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Defining a stem cell hierarchy in the intestine: markers, caveats and controversies

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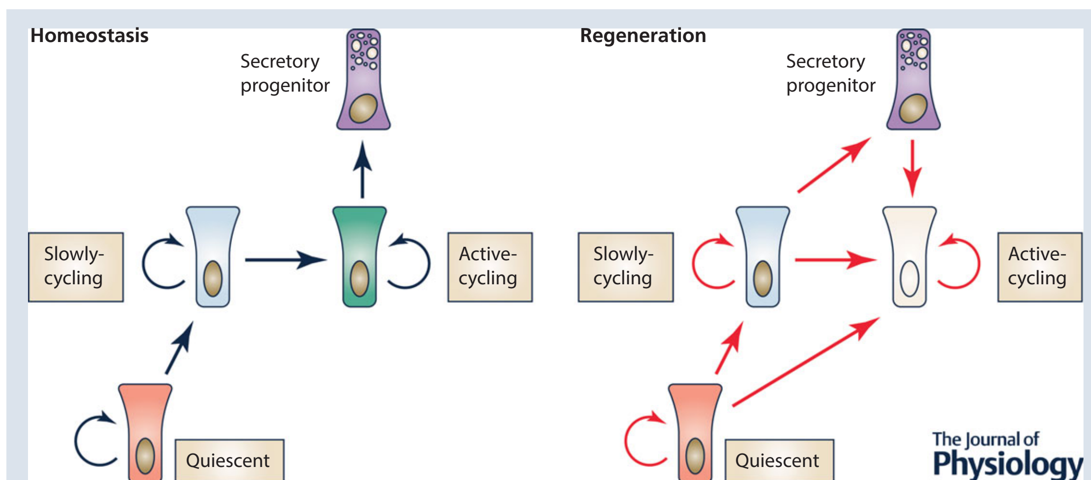

Abstract The past decade has appreciated rapid advance in identifying the once elusive intestinal stem cell (ISC) populations that fuel the continual renewal of the epithelial layer. This advance was largely driven by identification of novel stem cell marker genes, revealing the existence of quiescent, slowly- and active-cycling ISC populations. However, a critical barrier for translating this knowledge to human health and disease remains elucidating the functional interplay between diverse stem cell populations. Currently, the precise hierarchical and regulatory relationships between these ISC populations are under intense scrutiny. The classical theory of a linear hierarchy, where quiescent and slowly-cycling stem cells self-renew but replenish an active-cycling population, is well established in other rapidly renewing tissues such as the haematopoietic system. Efforts to definitively establish a similar stem cell hierarchy within the intestinal epithelium have yielded conflicting results, been difficult to interpret, and suggest non-conventional alternatives to a linear hierarchy. While these new and potentially paradigm-shifting discoveries are intriguing,

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the field will require development of a number of critical tools, including highly specific stem cell marker genes along with more rigorous experimental methodologies, to delineate the complex cellular relationships within this dynamic organ system.

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Abstract figure legend Proposed relationships between stem cell populations and progenitors in homeostasis and regeneration.

Abbreviations BrdU, 5-bromo-2′-deoxyuridine; CBC, crypt base columnar; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; ISC, intestinal stem cell; LRC, label-retaining cell; mTert, mouse telomerase reverse transcriptase; YFP, yellow fluorescent protein.

