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Review

Shifting paradigms in tissue stem cell biology: Insights from the intestine

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SUMMARY

The small intestinal epithelium represents the most rapidly self-renewing adult mammalian tissue, with a turnover time of 1–2 weeks. It contains ∼12 easily recognizable cell types with a wide diversity of functions, including nutrient absorption, mucus production, antimicrobial defense, and the regulation of metabolism by incretins like Glp1. The simple and repetitive crypt-villus architecture allows for easily interpretable experimentation in transgenic mice *in vivo*, while the human stem cell hierarchy is experimentally accessible in epithelial organoids *in vitro*. This review aims to comprehensively describe the design, the cellular constituents, and the molecular regulation of crypt-villus epithelial self-renewal. More generally, it highlights deviations from commonly held views on tissue stem cell biology: gut stem cells divide continually and symmetrically. They can be expanded indefinitely *in vitro*, while the plasticity of daughter cells can recreate stem cells during regeneration.

The small intestine and colon share a conserved epithelial architecture and stem cell-based renewal system, yet they differ in both cellular composition and physiological function. In both organs, a self-renewing pool of intestinal stem cells located at the crypt base continuously generates transit amplifying progenitors that differentiate into absorptive cells and secretory cells. Functionally, the small intestine specializes in nutrient absorption through the abundant villi, which dramatically increase its absorptive surface area (Figure 1). The colon lacks villi and resorbs water and electrolytes. Between rodents and primates, the overall stem cell architecture and signaling hierarchy are remarkably conserved; however, differences exist in turnover rates and specific marker expression. For instance, the newly discovered human bestrophin-4 (BEST4)$^+$ cell does not exist in the mouse. This review focuses primarily on the small intestinal crypt stem cell hierarchy in mouse and man. It is undoubtedly colored by the three decades that my lab has been involved in this field.

A brief historical recap (1745–1970)

The crypts of Lieberkuehn are named after their discoverer, Jonathan Nathanael Lieberkuehn, a young German scientist working at Leiden University. For his thesis, he created wax casts of intestinal blood vessels and described small indentations in the gut wall, which he termed "crypts" after the underground vaults beneath a church floor.[2] In 1887, the Viennese physiologist Joseph Paneth proposed that crypts and villi derive from the same embryological origin.[3] 6 years later, Bizzozero noticed that mitoses were abundantly present in adult crypts and correctly deduced a functional connection between the two compartments: new cells were generated in the crypts and migrated to the villus epithelium.[4] Two landmark papers by Leblond and Stevens[5,6] built on this concept showed that adult rats continuously produce large numbers of new crypt cells. Leblond and Stevens stated: "… the cells formed in the crypts of Lieberkühn move upward along the side of the villi to be ejected when they reach the villi tips." The authors drew the remarkable conclusion that the life span of an individual intestinal epithelial cell should be in the order of days. 10 years later, the conveyor belt mechanism was confirmed by tracing using radioactive DNA precursors and autoradiography.[7]

The elusive crypt stem cell: Two competing hypotheses (1970–2005)

The CBC stem cell

It was clear that a stem cell was hiding somewhere near the base of the crypts. Cheng and Leblond noted the presence of small, continuously cycling cells between the Paneth cells (the latter being described by Joseph Paneth to populate the crypt base[3]). They termed these as cells crypt base columnar (CBC) cells[8] (Figure 3). Upon 3H-thymidine injection, some CBC cells died and were phagocytosed by surviving CBC cells, yielding radioactive (and therefore traceable) phagosomes. Such "hot" phagosomes appeared within more differentiated cells at later time points. From this rudimentary lineage-tracing experiment, the authors concluded that the four main cell types (enterocytes, goblet cells, Paneth cells, and enteroendocrine cells [EECs]) are derived from CBC cells.[9] Much later, a clonal labeling strategy based on chemical mutagenesis demonstrated that crypts become clonal over just a few months. This approach also directly visualized the conveyor belt of cells, running from crypt bottoms to villus tips.[10]

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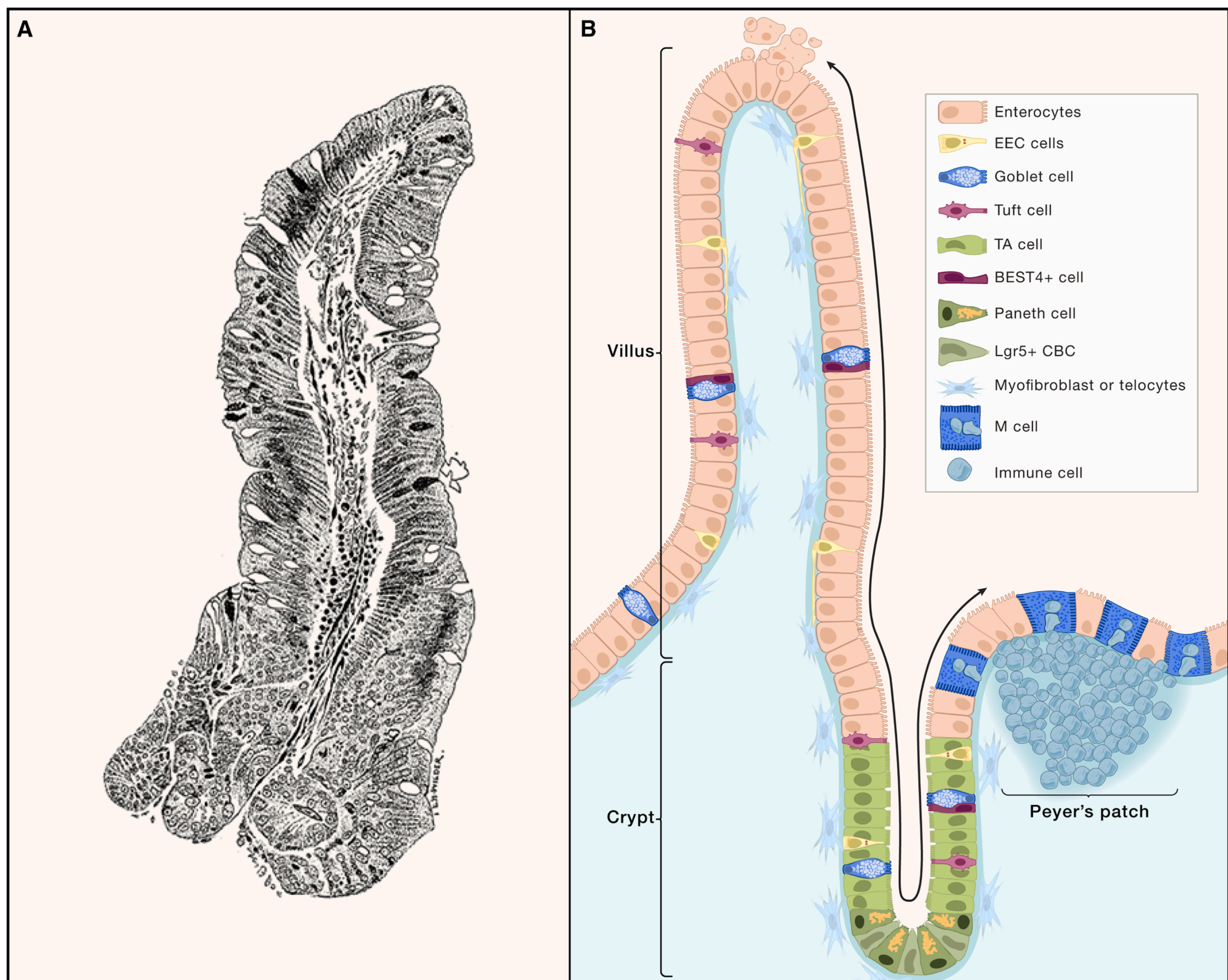

Figure 1. The small intestinal crypt-villus unit
(A) Hand-drawn image of a crypt-villus unit of the opossum (1906).[1]
(B) Schematic of a crypt-villus unit (left) and a crypt-Peyer's patch unit (right). Lgr5$^+$ CBC stem cells and Paneth cells reside at the crypt base, from which the various differentiating stem cell daughters move up the adjacent villus (left) or migrate over the Peyer's patch (right) to die at the villus tips or the center of the Peyer's patch's dome.

The "stem cell zone" model, as formulated by Bjerkness and Cheng, posits that CBC stem cells and Paneth cells reside at the crypt base.[11,12] Daughter cells migrate upward from this stem cell zone, and when they reach position 5 (directly above the uppermost Paneth cell), they commit toward individual differentiation fates. Paneth cells are unique in that they escape this upward flow and remain at the crypt base.[11]

The +4 stem cell

Potten and colleagues reported that rare DNA-label-retaining cells (LRCs) reside directly above the Paneth cells, also known as position +4.[13] Although DNA label retention has been widely used as a surrogate marker for quiescent stem cells, Potten's cells proposedly retained DNA labels for a different reason: the +4 cells are continuously cycling, yet the observed DNA label retention would result from segregation of old (labeled) immortal DNA strands into stem cells and new (unlabeled) DNA strands into their daughters.[14] Of note, the "immortal strand" hypothesis, postulated to protect stem cells against mutation, has not withstood the test of time.

