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REVIEW

Regulation of self-renewal and differentiation by the intestinal stem cell niche

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Abstract The gastrointestinal epithelium is a highly organised tissue that is constantly being renewed. In order to maintain homeostasis, the balance between intestinal stem cell (ISC) self-renewal and differentiation must be carefully regulated. In this review, we describe how the intestinal stem cell niche provides a unique environment to regulate self-renewal and differentiation of ISCs. It has traditionally been believed that the mesenchymal myofibroblasts play an important role in the crosstalk between ISCs and the niche. However, recent evidence in *Drosophila* and in vertebrates suggests that epithelial cells also contribute to the niche. We discuss the multiple signalling pathways that are utilised to regulate stemness within the niche, including members of the Wnt, BMP and Hedgehog pathways, and how aberrations in these signals lead to disruption of the normal crypt–villus axis. Finally, we also discuss how CDX1 and inhibition of the Notch pathway are important in specifying enterocyte and goblet cell differentiation respectively.

Keywords Intestinal · Stem cell · Niche · Differentiation · Wnt

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Introduction

The small and large intestine comprise the lower gastrointestinal tract. Both organs have a high cellular turnover and contain rapidly proliferating cells, which replace those that are shed into the lumen. The structure of the normal intestinal epithelium is highly organised and regulated to maintain tissue homeostasis. The small intestine luminal surface consists of simple columnar epithelium that is renewed by a small number of intestinal stem cells (ISCs) located in the crypts, formed from straight tubular invaginations of the epithelium. The ISCs give rise to a pool of multipotent progenitor cells (also known as transient-amplifying cells) that are highly proliferative and differentiate into one of four lineages: absorptive enterocytes, mucin-producing goblet cells, hormone-secreting enteroendocrine cells and Paneth cells [1, 2]. Whereas enterocytes, goblet and enteroendocrine cells migrate towards the lumen of the gut, Paneth cells move towards the bottom of the crypt. Paneth cells secrete alpha-defensins, lysozyme and phospholipase A2, and play a role in host defence against microorganisms [3, 4]. The large intestinal epithelium also contains ISCs located within the crypts, but lacks villi. In the colon, multipotent cells give rise to enterocytes, goblet cells and enteroendocrine cells [5], but not Paneth cells, except in the most proximal colon.

Identification of intestinal stem cells

Evidence that human colonic crypts are derived monoclonally is based on the finding that, in an XO/XY mosaic patient with FAP, individual colonic crypts were composed entirely of either XO or XY cells, not a combination of the two [6]. Further support came from the analysis of

Sardinian females heterozygous for the G6PD mutation [7]. Although crypts are clonal, this does not necessarily mean that crypts contain a single stem cell. The number of stem cells present within a crypt is heavily debated, and it has been suggested that as many as 4–16 actual stem cells and 30–40 potential stem cells may be present in the small intestinal crypt [8]. In contrast, there may be only three to four stem cells in the colonic crypt.

Initial labelling experiments by Cheng and Leblond in 1974 using ^{3}H-thymidine demonstrated that the four differentiated lineages of the adult murine intestinal epithelium originated from cells located between and immediately above the Paneth cells, termed crypt-base columnar (CBC) cells [9]. However, the position of these ISCs has been heavily debated for the last 40 years and other studies using DNA-label-retaining assays have concluded that they were positioned four to six cell diameters from the crypt base. This was based on the observation that these ISCs retained the template strand label during segregation of the DNA strands, thus providing a protective mechanism for ISCs by preventing replication errors from accumulating [10].

The +4 position stem cell model was further supported when the +4 cells were found to be sensitive to radiation, which was considered to be a protective mechanism to prevent stem cells from passing on damaged DNA to their progeny [11]. However, these studies have not shown that the +4 cells are capable of forming all the differentiated lineages in the intestinal crypt.

Further characterisation and functional analysis of these putative ISCs have been hampered by the lack of robust intestinal stem cell markers. Recently, the leucine-rich G protein-coupled receptor 5 (Lgr5) has been identified from a panel of intestinal Wnt target genes for its restricted crypt expression as a potential ISC marker. Also known as Gpr49, it has been elegantly demonstrated using knock-in mice and lineage-tracing to be exclusively found in cycling CBCs of murine small intestine and colon, and these cells are able to generate all epithelial lineages [12]. Lgr5+ cells are retained for at least 1 year and are able to self-renew to regenerate the epithelium. Consistent with their long-term ability to self-renew, Lgr5+ cells were found to have high telomerase activity, which was decreased in undifferentiated progeny and absent in differentiated cells [13]. Fate mapping of Lgr5+ cells demonstrated that most of them divided symmetrically, and that crypt homeostasis was maintained by neutral competition between the two daughter cells [14]. Lgr5 may be a pan-intestinal stem cell marker, as Lgr5+ gastric stem cells are also able to drive self-renewal in the stomach and build long-term organoids resembling mature pyloric epithelium in vitro [15]. Furthermore, Lgr5+ stem cells in the crypt could act as the origin of intestinal cancer as targeted deletion of APC in these cells in a murine model leads to the development of adenomas within 3–5 weeks [16].

