Identifying the Stem Cell of the Intestinal Crypt: Strategies and Pitfalls

Cell Stem Cell
Perspective

Identifying the Stem Cell of the Intestinal Crypt: Strategies and Pitfalls

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Decades ago, two nonoverlapping crypt stem cell populations were proposed: Leblond's Crypt Base Columnar (CBC) cell and Potten's +4 cell. The identification of CBC markers including Lgr5 has confirmed Leblond's predictions that CBC cells are anatomically distinct, long-lived stem cells that permanently cycle. While Potten originally described +4 cells as proliferative and unusually radiation-sensitive, recent efforts to identify +4 stem cells have focused on the identification of cells that are quiescent and radiation-resistant. Here, we describe commonalities and discrepancies between the individual studies and discuss challenges of marker-based lineage tracing.

Introduction

The intestinal tract consists of two anatomically and functionally distinct organs: the small intestine and the colon (Gregorieff and Clevers, 2005). The architecture of the epithelium that lines the lumen differs markedly between the two organs, reflecting their distinct functions. The epithelium of the small intestine maximizes available absorptive surface area by the presence of numerous finger-like protrusions that are called villi. Multiple invaginations, the crypts of Lieberkühn, surround the base of each villus. Colon epithelium lacks villi: from the flat surface epithelium, crypts penetrate the underlying submucosa.

The various differentiated cell types of the intestinal epithelium are well defined, both by morphology and in terms of marker expression. Absorptive enterocytes (which also produce hydrolytic enzymes) are abundant throughout the small intestine. They are columnar in shape, highly polarized, and carry an elaborate apical brush border. Mucus-secreting Goblet cells occur mostly in the distal small intestine (ileum) and the colon. Paneth cells, which secrete antimicrobial products and provide stem cell niche signals (Sato et al., 2011), are largely restricted to the crypts of the small intestine. Deep crypt secretory cells (Rothenberg et al., 2012) may represent the colon counterparts of Paneth cells. Other, more rare cell types can reside in crypts as well as villi and include hormone-secreting enteroendocrine cells, brush/tuft/caveolated cells, and cup cells. Finally, M cells reside on lymphoid Peyer's patches and transport antigens from the gut lumen to the underlying lymphoid tissue (de Lau et al., 2012).

The epithelium of small intestine and colon displays a remarkable self-renewal rate, likely necessitated by the constant barrage from physical, chemical, and biological insult. Indeed, the small intestinal epithelium of the mouse completely renews every 3–5 days. The intense proliferation that fuels this self-renewal process is confined to the crypts. Individual crypts comprise around 250 cells and generate a similar number of new cells each day. Resident stem cells have long been suspected to be located close to the crypt base (reviewed in Barker et al., 2010a). These stem cells produce vigorously proliferating progenitors called transit-amplifying (TA) cells, which move upward as coherent columns toward the crypt/villus border (Heath, 1996). During this upward migration, these TA cells begin to differentiate, and subsequently exit the crypt onto the villus 2 days after being "born." Their migration continues toward the villus tip, where they die and are shed into the lumen. Up to 10 crypts supply new cells to a single villus. The crypt-resident Paneth cells escape this upwardly mobile epithelial conveyer belt. Instead, they migrate downward to occupy the crypt base, where they live for 6–8 weeks. The combination of the stereotypical architecture of the crypt-villus unit and this intensive self-renewal process makes the intestinal tract an attractive model for the study of adult stem cell biology.

Early Studies on Intestinal Stem Cells

A minimal definition of adult stem cells comprises just two characteristics: longevity (stem cells persist for the lifetime of their owner) and multipotency (stem cells can produce all cell types of the tissue to which they belong). As argued elsewhere (Barker et al., 2010a; Barker and Clevers, 2007), two general experimental approaches can assess stemness at the level of a single stem cell: genetic lineage tracing and transplantation. In the intestine, two models of intestinal stem cell identity have been formulated: the stem cell zone model and the +4 model (Figure 1).

The stem cell zone model derives from the discovery by Cheng and Leblond that the crypt base is not exclusively populated by postmitotic Paneth cells. Using electron microscopy (EM) almost 4 decades ago, they revealed the existence of slender cells, wedged between the Paneth cells that divide once every day. These cells were referred to as CBC cells (Cheng and Leblond, 1974) (Figure 2). Following 3H-Thymidine exposure, many CBC cells died, which were subsequently phagocytosed by surviving CBC cells. The resulting radioactive phagosomes, initially restricted to CBC cells, were subsequently observed within more differentiated cells. This rudimentary lineage tracing result was interpreted as evidence for stemness of the CBC cells. However, since phagosome-labeled examples of the four cell

