Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration

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Abstract
Small populations of adult stem cells are responsible for the remarkable ability of the epithelial lining of the intestine to efficiently renew and repair itself throughout life. The recent discovery of specific markers for these stem cells, together with the development of new technologies to track endogenous stem cell activity in vivo and to exploit their ability to generate new epithelia *ex vivo*, has greatly improved our understanding of stem cell-driven homeostasis, regeneration and cancer in the intestine. These exciting new insights into the biology of intestinal stem cells have the potential to accelerate the development of stem cell-based therapies and ameliorate cancer treatments.

Introduction

The inner epithelial lining of the small intestine is a truly multitasking tissue. It must simultaneously achieve the efficient digestion and absorption of food contents released from the stomach, whilst maintaining an effective barrier to potentially lethal microbes and carcinogens present within the intestinal lumen. A persistent mechanical, biological and chemical aggression from the luminal contents induces a remarkably high rate of cell death, with up to 200g of epithelial cells being lost every day in humans[1]. This imposes a requirement for daily self-renewal throughout life, driven by small populations of adult stem cells that reside within specialized 'niches'. The ability of these stem cells to indefinitely self-renew while generating new functional epithelia makes them ideally suited for regenerative medicine applications. The same properties also make stem cells prime candidates for accumulating mutations that together initiate and sustain intestinal cancer growth – making them attractive therapeutic targets.

Decades of intensive study have successfully deciphered many key aspects of intestinal biology, including the approximate size and location of the adult stem cell population within the epithelium. However, their identity has long remained elusive due to a lack of specific markers and suitable methodologies for rigorous evaluation of endogenous stem cell function[2,3]. This inability to identify and isolate adult stem cells has been a major limitation to the study of their behavior during homeostasis and disease, as well as hampering efforts to exploit their undoubted therapeutic potential. However, the intestinal stem cell field has experienced something of a renaissance in recent years, driven largely by the discovery of robust adult intestinal stem cell (ISC) markers, together with the development of clonal fate mapping technologies (which evaluate stem cell behavior in vivo), and near-physiological *ex vivo* culture systems (which support the long-term growth of functional epithelia from patient biopsies for use in diagnostic and/or therapeutic applications)[4-6]. With these new tools, researchers

have made impressive breakthroughs in understanding how intestinal stem cells interact with their local niche to maintain homeostasis in healthy intestinal epithelia, and how they might contribute to intestinal cancer initiation and progression.

Other crucial findings include the identification of various sources of 'reserve' stem cells that can be rapidly recruited to maintain epithelial homeostasis following injury[7,8]. One major implication of this finding is that epithelial cells other than the small pool of regular stem cells harbor regenerative potential. Also noteworthy is the discovery of a nutrient-sensing mechanism within the stem cell niche that effectively regulates the size and activity of the intestinal stem cell pool[9]. Such information can potentially be exploited to maximize stem cell output for therapeutic applications.

Here, I review the current models of stem cell-driven epithelial homeostasis & repair in the intestine, highlighting the contributions of both pioneering research performed in the 1970's and 80's and more recent breakthroughs driven by the identification of robust stem cell markers and the development of near-physiological epithelial culture systems.

The adult intestinal epithelium

Cellular organization. Three weeks after birth, gut development is complete and the mouse is ready to tackle its first solid meal. In the small intestine, each villus is encircled by up to 6 crypts of Lieberkühn housing dedicated populations of stem and progenitor cells that provide the self-renewal capacity needed to maintain epithelial function throughout life (Fig. 1a).

The organization of the epithelium is tailored to meet the specific functional requirements of the different regions of the intestinal tract. In the duodenum, the villi are longest and the epithelium comprises mainly absorptive enterocytes that also secrete a cocktail of hydrolytic enzymes to facilitate the efficient breakdown of partly digested food exiting the stomach and the subsequent absorption of nutrients. Also present are goblet cells, which secrete mucus to help protect and lubricate the epithelium, together with much smaller populations of enteroendocrine cells producing hormones that regulate gut physiology (Fig. 1a). Goblet cells are most prevalent in the epithelium of the short villi of the distal small intestine, where they provide extra lubrication needed to facilitate the passage of increasingly compact stool towards the colon. Protection against the potentially hazardous microbial environment of the gut lumen is provided throughout the small intestine by crypt-resident Paneth cells, which secrete antimicrobial substances (such as cryptdins) and the hydrolytic enzyme lysozyme. At least three other cell types with poorly defined functions exist in the epithelium: cup cells, tuft cells and Peyers patch-associated M cells[10].

In the colon, which specializes in compacting stool for rapid excretion, the inner epithelial lining is arranged into multiple crypts associated with a flat luminal surface. In contrast to the small intestine, this epithelium is characterized by high density of goblet cells and absence of Paneth cells (Fig. 1b).

Rapid epithelial turnover. In the mouse, the adult epithelium renews itself every 3-5 days throughout life to maintain optimal function. Epithelial cells exposed to the harsh luminal environment die and are expelled from the tips of the villi via a complex cytoskeletal remodeling process that simultaneously seals the resulting gap to maintain barrier function[11]. Over 300 million new epithelial cells must be generated daily in the small intestine alone to compensate for this high rate of cell death on the villi. Regeneration relies on the crypt base, where small populations of adult stem cells regularly divide to produce highly proliferative progenitors known as transit-amplifying (TA) cells (Fig. 1). The nascent TA cells typically divide 2-3 times over a 1-2 day period and gradually commit to the absorptive or secretory cell lineages whilst migrating upwards towards the base of the villi. All proliferation ceases as the differentiated cells

exit the crypts, and these functional epithelial cells then continue migrating upwards along the villi over the next 48 hours.

The complete 3-5 day epithelial renewal cycle is repeated several hundred times during the average 2-year lifespan of an inbred mouse. Paneth cell turnover is the only exception to this rapid self-renewal. These cells are renewed every 3-6 weeks from dedicated secretory cell progenitors located at the base of the TA compartment, which mature into fully differentiated Paneth cells whilst following a downward migratory path to the crypt bottom[12,13].

Origin of the epithelium and its stem cells

Embryonic origin of the intestinal epithelium. In the mouse, the epithelium of the small intestine originates as a polarized, single-layered (pseudostratified) structure lining the inner surface of the primitive gut around embryonic day (E)9.5 [14]. At E14.5, following thickening of the epithelium, initiates a major remodeling process orchestrated by multiple signaling pathways, which results in the rapid conversion of the flat luminal surface into multiple finger-like projections called villi. The regenerative capacity of the adult epithelium is established between E16.5 and postnatal day 7, when mature crypts harbouring adult stem cells and progenitor cells develop from shallow pockets of proliferative cells restricted to the base of the embryonic villi[15].

Although the mechanics of these remodeling processes remain incompletely understood, bidirectional signals between the epithelium and the underlying mesenchyme are considered to be crucial orchestrators. In particular, Hedgehog signals from the developing epithelium are thought to direct embryonic villus formation and specify the site of crypt development at the villus base[16]. Furthermore, an essential role for Wnt signaling in establishing the regenerative capacity of the adult epithelium is evidenced by a loss of prospective crypts and impaired villus formation following embryonic ablation of the major intestinal Wnt effector protein, Tcf4[17]. Crypt numbers rapidly increase during postnatal development of the small intestine via a bifurcation mechanism known as crypt fission[18]. This process, which is most rapid around 2 weeks after birth in mice, is a poorly understood mechanism that may be triggered by a rapid increase in crypt size owing to increased stem and/or progenitor cell activity[18,19].

Adult stem cell origins in the intestine. Analysis of genetic marker expression patterns in newborn chimaeric mice has determined that nascent crypts are polyclonal, originating from a mixture of proliferative progenitor cells derived from both parents[20,21]. However, through a poorly understood stochastic process known as 'purification', a single progenitor cell gradually achieves dominance during the first 2 weeks of neonatal life, giving rise to a clonal pool of adult stem cells in each crypt that is maintained throughout life[22-24]. Also in humans, analyses of random mitochondrial mutation patterns within healthy small intestine and Y-chromosome inheritance patterns in the colon of a XO/XY chimeric patient have established that adult crypts throughout the intestinal tract are clonal[25-28]. Despite this phenotypic clonality, it is important to note that adult crypts are not maintained by a single stem cell. Indeed, multiple stem cells (estimated 3–16 depending on the methodology used to study them) sustain long-term epithelial homeostasis [29-31].

Formal proof of the existence of multipotent, self-renewing stem cells in the adult intestine was obtained by mapping the inheritance of genetic marks introduced at low frequency in crypt cells via random somatic mutation[24,32]. The appearance of rare, long-lived clones containing all major epithelial lineages identified the original marked crypt cells as multipotent adult stem cells. Although a seminal finding, these fate-mapping approaches were unable to reveal the location and identity of the stem cells within the crypt. Clues on the location of stem cells were already apparent back in the 1970's, when radioisotope and BrdU labeling of dividing cells was employed to track epithelial

renewal patterns in the intestine[33-35]. The cell migration patterns indicated a common origin close to the crypt base, hinting at this being a possible location for multipotent stem cells[1]. In support of this, ablation of the crypt base populations via targeted irradiation completely abolished the epithelial self-renewal capacity of the intestine, consistent with loss of the stem cell compartment[36]. Despite these advances, the identity of adult stem cells remained frustratingly elusive for more than 2 decades and is even today still the subject of intense debate.

