinct from +4 ISCs. That is, when isolating crypt populations based on Sox9<sup>GFP</sup> expression, as opposed to Lgr5<sup>GFP</sup> expression, the population expressing the highest levels of CBC ISC markers (Ascl2, Olfm4, and Lgr5) is genotypically distinct from the population expressing the highest levels of +4 ISC markers (Bmi1, mTert, and Hopx). Subsequent independent studies supported this observation, and demonstrated separation of CBC and +4 ISC marker genes between Sox9<sup>Low</sup> and Sox9<sup>High</sup> populations by RNA microarray, which also demonstrated differential clustering of the two populations by principal component analysis (PCA) (96). Compellingly, these studies also demonstrated that Sox9<sup>High</sup> cells were capable of forming enteroids in vitro following irradiation damage and partial gastrointestinal regeneration, consistent with Sox9<sup>High</sup> cells representing a reserve or facultative ISC population, the role classically attributed to the +4 ISC (96). Together, the emerging data on ISC biomarker and reported similarities and differences between CBC and +4 ISCs support a much more complex model for stemness and potency in the intestinal epithelium than originally postulated.

STEM CELL PLASTICITY AND INTERCONVERSION BETWEEN CBC AND +4 ISCS

Due to the complexities of reconciling myriad ISC biomarker studies, each focused on individual biomarkers, there has been intense interest in generating a unifying theory for how CBC and +4 ISCs contribute to maintenance of the intestinal epithelium, and if they function independently or in a cooperative manner. There is reasonable evidence that interconversion between CBCs and +4 ISCs occurs on a semiregulated basis under physiologically normal conditions, as Hopx+ +4 cells are capable of giving rise to Lgr5<sup>High</sup> CBCs both in vivo and in vitro (85). To further support the concept of ISC interconversion and distinct CBC and +4 populations, Tian et al. (86) demonstrated that Lgr5-negative, Bmi1+ cells were capable of maintaining intestinal epithelial homeostasis following complete ablation of Lgr5+ cells. The group developed a novel Lgr5<sup>EGFP</sup> allele that also expressed the diphtheria toxin receptor (DTR). Importantly, this model exhibited constitutive expression of the knock-in gene across all crypts, unlike the mosaicism of GFP expression seen in the original Lgr5<sup>EGFP</sup> model developed by Barker et al. (5, 86). The authors noted rare lineage tracing from Bmi1+ cells under physiological conditions, consistent with previous reports (73, 86). When mice were injected with diphtheria toxin, all Lgr5+ cells underwent apoptosis, and the number of lineage tracing events from Bmi1+ cells increased substantially (86). Surprisingly, there were no obvious defects in proliferation and differentiation following full ablation of Lgr5+ cells, although studies were limited to 5 days, due to organ failure in other tissues dependent on Lgr5+ populations (86). This study was the first definitive evidence that intestinal epithelial homeostasis is not dependent on the CBC ISC, and supported the existence of multiple populations with stemness potential in the intestinal crypt. The same mouse model was recently used to demonstrate that intestinal tumorigenesis in the APC<sup>min</sup> mouse model can occur in the absence of Lgr5+ ISCs, suggesting that the observed plasticity of normal ISC populations is conserved in cancer (56). Paradoxically, further studies using the Lgr5<sup>EGFP-DTR</sup> allele demonstrated that cells expressing Lgr5 are required for postirradiation regeneration and repair, as mice with ablated Lgr5+ cells failed to recover from gastrointestinal syndrome caused by high, but sublethal, doses of irradiation (56). At low doses of irradiation, Lgr5+ ISC-depleted crypts were able to regenerate similar to controls. In agreement with Potten’s initial observations of +4 cell behavior, this led the authors of the study to conclude that “+4/reserve” ISCs are highly radiosensitive and therefore not competent to restore epithelial function following high doses of radiation exposure (56, 64). Further experiments are needed to determine if regenerative failure following Lgr5+ cell ablation is mediated through a given population of ISCs, or whether the LGR5 receptor itself, which is known to facilitate proliferative signaling by R-spondins, is a critical genetic factor in intestinal regeneration (10, 20).

As an alternative explanation to the existence of two distinct ISC populations, emerging evidence is suggesting that the +4 population does not represent bona fide ISCs, but rather one or more populations of TA progenitor cells capable of dediffer-entiating and adopting stem cell fate following damage. Early studies theorized that TA cells may be able to function as ISCs, and recent experiments have begun to support these hypotheses (15, 64). In a recent study, the Notch ligand Dll1 was shown to mark a population of TA cells, concentrated around the +5 position (94). Under physiological conditions, lineage tracing from Dll1+ cells produced small, short-lived clones that contained secretory lineage cells, demonstrating that Dll1 marks TA cells committed to secretory differentiation (94). However, when Dll1 lineage tracing was conducted following exposure to irradiation damage, the cells were able to function as ISCs, producing long-lived clones that contained both secretory and absorptive lineage cells (94). Labeling of Dll1+ cells immediately before irradiation produced these long-lived clones, but labeling 5 or more days prior to irradiation failed to produce any significant lineage tracing events (94). This final observation revealed another important characteristic of this facultative Dll1 population, in that it is short-lived, and the window of time between cell “birth” and differentiation in which Dll1+ cells can dedifferentiate is limited to less than 5 days. While informative, this study did not address the possibility of a long-lived LRC that was capable of acting as an ISC.