Another potential marker is Bmi1, a member of the Polycomb group gene family, which was originally shown to regulate the self-renewal and proliferation of normal and leukaemic stem cells [17]. Sangiorgi and Capecchi used lineage-tracing experiments to show that Bmi1+ cells were located above the Paneth cells at the +4 position in the murine small intestine [18]. As with Lgr5, Bmi1+ cells are able to self-renew and survive for 12 months, and can differentiate into all of the lineages found within the small intestine. Bmi1+ cells were found in 10% of crypts throughout the first 10 cm of the murine small intestine, but this percentage decreased in a proximal–distal gradient, such that no Bmi1+ cells were detected in the ileum.

It is not clear whether Lgr5+ and Bmi1+ cells represent two distinct subpopulations, or overlap spatially as well as functionally as ISCs. Lgr5+ cells are potentially more proliferative than Bmi1+ cells, as there was more rapid expansion of Lgr5 lineage-traced cells in the crypt compared with Bmi1 [18]. Lgr5 is expressed in the small and large intestine, whereas Bmi1 expression is restricted to the proximal small intestine. There is increasing evidence that both actively cycling and quiescent stem cell subpopulations exist in tissues, including the haematopoietic system [19] and hair follicles [20, 21], where Lgr5+ cells are actively cycling. A similar arrangement may occur in the gastrointestinal tract, whereby Lgr5+ and Bmi1+ cells could represent actively cycling and quiescent ISCs, respectively, and the latter may be mobilised if the former is injured or destroyed.

Further analysis of Lgr5+ and Bmi1+ cells could yield the discovery of more ISC markers. For example, comparing Lgr5+ stem cells with their immediate daughter cells identified *Achaete scute-like 2 (Ascl2)* and *Olfactomedin-4 (Olfm4)* as specific ISC markers, coinciding with Lgr5 expression, marking CBCs located between Paneth cells [22, 23]. Another potential ISC marker is Musashi-1 (Msi-1), an RNA-binding protein first identified in early asymmetric cell division in sensory organ precursor *Drosophila* cells [24]. Msi-1 is expressed in a small number of cells at the +4/+5 position in the adult small intestine and in a few cells at the crypt base in the large intestine [25]. It is also expressed strongly in developing crypts, in regenerating crypts after radiation, and in early adenomas [25]. Similarly, Doublecortin and Ca Kinase-like 1 (DCAMKL-1) has been proposed as a putative ISC marker [26], as it is expressed in cells near the +4 position in intestinal crypts, and these cells were also Msi-1 positive [25]. Furthermore, DCAMKL-1 + cells have been shown to self-renew and form spheroids in culture [27]. However, in contrast to these findings, Gerbe et al. [28, 29] demonstrated that DCAMKL-1 is a marker of terminally differentiated and

rare tuft cells, a fifth type of differentiated intestinal cell, distinct from enterocytes, Paneth, goblet and enteroendocrince cells, and questioned the use of DCAMKL-1 as an intestinal stem cell marker.

ALDH1 (aldehyde dehydrogenase 1) has been used as a marker of normal and malignant mammary stem cells, and predicts poor clinical outcome [30]. Huang et al. [31] showed that ALDH1+ cells were limited to the normal crypt bottom, and during progression from normal epithelium to adenoma, ALDH1+ cells increased in number and were distributed higher up the crypt. Furthermore, as few as 25 ALDH1+ colorectal cancer cells were able to initiate tumours in NOD/SCID mice. Cell surface markers such as CD166 and CD24 have also been proposed as ISC markers [32, 33]. However, apart from Lgr5 and Bmi1, none of the above putative ISCs markers have yet been used in lineage tracing studies to functionally determine the ability of labelled cells to both self-renew and differentiate.

Attempts to characterise intestinal stem cells based on their maintenance of chromosomal telomeres and resistance of cellular senescence have been made. Breault et al. generated a mouse telomerase reverse transcriptase (mTert)-GFP-transgenic model and demonstrated that single GFP+ cells were located towards the lower part of the intestinal crypt [34]. However, out of 15,700 crypts analysed, only 1 in 157 contained single GFP+ cells and 1 in 25 contained single long-term BrdU+ cells, so mTert only labelled a small proportion of ISCs. Although the authors did not show lineage differentiation, a more recent study from the same group demonstrated that mTert+ cells could form all lineages in the intestine, and were able to respond to injury [35].