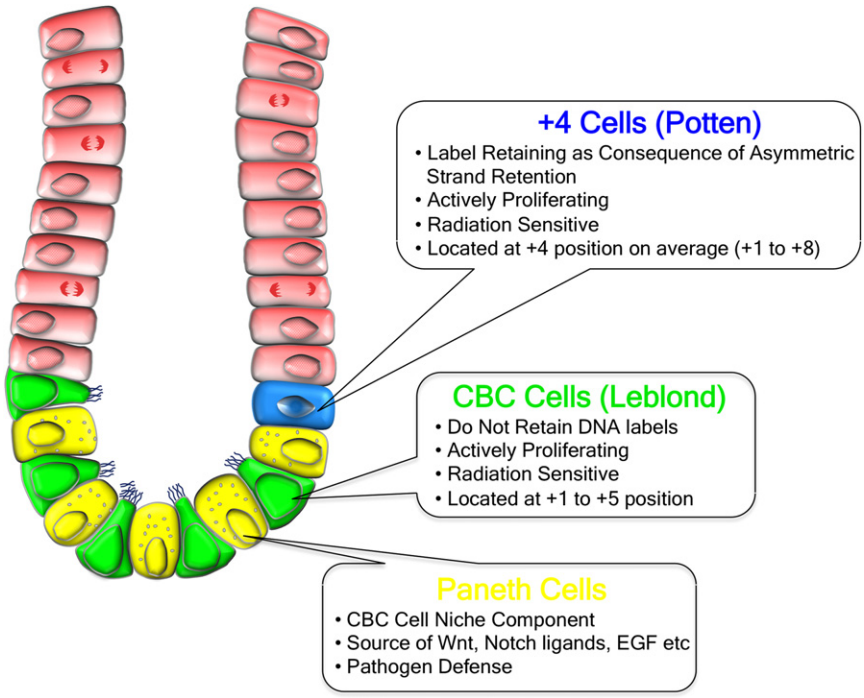

Figure 1. The Stem Cells of the Small Intestine: A Historical Perspective

lineages were observed in separate crypts, formal demonstration of CBC cell multipotency could not be given.

Bjerknes and Cheng continued to champion the CBC cell as the intestinal stem cell (Bjerknes and Cheng, 1999, 2002). While observing random mutagenesis, they noted both long-lived and short-lived clones of marked cells. Only the long-lived clones comprising all four major cell lineages consistently included a marked CBC cell. This was interpreted as further, yet still indirect, evidence for CBC cells as the self-renewing, multipotent stem cells. The stem cell zone model (Bjerknes and Cheng, 1981a, 1981b, 1999) states that the CBC stem cells reside in a stem-cell-permissive environment. These cycling stem cells regularly generate progeny, which subsequently exit the niche and pass through the "common origin of differentiation" around position +5, where they commit toward the various individual lineages. Progenitors mature as they migrate upward onto the villus. Maturing Paneth cell progenitors migrate downward, with the oldest Paneth cells residing at the very base of the crypt.

The +4 model was originally proposed when early cell tracking experiments predicted a common cell origin at position 4–5, directly above the differentiated Paneth cell compartment (Cairnie et al., 1965). Potten and colleagues then reported that radiation-sensitive, label-retaining cells (LRCs) reside immediately above the uppermost Paneth cell, at positions ranging from +2 to +7, but on average at +4 (Potten, 1977). The sensitivity to radiation was proposed to be beneficial for stem cells, preventing the accumulation of deleterious genome changes. Retention of DNA labels is widely considered as a reliable surrogate stem cell trait, indicative of quiescence under physiological conditions. Unknown to many in the field, however, Potten reported that the label-retaining +4 cells were actively proliferating with a cell cycle time of 24 hr (comparable to that of CBC cells). In Potten's words, "In a mouse, it divides approximately once a day, probably under the influence of circadian factors, and hence during the animal's full potential life span (e.g., in a laboratory) may undergo a thousand cell divisions" (Marshman et al., 2002). The LRC phenotype was instead proposed to result from asymmetric segregation of old (labeled) and new (unlabelled) DNA strands into stem cells and their daughters, respectively (Potten et al., 2009). This "immortal strand" phenomenon (Cairns, 1975) would protect the stem cell genome from accumulating mutations. No direct evidence for stemness of the +4 cell was put forward until 2008.

Acceleration in Intestinal Stem Cell Discovery

Markers for CBC Cells

A multitude of markers for the putative adult stem cell populations has been proposed, but most are not supported by direct evidence for stemness as assessed by transplantation or lineage tracing (Table 1). Instead, many studies have relied on positional information of marker expression alone, instigating some confusion and controversy in the ISC field.

The first marker to be discussed here is Lgr5, a specific CBC cell marker. The *Lgr5* gene is controlled by Wnt signals (van de Wetering et al., 2002; van der Flier et al., 2007) and itself encodes a facultative component of the Wnt receptor complex (Carmon et al., 2012; de Lau et al., 2011). Lgr5 is a 7TM protein, acting as the receptor for a small family of Wnt pathway agonists called R-spondins (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011).

The generation of an Lgr5-EGFP-ires-CreERT2/Rosa26RlacZ mouse model allowed visualization of live CBC cells, as well as their in vivo lineage tracing (Barker et al., 2007). Lgr5+ cells are highly uniform in morphology, invariably touch Paneth cells, and uniformly divide each day. They do not retain DNA labels (Escobar et al., 2011; Schepers et al., 2011). Each crypt harbors around 15 of these cells, some 10% of which occupy the +4 position. One day after stochastic induction of *Lgr5* locus-controlled Cre activity by tamoxifen, lacZ was observed in isolated CBC cells. At later time points, lacZ staining revealed clonal ribbons extending from crypt base to villus tip. These ribbons persisted throughout life and contained all cell lineages, demonstrating longevity and multipotency of the Lgr5+ CBC cells. Lgr5+ cells at the base of the colonic crypts were also identified as adult stem cells (Barker et al., 2007, 2008b).