A modern view on crypt stem cell identity

The CBC stem cell

In 1998, we reported an essential role for the Wnt pathway in the establishment of proliferative crypt stem cell compartments of mice mutant in the gene encoding the Wnt effector Tcf4/Tcf7l2.[15] The simultaneous discovery of activating Wnt pathway mutations in colorectal cancer (CRC)[16] implied a dominant role of this pathway (hitherto known as a key signaling cascade in developing animal embryos) in the patho-biology of the elusive adult gut stem cell. Later studies have indicated that maintenance of adult crypts continues to require Wnt, as observed upon transgenic expression of the Wnt receptor antagonist Dkk1[17] and upon conditional deletion of beta-catenin[18] or Tcf4/Tcf7l2[19] in

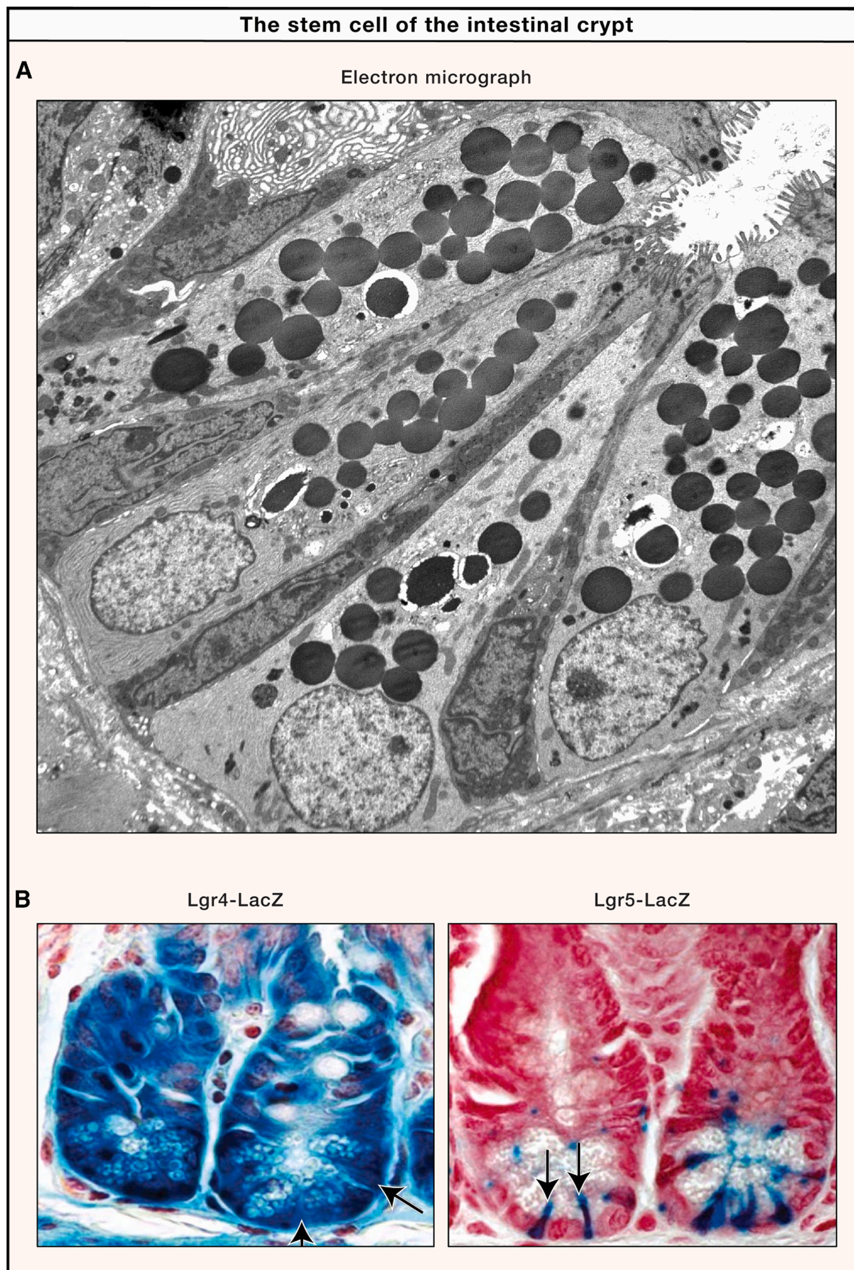

Figure 2. The stem cell of the intestinal crypt

(A) Transmission EM image of a healthy human jejunal crypt base, oriented upward and to the right. The large cells with prominent granules are Paneth cells. Squeezed between the Paneth cells are the slender CBC cells, not much larger than their nuclei. (Courtesy Joep Grootmans and Wim Buurman, Maastricht University, the Netherlands)

(B) Expression (blue) of Lgr4-LacZ and Lgr5-LacZ knockin alleles in mice. *Lgr4* is expressed in all crypt cells, whereas *Lgr5* marks CBC cells (from de Lau et al.[23]).

adult mice. DNA-arraying of mRNA extracted from a CRC cell line before and after inhibition of the Wnt pathway provided a first genome-wide glimpse of the intestinal Wnt target gene program.[20] One gene in this program, *Lgr5*, was revealed to be an exquisite marker for the CBC cell (Figures 2A and 2B). We generated an *Lgr5EGFP-ires-CreERT2* knockin mouse and crossed this with *R26R-lacZ* Cre reporter mice.[21] In these mice, each crypt harbors 12–15 Lgr5-GFP$^+$ cells, invariably in contact with Paneth cells.[22] As already reported by Leblond, murine Lgr5$^+$ CBC cells are continuously cycling, against the commonly held belief that stem cells should be quiescent. Tamoxifen-induced lineage tracing yielded "Winton/Ponder"-like[10] ribbons, extending from the crypt base to the villus tip. These ribbons persisted lifelong and contained all epithelial cell lineages. The "marker" Lgr5 turned out to have a functional role in stem cell biology as well. Like its homologs Lgr4 and Lgr6, Lgr5 is a 7-TM receptor for secreted Rspondins.[23,24] Rspondins are Wnt signal amplifiers that are secreted by subepithelial mesenchymal cells and act by simultaneously binding to an Lgr5 family member and to Rnf43/Znrf3.[25] The latter molecules are transmembrane E3 ubiquitin ligases that are themselves encoded by Wnt target genes and serve in a potent negative-feedback loop by targeting Wnt receptors for destruction.[26] Lgr5 and Rspondins break this negative feedback loop, thus amplifying signaling by active Wnt receptors. Much earlier, Rspondins had already been identified as Wnt pathway agonists,[27] capable of inducing dramatic crypt hyperplasia.[28]

Batlle and colleagues added a twist to the Lgr5 CBC model. They found that Lgr5$^+$ cells exist in two discrete states, one being slow-cycling and defined by Mex3a expression. During homeostasis, Mex3a$^+$/Lgr5$^+$ cells feed into the rapidly dividing Mex3a$^-$/Lgr5$^+$ pool. Mex3a-high/Lgr5$^+$ cells are much more resistant to chemotherapy and radiation than the rapidly dividing Lgr5$^+$ cells. Mex3a thus defines a reserve stem cell population within the Lgr5$^+$ compartment.[29] Easy experimental access to fluorescently labeled CBC cells was instrumental to the generation of a detailed definition of their gene expression profile.[30] Additional stem cell markers emerged from this study, including the transcription factor Ascl2 (which, like Lgr5, is encoded by a Wnt target gene[31]) and OlfM4 (encoded by a Notch target gene).[32] Ascl2 was subsequently shown to act as a master regulator of the Lgr5$^+$ stem cell,[33] and was also found to be essential for the regeneration of Lgr5$^+$ CBC cells from daughter cells during regeneration.[34]

A key feature of stem cells is their ability to limit the accumulation of somatic mutations. A study described that individual

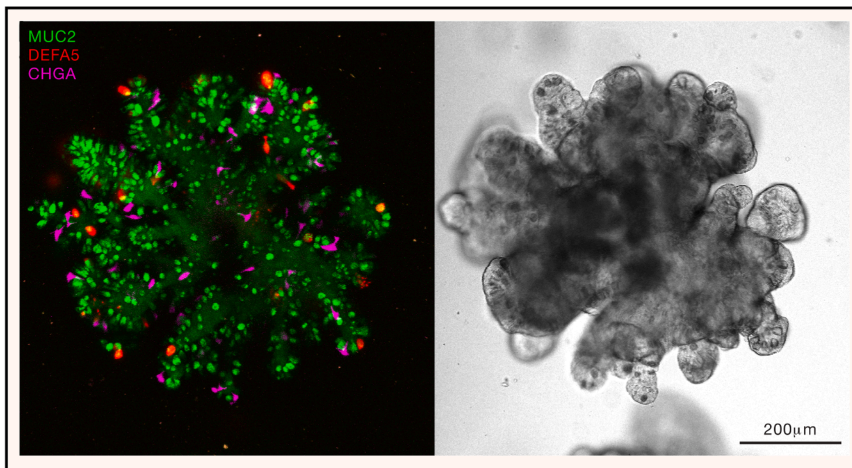

Figure 3. Human small intestinal organoid line, grown from one Lgr5⁺ CBC stem cell
This organoid line carries CRISPR-engineered genetic markers for goblet cells (Mucin-2; green), EECs (chromograninA; purple), and Paneth cells (DefensinA5; orange) from He et al.[45]

human intestinal stem cells accumulated single-base changes at a rate of 40 per year, with kinetics that remain stable with age.[35] Interestingly, stem cell mutation rates vary dramatically across species, such that short species lifespans correlate with high somatic mutation rates per year.[36]

Organoid technology complements transgenic mouse models to study CBC stem cells

In 2009, two studies reported the first evidence that primary intestinal tissue could be cultured long term. Kuo and colleagues described an approach to culturing mouse neonatal intestinal explants for up to a year by placing tissue fragments on top of a collagen gel at an air-liquid interface in the absence of added growth factors.[37] The presence of a robust underlying stroma was obligate for the persistence of the proliferative pockets in the outgrowing cystic structures, which contained cells of the main cell lineages as well as cells expressing the proposed stem cell markers Lgr5⁺ and Bmi1⁺. Addition of Rspo1 led to a significant increase in the number and size of intestinal spheres, whereas the Wnt inhibitor Dkk1 resulted in dose-dependent growth inhibition, pointing to the Wnt pathway as one key driver of epithelial expansion. The viability of adult intestinal explants was much less extensive than that of neonatal cultures.