It has been difficult to culture and therefore study the behaviour of human intestinal stem cells in vitro. However, over the last few years, considerable advances have been made in culturing murine intestinal stem cells. Sato et al. [36] demonstrated that a single Lgr5+ cell from murine intestine could form crypt–villus structures in vitro without a physical mesenchymal niche for long-term culture, but required supplementation with specific growth factors, including the Wnt agonist R-spondin1, EGF, Jagged and Noggin, suggesting that similar signals may be secreted by the stem cell niche to maintain ISCs in the normal crypt. Of note, the organoids produced by Sato et al. contained multiple lumens formed at the periphery, resembling the crypt, and Lgr5+ cells were found at the extremities of these lumen-like structures, replicating the crypt base. In another study, Ootani et al. [37] were able to sustain the proliferation and multi-lineage differentiation of murine intestinal epithelium for greater than 350 days in vitro by incorporating an air–liquid interface on a collagen framework. These spheroids contained endogenous Wnt and Notch activity, which was sufficient to support vigorous growth, and recapitulated the function of the in vivo intestinal stem cell niche. Proliferation of these spheroids was enhanced by the addition of a fusion protein between the Wnt agonist R-spondin-1 and immunoglobulin Fc (RSpo1-Fc). Conversely, addition of the Wnt antagonist Dickkopf-1 (Dkk1) inhibited spheroid growth [37, 38]. These spheroids were able to form different intestinal lineages, as inhibition of Notch pathway by the gamma secretase inhibitor dibenzazepine (DBZ) and overexpression of Neurogenin-3 (Ngn3) resulted in the development of goblet cells and enteroendocrine cells, respectively. Recently, Spence et al. [39] generated human intestinal tissue in vitro from induced pluripotent cells that contained Lgr5+ cells and was capable of multi-lineage differentiation. These advances in stem cell culture will no doubt help improve our understanding of how intestinal stem cells regulate self-renewal and differentiation.

The stem cell niche

The concept of a stem cell niche was first described by Schofield in 1978, who hypothesised that the microenvironment surrounding haematopoietic stem cells (HSCs) conferred the stem-like phenotype on HSCs [40]. It is believed that the components of the niche interact with stem cells and provide critical cues that regulate and maintain stemness.

The intestinal stem cell niche represents the specialised region of the crypt which provides all the environmental cues to maintain stem cells, supporting their ability to self-renew and differentiate to form all the lineages found within the crypt. The ISC niche comprises all the cellular and non-cellular components that regulate the fate of the ISCs, including adjacent epithelial cells, pericryptal myofibroblasts, enteric neurons, endothelial cells, intraepithelial lymphocytes and the basement membrane [41]. The pericryptal myofibroblasts have traditionally been believed to be the most influential mesenchymal niche cells as they are located adjacent to the ISCs [42, 43]. They secrete factors that can maintain the stem-like phenotype of stem cells in the niche. In particular, Wnt signalling appears to be important in the crosstalk between epithelial and mesenchymal cells in maintaining the survival of stem cells [38, 44–46]. As non-stem cells migrate away from the niche towards the lumen, they form different lineages (Fig. 1).

There is increasing evidence that ISCs require additional signals in order to grow in vitro which may not originate from the epithelium itself. For example, Sato et al. [36] demonstrated the ability of single Lgr5+ cells to grow in vitro in Matrigel, but only in the presence of additional factors, including R-Spondin1 (Wnt agonist), EGF, and Noggin (BMP antagonist). As Noggin and Gremlin 1,