Introduction

The intestinal epithelium serves critical functions for sustaining life: it provides an expansive surface area for nutrient uptake, mediates immune homeostasis, and maintains a contiguous barrier to the external environment (Peterson & Artis, 2014). The epithelial layer must be continuously renewed to safeguard against accumulation of physical and mutational injury (Stappenbeck *et al.* 1998; Wong *et al.* 1999). This renewal is fueled by a proliferative stem cell compartment tightly regulated to maintain discrete stem and progenitor cell pools. The proliferative zone of the small intestine's epithelial compartment is housed in protective invaginations – crypts of Lieberkühn – which line the floor of the organ and surround the base of the villus protrusions lined with differentiated epithelium (Wong *et al.* 1999, Henning & von Furstenberg, 2016). Within the protective crypt niche, the stem cell populations reside among regulatory crypt epithelium and surrounding stromal cells (Booth & Potten, 2000; Brittan & Wright, 2002). At least three types of stem cells have been identified in the intestine: quiescent ISCs (stem cells that do not divide at homeostasis; Montgomery *et al.* 2011), slowly-cycling ISCs (stem cells that rarely divide during homeostasis; Sangiorgi & Capecchi, 2008; Takeda *et al.* 2011) and active-cycling ISCs (stem cells that rapidly proliferate during homeostasis; Barker *et al.* 2007). These populations are hypothesized to be coordinately regulated, to exist in an ordered hierarchy, and to ultimately give rise to progenitor populations (immature cells with lineage commitment), transit-amplifying cells (rapidly proliferating cells that increase epithelial numbers), and differentiated epithelial lineages (enterocytes, goblet, enteroendocrine, tuft and Paneth cells) (Cheng & Leblond, 1974*c*; Karam, 1999; Barker *et al.* 2007; Sangiorgi & Capecchi, 2008; Gerbe *et al.* 2012) (Fig. 1). However, the exact relationship between identified ISC populations is not completely clear (Abstract figure). There are data supporting a structural hierarchy among stem and progenitor cells, but contradictory evidence also exists indicating that ISCs may reversibly transit between states of variable competency. Further, hierarchical relationships that exist during homeostasis may change in response to stimulation

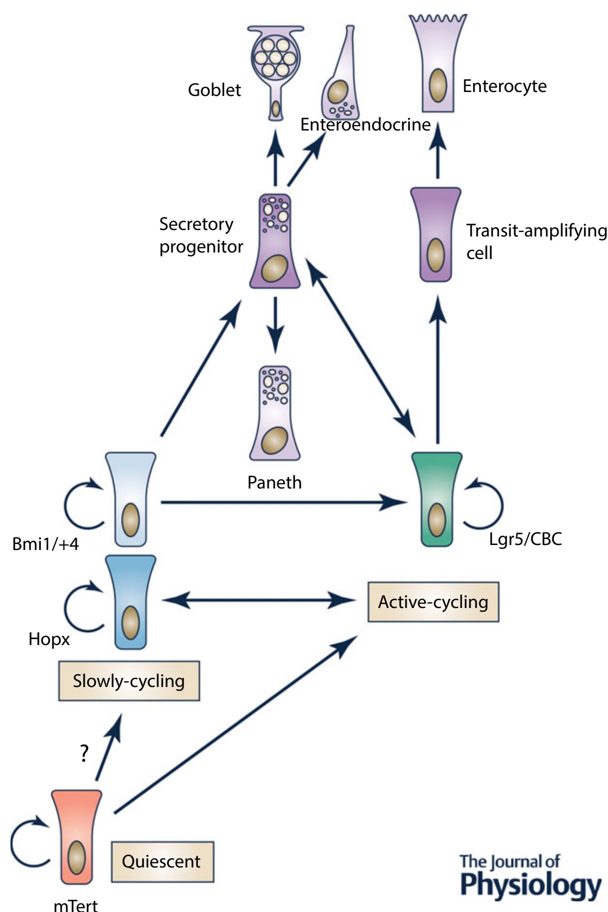

Figure 1. Intestinal stem cell hierarchy
Evidence for multiple functional populations of stem cells support the existence of quiescent, slowly-cycling, and active-cycling stem cell classifications. The dynamic relationships between intestinal stem cells, progenitors, and differentiated lineages has evolved to reflect potential plasticity of the Lgr5^{+} stem cell and secretory progenitors, and the Hopx stem cell population.

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by a regenerative microenvironment. These intriguing scientific challenges represent the frontier of ISC biology: to understand the complex cellular interplay within the ISC niche.

