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Understanding epithelial homeostasis in the intestine

An old battlefield of ideas, recent breakthroughs and remaining controversies

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Abbreviations: BP, basal process; CBC, crypt base columnar; COD, common origin of differentiation; Cp, cell position; DBA, Dolichos biflorus agglutinin; DOM, daughter of Mix; GLC, goblet-like cell; LOBA, longitudinally oriented basal asymmetry; LRC, label retaining cell; OCD, oriented cell division; PC, Paneth cell; SC, stem cell; SI, small intestine; TA, transit amplifying

The intestinal epithelium constitutes the barrier between the gut lumen and the rest of the body and a very actively renewing cell population. The crypt/villus and crypt/cuff units of the mouse small intestine and colon are its basic functional units. The field is confronted with competing concepts with regard to the nature of the cells that are responsible for all the day-to day cell replacement and those that act to regenerate the tissue upon injury and with two diametrically opposed models for lineage specification. The review revisits groundbreaking pioneering studies to provide non expert readers and crypt watchers with a factual analysis of the origins of the current models deduced from the latest spectacular advances. It also discusses recent progress made by addressing these issues in the crypts of the colon, which need to be better understood, since they are the preferred sites of major pathologies.

Introduction

The simple epithelium lining the gut is organized into millions of contiguous crypts of Lieberkühn[1] organized in crypt/villus and cypt/cuf units in respectively the small intestine (SI) and the colon. It is at the same time one of the most important tissue barriers in the body, the place of efficient absorption of nutrients and water and one of the most actively renewing tissues. The control of cell-cell adhesion during cell migration, division and morphogenesis is crucial for its maintenance in health, disease and regeneration.[2] The homeostasis of these remarkable stem cell (SC) driven multicellular proliferative units[3] requires the regulation of gene networks, signaling pathways and many dynamic processes.[4-7] Much of the knowledge on the biology of this tissue available today stems from works done on the crypt/villus unit of the mouse small intestine (SI). This review focuses on three fundamental questions. (1) What is the identity of the cells that can be attributed the characteristics of stemness and where are they located, (2) at what level in the stem cells > progenitor populations hierarchy, lineage specification is initiated and (3) to what extent and how are cell fate decisions timed in relation to the pattern of cell divisions? A special question addressed is: what can be learned from studies on the crypts in the colon? These crypts indeed display important differences in their cell kinetics and topological organization of SC populations and are the major site of carcinogenesis in human.

The intestine is now one of the organs where studies are the most active and competitive, also because of the gravity and incidence of the pathologies it develops and the hopes put in both preventive and regenerative medicine.[8]

For non-expert readers, we will first provide insight into key aspects of the architecture of a crypt of Lieberkühn in the SI and how this is investigated. We will also define some important notions with respect to cell stemness.

The Crypt, a Multicellular Proliferation Unit with a Tight Hierarchical Organization Under Steady State Conditions

Each crypt has a tubular part with a more or less constant length and width and is closed at the bottom by the semi-circular crypt base.[3] It contains ~250–300 apico-basally polarized epithelial cells. At their upper limit, the lumens of the crypt and the gut tubes communicate. Up to 10 crypts merge into fingerlike villi. This can be seen very nicely by scanning electron microscopy[9] on sheets of intact epithelium isolated by EDTA perfusion (see Fig. 4 in 10). The crypts are embedded in mesenchymal connective

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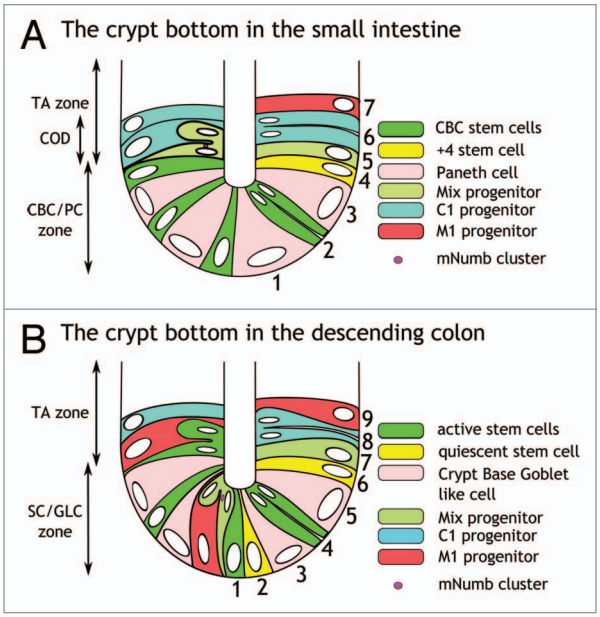

Figure 1. Topological organization of epithelial cells near the crypt bottom of crypts. (**A**) In the SI, the CBC/PC zone contains Paneth cells interspersed with actively proliferating CBC SCs. The latter divide symmetrically to yield two CBC cells. In positions around +4, more quiescent SCs are found. The light green cells are Mix progenitors which are CBC cells that have lost contact with the signals maintaining stemness. The dividing Mix cell displays anisotropic movement of the upper daughter. It also displays asymmetric distribution of a cluster of mNumb vesicles located in the daughter that has maintained a basal process with the extracellular matrix. (**B**) In the descending colon, actively proliferating SCs (vacuolated cells), quiescent Lrig1(+) SCs, crypt base Goblet-like cells, Mix and early C1 and M1 progenitors are all intermingled in the SC/GLC zone. The dividing dark green SC is represents the proposed mechanism by which anisotropic movement of the upper daughter places it outside the reach of the GLC its mother was contacting. This daughter will give rise to a Mix progenitor. This mechanism may also be used by CBC cells at the border between the CBC/PC zone and the OCD in the SI crypt. The dividing Mix progenitor near the crypt bottom is another example of the asymmetric distribution of transient mNumb clusters near the cleavage furrow that may initiate the symmetry breaking leading to lateral Delta-Notch inhibition between sister cells.

tissue separated from the latter by a basal lamina, rich in several species of extracellular matrix molecules,[11,12] and surrounded by a sleeve of myofibroblasts.[13] The gut epithelium is continuously and rapidly renewed. Around 10^9 new cells (~1 g) are produced and die every 5 d![14] This renewal is driven by multipotent intestinal SCs which comprise a small number of relatively slowly (average cycle time, 24–32 h) and about 15 frequently cycling cells (average cycle time, 12–16 h) at or near the crypt base.[1,15,16] They commit to and differentiate into three postmitotic epithelial lineages: the columnar, making up more than 90% of the cells, and the secretory/granulocyte comprising the mucous, enteroendocrine, brush/tuft and Paneth cells (PCs).[8,16-19] The SCs, PCs and a few other differentiated cells, are positioned at the lower cell positions of the crypt where the PCs survive for about 8 weeks[20] (**Fig. 1A**). PCs are major actors in innate immunity of the intestine by secreting antimicrobial peptides and the Cryptdins/Defensins and Lysozyme enzymes.[21] Slender, undifferentiated and proliferating Crypt Base Columnar cells (CBCs) are found interspersed between the PCs.[22] Most of the SC progeny migrate in columns from their site of origin upwards, at a velocity of around 1 cell diameter per hour.[23] The upper parts of crypts thus usually contain cells in various stages of differentiation and increasing age, each cell being linked to an ancestral "ultimate" SC.[1] By the time they leave the crypt, they are fully differentiated and continue their journey up to the top of the villi. The rate of programmed death at the end of the trajectory, after barely 2 to 3 d, dictates the size and complexity of the crypt/villus axis.[8,15-17] Crypts themselves undergo a cycle, since they multiply by crypt fission, starting at the crypt base. In mice, its frequency is high after weaning and becomes infrequent with aging.[24,25]