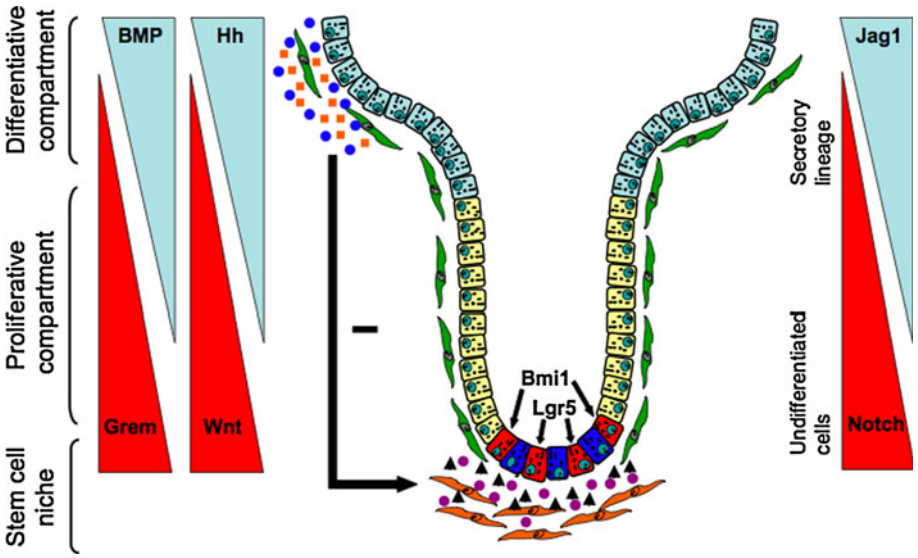

Fig. 1 Intestinal stem cells (ISCs, *red*) may be located at the base of the crypt (Lgr5+ cells) or at the +4 position (Bmi1+ cells). Paneth cells (*blue*) are located between Lgr5+ cells. There may be overlap between the Lgr5 and Bmi1 populations. The daughter cells of ISCs migrate up the crypt–villus axis and pass through a progenitor stage, forming transient amplifying (TA) cells (*yellow*), before becoming differentiated cells (*blue*). Myofibroblasts (*orange*) are an integral component of the stem cell niche intimately related to the ISCs and secrete factors that regulate ISC function, including Wnt ligands (*purple circles*) [104] and BMP antagonists, such as Gremlin 1 (*black triangles*) [48]. The Wnt protein family and BMP antagonists are expressed in a reciprocal gradient to the BMP (*blue circles*) and Hh (*yellow squares*) protein families along the crypt–villus axis. Hh is expressed by differentiated colonocytes and limits expression of Wnt target genes to the base of the crypts. Activation of Hh increases BMP signalling and inhibits the Wnt pathway. Myofibroblasts are Hh responsive and regulate the patterning of the crypt–villus axis. Inhibition of the Notch pathway leads to the differentiation of ISCs into the secretory lineage. The Notch ligand Jagged-1 is expressed in a reciprocal manner to the Notch-1, -2 and -3 receptors. Non-niche intestinal stromal cells are coloured *green*. Small intestine epithelium contains villi, but these are absent in the large intestine. (Adapted from Kosinski et al. [48])

another BMP antagonist, have been shown to be derived from stromal tissue [47, 48], this demonstrates that some of the growth factors required for organoid growth may well originate from the mesenchymal niche.

A further paper by Sato et al. [49] demonstrated that co-culture of Lgr5+ cells with Paneth cells increased organoid formation in vitro. However, these colonies still required the same three additional factors for growth, suggesting that other cells in addition to Paneth cells contribute to the ISC niche. Furthermore, Paneth cells are only found in the proximal colon in addition to the small intestine, so this raises the question of whether other cells take on the role of Paneth cells in the distal colon.

Under normal circumstances, the total number of stem cells within the niche remains constant, so these processes need to be highly regulated, most probably via negative feedback. Stem cells may divide asymmetrically, so that one stem cell remains within the niche, resulting in self-renewal, whilst the other daughter cell gives rise to progenitor cells that can migrate up the crypt and become more differentiated as they reach the top. (Fig. 1) Alternatively, stem cells may divide symmetrically, either forming two daughter stem cells (leading to expansion) or two daughter non-stem progenitor cells (leading to extinction), and this has been supported by two recent studies [14, 50].

If the processes regulating stem cell division and homeostasis are altered, for example by acquiring genetic mutations, then this could prevent stem cells produced by symmetrical division from exiting the niche, leading to an accumulation of stem cells and crypt expansion. Alternatively, stem cells could become niche independent, and lose the ability to respond to negative feedback control. This could be one of the first steps in neoplastic transformation. The exact nature of how the stem cell niche regulates the fate of stem cells is not clearly understood, but there is mounting evidence that several pathways play a role in maintaining and regulating stem ISCs, including Wnt, Bone Morphogenetic Protein (BMP), Hedgehog (Hh) and Notch.

Wnt pathway

The Wnt pathway proteins are a group of evolutionally conserved intercellular signalling molecules that regulate cellular fate along the crypt–villus axis in normal gut epithelium and have been implicated in stem cell

self-renewal. Myofibroblasts are located immediately beneath the basement membrane of basal crypt epithelial cells, and are believed to maintain the stem cell niche due to their location and their ability to secrete Wnt ligands, such as Wnt 5a [51]. In the canonical pathway, when Wnt ligand is not present, the destruction complex [composed of Adenomatous Polyposis Coli protein (APC), axin, and kinases CK1 and GSK3] phosphorylates cytoplasmic β-catenin, which then becomes degraded. In the presence of Wnt ligand, the formation of the destruction complex is inhibited, increasing the intracellular pool of β-catenin, which then translocates to the nucleus where it binds to transcription factors of the T-cell factor (Tcf) family and activates transcription of Wnt target genes, such as *c-myc*, involved with cell proliferation [52], *EphB2* and *EphB3*, which control crypt cellular segregation [53], *Sox9*, which regulates Paneth cell differentiation [54, 55], and *Lgr5* [12].