Historical ISC identification: label retention and morphology

The intestinal epithelium undergoes continual renewal (Leblond & Stevens, 1948), with the continuum of proliferation to differentiation represented along the crypt–villus axis. Early studies used radioisotopes to label proliferating cells within the crypt and track their fates (Leblond & Stevens, 1948; Leblond & Messier, 1958; Walker & Leblond, 1958). Such labelling techniques were designed to gain insight into the unknown cell population underlying epithelial turnover. The existence of a cell population capable of self-renewal (i.e. a stem cell pool) with multiple lineage potential residing among the undifferentiated cells within the crypt base was elegantly demonstrated in the mouse colon using radioautographic tracing (Chang & Leblond, 1971) and in the small intestine with ^{3}H-thymidine and 'phagosome' tracing (Cheng, 1974*a*,*b*; Cheng & Leblond, 1974*a*,*b*,*c*). The meticulous and detailed cataloguing of proliferative crypt base columnar cells (CBCs, slender cells localized between Paneth cells in the crypt base) and their direct progeny revealed that these CBCs were multipotent progenitors or stem cells (Cheng & Leblond, 1974*a*,*c*). Notably, these first experiments in the small intestine indicated that the immature proliferative cells occupy the nine lowest cell positions in the crypt. Not surprisingly, because of the short window of isotope labelling used in these studies, only active-cycling cells were analysed (therefore thought to be progenitors) and not long-lived stem cell populations in the crypt. Due to the general acceptance that a true stem cell must rarely divide, this active-cycling CBC population was largely dismissed as a candidate stem population for over 30 years.

In later studies by Christopher Potten, a label-retaining stem cell population was identified using a long-term labelling strategy with ^{3}H-thymidine and later with 5-bromo-2′-deoxyuridine (BrdU) (Potten *et al.* 1974), localized to the +4 cell position of the intestinal crypt, and hypothesized to be a facultative stem cell. In agreement with the classical stem cell characteristics, these cells rarely divided, were resistant to cytotoxic stress, and were capable of regenerating injured epithelium (Rizvi & Wong, 2005). Thus, for three decades, the ISC field was solely focused on the +4 stem cell population.

The era of marker-based identification of ISCs

The lack of functional stem cell markers and assays for demonstrating stemness stagnated the ISC field for years. A major breakthrough occurred when Hans Clevers' group, using an elegant microarray study to compare normal and tumour intestinal epithelium, identified the Wnt target gene *Lgr5* (Van der Flier *et al.* 2007) as a specific marker for the active-cycling intestinal CBC population (Barker *et al.* 2007). Lgr5, a G-protein-coupled receptor, serves a critical function in ISC regulation by binding the Wnt agonist, R-spondin, to amplify the local Wnt signal (Carmon *et al.* 2011). A knock-in mouse expressing green fluorescent protein (GFP) and inducible Cre recombinase from the endogenous *Lgr5* locus was generated and demonstrated strong GFP expression in the proliferative CBCs (Barker *et al.* 2007). Further, *in vivo* lineage tracing in the Lgr5 reporter mouse intestine demonstrated functional stemness of this cell population down the length of the intestine, as evidenced by stripes of LacZ-expressing epithelial cells resident on crypt–villus units which encompassed all of the differentiated epithelial cell lineages (Barker *et al.* 2007). With the development of *in vitro* growth conditions supporting proliferation and differentiation of single Lgr5-GFP-expressing intestinal epithelial cells, Clevers' group provided an *ex vivo* assay as a second approach to demonstrate a cell's stem potential (Sato *et al.* 2009). These critical advances energized the field, which resulted in the identification and validation of numerous additional markers of active-cycling ISCs (Table 1).

Establishing specific strategies to identify and isolate rare slowly-cycling ISCs, which were historically described to be located at the +4 cell position in the crypt, was more challenging. Characterization of a Bmi1-Cre;R26R-Yellow fluorescent protein (YFP) lineage mouse by Mario Capecchi's group revealed an expression pattern consistent with Potten's label-retaining, slowly-cycling ISC population (Sangiorgi & Capecchi, 2008). As expected, crypt-based Bmi1-expressing cells displayed slow cycling dynamics at homeostasis, but expanded and demonstrated enhanced lineage tracing capacity in response to regenerative injury (Yan *et al.* 2012). Fluorescence-activated cell sorting (FACS)-isolated Bmi1-expressing cells initiate growth of intestinal enteroids under conventional Sato culture conditions (Sato *et al.* 2009), further validating their role in stem cell homeostasis (Yan *et al.* 2012). As with the active-cycling stem cell population, this discovery set the stage for identification of numerous slowly-cycling ISC populations, including those expressing *Hopx* (Takeda *et al.* 2011), as well as one population that appears to be more quiescent than slowly-cycling, expressing mouse telomerase reverse transcriptase (*mTert*) (Montgomery *et al.* 2011) (Table 1). However, it remains unclear whether each of these stem cell populations, defined by marker gene expression, represents distinct or overlapping populations. Further, it remains controversial whether these populations exist in hierarchical relationships during

