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

Nick Barker

Abstract | Small populations of adult stem cells are responsible for the remarkable ability of the epithelial lining of the intestine to be efficiently renewed and repaired 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.

Niches
Specialized instructive microenvironments in which stem cells reside. Niches provide all of the factors necessary to regulate stem cell survival and function.

*A*STAR Institute of Medical Biology, 8A Biomedical Grove, 06-06 Immunos, 138648 Singapore.*
MRC Centre for Regenerative Medicine, The University of Edinburgh, 5 Little France Drive, Edinburgh EH16 4UU, UK.
Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, 117596 Singapore.
e-mail: nicholas.barker@imb.a-star.edu.sg
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The inner epithelial lining of the small intestine is a truly multitasking tissue. It must simultaneously achieve the efficient digestion (with the aid of enzymes released from the liver and pancreas) and the absorption of food contents released from the stomach while maintaining an effective barrier against potentially lethal microorganisms and carcinogens that are present in the intestinal lumen. Persistent aggression from the luminal contents induces a remarkably high rate of cell death, with up to 10^{11} epithelial cells (~200 g) being lost every day in humans[1]. This imposes a requirement for daily self-renewal throughout life, which is 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 promote cancer growth — making them attractive therapeutic targets.

Over decades of intensive study, researchers 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, the identity of the stem cell population has long remained elusive owing to a lack of specific markers and suitable methodologies for rigorous evaluation of endogenous stem cell function[2,3]. The inability to identify and isolate adult stem cells has been a major limitation to the study of their behaviour during homeostasis and disease. However, recent years have seen the discovery of robust adult intestinal stem cell markers, the development of clonal fate-mapping technologies (to evaluate stem cell behaviour *in vivo*), and the advent of 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.

Another crucial finding is the identification of ‘reserve’ stem cells that can be rapidly recruited to maintain epithelial homeostasis following injury[7,8]. This suggests that epithelial cells that are separate from the small pool of regular stem cells also harbour regenerative potential. Moreover, a nutrient-sensing mechanism within the stem cell niche (BOX 1) that regulates the size and activity of the intestinal stem cell pool was discovered[9], and these findings could be exploited for therapeutic applications.

This Review discusses the current models of stem cell-driven epithelial homeostasis and repair in the intestine, highlighting the contributions of pioneering research carried out in the 1970s and 1980s and of more recent breakthroughs driven by the identification of robust stem cell markers and the development of near-physiological epithelial culture systems.

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Box 1 | **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 neighbouring epithelial and stromal cells (FIG. 2b). Paneth cells, which are found in close association with the LGR5+ (Leu-rich repeat-containing G protein-coupled receptor 5-expressing) crypt base columnar (CBC) stem cells at the crypt base, are an important source of various niche factors, including epidermal growth factor (EGF), WNT3A and Notch ligand[86]. 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 mTOR complex 1 (mTORC1) signalling, 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[90–92] nor epithelial-specific loss of WNT3A expression[93] is sufficient to block stem cell-driven epithelial renewal *in vivo*, suggesting 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 KIT+ goblet cells that are located in close proximity to LGR5+ stem cells at the crypt base have been identified as probable niche components[94]. However, the major WNT source in the colon has yet to be identified.

The adult intestinal epithelium

Three weeks after birth, gut development is complete and the mouse is ready to tackle its first solid meal. The organization of intestinal tissue is highly adapted to its function and to maintaining tissue integrity.

Cellular organization. In the small intestine, each intestinal villus is encircled by at least six crypts of Lieberkühn, which house dedicated populations of stem and progenitor cells that self-renew 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 contains mainly absorptive enterocytes that also secrete hydrolytic enzymes to facilitate the efficient breakdown of partly digested food exiting the stomach and the absorption of nutrients. Also present are goblet cells, which secrete mucus, together with much smaller populations of enteroendocrine cells, which produce hormones (FIG. 1a). Goblet cells are most prevalent in the epithelium of the short villi of the distal small intestine, where they provide the extra lubrication needed to facilitate the passage of increasingly compact stool towards the colon. Intestinal crypt-resident Paneth cells, which secrete protective antimicrobial substances (such as cryptdins) and the hydrolytic enzyme lysozyme, are present throughout the small intestine. The epithelium contains at least three other cell types with poorly defined functions: cup cells, tuft cells and Peyer's patch-associated M (or microfold) 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 that of the small intestine, this epithelium is characterized by a high density of goblet cells and the absence of Paneth cells (FIG. 1b).

Intestinal villus
A finger-like structure covered in simple columnar epithelium that projects into the intestinal lumen to maximize the surface area for digestion and absorption.

Duodenum
The proximal third 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 invaginations of the epithelium harbouring the stem cells and their proliferating progeny; responsible for driving epithelial homeostasis and regeneration.

Rapid epithelial turnover. In the mouse, the adult epithelium undergoes rapid renewal to maintain optimal function. Epithelial cells exposed to the harsh luminal environment die and are expelled from the tips of the villi through a complex cytoskeletal remodelling process that simultaneously seals the resulting gap to maintain a barrier function[11]. More than 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 divide 2–3 times and gradually commit to the absorptive or secretory cell lineages while migrating upwards towards the base of the villi. Cell proliferation ceases when differentiated cells exit the crypt, and these epithelial cells then continue migrating upwards along the villi. This epithelial cell renewal cycle, which takes 3–5 days, 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 while 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 (REF. 14). At E14.5, following thickening of the epithelium, a major remodelling process initiates, resulting in the rapid conversion of the flat luminal surface into multiple 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 that are restricted to the base of the embryonic villi[15].

Although the mechanics of these remodelling processes remain incompletely understood, bidirectional signals between the epithelium and the underlying mesenchyme are considered to be crucial coordinators. 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]. Moreover, WNT signalling has an essential role in establishing the regenerative capacity of the adult epithelium, as evidenced by crypt loss and impaired villus formation following embryonic ablation of the major intestinal WNT effector protein, transcription factor 4 (TCF4)[17].

Crypt numbers rapidly increase during postnatal development of the small intestine through a bifurcation mechanism known as crypt fission[18]. This poorly understood process may be triggered by a rapid increase in crypt size owing to increased stem and/or progenitor cell activity, which peaks approximately 2 weeks after birth in mice[18,19].