My lab pursued a somewhat different route by aiming to grow single-sorted murine Lgr5-GFP cells autonomously and stroma-independently by providing all essential niche factors exogenously. For this, we exploited earlier insights into the niche growth factor- and integrin-dependencies of crypt stem cells: The Wnt agonist Rspondin1 was combined with the "generic" epithelial growth factor epidermal growth factor (EGF) and the BMP inhibitor Noggin, which we had previously observed to create a "crypt-permissive" environment in transgenic mice.[38] A personal advice from Mina Bissell led us to attempt this growth factor cocktail in 3D Matrigel, the latter providing an extracellular matrix (ECM)-like hydrogel, largely consisting of laminin-111 and collagen-IV.[39] Rather than the hoped-for production of clusters of stem cells from a single Lgr5-GFP stem cell, we observed the surprising formation of 3D structures with many features of the adult intestinal epithelium.[40] Thus, crypt-like structures appeared as buds, containing Paneth cells and intermingled Lgr5 stem cells. These buds surrounded a central lumen made of mature enterocytes, goblet cells, and occasional EECs (Figure 3). Another surprise involved the realization that these organoids could be expanded exponentially over years of culture: they showed no signs of senescence, and their telomeres remained long. Of interest, small intestinal organoids retain their regional identity (duodenum, jejunum, ileum) over these long time periods,[41] a property utilized elegantly by Sato and colleagues to generate a functional small-intestinalized colon by replacing the native colonic epithelium with ileum-derived organoids in a rat model for short bowel syndrome.[42] Organoid culturing approaches work equally well for human intestinal tract stem cells and malignancies derived thereof.[43,44]

To date, we have never observed the appearance of oncogenic changes in the genomes of long-term expanded organoids (unless specifically selecting for such mutations, see below). As definitive proof of the "normality" of extensively expanded Lgr5 organoids, the offspring of a single, murine Lgr5⁺ colon cell were transplanted to the inflamed colons of multiple mice. Long-term (>6 months) engraftment of the organoids ensued, with no sign of stem cell exhaustion or neoplastic changes.[46] Of note, human intestinal organoids can also be generated from pluripotent stem cells. Sato et al. used stepwise differentiation—first to definitive endoderm via activin A, then patterning to mid/hindgut by Wnt/fibroblast growth factor (FGF) signaling—to yield three-dimensional intestinal spheroids that could be expanded and matured using the growth factor cocktail described above.[40] Human intestinal stem cells form *de novo* during development of these organoids. These Lgr5⁺ stem cells give rise to functional enterocytes, as well as goblet, Paneth, and EECs.[47]

CRC mutations mirror the physiological growth factor dependencies of Lgr5⁺ stem cells

The emperically identified signaling pathways that are essential for physiological crypt self-renewal *in vivo* and for long-term organoid culture also appear as the mutational targets in intestinal tumors. Thus, two CRISPR-Cas9-based studies targeted the most commonly mutated genes in CRC (*APC*, *TP53*, *KRAS*/*PIK3CA*, and *SMAD4*) in normal human colon organoids. Mutation of *APC* removed the dependence on Wnt/Rspondin, mutation of *KRAS* or *PIK3CA* removed the dependence on EGF, while mutation of *SMAD4* removed the dependence on the BMP inhibitor Noggin. Organoids engineered to express all mutations grew independently of their niche factors *in vitro*, and formed metastasizing tumors upon xenografting.[48,49] A subsequent study utilized mismatch-repair-deficient

organoids to select spontaneous oncogenic mutations through the stepwise withdrawal of the Wnt agonists, EGF and Noggin. The organoids sequentially acquired mutations in AXIN1 or AXIN2 (Wnt pathway), TP53, ACVR2A or BMPR2 (BMP pathway), and NRAS (EGF pathway), gaining complete independence from stem cell niche factors. Again, quadruple-mutant organoids formed solid tumors upon xenotransplantation.[50]

Lgr5⁺ cells as the "workhorse" or "everyday" crypt stem cells

Taken together, observations of lifelong lineage tracing during which all cell types are produced from a single marked Lgr5⁺ stem cell *in vivo* were mirrored by the ability to grow transplantable organoids that contain all cell types of the intestinal epithelium from a single cell *in vitro*. This led us to propose that Lgr5 marks the CBC cells of Leblond and that these cells constitute the "workhorse" or "everyday" crypt stem cells, capable of driving the lifelong maintenance of the most rapidly self-renewing tissue in mammals. Yet, significant resistance to this concept persisted, most importantly because—at least in the mouse—Lgr5 CBC cells constantly cycle, whereas the quiescent state was—and sometimes still is—seen as the ultimate identifier of stemness. Of note, while LGR5⁺ stem cells in the mouse colon are predominantly cycling, their human colon counterparts appear to be largely quiescent.[51] They are more resistant to chemotherapy than their murine counterparts, reflecting differences in proliferative states. In mice, proliferating Lgr5⁺ cells are eliminated by proliferation-targeting therapies, and regeneration occurs through plasticity of differentiated cells. By contrast, human quiescent LGR5⁺ cells remain in the niche and prevent differentiated cells from reverting to stem cells under steady-state conditions. However, when human LGR5⁺ cells are genetically ablated, differentiated cells can indeed revert to LGR5⁺ stem cells. Thus, the human intestinal epithelium also possesses inherent plasticity. Of interest, *Lgr5⁺* cells in the crypts of the naked mole rat, a popular longevity model, similarly exhibit slower division rates compared with those of mice.[52]

Neutral competition rather than asymmetric division

Other surprising observations were made: Lgr5⁺ cells divide symmetrically, while their numbers remain constant through neutral competition for niche space. The textbook mechanism for maintenance of a constant stem cell population over a lifetime involves asymmetric cell division, where a dividing stem cell yields one stem cell and one fated daughter cell. A study in the mouse epidermis was the first to challenge this concept[53]: random genetic marking of the abundant proliferative cells of the basal epidermis yielded long-lived clones of ever-changing shapes and increasing sizes, implying that a single stem cell can generate multiple stem cells under steady-state conditions. A similar situation exists in crypts, again as originally revealed by lineage tracing.[54,55] The CBC stem cells, with a cell division time of ∼24 h, have the potential of generating a long-lived lineage of cellular offspring. However, these proliferating stem cells compete "neutrally" for niche space. Their long-term persistence within the niche is not guaranteed: the active division of one cell may lead to the displacement of its neighbor by a process that appears stochastic. Some CBC cells will persist in the niche, while others are pushed out, upon which they differentiate and are cleared from the system. This process was later directly visualized by *in vivo* live-imaging, and it was noted that stem cells at the crypt base held a competitive advantage over stem cells at the niche's upper border.[56] Building on this platform, it was subsequently noted that border stem cells can also move back toward the crypt base.[57] While crypts house 12–15 CBC stem cells, the neutral competition phenomenon will drive crypts to clonality, a process that takes only about 2 months in the mouse. The drive of crypts toward clonality had already been observed 20 years earlier.[58]

The +4 stem cell

Although the Lgr5⁺ CBC cell exhibits the two functional stem cell criteria (longevity and multipotency), it was argued that their relative abundance and proliferative nature define these cells more as progenitors. Four independent studies focused on slow-cycling/quiescent cells located around Chris Potten's +4 position. Thus, Bmi1 was observed to mark rare, slowly cycling cells at this position.[59] *In vivo* lineage tracing yielded the same ribbons that were seen in the Lgr5-driven model. A subsequent study proposed that Lgr5 stem cells execute homeostatic self-renewal, while Bmi1⁺ stem cells would become active upon injury.[60] A *Tert* promoter-driven GFP transgene revealed GFP⁺ cells in occasional crypts. Some of these Tert-GFP⁺ cells retained DNA labels.[61] A subsequent study showed that these transgene-expressing cells are radiation-resistant and distinct from Lgr5⁺ cells.[62] A LacZ knockin allele in the *Hopx* locus was expressed at the +4 position along the entire small intestine. Lineage tracing from these cells resulted in the tell-tale stem cell ribbons.[63] Finally, lineage tracing from an *Lrig1-CreERT2* allele[64] was also shown to yield these ribbons. A minority of Lrig1⁺ cells were DNA label-retaining. A simultaneous study using a different Lrig1 antibody raised doubts about the specificity of the Lrig1 marker: 30% of all small intestinal crypt cells express Lrig1, with Lgr5⁺ stem cells being the highest expressors.[65] Subsequent, careful expression analyses with state-of-the-art technologies have not confirmed the specificity of Bmi1, Tert, Hopx, and Lrig1 as markers of a stem cell located at the +4 position. Rather, it was shown that these markers are expressed broadly and are most abundant in Lgr5 stem cells (see, for instance Muñoz et al.,[30] Kim et al.,[66] and Itzkovitz et al.[67]). Thus, no specific marker for cells located at position +4 has been reported to date, whereas quiescent +4 cells appear to represent early secretory progenitors of CBC cells (see below).

