© 2020. Published by The Company of Biologists Ltd | Development (2020) 147, dev182675. doi:10.1242/dev.182675

REVIEW

Hallmarks of intestinal stem cells

Anna Baulies*, Nikolaos Angelis* and Vivian S. W. Li‡

ABSTRACT

Intestinal stem cells (ISCs) are highly proliferative cells that fuel the continuous renewal of the intestinal epithelium. Understanding their regulatory mechanisms during tissue homeostasis is key to delineating their roles in development and regeneration, as well as diseases such as bowel cancer and inflammatory bowel disease. Previous studies of ISCs focused mainly on the position of these cells along the intestinal crypt and their capacity for multipotency. However, evidence increasingly suggests that ISCs also exist in distinct cellular states, which can be an acquired rather than a hardwired intrinsic property. In this Review, we summarise the recent findings into how ISC identity can be defined by proliferation state, signalling crosstalk, epigenetics and metabolism, and propose an update on the hallmarks of ISCs. We further discuss how these properties contribute to intestinal development and the dynamics of injury-induced regeneration.

KEY WORDS: Development, Epigenetic, Intestinal stem cell, Metabolism, Plasticity, Signalling pathways

Introduction

The adult small intestine is crucial for executing multiple vital functions throughout life: food digestion, nutrient absorption, glucose homeostasis and energy maintenance. The intestinal epithelium consists of two different compartments: the proliferative crypts of Lieberkühn and long projections called villi. Villi are continuous structures that contain a single columnar layer of fully differentiated epithelial cells that are no longer capable of dividing. Intestinal stem cells (ISCs; see Glossary, Box 1) reside at the intestinal crypts and divide continuously to fuel the high turnover of the intestinal epithelium, giving rise to either another stem cell for self-renewal or a progenitor cell that enters the trans-amplifying (TA) compartment to rapidly divide before terminal differentiation (Fig. 1A). Two major types of progenitors exist within the intestinal epithelium: the absorptive and secretory progenitors (see Glossary, Box 1). Absorptive progenitors differentiate into enterocytes, the most prominent cell type of the intestinal epithelium, whereas secretory progenitors give rise to Paneth cells, enteroendocrine cells, tuft cells and goblet cells (Clevers, 2013). The differentiated cells arising from the TA zone migrate upwards towards the crypt-villus junction, and will eventually die and shed into the intestinal lumen when they reach the tip of the villus, approximately 5 days after differentiation. Paneth cells are the only differentiated cell type that migrates downwards to the stem cell zone (Barker et al., 2007; Sato et al., 2011; Clevers, 2013).

Cell positioning along the intestinal crypt has been the central discussion of ISC identity in the last few decades, based on histology and the ability of cells to retain the labelling reagents after pulse-chase experiments. Apart from physical position, the traditional text book definition of stem cells focuses mainly on functional properties, such as multipotency and ability to self-renew. However, studies have increasingly demonstrated that stem cells also display distinct cellular characteristics, including a defined chromatin landscape and metabolic states, which may potentially be used to define stemness and explain plasticity. In this Review, we will present an update on recent insights into the molecular definition of ISCs, which constitute an essential entity in the intestinal crypt, and provide an overview of ISC identity in various contexts during development and regeneration.

The regulation of ISC identity and fate

ISC position and cell cycle state

Previous debate on ISC identity focused mainly on the physical positioning in the intestinal crypt. Early studies proposed two distinct ISC populations: the crypt base columnar (CBC; see Glossary, Box 1) cells and the +4 cells (extensively reviewed by Barker et al., 2012; see Glossary, Box 1). Briefly, the CBC cells are slender cells that are wedged between Paneth cells at the crypt base and divide once every day (Cheng and Leblond, 1974). The +4 cells were proposed when early DNA labelling experiments showed long-term incorporation and retention of tritiated thymidine (^{3}HTdR) in the ISCs. These label-retaining cells (LRCs; see Glossary, Box 1) were reported to be slow-cycling and were located at around cell position 4, just above the differentiated Paneth cell (Potten, 1977; Potten et al., 1978, 1974). Advances in transcriptomics and lineage-tracing tools in the last decade have unveiled a number of putative stem cell-specific genes marking the CBC cells or +4 cells, and allowed direct tracing of their corresponding progenies for the first time. In 2007, a landmark study showed that the CBC-specific gene leucine rich repeat containing G protein coupled receptor 5 (Lgr5) is the bona fide ISC marker. Lineage-tracing experiments demonstrated that the Lgr5-expressing cells can generate all distinct cell types in the intestine: enterocytes, goblet cells, Paneth cells and enteroendocrine cells (Barker et al., 2007). Importantly, the Lgr5+ cells derived from intestinal crypts were able to establish long-term *ex vivo* organoid cultures that contain all of the mature intestinal cell types and recapitulate the structure and function of the intestine *in vivo* (Sato et al., 2009). Contrary to these findings, several studies have subsequently proposed a number of alternative ISC-markers, the expression of which is predominantly enriched at the +4 cells. These markers include polycomb complex protein Bmi1 (Sangiorgi and Capecchi, 2008), homeodomain-only protein (Hopx) (Takeda et al., 2011) and mouse telomerase reverse transcriptase (Tert) (Montgomery et al., 2011). Interestingly, despite the ability of these

Stem Cell and Cancer Biology Laboratory, The Francis Crick Institute, 1 Midland Road, London NW1 1AT, UK.
*These authors contributed equally to this work

‡Author for correspondence (Vivian.Li@crick.ac.uk)

A.B., 0000-0003-2041-0085; V.S.W.L., 0000-0003-2041-0085

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Glossary

+4 cells: slow-cycling stem cells located at +4 position directly above the Paneth cells as determined by counting cell nuclei from crypt bottom.
Absorptive progenitors: early progenitor precursors at positions +4/+5 that will differentiate to enterocytes.
Crypt base columnar cells (CBCs): actively cycling slender cells wedged between Paneth cells at the crypt base.
Intestinal stem cells (ISCs): cells residing at the proliferative crypt compartment of the intestinal epithelium that have high capacity to self-renew and give rise to all differentiated epithelial cells.
Label retaining cells (LRCs): radiation-resistant cells located between +2 and +7 position (on average at +4) that divide asymmetrically to retain DNA-labelling in the immortal strand of DNA.
Quiescent stem cells: slow-cycling or dormant population of stem cells located at +4/+5 position that can be re-activated in response to environmental challenges during homeostasis or upon injury.
Reserve stem cells: a reserve population of slow-cycling cells that are dedicated to replenish the loss of ISCs and support epithelial regeneration upon injury.
Secretory progenitors: early progenitor precursors at positions +4/+5 that will differentiate to the secretory lineages, including Paneth, goblet, enteroendocrine and tuft cells.

+4 cells to give rise to all intestinal cell lineages in these studies, the labelling efficiency of daughter cells in long-term lineage-tracing experiments using these +4 markers in tissue homeostasis is less robust when compared with Lgr5+ cells (Barker et al., 2007). Comprehensive expression analysis in later studies using single-molecule fluorescent *in situ* hybridization, transcriptomics and proteomics subsequently showed that, although these markers are enriched in +4 cells, they are likely also expressed in other cell positions in the crypt, including CBC cells (Itzkovitz et al., 2011; Schepers et al., 2011; Muñoz et al., 2012). This may explain the low lineage-tracing efficiency of these +4 cell markers in tissue homeostasis, as CBC and +4 cells have different proliferation states.