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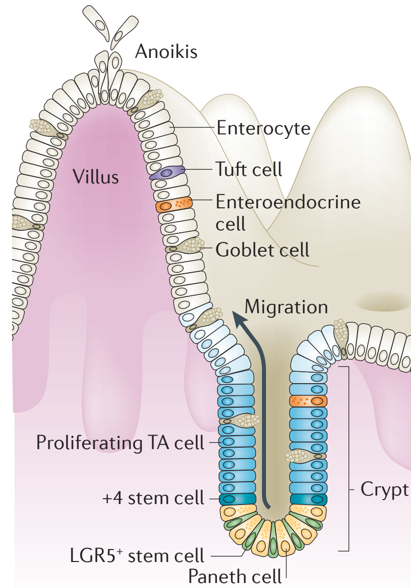
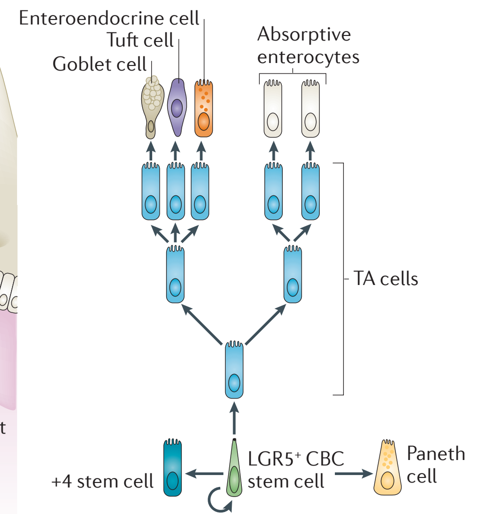
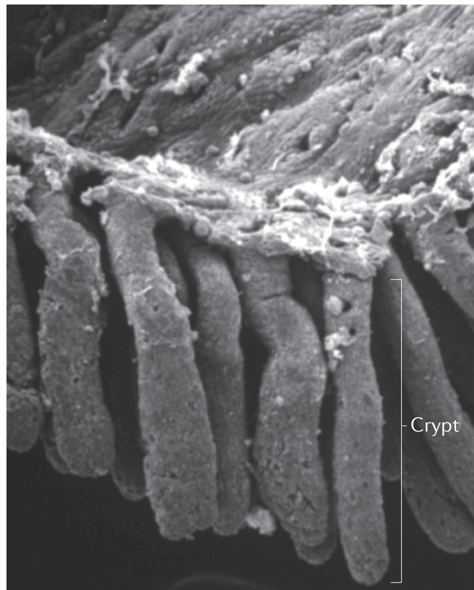
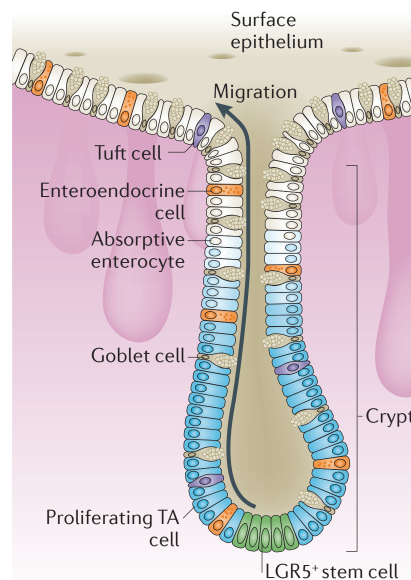
a Small intestine





b Colon





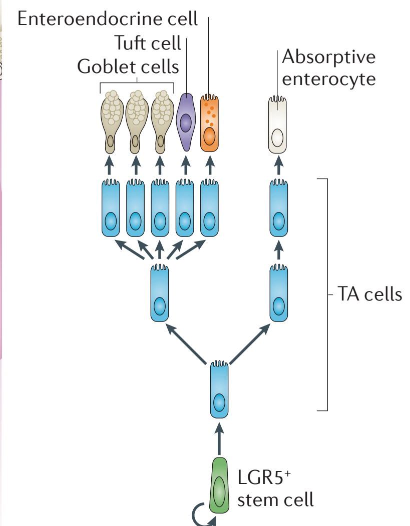


Figure 1 | **Epithelial self-renewal in the intestinal epithelium. a** | In the small intestine (the structural organization of which is shown in the scanning electron micrograph in the left panel), LGR5$^+$ (Leu-rich repeat-containing G protein-coupled receptor 5-expressing) crypt base columnar (CBC) stem cells are intercalated with Paneth cells at the crypt base (middle panel). These stem cells continuously generate rapidly proliferating transit-amplifying (TA) cells, which occupy the remainder of the crypt. TA cells differentiate into the various functional cells on the villi (enterocytes, tuft cells, goblet cells and enteroendocrine cells) to replace the epithelial cells being lost via anoikis at the villus tip. The +4 'reserve' stem cells (which occupy the fourth position from the crypt base) can restore the LGR5$^+$ CBC stem cell compartment following injury. This differentiating hierarchy is shown in the tree on the right panel. Epithelial turnover occurs every 3–5 days. New Paneth cells are supplied from the TA cells every 3–6 weeks. **b** | In the colon (the structural organization of which is shown in the scanning electron micrograph in left panel), LGR5$^+$ stem cells at the crypt base generate rapidly proliferating TA cells in the lower half of the crypt (middle panel). TA cells subsequently differentiate into the mature lineages of the surface epithelium (goblet cells, enterocytes, enteroendocrine cells and tuft cells), as shown in the lineage tree on the right panel. Epithelial turnover occurs every 5–7 days. Images in parts **a** and **b** are reproduced, with permission, from REF. 123 © Wiley (1986)

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

Adult stem cell origins in the intestine. Proliferating cells displaying stem cell characteristics are present in *ex vivo* cultures of E14 fetal intestinal epithelium[20–22]. Analysis of genetic marker expression patterns in newborn chimeric mice has shown that nascent crypts are polyclonal, as they develop from fetal stem cells derived from both parents[23,24]. During crypt morphogenesis in the first 2 weeks of life, these early stem cell populations

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initially undergo rapid expansion via symmetric cell division and then switch to an asymmetric cell division mode, which establishes the limited adult stem cell pool needed to sustain a functional epithelium[21]. During this period, a single progenitor cell gradually achieves dominance through a stochastic process known as purification, giving rise to a clonal pool of adult stem cells in each crypt that is maintained throughout life[25–27]. Additionally, analyses of random mitochondrial mutation patterns within healthy human small intestines and of Y-chromosome inheritance patterns in the colon of a XO/XY chimeric patient have established that adult crypts throughout the intestinal tract are clonal[28–31]. Despite this phenotypic clonality, it is important to note that adult crypts are not maintained by a single stem cell. Indeed, multiple stem cells sustain long-term epithelial homeostasis, although the exact number of them is not known (studies estimate between three and 16, depending on the methodology used)[32–34]. Perhaps the most precise calculation of crypt stem cell numbers was based on a continuous clonal labelling approach, which led to an estimate of five to seven stem cells driving daily epithelial renewal in the mouse small intestine[35].

Formal proof of the existence of multipotent, self-renewing stem cells in the crypt of the adult intestine was obtained by mapping the inheritance of genetic markers introduced at low frequency in crypt cells via random somatic mutation[27,36], as this led to the appearance of rare, long-lived clones containing all major epithelial lineages. However, these fate-mapping approaches were unable to reveal the location and identity of the stem cells within the crypt. Clues to the location of these stem cells were first obtained in the 1970s by radioisotope and 5-bromodeoxyuridine (BrdU) labelling of dividing intestinal cells[37–39]. The epithelial cell migration patterns indicated a common origin close to the crypt base, hinting at this being the location for multipotent stem cells[1]. In support of this, ablation of the crypt base populations through targeted irradiation completely abolished epithelial self-renewal in the intestine, which is consistent with loss of the stem cell compartment[40]. Despite these advances, the identity of adult stem cells remained frustratingly elusive for more than two decades and is 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 past four decades. The 'stem cell zone model', by Leblond, Cheng and Bjerknes[27], suggests that the columnar cells at the base of the crypt are the resident stem cells, whereas the '+4 model', by Potten[48], 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 proved difficult to definitively 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 publications by Cheng and Leblond in 1974, proposed that the CBC cells gave rise to the four major cell lineages in the intestinal epithelium[41–45]. 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 radiolabelled phagosomes as a consequence of engulfing neighbouring CBC cells that were killed via the incorporation of tritiated thymidine. Initially, only CBC cells were marked, but at later time points the cells from three major epithelial lineages became radiolabelled within the crypts. This was interpreted as CBC cells being the common ancestor of the differentiated epithelial cell types of the intestinal lining. However, because the three marked epithelial lineages were located in different crypts and no marked enteroendocrine cells were identified, formal identification of the CBC cells as multipotent stem cells was not possible.