Plasticity: Are all crypt cells facultative stem cells?

Reversion of secretory and absorptive progenitors replaces lost Lgr5 stem cells

The original papers from the 1970s by Cheng and Leblond[9] and Potten[68] already proposed that transit-amplifying daughter cells may fall back into the stem cell niche, thus regaining stemness. There is now ample experimental evidence for such plasticity.

As a first example, we used lineage tracing from the *Dll1* locus to demonstrate that the Notch ligand Dll1 marks a short-lived secretory progenitor, being a first-generation daughter of Lgr5⁺ stem cells.[69] Consequently, long-lived tracing was not achieved under homeostatic conditions. However, these Dll1⁺ secretory progenitors readily revert to Lgr5⁺ stem cells when the latter are killed by radiation, producing the characteristic stem cell

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ribbons. In an elegant strategy to specifically kill Lgr5+ cells, de Sauvage and colleagues expressed the receptor for diphtheria toxin (DTR) from the *Lgr5* locus.[70] Upon injection of DTR, the Lgr5+ cells succumbed, yet the self-renewal process was maintained. Upon stopping the toxin injections, Lgr5+ cells reemerged from other Bmi1+ cells.

Some years later, we employed this DTR mouse strain to perform a similar experiment using the *alkaline phosphatase intestinal* (*Alpi*) gene as an early, definitive lineage-tracing marker for the main cell lineage of the intestinal epithelium, the absorptive enterocyte.[71] These enterocyte precursors rapidly reverted into multipotent Lgr5+ cells when the latter were killed by DTR injection.

The +4 cell: A non-dividing secretory progenitor

Winton and colleagues specifically probed the identity of quiescent, DNA LRCs at crypt bottoms by briefly expressing the stable chromatin marker histone 2B-yellow fluorescent protein (YFP) throughout the crypt.[72] As expected, label retention was observed in the relatively long-lived Paneth cells. Yet, some non-Paneth cells also remained positive for this chromatin label for 2–3 weeks after the pulse. These quiescent cells co-expressed Lgr5, Paneth markers, as well as the +4 markers mentioned above, and were identified as secretory precursors. As seen above, the cells, marked for their quiescent nature, disappeared over time in healthy mice, likely due to their terminal differentiation. Upon crypt damage, however, the same cells generated the tell-tale stem cell ribbons.

Even fully differentiated cells revert to stem cells upon damage

The above examples all reflect the ability of fated stem cell daughters to revert to a stem cell phenotype. Several more dramatic examples of such plasticity involve fully differentiated cells. Located between the stem cells, Paneth cells are terminally differentiated and can live up to 6 weeks. Acute inflammation or irradiation of the mouse small intestine results in a severe loss of Lgr5+ stem cells. Subsequently, Paneth cells acquire stem-like properties, become proliferative, and thus contribute to a regenerative response.[73,74] Based on extensive marker analyses in single-cell RNA sequencing (scRNA-seq) datasets, it was proposed that the enteroendocrine lineage of hormone-secreting cells (EECs), including mature EECs, can also revert to a stem cell state.[75] And, finally, we very recently reported that single terminally differentiated tuft cells, when tested in a human small intestinal organoid setting, can form organoids that contain all intestinal epithelial cell types. Unlike stem and progenitor cells, human tuft cells survive irradiation damage and can regenerate organoids, containing all other epithelial cell types. Accordingly, organoids engineered to lack tuft cells fail to recover from radiation-induced damage. These observations indicate that human tuft cells—known for their role in innate immunity—"moonlight" as reserve stem cells.[76]

Other cells may also fall in this category. Gregorieff and colleagues have defined a revival stem cell (revSC) that is marked by clusterin (Clu).[77] Clu+ crypt cells are extremely rare under homeostatic conditions. Yet, upon intestinal damage, YAP signaling induces the swift emergence of large numbers of revSCs, which then reconstitute the Lgr5+ CBC compartment. The study[77] leaves open the question of whether damage-induced Clu+ cells arise from the pre-existing, minute Clu+ population. To date, no scRNA-seq dataset has been generated to allow cross-comparison of the pre-existing Clu+ cells with damage-induced Clu+ cells. It appears most likely that YAP signaling drives cells from one or more epithelial lineages into a Clu+ proliferative state, particularly in light of a recent flurry of studies describing a key role for a so-called "fetal-like" regenerative response in damaged crypts (see below).

Chromatin plasticity underlies cellular plasticity

Shivdasani and colleagues have studied chromatin states in various mouse crypt cell types in order to understand how stem cell daughters become fated to individual lineages while retaining a plastic state that allows rapid reversal toward an Lgr5+ stemness state. Transcript profiles of Lgr+ intestinal stem cells and their secretory and absorptive crypt daughters confirmed that each cell population was distinct and specified. Yet, both daughter types showed comparable levels of H3K4me2 and H3K27ac histone marks, as well as DNase I hypersensitivity. Enhancers uniquely active in lineage-specific progenitors were already demarcated in LGR5+ intestinal stem cells, revealing early priming for divergent transcriptional programs, while the active histone marks were well-retained after lineage specification.[78] A subsequent study probed how, upon the induced loss of Lgr5+ stem cells, absorptive and secretory populations revert toward an Lgr5+ cell identity. Although active histone marks were distributed similarly between Lgr5+ intestinal stem cells and the progenitors of the two major lineages, thousands of cis-elements selectively open in secretory cells converted to the (in-)accessibility pattern of Lgr5+ cells. These findings revealed how chromatin status underlies intestinal cell diversity and dedifferentiation to restore intestinal stem cell function.[79] In a follow-up study, gene activity and open chromatin were investigated at single-cell resolution using Neurog3-labeled mouse intestinal crypt cells, with a focus on the forward and reverse differentiation of the secretory lineage. Goblet, Paneth, and EECs arise by multilineage priming in common secretory precursors, followed by the opening of thousands of cell-type-restricted cis-elements. Selective killing of Lgr5+ cells induces the rapid reversal of chromatin and transcriptional features in the secretory daughter cells without obligate cell cycle re-entry. The daughter cells appear to simply reverse their forward trajectories without invoking extrinsic signaling programs, and lineage-related chromatin features are erased.[80] This type of reversal and dedifferentiation does not appear to occur in the haematopoietic system; it will be of interest to investigate whether similar levels of chromatin- and specification-plasticity exist in other solid tissues.

One isthmus stem cell to rule them all?

A wealth of experimentation from multiple labs thus supports the consensus that Lgr5+ CBC represent the everyday stem cells that maintain crypt homeostasis lifelong when unperturbed. When these cells are lost upon a biological, chemical, or physical insult, YAP-mediated plasticity can induce a fetal-like state in any crypt cell type to generate new Lgr5+ CBCs.

Two very recent papers in this journal challenge this model.[81,82] At variance with the literature and based on a claim of a higher sensitivity of the applied scRNA-seq strategy, it is stated that Lgr5 is not a specific CBC marker.[81] It is indeed true that low-level expression occurs in rare, fully differentiated

tuft cells (as previously reported by us[67,83]). Yet, histology,[21] single-molecule *in situ* hybridization,[67] lineage tracing,[21] deep bulk-mRNA sequencing of sorted Lgr5 cells,[30] as well as single-cell sequencing by multiple groups (e.g., Murata et al.,[34] Kim et al.,[66] Grün et al.,[83] Banjac et al.,[84] and Biton et al.[85]) all indicate that Lgr5 is an exquisite CBC marker. It is also claimed that the stem cell pool is not regenerated upon damage through the plasticity processes described above. Rather, it is postulated that the top of the crypt (isthmus) would harbor a population of stem cells that sit "above" the CBC cells in the stem cell hierarchy during both homeostatic self-renewal and regeneration. The two studies, unfortunately, do not utilize unique "isthmus cell" markers, essential for a definitive lineage-tracing experiment. *Ki67* and *Lgr4* are used as drivers for lineage tracing.[81] These two genes mark all proliferative crypt cells, including the CBC cells (see Figure 2B for *Lgr4*). *Fgfbp1*—similarly expressed by multiple crypt cell types—is used as driver of the lineage-tracing strategy of Capdevila et al.,[82] while the Lgr5$^+$ cells in this study were ablated in Lgr5-DTR mice by pulsed DTR injection. The appearance of stem cell ribbons upon lineage tracing initiated from the *Ki67*, *Lgr4*, *or Fgfbp1* loci is most readily explained by the extensive crypt cell plasticity described above. Neither of these markers can be used to substantiate a rare population of long-lived stem cells located at the isthmus.