Positional Analysis of Individual Cell Parameters and the Delineation of Zones

Techniques for analyzing the organization of the crypts play an important role in the field. Workers often use histological or optical sections obtained from confocal microscopy or Nomarski optics passing through the center of the longitudinal crypt axis to view the crypt as two cell columns of hierarchical lineage starting from cells positioned at or near the crypt base. This enables positional analysis of certain cellular capacities like performing DNA synthesis, undergoing mitosis and apoptosis, responding to injury,[16,26,27] or expressing genes.[15,18,28] Experiments using early time intervals after labeling cells in S phase were used to measure migration velocity and its variation with cell position. This showed that the extrapolated origin of this migration is about 4 cells up from the crypt base.[29] Bjerknes and Cheng referred to this zone as the COD (Common Zone of Differentiation). The positions above the Paneth cells where the bulk of the proliferation takes place is called TA zone (Transit Amplifying).[30] It contains the COD but extends further upwards. Most workers number the lowest cell in the column 1 and simply increment from there on upward. The Clevers lab names the first cell in the column 0 and uses 1, 2, 3 etc. for the Paneth Cells and 1', 2', 3' etc for the CBC cells.[31] As this may lead to some confusion, we will adopt here the common numbering system and use the terms CBC/PC zone, COD and TA zone when referring to topological aspects of crypt organization (**Fig. 1A**).

Stemness and Plasticity in the Gut Epithelium

Over the last decades, the field of adult SC biology has focused on the definition of what are features that define degrees of stemness of cell populations and when these become clonogenic progenitors, which are cells that have committed to differentiation but can replace SCs upon demand such as upon ablation of a SC population.[1] Potten and Loeffler have discussed in great detail the difficulties associated with defining what is an "actual" SC, the "SCs that are responsible for all the day-to day cell replacement".[17] They concluded that SCs must be defined by their function, and not by morphology or a gene expression profile and proposed that stemness is not necessarily a single property, but a number of properties or options which such a cell could execute depending

on the circumstances. These properties were defined as follows: the cell must be undifferentiated relative to the other epithelial cells in the tissue, capable of proliferation and self-maintenance, producing many differentiated progeny and regenerating the tissue after injury. It must also retain the ability to switch between these options when appropriate, meaning that at steady-state, the SC population may actually consist of apparently distinct cells that form a cell continuum, a concept now well established for hematopoiesis in the bone marrow.[32] Self-maintenance is a crucial ability of SCs to maintain their own population numbers, an important parameter of tissue homeostasis. This raises the issue of their mode of division, which under steady-state must comprise a degree of asymmetry so that on average, the probability of one daughter becoming a stem cell or going on to differentiate is 0.5. As will be discussed in this review, the way this asymmetry is obtained in the intestinal crypt is one of the most debated issues in the field. One school of thoughts proposed a major role for SC divisions asymmetric with respect to cell fate.[17] Recently, however, it has been shown that the majority of the SCs divide symmetrically with respect to cell fate and that maintenance is assured stochastically at the population level so that on average, each SC that is lost, is replaced by symmetric division of a neighboring one.[33]

In recent years, great progress has been achieved in understanding SC biology,[34,35] thanks also to insights provided by different model systems like hematopoiesis in the bone marrow,[36] hair follicles,[37] muscle,[38,39] skin,[40] germline cells,[41] and the development of the central nervous system[42,43] which include the equally rich fields of stem cell biology in invertebrate models.[44-46]

The Quest for Cells that can be Attributed the Characteristics of Stemness: The Undulating +4 Stem Cell Annulus vs. the Stem Cell Zone Hypothesis

In this section, we will review the groundbreaking studies initiated in the labs of Potten and the late Leblond, and more recently, in the lab of Bjerknes and Cheng. We will then summarize the works that have permitted to qualify as of now the Lgr5(hi) CBC cells as the "engines" of crypt self-renewal,[47] but also introduce and comment the ongoing debate on markers for the more quiescent cells of the +4 annulus (see below).[17,48,49] For a comprehensive review of the state of the art until 2005, see,[50] and for reviews of the same just after the discovery of the first markers for intestinal SCs, see.[6,51] Qualifying the field an old battlefield of ideas is not exaggerated, and the battle goes on today.

The Potten lab has extensively used selected longitudinally sectioned crypts to record information on a cell positional basis on markers for proliferation, differentiation, regeneration upon injury and death to try and define where the intestinal SCs were most likely located (summarized in 1). The first has been to measure the cell migration velocity at each position in the crypt and to determine the point of origin for all this movement. The apparent upward movement of Tritium-labeled cells was used to measure cell migration velocities. This produced graphs showing changing velocities with increasing position up the crypt whereby the best fitting line determined the "back extrapolate for all cell migration." This indicated that its origin was at cp (cell position) 4.9 ± 0.2, (of note: later corrected to cp 2–5[52]) and it was assumed that this was where the SCs were to be located (see Fig. 1E in 1). They then associated various parameters with the presumptive SC position. Thus, some cells there (1) had a slower cell cycle (24 h), (2) were specifically involved in regeneration following injury (see below), (3) exhibited a low level of spontaneous apoptosis and (4) died via a rapid form of apoptosis, 3–6 h upon a very low radiation dose (0.63 Gy whole body radiation). Their most characteristic approach was to use a range of increasing whole body radiation doses to successively ablate cells with decreasing radiation sensitivity and determine the cell positions at which apoptosis occurs. This led to the detection of 3–6 cells, largely confined to the crypt base, that belong to the most radiation sensitive cells of the body![53] When irradiated, they die by apoptosis, without leading to crypt destruction. Based on other data, the number of SCs was estimated to be around 20, and it was hypothesized that if they were SCs, they would be easily replaced by less radiosensitive SCs. The sum of his findings collected over almost two decades led Potten to propose a hierarchical SC compartment with three tiers (see Fig. 4D in 1). At the base of this hierarchy there are four to six "ultimate" lineage ancestor SCs. If these cells are all killed by a very low dose, their immediate, much more radioresistant daughter cells, can replace them and maintain the crypt. If these first two tiers of cells are killed by a much higher dose of radiation, there is a third tier of up to about 24 cells with even greater resistance that again can regenerate the earlier stem cell tiers, the crypt and the epithelium. So, about 36 cells were proposed to be actual SCs and potential, clonogenic SCs which are located in an undulating annulus covering cp 2–7, on average around cp +4. The remaining 120 proliferating cells possess no clonogenic SC properties. Potten and Loeffler subsequently refined this model to propose a cork-screw or spiral one, to take into account the options cells have at the population level, for example self-maintenance against commitment (see Fig. 4 and 5 in 17). More recently, the Potten and Epstein labs showed that some of the cp +4 cells were label retaining cells (LRCs)[54,55] which were cycling (shown by double BrdU and autoradiography labeling) and can commit suicide by apoptosis upon DNA damage. They showed pictures suggesting that when dividing, they asymmetrically segregate the template DNA strands.[54]