Additional evidence in support of CBC stem cell identity was provided more than two decades later, when Bjerknes and Cheng used a mutation approach to introduce heritable somatic marks within the crypts[27]. A small proportion of the resulting epithelial clones comprised cells of all major lineages that were maintained over a long period, indicating that the original mutation had occurred within an unidentified self-renewing stem cell. Only these persistent, multilineage clones invariably contained a CBC cell, which 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 and publish the stem cell zone model in 1999 (REF. 27). In this model, CBC cells are adult stem cells that reside in a specialised niche (which was named the 'stem cell permissive zone') at the very base of the crypt. These cells proliferate to generate daughter cells that exit the niche and commit to multilineage differentiation at the 'common origin of differentiation' around position +5 (that is, the fifth cell position from the crypt base). Paneth cell progenitors mature into functional lysozyme-secreting cells as they migrate downwards to occupy the base of the crypt, whereas the majority of the non-Paneth cell lineages mature into functional epithelia while migrating upwards onto the villus. 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 they 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[46]. These markers enabled *in vivo* lineage tracing experiments[46] and *ex vivo* assays[47] to provide more direct evidence of CBC stem cell function during epithelial homeostasis and disease (see below and see Supplementary information S1 (Box)).

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Box 2 | Additional CBC stem cell markers

The WNT target gene *Sox9* encodes a member of the sex-determining region Y (SRY) family of transcription factors, which regulate cell proliferation in the intestine[95,96]. Using transgenic mice expressing enhanced GFP (EGFP) under the control of the *Sox9* promoter (*Sox9–EGFP*), two distinct cell populations, expressing EGFP at low (*Sox9–EGFP*^{low}) and high (*Sox9–EGFP*^{hi}) levels, were identified at the crypt base[97]. EGFP^{hi} cells were enteroendocrine cells and candidate +4 stem cells, whereas EGFP^{low} cells overlapped substantially with the LGR5^{+} (Leu-rich repeat-containing G protein-coupled receptor 5-expressing) crypt base columnar (CBC) cell compartment[97,98]. *Sox9–ires–EGFP* and *Sox9–ires–lacZ* knock-in mice showed a similar *Sox9* expression profile at the crypt base but also reported endogenous *Sox9* expression in Paneth cells and, less frequently, in villus cells[99]. SOX9^{low} CBC cells were confirmed to have stem cell identity by their ability to generate multilineage intestinal 'organoids' in an *ex vivo* culture assay[98] (BOX 4). By contrast, SOX9^{hi} cells were only able to proliferate and generate intestinal organoids *ex vivo* following irradiation, 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/R26R–lacZ* model, when typical stem cell-driven tracing clones were generated and maintained throughout the small intestine and colon[99]. 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^{low} population did reveal CD24 as a cell-surface marker that can be used to enrich for CBC stem cells through cell cell-sorting approaches[100].

Musashi homologue 1 (*Msi1*) 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 labelled the crypt base in the small intestine and colon[101,102]. However, both single-molecule mRNA fluorescence *in situ* hybridization (FISH) analyses and *in vivo* reporter gene analyses using an *Msi1–EGFP* transgenic line indicate that *Msi1* expression may extend to the lower transit-amplifying compartment[51,78,103]. Definitive proof of the stem cell identity of MSI1^{+} cells could be obtained with *in vivo* lineage tracing experiments, which should now be feasible using a recently described *Msi1–CreERT2–KI* line[104].

Prominin 1 (*Prom1*; also known as CD133 in humans), which encodes a cell-surface glycoprotein, is a gene that has been controversially used as a marker of cancer stem cells in a variety of epithelial cancers[105,106]. It has also been reported as a stem cell marker of in healthy adult tissues, including the bone marrow and intestine[107,108]. In the intestine, *in situ* hybridization analysis revealed PROM1 expression at the crypt base, overlapping with the CBC stem cells. *In vivo* lineage tracing using a *Prom1–CreERT2–ires–nLacZ/R26R*–yellow fluorescent protein model identified at least some of these PROM1^{+} cells as being bona fide adult stem cells. However, a subsequent study using independent antibody staining and *in vivo* lineage tracing analyses found that *Prom1* 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[109].

***Lgr5–EGFP–ires–CreERT2/R26R–lacZ* mouse model**
Generated by crossing *Lgr5–EGFP–ires–CreERT2* (which encodes a tamoxifen-activatable Cre enzyme that catalyzes recombination across DNA sequences called loxP sites) and *R26R* (Rosa26 reporter construct)–*lacZ* mouse strains. Facilitates the conditional activation of the *lacZ* reporter gene in LGR5-expressing cells in living tissues to evaluate their stem cell identity via lineage tracing.

The +4 model. The first indication that intestinal epithelial cells may occupy position +4 (the fourth 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[37,38]. Potten and colleagues later documented the existence of cells with stem cell attributes at a similar location[48]. These cells were actively dividing (every 24 hours), but they were capable of retaining labels incorporated into their DNA during crypt neogenesis (when new stem cells are being generated). Although this label retention is generally considered to be a trait characteristic of cellular quiescence, the +4 label-retaining cells (LRCs) could be labelled subsequently with BrdU, confirming their active proliferation status[3]. These conflicting observations were attributed to the ability of the +4 cells to retain labelled template DNA strands selectively during mitosis while segregating newly synthesized DNA strands, which could contain potentially dangerous replication errors, to the short-lived TA daughter cells during asymmetric division[3]. Originally described in the 'immortal strand hypothesis' (REF. 39), this phenomenon 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 unfounded assumptions about +4 cells, including the presence of asymmetric cell division and DNA exchange between sister chromatids (that is, between template DNA and newly-synthesized DNA, as typically occurs in somatic cells)[49].

Moreover, cells that were highly sensitive to ionizing radiation, a surrogate stem cell trait believed to ensure that long-lived cells do not survive DNA damage that could eventually lead to cancer, were identified around the +4 position[48]. However, formal proof that label retention and radiation sensitivity can be attributed to the same +4 cell population is currently lacking. Studies to validate and refine the +4 stem cell model through the identification of specific marker genes that facilitate a direct evaluation of endogenous stem cell identity have been reported. However, their observations are difficult to reconcile with the original +4 model (as discussed later).

Validation of CBC cells as stem cells

Several approaches, including lineage tracing, gene expression studies and analysis of how stem cell activity is regulated, have been made to validate the stem cell nature of CBC cells (see also BOX 2 and FIG. 2).

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

The first marker for CBC cells, *Lgr5* (Leu-rich repeat-containing G protein-coupled receptor 5), was identified as a WNT target gene selectively expressed at the base of adult intestinal crypts[46] (FIG. 2a). CBC-specific expression of LGR5 was confirmed using *Lgr5–LacZ* and *Lgr5–EGFP* reporter mouse models. In these mice, approximately 14 LGR5-expressing (LGR5^{+}) CBC cells were found at positions 1–4 in the crypt base, evenly distributed between Paneth cells.