To investigate the physiological role of TCF4, Korinek et al. disrupted TCF7/2 (which encodes TCF4) by homologous recombination. TCF4 null mice died shortly after birth, but the embryonic epithelium was made entirely of differentiated, non-dividing villus cells with no proliferative compartments in the crypts. This suggested that TCF4 maintains the proliferation of stem cells in the murine small intestine [46]. Another study investigated the effects of deleting the Wnt/TCF4 target gene *c-Myc* in a murine model and found that this led to a loss of intestinal crypts [56].

The importance of the Wnt signalling pathway in maintaining the architecture and homeostasis of the adult intestinal epithelium was also elegantly shown by Kuhnert et al. in a murine model through adenoviral expression of Dickkopf1 (Dkk1), a potent secreted Wnt antagonist. This induced abrupt and fully conditional Wnt inhibition in fully adult mice, resulting in inhibition of proliferation in the small intestine and colon, with progressive loss of crypts, villi and glandular structure [38]. Pinto et al. [57] generated villin-Dkk1 transgenic mice ectopically expressing Dkk1 from embryogenesis onwards. While heterozygous transgenics exhibited no phenotype, homozygous transgenics exhibited greatly reduced epithelial proliferation together with a loss of small intestinal crypts. Although enterocyte differentiation was present, secretory cell lineages were largely absent. These results all support a role for Wnt and its downstream targets in maintaining the proliferation of intestinal stem cells. Furthermore, derangement of the Wnt pathway is one of the features seen in colorectal cancer. In particular, familial adenomatous polyposis (FAP) patients carry one mutant copy of *APC*, and *APC* mutations also occur in the majority of sporadic colorectal adenomas and adenocarcinomas [58].

Cell–cell interactions between cancer stem cells (CSCs) and the stromal tissue also appear to play an important role in regulating the stem-like phenotype of CSCs. Vermeulen et al. [59] recently demonstrated that high Wnt activity designates the colorectal CSC population, and in adenocarcinoma, high Wnt activity is located close to stromal myofibroblasts. Furthermore, they showed that myofibroblasts secrete HGF (hepatocyte growth factor) and could activate β-catenin transcription and subsequent CSC clonogenicity. These myofibroblasts could also restore the CSC phenotype in more differentiated tumour cells in vitro and in vivo.

Bone morphogenetic protein (BMP)

Bone morphogenetic proteins (BMP) are part of the transforming growth factor beta (TGF-β) superfamily and their signalling pathway is involved as a negative regulator of intestinal stem cell proliferation. BMP2 and BMP4 are strongly expressed in the intervillus mesenchyme near the villus tips, with a decreasing expression gradient towards the crypt [47, 60–62]. In addition, the BMP receptor BMPR1A and phosphorylated SMADs, the downstream mediators of BMP signalling, are found along the villus [47]. Conversely, stromal cells within the ISC niche surrounding the crypt based express BMP antagonists Noggin and Gremlin 1 [47, 48].

Haramis et al. [61] demonstrated that inhibition of BMP signalling by Noggin resulted in the formation of ectopic crypts. Furthermore, inhibition of the BMP receptor 1a led to an expansion of the stem and progenitor cellular populations leading to intestinal polyposis that resembled human juvenile polyposis syndrome [47]. He et al. also demonstrated that BMP signalling suppresses Wnt signalling, allowing a balanced control of stem cell self-renewal, thus highlighting the importance of crosstalk between different signalling pathways in maintaining the stem cell niche.

A study comparing the gene expression profile of human colon tops versus basal crypts identified BMP antagonists gremlin 1, gremlin 2, and chordin-like 1 as intestinal stem cell niche factors. In situ hybridisation and RT–PCR confirmed that these BMP antagonists were expressed by pericryptal myofibroblasts [48]. Gremlin 1 is able to partially inhibit cell differentiation of the colorectal cancer cell line Caco-2 in vitro and activates Wnt signalling in normal rat intestinal epithelial cells [48].

Hedgehog (Hh) signalling pathway

Interactions between the Hedgehog (Hh) and Wnt signalling pathways also appear to play an important role in regulating the colonic stem cell niche. Indian hedgehog (Ihh), a member of the mammalian hedgehog ligand

family, is expressed by differentiated colonocytes. Hh signalling limits the expression of Wnt targets to the base of the colonic crypt and inhibition of Hh signalling using cyclopamine leads to aberrations in epithelial cell differentiation [63]. Furthermore, transfection of Ihh into colon cancer cells downregulates the TCF/β-catenin signalling complex.