A central problem in studying the functional output of the putative quiescent LRC, or +4 ISC, lies in the fact that label-retaining methods rely on the incorporation of thymidine analogs into DNA during S-phase. Unfortunately, all of these techniques require detection methods that must be carried out on fixed cells or tissue, precluding the ability to isolate LRCs for downstream culture and gene expression analysis. Furthermore, despite rapid and substantial ISC biomarker discovery, the overlapping expression and conflicting reports of ISC biomarker specificity resulted in no specific genetic signature that could be used to isolate LRCs. In a recent landmark study, Buczacki et al. (9) employed an elegant strategy to genetically label LRCs through the use of a Cyp1a1-H2B<sup>YFP</sup> allele. In this model, expression of YFP is induced in all cells of the intestinal epithelium by injection of β-naphthoflavone (βNF), followed by a “washout” period, during which the YFP label is

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lost in short-lived, proliferating cells through multiple rounds of division and migration. The end result is retention of YFP expression exclusively in long-lived cells, effectively recapitulating label retaining studies that relied on thymidine analogs. Buczacki et al. (9) found that the majority of LRCs were localized to the +3 position following two weeks of post-induction washout. Interestingly, gene expression analysis of these cells revealed that while they expressed secretory lineage markers, they also shared significant overlap in highly expressed genes with Lgr5/H11001 cells, and in vitro culture demonstrated that they were capable of forming enteroendocrine cells in vivo (9). In order to meet the "gold standard" of in vivo lineage tracing to demonstrate stemness, the authors developed a novel mouse model, in which one-half of a dimerizable Cre recombinase was induced by βNF in the same manner as in the Cyp1a1-H2BYFP model ("induced Cre"), and the other half was constitutively expressed (constitutive Cre). The induced Cre was then present, but inactive, in LRCs following βNF administration and a washout period. Administration of a dimerizing agent, AP20187, caused the induced Cre (now expressed only in LRCs) to complex with the constitutive Cre in order to induce recombination of a reporter allele (9). Using this model, which allowed the authors to lineage trace from functionally identified LRCs in the absence of genetic biomarkers, it was shown that LRCs were capable of acting as ISCs following irradiation damage, but not under normal conditions (9). Together with evidence from Dll1 studies, these results suggest that there are at least two populations of TA progenitors that have secondary potential as facultative ISCs: one that is short-lived (Dll1+), and one that is long-lived (LRCs). However, the comprehensive genetic signatures of these populations and the degree to which they overlap with populations identified by single canonical ISC biomarkers (Lgr5, Ascl2, Olfm4, Bmi1, Hopx, mTert, Lrig, Sox9) remains unknown. Further research is needed into the functional role of ISC biomarkers in potency and stemness, and may shed light into the cellular dynamics of ISC biology. These emerging data in the ISC field increasingly support a model of population asymmetry, also referred to as neutral drift (54, 81). In population asymmetry, stem cells divide stochastically, resulting in asymmetric division or symmetric division, producing either two stem cells or two differentiated progeny (Fig. 4C) (45). To establish this experimentally, Snippert et al. generated a novel Cre reporter transgene, called R26-Confetti, based on the Thy1Brainbow allele. In both R26Confetti and Thy1Brainbow mice, expression of Cre recombinase results in the removal of a floxed stop sequence preceding a number of different fluorescent protein reporter cassettes (53, 81). Opposing flox sequences flank these cassettes, such that Cre activity results in the random expression of a single fluorescent reporter. In the R26Confetti model, cells that undergo recombination can be labeled by GFP, RFP, YFP, or BFP (Fig. 4A) (81). Importantly, as with lineage tracing from a single color reporter, all progeny of these cells will express the same color as their parent cell. To test the kinetics of ISC self-renewal, Lgr5/EGFP-CreERT2 mice were crossed to the R26Confetti allele, lineage tracing was initiated, and expansion of clonal units (independently identified by color differences) was observed at different time points following induction (81). Under the assumptions of the invariant asymmetry hypothesis, each ISC should expand clonally at approximately the same rate, such