The stem cell identity of LGR5^{+} CBC cells was confirmed via lineage tracing using an *Lgr5–EGFP–ires–CreERT2/R26R–lacZ* mouse model. Stochastic activation of the *lacZ* reporter gene at low frequency in LGR5^{+} CBC cells

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a Small intestine crypt

Enteroendocrine cell
Goblet cell
Enterocyte
Proliferating TA cell
Paneth cell

+4 stem cells	
Markers	Characteristics
• BMI1	• Label retaining
• LRIG1	• Quiescent?
• HOPX	• Damage resistant
• TERT	

CBC stem cells	
Markers	Characteristics
• LGR5	• Proliferative
• OLFM4	• Damage sensitive
• ASCL2	
• MSI1	
• SMOC2	

b

Proliferating TA cell
+4 stem cell
Paneth cell
CBC stem cell
Pericryptal cell
WNT2B

Niche factors
• WNT3
• DLL4
• EGF
• Noggin

c 2 days | 2 weeks | 6 weeks | 6 months

nGFP
YFP
RFP
mCFP

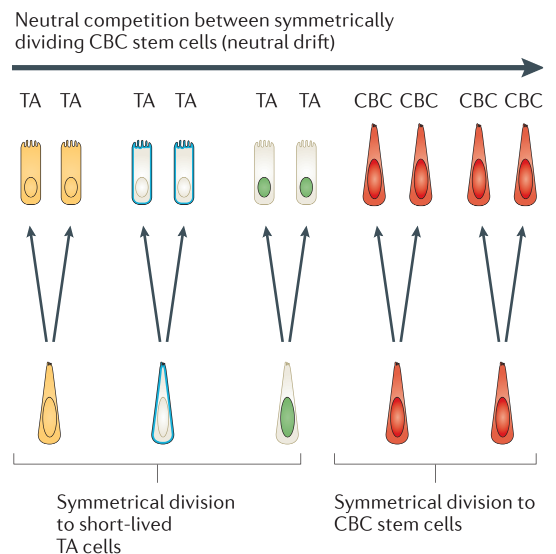

Figure 2 | **Intestinal stem cells and their defining niche. a** | Summary of the functional characteristics of the +4 stem cells and their markers and the crypt base columnar (CBC) stem cells and their markers. +4 stem cells (which occupy the fourth position from the crypt base) are generally considered to be relatively quiescent, are resistant to acute injury and display an inherent ability to retain DNA labels (owing to their infrequent replication or because of selective retention of labelled DNA strands during cell division). **b** | Cartoon depicting the components of the CBC stem cell niche at the crypt base. Both Paneth cells and pericryptal stromal cells supply essential factors (including WNT, the Notch ligand Delta-like 1 (DLL4), epidermal growth factor (EGF) and Noggin) to regulate the survival and function of the CBC stem cells *in vivo*. **c** | Neutral drift within the intestinal stem cell pool. Activation of multicolour lineage tracing (with yellow fluorescent protein (YFP), red fluorescent protein (RFP), nuclear GFP (nGFP) and membrane-bound cyan fluorescent protein (mCFP)) generates a population of distinctly labelled CBC stem cells in each crypt. Each labelled stem cell generates progeny of the corresponding colour, resulting in the appearance of 'rainbow' crypts over the next 2 weeks. However, over the following months, neutral competition between symmetrically dividing labelled stem cells causes the gradual conversion of multicolour crypts into single-coloured crypts. In this example, the RFP-labelled stem cell achieves dominance. ASCL2, Achaete–Scute homologue 2; HOPX, homeodomain-only; LGR5, Leu-rich repeat-containing G protein- coupled receptor 5; LRIG1, Leu-rich repeats and immunoglobulin-like domains 1; MSI1, Musashi homologue 1; OLFM4, olfactomedin 4; SMOC2, SPARC-related modular calcium-binding 2; TA cell, transit-amplifying cell; TERT, telomerase reverse transcriptase.

resulted in the appearance of clones expressing *lacZ* that rapidly expanded to form the epithelium spanning from the crypt base to the villus tip. These CBC cell-derived clones contained all major epithelial cell lineages and persisted over a long period, validating the LGR5$^+$ CBC cells as self-renewing, multipotent adult intestinal stem cells. Further proof of their stem cell identity came from a novel *ex vivo* culture method showing that single LGR5$^+$ CBC cells isolated by fluorescence-activated cell sorting (FACS) from *Lgr5–EGFP* reporter mice could

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generate self-renewing 'organoids' that recapitulated the architecture and cellular composition of a functional intestinal epithelium[47] (BOX 4). Of note, cells not expressing LGR5 (LGR5⁻ cells) lacked the ability to generate these epithelial organoids, highlighting the value of this culture system to assay endogenous 'stemness'.

The base of human intestinal crypts contains CBC-like cells, but the lack of antibodies against LGR5 has precluded a formal evaluation of their expression profile and their stem cell identity. However, epithelial cells from the very bottom of the crypt in the human colon have been isolated using antibodies against the surface-expressed WNT target gene ephrin type B receptor 2 (*EPHB2*). Cells expressing high levels of this gene (*EPHB2*^{hi} cells) behaved as stem cells in *ex vivo* organoid culture assays and expressed high levels of *LGR5*, suggesting a conservation of intestinal stem cell identity in mice and humans[50].

The LGR5⁺ CBC stem cell expression signature. Combinatorial microarray and proteomic approaches have established an accurate molecular signature for the LGR5⁺ CBC stem cells[51,52]. A comparison between stem cells and their progeny isolated by FACS from *Lgr5–EGFP* reporter mice (exploiting different levels of EGFP: high in stem cells and low in progeny) revealed approximately 500 genes preferentially expressed in stem cells. Consistent with the known function of WNT signalling on stem cell-driven epithelial homeostasis in the intestine[17], a strong WNT signature was present, including many WNT target genes, such as *Sox9*, Achaete–Scute homologue 2 (*Ascl2*), *EphB2*, *Troy* (also known as *Tnfrsf19*) and axis inhibition 2 (*Axin2*). This approach also revealed novel markers of the LGR5⁺ CBC stem cells, including olfactomedin 4 (*Olfm4*), SPARC-related modular calcium-binding 2 (*Smoc2*) and ring finger 43 (*Rnf43*) (FIG. 2). Selective expression of the bone morphogenetic protein inhibitor *Smoc2* on the stem cell compartment was validated through *in vivo* lineage tracing[51]. Surprisingly, many +4 stem cell markers (including *Bmi1*, Leu-rich repeats and immunoglobulin-like domains 1 (*Lrig1*), telomerase reverse transcriptase (*Tert*) and homeodomain-only (*Hopx*)) were strongly expressed in LGR5⁺ stem cells, casting doubt onto whether these are truly independent intestinal stem cell pools (as 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* (FIG. 2). ASCL2, a transcription factor that promotes neuroblast differentiation, was identified as a critical regulator of intestinal stem cell fate, as its conditional ablation in the intestinal epithelium resulted in a rapid and selective loss of the LGR5⁺ stem cell compartment[53]. *Lgr5*, which encodes a receptor with seven transmembrane segments, was itself linked to stem cell homeostasis, as conditional ablation of *Lgr5* and its close homologue *Lgr4* resulted in the suppression of WNT signalling and stem cell death[54]. The direct role for LGR5 in modulating WNT signalling in intestinal stem cells was confirmed when LGR5 was identified as a facultative component of the WNT signalling complex at the plasma membrane. LGR5 recruits secreted WNT agonists (roof plate-specific spondin (R-spondin) 1–4), thus amplifying canonical WNT signalling and ensuring stem cell homeostasis *in vivo*[54–57]. Two other WNT target genes that are highly expressed in stem cells, *Rnf43* and *Troy*, also regulate endogenous WNT signalling. RNF43 suppresses WNT signalling in stem cells by ubiquitylating Frizzled receptor proteins to promote their degradation[58,59], whereas TROY exerts an inhibitory function by destabilizing the low-density lipoprotein receptor-related protein 6 (LRP6) co-receptor protein (after it is recruited to the WNT receptor complex by LGR5)[60]. These observations highlight the importance of maintaining an optimal level of WNT signalling for intestinal stem cell homeostasis *in vivo* (see Supplementary information S1 (Box)).The recent elucidation of the structure of the WNT receptor complex containing LGR5, R-spondin and other components should increase our understanding of the mechanism of action of LGR5 in intestinal stem cells[61–63].