An even more recent study has revisited the stem cell identity of Lgr5$^+$ versus Fgfbp1$^+$ cells, applying single-cell transcriptomic-, chromatin accessibility-, spatial-, and *Lgr5*-based lineage-tracing analyses along the small intestinal tract. *Fgfbp1* is described to be widely expressed, including in Lgr5$^+$ stem cells. It is concluded that the Lgr5$^+$ CBC cells occupy the "apex" of the stem cell hierarchy of the intestinal epithelium in homeostasis and injury, and that plasticity and fetal-like reprogramming represent transient compensatory mechanisms to restore a functional Lgr5$^+$ stem cell population.[86]

Independent of this, there is a general argument against a stem cell that would hierarchically sit above the CBC cell. The offspring of such a stem cell would replace CBC cells during homeostasis over time. This should become evident in long-term lineage-tracing experiments initiated in CBC cells, as the labeled CBC cells would be replaced by unlabeled offspring derived from the hypothetical isthmus stem cell. However, we have consistently observed that homeostatic crypts remain marked for at least 14 months upon tracing from the Lgr5 locus.[87]

A summary of crypt-villus developmental signaling pathways

Paneth cells, as well as specialized crypt-adjacent mesenchymal cells (also known as telocytes or trophocytes, reviewed in Shoshkes-Carmel[88]), together build the crypt stem cell niche. The role of the WNT and BMP signals in crypt biology and in CRC has been extensively documented. FOXL1$^+$ telocytes form a subepithelial network that extends from the crypt bottom to the villus tip and compartmentalizes the production of Wnts, Rspondins, and BMP ligands and inhibitors (such as Gremlins) in such a way that two countergradients are set up. Another subepithelial niche cell type is the lymphatic endothelial cell. Surrounded by Rspondin3$^+$Gremlin1$^+$ fibroblasts and located near the crypt base, these specialized endothelial cells provide Rspondin3 to the stem cells in homeostasis and regeneration.[89]

Wnts and Rspondins

Wnt signals exist in a steep gradient, with their highest activity at the crypt base. Murine (but not human) Paneth cells produce Wnt3,[22] whereas mesenchymal cells located around the crypt produce Wnt2B.[90–92] Wnts are lipid-modified,[93] which limits their diffusion to a distance of no more than one cell radius.[94] Although Wnt3 secreted from Paneth cells can directly engage with Wnt receptors in stem cells, the subepithelial stromal cells are separated from the stem cells by the basement membrane, which is presumably impenetrable by the hydrophobic Wnt lipoproteins. Telocytes project specialized extensions that make intimate contacts with epithelial cells, allowing them to locally deliver Wnts on microvesicles.[95] In a particularly striking experiment, conditional ablation of porcupine (essential for functional maturation of all Wnts) in telocytes resulted in the rapid demise of all intestinal crypts.[96] Frizzled5 is the primary Wnt receptor in crypts.[97]

Secreted Rspondins, produced by a variety of subepithelial cells, potently amplify the pre-existing Wnt gradient in crypts: stem cell maintenance always requires R-spondin activity and cannot be achieved with high WNT ligand levels alone. Indeed, deletion of the Rspondin receptor Lgr4 results in a catastrophic collapse of crypts.[23] Finally, Wnt signaling not only drives the proliferative stem cells compartment, but paradoxically also is key for terminal differentiation and positioning of Paneth cells.[91,98] Wnt signaling drives expression of EphB2 and EphB3 in CBC and Paneth cells. EphrinB1, the counter-structure of the EphB sorting receptors, is expressed on more differentiated cells. The resulting repulsive forces between EphB- and EphrinB-positive cells allow CBCs and Paneth cells to escape the upward flow, restricting their location to the crypt base and creating a tight niche.[99]

The BMP gradient

Two early studies in genetically modified mice pointed to the relevance of a crypt-villus BMP gradient, running counter to the Wnt gradient. Genetic deletion of Bmpr1a in mice leads to an expansion of the stem and progenitor cell populations,[100] whereas transgenic expression of the soluble BMP inhibitor Noggin in the intestine induced the appearance of multiple mini-crypts along the flanks of the villi.[38] Indeed, BMP signals are high in the villus yet are inhibited at the crypt base. Multiple BMPs (e.g., BMP2, BMP4, BMP5, and BMP7) are produced by telocytes and other mesenchymal cells located beneath the villus epithelium,[92,101] while stromal cells (telocytes/trophocytes, myofibroblasts, and smooth muscle cells) surrounding the crypt bottom secrete BMP inhibitors, most notably Gremlin1.[92,102] It was recently shown that sub-cryptal CD81$^+$ PDGFRA^{lo} trophocytes are a main source of crucial canonical Wnt ligands.[103] The localized expression of these Wnts is dictated by the repressive effect of BMP-secreting PDGFRA^{hi} myofibroblasts, which are located higher up along the crypt-villus axis.

High BMP signals drive terminal differentiation of the various epithelial cell types. Villus enterocytes execute the uptake of carbohydrates, peptides, and lipids in a zonated fashion, with the first two functions located toward the villus base and mid-villus, while fat uptake occurs near the villus tip.[104] This zonation (and

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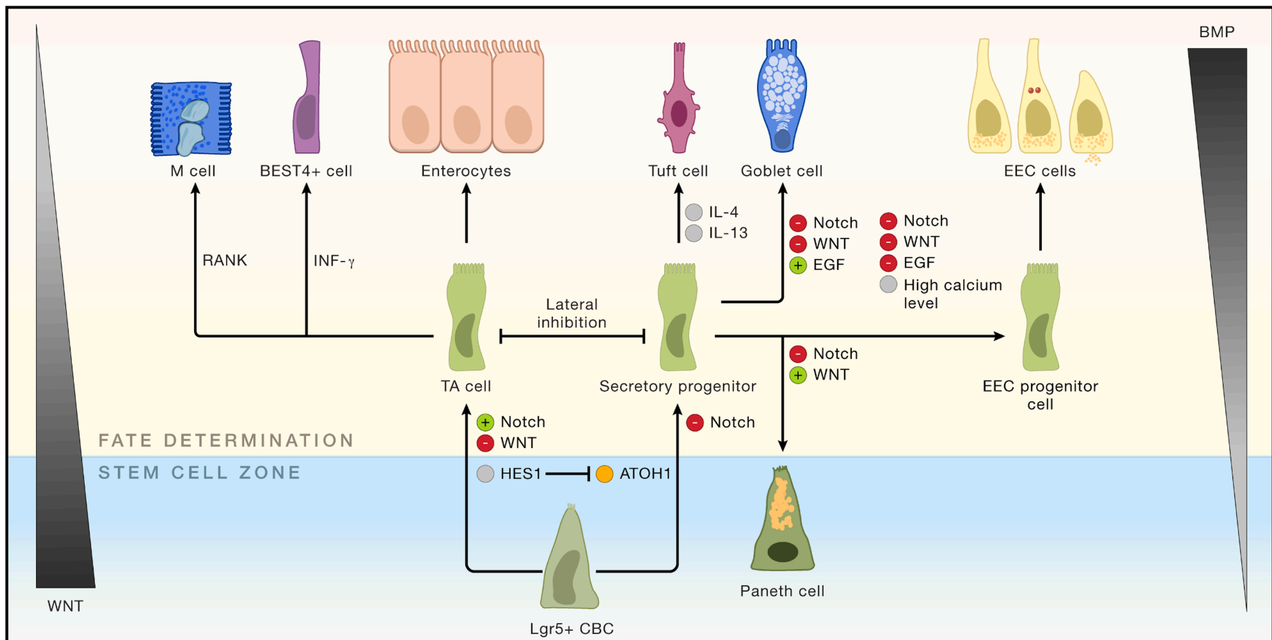

Figure 4. Cell types of the crypt stem cell hierarchy and their fate-determining signals
Notch signaling drives daughter cells through a highly mitotically active transit-amplifying state toward an absorptive fate (enterocytes, BEST4^{+} cells, M cells). Notch/HES inhibition leads to Atoh1 expression, driving daughter cells out of the cell cycle toward a secretory fate (goblet cells, Paneth cells, tuft cells, EECs). The various known signals that set individual fates are indicated in the figure. A Wnt gradient, high at the crypt base, maintains stem cell state and drives Paneth cell fates. A BMP counter-gradient, high at the villus tip, drives terminal differentiation of enterocytes, goblet cells, EECs, and tuft cells.

also that of secretory cells[105]) is set up by the crypt-villus BMP gradient.[106] BMP signals similarly drive a switch in hormone expression within the different EEC lineages when these cells exit the crypts onto the villus. For instance, L cells produce Glp1 in crypts, yet switch to Pyy and Nts upon entering the villus domain.[107]