FATE DECISIONS AND CLONAL EXPANSION OF INTESTINAL STEM CELLS

In addition to efforts focused on the identification and characterization of ISC biomarkers, there is also a strong interest in understanding the proliferative dynamics and clonal behavior of ISCs. As stemness is characterized by a cell’s ability to self-renew and differentiate in a multipotent manner, the clonal behavior of a single ISC is the functional outcome of these processes as they pertain to the production and maintenance of a given tissue area. The survival of an ISC clone is entirely dependent on the ability of the parental ISC to self-renew and produce daughter ISCs that can continue to proliferate, differentiate, and maintain the epithelial monolayer. Since inadequate or excessive self-renewal could theoretically lead to a compromised epithelium or tumorigenesis, respectively, the cellular dynamics underlying ISC self-renewal and differentiation are critical in understanding intestinal epithelial homeostasis and disease. The classical model for self-renewal is invariant asymmetry, in which ISCs consistently divide to give rise to one progenitor cell, which will undergo one or more cycles of division and terminal differentiation; and one stem cell, which will serve the purpose of replacing the parent cell and maintaining homeostasis of the stem cell population (Fig. 4B). This model was proposed as the mechanism for self-renewal in ISC populations, largely based on theoretical models of tissue renewal and maintenance of the stem cell pool (67). Potten argued against the term “self-renewal,” in that it could imply expansion of stem cell numbers, and instead employed the term “self-maintenance” to describe the production of new stem cells without an overall net change in stem cell numbers (67). Additionally, Potten supported his model of invariant asymmetry by arguing that expansion of ISC numbers by symmetric division, while a feasible possibility in the context of tissue regeneration following damage, would be impractical under physiological conditions, as it would lead to aberrant tissue growth and, perhaps, tumorigenesis (67). It was acknowledged that an alternative model could exist, in which some ISCs divide symmetrically to produce two daughter ISCs. This expansion could exist in homeostatic conditions under which the symmetric division of other ISCs toward differentiated lineages compensated for it, although Potten argued that this stochastic process would be subject to random and aberrant tissue loss and/or expansion since random shift in ISC population dynamics could be difficult to balance (67). Since these theories about ISC population dynamics and self-renewal were formed long before established ISC biomarkers, they remained impossible to test for some time.

Taking advantage of the validated Lgr5 biomarker, recent studies have tested Potten’s hypothesis regarding invariant asymmetry and, surprisingly, demonstrated that the kinetics associated with clonal expansion driven by ISCs support a model of population asymmetry, also referred to as neutral drift (54, 81). In population asymmetry, stem cells divide stochastically, resulting in asymmetric division or symmetric division, producing either two stem cells or two differentiated progeny (Fig. 4C) (45). To establish this experimentally, Snippert et al. generated a novel Cre reporter transgene, called R26-Confetti, based on the Thy1Brainbow allele. In both R26Confetti and Thy1Brainbow mice, expression of Cre recombinase results in the removal of a floxed stop sequence preceding a number of different fluorescent protein reporter cassettes (53, 81). Opposing flox sequences flank these cassettes, such that Cre activity results in the random expression of a single fluorescent reporter. In the R26Confetti model, cells that undergo recombination can be labeled by GFP, RFP, YFP, or BFP (Fig. 4A) (81). Importantly, as with lineage tracing from a single color reporter, all progeny of these cells will express the same color as their parent cell. To test the kinetics of ISC self-renewal, Lgr5/EGFP-CreERT2 mice were crossed to the R26Confetti allele, lineage tracing was initiated, and expansion of clonal units (independently identified by color differences) was observed at different time points following induction (81). Under the assumptions of the invariant asymmetry hypothesis, each ISC should expand clonally at approximately the same rate, such.
that each clone always contains a single ISC and persists over a long period of time to produce labeled, differentiated progeny. In this model, the number of clones observed at early time points postinduction would be equivalent to that observed at late time points, and all clones would expand at approximately the same rate (Fig. 4B). Surprisingly, Snippert et al. (81) observed a decreasing number of clones over time, with increasing diversification of clone size (Fig. 4C). Through the application of mathematical modeling, the authors showed that the dynamics of ISC clone expansion followed neutral drift, an observation that was independently confirmed by another group that conducted similar experiments using a different genetic model for clone expansion (54, 81). These data are in direct conflict with Potten’s model of invariant asymmetry, and instead demonstrate that stochasticity in self-renewal is the predominant mode of ISC maintenance (67). Although some stem cells are lost to differentiation under this model, the net outcome of all stem cell divisions is the same as it would be under invariant asymmetry and is sufficient to maintain homeostasis in the stem cell pool. In the absence of the present experimental validation of neutral drift ISC dynamics, logic would follow Potten’s original postulation that stochasticity in ISC self-renewal seems impractical (67). However, despite seemingly random expansion and loss of ISC clones, there appears to be no pathological consequences to intestinal epithelial homeostasis. Therefore, rather than behaving in a manner consistent with cell autonomous asymmetry, ISCs exhibit the property of population asymmetry, in that the net outcome of all stochastic symmetric and asymmetric divisions is such that the overall number of ISCs in the intestinal epithelium remains at equilibrium. In further support of this model, a recent study has demonstrated that only a minority of ISCs identified by the expression of ISC markers actually exhibits functional stemness in vivo (49). Clonal populations were observed through the use of a mutation-based reporter mouse, and were labeled in response to DNA-replication specific frameshift mutations in a microsatellite repeat region placed upstream of a reporter gene. Unlike lineage tracing studies reliant on Cre recombinase, this “continuous labeling” was not subject to caveats of marker specificity or Cre induction, and revealed that there are approximately 5–7 functional ISCs per crypt unit (49). These data provide experimental validation of the self-limiting nature of population asymmetry in the intestinal epithelium.