A model of CBC stem cell-driven homeostasis

Insights into how actively cycling LGR5⁺ CBC cells balance stem cell maintenance and differentiation to maintain epithelial homeostasis *in vivo* were obtained using a multicolour lineage tracing approach[64]. These clonal fate-mapping experiments unexpectedly revealed that individual LGR5⁺ crypt cells typically undergo symmetrical division to generate progeny with identical fates (either two stem cells or two TA cells), rather than undergoing asymmetric division like most somatic stem cells. Although this division mode suggests 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 multicoloured LGR5⁺ stem cell populations gradually became monochromatic, through a process of stochastic refinement known as neutral drift (FIG. 2c). These observations indicated that homeostasis of the epithelial and stem cell compartments is achieved by neutral competition among 14 symmetrically dividing stem cells for restricted niche space at the crypt base. A separate mathematical modelling approach provided additional support for this model[65]. By contrast, analysis of the relationship between mitotic spindle orientation and DNA segregation during LGR5⁺ stem cell division indicated that asymmetric division predominates, with template strands being preferentially distributed to progeny adopting a stem cell fate during mitosis[66]. However, these results were challenged in later studies analysing spindle orientation in crypt base cells[67] and DNA label distribution kinetics in dividing LGR5⁺ CBC stem cells, which suggested that chromosomes randomly segregate to daughter cells[68,69]. More recently, highly sensitive multi-isotope imaging mass spectrometry analyses failed to identify any evidence of label retention during crypt cell division, confirming a model of random DNA strand segregation, which is inconsistent with the immortal strand hypothesis[70].

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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 past decade[110]. Unlike *Bmi1*, Leu-rich repeats and immunoglobulin-like domains 1 (*Lrig1*) and telomerase reverse transcriptase (*Tert*), 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.

Immunohistochemistry analyses have detected enriched phospho-phosphatase and tensin homologue expression on label-retaining cells (LRCs) residing just above the Paneth cell compartment around positions +4 and +5 (that is, the fourth and fifth position from the crypt base)[111,112]. 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[113].

Expression of the phosphatase *Wip1* (also known as *Ppm1d*) was also reported to be predominantly restricted to position +4, although WIP1+ cells intercalated with the Paneth cells were also readily apparent[114]. Somewhat surprisingly, the observed depletion of this candidate stem cell population in *Wip1*-knockout mice had no discernible effect on epithelial homeostasis.

Interest in doublecortin-like and CAM kinase-like 1 (*Dcamkl1*; also known as *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[115]. Immunohistochemical analysis subsequently documented expression on rare, quiescent cells predominantly located around position +4 (REFS 116,117). 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 and villus epithelium and phenotyped them as a rare population of tuft (caveolated) cells, a differentiated cell type displaying thick tufts of microvilli the *in vivo* function of which is still poorly understood[10,95,118]. This was confirmed by *in vivo* lineage tracing using a new *Dcamkl1*-driven CreERT2 allele[119].

Although symmetrical division seems to predominate in the LGR5+ CBC stem cell compartment, it is currently unknown whether the choice of adopting stem cell or TA cell fates is truly stochastic. It is plausible that local niche and biomechanical factors could direct post-mitotic fate choices of crypt LGR5+ stem cells in a position-dependent manner. In theory, this could be investigated by fate-mapping LGR5+ stem cells at different crypt locations through multicolour lineage tracing, but this is technically challenging. Alternatively, robust, predictive computer models of crypt homeostasis can be used to evaluate experimental predictions of *in vivo* stem cell behaviour[71,72].

Recent studies are shedding light onto the regulation of CBC stem cell fate choices *in vivo*. Surprisingly, epigenetic regulation via DNA methylation does not seem to play a major part in directing their fate[73]. Conversely, the GTPases CDC42 and RAB8A, together with the endoplasmic stress response pathway, seem to be important mediators of both stem cell division and differentiation[74,75].

Bmi1–ires–CreERT2/R26R–lacZ **mouse model**
Generated by crossing *Bmi1-ires-CreERT2* and *R26R-lacZ* mouse strains. Facilitates the conditional activation of the *lacZ* reporter gene in BMI1-expressing cells in living tissues to evaluate their stem cell identity via lineage tracing.

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

Validation of +4 cells as stem cells

Several genes have been reported to be selectively expressed in the candidate +4 stem cells (BOX 3; FIG. 2a). Although some of these markers have been validated by *in vivo* lineage tracing, many of them seem to mark distinct epithelial cell populations with different cellular features distinct from those of the LRC population originally described by Potten. This may indeed reflect the existence of multiple phenotypically distinct stem cell populations residing at the +4 position. However, it is also becoming increasingly apparent that some mouse models do not faithfully report endogenous expression patterns of candidate stem cell markers, and this has caused some controversy regarding the validity of some of the +4 stem cell markers, which we briefly discuss below. Here, we discuss findings pertinent to markers evaluated by *in vivo* lineage tracing.

BMI1. The *Bmi1* gene encodes a component of the Polycomb repressor complex that is implicated in the regulation of haematopoietic and neural stem cell replication[76]. *In situ* hybridization analyses documented Bmi1 expression predominantly at the +4 position within the proximal small intestine, which was confirmed using a *Bmi1–EGFP* reporter mouse model[8]. *In vivo* fate-mapping experiments using a *Bmi1–ires–CreERT2/R26R–lacZ* mouse model revealed that these proximal BMI1+ populations harbour self-renewing, multipotent stem cells contributing to long-term epithelial homeostasis. Additional support for these BMI1+ cells having stem cell identity was provided by *in vivo* ablation of the BMI1+ population, which blocked epithelial renewal, and by showing that isolated BMI1+ cells can generate epithelial organoids in culture that are reminiscent of functional intestinal tissue[8,77].

The BMI1+ population was found to be relatively quiescent, radiation resistant and not regulated by WNT signalling, prompting the conclusion that BMI1 marks a +4-restricted reserve stem cell population that is different from that in the LGR5+ CBC compartment at the crypt base. However, further expression analyses have documented robust *Bmi1* expression throughout the proliferative zone of the crypt, including the LGR5+ CBC compartment[51,78–81]. In agreement with such a broad endogenous expression pattern, a recent fate-mapping study using a *Bmi–CreERT2* transgenic model noted the initiation of *Bmi1*-driven lineage tracing at random locations throughout the crypts[8]. Moreover, efforts to reproduce the original findings using the *Bmi–ires–CreERT2* mouse model led to similar conclusions: in contrast to previous reports of lineage tracing being predominantly initiated at the +4 position, tracing initiation occurred at random positions throughout the crypt, including the CBC compartment[51]. Over time, most of these lineage tracing events were lost, which is consistent with them having originated within the short-lived TA progenitor cell compartment. Long-term tracing events were postulated to arise from CBC stem cells expressing both *Lgr5* and *Bmi1* (REF. 51).

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 using *Bmi1*-driven lineage tracing for studying the contribution of +4 reserve stem cells to crypt regeneration and disease.