Models of adult stem cell identity

Two models of intestinal stem cell identity have historically competed over the last 4 decades. The 'stem cell zone model' by Charles Leblond, Hazel Cheng and Matthew Bjerknes suggests that the columnar cells at the base of the crypt are the resident stem cells, whereas the '+4 model' by Chris Potten and colleagues proposes that stem cells reside within a ring of 16 cells immediately above the Paneth cells. Despite the recent discovery of specific markers for these two candidate stem cell populations, it has proven difficult to definitely determine which of these models is correct. Instead, a unifying theory that incorporates aspects of both models is emerging, based on the existence of distinct stem cell pools involved in epithelial homeostasis and regeneration.

The stem cell zone model. The identification of undifferentiated, mitotically active crypt base columnar (CBC) cells intercalated with Paneth cells at the bottom of crypts lead to the formulation of the 'Unitarian theory of the origin of the four epithelial cell types'. This model, outlined in a series of elegant papers by Hazel Cheng and Charles Leblond published in 1974, proposed that the CBC cells gave rise to the four major cell lineages in the intestinal epithelium[37-41]. CBC cells were described as being actively phagocytic, helping to clear dead cells from the crypt base. Taking advantage of this trait, Cheng irradiated mice and showed that surviving CBC cells contained radiolabeled phagosomes as a consequence of engulfing neighbouring CBC cells killed via incorporation of tritiated thymidine. Initially, only CBC cells were marked, but at later time points, examples of 3 major epithelial cell lineages became radiolabeled within the crypts. This rudimentary fate-mapping result was interpreted as CBC cells being the common ancestor of the differentiated epithelial cell types of the intestinal lining. However, the fact that the 3 marked epithelial lineages were located in different crypts and that no marked enteroendocrine cells were identified precluded a formal identification of the CBC cells as multipotent stem cells.

Additional evidence in support of CBC stem cell identity was provided over 2 decades later, when Bjerknes and Cheng employed a mutation approach to introduce heritable somatic marks within the crypts[24]. A small proportion of the resulting epithelial clones comprised cells of all major lineages that were maintained long-term, indicating that the original mutation had occurred within a (unidentified) self-renewing stem cell. The fact that only these persistent, multi-lineage clones invariably contained a CBC cell was interpreted as further evidence of a CBC stem cell identity.

This prompted Cheng and Bjerknes to refine the original Unitarian theory of intestinal cell origins, resulting in the publication of the 'stem cell zone model' in 1999[24]. This model proposes that CBC cells are adult stem cells that reside in a stem cell permissive zone (the niche) at the very base of the crypt. These cells proliferate to generate daughter cells that exit the niche and commit to multi-lineage differentiation at the 'common origin of differentiation' around position +5. Paneth cell progenitors mature into functional lyzozyme-secreting cells as they migrate downwards to occupy the base of the crypt, whilst the majority of the non-Paneth cell lineages mature into functional epithelia whilst migrating upwards onto the villus epithelium. However, it is important to note that the crypt base is not exclusively populated by Paneth cells and CBC cells - rare enteroendocrine cells and goblet cells are also present, but are post-mitotic and are therefore thought to be unresponsive to stem cell niche signals. Despite

a substantial body of indirect evidence in support of the stem cell zone model, it failed to gain a general acceptance until 2007, when CBC cell-specific markers were first identified[42] (Box 1). These markers enabled *in vivo* lineage tracing experiments[42] and *ex vivo* assays[43], to provide more direct evidence of CBC stem cell function during epithelial homeostasis and disease (Box 2).

The +4 model. The first indication that intestinal epithelial cells may occupy position '+4', (defined as the fourth cell position from the crypt base) immediately above the Paneth cell compartment, came from cell tracking experiments evaluating the migration rates of radiolabelled cells at various positions within the crypt[33,34]. Potten and colleagues later documented the existence of cells with stem cell attributes at a similar location[44]. These cells were actively dividing (every 24 hours), yet capable of retaining labels incorporated into their DNA during crypt neogenesis (when new stem cells are being generated). Although the latter is generally considered to be a trait characteristic of cellular quiescence, the +4 label-retaining cells (LRCs) could be subsequently labeled with BrdU, confirming their active proliferation status[3]. These conflicting observations were attributed to the ability of the +4 cells to selectively retain labeled template DNA strands during mitosis, while newly-synthesized DNA strands that could contain potentially dangerous replication errors are segregated to the short-lived transit-amplifying daughter cells during asymmetric division[3]. This phenomenon, originally described in the 'immortal stand hypothesis' by Cairns,[38,39] was proposed to limit the accumulation of DNA damage in long-lived adult stem cells. However, this model has been challenged, as it makes several assumptions about +4 cells that are not supported by experimental evidence, including the absence of symmetrical cell divisions and DNA exchange between sister chromatids (that is, between template and newly-synthesized DNA as it typically occurs in somatic cells)[45].

Independent experiments also identified cells located around the +4 position that were highly sensitive to ionizing radiation[44], a surrogate stem cell trait believed to ensure that these long-lived cells do not survive DNA damage that could eventually lead to cancer. However, formal proof that label-retention and radiation-sensitivity can be attributed to the same +4 cell population is currently lacking. Efforts to validate and refine the +4 stem cell model through identification of specific marker genes that facilitate a direct evaluation of endogenous stem cell identity have been reported. Although these studies increase our understanding of the biology of intestinal stem cells, their observations are difficult to reconcile with the original +4 model (as discussed later).

Validation of CBC cells as stem cells

In vivo lineage tracing using the Lgr5 marker. *In vivo* lineage tracing is a cell fate mapping tool used for evaluating the stem cell identity of candidate populations in their native microenvironments[4]. It is performed by introducing permanent, heritable genetic marks into candidate stem cells. As the descendants of these cells inherit the marks, this facilitates their identification and characterization within the epithelium. If all differentiated cell-lineages can be traced back to a single candidate stem cell, then this cell is considered to be multipotent. Long-term generation of marked cell lineages within the epithelium is indicative of self-renewal capacity of the stem cell candidate. Any candidate demonstrating both multipotency and self-renewal capacity is considered to fulfill the minimal definition of an epithelial stem cell.

The first marker for CBC cells, *Lgr5* (Leucine-rich G protein-coupled receptor 5), was identified as a Wnt target gene selectively expressed at the base of adult intestinal crypts [42] (Fig 2). CBC-specific expression of *Lgr5* was confirmed using Lgr5-lacZ/EGFP reporter mice. In these mice, approximately 14 Lgr5-expressing (Lgr5+) CBC cells were

uniformly distributed amongst the Paneth cells throughout positions 1–4 of the crypt base.

The stem cell identity of Lgr5+ CBC cells was confirmed via lineage tracing using an Lgr5-EGFP-ires-CreERT2/R26R-lacZ model. Stochastic activation of *lacZ* reporter gene expression at low frequency in the Lgr5+ CBC cells resulted in the appearance of lacZ+ clones that rapidly expanded to encompass epithelia spanning the crypt base to the villus tip. These CBC cell-derived clones contained all major cell lineages and generally persisted long-term, validating the Lgr5+ CBC cells as self-renewing, multipotent adult stem cells contributing to epithelial homeostasis in the intestine. Further proof of their stem cell identity was provided when single Lgr5+ CBC cells isolated by FACS-sorting from Lgr5-EGFP reporter mice were shown to generate self-renewing epithelial "organoids" that recapitulated the general architecture and cellular composition of functional intestinal epithelium using a novel ex vivo culture method[43]. Of note, Lgr5- cells lacked the ability to generate these epithelial organoids, highlighting the value of this ex vivo culture system as a surrogate assay of endogenous stemness.

CBC-like cells are also present at the base of human intestinal crypts, but the current lack of validated Lgr5 antibodies has precluded a formal evaluation of their global expression profile and their stem cell identity. However, epithelial cells residing at the very crypt base in the human colon have been isolated using antibodies against the surface-expressed Wnt target gene *EphB2*. These EphB2^{hi} cells behaved as stem cells in ex vivo organoid culture assays and demonstrated a marked enrichment of *Lgr5* expression, indicating a likely conservation of intestinal stem cell identity in mice and humans[46].

The Lgr5+ CBC stem cell expression signature. Combinatorial microarray and proteomic approaches have been used to establish an accurate molecular signature for the Lgr5+ CBC stem cells[47,48]. A comparison of EGFP^{hi} stem cells and EGFP^{lo} progeny isolated by FACS from Lgr5-EGFP reporter mice revealed approximately 500 stem cell enriched genes. Consistent with the known influence of Wnt signaling on stem cell driven epithelial homeostasis in the intestine[17], a strong Wnt signature was present, including many established Wnt target genes such as *Sox9, Ascl2, EphB2, Troy* and *Axin2*. This approach also revealed novel markers of the Lgr5+ CBC stem cells including OLFM4, Smoc2 and Rnf43 (Fig 2). Selective expression of the Bmp inhibitor, *Smoc2*, on the stem cell compartment was validated via in vivo lineage tracing[47]. Surprisingly robust expression of many published +4 stem cell markers (*Bmi1*, *Lrig1*, *mTert*, & *Hopx*) in the Lgr5+ stem cells was also noted, casting doubt onto claims that these represent truly independent intestinal stem cell pools (discussed below).