While these recent studies reveal that ISCs undergo clonal expansion patterns that are consistent with population asymmetry and neutral drift, it remains unproven whether the processes leading to this behavior are cell-autonomous or entirely influenced by the stem cell niche, the complex network of support cells that may provide fate-deterministic signals to ISCs. Although niches have been well described in other stem cell populations, especially in the hematopoietic system, little is known about the ISC niche (59, 82). Recent studies on the interaction between Paneth cells and ISCs have suggested that the former may provide critical mitogenic and morphogenic signals to the latter and greatly enhance ISC survival and...
growth in vitro (75). This observation provides proof of principle that extrinsic factors can, at the very least, significantly modulate ISC behavior.

REGULATION OF PROLIFERATION AND DIFFERENTIATION IN THE INTESTINAL STEM CELL NICHE

Although much of our current knowledge surrounding molecular signaling networks in the intestinal epithelium precedes the advent of ISC biomarkers, there is a considerable amount of data concerning the various pathways that regulate proliferation and differentiation in the intestinal epithelium. The most substantially studied pathways pertaining to intestinal epithelial proliferation and differentiation are Wnt, Notch, and Bmp (16). The molecular functions of these pathways are reviewed at great length elsewhere, and will be summarized here (17, 19, 62).

Wnt signaling. The Wnt signaling pathway is one of the most comprehensively studied pathways and provides many of the critical proliferative signals to stem and progenitor cell populations throughout a wide range of cell populations and tissues, both during development and in adult organisms. Canonical Wnt signaling occurs when WNT ligands interact with FZD or LRP receptors. This ligand-receptor interaction stabilizes the cytoplasmic protein β-catenin, which is bound to a destruction complex consisting of APC, GSKβ, AXIN, DVL, and CK1, which targets it for ubiquitination and degradation by the proteasome. Receptor activation sequesters the destruction complex through direct interaction with LRP, and allows the accumulation of unbound β-catenin in the cytoplasm. This free β-catenin then translocates to the nucleus, where it derepresses the transcription factor TCF, and recruits myriad cofactors to activate context-dependent targets, generally associated with cellular growth and proliferation (reviewed in Ref. 17). In addition to canonical Wnt signaling, noncanonical Wnt signaling can be activated by WNT ligands, but induces downstream effects that are unique and independent from stabilization of β-catenin (84). Because proliferation is an important steady-state process in the intestinal epithelium, the Wnt pathway is especially active throughout adult life and has been the subject of many studies aimed at understanding ISC biology. Additionally, mutations in the Wnt pathway are common across a wide range of cancers. Inactivating mutations in Apc are one of the most common mutations found in colorectal cancer (CRC), being detected in 85% of sporadic CRCs, and resulting in the aberrant accumulation of β-catenin due to defective destruction complexes (48, 55, 58, 61).

Numerous studies have demonstrated that WNT is essential for the maintenance of ISC populations, and genomic analyses of the effects of disruption in Wnt signaling were the basis for early ISC biomarker discovery, as many ISC biomarkers are downstream targets of WNT/β-catenin (84). Because proliferation is an important steady-state process in the intestinal epithelium, the Wnt pathway is especially active throughout adult life and has been the subject of many studies aimed at understanding ISC biology. Additionally, mutations in the Wnt pathway are common across a wide range of cancers. Inactivating mutations in Apc are one of the most common mutations found in colorectal cancer (CRC), being detected in 85% of sporadic CRCs, and resulting in the aberrant accumulation of β-catenin due to defective destruction complexes (48, 55, 58, 61).

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Interestingly, the authors note that transition from endoderm to intestinal epithelium occurs normally in both wild-type and Tcf7l2−/− mice at E14.5, demonstrating that Wnt signaling is required for the establishment of ISCs and TAs, but dispensable for the formation of a specified epithelium (47). Later studies examined the role of Tcf1, Tcf3, and Tcf4 in adult murine intestine using conditional, floxed alleles, and demonstrated that loss of Wnt signaling through Tcf results in the loss of Lgr5/1Ofm4+ ISCs in adult intestine (92). In addition to the establishment of intestinal crypts, it was shown that Wnt signaling is required for CRC cell proliferation through the expression of dominant-negative TCF1 and TCF4 in CRC cell lines (88). Inducible expression of the dominant-negative proteins was used to show that loss of functional TCF was due to decreased TCF4 signaling causing a decrease in C-MYC and subsequent derepression of p21WAF1, a cyclin-dependent kinase (CDK) inhibitor (88). Importantly, these studies identified a number of direct genetic targets of Wnt signaling, which were in turn associated with ISC identification and function.