Gene expression and proteome profiling of FACS-sorted Lgr5-EGFP cells has revealed an Lgr5 stem cell "signature" (Muñoz et al., 2012; van der Flier et al., 2007). Follow-up research on several of the signature genes has shown how these genes contribute to stemness. Genetic ablation or overexpression of

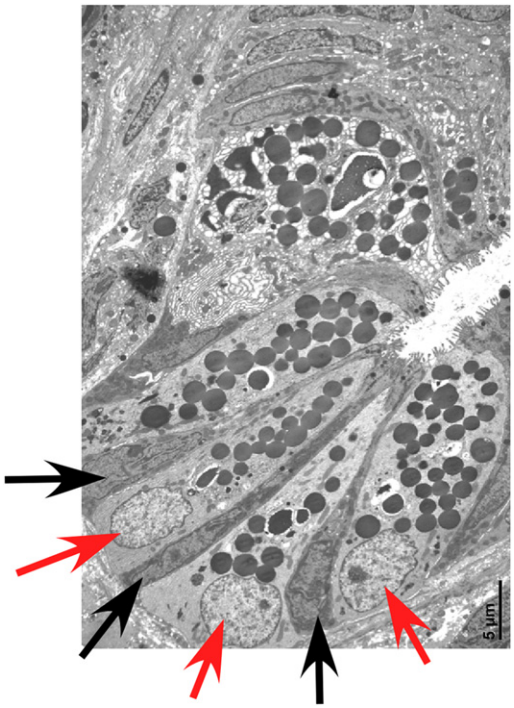

Figure 2. A Closer Look at the Human CBC Stem Cells
CBC stem cells (black arrows) are readily identifiable as slender cells with large basal nuclei and luminal microvilli. Note the intimate association with the Paneth cells (red arrows), which supply many of the niche factors.

Ascl2 expression in vivo results in rapid stem cell death, or in dramatic expansion of the stem cell compartment, respectively, identifying *Ascl2* as a master regulator of the CBC stem cell (van der Flier et al., 2009b). Another gene in this signature, *Smoc2*, has been used to create a lineage tracing allele with equivalent results (Muñoz et al., 2012). In addition, *OlfM4* has emerged as a robust marker of Lgr5+ cells (van der Flier et al., 2009a). Rnf34 and Znrf3 are stem-cell-specific transmembrane E3 ligases that remove Frizzleds from the cell surface (Hao et al., 2012; Koo et al., 2012). Their induced deletion results in adenomas comprising rapidly expanding stem/Paneth compartments, indicating that these proteins serve to restrict the stem cell zone by decreasing Wnt signal strength.

Lgr5+ cells isolated from the small intestine, colon, or stomach can form organoids in long-term culture (Barker et al., 2010b; Sato et al., 2009). An essential component of these cultures is R-spondin, which (as recently unveiled) is the ligand of Lgr5. These epithelial organoids faithfully recapitulate many features of normal gut epithelium, including crypts with resident Lgr5+ cells and Paneth cells, and villus domains with mature epithelial cells of all lineages. Using these culture systems, it was demonstrated that the Paneth cells provide EGF, Wnt, and Notch signals to the stem cells and thus constitute an important part of the stem cell niche, at least in vitro (Durand et al., 2012; Sato et al., 2011). Clonal organoids, expanded from a single Lgr5+ cell from an adult mouse colon, have been used for transplantation into multiple recipient mice in which epithelial damage had been induced by DSS treatment. Grafts were healthy and functional for at least 6 months after transplantation (Yui et al., 2012).

Musashi-1 (He et al., 2007) and Prominin1 (Zhu et al., 2009) also mark CBC cells, but their expression may extend into the lower TA compartment (Snippert et al., 2009). Van Oudenaarden and colleagues recently developed multicolor-fluorescent in situ hybridization for single mRNA molecules, which allows the simultaneous, quantitative measurement of three mRNAs in individual cells in histological sections. They applied this to a series of CBC and +4 cell markers (Itzkovitz et al., 2012; Muñoz et al., 2012). These studies confirm the CBC-specific expression pattern of *Lgr5* and *Ascl2* (Mash2) and reveal a somewhat broader expression for *OlfM4* and *Musashi-1*.

The ability of the intestine to survive the acute loss of its active stem cell pool may in fact relate to the general plasticity of the TA progenitor compartment, with the earliest TA cell generations harboring the capacity to fall back into the stem cell niche and quickly assume stem cell functions as originally proposed both by Leblond (Cheng and Leblond, 1974) and Potten (Potten, 1977) (Figure 3). Indeed, the Lgr5+ stem cell phenotype appears to be by no means hard-wired. The stem cell zone model already proposed that, during their upward migration, CBC stem cells would only gradually lose their self-renewal capacity. In vitro, Lgr5− crypt cells can be turned into organoid-forming Lgr5+ cells by a brief pulse of Wnt3A (Sato et al., 2011). In another example of such plasticity, Dll1 was recently shown to mark an early daughter of Lgr5+ stem cells residing around position +5, corresponding to the "common origin of differentiation" of Bjerknes and Cheng (van Es et al., 2012). Lineage tracing using CreERT2 expressed from the Dll1 locus showed that these Dll1+ cells represent short-lived progenitors that, under physiological conditions, produce small, mixed clones of secretory cells. However, when Lgr5+ cells are killed by radiation 1 day after induction of Dll1 lineage tracing, these Dll1+ secretory progenitors readily revert to Lgr5+ stem cells during the regeneration process.