Notch signaling

The third developmental signaling pathway is Notch, which, unlike the previous pathways, depends on physical contact between a cell expressing Notch receptors and a neighbor expressing Notch ligands. In the crypt, Notch1 and Notch2 are expressed by stem cells and proliferative enterocyte progenitors, whereas the ligands Delta-like1 and Delta-like 4 are expressed on all secretory lineage cells. The wiring of this pathway is simple: active Notch signaling activates expression of hairy and enhancer of split (Hes) transcriptional repressors. Hes1, in turn, represses the transcription factor Atoh1, which is the master regulator of all secretory cells.[108,109] Genetic or chemical inhibition of Notch signaling[32,110–112] causes the induction of Atoh1and the acute conversion of all proliferative stem and progenitor cells into goblet cells. Intestinal stem and progenitor cells thus depend on Notch1/2-generated signals, activated by Notch ligands expressed on neighboring Paneth cells. In the absence of Paneth cells, other secretory lineage cells can provide Notch ligands.[113]

The EGF signaling pathway

The earliest *in vivo* studies describing the mitogenic effects of EGF on rodent intestinal epithelial cells date back to the early 80–90s.[114–116] With the advent of mouse knockout technology, it was shown that germline mutation of murine EGFR led to severe early postnatal abnormalities along the intestinal tract, due to impaired proliferation in the crypts.[117] Mice simultaneously lacking EGF, transforming growth factor-alpha, and amphiregulin (all ligands of EGFR) showed similar proliferation defects in the intestinal epithelium.[118] Abud and colleagues then established an embryonic gut culture system that maintains the three-dimensional architecture of the gut and showed that inhibition of EGFR reduced the proliferation and survival of cells within the epithelial cell layer of the explants.[119] Inspired by these studies, we established EGF as one of the key growth factors for long-term 3D culturing of intestinal organoids.[40]

Several groups have since addressed the activities of EGF family members in organoid culture. Thus, Sato and colleagues improved human organoid cultures based on the expression of tyrosine kinase receptors by crypt cells; their optimized cocktail contains EGF and HB-EGF in conjunction with insulin growth factor (IGF)-1 and several FGFs.[120] Several studies point to neuregulin (NRG1) as the key physiological EGFR ligand. Thus, NRG1 (but not EGF), expressed in stromal cells, macrophages, and Paneth cells, was found to be upregulated upon damage and shown to robustly stimulate proliferation of crypts and support organoid growth.[121] Using advanced single-cell mRNA expression analyses, it was then documented that EGF is expressed in the villus, away from crypts. Instead, a PDGFRA^{HI}/F3^{HI}/DLL1^{HI} mesenchymal population was identified as the source of NRG1. In organoid cultures, NRG1 was superior to EGF in generating a broad diversity of differentiated cell types.[122] In a linked study on human pluripotent stem cell (hPSC)-derived

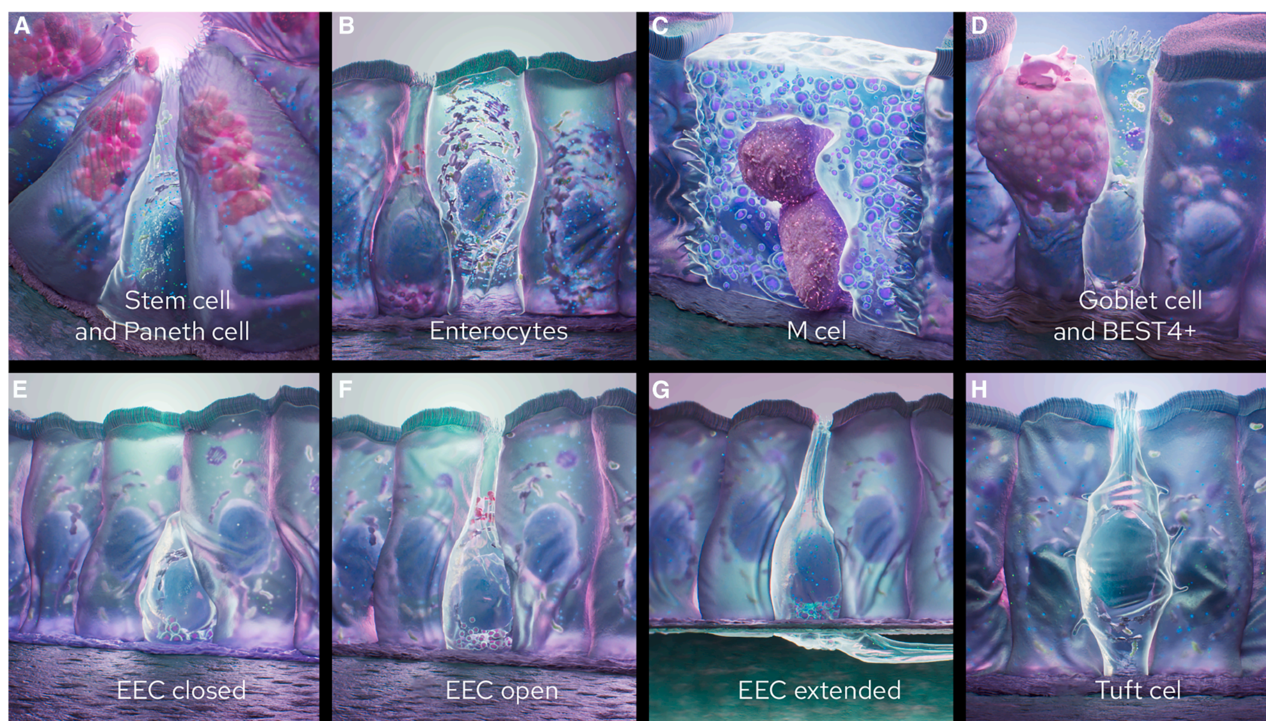

Figure 5. Morphology of the cell types of the crypt stem cell hierarchy
See Box 1 for a short description of each cell type.

intestinal organoids, NRG1 again emerged as a key factor to enhance intestinal stem cell maturation *in vitro*.[123]

Diet and the microbiome

Although beyond the intended scope of this review, it should be mentioned that diet and the microbiome play a prominent role in modulating intestinal stem cell function. For example, research from the Yilmaz lab[124] has illustrated how specific dietary fats promote the growth of beneficial microbial communities, enhancing stem cell regeneration under inflammatory conditions. Another study from this lab has demonstrated that a fiber-rich diet can reshape the microbial ecosystem, thereby optimizing intestinal stem cell responses and promoting epithelial integrity.[125] I refer the reader to an excellent review discussing how dietary components and microbiota metabolites influence microbiome composition, in turn affecting stem cell behavior and tissue regeneration.[126] Another review emphasizes the mechanisms through which gut microbiome metabolites interact with host cellular pathways, providing insights into their protective roles in stem cell maintenance and differentiation.[127]

Signals controlling individual cell fates

Thus, Notch signals control the first developmental decision, between the secretory lineage and the absorptive/enterocyte lineage. The enterocyte fate appears to be the default fate of stem cell daughters. Enterocyte lineage progenitors undergo multiple rounds of cell division during their upward migration, whereas secretory fates appear to be set by the direct conversion of $Lgr5^+$ stem cells just above the crypt base, concomitant with cell cycle arrest.[84] Subsequent signals specify further fate decisions. Within the enterocyte lineage, the migration of precursors over a Peyer's patch exposes the cells to RankL, which induces the M cell fate.[128] The number of recently identified $Best4^+$ cells is increased by interferon-γ;[129,130] yet, the signals driving the actual fate decisions remain unknown. Within the secretory lineage, Goblet cells appear to represent the default state as they appear in massive numbers upon inhibition of Notch signals.[110] Tuft cells require interleukin (IL)-4/-13 for their formation, and they do so in a positive feedback loop: Murine tuft cells express the cytokine IL-25, which triggers resident type 2 innate lymphoid cells to secrete IL-13. This cytokine promotes the *de novo* generation of tuft cells in a positive feedback loop.[131–133] A recent study has shown that the tuft-2 subtype develops from the tuft-1 subtype under the control of the crypt-villus BMP gradient.[134] The transcription factor Ngn3 is the master regulator of all EEC lineages;[135] however, it remains unknown which (if any) signals initiate its expression and, similarly, which signals specify the individual EEC lineages. Paneth cells require Wnt signals in the absence of Notch signals.[98] Although IL-22 was originally proposed to directly activate CBC stem cells to support epithelial regeneration,[136] subsequent studies have failed to confirm this notion.[45,137,138] Rather, IL-22 induces Paneth cell maturation and enforces antimicrobial defense. Of interest, a recent study has shown the existence of a Paneth/goblet cell bipotent progenitor.[139] The signals controlling individual cell fates are summarized in Figure 4, while cell types are described in Figure 5 and Box 1.

Box 1. Differentiated cell types of the small intestinal epithelium

Two main cell lineages are currently discerned in this rapidly self-renewing tissue, the absorptive enterocyte lineage (enterocytes, M(icrofold) cells, and BEST4$^+$ cells) and the secretory lineage (goblet cells, Paneth cells, EECs, and tuft cells). See Figure 6. A short description of each of these cell types is given in this box.

THE ENTEROCYTE

The most populous cell on the villus is the absorptive enterocyte, a highly polarized columnar cell, characterized by an elaborate luminal brush border, which vastly increases the surface area for efficient absorption of sugars, amino acids, fats, vitamins, and electrolytes.[157] It is the only cell type of the small intestine that executes the gut's primary function. Enterocytes also play a critical role in digestion by harboring enzymes on their brush border and contribute to the intestinal barrier by forming tight junctions to prevent harmful substances from entering the body.