HOPX. The *Hopx* gene encodes an atypical homeobox protein predominantly expressed at the +4 position throughout the entire intestine, as shown using

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Hopx–lacZ reporter mice (in which *LacZ* is inserted in the endogenous *Hopx* locus)[81]. These HOPX+ cells are relatively quiescent and radiation resistant, and they harbour 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 at the +4 position produced persistent, multipotent stem cell signature tracings throughout the intestine.

Comparative expression profiling of laser-microdissected HOPX+ cells and their immediate crypt base progeny showed that HOPX+ cell descendants have higher expression of *Lgr5* and other CBC marker genes. Conversely, organoid culture assays confirmed that isolated LGR5+ CBC stem cells give rise to HOPX+ cells *ex vivo*[81].

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. Such functional interplay between +4 cells and CBC cells at lower crypt positions helps to unify the two models of intestinal stem cell identity. However, it should be noted that a different *Hopx* expression profile was reported in a separate study, in which single-molecule RNA fluorescence *in situ* hybridization (FISH) and LGR5+ CBC expression profiling analyses detected endogenous *Hopx* expression throughout the crypt, with highest levels in the LGR5+ CBC cells at the crypt base[51].

LRIG1. The *Lrig1* gene encodes a single-pass transmembrane receptor that functions as a conditional inhibitor of ERB proteins in several adult tissues. Its discovery as a marker and proliferation regulator of stem cells in the epidermis[82] prompted efforts by two groups to investigate its value as a marker of intestinal stem cells.

In one 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[80]. Reporter gene activation was most commonly observed within positions +2 to +5, although tracing was also initiated throughout the lower portion of the TA compartment, which is 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 bottom of the crypt, a minority of which co-expressed *Lgr5*. Although this was interpreted as evidence of very limited overlap between the LRIG1+ and LGR5+ stem cell populations, the mosaic expression of the *Lgr5–EGFP* reporter allele in the colon invalidates this claim. Fate mapping indicated that at least some of the cells 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 labelled 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, providing further evidence of their independence. Although both populations expressed equivalent levels of reported +4 markers, including BMI1, TERT and prominin 1, the LRIG1+ cells selectively expressed gene signatures involved in oxidative damage responses and negative regulation of cell proliferation. LRIG1 was itself identified as tumour suppressor in the intestine when genetic ablation resulted in adenoma formation within 6 months[80]. This prompted speculation that LRIG1 functions to regulate ERBB signalling 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[83]. *In situ* and immunohistochemistry analyses confirmed LRIG1 expression throughout the lower third of the small intestine crypts, but the LGR5+ stem cell transcriptome revealed a significant enrichment of *Lrig1* expression within this CBC population[51]. An independent study using single-molecule mRNA FISH also documented *Lrig1* as being expressed in a broad gradient, with the highest levels being present in LGR5+ CBC stem cells[51]. This extensive overlap of the LRIG1+ and LGR5+ crypt populations was confirmed at the protein level through flow cytometric analysis using an independent LRIG1-specific antibody[51]. 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 in 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, which was directly linked to deregulated *Erbb* expression in the stem cell compartment (as treatment of *Lrig1*-knockout mice with an ERBB inhibitor restored crypt homeostasis).

Collectively, these studies suggest that LRIG1 has important functions in regulating ERBB signalling in the intestinal stem cells to prevent disease. However, the broad expression gradient of *Lrig1* in crypts of the small intestine and its enrichment in the LGR5+ CBC cell pool seem to disqualify it as a specific marker for intestinal stem populations.

TERT. Elevated telomerase expression is considered to be a stem cell trait that protects against replication-induced senescence. Using *Tert–GFP* reporter mice, rare, predominantly quiescent *Tert*+ cells were detected around the +4 position[84]. These TERT+ cells were later shown to be independent of the LGR5+ CBC stem cells and other +4 stem cell pools, and resistant to ionizing irradiation[79]. *In vivo* lineage tracing demonstrated that a small fraction of the TERT+ crypt cells were actively cycling stem cells contributing to epithelial homeostasis in both the small intestine and colon[79]. The quiescent TERT+ cells that did not participate in daily tissue homeostasis could be activated and proliferate to contribute to damage-induced epithelial regeneration. Collectively, these observations seem

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to support TERT 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 contrasting conclusions[69,78]. *Tert* mRNA and telomerase activity were readily detected in all proliferative cells of the crypt, with highest levels present within LGR5+ CBC stem cells sorted by FACS. Single-molecule mRNA FISH analysis confirmed this expression pattern in proximal small intestine. Such an elevated telomerase activity would probably 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 seen major advances over the past 5 years, mostly driven 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 cells versus LGR5+ CBC cells) of adult intestinal stem cells. Recent attempts to reconcile the opposing views have led to the formulation of a more plastic model of stem cell identity. This model describes the intestinal crypt base as a specialized niche environment harbouring 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 TA cell compartment can acquire stem cell identity to ensure maintenance of epithelial homeostasis in the eventuality of a catastrophic loss of stem cell populations resident in the crypt base (FIG. 3).

Epithelial regeneration and crypt plasticity

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' (or 'actual') and 'reserve' (or 'potential') stem cell populations within the lower regions of the crypt. It has been proposed that many of the +4 markers discussed above 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 committed progenitors into functional stem cells following tissue damage.

Dedicated reserve stem cells. Actively proliferating LGR5+ CBC cells drive intestinal epithelial homeostasis under physiological conditions[46] (FIG. 3a). However, a study using a targeted conditional ablation strategy has shown that the intestine can survive short-term loss of the CBC stem cell pool *in vivo*, despite CBC cells having such a crucial role, hinting at the existence of additional, damage-resistant stem cells[8] (FIG. 3b).

Based on traits such as cellular quiescence and resistance to radiation, +4-resident cells are reserve stem cell candidates[77]. Although the +4 populations marked by HOPX and LRIG1 remain to be functionally identified as reserve stem cells, the exquisite radiation-sensitivity of the +4 LRC population originally described by Potten would seem to disqualify it from serious consideration[48]. Strong support for the existence of a BMI+ reserve stem cell pool was provided by *in vivo* lineage tracing from the *Bmi1* locus following acute ablation of the LGR5+ CBC stem cell compartment, revealing that *Bmi1*+ cells contribute to the repopulation of the LGR5+ stem cell pool during the subsequent regeneration phase[8] (FIG. 3c). However, the fact that *Bmi1* is expressed throughout (and traces from) the entire proliferative zone of the crypt precludes a formal identification of these as reserve stem cells[51].

Committed progenitors as potential stem cells. An alternative, although not necessarily mutually exclusive, explanation for the robust regenerative capacity of the intestinal epithelium may be related to the plasticity of early stem cell progeny. Clonal regeneration assays have identified up to 30–40 cells in the lower third of the intestinal crypts with the ability to survive exposure to varying doses of radiation or cytotoxic drugs and contribute to crypt regeneration[40,85]. These observations 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 progenitor populations 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[48]. Such niche-driven plasticity has been demonstrated using *ex vivo* organoid cultures (BOX 4). Under normal conditions, LGR5− TA cells isolated from intestinal crypts are incapable of generating epithelial organoids in culture. However, brief exposure to one of the major *in vivo* niche signals, WNT3A, efficiently converts these LGR5− cells into organoid-proficient LGR5+ stem cells[86].