Extending these findings toward the identification of broader genetic programs underlying the formation of ISCs, the Clevens lab (90) conducted gene expression microarray on human colonic adenomas, which are known to have increased levels of Wnt signaling, and normal human colon, as well as several CRC cell lines expressing dominant-negative forms of TCF4. By comparing these analyses and localizing anatomical expression of significantly regulated genes to the crypt base, the authors generated a list of 17 genes that were regulated by Wnt signaling and represented a putative ISC signature (90). Included in this list were Lgr5 and Ascl2, which are now validated markers of active CBC ISCs (90).

Further studies by the Clevens group demonstrated that, in addition to its role in ISC establishment and proliferation, Wnt signaling exerts an influence on cell position in the crypts. Dominant-negative TCF experiments by van de Wetering et al. (6, 88) demonstrated that Wnt signaling positively regulated the expression of EphB receptors, while negatively regulating the expression of their ligands, ephrinB. In normal tissues, the Eph-ephrin interactions result in repulsive forces that effectively “sort” cells into discreet compartments. While EphBs and ephrinBs were expressed as an opposing gradient in the intercellular regions of normal embryonic intestines and adult intestinal crypts, they were absent in Tcf7l2−/− embryonic intestines (6). Genetic or pharmacological disruption of EphB expression in the intestine resulted in the displacement of Paneth cells, as well as a decrease in crypt proliferation, and less clearly separated proliferative and differentiated zones between the crypts and villi (6, 34).

Recent studies have demonstrated that multiple sources in the intestinal epithelium produce WNT ligands, including the Paneth cells and mesenchymal cells of the lamina propria (24, 75). Paneth cells have been shown to produce sufficient niche signals, including WNT3, to support the growth of isolated ISCs in vitro, but appear to be dispensable for ISC survival in vivo (21, 43). A series of in vitro coculture experiments demonstrated that Wnt signaling can also be supplied to ISCs by intestinal subepithelial myofibroblasts (ISEMFs), providing a partial explanation for the persistence of ISCs in the absence
of Paneth cells (24). Interestingly, Wnt signaling also appears to drive differentiation of Paneth cells, an unexpected result since Wnt/β-catenin is most often associated with proliferative cell populations (24, 93). Several Paneth cell genes are dependent on Tcf4 signaling, and proper Paneth cell differentiation and localization to the crypt base is dependent on expression of the WNT receptor Fzd5 (93). Together, these data demonstrate that WNT provides a significant mitogenic and morphogenic signal in the intestinal epithelium that is critical to ISC survival and proliferation. Additionally, Wnt signaling works in a highly context-dependent manner, directing ISC self-renewal and proliferation, while simultaneously controlling Paneth cell differentiation, and cell position in the crypt niche. Wnt signaling appears to arise from cells that constitute the ISC niche, including ISEMFs and Paneth cells, suggesting that dynamic crossstalk between the intestinal epithelium and its underlying mesenchyme is critical for controlling ISC self-renewal and differentiation.

**Bmp signaling.** While much less is known about the role of Bmp signaling in the intestinal epithelium, evidence suggests that this pathway provides prodifferentiation cues that serve as a counterbalance to Wnt-induced proliferation. BMP proteins belong to the TGF-β superfamily, and act by binding to type II and type I serine/threonine kinase receptors, which form heteromeric complexes and activate downstream transcriptional effectors belonging to the Smad family (39). Mutations in the Bmp pathway, both at the receptor (BMPR1A) and effector (SMAD4) level, are associated with juvenile polyposis syndrome, a heritable disorder that predisposes individuals to gastrointestinal cancer (35, 36). In the intestine, Bmp4 is strongly expressed by the mesenchymal cells immediately underlying the epithelium, suggesting that these stromal cells play an important role in the induction of epithelial differentiation (32). As expected, Bmp4 is most strongly expressed in the villus mesenchyme, with only a few ISEMFs expressing the transcript in the crypt mesenchyme (32, 33). One of the most compelling studies demonstrating the role of Bmp signaling in the intestinal epithelium employed the use of transgenic mice that overexpressed Noggin in epithelial cells (32). TGF-β inhibitory proteins, such as Noggin, can negatively regulate Bmp signaling. Upon overexpression of Noggin in the epithelium, Bmp4 signals from the mesenchyme were effectually inactivated, as measured by a reduction in SMAD phosphorylation, a downstream effect of Bmp4 signaling (32). The striking physiological consequence of this inhibition was the de novo formation of crypts along the length of the intestinal villi, which the authors likened to the hyperplastic growth observed in patients with juvenile polyposis syndrome (32). Further studies examined the role of Bmpr1a in the intestinal epithelium through genetic ablation. Surprisingly, while these studies produced defects that included increased epithelial proliferation and decreased differentiation that eventually led to a polyposis phenotype, they did not phenocopy the severe de novo crypt phenotype observed by Haramis et al. (3, 32). Together, these studies suggest differential effects for epithelial (Bmpr1a) and nonepithelial (Noggin) sources of Bmp signaling during tumorigenesis. Alternatively, the severe phenotypes associated with clinical polyposis disorders may be the result of multiple mutations in the Bmp signaling pathway, which plays a major role in regulating intestinal epithelial proliferation through the induction of differentiation (3, 32, 33, 35, 36). Genetic evidence indicates that the opposing regulatory elements of Wnt and Bmp signaling may act through extensive crosstalk, as a number of loci containing regulatory response elements for both Wnt (Tcf4/Lef) and Bmp (Smad) have been identified (38). Ablation of Bmpr1a also results in a PTEN/AKT-mediated increase in nuclear β-catenin, demonstrating that Bmp signaling can negatively regulate canonical Wnt signaling (33).