A recent study applied an elegant strategy to inducibly kill Lgr5+ cells through transgenic expression of the receptor for diphtheria toxin from the Lgr5 locus (Tian et al., 2011). Upon injection of diphtheria toxin, the Lgr5+ cells die, yet remarkably, the crypts remain intact for at least 1 week (after which the animals succumb to liver-related pathology), implying that the self-renewal process can be maintained in the absence of Lgr5+ cells over this period. As soon as the toxin injections are stopped, Lgr5+ cells reappear. Using lineage tracing from the Bmi1 locus, it was shown that these new CBC cells derive from Bmi1+ cells, suggestive of a stem cell hierarchy (Tian et al., 2011). In a comment subsequently added to this study, the authors report that they observe proliferation at the crypt base in non-Lgr5+ cells 24 hr after toxin treatment. They hypothesize that, "...the observed proliferation is due to the Transiently Amplifying compartment collapsing to the bottom of the crypts" (http://www.nature.com/nature/journal/v478/n7368/full/nature10408.html).

To summarize, the CBC cell is long-lived and multipotent, as demonstrated by lineage tracing, by culture, and by transplantation. It is readily identified by its unique morphology and location (which includes the +4 position), and by the expression of markers such as Lgr5, Ascl2, OlfM4, and Smoc2. It can be cultured and transplanted at the clonal level. Importantly, the Lgr5+ CBC phenotype appears not to be hard-wired, but is

Table 1. An Overview of the Strengths and Weaknesses of Current +4 Markers

Marker	Reporter Gene Expression	Reported Population Characteristics	Supporting Evidence	Conflicting Evidence
Bmi1 (Sangiorgi and Capecchi, 2008; Yan et al., 2012)	proximal SI; predominantly at +4 position; minimal overlap with Lgr5+ CBC cells	retain DNA labels as consequence of relative quiescence (<2% proliferating); radiation resistant; induced to proliferate upon damage (20-fold); Wnt-independent; independent reserve stem cells	Bmi1-CreER-driven in vivo lineage tracing originates exclusively at +4 position; increased proliferation and frequency of lineage tracing following injury; Bmi1-driven lineage tracing observed following Lgr5+ stem cell ablation; Bmi1^{+ve} cells can generate intestinal organoids ex vivo	enriched in sorted Lgr5+ stem cells + TA progeny; endogenous expression throughout crypt (via FISH and IHC); original lineage tracing data nonreproducible: Bmi1-driven tracing events originate at random throughout the crypt
Hopx (Takeda et al., 2011)	entire SI + colon; predominantly around +4 position	retain DNA labels as consequence of relative quiescence; coexpress high levels of other +4 genes, including Bmi1 and mTERT; interconversion observed between Lgr5^{+ve} CBC and Hopx stem cells; independent reserve stem population?	Hopx-CreER-driven in vivo lineage tracing originates at +4 position; single Hopx^{+ve} cells typically remain quiescent in ex vivo culture; Hopx+ cells are phenotypically distinct from Lgr5+ stem cells; early progeny express CBC stem cell marker genes (Lgr5 and OlfM4)	Hopx expression enriched in sorted Lgr5+ stem cells + TA progeny; endogenous expression present throughout CBC stem cell zone and TA compartment (via FISH and IHC)
mTERT (Montgomery et al., 2011)	entire SI; predominantly located at +4 position; no expression detectable in majority of crypts	typically quiescent (<6% proliferating); radiation resistant; phenotypically distinct from both Lgr5+ stem cells and other purported +4 stem cell populations	mTERT-CreER-driven in vivo lineage tracing originates at +4 position; mTERT+ cells contribute to crypt regeneration in vivo; do not express Lgr5; do not coexpress other +4 markers (with exception of Bmi1, which was found to be expressed in both mTERT+ and mTERT− cells)	mTERT expression enriched in sorted Lgr5+ stem cells; also expressed in TA progeny; endogenous expression present throughout CBC stem cell zone and TA compartment (via FISH)
Lrig1 (Powell et al., 2012)	entire SI and colon; predominantly localized to crypt base (+1 to +5; excluding Paneth cells)	typically quiescent; radiation resistant; stimulated to proliferate following injury; independent from Lgr5+ CBC cells; independent from +4 populations?	Lrig1-CreER-driven in vivo lineage tracing originates at crypt base; increased proliferation and frequency of lineage tracing following injury; limited physical overlap with Lgr5-EGFP+ cells (yet demonstrate 3-fold enrichment of Lgr5 by microarray); no enrichment of other +4 markers (using mAb to isolate Lrig+ cells)	endogenous transcripts detected throughout crypts (via FISH); expression enriched in Lgr5+ stem cells and progeny via microarray; contradictory observations re. Lrig1+ IHC profile (due to different mAbs employed?); conflicting conclusions re. proliferation status of Lrig1+ cells: methodology issues?
Lrig1 (Wong et al., 2012)	entire SI and colon; expression gradient, with highest levels in Lgr5+ CBC compartment	typically proliferating; overlaps with, but is not restricted to the Lgr5+ stem cell compartment	both microarray and IHC analysis reveal expression gradient, with highest levels in Lgr5+ CBC compartment; no label retention observed in pulse-chase experiments	conflicting conclusions re. proliferation status of Lrig1+ cells: methodology issues?

readily inducible by Wnt signaling in vitro. In vivo, TA cells can revert to Lgr5+ stem cells after damage, presumably by direct contact with Paneth cells.