As a follow-up to the previous study, von Dop et al. [64] demonstrated that activation of the Hh pathway resulted in increased BMP signalling in the epithelium, as well as inhibition of the Wnt pathway. This was also associated with a reduction in the number of epithelial precursor cells and premature differentiation into enterocytes. One possible mechanism of crosstalk is that Ihh signals from the colonocyte to the mesenchyme resulting in the accumulation of myofibroblasts, which then reduce Wnt signalling, possibly indirectly via increased expression of BMP. This leads to a reduction in the number of precursor colonic crypt cells. In this manner, Ihh is able to act as a paracrine negative feedback signal from differentiated colonocytes to regulate precursor cells in the colonic crypt. In support of this hypothesis, myofibroblasts within the mesenchyme are Hh-responsive, as indicated by the expression of Hh target genes Patched and Gli [65]. In addition, a recent study by Kosinski et al. [66] demonstrated that intestinal epithelial Ihh signals to mesenchymal cells to regulate their formation and proliferation and in turn, this affected epithelial proliferation and differentiation. Further evidence that Hh signals pattern the crypt–villus axis through their interactions with myofibroblasts stems from a study by Madison et al. [67] who used a villin promoter to overexpress Hedgehog interacting protein (Hhip), a negative regulator of the Hh pathway, to block all Hh signals. These mice demonstrated defective villus formation, increased proliferation, aberrant crypt structures and mislocalised myofibroblasts.

The interaction between Wnt and Hh pathways is also important in regulating the behaviour of adult hindgut stem cells in *Drosophila melanogaster*. Intestinal stem cells (ISCs) of the posterior intestine are located within the hindgut proliferation zone (HPZ). Anterior expression of Wingless (Wg, a *Drosophila* Wnt homologue) acts as a niche signal to maintain the self-renewal of ISCs. As cells divide and move posteriorly away from the Wg source, they become more proliferative. Hh signal is then required for the cells to exit the cell cycle and differentiate [68].

Notch pathway

The Notch signalling pathway is a highly conserved signalling system, present in almost all multicellular organisms and used to control cell fate through local cell interactions [69]. In mammals, it consists of four different receptors (Notch1, Notch2, Notch3 and Notch4) and its ligands (Jagged1, Jagged3, DLL1, DLL3 and DLL4) [70]. The Notch pathway is activated when Notch ligands bind to Notch receptors on an adjacent cell, which then activates the gamma–secretase protein complex. This then cleaves the transmembrane Notch receptor, liberating the constitutively active domain NICD (Notch intracellular domain). NICD then translocates to the nucleus, forms a complex with one of three transcriptional regulators (CSL, MAML-1 or p300/CBP) [71] and induces the expression of downstream Notch transcriptional factors, in particular Hes-1.

The Notch pathway plays a role in maintaining the progenitor cell population, as well as regulating the secretory lineage of intestinal cells, namely goblet, enteroendocrine and Paneth cells. Using the villin promoter to drive the expression of a constitutively active form of mouse Notch 1 receptor (N1ic), Fre et al. [72] noticed an expansion of proliferating intestinal progenitor cells. However, villin is mainly expressed at the brush border of differentiated columnar cells in the epithelium, so it is not clear how Notch was expressed in the progenitor cells. In addition, an increase in progenitor cell numbers may reflect the block on the exit of these cells into more differentiated cells, rather than a direct effect of Notch on self-renewal per se. The authors also noted that Notch activation inhibited the differentiation of secretory cells in the mouse intestine, as there was a complete depletion of goblet cells, marked reduction in enteroendocrine cells, and low expression of Cryptidin-1 mRNA, an early marker for Paneth cells. Notch activation upregulated Hes1, a basic helix–loop–helix (bHLH) transcription factor that is part of the Notch signalling pathway. It also repressed the bHLH transcription factor *Drosophila* Atonal Homolog 1 (Atoh1, also known as Math1/Hath1 in mouse and humans, respectively), suggesting that Math1 may be important in regulating secretory cell lineages. Conversely, conditional removal of the Notch pathway transcription factor CSL/RBP-J increases the proportion of goblet cells in the murine intestine, and a similar phenotype was observed with the addition of DBZ, a gamma secretase inhibitor [73].

The non-mesenchymal niche

Although the mesenchyme has traditionally been viewed as playing a crucial role in regulating the function of ISCs, studies in *Drosophila* have raised the possibility that epithelial tissues also play an important role in ISC self-renewal and differentiation. Ohlstein and Spradling [74] demonstrated that differential expression of the Notch

ligand Delta by ISCs determines the fate of adjacent daughter cells. ISCs that express a high level of Delta downregulate Delta in their daughters, resulting in enterocyte differentiation. Conversely, ISCs that express a low level of Delta specify their daughters to become enteroendocrine cells. During organogenesis, the adult midgut progenitor (AMP) divides asymmetrically and signals via Notch to direct its first daughter cell to become a peripheral cell, which then acts as a niche to keep other AMPs undifferentiated, using the bone morphogenetic protein 2/4 homolog, decapentaplegic [75]. During morphogenesis, this niche breaks down, allowing the AMPs to respond to Notch signalling and differentiate into enterocytes.