Regulation of CBC stem cell activity and fate. A closer look at the Lgr5+ stem cell expression signature has provided new mechanistic insight into the regulation of intestinal stem cell activity and fate in vivo. Ascl2, a transcription factor implicated in promoting neuroblast differentiation, was identified as a critical regulator of intestinal stem cell fate when its conditional ablation on the intestinal epithelium resulted in a rapid and selective loss of the Lgr5+ stem cell compartment[49]. *Lgr5*, which encodes a 7-transmembrane receptor, was itself linked to stem cell homeostasis when its conditional ablation, together with a close homolog *Lgr4*, resulted in a rapid suppression of Wnt signaling and consequent stem cell death[50]. A direct role for Lgr5 in modulating Wnt signaling on the intestinal stem cells was confirmed when it was identified as a facultative component of the Wnt signaling complex at the membrane, effecting the recruitment of secreted Wnt agonists, Rspondin1-4, to selectively amplify existing canonical Wnt signals above a threshold compatible with stem cell homeostasis in vivo[50-53]. Two additional Wnt target genes enriched in the stem cells, *Rnf43* and *Troy* were also found to be instrumental in regulating endogenous Wnt signaling activity. Rnf43 moderates Wnt signals on the stem cells by ubiquitylating Fzd receptor proteins

to promote their turnover[54,55]. Troy also dampens Wnt signaling on the stem cells by destabilizing the Lrp6 coreceptor protein following its recruitment to the Wnt receptor complex by Lgr5[56]. Collectively these observations highlight the importance of maintaining an optimal level of Wnt signaling for intestinal stem cell homeostasis in vivo. The recent elucidation of the complex structure of Lgr5 with R-spondin and other Wnt receptor components will undoubtedly deliver additional insights into the mechanism of action of Lgr5 on the intestinal stem cells in the near future[57-59]

A model of CBC stem cell-driven homeostasis

Important insights into how the actively cycling Lgr5^{+} CBC population balances epithelial homeostasis and stem cell maintenance in vivo were obtained by documenting the output of individual Lgr5^{+} cells using a multicolor lineage tracing approach[60]. These clonal fate-mapping experiments unexpectedly revealed that individual Lgr5^{+ve} cells within a single crypt population typically undergo symmetrical division to generate progeny with identical fates (either 2 stem cells or 2 TA cells), rather than adopting the standard asymmetric division mode attributed to most somatic stem cell populations. Although this division mode implies a regular loss of individual Lgr5+ stem cells, a balanced supply of new stem cells and TA cells is achieved at the population level. Over time, the multicolored Lgr5 stem cell populations gradually became monochromatic through a process of stochastic refinement known as "neutral drift" (Fig 2c). These observations collectively indicated that a balanced homeostasis of the epithelial and stem cell compartments is achieved by neutral competition between 14 symmetrically dividing stem cells for restricted niche space at the crypt base. A separate study employing a predominantly mathematical modeling approach provided additional support for this model[61]. In contrast, a study analyzing the relationship between mitotic spindle orientation and DNA segregation during Lgr5+ stem cell division concluded that asymmetric division predominates, with template strands being preferentially distributed to progeny adopting a stem cell fate during mitosis[62]. However, this was challenged in later studies analyzing spindle orientation in crypt base cells[63] and DNA label distribution kinetics during Lgr5+ CBC stem cell division, which conclusively demonstrated a random mode of chromosome segregation to daughter cells[64,65].

Although symmetrical division seems to predominate within the Lgr5+ CBC stem cell compartment, it is currently unknown whether the choice between adopting stem cell and transit-amplifying cell fates is truly stochastic. It is plausible that local niche and biomechanical influences can direct post-mitotic fate choices of Lgr5+ stem cells in a position-dependent manner at the crypt base. In theory this could be investigated by fate mapping Lgr5+ stem cells at different crypt locations via multicolor lineage tracing, although this is technically challenging. Alternatively, robust, predictive computer models of crypt homeostasis can be employed to evaluate experimental predictions of in vivo stem cell behavior[66,67].

Regulation of CBC stem cell activity and fate.

Recent studies have begun to shed light onto the regulation of CBC stem cell activity in vivo. Surprisingly, epigenetic regulation via DNA methylation does not appear to play a major role in directing stem cell fate[68]. However, the GTP'ases CDC42 and Rab8a, together with the endoplasmic stress response pathway appear to be important mediators of both stem cell division and differentiation[69,70].

Validation of +4 cells as stem cells

A number of genes have recently been reported as being selectively expressed on the candidate +4 stem cells (Fig 2a & Box 3). Although some of these markers have been validated by in vivo lineage tracing, many of them appear to mark epithelial populations with quite distinct cellular characteristics to each other and to the original "Potten" LRC

population. This may indeed reflect the existence of multiple, phenotypically distinct, stem cell populations residing at the +4 position. However, it is also becoming increasingly obvious that some published mouse models do not faithfully report endogenous expression patterns of candidate stem cell markers – this has caused some controversy regarding the validity of some of the +4 stem cell markers, which we briefly discuss below.

Markers evaluated by in vivo lineage tracing

Bmi1. This encodes a polycomb repressor component that has previously been implicated in the regulation of hematopoietic and neural stem cell replication[71]. Standard in-situ hybridization analyses documented restricted *Bmi1* expression at the +4 position within the proximal small intestine, a finding corroborated using a Bmi1-EGFP reporter mouse model[8]. In vivo fate mapping experiments using a Bmi1-ires-CreERT2/ROSA26RlacZ model revealed these proximal Bmi1+ populations to harbor self-renewing, multipotent stem cells contributing to long-term epithelial homeostasis. Additional support for a stem cell identity of these Bmi1+ cells was provided when in vivo ablation of the Bmi1+ population via targeted expression of diptheria toxin effectively blocked epithelial renewal, and isolated Bmi1+ cells were found to be capable of generating epithelial organoids reminiscent of functional intestinal epithelia in culture [8,72].

The Bmi1+ population was further characterized as being relatively quiescent, Wnt-independent and radiation resistant, prompting the conclusion that Bmi1 marks a +4-restricted "reserve" stem cell population independent from the crypt-base resident Lgr5+ CBC compartment. However, more recent independent expression analyses have documented robust *Bmi1* expression throughout the proliferative zone of the crypt, including the Lgr5+ CBC compartment[47,73-75,76]. In agreement with such a broad endogenous expression pattern, a recent fate mapping study employing a new Bmi-CreERT2 transgenic model noted the initiation of Bmi1-driven lineage tracing at random locations throughout the crypts[8]. Moreover, efforts to independently reproduce the original findings of Sangiorgi and colleagues using their Bmi-ires-CreERT2 mouse model yielded surprisingly contrasting conclusions - rather than lineage tracing being predominantly initiated at the +4 position as published, tracing initiation was instead documented at random positions throughout the crypt, including the Lgr5+ CBC compartment[47]. Over time, the majority of these lineage tracing events were lost, consistent with them having originated within the short-lived TA progenitor cell compartment. Long-term tracing events were postulated to have arisen from the Bmi1+/Lgr5+ CBC stem cell compartment[47].

Taken together, these latest findings are incompatible with Bmi1 being a selective marker of the +4 stem cells in the small intestine, casting doubt on the value of employing Bmi1-driven lineage tracing for studying the contribution of +4 reserve stem cells to crypt regeneration and disease.

Hopx. This encodes an atypical homeobox protein found to be predominantly expressed at the +4 position throughout the entire intestine using Hopx-lacZ reporter mice[76]. These Hopx-lacZ+ cells were characterized as being relatively quiescent, radiation resistant, and harboring the capacity to rapidly proliferate in response to irradiation-induced injury. A formal demonstration of Hopx+ stem cell identity was provided by in vivo lineage tracing using a Hopx-ires-CreERT2 mouse model, when reporter gene activation around the +4 position resulted in the formation of persistent, multipotent stem cell signature tracings throughout the intestine.

Comparative expression profiling of laser-microdissected Hopx+ cells and their immediate crypt base progeny documented a marked enrichment of Lgr5 and other CBC

marker genes in the presumptive Hopx cell descendents. Conversely, organoid culture assays confirmed that isolated Lgr5+ CBC stem cells give rise to Hopx+ cells ex vivo.

These observations support a model in which proliferating Lgr5+ stem cells and quiescent Hopx+ stem cells located at distinct anatomical locations within the crypt efficiently interconvert during epithelial homeostasis. In agreement with the findings of Munoz et al., this study also documented robust *Bmi1* expression within the Lgr5+ CBC stem cells. However, it should be noted that a very different *Hopx* expression profile was reported in a separate study, when single RNA molecule FISH and Lgr5+ CBC expression profiling analyses detected endogenous expression throughout the crypt, with highest levels in the Lgr5+ stem cells at the crypt base[47].

Lrig1. This encodes a single-pass transmembrane receptor that functions as a conditional panErB inhibitor in a range of adult tissues. Its discovery as a marker and proliferation regulator of stem cells in the epidermis[77] prompted efforts by two independent groups to investigate its value as a marker of intestinal stem cells.

In the first study, in vivo lineage tracing using an Lrig1-ires-CreERT2 mouse model generated long-term tracing units typical of multipotent stem cell output throughout the small intestine[75]. Reporter gene activation was most commonly observed within positions 2-5, although tracing was also initiated throughout the lower portion of the transit-amplifying compartment, consistent with a gradient of *Lrig1* expression emanating from the crypt base.