Markers of + 4 Cells

The original definition by Chris Potten of an LRC located primarily (although not exclusively) at the +4 position has prompted the quest for a "reserve" stem cell (Li and Clevers, 2010). Early studies, discussed below, have focused on +4 position-specific markers in conjunction with DNA-label retention.

Phospho-PTEN is reportedly enriched on LRCs located at crypt position +4/+5 (He et al., 2004, 2007). An independent study cast doubt on the validity of this putative +4 stem cell

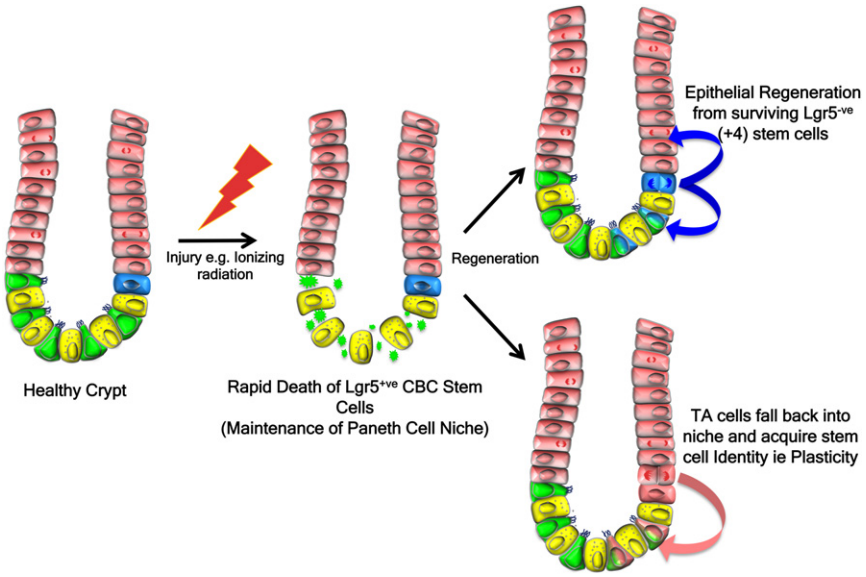

Figure 3. Crypt Regeneration following Injury: Reserve Stem Cells versus Plasticity
CBC stem cells rapidly die following acute injury such as irradiation. Crypt survival/regeneration may result from either reactivation of a quiescent Lgr5^{-ve} (+4) stem cell population (upper panel) or from dedifferentiation of non-stem cells to generate new CBC stem cells (lower panel).

marker by pointing out that the antibody crossreacts with rare enteroendocrine cells in crypts (Bjerknes and Cheng, 2005). The Wip1 phosphatase was proposed as another marker for the +4 cell (Demidov et al., 2007). Although Wip1+ cells were most commonly found at position +4, they also occurred within the CBC compartment. Depletion by apoptosis of these Wip1+ cells was observed in Wip1 knockout mice, but this had no detrimental effect on epithelial homeostasis. Other +4 markers identified solely on the basis of location include Sox4 (van der Flier et al., 2007), sFRP5 (Gregorieff et al., 2005), and DCAMKL-1 (Giannakis et al., 2006). The latter protein is a microtubule-associated kinase that was originally identified in the developing brain. Immunohistochemistry (IHC) revealed the presence of rare, nonproliferating DCAMKL-1 cells around position +4. These cells were negative for all known markers of differentiated cells, prompting the authors to identify them as likely +4 stem cells. Independent studies by May and colleagues confirmed the +4 localization, but also noted DCAMKL-1+ cells on villi (May et al., 2008, 2009). Jay and colleagues subsequently provided definitive evidence that DCAMKL1+ cells were not stem cells, but postmitotic Tuft cells, equally distributed along the crypt-villus axis (Gerbe et al., 2009); (Bezençon et al., 2008).

From these studies, it became evident that the quest for reliable markers for +4 stem cells should go beyond specific staining patterns, or DNA-label retention. The first +4 stem cell marker investigated by lineage tracing was Bmi1 (Sangiorgi and Capecchi, 2008) (Figure 4). The *Bmi1* gene encodes a component of a Polycomb transcriptional repressor complex, proposed to regulate self-renewal of neural and hematopoietic progenitors. By mRNA in situ hybridization, Bmi1 was found to mark rare cells at the +4 cell position uniquely in the proximal small intestine. In vivo lineage tracing using a Bmi1-ires-CreER/Rosa26RlacZ mouse model yielded ribbons under noninjury conditions that resembled those obtained in the Lgr5 model. Moreover, ablation of the Bmi1-Cre+ population using targeted expression of diptheria toxin caused crypt death, consistent with loss of the stem cell compartment. A follow-up study confirmed the notion that the Bmi1+ population is distinct from the Lgr5+ population in being highly radiation-resistant and quiescent, yet activated upon damage (Yan et al., 2012). The latter study supports a model in which Lgr5+ cells facilitate homeostatic self-renewal, whereas Bmi1+ cells mediate injury-induced regeneration (Yan et al., 2012).