THE M(ICROFOLD) CELL

M cells reside in the specialized epithelium that overlies the Peyer's patches, lymphoid accumulations.[158] A pocket inside an M cell can contain a few lymphocytes or dendritic cells. M cells are believed to serve as portals for luminal antigens and have been shown to process and present gluten antigens to T cells.[159]

THE BEST4$^+$ CELL

The newest kid on the block. These rare, slender cells were originally identified by scRNA-seq and are defined by the expression of electrolyte and water transporters and channels, including BEST4, OTOP2 (otopetrin2), and CFTR (cystic fibrosis transmembrane conductance regulator).[160] They do not exist in mice. BEST4$^+$ cells belong to the enterocyte lineage and often sit directly adjacent to a goblet cell. They are primarily involved in regulating luminal pH, hydrating mucus, and secreting electrolytes, and appear to be the major drivers of watery diarrhoea upon bacterial infection.

THE GOBLET CELL

Goblet cells are characterized by a distinct "goblet" or cup-like shape, with a narrow base and a wider, apical portion filled with mucin granules.[161] Their primary function is to secrete mucus, which forms a protective barrier and lubricant layer over the intestinal epithelium, facilitating the passage of food and preventing the adherence of pathogens. Goblet cells also secrete antimicrobial proteins, chemokines, and cytokines, demonstrating functions in innate immunity beyond barrier maintenance.

THE PANETH CELL

The pyramid-shaped Paneth cells are located at the base of the small intestine's crypts of Lieberkühn. They are characterized by dense, apical secretory granules containing lysozyme and antimicrobial peptides (e.g., defensins).[162] They defend the stem cell niche against pathogens through the secretion of these antimicrobial factors, while also providing signals that support the proliferation and differentiation of nearby stem cells.

THE EECs

EECs are rare cells that secrete over 20 different gut hormones in response to changes in the gut environment.[163] In the small intestine, ~6 independent lineages are recognized. Although EECs are polyhormonal cells, they are named with letters and are defined by their main hormone(s): EC cells (enterochromaffin cells producing serotonin), L cells (glucagon-like peptide-1 [GLP1], PYY, NTS), MX cells (ghrelin, motilin), K cells (glucose-dependent insulinotropic polypeptide [GIP]), I cells (CCK), and D cells (somatostatin). EECs come in different shapes and detect nutritional stimuli through a range of nutrient-sensing G-protein-coupled receptors and electrogenic nutrient cotransport, which result in secretion into the bloodstream. Through these secreted factors, EECs modulate pancreatic insulin secretion, levels of satiety and hunger, gut peristalsis, and many other metabolic phenomena throughout the body. Two EEC hormones (GLP1 and GIP) have inspired the development of a rapidly increasing number of weight-loss drugs.

THE TUFT CELL

Tuft cells are bottle-shaped chemosensory cells that act as immune sensors and effectors.[164] The recently identified tuft-1 and tuft-2 cell subtypes have differential functions: tuft-1 cells are associated with a neuron-related transcriptional program and may mediate interactions with the nervous system, whereas tuft-2 cells exhibit an immune-related program, secreting immune-activating molecules, such as TSLP and IL-25. Tuft-2 cells arise from tuft-1 cells in the high BMP signaling environment on the villus.[134]

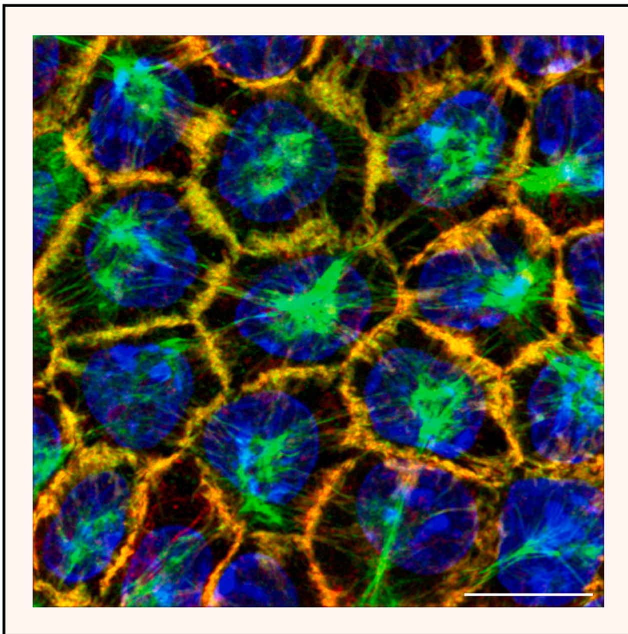

Figure 6. Basal actin forms a multicellular star-shaped network in enterocyte monolayers

Confocal Z-projection showing F-actin (green, phalloidin-Alexa Fluor 488), membranes (red, villin-tdTomato), and nuclei (blue, Hoechst 33342), imaged at the basal surface. Scale bar, 10 μm. Image courtesy of the Delacour Team, Institut de Biologie du Développement de Marseille (IBDM) from Barai et al.[155]

The fetal-like regenerative state

The endoderm-derived gut epithelium undergoes massive expansion in the third gestational week in mice, after which proliferative crypts and differentiated villi are formed, just days before birth. In Tcf4 knockout mice, this expansion occurs normally, yet all proliferation halts when crypts should appear (a few days prior to birth).[15] Apparently, expansion of fetal intestinal epithelium is Wnt-independent, whereas proliferative crypts require Wnt signals. In agreement with this, fetal organoids do not require Wnt-amplifying R-spondin1 for their growth.[140,141]

Fetal reversion of adult epithelium was first reported in the intestines of mice infected with the worm *Heligmosomoides polygyrus*.[142] Crypts close to worm-induced granulomas downregulate the expression of canonical stem cell markers like Lgr5 and Olfm4, while upregulating genes like Ly6a/Sca1, Il33, Gja1, Spp1, Tacstd2, and Sprr1, typically enriched in organoids grown from fetal gut.[140,141] A key driver of this granuloma-induced state is interferon-γ.[142] Similar observations were made in dextran sulfate sodium (DSS)-treated mice, a model for inflammatory bowel disease.[143] Again, the fetal-like markers (e.g., Ly6a, Anxa1, and Tacstd2) were upregulated, which was subsequently also noted in tissue samples from ulcerative colitis patients. This response was noted in multiple other damage states[77,80,144,145] (reviewed in Viragova et al.[146]). There has been some discussion as to whether the term fetal-like describes this damage response state well, since multiple genes in this signature are expressed in healthy adult gut epithelium. For convenience, this review uses the term "fetal-like regenerative state."

Which signaling pathways drive this state? Klein and colleagues have written an excellent recent review on the fetal-like generation and the factors that drive this process.[146] YAP/TAZ, the transcriptional mediators of the mechanosensing Hippo signaling pathway, were observed to be activated (i.e., YAP displayed nuclear localization) in the regenerating epithelium. Culturing small intestinal organoids in collagen I resulted in a fetal-like spheroid phenotype *in vitro*, expressing Sca1 and Anxa1.[143] After radiation damage, regenerating crypts display high levels of nuclear YAP, leading to expression of YAP targets such as Clu (see above), Il1rn, and Areg, genes that are often co-expressed with the fetal culture markers described above.[77] Overall, these observations are consistent with data demonstrating that activation of YAP is required for the maintenance of the fetal epithelial state and that its expression is transiently required in intestinal development and regeneration.[147–151]

Other factors that have been described to be involved are interferon-γ (see above), TGF-β, prostaglandin E2, and NRG1 (reviewed in Viragova et al.[146]). Asporin, an ECM-bound proteoglycan produced by mesenchymal cells, also appears as a significant player through its action on epithelial TGF-β signaling. It is transiently increased upon chemotherapy-induced damage and is key for the induction of the fetal-like state and thus for tissue regeneration.[145]

Taken together, the regenerative response of intestinal epithelium represents a state that differs from the homeostatic self-renewal state, in that it is not driven by Wnt and does not involve cells marked by Lgr5^{+}. Rather, the fetal-like regenerative state appears primarily driven by YAP signaling, involving cells expressing Clu, Sca1, and other marker genes that were first noted in organoids grown from fetal intestine.