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Table 1. Newly discovered stem cell populations

Active-cycling		Overlapping expression with other cell populations
Lgr5	(Barker *et al.* 2007)	Y
Ascl2	(Van der Flier *et al.* 2009)	NR
Olfm4	(Van der Flier *et al.* 2009)	NR
Lrig1	(Wong *et al.* 2012)	Y
$Sox9^{lo}$	(Formeister *et al.* 2009)	NR
$CD24^{lo}$	(von Furstenburg *et al.* 2011)	NR
Upper SP	(von Furstenburg *et al.* 2014)	NR
$CD44^{+}CD24^{lo}CD166^{+}GRP78^{lo}$	(Wang *et al.* 2013)	NR
Smoc2	(Munoz *et al.* 2012)	NR
Troy	(Fafilek *et al.* 2013)	NR
Slowly-cycling/quiescent		**Overlapping expression with other cell populations**
Bmi1	(Sangiorgi & Capecchi, 2008)	Y
mTert	(Montgomery *et al.* 2011)	NR
Hopx	(Takeda *et al.* 2011)	Y
Lrig1	(Powell*et al.* 2012)	NR
Dclk1	(May *et al.* 2008)	Y
$Sox9^{hi}$	(Roche *et al.* 2015)	NR
Lower SP	(von Furstenburg *et al.* 2014)	NR
LRC	(Buczacki *et al.* 2013)	NR
Wip1	(Demidov *et al.* 2007)	NR
Krt19	(Asfaha *et al.* 2015)	NR

NR, not reported; Y, yes.

homeostatic conditions and if these relationships are maintained during tissue regeneration.

Hierarchical relationships between ISC populations

To begin to explore hierarchical relationships between the numerous newly discovered stem cell populations within the intestinal epithelium, we first turn to other organ systems with similar dynamic renewal properties, such as the skin (Hsu *et al.* 2014), germ cells (Nakagawa *et al.* 2010), and the haematopoietic system (Kondo *et al.* 2003). In these systems, a classical stem cell hierarchy is well established. Rare slowly-cycling stem cells are positioned 'upstream' of both the active-cycling stem cell populations and committed lineage progenitors. This general strategy, where a subset of stem cells are slowly-cycling but capable of rejuvenating an active-cycling population, protects against accumulated mutations and transformation of the active-cycling stem cells, which are more prone to genotoxic and cytotoxic stresses (Li & Clevers, 2010). Because the intestinal epithelium has active- (CBC, Lgr5), slowly-cycling (Bmi1, Hopx), and quiescent (mTert) stem cell populations, it is likely that such a hierarchy exists, but this theory has proven difficult to conclusively demonstrate.

A recent landmark study provides compelling evidence that a stem cell hierarchy exists between two of the identified ISC populations, $Bmi1^{+}$ and $Lgr5^{+}$. This study employed an elegant diphtheria toxin approach – harnessing expression of the extracellular protein of *Corynebacterium dipththeriae* that inhibits protein synthesis and kills susceptible cells – to specifically ablate the $Lgr5^{+}$ ISC population. The approach revealed that this active-cycling pool is dispensable for maintenance of normal epithelial architecture and homeostasis. Remarkably, ablation of the $Lgr5^{+}$ ISC population resulted in both expansion and enhanced lineage tracing capacity from the $Bmi1^{+}$ ISC population (Tian *et al.* 2011). The authors went on to demonstrate direct and definitive lineage tracing of the $Bmi1^{+}$ ISC to an $Lgr5^{+}$ ISC under homeostatic conditions, using a β-gal indicator for $Bmi1^{+}$ ISC lineage tracing and GFP to mark the $Lgr5^{+}$ population. These findings hierarchically position the $Bmi1^{+}$ ISC upstream of the $Lgr5^{+}$ ISC under homeostatic conditions (Fig. 1). Interestingly, earlier studies exploiting diphtheria toxin to ablate the $Bmi1^{+}$ ISC resulted in collapse of crypt architecture in a subset of crypts (Sangiorgi & Capecchi, 2008). In retrospect, these findings may indicate that the $Bmi1^{+}$ population provides important renewal of the active-cycling ISC population.