Thus, Bmi1-based lineage tracing clearly yields "signature" stem cell tracings, but how specific is *Bmi1* expression for rare cells located at the +4 position? We have noted robust expression of Bmi1 mRNA in sorted Lgr5+ stem cells (Muñoz et al., 2012; van der Flier et al., 2009b), a finding independently confirmed by Breault and colleagues (Montgomery et al., 2011) and Coffey and colleagues (Powell et al., 2012). Using the single mRNA molecule FISH approach, *Bmi1* was found to be broadly expressed at roughly equal levels by all proliferative crypt cells, including the Lgr5+ CBC cells (Itzkovitz et al., 2012) (Figure 4). Staining for Bmi1 protein, using a Bmi1 knockout as a control, confirmed this broad expression pattern (Muñoz et al., 2012). Takeda et al. (2011) in their study on Hopx presented similar results for Bmi1 protein expression. De Sauvage and colleagues have quantified at which cell positions Bmi1 tracing initiates. In contrast to the original report (Sangiorgi and Capecchi, 2008), these authors found that tracing can initiate anywhere in the crypt, including rather frequently in Lgr5+ cells (Tian et al., 2011). In a repeat of the original Bmi1 tracing experiment (Sangiorgi and Capecchi, 2008), we have confirmed that Bmi1-CreER tracings can initiate in Lgr5+ cells, but we additionally document that most tracing events initiate in TA cells that are "washed out" within days of tracing initiation (Muñoz et al., 2012). Collectively, these studies would indicate that Bmi1 is not a specific marker for a +4 cell, but is broadly expressed in crypts. If true, Bmi1-based lineage tracing therefore would not report unique characteristics of a quiescent +4 cell. Rather, it reports a combination of behaviors of Lgr5 stem cells, TA cells, and, potentially, a quiescent stem cell type. As an additional complication, the Dll1+ secretory progenitor cell that can revert to Lgr5+ stem cells upon damage also expresses *Bmi1* (van Es et al., 2012). Thus, the contribution of quiescent stem cells to the complex pattern of Bmi1-controlled lineage tracing during homeostatic self-renewal (Sangiorgi and Capecchi, 2008) or upon damage (Yan et al., 2012) would not be easily discernable.

High telomerase levels may be a general feature of adult stem cells. Breault and colleagues reported rare cells (1 in about 150 crypts) expressing GFP from an mTert promoter-GFP transgene. Seventeen percent of these Tert-GFP+ cells were LRCs (Breault

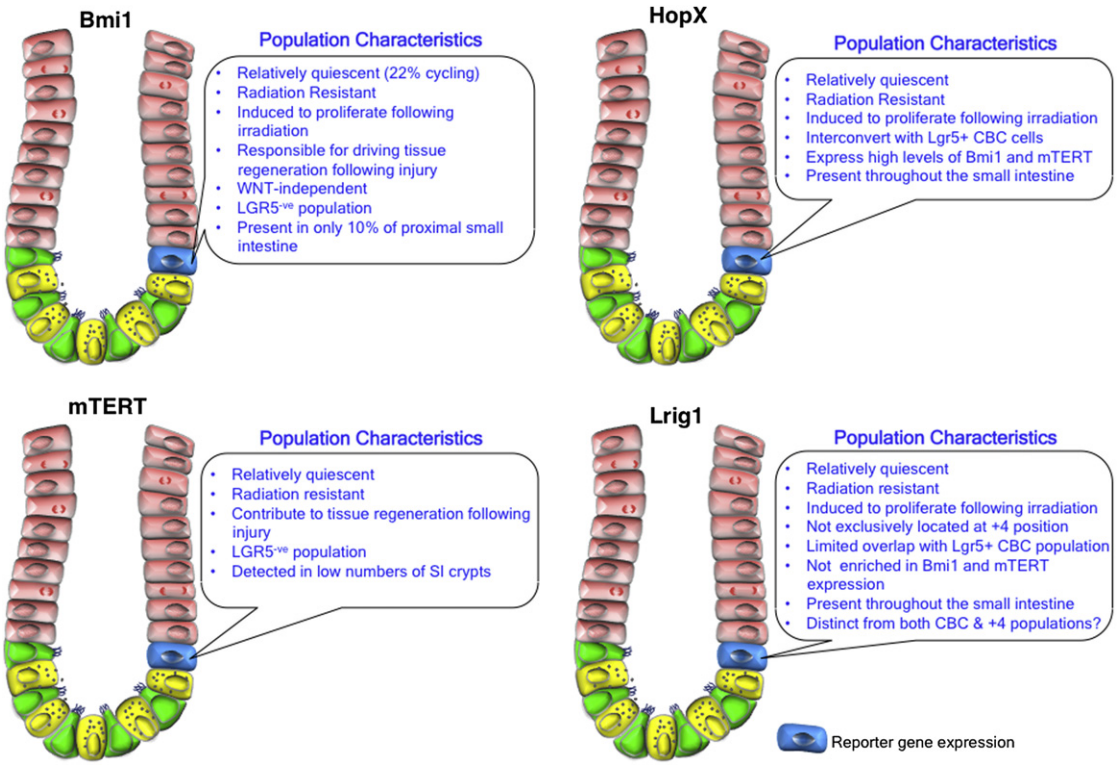

Figure 4. The +4 Stem Cells of the Small Intestine: A Current Perspective

et al., 2008). A follow-up study showed that mTert expression marks a radiation-resistant ISC population distinct from Lgr5+ cells (Montgomery et al., 2011). Using an mTert-CreER allele in a lineage tracing experiment, the mTert+ cells were found to generate all differentiated intestinal cell types as well as Lgr5+ stem cells. The quiescent mTert+ cells could be activated by damage. Thus, the mTert transgene alleles mark a very rare, quiescent, and radiation-resistant stem cell.