Taking a different approach, the Leblond lab applied radioautography of tritium-labeled cells to optimally oriented semi-thin sections, cut through the center of the crypt axis, of tissue fixed at various time points after administration of a single or continuous administration of tritiated thymidine.[56] The thinness of these sections was a crucial innovation, since it allowed very accurate allocation and counting of the silver grains to a given cell and to clearly detect the CBC cells, which was less the case in the 5 μm thick paraffin sections used in the Potten lab. It was observed that these have a higher capacity to phagocytose the few cells that died upon ingestion of tritiated thymidine, and this was used as a primitive tracing technique to follow their fate. Twelve hours after administration, several midcrypt cells of all the gut types were found to contain ingested radioactive debris, thus providing

first evidence in favor of them being the descendants of the CBC cells. This gave rise to the concept of the Unitarian Origin of the four intestinal epithelial cell types, namely that CBC cells are multipotent SCs.[22] Adding to this technique precise positional cell type assignment analysis of cells, Bjerknes and Cheng found that (1) the only cells to cycle in the cp 1–5 were the roughly 14 CBC cells, (2) all their differentiated offspring originate in cp 5–8, whereby most cells migrate upwards, but all PCs, some columnar, and a few mucous and enteroendocrine progenitors migrate downward into cp 1-4 while differentiating and losing their proliferative ability, (3) importantly, this and not SC differentiation in situ, results in the presence of differentiated cells in cp 1-4.[30] Cp 5–8, were therefore defined as "Common Origin of Differentiation" (COD) and cp 1-4 the SC zone. According to this Stem Cell zone hypothesis, all the CBC cells were predicted to be equivalent SCs which persist as such throughout life. Accordingly, they deduced from this that the microenvironment of this zone does not induce CBC cells to differentiate, but is at the same time compatible with differentiation of other cell lineages. Although they did not use terminology like "actual" SCs, it is very likely that they considered the CBC cells as the SCs that are responsible for all the day-to day cell replacement. The COD and the +4 annulus overlap in space, the latter extending into lower cp.

The proposal that CBC cells could be "actual" SCs, was challenged by Potten and Loeffler.[17] These authors noted that they rarely appeared as vertical pairs. Because of that, they proposed that instead, they may be part of the PC lineage or even represent an entirely separate population of SCs: that for the PCs. They also noted that CBC cells do not apparently displace functional PCs to higher positions in the crypt. So, they argued that, unless the CBC cells possessed some remarkably versatile movement abilities, they could not be the origin of the predominant crypt columnar cells. In fact, a pair of CBC cells seen in an EM thin section is visible in **Figure 1N** in reference 57 and Bjerknes and Cheng had also deduced from their data that the migration of CBC cells was likely "turbulent" in nature.[58] Direct evidence for this was later provided by the Clevers lab in time-lapse videos of CBC cells expressing GFP![59] Yet, as detailed in,[51] the initial proof from Cheng and Leblond and from Bjerknes and Cheng that CBC cells are the SCs was somewhat weak. First, since their approach did not detect the rarer putative SCs in the +4 annulus, some of their observations could have been interpreted differently if they had done so. Second, because the ingested cell remnants did not contain any type of granules, Leblond assumed that they were from killed CBC cells, but recently, it has been shown that CBC cells are rather radioresistant.[60] It is at least as likely, if not more, they were Potten's hypersensitive cp +4 actual SCs! Since the injected tritiated thymidine induced some injury, there is some concern about the consequences of this perturbation on the interpretation of the observations.

Bjerknes and Cheng, subsequently developed an original clonal cell tracking technique, in order to test their hypothesis that CBC cells can give rise to all the intestinal cell lines.[61] The *Dlb-1* locus generates an intestinal binding site for the Dolichos biflorus agglutinin (DBA) lectin and the mutagen, N-nitroso-N-ethylurea (NEU) induces random genetically marked clones of DBA-positive cells in epithelia of Dlb-$1^{-/-}$ SWR mice whose cells are otherwise unlabeled by DBA.[62] They applied this to the SI epithelium using isolated intact crypt/villus preparations,[9] which had two crucial advantages: they provided a large sample of crypts (thousands) so that statistical analysis became possible, even of rare incidences, and they offered superior cytological detail and accurate cell positional information by Nomarski microscopy.[10] Starting three days after NEU treatment and at intervals up to 154 d, they analyzed in crypts labeled with peroxidase-labeled DBA the position, composition with respect to cell types and longevity of the observed DBA+ cell clones. This yielded graphs showing the dynamics of clones. After 3 d, they detected DBA+ clones in 3 to 5% of the crypts the majority of which were extinct within 14 d, suggesting mutation of short-lived committed differentiating cells. Those persistent over months contained either columnar cells or mucus cells (see below) although a small number of clones were mixed. They observed that 90% of the latter included mutant CBC cells in cp 1–4 (for an example, see **Fig. 3** in 10), thus providing the first evidence in favor of the multipotent SC nature of the cycling CBC cells and their SC zone hypothesis.[30,58]

Clevers judged that "unfortunately with this method, it was not clear which cell sustains the first clonal mutation"[6] and his group moved forward to try and identify genetic markers for intestinal SCs. They started this search by showing the relevance of canonical Wnt signaling which instructs intestinal cells to adopt a proliferative progenitor phenotype and functions as a master switch controlling proliferation vs. differentiation in the intestinal epithelium.[63,64] They then went on performing experiments leading to the definition of the Wnt-target gene program and identified a module of 17 genes that were specifically expressed at the position of SCs, distinct of the PCs, notably including *Lgr5*.[65] Direct proof that CBC cells display several stemness properties was obtained by the use of this marker in genetically marked lineage tracing[31] and by cell sorting, culturing and genetic profiling experiments (reviewed in refs. 6, 15). Recently this culminated in defining a definitive *Lgr5* intestinal stem cell signature by using improved cell isolation and state of the art transcriptomic and proteomic techniques.[66] This supported and extended the concept of the SC zone and of the CBC cells as a pool of clonogenic intestinal SCs during normal gut homeostasis. The potential of the CBC cells is highlighted by the fact that a single fluorescently sorted CBC cell is capable of producing in vitro intestine-like organoids which reproduce the typical organization comprising compartments of proliferation (CBC/PC and TA zones) and differentiation, providing that cell culture is performed in the presence of a precise mix of activators and inhibitors of signaling pathways.[59] CBC cells strongly express *Lgr5*, now known to be the G protein coupled R-Spondin 1 receptor,[8,67] a direct Wnt/Tcf-4 target gene,[65] which amplifies their response to Wnt ligands (reviewed in ref. 6).