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In addition to direct hierarchical relationships, studies in regenerating crypts also revealed that apparent lineage-committed progenitor cells possess plasticity. In response to injury, these cells display the ability to dedifferentiate toward a stem cell fate. The idea that committed lineage progenitors can be coaxed back into a stem cell fate is not entirely novel, as it has been described and studied in other systems such as the haematopoietic system (Graf, 2002). Indeed, within the intestine, it appears that chromatin marks between active-cycling Lgr5$^+$ ISCs and progenitor cell types are nearly identical, supporting the notion that various intestinal epithelial cells, regardless of their differentiation status, have the capacity to rapidly change their cellular expression programme in response to environmental stimuli (Kim *et al.* 2014). In one such example, Doug Winton's group demonstrated that intestinal H2B-YFP label-retaining cells (LRCs), thought to represent quiescent secretory lineage precursors, are committed to mature into differentiated secretory cells (Paneth and enteroendocrine) rather than an active-cycling ISC (Buczacki *et al.* 2013). However, using a novel genetic mouse model, the authors demonstrate that these LRCs can be coaxed into re-acquiring stem cell function after injury. Supporting this concept, an independent study from the Clevers' group found that secretory progenitors marked by the Notch ligand Dll1 have a similar stem cell capacity after tissue damage (van Es *et al.* 2012). These studies highlight that hierarchical relationships between different ISC pools may not be simplistically linear and therefore is not easily defined. Furthermore, an exciting study by Jonathan Epstein's laboratory provides the first evidence that hierarchical relationships between ISC populations can be bidirectional and not merely linear. Using a complex tri-transgenic mouse cross to lineage trace from the *Hopx* loci, while maintaining the ability to detect Lgr5$^+$ populations, the authors identified double-marked cells within the crypt epithelium indicating that Hopx$^+$ ISCs could give rise to Lgr5$^+$ ISCs and vice versa (Takeda *et al.* 2011; Li *et al.* 2014). This intriguing concept suggests that the intestinal epithelium has built in contingency plans for robust maintenance of tissue homeostasis in the event of ablation of discrete ISC populations.

Challenges with marker-based definitions for stem cell populations

The majority of currently identified stem cell populations within the intestinal epithelium are based solely upon gene or protein expression (Table 1). Multiple challenges exist with this approach in clarifying relationships between different populations. Unlike the haematopoietic field where the development and organization of multiplexed antibodies to cell surface antigens facilitated the identification, characterization and manipulation of discrete populations and related subpopulations (Kondo *et al.* 2003), these types of tools have drastically lagged behind in the ISC field. To compensate, the field has relied upon the generation of stem cell-reporter mouse lines, a cumbersome and expensive approach to exploring relationships between ISC populations. The fact that the majority of ISC-reporter mice were generated with GFP prevents combinatorial analyses of multiple stem cell populations. Further, unlike in the haematopoietic system, intestinal lineage progenitors have been primarily identified by histological hallmarks (Cheng, 1974*b*). The inability to dissect populations down a lineage differentiation pathway and identify intermediate populations prevents the resolution of underlying mechanisms that drive these processes. Furthermore, understanding whether or not inter-convertible relationships between progenitors, stem cells and differentiated lineages underlies homeostasis or regenerative mechanisms cannot be adequately and clearly addressed.