It is not intuitively clear why a quiescent cell, and not the cycling crypt cells, would express mTert. We have observed significant levels of active telomerase in all proliferative crypt cells, with the highest activity in Lgr5+ cells (Schepers et al., 2011). Similar results were obtained by single mRNA molecule FISH (Itzkovitz et al., 2012). An explanation for this discrepancy could be that the mTert transgenes fortuitously mark stem cells, but do not report the much broader endogenous mTert expression pattern. Alternatively, the mTert-GFP+ cells may express unusually high mTert levels. Such very rare cells (<1 per 150 crypts) could be missed in the FACS or FISH approach of the latter studies.

Hopx

Epstein and colleagues propose Hopx, an atypical homeobox protein, as a marker of +4 cells (Takeda et al., 2011). A Hopx LacZ knockin allele is expressed along the entire intestinal tract with strongest expression in the +4 position, and the majority of these were LRCs. Upon lineage tracing using a novel Hopx CreER allele, initiating events were preferentially seen around the +4 position and resulted in long-lived signature stem cell tracings. The study provided compelling evidence that Hopx+ cells can yield Lgr5+ cells and vice versa, leading to the notion that the two populations represent slow-cycling and fast-cycling stem cell populations that are interconnected. In contrast to this study, our *Lgr5* gene signature, confirmed by single RNA molecule FISH analysis, implies that *Hopx* is expressed in a broad gradient with highest Hopx levels occurring in Lgr5+ cells (Muñoz et al., 2012). Of note, Coffey and colleagues also report high *Hopx* expression levels in sorted Lgr5+ cells (Powell et al., 2012).

Lrig1

Lrig1 is a transmembrane molecule that acts as a pan-ErbB inhibitor. Two recent papers on Lrig1 report conflicting expression data (Powell et al., 2012; Wong et al., 2012). Coffey and colleagues have generated an Lrig1-CreERT2 allele (Powell et al., 2012). Lineage tracing initiated in the bottom one-third of crypts along the entire length of the intestinal tract and yielded signature stem cell ribbons by 7 days in small intestine and colon. Lrig1+ cells are at least as frequent as Lgr5-GFP+ cells, yet although these cells occupied the same positions (1–5) in crypts, little if any overlap was seen between *Lrig1* expression and Lgr5-GFP in the colon. Around 20% of the Lrig1+ cells were LRCs, whereas around 25% were KI67+. The authors noted that a low percentage of crypts (8%) after long-term tracing contained a single LacZ+ cell. These noncycling cells became proliferative upon irradiation. Microarray profiling revealed that sorted Lrig1+ as well as Lgr5+ cells from colon expressed the CBC marker Prominin/CD133 and the +4 markers mTert and Bmi1. Another proposed marker for quiescent +4 cells, *Hopx*, was expressed at 2-fold higher levels in Lgr5+ cells than in Lrig1+ cells. Lgr5+ cells showed an active cell cycle gene

signature, whereas the Lrig1+ population showed signs of being "in the process of downregulating the cell cycle."

While Lgr5 was 20-fold enriched in the Lgr5+ cells versus Lrig1 cells, Lrig1 was also 3-fold enriched in Lgr5+ versus Lrig1+ cells. The latter observation appears paradoxical, but can be explained by the notion that Lgr5+ cells are contained within the Lrig1+ population and represent the highest *Lrig1* expressors within this population. Indeed, Kim Jensen and coworkers report that approximately one-third of all crypt cells express *Lrig1* with highest levels in the Lgr5+ stem cells (Wong et al., 2012). Our *Lgr5* gene signature as well as the single mRNA molecule FISH have confirmed that *Lrig1* is expressed in a broad gradient with highest Lrig1 levels occurring in Lgr5+ cells (Muñoz et al., 2012).

To summarize, four markers are now available for the study of the +4 cell (Figure 4). The original studies on these markers have in common that the marked cells have features of quiescence/LRCs and are preferentially located at Potten's +4 position. An important aspect in the definition of the +4 markers has been their shared propensity to identify cells that are distinct from the Lgr5+ CBC cells. Together, these observations have led to the perception of the +4 cell as a homogeneous stem cell class, identifiable by label retention, location, and multiple molecular markers.

However, direct comparison of the description of +4 cells between the original studies reveals three major differences that have not been emphasized previously. (1) The recent marker-supported studies on the +4 cell do not recognize that in the original description of Potten, the +4 cell is extremely radiation-sensitive and cycles every 24 hr. The Potten +4 cell appears therefore to be of a separate class, not to be confused with the more recent "marker-identified" +4 cells. (2) The frequency of +4 cells as identified by the different markers is very divergent. The Tert+ cell occurs in 1 in every 150 crypts (Montgomery et al., 2011), while the Lrig1+ cell is more frequent than the Lgr5+ CBC cell (of note, there are 15 CBC cells in each crypt) (Powell et al., 2012), a difference of >2,250-fold. (3) Location along the intestinal tract differs; Bmi1+ cells are confined to the proximal small intestine (Sangiorgi and Capecchi, 2008), while Lrig1+ cells are found along the entire length of the intestine (Powell et al., 2012).