In hindsight, it is remarkable that, because Potten's considerations were widely accepted, it was not until very recently that the CBC cells were considered good candidates for being self-maintaining multipotent SCs.[67] Notwithstanding, a group of workers expressed skepticism on the notion that CBC cells are

the "actual" SCs of the SI crypts.[51] They argued that in the +4 annulus, there are enough Lgr5(+) cells that could be part of the three tier SC hierarchy they championed.[51]

This skepticism was also based on reports proposing that beside the actively cycling CBC cells, less numerous, relatively quiescent cells expressing a series of genetically defined markers are located in the +4 annulus and contribute to the production of progeny, possibly upstream of CBC cells.

We will first review these papers and then those of other groups casting doubt on this concept and conclude by considering possible reasons for this serious controversy.

The first marker was *Bmi1*, a member of the polycomb-repressing complex 1 (PRC1) family which has an essential role in maintaining chromatin silencing and was known to be involved in the renewal of SCs in other tissues.[68] *Bmi1*(+) cells, visualized as for *Lgr5* by a reporter (LacZ) driven by the *Bmi1* locus are much rarer then CBC cells and more prevalent in the duodenum. They are cycling, but significantly less actively then CBC cells,[49] display self-renewal and give rise to all the cell lineages of the SI epithelium. Targeted toxin-ablation of *Bmi1*(+) cells led to crypt loss, which was interpreted as indicating that they are crucial for crypt maintenance. In a follow-up study,[49] *Lgr5*(+) CBC and *Bmi1*(+) cells (visualized by β-Gal expression) were shown to display striking differences in the functional contribution to progeny generation under steady-state and response to canonical Wnt modulation, both to the advantage of CBC cells. Whereas a high 12 Gy whole body radiation led to almost complete loss of CBC cells and had eradicated their progeny 4.5 and 7 d after irradiation, most *Bmi1*(+) cells survived and were induced to cycle actively and expand 5-fold by 4.5 d after irradiation. In vitro, isolated *Bmi1*(+) cells are multipotent and give rise to CBC cells in organoids. From these studies, the Capecchi and Kuo labs concluded that *Bmi1* marks quiescent, injury-inducible reserve intestinal SCs that exhibit striking functional distinctions from *Lgr5*(+) SCs supporting a model whereby distinct populations of SCs facilitate homeostatic vs. injury-induced regeneration.

In support of these findings, the results of a study using elegant strategies to specifically and totally, but also reversibly ablate CBC cells suggested that *Bmi1*(+) cells are a reserve SC population that plays a large role during epithelial repair, whereas *Lgr5*(+) CBC cells are the major SC population for steady-state renewal.[48] It also raised the possibility that there is a hierarchical order whereby slowly cycling SCs could give rise to actively cycling CBC cells during normal homeostasis, albeit at a low level. Indeed, it was shown that when *Lgr5*(+) CBC cells are specifically toxin-ablated, the crypts were not lost, but instead, *Bmi1*(+) cells (expressing GFP from its locus) reentered proliferation, tripled and replaced them to give rise to all the types of differentiated cells, whereby *Bmi1*(+) cells also replenish the CBC cells in vivo when the inducible toxin-expression is halted. Of note, *Bmi1*(+) cells remained isolated and did not intersperse between PCs. These findings also showed that upon injury, it is the activation of *Bmi1*(+) cells that renders CBC cells dispensable.

Telomerase reverse transcriptase *mTert*(+) cells, a subpopulation of *Bmi1*(+) cells distinct from CBC cells, with full SC competence are also able to give rise, albeit at low frequency, to CBC cells under steady-state.[69] Telomerase helps maintaining the telomeric ends of chromosomes and had previously been shown to mark long-term label-retaining cells (LRCs) within SI crypts.[70] This was also the case for other cells of the +4 annulus expressing *Hopx*, an atypical homeodomain-containing protein with a role in heart and neural stem cells.[55] It is robustly expressed along the entire length of the intestine, and in LRCs of the +4 annulus they were shown to display full SC competence. The CBC cells and the *Hopx*(+) cells can regenerate one another and dynamically interconvert during steady-state, both in cultured organoids and in vivo conditions.

Collectively, the above findings raise the question whether the low frequency at which the *Bmi1*(+), *Hopx*(+) or *mTert*(+) cells are thought to convert into *Lgr5*(hi) CBCs would match the rate of CBC loss. This may be feasible, in view of the high proliferation rate of CBC cells and the symmetric, therefore expansive, divisions they mainly undergo.[71] It follows that CBC cells arisen from presumptive SCs in the +4 annulus would have to migrate down into the CBC/PC zone! This too may be feasible. Slowly cycling columnar cells that migrated down into the CBC/PC zone were reported,[30] but thought to die there after a few days. The +4 annulus being spread out over cp 2–8, occasional Bmi1(+) and mTert(+) cells are seen in the CBC/PC zone. Could these be the same and have given rise to CBC cells instead?

They also raise the question whether some of the more quiescent cells discussed here could be the "ultimate SCs" of the three tier model of Potten's model. This seems improbable since they belong to the more radiation-resistant tier(s) of clonogenic SCs of the model. Accordingly, the CBC cells, in spite of being less resistant then *Bmi1*(+) and *mTert*(+) cells, are still rather resistant[48,60] and would give rise to the more radioresistant +4 cells, as recently suggested by Clevers.[47] The nature of the ultra-sensitive "ultimate" SI SCs thus remains a total mystery, and formal proof for them having the attributes of stemness, as for CBC and *Bmi1*(+) cells has never been presented! This could possibly be done by showing in whole, isolated crypts of animals irradiated with < 1 Gy the cells that within a few hours show early signs of apoptosis and checking out whether they differentially express the current markers for stemness, preferentially at the protein level (see below) and by studying their mode of division and fate.