In the colon, *Lrig1* expression marked a small population of cells at the very crypt base, a minority of which co-expressed *Lgr5*. Fate mapping indicated that at least some of them were actively cycling stem cells contributing to daily epithelial homeostasis. However, a proportion of Lrig1+ cells that underwent Cre-mediated reporter gene activation failed to generate labeled progeny over a long period, indicating that these might be quiescent. These quiescent cells were induced to proliferate and contribute epithelial progeny following irradiation-induced damage to the colonic epithelia.

Comparative expression profiling of Lrig1+ and Lgr5+ populations from the colon yielded markedly different transcriptomes, interpreted as further evidence of their independence. Whilst both populations expressed equivalent levels of reported +4 markers, including Bmi1, mTERT and Prominin1, the Lrig1+ cells selectively expressed gene signatures involved in oxidative damage responses and negative regulation of cell proliferation. Lrig1 was itself identified as tumor suppressor in the intestine, when genetic ablation resulted in adenoma formation within 6 months[75]. This prompted speculation that Lrig1 functions to regulate ErbB signaling on the intestinal stem cell compartment to prevent aberrant stem cell activities that could lead to cancer formation.

A second study yielded markedly contrasting conclusions in the small intestine[78]. Independent in situ and IHC analyses confirmed *Lrig1* expression throughout the lower 1/3rd of the small intestine crypts, but the Lgr5+ stem cell transcriptome revealed a significant enrichment of *Lrig1* expression within this CBC population[47]. An independent study employing single mRNA molecule FISH also documented *Lrig1* as being expressed in a broad gradient, with highest levels present in Lgr5+ CBC stem cells[47]. This extensive overlap of the Lrig1+ and Lgr5+ crypt populations was confirmed at the protein level via flow cytometric analysis using an independent Lrig1 antibody[47]. The Lrig1+ cells were also characterized as being actively proliferating - again in stark contrast to the colon-resident Lrig1+ cells that were documented as being quiescent by Powell and colleagues.

A clue to the function of Lrig1 on the Lgr5+ stem cells was revealed by phenotypic analysis of Lrig1 knockout mice intestines – extensive crypt hyperplasia was evident soon after birth and the Lgr5+ stem cell compartment was markedly expanded. This phenotype was directly linked to deregulated *ErbB* expression on the stem cell

compartment when treatment of Lrig1 KO mice with an ErbB inhibitor restored crypt homeostasis.

Collectively, these studies agree that Lrig1 has important functions in regulating ErbB signaling on the intestinal stem cell populations as a prerequisite to preventing disease. However, at least in the small intestine, the broad expression gradient of *Lrig1* within the crypt and its enrichment in the Lgr5+ CBC cell pool would appear to disqualify it as a specific marker of an intestinal stem population.

mTERT. Elevated telomerase expression is considered to be a stem cell trait that protects against replication-induced senescence. Using mTERT-GFP reporter mice, rare, predominantly quiescent mTert+ cells were detected around the +4 position[79]. These mTert+ cells were later shown to be independent from the Lgr5+ CBC stem cells and other +4 stem cell pools, and are resistant to ionizing irradiation[74]. In vivo lineage-tracing subsequently demonstrated that a small fraction of the mTert+ crypt cells were actively cycling stem cells contributing to epithelial homeostasis in both the small intestine and colon[74]. The quiescent mTert+ cells that did not participate in daily tissue homeostasis could be efficiently activated to proliferate and contribute epithelial progeny during damage-induced epithelial regeneration. Collectively, these observations appear to support mTert as being a marker of an independent quiescent, damage-inducible pool of intestinal stem cells. However, other studies evaluating endogenous telomerase expression and activity levels in the crypt have reached quite contrasting conclusions[65,73]. Both mTert mRNA and telomerase activity was readily detected in all proliferative cells of the crypt, with highest levels present within FACS-sorted Lgr5+ CBC stem cells. Single molecule mRNA FISH analysis confirmed this expression pattern in proximal small intestine. Such an elevated telomerase activity would likely benefit the highly proliferative Lgr5+ stem cells, but it remains unclear what advantage this would bestow on quiescent populations that by definition rarely divide

A current view of adult stem cell identity

The intestinal stem cell field has made major advances over the last 5 years, driven largely by the identification of new stem cell markers, and the development of near-physiological assays to rigorously evaluate endogenous stem cell identity *in vivo*. Despite these technological breakthroughs, there is still no consensus on the true identity (+4 versus CBC cell) of adult intestinal stem cells. Recent attempts to reconcile the opposing views have lead to the formulation of a more plastic model of stem cell identity. This model describes the intestinal crypt base as a specialized niche environment harboring both dedicated active stem cells (the CBC cells), responsible for daily epithelial homeostasis, and more quiescent, reserve stem cells (collectively known as +4 cells) that can be activated to effect tissue repair following injury. This new model also suggests that cells residing in the lower positions of the transit-amplifying cell compartment can assume stem cell identity to ensure maintenance of epithelial homeostasis in the eventuality of a catastrophic loss of crypt base-resident stem cell populations (as discussed below).

Stem cell roles in epithelial regeneration

The intestinal epithelium has a remarkably ability to survive acute injury and to effect rapid regeneration to restore function. Although not strictly physiological, irradiation-induced injury models have provided important mechanistic insight into the regeneration process, revealing the existence of 'regular' (also known as 'actual') and 'reserve' (also known as 'potential') stem cell populations within the lower regions of the crypt. Many of the +4 markers discussed above are proposed to define dedicated pools of damage-resistant reserve stem cells that are selectively activated in response to acute damage to the regular stem cell pool. However, there is growing support for a

more general model of crypt plasticity, which describes a niche-induced conversion of early stem cell progeny into functional stem cells following tissue damage.

Dedicated 'Reserve' Stem Cells. Actively proliferating Lgr5+ CBC cells drive intestinal epithelial homeostasis under physiological conditions[42](Fig 3a). Despite having such a crucial role, a recent study employing a targeted conditional ablation strategy has shown that the intestine can survive the short-term loss of the CBC stem cell pool *in vivo*, hinting at the existence of additional, damage-resistant stem cells[8] (Fig 3b).

+4-resident cells have recently been championed as likely reserve stem cell candidates, based on traits such as cellular quiescence and resistance to radiation[72]. Although the +4 populations marked by Hopx and Lrig1 remain to be functionally identified as reserve stem cells, the exquisite radiation-sensitivity of the original +4 LRC population described by Chris Potten would appear to disqualify it from serious consideration[44]. Strong support for the existence of a Bmi1+ reserve stem cell pool was provided when in vivo lineage tracing from the Bmi1 locus following acute ablation of the Lgr5+ CBC stem cell compartment revealed that Bmi1+ cells contributed to the repopulation of the Lgr5+ stem cell pool during the subsequent regeneration phase[8]. However, the fact that *Bmi1* is expressed throughout (and traces from) the entire proliferative zone of the crypt precludes a formal identification of these reserve stem cells[47].

Label-retention has long been employed as a surrogate marker of quiescent "reserve" stem cell populations that are activated in response to injury stimuli in the small intestine[80]. In an effort to directly characterize this population without the use of markers, conditional H2B-GFP/YFP pulse chase approaches were used to mark the LRCs and facilitate their identification and isolation[7,81].

Long-lived LRC's (4-9 weeks) were shown to comprise exclusively mature Paneth cells, consistent with the known lifespan (6-8 weeks) of this terminally differentiated population[13]. However, an independent, shorter-lived LRC population (< 4 weeks) presented a mixed phenotype, expressing a collection of markers for paneth cells, enteroendocrine cells and Lgr5+CBC/+4 stem cells. Using an elegant split-Cre recombinase strategy to perform lineage tracing from these LRC's in vivo it was shown that under physiological conditions, the mixed phenotype LRC population functioned as bipotent progenitors of the mature Paneth cell and enteroendocrine cell lineages[7]. However, following irradiation-induced damage, the same LRC population was rapidly converted into self-renewing, multipotent stem cells contributing to epithelial regeneration (Fig 3c). This LRC population was further characterized as being a subset of the Lgr5+ CBC cells, comprising 20% of the total Lgr5+ pool at the crypt base.

A major implication of this finding is that Lgr5 is marking a mixed population of "actual" and "potential" stem cells in the small intestine. However, if true, then Lgr5-driven lineage tracing should result in the generation of a substantial proportion of Paneth cell-restricted clones under physiological conditions – this has never been reported. Since the regeneration capacity of the small intestinal epithelium is apparently maintained following short-term ablation of the entire *Lgr5*-expressing crypt population, including both the regular CBC and reserve LRC stem cell pools, other Lgr5-cell populations capable of functioning as stem cells must exist.

In a separate study, LRC's were characterized after 7 weeks of pulse-chase – in agreement with the findings of the study described above, these long-lived LRC's were found to be differentiated Paneth cells[81]. These Paneth cell LRC's did not express *Lgr5* and could be activated to proliferate in response to irradiation injury, generating Bmi1+ cells proposed to function as stem cells during the subsequent tissue regeneration process. These findings contrast with those of the earlier study, which documented the Paneth cell LRC's as being a terminally differentiated population incapable of injury-induced stimulation[7]. The colonic epithelium, which is devoid of both Paneth cells and

LRC populations, is also capable of surviving acute injury, implying the existence of actively cycling cells capable of functioning as stem cells during regeneration.