Although non-mesenchyme cells are important in regulating the *Drosophila* ISC niche, epithelial cells may also contribute to the mammalian ISC niche. A recent paper by Sato et al. [49] demonstrated that Paneth cells contributed to the niche for murine Lgr5+ cells, and co-culture of Paneth cells with Lgr5+ greatly enhanced in vitro outgrowth of intestinal organoids, although exogenous factors such as R-Spondin, EGF and Noggin were still required. As Paneth cells are only found in the small intestine and proximal large intestine, other epithelial or mesenchymal cell subpopulations may contribute to the ISC niche in the colon.

Lineage specification and regulation of stem cell differentiation

Enterocyte lineage and CDX1

CDX1 (Caudal-type homeobox protein 1) is a homeobox transcription factor that regulates intestinal development and is expressed in both the murine and human gastrointestinal tract [76]. In normal epithelium, nuclear CDX1 expression is most prominent in enterocytes lining the crypt [77]. Wnt directly regulates the expression of CDX1 in the murine embryonic intestine. Lickert et al. [78] demonstrated that, upon Wnt stimulation, *Cdx1* was induced in mouse embryonic stem cells. Furthermore, Tcf4-deficient mouse embryos no longer expressed CDX1 protein in the small intestinal epithelium. As the Cdx1 gene promoter region contains several Tcf binding domains, Tcf4 is the likely candidate mediating the effects of the Wnt signalling pathway on CDX1 expression in the developing small intestine. Beland et al. [79] showed that CDX1 and LEF-1, a nuclear effector of Wnt signalling, synergised to induce expression from the *Cdx1* promoter through LEF/T-cell factor response elements. Among the Wnt family members, Wnt3a appears to regulate CDX1 as Wnt3a murine mutants have reduced CDX1 expression [80]. In addition to Wnt, retinoic acid (RA), the oxidised form of vitamin A, also directly regulates CDX1. RA has been implicated in vertebral patterning, and CDX1 null mice show similar homeotic transformations as RA receptor null mice [81]. Prinos et al. [82] showed that RA and Wnt3a synergise to initiate CDX1 expression during development. Interestingly, CDX1 is able to negatively feedback and block β-catenin/TCF transcriptional activity [83].

CDX1 has several downstream targets that are specific to enterocyte differentiation. CDX1 induces the expression of intestinal alkaline phosphatase [84], a marker of intestinal enterocyte differentiation, and regulates the expression of ApoB mRNA editing protein, which is located in the differentiated villi [85]. CDX1 also directly regulates the expression of the gastrointestinal differentiation marker, cytokeratin 20 (CK20), by binding to its promoter region [86]. Expression of CDX1 is lost or reduced in a significant proportion of colorectal cancer cell lines and this is primarily regulated by promoter methylation [87]. As the development of a cancer is associated with a disruption to the normal balance between proliferation and differentiation, loss of CDX1 expression may be a selective advantage in maintaining an undifferentiated state, and increase the proportion of undifferentiated stem cells within a tumour [88].

Secretory lineage: goblet, enteroendocrine and Paneth cells

Whilst CDX1 plays an important role in enterocyte differentiation, inhibition of the Notch pathway regulates the secretory lineage. As mentioned earlier, one of the Notch downstream targets is Hes1, which has a reciprocal relationship with Atoh1/Math1. Hes1 null mice were found to have increased expression of Math1 on RT-PCR in the small bowel, but not in the colon. In addition, Hes1 null mice had increased numbers of goblet cells and decreased numbers of enterocytes in the duodenum [89]. Yang et al. [90] examined the small and large intestines of Math1 knockout mice, which showed a complete lack of goblet and enteroendocrine cells, and no Cryptdin-1 mRNA expression. These results suggested that Math1 was important in specifying the secretory lineage in the intestine, also confirmed by Vandussen et al. [91] who demonstrated that Math1 directs cells to the secretory rather than the absorptive lineage.

Due to difficulties in culturing human intestinal stem cells in vitro, the regulation of Notch, Hath1 and the secretory cell lineage is less well understood in humans. Instead, studies have been based on human colorectal cancer cell lines. Hath1 undergoes proteosomal destruction by GSK3β, and inactivation of the Wnt signalling pathway

is required for Hath1 stabilisation [92]. Cotransfection of APC2, a mutant that destabilises β-catenin, together with Hath1 in the SW480 colorectal cancer cell line, induced MUC2 expression. Similarly, when a mutant form of Hath1 protein (serine residues replaced with alanine) was stably expressed in SW480 and DLD1 cell lines, this also increased MUC2 mRNA expression [93]. These results suggest that Hath1, like its homologues Math1/Atoh1, is important in mediating goblet cell differentiation.