**Notch signaling.** In addition to the relatively long-range cell-cell signaling provided by Wnt and Bmp pathways in the intestinal epithelium, Notch provides localized signals that regulate cell fate and differentiation through direct cell-cell contact. Notch receptors and their ligands, which belong to the Delta/Serrate/Lag-2 (DSL) family, are both expressed on the cell surface and, unlike Wnt and Bmp signaling, require direct contact between neighboring cells to induce regulatory responses (reviewed in Ref. 62). Interaction of Notch receptors and DSL ligands induces the release of the Notch intracellular domain (NICD) through cleavage by ADAM family metalloproteases and gamma-secretase proteases, which allows NICD to localize to the nucleus and interact with cofactors to induce transcriptional responses (62). In addition to trans signaling, Notch receptors and ligands expressed on the same cell exhibit cis inhibition through unknown mechanisms (62). Cis inhibition is thought to preserve the integrity of the trans Notch signal, by ensuring that both neighboring cells do not activate the downstream pathway.

In the intestinal epithelium, Notch signaling is mainly restricted to the crypts, which express Notch1 and Notch2 receptors, as well as Dll1, Dll4, and Jag1 ligands (77). Notch1/2 receptors are associated with ISCs, and appear to play important roles in ISC maintenance (26). Ablation of Notch signaling in the intestine, through genetic deletion of the Rhyp transcriptional cofactor, or pharmacological inhibition of γ-secretase, which acts upstream of NICD, results in a complete loss of ISCs, and conversion of a majority of crypt cells into differentiated Goblet cells (95). However, there appears to be some redundancy in Notch receptors and ligands, as dual deletion of Notch1 and Notch2 is required to convert proliferating cells to secretory lineages, and dual deletion of Dll1 and Dll4 is required to achieve the same effect through modulation of ligand expression (63, 71). These results point to a role for Notch signaling in ISC maintenance as well as secretory differentiation.

Indeed, active Notch signaling results in the expression of the target gene Hes1, the loss of which results in increased secretory lineage differentiation and decreased production of absorptive enterocytes (40). Loss of Hes1 can be partially compensated for by Hes3 and Hes5, and loss of all three intestinal Hes genes results in decreased proliferation, but does not fully ablate ISCs (87). Taken in context of the conversion of all proliferating cells to secretory fates following broad ablation of Notch signaling, these data suggest that other Notch targets may sustain ISC cell fate, while Hes1 is more important for absorptive lineage commitment. Olfm4, which is a CBC ISC marker, is also a target of Notch signaling and may be a candidate Notch-regulated effector of ISC fate (97). In addition to participating in absorptive fate specification, expression of Hes1 also results in the repression of transcription factor Atoh1, which drives cells toward enteroendocrine, goblet, or Paneth cell secretory fates in the absence of Notch signaling.
(40, 107). Loss of Atoh1 results in defective secretory lineage differentiation, and represents a reciprocal phenotype compared with loss of Hes1, strengthening the evidence for a balanced interaction between the two pathways in normal intestinal physiology (107). Therefore, the repression of Notch through cis inhibition or pharmacological intervention results in early specification toward a secretory cell fate. Through this mechanism, Notch may provide contact-dependent regulation of ISC numbers in the intestinal crypts, by preventing two neighboring cells from adopting ISC fates. Interestingly, Paneth cells, which are intercalated between CBC ISCs, express DSL ligands, suggesting that they may contribute to the establishment of an ISC niche by providing trans-activation of Notch in ISCs (75). Additionally, cis inhibition by receptor/ligand interaction may help maintain or establish Paneth cell fates. When taken in context of the newfound understanding of neutral drift dynamics in the intestinal epithelium, earlier studies on Notch signaling in the intestine may shed light onto how the epithelium is capable of maintaining an appropriately sized ISC pool despite seemingly stochastic expansion and loss of clones.