Role of niche plasticity. An alternative, though not necessarily mutually exclusive, explanation for the robust regenerative capacity of the intestinal epithelium may be related to the general plasticity of relatively undifferentiated cells within the transit-amplifying compartment. Early clonal regeneration assays have identified up to 30-40 cells in the lower 1/3rd of the intestinal crypts with the ability to survive exposure to varying doses of radiation or cytotoxic drugs and contribute to crypt regeneration[36,82]. This observation hinted at the existence of a "stemness" hierarchy in the lower crypt region, with early Lgr5+ CBC cell progeny gradually losing their stem cell features during successive rounds of cell division as they progressively switch on differentiation programs during their migration up the crypts. Following acute loss of the regular stem cell pool, the surviving undifferentiated stem cell progeny are thought to fall back into the vacant stem cell niche at the crypt base, where they quickly re-acquire stem cell identity and re-establish epithelial renewal[44]. Such niche-induced plasticity has been demonstrated using an ex-vivo organoid culture system as a read-out of endogenous stem cell activity. Under normal conditions, Lgr5- TA cells isolated from intestinal crypts are incapable of generating epithelial organoids in culture. However, a brief exposure to one of the major in vivo niche signals, Wnt3A, efficiently converts these Lgr5- cells into organoid-proficient Lgr5+ stem cells[83].

More recently, in vivo lineage tracing was used to demonstrate the plasticity of early stem cell progeny expressing the Notch ligand Dll1[84]. Under physiological conditions, Dll1+ cells restricted to the proposed "common origin of differentiation" position at +5 were shown to function as secretory progenitors, generating short-lived clones comprising goblet cells, enteroendocrine cell, Paneth cells and tuft cells. However, irradiation-induced depletion of the Lgr5+ stem cell compartment resulted in conversion of the Dll1+ cells into multipotent Lgr5+ CBC stem cells contributing to the subsequent epithelial regeneration process[84] (Fig 3d).

The observed plasticity of the lower crypt cell populations during epithelial regeneration is likely dependent upon instructive signals emanating from the surviving stem cell niche. Such niche-driven plasticity may also exist within gastrointestinal tumors, which, if proven, would have important implications for therapies targeting the elimination of cancer stem cell populations. Evidently, the intestinal niche is a critical component in governing stem cell behavior not only during epithelial homeostasis, but also during tissue regeneration following injury.

Biomedical applications of ISC's

Efforts to exploit intestinal stem cells for regenerative medicine have accelerated over the last decade, driven largely by the identification of cell-surface markers facilitating the isolation of pure stem cell populations and the development of near physiological culture methods supporting stem cell expansion and epithelial growth. Recent characterization of the complex niche signals that regulate stem cell function in vivo has also been of fundamental importance to these translational efforts, ensuring maintenance of the regenerative capacity of isolated stem cells and minimizing the risk of introducing undesirable heritable traits that could compromise the safety of patients.

A glance at the intestinal stem cell niche. Stem cell activity at the crypt base is strictly regulated during regular homeostasis by a complex array of signals delivered by neighboring epithelial/stromal cells (Fig 2b). Paneth cells, which are found in close association with the Lgr5+ CBC stem cells at the crypt base, are an important source of various niche factors, including EGF, Wnt3A and Notch ligand[83]. These niche cells also enable the intestine to tailor the output of its stem cell compartment to nutrient availability[9]. Paneth cells respond to calorie restriction by reducing mTORC1 signaling,

initiating a signal cascade that results in a rapid reduction in the size of the Lgr5+ stem cell pool.

However, Paneth cells are unlikely to be the sole niche component in vivo. Indeed, neither Paneth cell ablation[85-87] nor epithelial-specific loss of *Wnt3A* expression[88] is sufficient to block stem cell-driven epithelial renewal in vivo, implying the existence of redundant, non-epithelial sources of Wnt and other key niche signals.

The colonic stem cell niche is less well defined. Although Paneth cells are generally absent from the majority of the colon, CD24+ and c-Kit+ goblet cells located in close proximity to Lgr5+ stem cells at the crypt base have been identified as likely niche components[89]. However, the major Wnt source in the colon has yet to be identified.

Growing intestinal epithelia ex vivo. Ex vivo culture systems capable of efficiently and safely maintaining the long-term regenerative capacity of purified stem cells following their extraction from endogenous niches are of crucial importance for realizing the clinical potential of adult stem cells. Although it is currently not possible to maintain pure populations of intestinal stem cells *ex vivo*, major advances have recently been made in establishing culture systems that support the long-term growth and expansion of near-physiological intestinal epithelia from purified stem cells. To date, several systems have been described for the long-term growth of "organoids" from the small intestine[90], but arguably the most successful method is a matrigel-based system that supports the growth of self-renewing, near-native intestinal epithelia in the absence of stromal niche components[43] (Box 4).

In a beautiful example of their clinical potential, mouse colonic "organoids" were successfully used to repair damaged colonic epithelia in vivo when delivered via a simple enema[91]. Importantly, this system has recently been successfully adapted for routinely growing human epithelia from small intestine and colon[46,92]. This development opens up many exciting translational opportunities in the biomedical sectors, including the de novo growth of isotype-matched human epithelia for treating human GI tract diseases such as ulcers, and the expansion of matched healthy and tumor epithelia from patient biopsies for use in drug screening programs and to facilitate deep sequencing efforts as a prerequisite to the development of personalized treatment regimes.

Summary and future outlook

The intestinal stem cell field has made impressive advances over the last decade, fuelled by the identification of specific biomarkers, better characterization of the endogenous niche and the development of new in vivo and ex-vivo models for rigorously evaluating stem cell identity and function during homeostasis and disease. After several years of intense debate over the precise identity and function of the intestinal stem cell, it is becoming apparent that there is perhaps no single, definitive answer. Rather than relying on a single, hard-wired stem cell compartment to maintain epithelial homeostasis and effect tissue regeneration following injury, the intestine appears capable of drawing on several pools of highly plastic, "potential" stem cell populations in the lower regions of the crypt. Such plasticity is likely to be endowed by the specialized niche environment at the crypt base, which provides the requisite signals to efficiently convert committed progenitor populations into multipotent adult stem cells. Such niche-driven plasticity would readily explain the impressive ability of the intestinal epithelium to survive and regenerate following major injury and could potentially be exploited for regenerative medicine applications in the clinic.

The next major challenge for the intestinal stem cell field is to rapidly translate our knowledge of mouse stem cells into the human arena to ensure that the clinical potential of intestinal stem cells is quickly realized. Lgr5 is one of the few cell-surface stem cell markers that could potentially be used to isolate human intestinal stem cells, but has proven to be a difficult target for antibody generation. However, next generation strategies such as genetic immunization are starting to deliver antibodies capable of

effecting the isolation of live human intestinal stem cells. The available human intestinal organoid culture systems will be instrumental in evaluating and characterizing these candidate human stem cells as a prerequisite to exploiting their clinical potential in the near future.

Box 1: Additional CBC stem cell markers

Sox9 is a Wnt target gene encoding a member of the SRY family of transcription factors, which regulate cell proliferation in the intestine[93,94]. Using transgenic mice expressing EGFP under the control of the *Sox9* promoter (Sox9-EGFP), two distinct cell populations, expressing EGFP at low (Sox9-EGFP^{lo}) and high (Sox9-EGFP^{hi}) levels, were identified at the crypt base[95]. EGFP^{hi} cells were enteroendocrine cells and candidate +4 stem cells, whereas EGFP^{lo} cells overlapped substantially with the Lgr5+ CBC compartment[95,96]. An independent study employing Sox9-ires-EGFP & Sox9-ires-lacZ KI mice identified a similar expression profile at the crypt base, but also reported the well-documented endogenous Sox9 expression in Paneth cells and, less frequently, in villus cells[97].CBC Sox9^{lo} cells were confirmed to have stem cell identity by their ability to generate multi-lineage intestinal organoids in an *ex vivo* culture assay[96]. In contrast, Sox9^{hi} cells were only able to proliferate and generate intestinal organoids ex vivo following irradiation,[Au:Can you include a reference here?] prompting speculation that this population includes damage-responsive reserve +4 stem cells. The most compelling evidence in support of *Sox9*-expressing cells being stem cells was provided by *in vivo* lineage tracing using a Sox9-ires-CreERT/R26RlacZ model, when typical stem cell-driven tracing clones were generated and maintained throughout the small intestine and colon[97]. Although the expression of Sox9 in various differentiated epithelial lineages reduces its value as a selective marker of endogenous intestinal stem cells, expression profiling of the Sox9^{lo} population did reveal CD24 as a cell-surface marker that can be used to enrich for CBC stem cells via cell cell-sorting approaches[98].

Musashi-1 encodes an RNA-binding protein implicated in neural stem cell homeostasis. It was originally proposed as a CBC cell marker on the basis of antibody staining patterns, which documented a restricted expression pattern at the crypt base in both the small intestine and colon[99,100]. However, both single molecule mRNA fluorescence in situ hybridization (FISH) analyses and in vivo reporter gene analyses using a Musashi1-EGFP transgenic line indicate that *Musashi-1* expression may extend to the lower transit-amplifying compartment[47,73,101]. Definitive proof of the stem cell identity of Msh-1+ cells could be obtained with in vivo lineage tracing experiments, which should now be feasible using a recently described Musashi1-CreERT2 KI line[102].