Full molecular signatures for each of the four +4 cell classes could shed light on their relatedness and on their relation to Lgr5+ CBC cells. So far, microarray expression profiling has been performed for Lrig1+ cells in comparison to Lgr5+ cells (Powell et al., 2012). This study reveals that Hopx is significantly enriched in Lgr5+ cells relative to Lrig1+ cells, whereas Bmi1 and Tert are expressed to somewhat higher levels in Lgr5+ cells than in Lrig1+ cells. While this study emphasizes that the +4 markers appear not to define a single class of cells, it also underscores that all four +4 markers show very significant expression in Lgr5+ cells.

Pitfalls

It is clear from the examples given above that the definitive identification of stem cells by unique markers is less straightforward than it may appear. A number of considerations and pitfalls are listed below.

The mere expression of a marker at a specific location is not sufficient to establish a marker and a new stem cell type. The evidence should always involve lineage tracing, for which the gut is ideally suited given its architecture.

DNA-label retention is not restricted to quiescent stem cells, but is also a hallmark of postmitotic cells. Examples of the latter are Paneth cells, enteroendocrine cells, and tuft cells. Because tuft cells and enteroendocrine cells are rare and do occur in crypts, they can easily be mistaken for LRC stem cells.

Marker expression should be very carefully evaluated. Pitfalls are myriad. The use of antibody staining on gut sections is notorious for its propensity to yield false positive signals on any of the (rare) secretory cell types in the gut. This problem can be easily circumvented by showing images of the entire crypt-villus axis and by showing multiple crypts in the same image. A very good control for in situ hybridization or IHC is the side-by-side analysis of WT and knockout tissue. Examples are available for Bmi1 (Muñoz et al., 2012) and Lrig1 (Wong et al., 2012).

Given that a series of markers have now been proposed, this should facilitate the determination and comparison of genome-wide expression signatures by microarray analyses of purified marker+ populations. This has now been done for Lgr5 and for Lrig1 (Muñoz et al., 2012; Powell et al., 2012; Wong et al., 2012).

Multicolor single mRNA FISH appears to be an excellent method for analyzing coexpression of candidate genes at the single cell level, as is the single cell PCR-based approach of Clarke (Guo et al., 2010; Itzkovitz et al., 2012).

In lineage tracing, it is of paramount importance to ensure that the site of tracing initiation is very carefully mapped, as discussed above for CD133 (Zhu et al., 2009 versus Snippert et al., 2009) and Bmi1 (Sangiorgi and Capecchi, 2008 versus Muñoz et al., 2012; Tian et al., 2011).

We have realized that recombinant alleles or transgenes are often mosaically expressed between groups of crypts, while expression is most consistent in the proximal small intestine. It appears that the Bmi1 alleles (Sangiorgi and Capecchi, 2008) are subject to this phenomenon, as may be the mTert-CreER allele (Montgomery et al., 2011). Our original Lgr5 allele is similarly silenced in the majority of distal small intestinal and colon crypts. When sorting Lgr5-GFP− fractions from these Lgr5-knockin mice, these fractions will contain large numbers of genuine CBC cells in which the recombinant Lgr5 allele is silenced. We have circumvented this in the past by sorting and comparing Lgr5-GFP^{hi} stem cells and Lgr5-GFP^{lo} daughters. Our Lgr5-LacZ mice (Barker et al., 2007) and the Lgr5 knockin mice generated by de Sauvage and colleagues (Tian et al., 2011) do not display this problem.

Epilogue

While our view is undoubtedly biased toward Leblond's CBC cells, we feel that the verdict is still out as to the existence of a reserve +4 cell (or of several classes of such cells). As discussed above, several independent studies report that the +4 markers are robustly expressed by Lgr5+ CBC stem cells. If true, this complicates the interpretation of lineage tracing experiments based on these markers, because such lineage tracing can neither prove nor disprove definitively the existence of an

Lgr5− +4 stem cell. In the absence of a unique +4 marker (or combinations of markers), neither a head-to-head comparison to the CBC cell nor definitive lineage tracing or transplantation can be performed.

Multi-isotope imaging mass spectrometry (Steinhauser et al., 2012) has been used very recently to search for label-retaining cells in the small intestine. No long-term label-retaining cells other than Paneth cells were found by this exquisitely sensitive assay. Of course, if stem cells would be slowly cycling, rather than be deeply quiescent, these would be missed by this approach.

The definitive demonstration of a quiescent stem cell, distinct from the CBC cell, may exploit strategies that lean on the elegant H2B-GFP in vivo chromatin-label retention approach of Fuchs and colleagues (Tumbar et al., 2004). Such a study was recently conducted by Fodde and colleagues (Roth et al., 2012), who characterize small intestinal LRCs persisting for up to 100 days. These LRCs are postmitotic and are positive for Paneth cell markers, yet can switch to a proliferative state upon tissue injury. Possibly, the distinction between differentiated cells and quiescent stem cells is less absolute than generally believed.

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