Notwithstanding the elegance of some of the above studies, a number of observations have cast doubts on the validity of *Bmi1*, *Hopx* and *mTert* as markers for SCs of the +4 annulus. First, isolated *Lgr5*(hi) CBC cells have high telomerase activity which rapidly decreases in their undifferentiated progeny.[72] They also express slightly increased levels of *Bmi1* mRNA.[73] In agreement with this, in duodenal crypts, tamoxifen induced *Bmi1*(+) cells (visualized by β-Gal expression) displayed a broad distribution (cp 1–15), peaking at cp 4–6, and in cp 1–6 up to half of them showed overlap with *Lgr5*(+) cells, peaking at cp 3 and 4.[48] These observations were extended by showing that the +4 cell markers are all robustly expressed in isolated CBC cells (of note: not at the protein level for Bmi1 and mTert).[66] Single molecule mRNA hybridizations for all the known intestinal SC markers confirmed *Lgr5* as the most exclusively expressed gene in CBC cells, whereas

the expression of *Hopx* was also enriched in these cells, but its expression gradient extended into the TA zone. *mTert* and *Bmi1* were confirmed to be expressed at low levels throughout the crypt, but no specific enrichment of mRNA molecules of any of the markers was detected at the +4 annulus.[28,66] Revisiting the *Bmi1-ires-CreER* knock-in mouse model revealed that 20 h after tamoxifen induced *Cre* activation single marked cells appeared at any position along the crypt-villus axis, in agreement with its crypt-wide expression.[66] From these findings, it was concluded that the lineage tracing or organoid-culturing experiments using the *Bmi1*, *Hopx* and *mTert* mouse models discussed above likely had reported characteristics of *Lgr5*(hi) CBC SCs.

The strongest evidence supporting the proposal that *Bmi1*(+) cells serve as clonogenic SCs, upstream of CBC cells, have been provided by experiments in the de Sauvage lab showing that *Lgr5*(–)/Bmi1(+) cells can replenish selectively ablated CBC cells.[48] But because of the observation that all cells in a duodenal crypt express *Bmi1*[28,55,66] the Clevers lab declined to consider this protein as a marker for any particular cell type. They believe that this explained why targeted *Bmi1* toxin-ablation leads to crypt loss[68] and proposed an alternative explanation based on reviving older proposals[10,17,22] that early progenitors in the TA zone serve as reserve cells to the SC compartment by using their plasticity upon damage and revert to CBC SCs. In support of this, secretory progenitors located above the CBC/PC zone exhibit plasticity by regaining stemness (Lgr5-positivity) on damage.[74] Although indeed attractive, this possibility would still imply these reprogrammed cells do not express *Lgr5* and are capable of self-renewal or else, escape in time the deadly toxin.

Data from the Kuo lab had suggested that the *Bmi1*(hi) cells of the +4 annulus cycle, albeit less frequently then CBC cells,[49] whereas the immature Paneth cell LRCs did not at all.[75] In view however, of the uncertainties with regard to the status of the cells expressing a *Bmi1* locus-driven reporter with regard to *Lgr5* expression (*Lgr5*(+ or –)), it is possible that the data in[49] measured a mix of proliferating and non-proliferating cells. Here too, clarification of the functional status and proliferative state of *Bmi1*(hi)/*Lgr5*(–) cells is needed urgently.

The above controversies leave crypt watchers like us with confusion and some concerns. One question we pose is to what extent methodological limitations of the methods used contributed to this situation.

For example, in the de Sauvage lab, two distinct locus driven *Bmi1* marker proteins showed a broad distribution of *Bmi1*(+) cells, but a clear peaking in positions 4–6.[48] Of note, the images show rare isolated cells with much higher *Bmi1*-reporter protein content. The results from DNA-arrays and proteomics also do not always correlate. Whereas *Lgr5*(hi) cells displayed slightly increased *Bmi1* mRNA levels, the protein itself could not be detected presumably due to its low level. This raises the question which level of Bmi1 protein is required to provide a cell with a significant functional outcome such as contribution to progeny generation under steady-state and weak response to canonical Wnt modulation.[49] It could therefore be worthwhile to assess whether in analogy with Lgr5(hi&lo) cells, there exists *Bmi1*(hi&lo) cells with different functional characteristics, since this would leave the possibility that the rare *Bmi1*(hi) cells in duodenal and proximal SI crypts really are functionally distinct cells as originally claimed.[49,68]

The powerful single mRNA in situ hybridization indicated that *Bim1* (and *mTert*) were expressed at low levels throughout the crypt, including the CBC/PC zone, and did not detect specific enrichment of mRNA molecules of any of the cp +4 markers.[66] However, looking in detail at how this powerful method was applied, one can question whether it was able to detect such enrichment. Potten's three tier SC model indeed proposes that between four and six cells in an undulating +4 annulus represent the "actual" extremely radiosensitive stem cells and that about 6 much more radioresistant daughters of these would stay parked in the same annulus.[1] It is likely that the latter would correspond to the observed *Bmi1*(hi) cells. It was pointed out, however, that individual cells in this undulating +4 annulus could be located anywhere between cell position 3 and as high as cell position 8 or 9, as indeed reported.[48] As pointed out in,[51] the tubular part of SI crypts contains 16 cells in circumference, and the crypt is about 30–35 μm wide. With only six *Bmi1*(hi) cells in the +4 annulus, only relatively rare 5 μm longitudinal sections will "hit" such a cell. In the study discussed here[66] 168 stacks corresponding to 4 μ thick sections were analyzed for the distribution of *Bmi1* mRNA. Here too, many would not contain a single *Bmi1*(hi) cell. It is therefore likely that this prevented them from finding any enrichment of *Bmi1*-encoding mRNA at or around cp +4. Using 30 stacks for the other markers was certainly largely insufficient.

The powerful mice models in which expression of genes like *Lgr5*, *Bmi1* or *Hopx* are shown by locus-driven reporter-protein expression have proven very instrumental in making progress, but they may also have been a source of inaccuracies. As pointed out elsewhere,[10,51] the induction may be influenced by bioavailability of the small molecule, like Tamoxiphen, administrated through injection or the recombination kinetics in different cell types. GFPs have a half-life of 26 h, which is significant with respect to the cell migration velocity of 1 cell perimeter/hour and cell cycles of 12 to 36 h. The fluorescence intensities obtained do not reflect protein levels or localization and do not necessarily reflect the actual level of gene expression. In addition, from a threshold of fluorescence intensity on, distinction between cells expressing lower or higher mRNA levels may no longer be possible. This may be the reason why certain locus-driven reporter mice indicate broader spatial expression then others. Examples are the *Bim1*[68,75] and the *Prominin/CD133*[76,77] models. Future work will have to clarify to which extent, if any, some conflicting results could be a consequence of the above limitations and that these may have influenced the purity of cells obtained after fluorescent activated cell sorting and the outcome of signature tracing experiments. They also complicate the assessment of the reality of cells displaying overlapping protein expressions, for example for *Lgr5*(hi) and *Bmi1*(hi) cells in.[48]

In conclusion, more work is needed to assess whether certain of the *Bim1*(hi) cells represent a SC population with properties distinct from *Lgr5*(hi) CBC cells. The availability of well characterized antibodies against both Lgr5 and Bmi1 would allow marking these proteins. Using tissue preparation of intact crypts and

imaging techniques as in[78,79] could increase the chances to detect them. The study of Lrig1 at the mRNA and protein levels in the colon[80] shows the potential (see below). *Ascl2* (*Mash2/HASH2*) is homologous to the Drosophila Achaete-scute complex genes and a direct Wnt-target and encodes a basic helix-loop-helix (bHLH) transcription factor. In SI crypts, antibodies to Ascl2 localize it in the nuclei of CBC cells, but not in their immediate daughters.[73] Broad use of a standardized panel of antibodies and labeling techniques would be beneficial when used in complement with the existing, locus-driven reporter models. Eventually, KO-KI mice successively expressing distinctly fluorescently-marked SC markers placed under their natural promoters and 4D video time-lapse tracking of single cells in isolated intact crypts may be needed to reveal the directionality and the kinetics of the communication between different types of clonogenic SCs.