Reliance on gene or protein expression complicates and limits useful tracking of stem cell populations. Gene expression within discrete stem cell populations may reflect a physiological response to their environment. For example, while Lgr5 expression on the active-cycling CBC functions to mediate a proliferative Wnt signal to this cell population, homozygous knockout of this protein in embryonic intestines that were transplanted and matured in kidney capsule xenografts retained normal tissue architecture, despite complete loss of Lgr5 expression (Tian *et al.* 2011). Interestingly the void of cells was filled with unknown, non-Lgr5-expressing CBC populations (Tian *et al.* 2011). This finding strongly suggests that either a subpopulation non-Lgr5-expressing CBCs can compensate, or that CBCs can harness a bypass mechanism to gain independence from Lgr5-mediated Wnt signalling. This bypass mechanism could involve either upregulation of different Wnt-regulatory machinery or transition into an entirely different cell state that can proliferate independently of Wnt. For example, Lrig1 is reported to be expressed on a variety of cells within the crypt, with overlapping expression in Lgr5$^+$ (Wong *et al.* 2012) and +4-positioned (Powell *et al.* 2012) ISCs. Because of its broader expression pattern, as reported by Wong *et al.*, and its biological function as a negative regulator of the ErbB receptor family, it is likely that Lrig1 expression reflects a transient cell signalling state rather than marking a discrete homogeneous stem cell population.

Further complicating marker-based identification of cells, studies interchangeably define populations based on their RNA expression or protein expression (Munoz *et al.* 2012). While Bmi1 RNA may be expressed at very low levels in most cells within the crypt base (Munoz *et al.* 2012),

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the Bmi1 protein is expressed in +4-positioned cells, as determined within the Bmi1-reporter mouse intestine (Tian *et al.* 2011). A cell's identity might be based *both* on its function from its protein expression, and its potential as reflected by its RNA status, but this notion has not been formally addressed.

Finally, many of the protein markers have broad expression patterns that are not restricted to a single 'population' of cells within the intestinal epithelium. Prime examples of this are that the stem cell marker Dclk1, which is also expressed on the villus and in cells co-expressing differentiated enteroendocrine or tuft markers (Levin *et al.* 2010; Gerbe *et al.* 2012). Additionally, Bmi1 is not only expressed within the crypt compartment, but also by a subset of differentiated villus cells (Takeda *et al.* 2011; Munoz *et al.* 2012; Li *et al.* 2014). This heterogeneous expression found from many stem cell markers complicates the ability to fine tune characterization of discrete functional ISC populations. For instance, Lgr5 is expressed on active-cycling stem cell populations, but was recently described to be expressed on secretory progenitor cells (Buczacki *et al.* 2013; Grun *et al.* 2015). Hopx, which is expressed in a subset of active-cycling ISCs, the slowly-cycling ISC population, secretory progenitors *and* mature Paneth cells, serves as an additional example of heterogeneous expression (Li *et al.* 2014). Current experimental methodologies cannot easily differentiate two populations in the *in vivo* studies involving lineage tracing. While marker-based identification of ISCs has moved the field forward, the next step for ISC manipulation should be towards a unified, cell surface antigen-based approach for isolation of ISC populations. This approach has served the haematopoietic stem cell field well in defining discrete populations, their relationships and their overlapping function within the blood.

Caveats with current assays to determine stemness

In addition to challenges identifying and manipulating ISC populations, undoubtedly the biggest hurdle is the lack of definitive assays to determine functional stemness of discrete, putative stem cell populations. Four primary ISC assays are routinely employed by the field: (a) reporter mice, (b) lineage tracing, (c) gene expression profiling, and (d) *in vitro* enteroid cultures. The location of the proliferative stem cell niche in relation to the differentiated cells on the adjacent villi provide a convenient secondary architecture to readily appreciate the continuum of cell proliferation to differentiation (Stappenbeck *et al.* 1998), and to discover new progenitor and ISC populations based on protein expression. These studies are typically initiated with observations from RNA or protein expression patterns discovered in immunohistochemical analyses, but then followed with the generation of a reporter mouse line for confirmation. While these studies function to identify putative populations, they do not definitively confirm stem cell behaviour.