Prominin 1 (also known as CD133 in human), which encodes a cell-surface glycoprotein, is a controversial marker of cancer stem cells in a variety of epithelial cancers[103,104]. It has also been reported as a stem cell marker of in healthy adult tissues, including the bone marrow and intestine[105,106]. In the intestine, in-situ hybridization analysis revealed *Prominin1* expression at the crypt base, overlapping with the CBC stem cells. In vivo lineage tracing using a Prom1-CreERT2-ires-nLacZ/R26R-YFP model identified at least some of these Prominin1+ cells as being bona-fide adult stem cells. However, a subsequent study employing independent antibody staining and in vivo lineage tracing analyses found that *Prominin1* is expressed throughout the proliferative zone of the crypt (including the CBC cell compartment) and that the majority of marked cells originated from short-lived transient-amplifying cells and thus were rapidly lost[107].

Box 2: **Stem cells in intestinal cancer**

Aberrant activation of Wnt signaling is known to be a major initiating event in intestinal cancer, driving the formation of benign polyps or adenomas that gradually accumulate additional mutations during their progression towards metastatic disease[108]. Stem cells have long been considered a likely cell of origin of intestinal cancer, simply because of their relative longevity compared to most other epithelial lineages of the intestine. Indeed, the targeted ablation of the tumor suppressor gene APC, a negative regulator of Wnt signaling, within the Lgr5+ stem cell compartment efficiently drives adenoma formation in vivo[109]. In contrast, ablation of APC function within the transit-amplifying cell compartment fails to sustain adenoma formation. Similar results were obtained following conditional activation of oncogenic beta-catenin on the *Bmi1*- and *Prominin1*-

expressing stem cell compartment, strongly implicating the intestinal stem cells as being a major cell of origin of intestinal cancer in mice[71,106]. However, a recent study showed that Wnt-activated transit-amplifying cells could be efficiently converted into tumor-initiating stem cells in response to NF-κB-driven inflammation in the adjacent stroma, highlighting the influence of the local microenvironment on cancer formation[110].

Adenomas generated as a consequence of aberrant Wnt pathway activation were found to harbor pockets of *Lgr5*-expressing cells in close association with Paneth-like cells, an organization that was reminiscent of the stem cell zone at the base of healthy intestinal crypts. These tumor resident Lgr5+ populations were subsequently shown to actively contribute to tumor growth in vivo and their human colon cancer equivalents were capable of generating tumor organoids ex vivo, indicative of a cancer stem cell identity[111,112]. More recently, adenoma-resident cells expressing another putative intestine stem cell marker, Dclk1, were shown to function as cancer stem cells in vivo [113]. Of note, this study also concluded that *Dclk1* expression distinguished cancer stem cells from regular intestinal stem cells, highlighting its value as a potential selective therapeutic target.

Box 3: **Other reported +4 markers**

Many other purported markers of intestinal stem cells, often collectively referred to as "+4 stem cell markers" despite their poor characterization, have been published over the last decade[114]. Unlike Bmi1, Lrig1 and mTert, these have not been validated by in vivo lineage tracing, but have instead been proposed on the basis of their localization within the crypt, or their selective expression on cells displaying presumptive stem cell attributes such as DNA label retention or cellular quiescence.

IHC analyses have detected enriched Phospho-PTEN expression on LRC's residing just above the Paneth cell compartment around position +4/+5[115,116]. However, these findings were subsequently questioned when the same antibody was shown to detect a subset of post-mitotic enteroendocrine cells at the crypt base[117].

Expression of the phosphatase *Wip1* was also reported to be predominantly restricted to position +4, although Wip1+ cells intercalated with the Paneth cells were also readily apparent[118]. Somewhat surprisingly, the observed depletion of this candidate stem cell population in Wip1 knockout mice had no discernable effect on epithelial homeostasis.

Interest in *DCAMKL-1* (*Dclk1*) as a potential stem cell marker resulted from its identification as a highly expressed gene in stem cell-enriched zones micro-dissected from the crypt base[119]. IHC analysis subsequently documented expression on rare, quiescent cells predominantly located around position +4[120,121]. Their potential stem cell status was further enhanced when lineage marker studies failed to document any overlap with the major differentiated epithelial cell types. However, more recent studies detected DCAMKL1+ cells throughout the crypt/villus epithelium and phenotyped them as a rare population of Tuft (caveolate cells), a differentiated cell type displaying thick "tufts" of microvilli whose in vivo function is still poorly understood[10,93,122]. This was confirmed by in vivo lineage tracing using a new DCAMKL1-driven CreERT2 allele[113]

Box 4: **The intestinal epithelial organoid culture system**

Intestinal epithelial organoids are an excellent resource for studying stem cell biology and for biomedical applications. (a) Intact intestinal crypts or purified Lgr5+ CBC stem cells are plated into laminin-rich Matrigel supplemented with a cocktail of growth factors known to be present within the endogenous stem cell niche, including a BMP inhibitor Noggin, the Wnt agonist R-Spondin1, epidermal growth factor and Notch ligand[43]. This 3D culture system supports the generation of self-renewing epithelial organoids organized into discrete crypts harboring intercalated stem cell/Paneth cell populations at their base and associated villus-like regions comprising the various

differentiated cell lineages. It was subsequently adapted for use in culturing colonic epithelia, although this required exogenous Wnt3A, likely reflecting subtle differences in the endogenous stem cell niches of the small intestine and colon[92]. (b) Single Lgr5+ stem cells carrying a permanent, heritable RFP mark generate RFP+ colonic organoids in ex vivo culture. (c) The cultured RFP+ colonic organoids can be used to effectively repair damaged colonic epithelia in vivo via orthotopic transplantation in the mouse. This is supported by the appearance and maintenance of patches of RFP+ colonic epithelia in the recipient wild-type mouse (d) Intestinal epithelial organoids grown from humans can potentially be used for basic research into epithelial biology and a variety of biomedical applications including drug screening, mutation analyses and tissue repair in vivo.

Figure legends

Figure 1 Epithelial self-renewal in the intestinal epithelium. (a) In the small intestine, stem cells intercalated with Paneth cells at the crypt base continuously generate rapidly proliferating transit-amplifying (TA) cells, which occupy the remainder of the crypt. These TA cells differentiate into the various functional cells on the villi (enterocytes, Tuft cells, Goblet cells & enteroendocrine cells to replace the epithelial cells being lost via anoikis at the villus tip. Epithelial turnover occurs every 3-5 days. New Paneth cells are supplied from the TA cells every 3-6 weeks (b) In the colon, stem cells at the crypt base generate rapidly proliferating TA cells (blue) in the lower half of the crypt. TA cells subsequently differentiate into the mature lineages of the surface epithelium (Goblet cells, enterocyes, enteroendocrine cells and Tuft cells). Epithelial turnover occurs every 5-7 days.

Figure 2 Intestinal stem cells and their defining niche. (a) A summary of the published functional characteristics of the +4 stem cell markers (dark blue) and the CBC stem cell markers (green). +4 stem cells are generally considered to be relatively quiescent and resistant to acute injury (b) Cartoon depicting the various CBC stem cell niche components at the crypt base. Both Paneth cells and pericryptal stromal cells supply essential factors (Wnt, Notch ligand, EGF, Noggin, TGFβ) to regulate the survival and function of the CBC stem cells *in vivo*. (c) Neutral drift within the intestinal stem cell pool. Activation of multicolor lineage tracing generates a population of distinctly labeled CBC stem cells in each crypt. Each labeled stem cell generates progeny of the corresponding colour, resulting in the appearance of "rainbow" crypts over the next 2 weeks. Over the next few months, neutral competition between symmetrically dividing labeled stem cells causes the gradual conversion of multicolor crypts into single-colored crypts. In this example, the RFP-labelled stem cell achieves dominance.

Figure 3 Models of epithelial regeneration in the small intestine. (a) During homeostasis, multipotent Lgr5+ CBC stem cells drive regular epithelial renewal. Lgr5+ Paneth cell precursors (dark brown) exclusively supply mature Paneth cells. Dll-1+ TA cells (green) are secretory cell progenitors supplying goblet cells, endocrine cells and tuft cells. (b) Acute injury results in the loss of the proliferating Lgr5+ stem cells, but leaves the damage-resistant Paneth cell precursors, +4 stem cells and the niche intact. (c) Surviving Lgr5+ Paneth cell precursors and/or +4 cell populations function as dedicated reserve stem cells to rapidly regenerate the Lgr5+ CBC stem cell pool and restore epithelial renewal. (d) Surviving Dll1+ secretory progenitors or other early TA cells fall back into the surviving niche at the crypt base and are consequently converted into Lgr5+ stem cells to restore epithelial renewal.

Glossary terms

Niche. The specialized instructive microenvironment in which stem cells reside. Provides all necessary factors to regulate stem cell survival and function.

Duodenum. The proximal 1/3rd of the small intestine closest to the stomach. Characterized by the presence of long villi to ensure maximal nutrient digestion and absorption.

Intestinal Crypt. Tubular invagination of the epithelium harboring the stem cells and their proliferating progeny. Responsible for driving epithelial regeneration.

Intestinal Villus. Finger-like structure covered in simple, columnar epitheliium that projects into the intestinal lumen to maximize the surface area for digestion and absorption.

Ires (Internal Ribosome Entry Site). A ribosome binding site present in the middle of a mRNA that facilitates internal translation initiation to generate an independent protein.