Long-Lived, Cycle Arrested, Label Retaining Progenitors Reprogram into Cycling SCs upon Injury

Using a pulse-chase histone H2B-GFP in vivo chromatin-label retention approach to characterize long-term LRCs, a study from the Fodde lab reported that the **CBC/PC zone** contains about 7 quiescent LRCs.[81] Cell-sorter purified LRCs were characterized as PC-like cells, with a life span of up to 100 d, and were not enriched for any of the SC markers. They were also capable of producing in vitro organoids and to support isolated CBC cells in producing them, thus functioning as normal PCs (see below). Upon tissue injury from whole body irradiation, they switched to a proliferative state and expressed the SC marker *Bmi-1*, but not *Lgr5*, while silencing PC genes. This was said to indicate that *Bmi1*(+) cells, and not CBC cells are direct descendents from LRCs upon regenerating from injury.

The Winton lab used a similar approach modified in some important aspects.[75] They indeed used a much shorter pulse of Tamoxiphen to obtain LRCs after only a few weeks. These contained beside normal PCs, also cells that were identified as non-dividing immature PCs which expressed besides PC markers, also Lgr5 and +4 cell markers. They created a novel transgenic mouse that expressed an inducible H2B-GFP protein fused to the N-terminal domain of the Cre-recombinaseA enzyme, in mice that expressed the Cre-recombinase B constitutively in all the cell types. Upon Tamoxiphen administration, H2B-GFP LRCs expressing intact Cre-recombinase,remained quiescent in healthy mice. Upon crypt injury, however, they gave rise to persistent large crypt-villus ribbons, the signature of intestinal SCs. These LRCs too formed organoids in vitro upon isolation.

These important findings at last provided direct proof for the decades old hypothesis that committed progenitors and even fully differentiated progeny can reacquire clonogenic properties, meaning that the distinction between committed/differentiated cells and quiescent SCs is less absolute than generally believed. Generally overlooked, the results of the initial clonal analysis made by Bjerknes and Cheng indeed indicated that CBC cells also give rise to some long-lived (months) unipotent progenitors with secretory and columnar phenotypes, respectively (M0 and C0 in Fig. 1 in 61), and which were thought to likely reside in the COD (author's note: or the undulating +4 annulus?). They were originally thought to directly give rise to M1 and C1 progenitors (**Fig. 2B**). We speculate that the LRCs discussed in[81] and the long-lived M0 progenitors are identical cells, which would imply that they first give rise to +4 annulus SCs and then Mix progenitors (see below).

As discussed before, by the end of 2010, workers in the field had produced evidence that in the crypts of the SI, like in other SC niches, at least two populations of intestinal SCs co-existed,[82] but subsequent studies had cast doubts that the more quiescent +4 cells visualized by +4 cell markers represent a unique cell population. In a commentary paper, Clevers stated that the study from the Winton lab unifies earlier theories about the identity of crypt SCs.[47] He based this on the finding that the LRCs with immature PCs properties, expressed +4 cell markers besides *Lgr5*. He proposed a simple model in which cycling CBCs are the "engines" of crypt self-renewal. Accordingly, they also generate a transient population of non-dividing immature PCs that will further differentiate into long-lived PCs (**Fig. 2A**) (author's note: according to,[81] these are PC-like cells expressing none of the SC markers). Since these early *Lgr5*(+) progeny also express markers of the +4 cells, he proposed that they probably represent the actual +4 annulus cells.

Paneth Cells as Niche-Supporting Cells of the Intestinal SC-niche?

The CBC cells were initially identified as interspersed between PCs[22] (**Fig. 1A**). The finding that CBC cells were clonogenic SCs opened the possibility that PCs may function in providing CBC cells with signals for stemness maintenance. Analyzing the spatial and topological aspects of PC progenitor appearance and maturation Bjerknes and Cheng deduced that there is a gradient of PC age in the crypt base, with the oldest PCs at the bottom and the youngest at the top, at cp 5–8.[58] This population top to bottom density gradient of PCs led to the proposal that PCs can be considered functional when arrived in position 4 and lower and that CBC cells leaving this mature PC zone commit to differentiation in the COD, containing immature PCs. This hypothesis was not supported by the observation that PCs are absent in SI crypts of dogs and pigs, even if cells of the secretory lineage are present at the crypt bottom in these species.[27] In addition, when up to 90% of the PCs were ablated by forcing them to produce a diphtheria toxin A fragment, an increase in CBCs and no effect on crypt homeostasis was observed.[83] More recently, however, PCs and CBCs were shown to be arranged in an almost geometrical distribution that optimizes PC-CBC contact area at the expense of the homotypic one, both in vivo and in organoids grown from single CBCs.[84] Recombining in a defined cell culture medium purified CBC cells and PCs dramatically increased the efficiency of organoid formation compared with using single CBC cells. The way these organoids formed indicated a requirement for cell-cell contact. Gene expression profiling then revealed that Paneth cells indeed provide essential signals for CBC-growth support. In three transgenic mice models in which the number of PCs was drastically decreased, the CBC cells decreased coincident with