The ability to lineage trace from a discrete cell population, that is to identify all progeny derived from a single cell, was originally established as a developmental biology tool but has more recently been harnessed in stem cell biology to assay the stem cell potential of various populations within a tissue (Kretzschmar & Watt, 2012). To investigate the lineage-propagating potential of a putative ISC, a mouse line that expresses inducible Cre recombinase from the putative ISC promoter is generated and then crossed to a Cre-reporter mouse line (for lineage tracing) or an inducible diphtheria toxin mouse line (for ablation of the population). While the ability to inducibly activate Cre provides an important scientific tool, most of these reporters rely on tamoxifen for activation, which is known to differentially affect the viability of various ISC pools (Zhu *et al.* 2013). Therefore, results using such approaches must be interpreted with caution. Further, most of these mice require complicated breeding schemes to generate bi- or tri-transgenic/knock-in mouse lines. Therefore, these studies are inefficient, costly, time consuming, and complex to analyse. One primary caveat with analyses of the *in vivo* lineage studies is that many of the ISC markers harbour heterogeneous expression patterns (e.g. expressed in differentiated, progenitor, and ISCs). Specifically, if the marker is expressed in slowly-cycling *and* differentiated populations, the interpretation of the results may be different from that if the marker were represented in a single population. These studies complicate interpretation, as it is difficult to determine if lineage tracing originates from a differentiated population (i.e. suggesting that differentiated cells have the plasticity to convert to a stem cell state) or if it originates from a rare undifferentiated subpopulation with stem cell capacity. Often the extent of marker heterogeneity is not appreciated in publications describing the initial 'validation' of a stem cell marker.

Bioinformatics of gene expression data sets have been heavily leveraged to gain insight into the relationships between stem cell populations (Munoz *et al.* 2012; Li *et al.* 2014; Grun *et al.* 2015). While these types of studies have yielded amazing breakthroughs in the ISC field (Barker *et al.* 2007; Van der Flier *et al.* 2007), most of the analyses are based upon RNA expression and this raises the concern about the validity of RNA *vs.* protein expression patterns. Further, interpretation of the clustering and modelling is always superimposed upon what appears logical within the current state of stem cell interactions and could carry some bias.

Due to the lack of available experimental tools, very few studies demonstrate direct lineage relationships through temporal analysis. Instead, they rely on circumstantial

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evidence to make inferences regarding hierarchy. For example, thorough gene and protein expression analyses of five FACS-isolated Lgr5-GFP populations based on GFP signal intensity compared levels of quiescent (*mTert*) and slowly-cycling ISC marker gene expression (including *Lrig1*, *Bmi1* and *Hopx*). Relative gene expression levels (but not protein levels) between these arbitrary populations led to the conclusion that all Lgr5^{+} ISCs express Bmi1 (van der Flier *et al.* 2009; Munoz *et al.* 2012).

Finally, while the field is fortunate to have an *in vitro* assay system to grow single FACS-isolated cells into 3-dimensional enteroid structures that recapitulate stem and differentiated domains (Sato *et al.* 2009), it must be acknowledged that the growth conditions mimic an activated, regenerative or cancer-like microenvironment. Therefore, this assay system cannot be used to address questions involving normal homeostasis (due to lack of signalling gradients and domains) or to resolve regulation of signalling pathways to discrete populations within the enteroids due to excess growth factors that are primarily geared towards supporting the Wnt-dependent active-cycling Lgr5^{+} ISC pool (Sato *et al.* 2009). Further, we lack the necessary culture conditions to understand the regulatory microenvironment for quiescent and slowly-cycling ISCs. Importantly, taken together, the field lacks an *in vivo* reconstruction assay, which was instrumental in elevating the level of discovery for the haematopoietic stem cell system (Jacobson *et al.* 1951; Becker *et al.* 1963; Weissman & Shizuru, 2008).

Future directions for the ISC field

Over the past decade, breakthrough discoveries have elevated the ISC field, facilitating rapid advancement in identifying markers of the once elusive ISC populations – quiescent, slowly- and active-cycling – and have laid the foundation for unravelling their complex interrelationships during homeostasis, in response to regenerative cues, and in disease. Understanding the dynamic interplay between ISC populations and how they are related and inter-regulated represents a critical threshold for translating our stem cell-based knowledge to patient therapeutics. Understandably, evolution of this nascent field brings with it rekindled controversy as we seek clarity into ISC relationships and hierarchy, highlighting that there are still many unanswered questions.