Cell death at the villus tip

It has long remained elusive what drives villus tip cells to slough off. The process is unaffected in mice carrying mutations in cell death genes, such as *Bcl-2*, *Bax*, *Bcl-w*, and *caspase-3/7/8* (reviewed in Krueger et al.[152] and Pérez-González et al.[153]). Together with evidence from human colon showing that most extrusions are non-apoptotic,[154] a focus on live cell extrusion developed. In Madin-Darby canine kidney cell monolayers, zebrafish epidermis, and the *Drosophila* notum, extrusion is driven by increased cell density. These findings have inspired a model for the mammalian intestinal epithelium, in which the flow of cells to the villus tip leads to cell crowding. We have recently reported a very different, epithelial tension-based mechanism, which involves a local "tug-of-war" competition between contractile cells at villus tips.[152] By combining quantitative live microscopy, optogenetic induction of tissue tension, genetic perturbation of myosin II activity, and local disruption of the basal cortex in mouse intestines and intestinal organoids, we observed that a dynamic actomyosin network generates tension throughout the intestinal villi, including the villus tip region. This is supported by high-resolution studies showing star-shaped, basal actomyosin structures that place epithelial cells under tension and ensure epithelial connectivity[155] (Figure 6). Mechanically weak cells, unable to maintain this tension, are extruded. In an independent study, Trepat and colleagues reported a very similar mechanism, in which extrusion is initiated by the sudden

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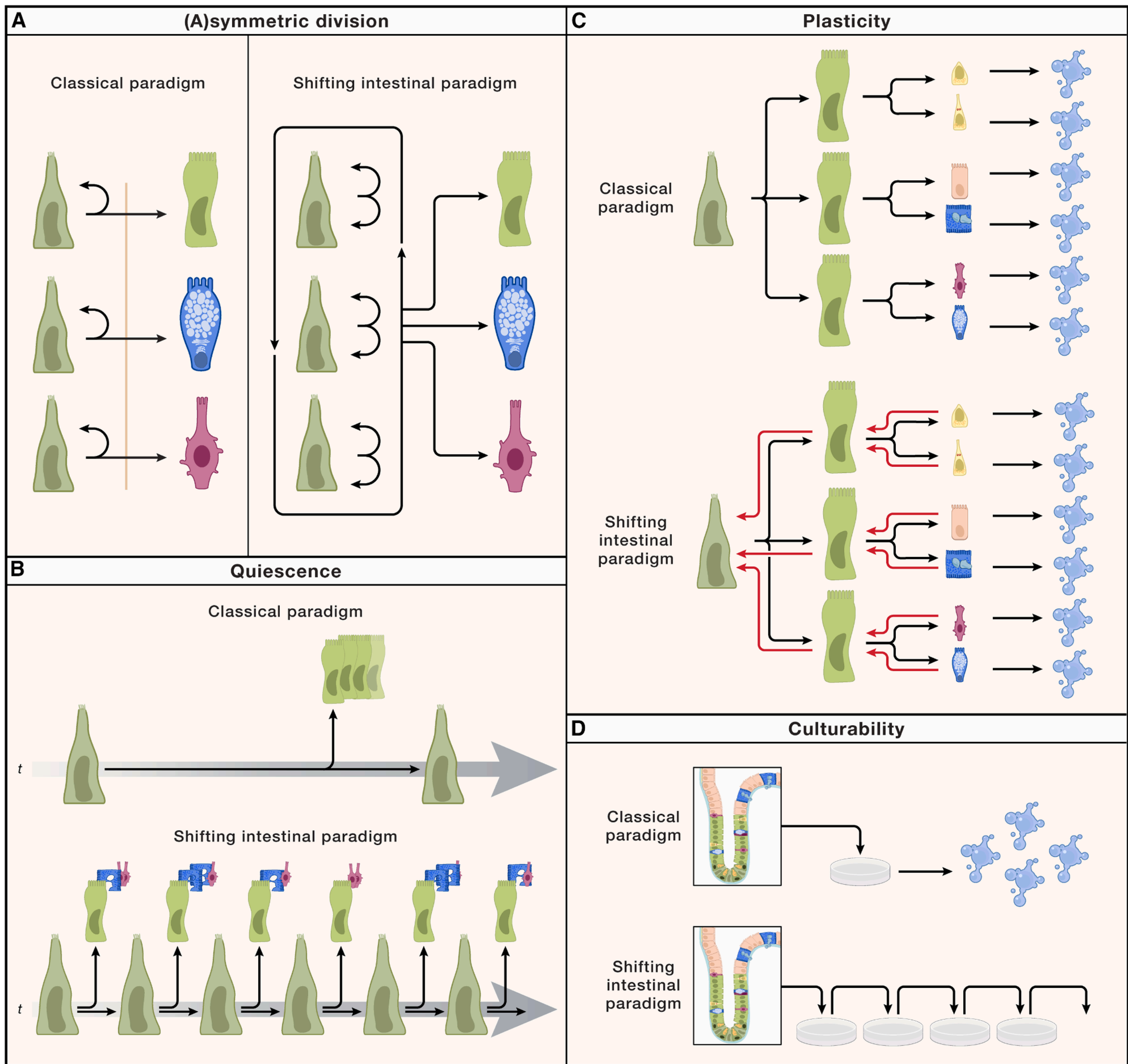

Figure 7. Shifting paradigms in tissue stem cell biology

(A) Classical paradigm: stem cells divide asymmetrically, which keeps the size of the stem cell population fixed: Each division yields one new stem cell and one daughter cell. When a stem cell is lost, another stem cell needs to shift to a symmetric division mode to replace its lost neighbor, after which the stem cell switches back to asymmetric division.

Intestinal paradigm: stem cells divide symmetrically. They are in continuous "neutral" competition for niche occupancy. The size of the niche decides the size of the stem cell population. Lost stem cells are automatically replaced, whereas supernumerary stem cells are pushed out of the niche and become daughter cells.

(B) Classical paradigm: stem cells are quiescent. They divide rarely and can be identified because their quiescent state allows them to retain DNA labels over long time periods.

Intestinal paradigm: stem cells divide continually. While it is generally believed that this increases the risk of somatic mutation, this is not confirmed by comparison of quiescent liver with gut stem cells.[35]

(C) Classical paradigm: plasticity of daughter cells can not generate stem cells. Stem cells are hardwired. All arrows in stem cell hierarchies point toward the terminally differentiated states.

Intestinal paradigm: plasticity of daughter cells is widespread. Stem cells are easily recognizable, yet progenitor or even differentiated cells can revert to stemness under regenerative conditions.

(D) Classical paradigm: stem cells cannot be expanded in culture. This notion still holds for hematopoietic stem cells and has long hindered the establishment of *in vitro* culture approaches. Related to this are the notions that there is a limit ("Hayflick") to the number of cell divisions, that telomeres invariably shorten with each division, and that senescence and stem cell exhaustion are inevitable.

Intestinal paradigm: stem cells can be expanded seemingly indefinitely when cultured in the appropriate environment. Telomers do not shorten, cell cycle times remain constant over long-culture periods, while stem cell exhaustion or senescence are not observed in organoid cultures.

dissolution of a contractile myosin 2A meshwork triggered by a calcium influx. The extruding cell and its neighbors generate an upward traction force that requires myosin contractility, but is generated by lamellipodial protrusions in neighboring cells, which are responsible for the directionality of the extrusion.[156] Both studies show that intestinal cell extrusion involves a unique mechanism that relies on tension rather than crowding, does not require a convex curvature, and depends on myosin.

Epilogue

In recent years, the intestinal crypt has become a model system that reshapes our understanding of adult stem cell biology. A detailed map of the crypt hierarchy has emerged, encompassing the identities of stem and progenitor populations, their modes of division, and the differentiated lineages they sustain. Strikingly, this hierarchy deviates in multiple respects from traditional models, which are mostly derived from hematopoietic stem cells (see Figure 7). Intestinal stem cells are not quiescent but continually proliferate; they do not rely on asymmetric divisions but instead divide symmetrically and undergo neutral competition for niche occupancy. Furthermore, plasticity pervades the system, with progenitor- or even differentiated cells capable of reverting to stemness under regenerative conditions. And finally, intestinal stem cells can be expanded indefinitely *in vitro* in the form of organoids.

Cancer biology has echoed these insights. Intestinal neoplasms co-opt the same signaling pathways that crypt stem cells rely on for their normal function: activation of Wnt and EGFR signaling, and inhibition of BMP signaling, are hallmarks of both normal renewal and malignant transformation. It has been a surprising discovery that the rules of growth and self-renewal can be harnessed *in vitro*. The advent of human intestinal organoids—a long-term culture system starting from adult stem cells—is now complementing genetic mouse models, enabling mechanistic studies, disease modeling, and translational applications.

Despite this progress, fundamental questions remain unresolved. In particular, the functions of the rare epithelial cell types—BEST4$^+$ absorptive cells, tuft cells, Paneth cells, and M cells—are incompletely understood. Although their developmental origins and plasticity are beginning to be charted, these cells are not typically seen as immune cells. Their physiological contributions to host-microbe interactions and immune surveillance may inspire new approaches to incompletely understood conditions such as Inflammatory Bowel Diseases or chronic bowel infections. Similarly, research on EEC biology is growing rapidly and will increasingly play a role in the understanding and treatment of metabolic diseases.

The paradigm-shifting principles that have emerged from the intestinal crypt are readily applicable to stem cell hierarchies in other organs. Meanwhile, crypt research has been providing experimental tools that are similarly translatable to research on other organs. Organoid technology is emerging as an *in vitro* experimental platform for studying human biology, which complements animal research. The crypt continues to pose critical questions, particularly regarding the integration of the rare epithelial cell types into human tissue physiology and pathology. Addressing these gaps will be essential to achieve a comprehensive understanding of the intestinal epithelium in both health and disease.

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DECLARATION OF INTERESTS

H.C. is a member of the scientific advisory board of *Cell*. H.C. is an inventor on multiple patents related to organoid technology. His full disclosure is given at www.uu.nl/staff/JCClevers/Additionalfunctions. H.C. currently holds no advisorships to commercial entities, yet holds stock in Roche, in D1Med (Shanghai), in Xilis, and in Surrozen.

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