Chimaeric mice. Mice that are comprised of two or more populations of genetically distinct cells

Pulse-chase. A method for detecting quiescent (label-retaining) cells in vivo. A nucleotide analogue (label) is administered to the mouse for a short period (pulse), followed by an extended chase period when no further nucleotide analogue is given. Actively dividing cells rapidly dilute out the label during DNA replication, resulting in the loss of labeled cells within 3-4 rounds of cell division. In contrast, non-dividing (quiescent) cells do not dilute out the label and thus remain detectable as label-retaining cells.

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Box 1

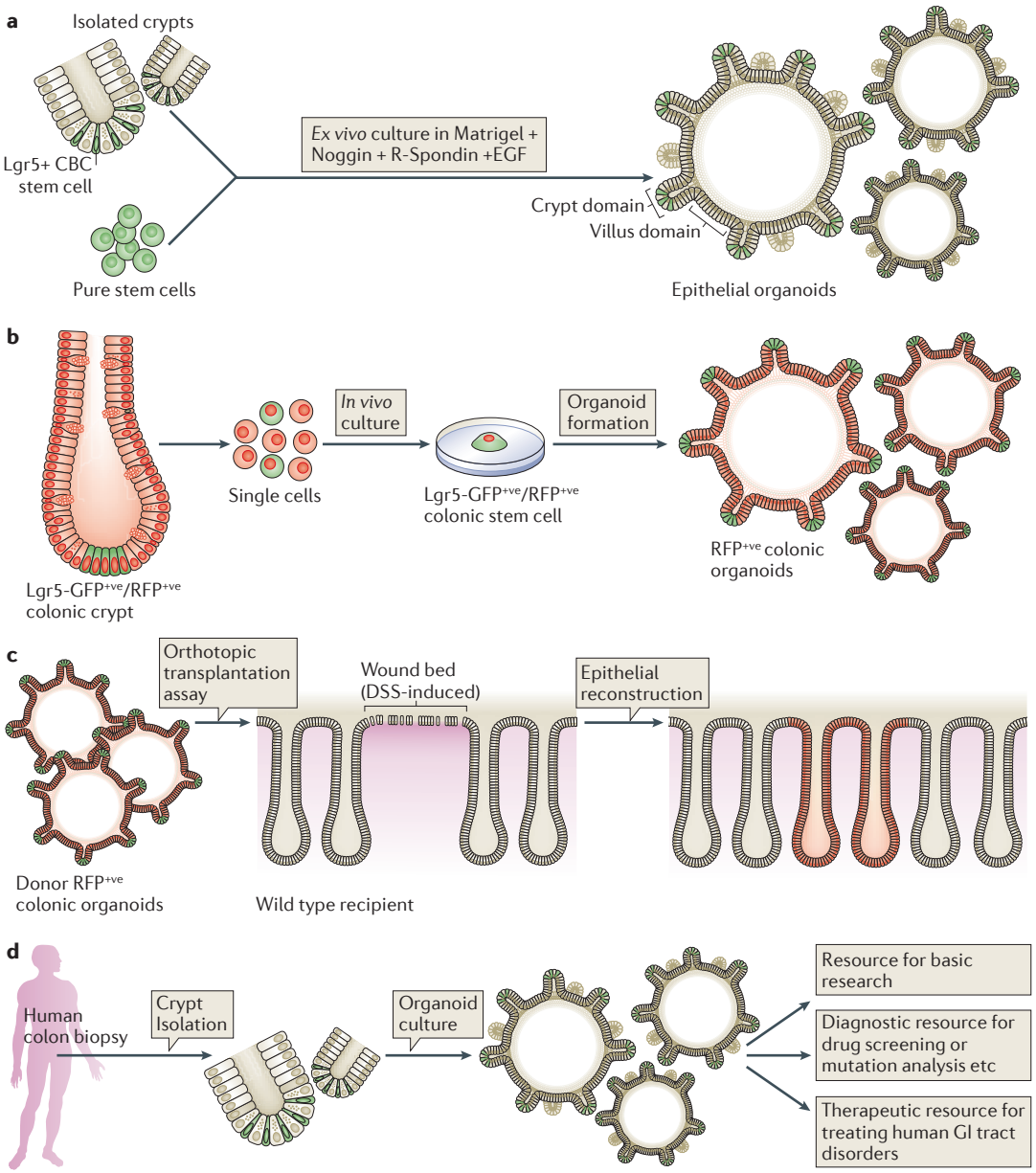

a
Isolated crypts
Lgr5+ CBC stem cell
Pure stem cells
Ex vivo culture in Matrigel + Noggin + R-Spondin +EGF
Crypt domain
Villus domain
Epithelial organoids
b
Lgr5-GFP+ve/RFP+ve colonic crypt
Single cells
In vivo culture
Lgr5-GFP+ve/RFP+ve colonic stem cell
Organoid formation
RFP+ve colonic organoids
c
Donor RFP+ve colonic organoids
Orthotopic transplantation assay
Wound bed (DSS-induced)
Wild type recipient
Epithelial reconstruction
d
Human colon biopsy
Crypt Isolation
Organoid culture
Resource for basic research
Diagnostic resource for drug screening or mutation analysis etc
Therapeutic resource for treating human GI tract disorders

Fig 1

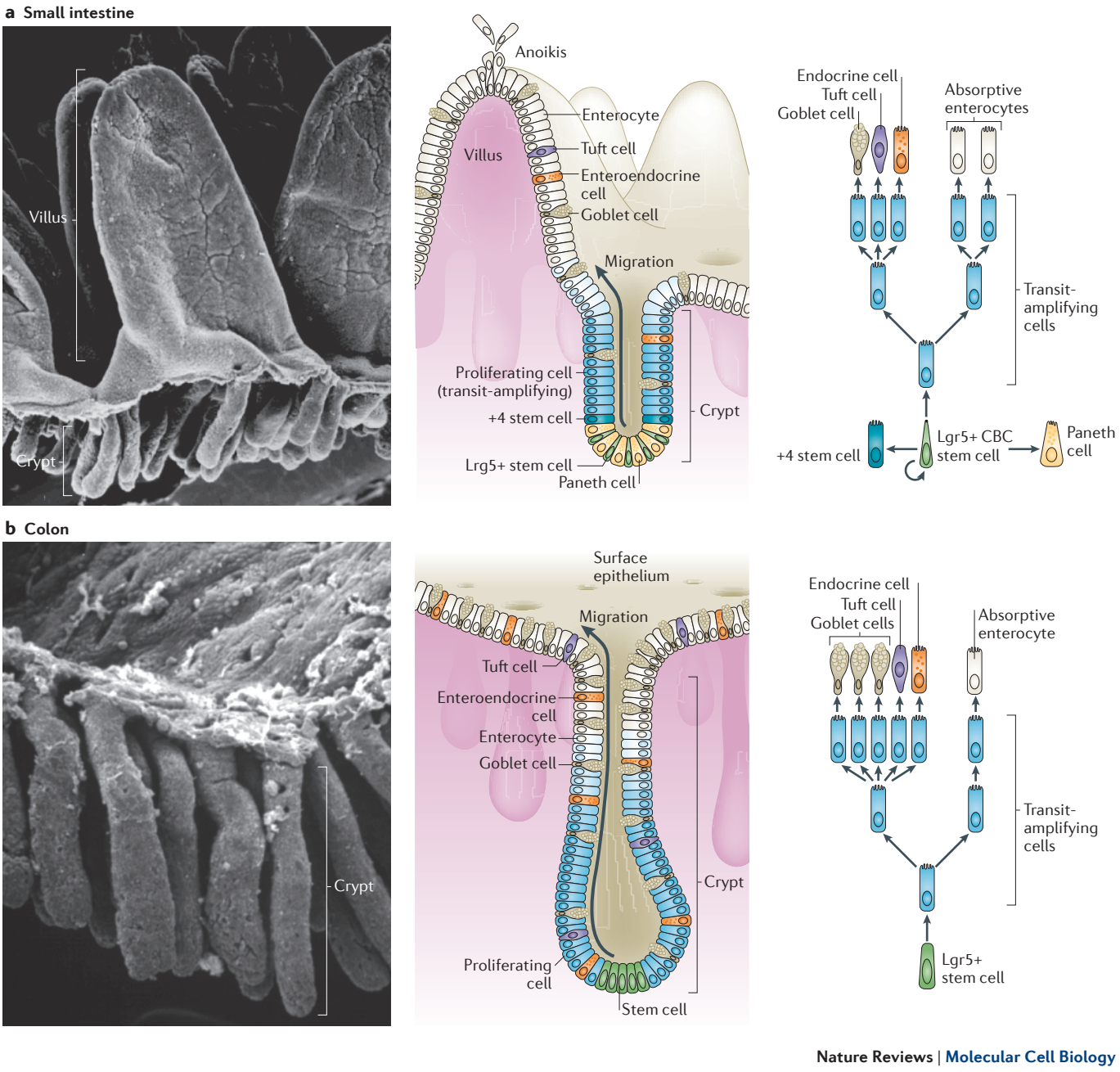

a Small intestine
Villus
Crypt
Anoikis
Enterocyte
Tuft cell
Enteroendocrine cell
Goblet cell
Villus
Migration
Proliferating cell (transit-amplifying)
+4 stem cell
Lrg5+ stem cell
Paneth cell
Crypt
Endocrine cell
Tuft cell
Goblet cell
Absorptive enterocytes
Transit-amplifying cells
+4 stem cell
Lgr5+ CBC stem cell
Paneth cell
b Colon
Crypt
Surface epithelium
Migration
Tuft cell
Enteroendocrine cell
Enterocyte
Goblet cell
Crypt
Proliferating cell
Stem cell
Endocrine cell
Tuft cell
Goblet cells
Absorptive enterocyte
Transit-amplifying cells
Lgr5+ stem cell