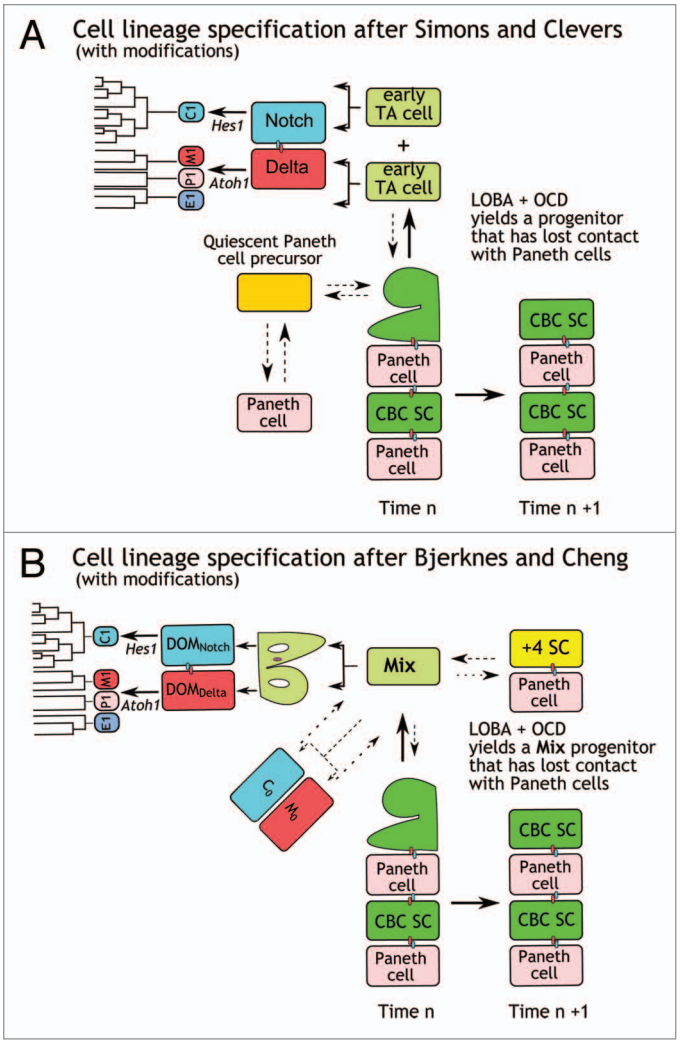

Figure 2. Two competing models for lineage specification in crypts of the SI. (**A**) According to Simons and Clevers,[33] CBC SCs divide symmetrically to yield two CBC cells and self-produce a niche with one of their immediate progeny, the PCs. Shown here is the proposal of De Mey, Freund and coll. that the anisotropic movement of one daughter cell at the border between the CBC/PC zone and the OCD generates early TA cells that continue dividing symmetrically. Stochastic Delta-Notch signaling between progeny that are not necessarily siblings and additional gene network expression generate progenitors of each of the differentiated cell types. The dark yellow cell is a quiescent Paneth cell precursor that will give rise to PC-like cells. These can be reprogrammed into CBC cells upon tissue injury. This was interpreted as meaning that these LRCs are the same cells as the quiescent +4 SCs described by others. (**B**) According to Bjerknes and Cheng, CBC SCs that leave the CBC/PC zone become committed Mix progenitors. Divisions asymmetric with respect to cell fate generate DOMNotch and DOMDelta daughters that set up between them Delta/Notch lateral inhibition. Added here is the suggested possibility that asymmetric mNumb distribution initiates this process. To the right is shown a yellow cell that stands for all the proposed types of quiescent SCs found in the undulating +4 annulus. These cells may display their own hierarchy that is not shown here. Also shown are C0 and M0 long-lived and quiescent progenitors which can be reprogrammed to become either +4SCs or CBC cells. Shown here is the possibility that this involves passage through a Mix progenitor, but no evidence for this exists.

PCs and remaining CBCs crowded around the remaining PCs. From this, it was concluded that the CBC SCs receive niche support from their own specialized progeny, the PCs, much like in the SC niche of the fly testis.[85]

Notwithstanding, a number of observations have tended to question this notion. First, in neonatal mice, before the appearance of PCs, CBC cells also occur as clusters and function as SCs which can be induced to differentiate into secretary cells prematurely by *Hes1* ablation.[86,87] Hes1 indeed represses the expression of Atoh1/Math1, a transcription factor involved in secretory lineage commitment, hence that of PCs.[88] Second, PC ablation by conditionally knocking out *Atoh1* did not lead to CBC cell malfunctioning or delocalization.[57,86] With this respect, it may be of relevance that Bjerknes and Cheng noted that progenitors with an immature secretory cell phenotype appeared in the **COD** and moved downward while differentiating into mucus secretory cells[30] indicating that beside PCs, additional crypt base secretory cells may play a niche-supporting role and may do this alone or in combination with mesenchymal cells in certain species.

Refining and completing these findings, another study indicated that the intestinal SC niche also comprises surrounding mesenchymal myofibroblasts separated from the epithelium by the basal lamina, which provide a redundant Wnt, namely Wnt2b, to the CBC cells.[89] In agreement with this, it was noted before that the need for PCs in organoid culture could be overcome by supplying the medium with Wnt3a during the first three days (Wnt2b was not tested, however).[84] These authors also argued that the loss of *Atoh1* may render Delta/Notch signaling to CBC cells dispensable,[90] and that in crypts that possess PCs, these indeed function as niche-supporting cells. PCs also contribute other factors besides Wnts, such as Dll1, the ligand of the Notch receptor, present on the surface of CBC cells (see below).

In conclusion, it appears well established that the CBC and mature PCs support each other to build a SC niche and that CBCs compete for contact with PCs in order to maintain stemness.

New Findings in the Crypts of the Ascending and Descending Colon

The mouse colon is divided in the cecum and ascending and descending segments each of them showing distinct morphological features. They for example display complex mucosal folds in the descending segment and simpler longitudinal folds in the descending one.[91] With regard to the issues discussed above, until very recently few attempts to assess whether the models developed with regard to cell stemness can be extended to the crypts of the ascending and descending colon, had been made. We will now comment recent work done on the crypts of the descending colon[78,80,92-94] which addressed that question.

In the crypts of the ascending segment PCs are replaced by Deep Secretory Cells (DSCs) that are cKit and CD24-positive.[92,95] They also display intercalated cells that are, however, mostly quiescent.[95,96] Most of the proliferation takes place above this DSC zone and the SCs are assumed to be localized there.[93-96] It has been proposed that both the DSCs and intercalated cells

appear in this zone of differentiation and migrate down from it.[95,97] Two recent studies have improved our understanding of the differences between the crypts of ascending and descending segments by showing clear regional differences in the organization of the SC zone and of the sensitivity of SCs to radiation.[93,94] In crypts of the ascending colon, only few cells were cycling at cp 1–4, the bulk being in cp 4–9. Interestingly, *Lgr5*(hi) cells seen by GFP-microscopy, occupied these positions, meaning that those at lower positions were not cycling actively. Intriguingly, the Lgr5hi cells at the lower positions also were CD24-(+). This suggests that DSCs may be cycle arrested reserve SCs. It is not clear whether these crypts also contain crypt base goblet-like cells (GLCs) found in the descending colon.

The descending segment is of particular interest, since it is the preferred site of tumorigenesis in man. This segment contains only very few, if any, DSCs.[95] Instead, they display at their bottom mature Muc2-secreting cells, called crypt base Goblet-Like Cells (GLC) that are also cKit(+) and CD24(+).[78,92,93,95] Intermingled with these exist proliferating columnar "vacuolated" cells[95,98] that were in fact the very first cells proposed to act as intestinal SCs (**Fig. 1B**). Recent studies have shown that they indeed are *Lgr5*(+) and possess full SC competence.[31,80,93] Proliferating *Lgr5*(–) "vacuolated" cells occur up to cp 18, whereas proliferating immature Mucus2-producing Goblet-cell precursors are found in cp 1–6.[78,99] These cells contain fewer mucous granules, are not displaying the morphology of goblet cells and express *Spdef*, encoding an ets-domain transcription factor that promotes maturation of goblet and Paneth cells.[100] They also express *Atoh1* which rapidly leads to cell cycle arrest.[101] The crypts further contain large numbers of mucous secreting goblet cells, whereas columnar cells are most numerous in the flat epithelial cuff.[98]

We will use in the following the term SC/GLC zone to designate the zone in crypts of the descending colon of mice where all the SC populations and the GLC co-exist. It occupies cp 1–7, whereby the GLCs are denser at the first positions.[78,93] Contrary to the crypts of the SI, there is no COD, since the actively cycling SCs, their first progenitors and some less frequently cycling SCs are all co-localized in the SC/GLC zone[78,80,93,99] (see below). Above the SC/GLC zone there is the TA zone in which progenitors, probably corresponding to a majority of C1 and fewer M1 progenitors (see below) proliferate while becoming more and more differentiated **Figure 2B**.