Early studies have suggested that most CBC cells are actively cycling, whereas LRCs are quiescent (see Glossary, Box 1) at the +4 cell position (Leblond and Stevens, 1948; Potten et al., 1978). Consistently, a study of telomerase activity in the intestine has demonstrated that Lgr5+ CBC stem cells have significantly higher telomerase activity than their progeny, apparently to avoid telomere shortening in these continuously cycling ISCs (Schepers et al., 2011). Analysis of S-phase and mitosis-specific markers at various time points showed that Lgr5+ ISCs divide on average every 21.5 h (Schepers et al., 2011). Contrary to this finding, it was later reported that ∼20% of Lgr5+ cells are quiescent, with a combined secretory and stem-cell signature, thus challenging the theory that all Lgr5+ cells are actively cycling (Buczacki et al., 2013). Another study subsequently showed that Lgr5-high ISCs are continuously cycling, whereas a fraction of Lgr5-low cells that locate predominantly to the +4 position are quiescent (Basak et al., 2014). This study suggested that the quiescent population in the +4 position resembles the previously reported LRCs (Buczacki et al., 2013) and Dll1+ secretory progenitors (van Es et al., 2012), supporting the notion that CBC cells are indeed actively cycling whereas +4 cells are quiescent. In line with this observation, Lgr5-high/Sox9-low ISCs have a higher proliferation rate than Lgr5-low/Sox9-high LRCs, which show quiescence and resilience to DNA damaging agents (Roche et al., 2015). Finally, expression of leucine-rich repeats and immunoglobulin-like domains 1 (Lrig1) has been found in both CBC and +4 ISCs (Powell et al., 2012; Wong et al., 2012). Powell et al. proposed that Lrig1 marks a distinct class of label-retaining and slow-cycling ISCs in the lower crypts (between +2 to +5 positions), which can proliferate and give rise to daughter cells in response to irradiation (Powell et al., 2012).

The regulation of ISC cell cycle exit at the +4 position and progenitor lineage specification could possibly be explained by the complex signals and epigenetic cues released by the surrounding niche cells in the crypt. For example, Paneth cells constitute the crucial niche at the crypt bottom to define ISC stemness by secreting essential niche signals such as EGF and Wnt for ISC maintenance and proliferation (Sato et al., 2011; Farin et al., 2012). Notably, circadian secretion of Wnt3 in Paneth cells is believed to regulate the timing of cell divisions in ISCs and rhythmic budding of crypts in organoids (Matsu-Ura et al., 2016). Conversely, ablation of Paneth cells has no effect on ISC maintenance *in vivo* (Durand et al., 2012; Kim et al., 2012), indicating that the surrounding subepithelial mesenchyme also plays an indispensable role in ISC maintenance. Understanding how signalling cues coordinate between epithelium and mesenchyme is important to advance our knowledge on ISC regulation and maintenance.

Signalling pathways involved in regulating ISCs

The stem cell-to-daughter cell transition in the intestinal epithelium is believed to be a highly dynamic and plastic process. Stem cell maintenance and the fine-tuning of differentiation into all different lineages are controlled by the surrounding microenvironment, including the cellular and extracellular matrix niche (Meran et al., 2017). On a molecular level, multiple signalling pathways together orchestrate cell-cell communication to achieve tissue homeostasis of the gut, and dysregulation of these cascades is commonly associated with intestinal diseases. Below, we highlight the role of several key signalling pathways orchestrated by the epithelium and mesenchyme to define ISC identity and fate (Fig. 1B).

Wnt signalling

The Wnt gradient from the crypt bottom to the crypt-villus junction is pivotal for the proliferation and maintenance of ISCs (Clevers, 2013; Clevers and Nusse, 2012; Farin et al., 2016). Wnt ligands that activate both the canonical and non-canonical pathway are secreted at the surrounding niche by both epithelial (Wnt3/6/9b) and stromal cells (Wnt2b/4/5a/5b) (Farin et al., 2012; Gregorieff et al., 2005; Kabiri et al., 2014). The epithelial origin of Wnt is the Paneth cells that secrete short-range Wnt3 locally to shape the epithelial Wnt gradient for ISC maintenance (Farin et al., 2016). In addition, the Paneth cell-mediated Wnt3a expression shows circadian oscillations that couple the circadian clock with the cell cycle by facilitating the transition of G1-S via cyclin D1 in mouse intestinal organoids (Matsu-Ura et al., 2016). On the other hand, Foxl1+ telocytes, as well as other Gli1+ and CD34+ subepithelial mesenchymal cells, have been identified as the main sources of stromal-derived Wnts (Degirmenci et al., 2018; Shoshkes-Carmel et al., 2018; Aoki et al., 2016; Stzepourginski et al., 2017; Valenta et al., 2016). Blocking Wnt ligands secretion or genetic ablation of these mesenchymal cells causes rapid cessation of ISC proliferation followed by crypt loss, highlighting the indispensable role of stromal-derived Wnts in ISC maintenance. These mesenchymal cells also secrete the Wnt agonists R-spondins (Rspos) that enhance Wnt activity in the crypt by binding to the Lgr family of receptors (de Lau et al., 2014; Yan et al., 2017b). Among the four members of the Rspo family, Rspo2 and Rspo3 are absolutely essential for Lgr5+ stem cell maintenance (Storm et al., 2016). Notably, Rspo3 is secreted by the PDGFRα+ pericryptal stromal cells, which constitute a crucial component of the stem cell niche (Greicius et al., 2018). A more recent study