Fig 2

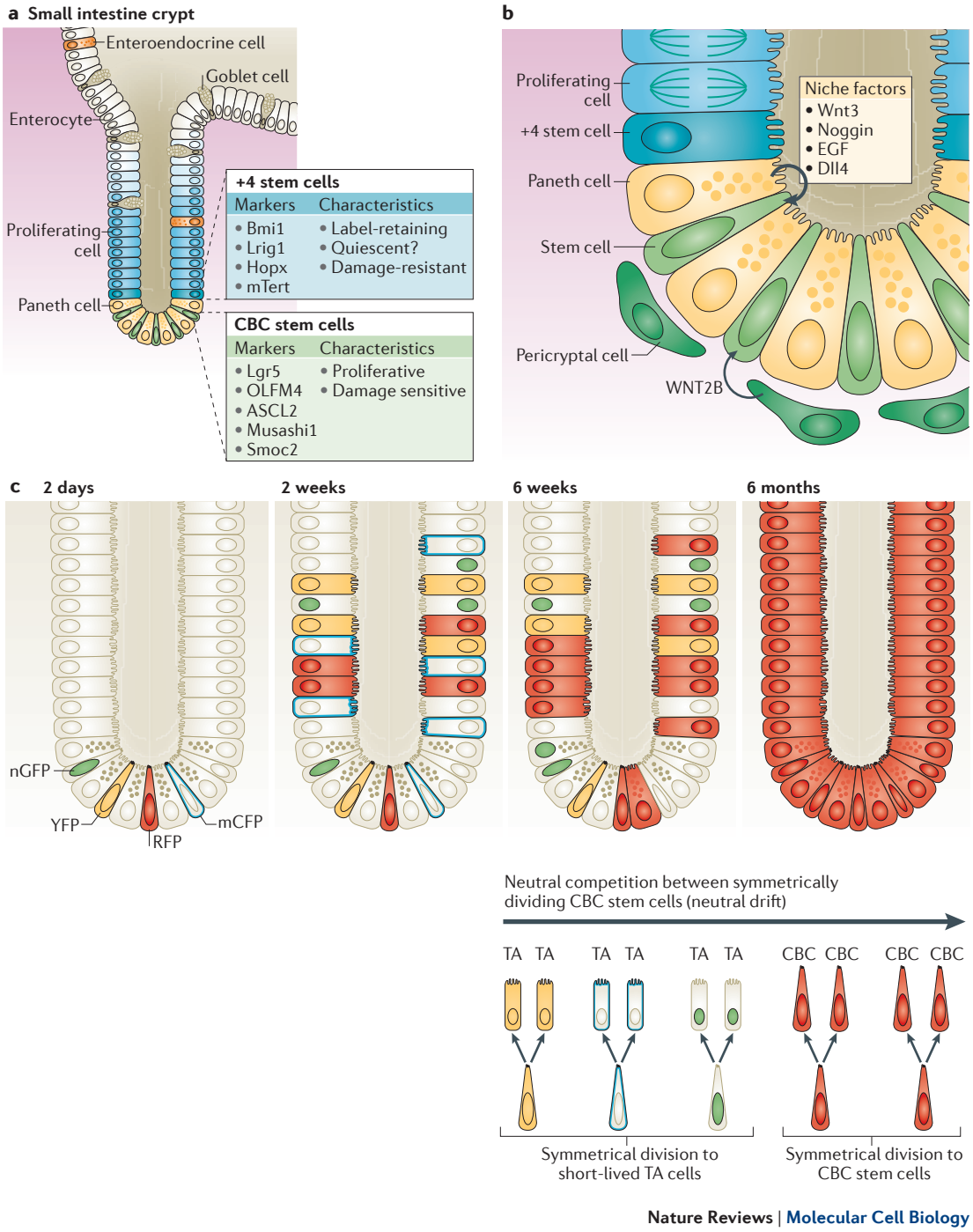
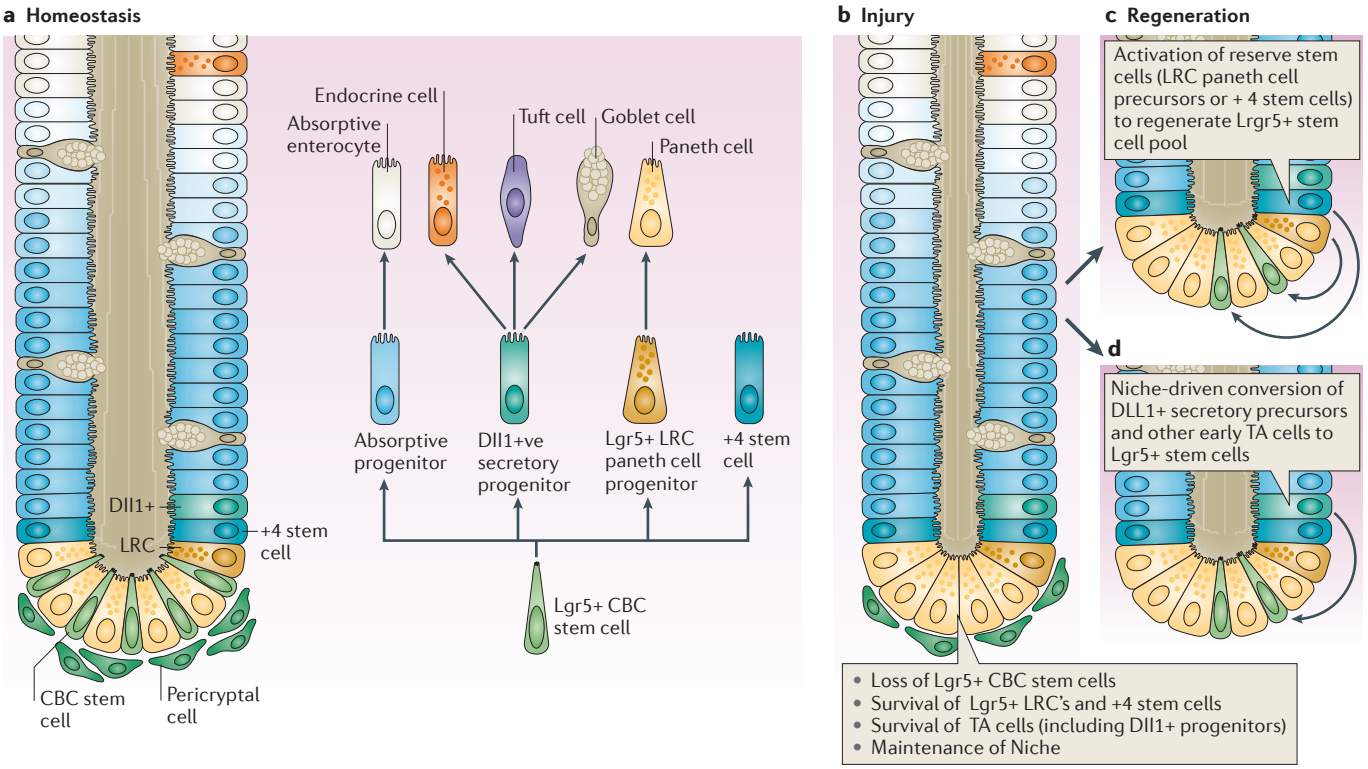

a Small intestine crypt
Enteroendocrine cell
Goblet cell
Enterocyte
Proliferating cell
Paneth cell
+4 stem cells
Markers
Characteristics
• Bmi1
• Lrig1
• Hopx
• mTert
• Label-retaining
• Quiescent?
• Damage-resistant
CBC stem cells
Markers
Characteristics
• Lgr5
• OLFM4
• ASCL2
• Musashi1
• Smoc2
• Proliferative
• Damage sensitive
b
Proliferating cell
+4 stem cell
Paneth cell
Stem cell
Pericryptal cell
WNT2B
Niche factors
• Wnt3
• Noggin
• EGF
• Dll4
c
2 days
2 weeks
6 weeks
6 months
nGFP
YFP
RFP
mCFP
Neutral competition between symmetrically dividing CBC stem cells (neutral drift)
TA TA
TA TA
TA TA
CBC CBC
CBC CBC
Symmetrical division to short-lived TA cells
Symmetrical division to CBC stem cells

Fig 3


a Homeostasis
Endocrine cell
Absorptive enterocyte
Tuft cell
Goblet cell
Paneth cell
Absorptive progenitor
Dll1+ve secretory progenitor
Lgr5+ LRC paneth cell progenitor
+4 stem cell
Lgr5+ CBC stem cell
Dll1+
LRC
+4 stem cell
CBC stem cell
Pericryptal cell
b Injury
c Regeneration
Activation of reserve stem cells (LRC paneth cell precursors or + 4 stem cells) to regenerate Lrgr5+ stem cell pool
d
Niche-driven conversion of DLL1+ secretory precursors and other early TA cells to Lgr5+ stem cells
• Loss of Lgr5+ CBC stem cells
• Survival of Lgr5+ LRC's and +4 stem cells
• Survival of TA cells (including Dll1+ progenitors)
• Maintenance of Niche