Emerging issues with ISC population heterogeneity and overlap of ISC markers require elucidation. While it is clear that ISC populations distinct from the active-cycling Lgr5^{+} pool exist, their exact identities and functions during tissue homeostasis and repair remain unclear. Multiple markers of putative quiescent and slowly-cycling ISCs have been identified (Table 1), but it is increasingly clear that these markers represent heterogeneous populations. Currently, it appears that none of the identified +4 ISC markers successfully distinguish a homogeneous population, as marker-positive cells are located on the differentiated villi and do not lineage trace. This indicates that a rare subset of marker-positive, crypt-based cells may harbour stem cell characteristics. Therefore, development of novel single cell technologies to dissect this heterogeneity will address the degree of overlap between all ISC markers. These approaches will uncover new ISC markers, that when implemented in a combinatorial fashion with current markers, will support identification schemes for increased specificity for homogeneous ISC populations. Advances have been made in these technologies in recent years, including single cell gene expression analysis of FACS-isolated ISC and crypt cell populations (Dalerba *et al.* 2011; Rothenberg *et al.* 2012; Grun *et al.* 2015; Roche *et al.* 2015), as well as developing platforms to assay stemness of single ISC populations (Gracz *et al.* 2015). Unfortunately, the majority of ISC markers are represented by intracellular proteins or proteins where functional antibodies have not been successfully derived. Therefore, the field requires a focused development of ISC population-specific cell surface antibodies to translate discoveries in mice to humans. Overall, these approaches will lead to greater population specificity and facilitate future studies to address the functional relevance of marker expression to ultimately illuminate the hierarchical relationships between distinct ISCs.

Complicating the traditional views of a stem cell hierarchy is the recent evidence that lineage-committed progenitors possess a level of plasticity allowing them to revert to a stem cell state. In the intestine, this plasticity has been demonstrated by lineage-tracing of 'committed cells' (van Es *et al.* 2012; Buczacki *et al.* 2013) and highlights the existence of alternative cellular mechanisms to regenerate the stem cell niche after injury. It is important to note that these exciting findings do not disprove the existence of a more traditional stem cell hierarchy, as both mechanisms may co-exist. Instead, these discoveries may allude to the amazing redundancy orchestrated within the ISC niche to ensure maintenance of a functional epithelium. We propose that these different views of 'hierarchy' likely depend upon microenvironmental context (i.e. homeostasis *vs.* injury or disease).

With current experimental tools and methodologies, it is impossible to distinguish the relative contribution of direct hierarchy *vs.* progenitor plasticity in restoration of tissue homeostasis after injury. In order to elucidate the functional importance of these diverse cellular mechanisms, a greater understanding of the cell–cell signalling interactions within the niche responsible for regulating the maintenance or stimulation of the quiescent and slowly-cycling ISC populations is paramount. This

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elucidation will require continued development of novel technologies for querying multiple cell signalling readouts derived from discrete cell populations (Simmons *et al.* 2015), with further advancement of real-time *in vivo* intravital imaging (Ritsma *et al.* 2014), *in vitro* culture methods that support quiescent and slowly-cycling ISC propagation, and identification of specific culture conditions that stimulate interconversion between ISC populations.

With the exciting advancements in ISC marker identification and initial studies into the hierarchical relationships between distinct ISC populations, a complex picture is emerging. It appears that the intestinal epithelium does not follow a simplistic linear stem cell hierarchy model. Instead, a model of hierarchy with built-in redundancy likely exists (Abstract figure) whereby different types of cells with stem potential are called upon depending on the physiological context. For example, during homeostasis, the slowly-cycling ISC population provides moderate renewal of the active-cycling niche. However, in response to injury, this activity is enhanced and plasticity of lineage-committed progenitors is appreciated (Abstract figure). These diverse mechanisms illustrate an exquisite evolutionary safety net built into a dynamic epithelium to ensure robust tissue renewal and rapid restoration of homeostasis after insult, properties that are absolutely essential for survival.

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Additional information

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