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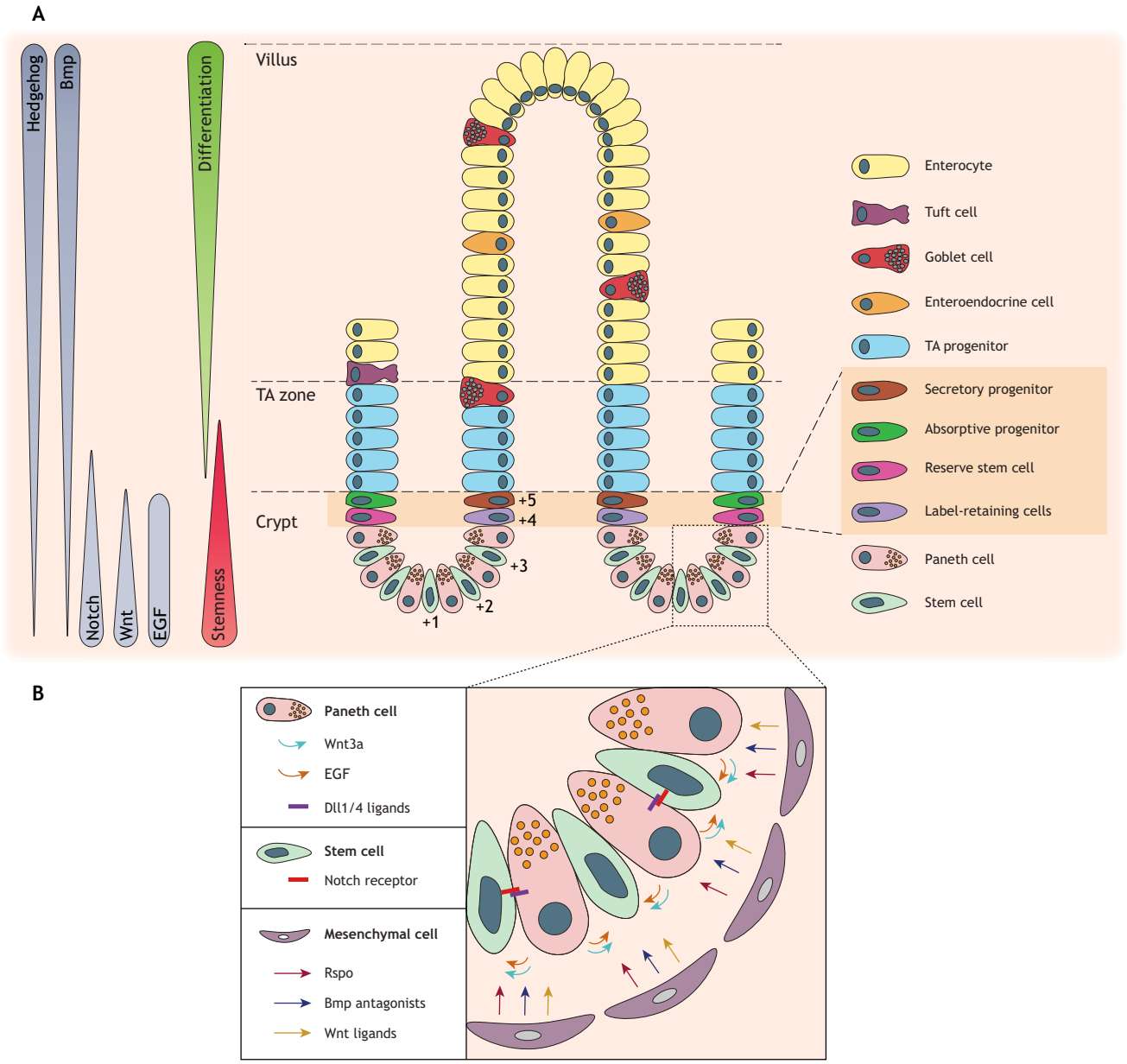

Fig. 1. The small intestinal epithelium. (A) Rapidly cycling intestinal stem cells reside at the bottom of the crypt (position 1-3), whereas all differentiated cells migrate towards the villus. Absorptive and secretory progenitors are positioned directly above the stem cells and are highly plastic. Paneth cells are the only differentiated cells at the crypt and they serve as a stem cell niche. Wnt, Notch, EGF, Bmp and Shh signalling pathways are collectively regulating stem cell fate. (B) Snapshot of the intestinal stem cell niche. Both epithelial Paneth cells and mesenchymal cells provide essential ligands, agonists and antagonists to drive ISC self-renewal.

suggested that Wnt plays a priming role by regulating Lgr expression in ISCs, whereas Rspo (not Wnt) regulates ISC self-renewal (Yan et al., 2017b).

EGF signalling

Epidermal growth factor (EGF) is another essential factor required for ISC proliferation. Similar to Wnt3, EGF ligand is also secreted by Paneth cells and is one of the key components that is required for establishing an *ex vivo* organoid culture (Sato et al., 2011, 2009). A recent study further showed that EGF signalling is an indispensable driver of Lgr5+ stem cell proliferation in mouse intestinal organoids (Basak et al., 2017). Inhibition of EGFR or its downstream mitogen-activated protein kinases (MAPK) signalling pathway is sufficient to induce Lgr5+ ISC quiescence while maintaining the Wnt transcriptional programme in the stem cells. Restoration of EGF signalling readily reverts the quiescent ISCs back into their active cycling state (Basak et al., 2017). These data suggest that Wnt defines ISC identity, whereas the combination of Wnt and EGF signalling is required for stem cell proliferation. Interestingly, replacement of EGF and p38 MAPK inhibitor with insulin-like growth factor 1 (IGF1) and fibroblast growth factor 2 (FGF2) in human intestinal organoid cultures promotes differentiation without compromising the proliferative state of the organoids (Fujii et al., 2018). These results highlight the important roles of various receptor tyrosine kinase ligands in ISC regulation.

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Notch signalling

Notch signalling is required for the maintenance of the ISC pool, whereas Notch inhibition leads to reduced Lgr5+ cell numbers and proliferation (Demitrack and Samuelson, 2016). Multiple studies have previously shown that Paneth cells release the Notch delta-like family of ligands (Dll1/4) to activate Notch signalling in the neighbouring ISCs and promote stem cell maintenance (Kim et al., 2014; Shroyer et al., 2007; Stamataki et al., 2011; van Es et al., 2005; Yang et al., 2001). Indeed, transgenic activation of *Notch1* increases ISC proliferation (Fre et al., 2005), whereas compound deletion of *Notch1* and *Notch2* induces ISC loss and impairs regeneration after radiation (Carulli et al., 2015). Likewise, deletion of either the Notch ligands (*Dll1* and *Dll4*) or the Notch effectors (*Hes1*, *Hes3* and *Hes5*) results in loss of crypt proliferation (Pellegrinet et al., 2011; Ueo et al., 2012), indicating that Notch signalling is required for ISC maintenance. In addition, Notch is the key driver of the progenitor cell fate decision that takes place at the +4/+5 position of the crypt by regulating the expression of the master transcriptional regulator *Atoh1* and lateral inhibition in the neighbouring progenitors (Philpott and Winton, 2014; Sancho et al., 2015). According to the proposed model, Notch inactivation in progenitor cells will upregulate *Atoh1* expression and drive differentiation towards the secretory lineage. This is followed by Atoh1-dependent *Dll1/4* expression in these progenitors (Kim et al., 2014; Lo et al., 2017), which leads to the activation of Notch in neighbouring progenitor cells and the subsequent differentiation towards the absorptive lineage. It is worth noting that Atoh1 is also required for regulating ISC proliferation in addition to its crucial role in cell fate decision. Suppression of ISC proliferation mediated by pharmacological inhibition of the Notch pathway is dependent on Atoh1 (van Es et al., 2010), whereas deletion of *Atoh1* is sufficient to restore the expression of Notch target gene *Hes1* and ISC proliferation in the recombining binding protein suppressor of hairless (Rbpj)-deficient intestine (Kim and Shivdasani, 2011). Together, the data highlight the indispensable role of Notch signalling in ISC maintenance.