Strong evidence in support of this model was recently provided by approaches using inducible histone H2B–GFP (or histone H2B–yellow fluorescent protein) to identify and isolate LRCs in the small intestine[7,87]. Long-lived LRCs (4–9 weeks old) comprised exclusively mature Paneth cells, which is consistent with the lifespan (6–8 weeks) of this terminally differentiated population[13]. However, a different, shorter lived (<4 weeks) LRC population presented a mixed phenotype, expressing a collection of markers for Paneth cells, enteroendocrine cells and LGR5+ CBC and +4 stem cells. Using an elegant split-Cre recombinase strategy to carry out lineage tracing from these LRCs 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,

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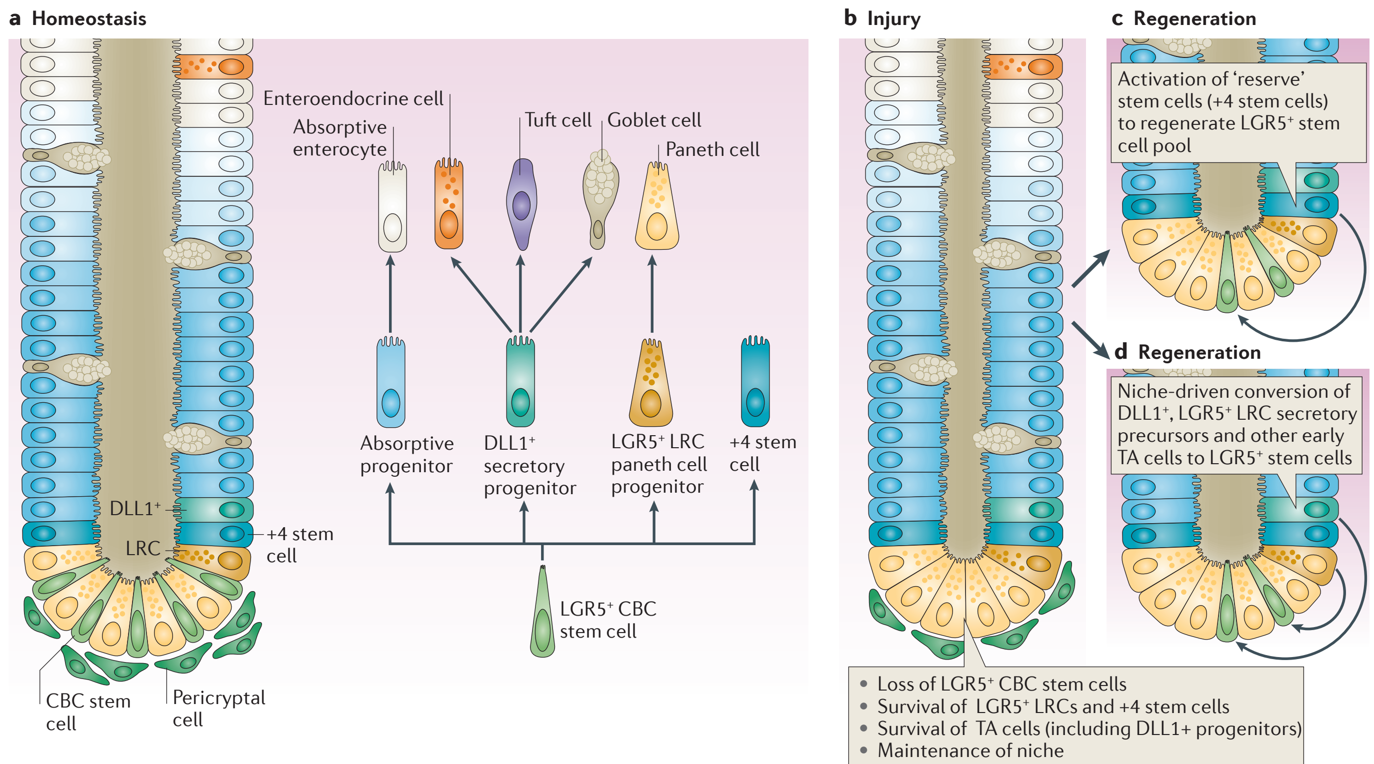

Figure 3 | **Models of epithelial regeneration in the small intestine.** **a** | During homeostasis, multipotent LGR5⁺ (Leu-rich repeat-containing G protein-coupled receptor 5-expressing) crypt base columnar (CBC) stem cells drive regular epithelial renewal. LGR5⁺ label-retaining cells (LRCs) are Paneth cell precursors that exclusively supply mature Paneth cells. Delta-like 1-expressing (DLL1⁺) transit-amplifying (TA) cells 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 (which occupy the fourth position from the crypt base) and the niche intact. **c** | Surviving +4 cell populations function as 'reserve' stem cells to rapidly regenerate the LGR5⁺ CBC stem cell pool and restore epithelial renewal. **d** | Surviving DLL1⁺ and LGR5⁺ LRC 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.

cells of the same LRC population were rapidly converted into self-renewing, multipotent stem cells contributing to epithelial regeneration (FIG. 3d). This LRC population was further characterized as being a subset of the LGR5+ CBC cells, constituting 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 regular and reserve stem cells in the small intestine. However, if this were true then *Lgr5*-driven lineage tracing would result in the generation of a substantial proportion of Paneth cell-restricted clones under physiological conditions — this has never been reported. As the regeneration capacity of the small intestinal epithelium is maintained following short-term ablation of the entire LGR5-expressing crypt population, including both regular CBC and reserve LRC stem cell pools, other LGR5⁻ cell populations capable of functioning as stem cells must exist.

In a separate study, LRCs were characterized after 7 weeks of pulse–chase. In agreement with the findings described above, these long-lived LRCs were differentiated Paneth cells[87]. These label-retaining Paneth cells 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 are in contrast to those of the earlier study, which documented the Paneth cell LRCs as being a terminally differentiated population incapable of injury-induced stimulation[7]. The colonic epithelium, which is devoid of Paneth cells and LRC populations, is also capable of surviving acute injury, suggesting the existence of actively cycling cells capable of functioning as stem cells during regeneration.

In another study, *in vivo* lineage tracing was used to demonstrate the plasticity of early stem cell progeny expressing the Notch ligand Delta-like 1 (DLL1)[88]. Under physiological conditions, DLL1⁺ cells at the +5 position (the proposed 'common origin of differentiation') function as secretory progenitors, generating short-lived clones comprising goblet cells, enteroendocrine cells, Paneth cells and tuft cells. However, irradiation-induced depletion of the LGR5⁺ CBC stem cell compartment resulted in conversion of the DLL1⁺ cells into multipotent LGR5⁺ CBC stem cells contributing to the subsequent epithelial regeneration process[88] (FIG. 3d).

Pulse–chase
A method for detecting quiescent label-retaining cells (LRCs) *in vivo*. A nucleotide analogue (the label) is administered to the mouse for a short period (the pulse); this is followed by an extended period when no further nucleotide analogue is given (the chase). Actively dividing cells rapidly dilute out the label during DNA replication, and labelled cells are lost within 3–4 rounds of cell division. Non-dividing (quiescent) cells do not dilute out the label and thus remain detectable as LRCs.