STUDYING INTESTINAL STEM CELLS IN VITRO

The inability to culture primary intestinal tissue, including ISCs, was a significant hurdle in the field of intestinal biology for decades. Due to the lack of an in vitro model system for studying ISCs, much of the early data regarding molecular signaling in the intestine relied on expensive and time-consuming in vivo mouse models, or CRC cell lines, which often fail to accurately recapitulate physiologically relevant properties. However, the increased understanding of signaling networks in the intestinal epithelium led to the development of a novel, three-dimensional culture system for intestinal crypts and primary-isolated ISCs (76). The culture conditions rely on the extensive use of small molecules and growth factors targeting the Wnt, Bmp, and Notch pathways. While originally established for murine small intestinal epithelium, these conditions have now been adapted and expanded to support the growth of murine colon and single colonic stem cells, human small intestine and colonic crypts and stem cells, murine stomach, and adenomatous tissue (30, 41, 74, 76). Single isolated ISCs are capable of producing complex, multicellular organoids without the need for coculture with ISEMFs (76). These long-lived structures, termed enteroids, produce cryptlike proliferative buds and contain all of the postmitotic lineages found in the intestinal epithelium (76, 83). Significant modifications to the original growth factor cocktail used to support ISC growth has resulted in increased survival rates that potentiates expanded in vitro studies (101).

The ISC culture system is an invaluable technology that has been applied to elegant in vitro studies clarifying and supporting observations made in vivo. Our group recently used the system to demonstrate that specific levels of the transcription factor Sox9 are associated with ISCs, TAs, and enteroendocrine cells (31). While in vivo lineage tracing remains the gold standard for establishing the stemness of cellular populations, the in vitro assay adds an additional level of precision for genes like Sox9, which exhibit broad but variable expression levels, in that lineage tracing is unable to distinguish between cell types based on gene expression level. Since the in vitro conditions result in the production of multipotent enteroids that can be maintained through multiple rounds of serial passaging, they provide an alternative platform that can be used to establish the stemness criteria of multipotency and self-renewal in isolated cells (31, 76).

Additionally, the culture system has been used to demonstrate that Paneth cells are capable of supporting the development of ISCs into enteroids in vitro, suggesting that Paneth cells constitute a niche cell for ISCs (75). These experiments were in turn used as a basis to assess the effect of calorie restriction on ISC-niche interactions, which demonstrated that decreased caloric intake correlated with an increased ability of Paneth cells to promote enteroid formation from isolated ISCs (108). However, while the conventional system has facilitated a handful of functional experiments examining ISC biology, significant problems prevent it from being applied to elegant molecular and genetic assays (75, 96, 97). For one, simple quantification of ISC development into enteroids is significantly hindered by the three-dimensional nature of the system and the tendency of cells to move through the extracellular matrix and fuse to form single enteroids from multiple cells (75). Additionally, Paneth cell co-culture experiments provide proof of principle that non-ISC cell types can considerably influence ISC behavior in vitro, raising significant concerns about cell-cell signaling in ISC culture assays and highlighting the need for advanced technology aimed at studying ISCs in vitro (75). Despite these limitations, the development of an in vitro culture system for ISCs has provided the field with a critical tool for examining and modulating the effect of extrinsic signaling on ISC stemness and differentiation.

BIOMARKERS AND SIGNALING NETWORKS, BENCH TO BEDSIDE

The recent growth in understanding of ISC signaling networks, cellular dynamics, and biomarkers has driven considerable effort to translate these basic science findings into clinically applicable therapies. As a first step toward bringing ISC-focused therapies to clinic, several recent studies have utilized the groundwork laid by ISC biomarker discovery in mouse models to identify, isolate, and culture intestinal crypts and ISCs from human tissue (30, 74, 101). Human ISCs have been isolated from adult tissues using a combinatorial surface marker approach, based on previous observations in mice demonstrating enrichment for functional Lgr5+ ISCs in cell populations FACS-sorted for CD24 (31, 99). Interestingly, in human intestinal epithelium, LGR5 is most significantly enriched in CD44+/CD24− or CD44+/CD24−/CD166+ populations, which also demonstrate functional stemness in basal culture conditions (30, 101). CD44+/CD24+ populations are most significantly enriched for HOX, express lower levels of LGR5, and do not form enteroids unless cocultured with ISEMFs (30). These data suggest that ISC populations may be more phenotypically distinct in humans than mice, and warrant further investigation to determine the clinical relevance of these observations and identify possible therapeutic targets in different human cell populations with ISC potential.