Bmp signalling

Bone morphogenetic protein (Bmp) signalling plays a crucial role in intestinal epithelial differentiation in the post-mitotic villi by forming an opposing gradient to Wnt signalling along the crypt/villus axis. This is achieved by ligand secretion (such as Bmp4) from the mesenchyme and expression of several Bmp antagonists (such as noggin and Grem1/2) at the pericryptal stromal cells to repress Bmp-induced differentiation at the crypt region (He et al., 2004; Kosinski et al., 2007). Transgenic expression of noggin or Grem1 in the villi induces ectopic crypt formation, indicating that the inactivation of Bmp signalling is crucial and sufficient for defining ISC stemness (Davis et al., 2015; Haramis et al., 2004). In addition, it has also been reported that Bmp signalling represses ISCs via its downstream effector Smad4, which inhibits the expression of *Lgr5* and other core stem cell signature genes, and directly suppresses crypt proliferation (Qi et al., 2017). The opposing gradients of Bmp and Wnt together maintain intestinal homeostasis, in which Wnt promotes stemness at the crypt bottom and Bmp represses proliferation and drives differentiation at the villi. Notably, the Bmp inhibitor noggin and Wnt agonist Rspo are both essential for the establishment of intestinal organoid culture, highlighting the requirement of both Bmp inhibition and Wnt activation for maintaining stemness (Sato et al., 2009).

Hedgehog signalling

The two Hedgehog ligands sonic hedgehog (Shh) and Indian hedgehog (Ihh) are expressed in the intestinal epithelial cells, whereas the receptors patched 1 and 2 (Ptch1/2) are expressed in the mesenchymal cells. Binding of the Hedgehog ligands to Ptch1/2 releases the seven-span transmembrane protein smoothened (Smo), which in turn facilitates the activation of the glioblastoma family of transcription factors (Gli1/2/3) to drive expression of the Hedgehog target genes (Madison et al., 2005; Taipale et al., 2002). In adult intestine, the epithelium indirectly affects its own proliferative status through secretion of Hedgehog ligands, which regulate secretion of the Bmp ligands from the surrounding fibroblasts, mesenchymal and muscle cells (Kosinski et al., 2010). Reduced Hedgehog signalling induces crypt hyperproliferation and reduces differentiation (Madison et al., 2005; van den Brink et al., 2004). Interestingly, the Gli1+ subepithelial myofibroblasts have been reported to be an essential ISC niche by secreting Wnt ligands (Degirmenci et al., 2018; Valenta et al., 2016). Loss of epithelial Wnt secretion from Paneth cells induces Hedgehog signalling to increase the number of Gli1+ mesenchymal cells, which promote ISC maintenance (Degirmenci et al., 2018), indicating that Hedgehog signalling plays a unique role in ISC regulation via dynamic epithelial-mesenchymal interactions.

Epigenetic regulation of ISCs

Although signalling-mediated transcriptional regulation is undoubtedly crucial for defining stem cell identity and fate decisions, increasing evidence suggests that it is coordinated with cellular epigenetic states. Epigenetic events, such as histone acetylation or methylation, regulate chromatin conformational changes that reinforce or repress transcriptional activity at a given locus in response to intrinsic or extrinsic signals, which in turn orchestrate cellular processes such as proliferation, differentiation and reprogramming (Martinez-Redondo and Izpisua Belmonte, 2019). In particular, simultaneous loss of histone deacetylases Hdac1 and Hdac2 has been found to impair intestinal crypt proliferation and stem cell gene expression (Zimberlin et al., 2015). Interestingly, it has also been reported that Smad4 represses ISC gene expression via recruitment of Hdac1 to gene loci upon Bmp-induced differentiation (Qi et al., 2017), highlighting the important role of histone acetylation in ISC maintenance and differentiation. DNA methylation levels have also been shown to drive ISC differentiation, in this case by regulating the accessibility of gene enhancers, a process that is apparently guided by the microbiome postnatally (Yu et al., 2015). Deletion of DNA methyltransferase 1 (Dnmt1) leads to the expansion of stem cells in the crypt and a reduction in differentiation (Sheaffer et al., 2014). Notably, Tcf4, the core transcriptional regulator of Wnt signalling, binds in close proximity to hypo-methylated genomic sites during ISC differentiation, possibly by interacting with other additional factors that affect DNA methylation and the formation of differentially methylated regions (DMRs) (Kaaij et al., 2013). In human intestinal organoids, it was further shown that stable DNA methylation signatures define the regional identity of the intestinal epithelium by regulating mRNA expression, and this process is regulated by the DNA demethylating enzyme tet methylcytosine dioxygenase 1 (TET1) (Kraiczy et al., 2019). These methylation signatures can be maintained in organoid cultures derived from different regions of the intestine, indicating that ISC-intrinsic DNA methylation patterns establish and maintain regional gut specification. These data together highlight the essential role of epigenetic modulation in defining ISC identity.

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Indeed, recent studies using genome-wide epigenetic analysis further revealed changes of the chromatin landscape during ISC differentiation. Comparison of chromatin enhancer accessibility by assessing the levels of the repressive H3K4me2 and activating H3K27Ac epigenetic markers in Lgr5^{+} ISCs and early progenitor cells showed distinct epigenetic profiles in the two populations, but signatures were comparable between secretory and absorptive progenitors (Kim et al., 2014). This study suggested that early intestinal progenitors have a broadly permissive chromatin background to establish multipotency. Another study demonstrated that repression of ISC signatures and activation of enterocyte-specific genes strongly correlates with loss of the embryonic histone variant H2A.Z during ISC differentiation, supporting the notion that chromatin modification regulates ISC specification and differentiation (Kazakevych et al., 2017). In addition, distinct active chromatin enhancer signatures are selectively open in Lgr5+ ISCs and progenitor cells to control expression of stem cell- and lineage-specific genes, respectively, indicating that chromatin accessibility is highly dynamic (Jadhav et al., 2017).

Among the various chromatin modifiers, the polycomb repressive complexes (PRCs) play a pivotal role in embryonic development, stem cell maintenance and differentiation by maintaining repression of target genes via mono-ubiquitination of H2A or di- and tri-methylation of H3K27 (Aloia et al., 2013). In particular, global Prc1 activity is required to preserve ISC identity, whereas Prc2 is required for radiation-induced epithelial regeneration (Chiacchiera et al., 2016a,b). Intestinal-specific ablation of the core Prc2 component Eed further shows a marked increase in goblet cells and enteroendocrine cells and aberrant enterocyte differentiation, indicating that Prc2 activity is also required to restrict secretory lineage specification (Chiacchiera et al., 2016b; Jadhav et al., 2016).

In summary, the data generated from recent work indicate that epigenetic modifications, such as DNA methylation and histone acetylation and methylation, are actively involved in defining ISC identity and regulating lineage specification by controlling gene expression on a genome-wide scale. This is coordinated via a combination of transcriptional regulation and modifications of the chromatin landscape to achieve a precise gene expression signature and define cellular identity. Importantly, such epigenetic changes are highly dynamic: the histone marks of the ISC signature genes are rapidly changed during the transition from embryonic progenitors to adult ISCs and upon crypt regeneration to allow dynamic chromatin rearrangement and gene transcription (Jadhav et al., 2017; Kazakevych et al., 2017). This may explain the dynamic nature of the early progenitor populations for lineage selection and plasticity during regeneration.