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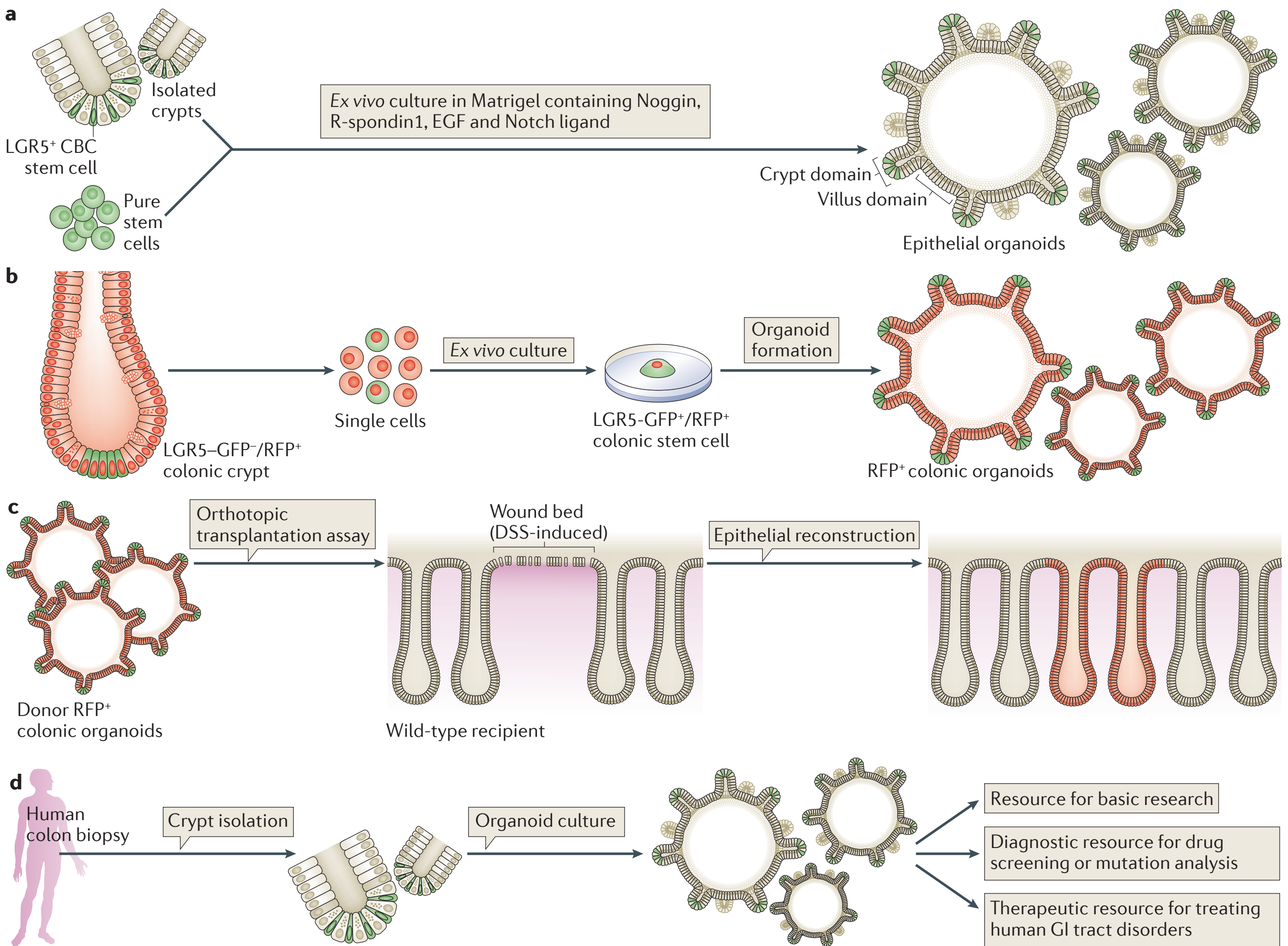
Box 4 | **Biomedical applications of intestinal stem cells: the epithelial organoid culture system**



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. Major advances have been made in establishing culture systems that support the long-term growth and expansion of near-physiological intestinal epithelia from purified stem cells[20,22,47,120]. To date, several methods have been used to grow 'organoids' from the small intestine[121], but the most successful method is a Matrigel-based three-dimensional culture system that supports the growth of self-renewing, near-native intestinal epithelia in the absence of stromal niche components[47] (see the figure, part **a**). In this system, intact intestinal crypts or purified LGR5⁺ (Leu-rich repeat-containing G protein-coupled receptor 5-expressing) crypt base columnar (CBC) stem cells are plated into laminin-rich Matrigel supplemented with a cocktail of growth factors found in the endogenous stem cell niche, including the bone morphogenetic protein inhibitor Noggin, the WNT agonist roof plate-specific spondin 1 (R-spondin1), epidermal growth factor (EGF) and Notch ligand[47]. This generates self-renewing epithelial organoids organized into discrete crypts harbouring intercalated stem cells and Paneth cells at their base and associated villus-like regions comprising the various differentiated cell lineages. This system was adapted for use in culturing colonic epithelia, although this required exogenous WNT3A, probably reflecting subtle differences in the endogenous stem cell niches of the small intestine and colon[120] (see the figure part **b,** which depicts the generation of red fluorescent protein-expressing (RFP⁺) colonic organoids from a single LGR5⁺ stem cell carrying a permanent, heritable RFP mark). In a beautiful example of their clinical potential, both mouse colonic organoids and intestinal 'spheroids' were successfully used to repair damaged colonic epithelia *in vivo* when delivered via a simple enema[22,122] (see the figure, part **c**, which shows that cultured RFP⁺ colonic organoids can be used to effectively repair damaged colonic epithelia *in vivo* via orthotopic transplantation in the mouse, as supported by the appearance and maintenance of patches of RFP⁺ colonic epithelia in the recipient wild-type mouse). Importantly, the organoid culture system has recently been successfully adapted for routinely growing human epithelia from the small intestine and colon[50,120]. 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 gastrointestinal (GI) tract diseases, such as ulcers, and the expansion of matched healthy and tumour epithelia from patient biopsies for use in basic research into epithelial biology and various biomedical applications, including drug screening and deep-sequencing efforts as a prerequisite to the development of personalized treatment regimens (see the figure, part **d**).

An alternative source of intestinal stem cells for therapeutic applications was revealed recently, when it was shown that abundant progenitor cells isolated from both mouse and human fetal intestine could be expanded as self-renewing spheroids in an *ex vivo* culture system. Exposure to exogenous WNT ligands efficiently converted these progenitor cultures into mature organoid structures, highlighting their value as a source of functional intestinal epithelia[22].

DSS, dextran sodium sulphate.

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This plasticity of the lower crypt cell populations during epithelial regeneration is probably dependent on instructive signals emanating from the surviving stem cell niche. Such niche-driven plasticity may also exist within gastrointestinal tumours, which would have important implications for therapies targeting the elimination of cancer stem cell populations (see Supplementary information S1 (Box)). The intestinal niche is a critical component in governing stem cell behaviour not only during epithelial homeostasis but also during tissue regeneration following injury.

Summary and future outlook

The intestinal stem cell field has seen impressive advances over the past 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 (BOX 4). 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 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 seems capable of drawing on several pools of highly plastic, reserve stem cell populations in the lower regions of the crypt (FIG. 3). Such plasticity is likely to be endowed by the specialized niche environment at the crypt base (BOX 1; FIG. 2b), which provides the requisite signals to efficiently convert committed progenitor populations into multipotent adult stem cells. Such niche-driven plasticity would 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. Efforts to exploit intestinal stem cells for regenerative medicine have accelerated over the past decade, driven mostly 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 (BOX 4). LGR5 is one of the few cell-surface stem cell markers that could potentially be used to isolate human intestinal stem cells, but it has proved 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[89]. 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. 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.

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