One of the most exciting potential applications of ISC-based clinical therapy is the opportunity to replace diseased or necrotic epithelium with healthy donor epithelium that can be expanded in vitro from a small amount of source material.
While not yet demonstrated using human donor tissue or small intestinal stem cells, studies in mice have expanded single \Lgr5^{+}\ colonic stem cells in vitro and successfully grafted the donor epithelium into damaged colons. Once transplanted, the donor tissue was able to form functional crypt units that demonstrated stability for greater than 6 mo (109). Organ-on-a-chip technologies have also recently been used to expand colonic epithelium on microfabricated scaffolds in vitro, laying the foundation for biomimetic platforms as sources of engineered epithelium that can be used for transplantation-based therapy (2, 102, 103).

In addition to direct applications to gastrointestinal disease, advances in intestinal epithelial culture and genetic manipulation have the potential to facilitate patient-specific diagnostics and experimental medicine. The Clevers group has established several different techniques for modulating gene expression in enteroids in vitro, including viral transfection and insertion of large BAC transgenes (46, 78). Using these techniques, the group recently demonstrated CRISPR/Cas9-mediated correction of the \textit{CFTR} locus in rectal enteroids derived from patients with cystic fibrosis (79). This study provided important proof of principle for the combination of cutting edge genomic engineering methodology and primary ISC/enteroid culture.

**NEXT STEPS: TURNING TO FUNCTIONAL EVIDENCE TO UNDERSTAND ISC HIERARCHIES**

A rapid expansion of knowledge in the ISC field over the past 5–10 years has been driven largely by an increase in the tools available to identify and isolate ISCs. Further efforts are required in order to understand the precise molecular function of the newly characterized ISC markers and may also shed light onto the association of these markers with similar or distinct populations with ISC potential. Given the number of newly discovered ISC biomarkers, determining the phenotypic characteristics of their associated populations also remains as a significant hurdle in the field. As opposed to classical views of ISC hierarchies, which focused mainly on active and “quiescent” populations, emerging evidence suggests that stem cell potential is a context-dependent characteristic shared between many phenotypically distinct cell populations in the intestinal crypts. Shifting ISC potential may be guided largely by extrinsic signaling from the niche, and a greater understanding of the cell types and signaling components that comprise the niche will be critical in furthering our knowledge of ISC behavior in physiology and disease. Compelling studies in the hair follicle niche have demonstrated that stem cell position can dictate whether or not a hair follicle stem cell will contribute to a long-lived clonal unit, providing validated proof of concept for control of potency by localized niche signals (72). Similar efforts in the intestine, perhaps facilitated by in vitro models, would be informative.

As the ISC field moves toward a more advanced understanding of extrinsic regulation of stemness, concomitant efforts are required in order to understand the intrinsic mechanisms that allow ISCs to make fate decisions in response to cues from the niche. Two very recent studies have extended the neutral drift models observed in ISC physiology to conventional murine models of intestinal tumorigenesis. As expected, mutations commonly associated with tumor development, such as \textit{K-ras} overexpression and loss of \textit{Apc}, conferred selective advantages in ISC clones, leading to increased expansion of mutant ISCs over their wild-type counterparts (80, 98). These studies suggest that, in certain cases, ISCs can subvert the balancing influence of the niche, and carry important implications for our understanding of intestinal pathologies. Recent mapping of epigenetic marks and DNase I-hypersensitivity sites in \Lgr5^{+}\ ISCs and progenitor populations demonstrates that overall chromatin accessibility is surprisingly similar across lineage hierarchies in the intestinal epithelium (44). Combined lineage tracing and gene knockout studies show that \textit{Atoh1} is critical in resolving cells with relatively homologous chromatin into secretory lineages and suggests similar roles for other \textit{trans} activating factors (44). Together, these data suggest that plasticity in the intestinal epithelium extends beyond the ISC-progenitor level and applies to absorptive-secretory plasticity in transit amplifying populations as well (Fig. 2).

As more studies suggest that stem and progenitor cell potency in the intestine exists in a population-averaged state of flux, as opposed to a fixed linear hierarchy, the demand for novel technological approaches to basic intestinal epithelial physiology increases. The rapid turnover and wide-range of distinct cellular lineages in the intestine provide many advantages in terms of studying stem cell behavior. However, they also present challenges in that isolating cells at distinct stages of proliferation and differentiation remains challenging despite breakthroughs in biomarker discovery. New methodologies for studying single cells may address underlying limitations of isolating ISCs and progenitors by single biomarkers and aid in the understanding of lineage progression in the intestinal epithelium. Additionally, advances in genomic and epigenetic regulation in other stem cell fields may inform our understanding of ISCs, and could facilitate studies aimed at gaining new functional insights into intestinal populations associated with established biomarkers.

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**AUTHOR CONTRIBUTIONS**

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CELLULAR HIERARCHIES IN THE INTESTINAL EPITHELIUM