Metabolism in ISCs

Although the role of signalling pathways and epigenetic modifications in stem cell maintenance have been studied extensively, little is known about how metabolism contributes to ISC homeostasis. One of the first studies providing evidence of non-cell autonomous metabolic control of ISCs showed that calorie restriction inhibits mTORC1 signalling in Paneth cells, but not in ISCs, which in turn promotes stem cell self-renewal and proliferation (Yilmaz et al., 2012). Short-term fasting can also intrinsically enhance ISC function by inducing the nuclear receptor peroxisome proliferator activated receptor (Ppar)-mediated fatty acid oxidation programme (Mihaylova et al., 2018). Conversely, high-fat diet (HFD)-induced obesity has also been shown to activate Ppar-δ signalling directly in ISCs to promote Lgr5+ ISC regenerative capacity and increase their numbers, thus enhancing their capacity to form tumours upon Apc loss (Beyaz et al., 2016). Ppar-δ has been previously shown to activate Wnt/β-catenin signalling in bone (Scholtysek et al., 2013), suggesting that HFD-induced Ppar-δ might amplify Wnt signalling in ISCs. Moreover, membrane phospholipid remodelling can also increase ISC proliferation by stimulating cholesterol biosynthesis, and excess cholesterol is sufficient to promote organoid growth and ISC proliferation (Wang et al., 2018). In addition, a ketogenic diet enhances ISC function through the ketone body beta-hydroxybutyrate-mediated Hdac inhibition and Notch activation, whereas a glucose-supplemented diet supresses ketogenesis and has the opposite effects on ISC stemness (Cheng et al., 2019). Another nutrient-sensing nuclear receptor and master regulator of bile acid (BA) homeostasis, farnesoid X receptor (Fxr; Nr1h4), has also been recently linked to ISC. The combination of dietary factors (e.g. HFD) with dysregulation of Wnt signalling increases BA production, which in turn antagonises the function of intestinal Fxr in Lgr5+ ISCs, leading to increased ISC proliferation and tumour progression (Fu et al., 2019). Indeed, Fxr deficiency has previously been reported to promote intestinal cell proliferation and tumour development (Maran et al., 2009), implicating the nutrient cue sensor Fxr in coordinating ISC homeostasis.

Extensive studies in haematopoietic stem cells or embryonic stem cells suggest that stem cells have different metabolic requirements from their differentiated progeny (Ochocki and Simon, 2013). Metabolomic analysis of adult intestinal Lgr5+ ISCs and Paneth cells revealed increased glycolysis in Paneth cells and increased mitochondrial oxidation in ISCs (Rodriguez-Colman et al., 2017). The study provided evidence that Paneth cells constitute a metabolic niche to produce lactate, which is converted into pyruvate in ISCs and fuels mitochondrial oxidative phosphorylation (OXPHOS) during homeostasis. In addition to Paneth cells, lactate can also be produced by the microbiota to promote ISC proliferation (Lee et al., 2018). The mitochondrial pyruvate carrier (Mpc) finely balances the import of the cytoplasmic pyruvate generated from glycolysis to mitochondria for OXPHOS. Inhibition or genetic deletion of Mpc in Lgr5+ ISCs increases crypt proliferation and expands the stem cell compartment in both mouse intestine and isolated organoids, indicating that restricting mitochondrial pyruvate metabolism is essential and sufficient to maintain ISC proliferation (Schell et al., 2017). Interestingly, Mpc expression is low in ISCs and high in differentiated cells, suggesting an increased demand of macromolecule biosynthesis to ATP production during the differentiation process. Reactive oxygen species (ROS) production during mitochondrial OXPHOS could lead to oxidative stress and cellular dysfunction. Interestingly, a recent study reported that mice lacking autophagy protein 5 (Atg5), an E3 ubiquitin ligase essential for autophagosome elongation, show deficient autophagy, accumulation of dysfunctional mitochondria and increased ROS levels in ISCs, leading to impaired irradiation-induced intestinal regeneration (Asano et al., 2017). Antioxidant treatment in these autophagy-deficient animals is able to restore their regeneration capacity, suggesting that intrinsic autophagy is important for ISC maintenance by preventing ROS accumulation. Overall, a fine balance of pyruvate metabolism and OXPHOS is important for ISC homeostasis, not only to maintain ISC proliferation, but also to control oxidative stress by modulating the ISC metabolic programme. Further studies will be needed to investigate the potential metabolic flexibility of ISCs and their progenitors and its role in cell fate regulation.

The traditional hallmarks of stem cells include multipotency and the ability to self-renew. By integrating new insights from recent

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ISC studies, we propose to add four additional hallmarks to the list – mitotic cell cycle (active cycling versus slow-cycling/quiescent ISCs), signalling crosstalk (high Wnt and Notch, low Bmp), epigenetic landscape (increased chromatin accessibility in ISC signature genes) and metabolic state (high mitochondrial respiration and dietary-adaptation capacity) (Fig. 2). These six hallmarks together constitute the functional and molecular identity of ISCs. Importantly, crosstalk between these characteristics drives ISC stemness and fate. For example, signalling pathways directly control proliferation and metabolic state via gene regulatory networks, whereas the epigenetic/chromatin landscape can shape both transcriptional and metabolic programmes.

ISCs in development

During development, the endoderm layer will give rise to the adult gastrointestinal (GI) tract (de Santa Barbara et al., 2003). This is a very complex process that involves turning what is a single tube in early development into a highly-organized adult tissue, which contains specialized cells for barrier function, food digestion and absorption. Fetal development of the intestinal epithelium is a multi-step process. In mice, the primitive gut tube is first formed between embryonic day (E) 1.0 and E9.5 and consists of three distinct sections – foregut, midgut and hindgut (Guiu and Jensen, 2015). The small intestine arises mainly from the midgut. From E9.5-E14.5, the tube elongates and increases in size, forming a pseudostratified epithelium full of highly proliferative cells. By E16.5, non-proliferative villus and proliferative intervillus regions are clearly formed, and these developmentally premature regions subsequently give rise to crypts of Lieberkühn and all differentiated adult epithelial cells. Maturation of the gut is completed by postnatal day (P) 15 (Guiu and Jensen, 2015; Wells and Spence, 2014). Unlike in the adult intestine, little is known about how ISC identity is established during these developmental milestones. Recently, it has been shown that Lgr5+ progenitors first emerge at E13.5, with stronger expression at the posterior end of the premature intestine, indicating that developing progenitors acquire Lgr5 expression during embryogenesis (Nigmatullina et al., 2017). The Lgr5+ cells gradually become more localised to intervillus regions at E16.5 and to emerging crypts at P5, and are eventually confined to CBC cells, which are intermingled between Paneth cells at the crypt base by P21 (Kim et al., 2012) (Fig. 3).