The regulation of the differentiation of enteroendocrine and Paneth cells from the common secretory progenitor cell is not well understood. Both neurogenin-3 (a bHLH transcription factor involved the development of endocrine cells in the pancreas) [94] and PDX-1 (pancreatic and duodenal homeobox 1) have been implicated in the differentiation of enteroendocrine cells. Neurogenin-3 overexpression in mice driven by the villin promoter increases the numbers of enteroendocrine cells and decreases the proportion of goblet cells, suggesting that Neurogenin-3 directs differentiation of secretory progenitors to endocrine rather than goblet cells [95]. Furthermore, Neurogenin-3 overexpression leads to enteroendocrine cell differentiation in murine intestinal spheroids long-term culture in vitro [37]. PDX-1 is a transcription factor required for pancreatic development and islet cell function in adulthood [96]. It is also required for appropriate gene expression in enteroendocrine cells in the proximal small intestine [97]. Yamada et al. [98] demonstrated that PDX-1 overexpression could cause immature rat intestinal epithelial cells (IEC-6) to differentiate into enteroendocrine cells.

As mentioned previously, Paneth cells are usually found in the small intestine, and only occasionally in the proximal colon. A variety of different genes have been implicated in regulating the development of Paneth cells in the murine small intestine, including FGF3 [99], LKB1 [100], SOX9 [101] and SPDEF [102]. Intriguingly, SPDEF promotes the maturation of both goblet cells and Paneth cells downstream of Math1 [102], and LKB1 deficiency alters both goblet and Paneth cell differentiation. These results suggest that goblet cells and Paneth cells share a similar origin.

Thus, our current understanding of lineage specification can be summarised by Fig. 2. An intestinal stem cell gives rise to progenitor cells that can form either the enterocyte lineage (regulated by CDX1) or the secretory lineage (via inhibition of the Notch pathway). This model is likely to be an oversimplification, and the caveat is that most data are from murine studies, not humans. In particular, there may not be a common secretory lineage progenitor cell that gives rise to goblet, enteroendocrine and Paneth cells; instead, it has been suggested that a bipotential progenitor gives rise to enterocyte and Neurogenin-3+ enteroendocrine cells [103].

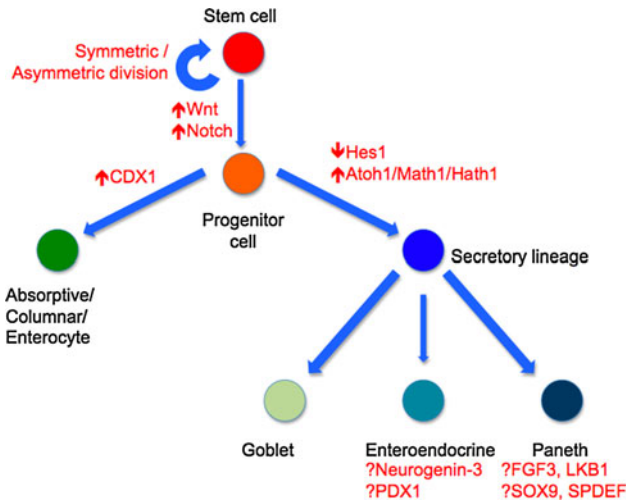

Fig. 2 Lineage specification of an intestinal stem cell (ISC). Stem cells may divide either symmetrically or asymmetrically, although recent evidence suggest the former is most likely [14, 50]. Wnt signals maintain the stem-like phenotype of ISCs, whilst Notch maintains the proliferation of progenitor cells. The intestinal stem cell gives rise to progenitor cells, which can either form enterocytes (via CDX1) or the secretory lineage, mediated by inhibition of the Notch pathway, downregulation of Hes-1 and upregulation of Atoh1/Math1/Hath1 homologues. Paneth cells are found in the small intestine, but not normally in the large intestine apart from the proximal colon. The mechanisms that regulate specification of enteroendocrine and Paneth cells are not fully understood. Candidate genes include neurogenin-3 and PDX1 (enteroendocrine) and FGF3, LKB1, SOX9 and SPDEF (Paneth cells)

Conclusions

The gastrointestinal tract is a highly organised tissue with a crypt–villus axis that is tightly regulated in order to maintain homeostasis. Lineage tracing experiments have demonstrated that single ISCs, as exemplified by Lgr5+ or Bmi1+ populations, can self-renew and give rise to all the differentiated lineages found in the crypt. The ISC niche is a specialised environment that provides crosstalk to regulate the function of stem cells, and emerging evidence that pericryptal myofibroblasts as well as the epithelium may be important constituents of this niche. Further research is warranted to determine the exact location of ISCs, to investigate if there are multiple subpopulations of ISCs that are either actively cycling or quiescent and the interrelationships between these populations, and to assess the relative functional contributions of the potential components of the ISC niche. A greater understanding of how ISC self-renewal and differentiation are regulated will have a significant impact in the fields of stem cell biology, tissue regeneration and cancer.

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