These developmental milestones and ISC specification are tightly regulated by signalling pathways and tissue-specific transcriptional control. For example, it is well characterised that the gut-specific transcription factor caudal type homeobox 2 (Cdx2) is required for the normal development of the primitive gut, as loss of Cdx2 in early fetal stages results in defective villus morphogenesis (Gao et al., 2009). Cdx2 has been shown to have distinct chromatin binding sites and target genes in the embryonic versus the adult intestine (Kumar et al., 2019). It is believed that transcription factors, including Cdx2, interact with the chromatin as its accessibility shifts in order to define intestinal identity during development (Banerjee et al., 2018). Moreover, signalling pathways involved in adult intestinal homeostasis, such as Wnt and Notch, also play a crucial role during development. Lgr4/5-Rspo-mediated Wnt activation, for example, has been reported to be important during early endoderm differentiation (Tsai et al., 2016). Deletion of mesenchymally secreted Wnt5a leads to a failure of single tube formation, highlighting its crucial role in endoderm elongation and mid-gut formation (Cervantes et al., 2009). Wnt signalling is also required for intestinal proliferation and villus morphogenesis at E15.5 (Chin et al., 2016). Wnt activation at these early stages has been shown to be tightly controlled by the transcriptional repressor Id2, which suppresses proliferation of the early Lgr5+ progenitors by direct inhibition of Wnt/β-catenin signalling (Nigmatullina et al., 2017). Notch signalling is also important during intestinal development. Deletion of the transcription factors Gata4/6 has been shown to disrupt intestinal epithelial architecture and cell proliferation and differentiation in the developing gut through reduced Notch signalling (Walker et al., 2014). This is caused by

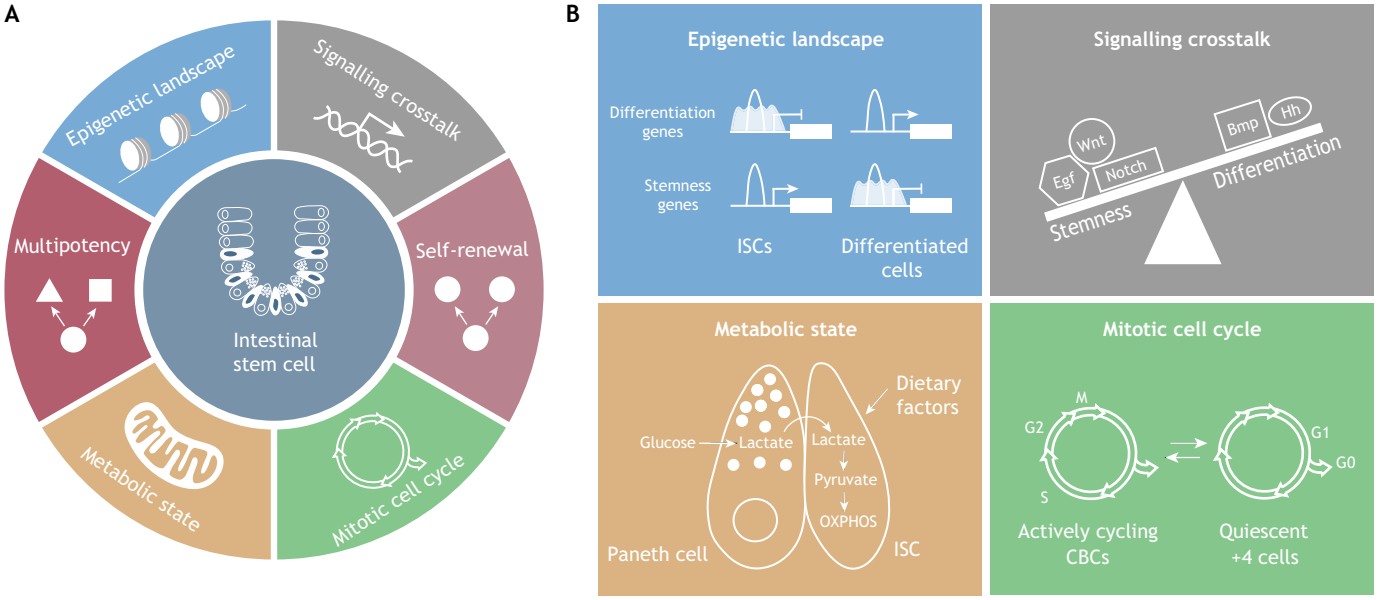

Fig. 2. Hallmarks of intestinal stem cells. (A) Schematic of six functional and molecular hallmarks of ISC. (B) ISCs not only have the capability to self-renew and give rise to all differentiated cell types, but they also have a unique epigenetic landscape (increased chromatin accessibility in ISC signature genes), signalling crosstalk (high Wnt and Notch, low Bmp), metabolic state (high mitochondrial respiration and dietary-adaptation capacity) and mitotic cell cycle (active cycling ISCs versus slow-cycling/quiescent stem cells) that define their identity.

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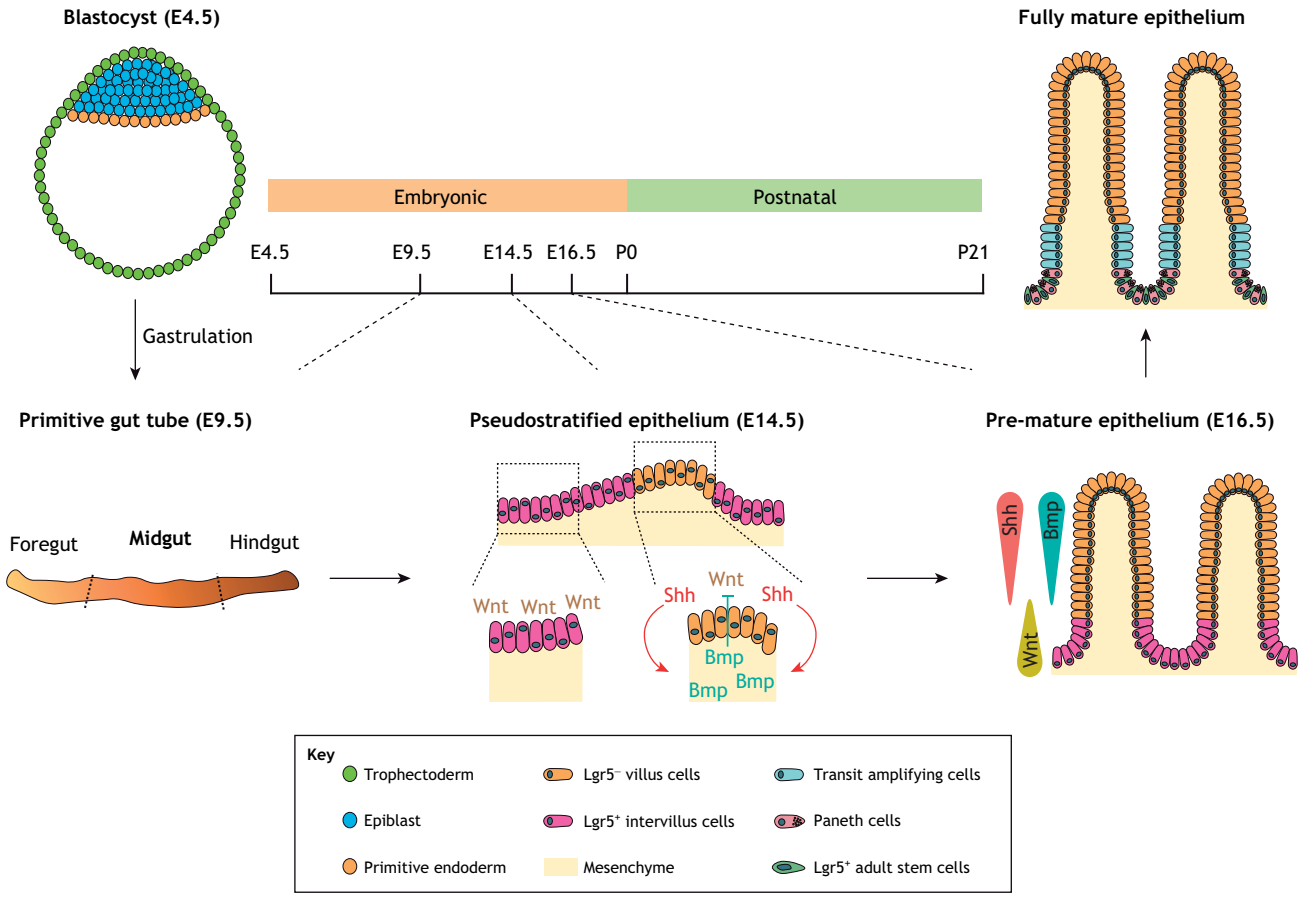

Fig. 3. Intestinal stem cells during mouse development. The late blastocyst undergoes gastrulation and forms the primitive gut tube by E9.5. The small intestine originates from the midgut of the tube and a pre-mature pseudostratified epithelium is formed by E14.5. Epithelial Shh expression during villus formation activates the secretion of Bmp ligands from stromal cells, which in turn directly supress Wnt and restrict stemness at the intervillus regions (Shyer et al., 2015). Postnatal intestinal crypts arise as Paneth cells mature. These crypts are characterised by adult Lgr5+ intestinal stem cells that intermingle between Paneth cells at the crypt bottom to fuel the continuous renewal of the intestinal epithelium.

Gata4-mediated Dll1 expression via direct transcriptional control, which in turn controls and fine-tunes Notch activity during development. In addition, epithelial Shh signal has been shown to induce mesenchymal Bmp expression in the emerging villi, where the Hedgehog-Bmp axis directly suppresses Wnt activity at the villi and restricts the presence of Lgr5+ cells in the proliferative fetal intervillus region at E15.5 (Shyer et al., 2015).

Despite the substantial effort towards understanding the embryonic intestine, it remains unclear how and when ISC identity is defined during development. Lgr5+ ISCs in the fetal and adult intestine appear to have distinct chromatin and gene expression signatures (Kazakevych et al., 2017), suggesting that ISC identity may be defined after birth. A recent study further suggested that all cells of the fetal mouse intestinal epithelium (at E16.5), regardless of their position and Lgr5 expression, contribute to the adult ISC pool (Guiu et al., 2019). Using an unbiased lineage-tracing approach, the study showed that both Lgr5+ and Lgr5− cells in the fetal intestine are equipotent in contributing to the formation of the adult ISCs. This is caused by the continuous remodelling and fission events of the villus during fetal development, which bring the post-mitotic Lgr5− cells in the villus back to the proliferative intervillus region to regain Lgr5+ identity (Guiu et al., 2019). These results suggest that the fetal intestinal epithelium is highly plastic, and stem cell identity is induced by the surrounding niche rather than being defined by an intrinsic genetic programme.

Plasticity and regeneration of ISCs upon injury

Apart from the developing gut, epithelial plasticity in the adult intestine has also been reported by multiple studies of injury-induced regeneration and involves dedifferentiation of committed/differentiated cells, which consequently regain a more immature or progenitor-like cell fate (de Sousa e Melo and de Sauvage, 2019). One common observation across these studies is that the reported plasticity event often occurs in the early progenitor cells or reserve stem cells (see Glossary, Box 1) located at +4/5 cell position (summarised in Table 1). Cells expressing Tert, located predominantly at +5/6 positions, are slow-cycling and contribute to the regenerative response upon irradiation (Montgomery et al., 2011). Similarly, Bmi1-expressing cells (at +4/5 position) can give rise to Lgr5+ cells for regeneration after genetic ablation of Lgr5+ ISCs through administration of diphtheria toxin to mice in which Lgr5 had been replaced with the diphtheria toxin receptor (Lgr5-DTR-EGFP) (Tian et al., 2011). In fact, such capacity for plasticity is not unique to Tert- or Bmi1-expressing cells. Multiple studies have subsequently reported similar findings on injury-induced regeneration using different markers (see Table 1 for details). These include Hopx-expressing cells at the +4 position (co-expressing Tert and Bmi1) (Takeda et al., 2011), Dll+ secretory progenitors at the +5 position (co-expressing Bmi1) (van Es et al., 2012), LRCs via H2B nuclear labelling (secretory precursors at the +3 position co-expressing Lgr5) (Buczacki et al., 2013), radioresistant Krt19+

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Table 1. Summary of intestinal cell types involved in injury-induced plasticity

Cell type	Cell marker	Experimental model	Injury model	References	Mechanistic insights
Secretory progenitors	Dll1	Lineage tracing	IR	van Es et al., 2012	Dll1+ secretory progenitors revert to stem cells.
	Cd69+ Cd274+	FACS/ Transcriptomics	DTR, IR	Jadhav et al., 2017	Goblet cell precursors with a unique epigenetic signature regenerate Lgr5+cells.
	P-Atoh1	Lineage tracing	DSS, DTR, IR	Tomic et al., 2018	Post-translational phosphorylation of Atoh1 is required for the secretory progenitors to regain stemness.
	NeuroD1 Tph1	Lineage tracing	IR	Sei et al., 2018	A subset of NeuroD1- and Tph1-expressing enteroendocrine cells revert to stem cells.
	Atoh1	Lineage tracing	DSS, DTR	Castillo-Azofeifa et al., 2019	Secretory but not absorptive progenitors regain stemness.
Paneth cells	Lyz1	Lineage tracing	IR	Yu et al., 2018	Notch activation through nuclear Yap and P-Stat3.
	Lyz	Lineage tracing	DSS	Schmitt et al., 2018	Activation of SCF/cKit/Wnt signalling.
	Defa4	Lineage tracing	DXR	Jones et al., 2019	Adam10-dependent Notch activation is required for Defa4-expressing Paneth cells to dedifferentiate after injury.
Enterocytes	Alpi	Lineage tracing	DTR	Tetteh et al., 2016	Fth1 overexpression to mitigate oxidative stress.
Reserve or quiescent stem cell populations at +4/5 position	Bmi1	Lineage tracing	DTR	Tian et al., 2011	Bmi1-expressing cells give rise to Lgr5-expressing cells.
	Tert	Lineage tracing	IR	Montgomery et al., 2011	Tert marks a slow cycling population of stem cells resistant to irradiation.
	Hopx	Lineage tracing	IR	Takeda et al., 2011	Hopx-expressing slow cycling cells can interconvert to a more rapidly cycling stem cell population.
	LRCs-H2B	Lineage tracing	IR, Drugs	Buczacki et al., 2013	LRCs are slow cycling cells expressing Lgr5 and possessing regenerative capacity.
	Krt19	Lineage tracing	DTR, IR	Asfaha et al., 2015	Krt19+ Lgr5− mark colonic and intestinal progenitors that render Lgr5+ ISCs dispensable.
	Prox1	FACS/ Transcriptomics	IR	Yan et al., 2017a	Prox1+ enteroendocrine lineage facilitates epithelial regeneration.
	Clu	Lineage tracing/ Transcriptomics	DSS, DTR, IR	Ayyaz et al., 2019	Damage-induced quiescent stem cells arise following Yap1-dependent signalling and reconstitute Lgr5+ ISCs.
Epithelium with induced fetal-like state	Sca1+ Lgr5−	FACS/ Transcriptomics	INF, DTR, IR	Nusse et al., 2018	Injury-induced developmental programme via Ifn-γ-mediated Sca1 overexpression.
	Sca1	FACS/ Transcriptomics	DSS	Yui et al., 2018	Extracellular matrix-mediated activation of fetal signature through Sca1/Yap/Taz.

DSS, dextran sulphate sodium; DTR, diphtheria toxin receptor; DXR, doxorubicin; FACS, fluorescence-activated cell sorting; INF, infection; IR, irradiation.

Lgr5− cells at the +4 position (co-expressing Bmi1) (Asfaha et al., 2015), Alpi+ enterocytes from the +6 position (co-expressing Hopx) (Tetteh et al., 2016), Prox1+ cells at the +4 position (co-expressing Bmi1 and NeuroD1) (Yan et al., 2017a), NeuroD1-and Tph1-expressing enteroendocrine cells at the +4 position (co-expressing Hopx) (Sei et al., 2018), Atoh1+ secretory progenitors at the +4 position (co-expressing Krt19) (Castillo-Azofeifa et al., 2019) and clusterin-expressing cells at the +4 position (Ayyaz et al., 2019). Such a remarkable number of findings in a similar context with diverging and overlapping markers supports the notion that stemness in the intestine is not hardwired, where +4/5 progenitor cells (regardless of the markers they express) are highly plastic to regain ISC identity in response to injury. In addition to +4/5 progenitors, recent studies have further suggested Paneth cell plasticity upon intestinal damage. Lineage tracing and transcriptomic analyses showed that lysozyme (Lyz1)-expressing Paneth cells gain a stem cell-like signature and acquire multipotency upon irradiation-induced Notch activation (Yu et al., 2018). Consistently, defensin-α4 (Defa4)-expressing Paneth cells can also dedifferentiate into multipotent stem cells following acute doxorubicin (DXR)-induced intestinal injury via Notch activation (Jones et al., 2019). Another study also demonstrated that Paneth cells re-enter the cell cycle and dedifferentiate in response to dextran sulphate sodium (DSS)-induced inflammation, which is mediated by Wnt activation (Schmitt et al., 2018). These findings indicate that the plasticity of the intestinal epithelium is not limited to early progenitor cells but extends to the post-mitotic and fully differentiated Paneth cells.

As discussed above, distinct active enhancer signatures have been reported in Lgr5+ ISCs and +4 cells (Jadhav et al., 2017). This study further showed that specialized Cd69+ Cd274+ goblet cell precursors dedifferentiate to stem cells by reorganising their chromatin accessibility signature to resemble Lgr5+ ISCs after irradiation or genetic ablation of Lgr5+ ISCs, highlighting the dynamic property of the epigenetic landscape in both intestinal homeostasis and regeneration. In addition, a more recent study demonstrated that Atoh1+ progenitors are further regulated by post-translational modifications (Tomic et al., 2018). The study showed that Atoh1 can be phosphorylated at nine serine-proline or threonine-proline sites by cyclin-dependent kinases; a phospho-null mutant of Atoh1 promotes secretory differentiation and reduces clonogenic capacity of Atoh1+ cells in response to DSS-induced colitis. The data suggest that plasticity can be modulated by post-translational modifications to facilitate the conversion of secretory progenitors to ISCs. It will be worth comparing the chromatin accessibility in Atoh1 wild-type and phospho-mutant cells to examine whether the chromatin landscape can potentially be remodelled by post-translational modification.

Interestingly, recent studies have proposed an alternative regeneration model upon loss of tissue integrity. Infection by the intestinal roundworm *Heligmosomoides polygyrus* disrupts the intestinal mucosal barrier, which then activates an interferon-gamma (Ifn-γ) transcriptional programme and induces an Lgr5− programme in the affected crypts (Nusse et al., 2018). Further characterisation revealed that those affected crypts acquire a fetal-like transcription programme, marked by high Sca1 (Atxn1) expression (a cell surface marker expressed in proliferative cells and the fetal intestine) (Yui et al., 2018), to regenerate and restore

tissue integrity. Similarly, the colonic epithelium undergoes reprogramming to acquire a more primitive fetal-like state (Sca1+ Lgr5−) in the DSS-induced colitis model (Yui et al., 2018). This study further showed that activation of yes-associated protein/tafazzin (Yap/Taz) by extracellular matrix remodelling is required to induce reprogramming and regeneration. A more recent study, using single cell transcriptomic analysis, has also highlighted the reprogramming capacity of ileal enterocytes toward a proximal identity after a 50% proximal small bowel resection (SBR) (Seiler et al., 2019). Further characterisation of this model will be needed to elucidate whether such adaptation capacity of enterocytes is caused by reprogramming of the ISCs, which give rise to proximal enterocytes instead of distal enterocytes following SBR. Collectively, these findings suggest that re-initiation of the developmental programme represents a fundamental repair mechanism after epithelial integrity is compromised or in response to SBR. It is currently unclear how the intestinal epithelium chooses different regeneration models, which include dedifferentiation of +4/5 progenitors or differentiated cells to adult Lgr5+ ISCs, or transient reprogramming of the epithelium into a fetal-like state, upon injury. Conceivably, the adult intestinal epithelium acquires different repair mechanisms in response to different injuries: stem cell-specific ablation will signal to the neighbouring healthy progenitors (or differentiated cells) to induce dedifferentiation, whereas catastrophic loss of intestinal epithelium will induce transient reprogramming. Additional studies will be needed to better understand the causal factors driving these repair mechanisms.

Conclusions

Recent advances in genetic tools and technologies enable us to delineate the molecular profile of the intestinal epithelium at a single cell level, which offers new insights in defining ISC identity. In addition to the textbook definition of stem cells as multipotent with an ability to self-renew, ISCs have also been shown to actively proliferate and have distinct signalling activities, as well as epigenetic and metabolic profiles. All these six properties together contribute to the updated hallmarks of ISCs. Importantly, these hallmarks are closely related to one another: signalling activities alter the proliferation state and drive epigenetic and metabolic changes, which in turn regulate multipotency and self-renewal capacity. Evidence collected from early developmental events and injury-induced regeneration in adult tissues further suggests that these ISC hallmarks are dynamic rather than hardwired, although the cause of cellular plasticity remains unclear. Moving forward, it will be important to further characterise the environmental cues contributing to plasticity in the context of signalling pathways, chromatin and metabolic profiles. The ISC niche is key to tissue homeostasis. Thus, it will be worth exploring how the stem cell niche defines ISC identity and, possibly, drives plasticity upon injury.

Acknowledgements

This Review is a snapshot of the current state of intestinal stem cell biology during development, homeostasis and regeneration and we apologize to the many colleagues whose work could not be cited here due to space limitations.

Competing interests

The authors declare no competing or financial interests.

Funding

The authors' research is supported by the European Union's Horizon 2020 research and innovation programme (668,294) and the Francis Crick Institute, which receives its core funding from Cancer Research UK (FC001105), the UK Medical Research Council (FC001105) and the Wellcome Trust (FC001105).

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