The mechanism by which quiescent SCs can become actively cycling CBC cells has become a field of active research, also in view of the potential in cancer therapy. *Lrig1* controls SC proliferation in the epidermis[102] and is a negative regulator of the Egf-receptor, a tyrosine receptor kinase of the ErbB family.[103] It is also a direct target of *c-Myc*, downstream of the pEgfr/Akt/Pi3K pathway.[102] Increased c-Myc activity will therefore induce a negative feedback loop by increasing the amount of Lrig1 at the membrane. Indications for the existence of less frequently cycling SCs, also in the crypts of the descending colon were reported,[31] and recently, the Coffey lab reported that these correspond to Lrig1-positive cells.[80] As a population, the latter are predominantly quiescent, long-lived LRCs, but a minority of them is slowly cycling and can expand upon receiving appropriate signals, such as from injury. Distinct $Lgr5_{hi}$ cells and Lrig1-positive cells (the latter by immunofluorescence!) co-existed in the first cell positions of the crypt. Their transcriptome profiles obtained by transcriptome analysis of Lrig1-cells (sorted from antibody-labeled cells) and *Lrg5*(hi) cells (from sorted *lgr5* locus-driven GFP expressing cells) were distinct, but related. Both cell types expressed low levels of *Bmi1*, *Prominin1* and *mTert*. *Hopx* was 2x more expressed in *Lgr5*hi cells then in Lrig1-cells. Only the *Lgr5*hi cells expressed *Lgr5* and *Olfactomedin4*, whereas Lrig1(+) cells highly expressed *Ly6a/Sca-1*, a cell-surface SC and progenitor marker in various tissues, associated with inhibition of growth and differentiation.[104,105] Genetic ablation of *Apc* in Lrig1-cells led to adenoma formation. Coffey proposed that calibrated ErbB signaling maintains Lrig1-SC quiescence during normal homeostasis, much like in the epidermis.[102] Of note, *Lrig1* is also a tumor suppressor gene, since its loss led to adenomas in the duodenum.[80] These findings led to a model of a continuum between SC populations whereby Lrig1-cells are downstream of other, more quiescent SCs and upstream to progenitors of the differentiated cells. The direct transition between $Lrig1_{hi}$ and *Lgr5*(hi) cells was proposed to be infrequent under normal homeostasis, only taking place when needed (see Fig. S7 in 80). The regulation of the expression of *Lrig1* could thus be a key event in the transitions between quiescent and proliferating SCs, but this remains to be proven. These data seem to strengthen the notion that *Bmi1*, *mTert* and *Hopx* may not be robust markers for more quiescent SCs, but identify the Lrig1-cells as such, at least in the descending colon.

Progress with Understanding the Control of the Expansion of the SC Zone

In the SI, on the contrary, and adding to the list of differences between colon and SI, *Lrig1* is highly expressed in a bottom to top gradient in the CBC/PC and COD, with the highest expression in CBC cells and none in PCs.[14] The *Lrig1* gradient is opposite to the top to bottom gradient of pEgfr, the phosphorylated, active form of this receptor. Its loss leads to massive ErbB dependent Egfr phosphorylation and *c-Myc* activation, which constitutes a strong inductive signal for SC-proliferation and expansion of the CBC/PC zone.[106] In the SI therefore, *Lrig1*'s role appears to be to control the size of the SC compartment. Knockout of *Lrig1* in the colon, did not notably change the size of the SC/GLC zone, but led to increased crypt fission,[80] known to occur when the number of SCs augments.[107-109]

Control of the expansion of the CBC/PC zone, during both steady-state and tissue regeneration, requires the ability to return proliferation to normal levels in cells that leave the zone. This involves counterbalancing the Wnt-signaling pathway, which is central to SC-renewal and regeneration. Wnt ligands normally signal by interacting with a Frizled (Fz) receptor to Disheveled (Dvl), which leads to inhibition of the β-Catenin destruction complex composed of Axin, Apc, GSK-3beta and β-Catenin.[110] The now stable β-catenin translocates in the nucleus and activates the transcription factor TCF4. This also involves Dvl nuclear translocation.[111] A recent study has shown that the yes-associated protein 1 (Yap1), a direct Wnt-target, counterbalances canonical

Wnt-signaling during intestinal regeneration, in part by limiting the Dvl signaling to the nucleus.[112] This study indeed showed that the cytoplasmic pool of Yap1 binds to Dvl and inhibits its nuclear translocation independently of the β-Catenin destruction complex. Conditional *Yap1* KO (cKO) induces the rapid loss of crypts, associated with reduced Wnt-signaling. Paneth cells lost their anchored location at the crypt bottom and disappeared, together with CBC cells. No regeneration from other SC populations occurred. Manipulation of Wnt-signaling by stimulators led to a much higher expansion of the CBC/PC zone in *Yap1* cKO mice, which together with other data strongly indicated that *Yap1* contributes to controlling the size of this zone. Using immunohistochemistry, Yap was found to be nuclear in CBC cells, but cytoplasmic in progenitors in the TA zone. This suggested that cytoplasmic Yap1 may be regulating the progress from a proliferative CBC cell to an early progenitor (**Mix** or committed TA cell depending on the model one prefers), which indeed downregulate their level of Wnt-signaling.[6] These findings are also important because Yap1 is best known as a critical component of the Hippo signaling pathway, which in mammals, controls organ size. Activation of this pathway leads to Yap1 phosphorylation, which prevents its nuclear translocation, where it normally functions as a transcriptional co-activator.

In conclusion, most models for explaining the mechanism of growth and tumor suppression by the Hippo pathway are based on restricting the transcriptional activity of Yap1. This study now indicates that this role may very well be due to the inhibition of Wnt-signaling by Yap1 via restricting nuclear Dvl signaling!

Oriented Cell Division (OCD) in the Intestinal Colon and SI Crypt Indicates Planar Cell Polarity Signaling

Spindle orientation sets up the orientation of the cell cleavage plane, and in most simple epithelia, this is essential for keeping the epithelial intact and to assure that both sister cells contribute to epithelium homeostasis. During M-phase of the cell cycle, polarized epithelial cells lift their nuclei toward the apical pole and round up while retracting from the basal lamina, often keeping a connection with the basal lamina in the form of an actin-rich basal process.[113,114] In order to maintain intact their tissue barrier function, the intercellular junctional complexes stay intact. In divisions symmetric with respect to cell fate, the movements of the separating centrosomes during prophase and pro-metaphase follow a peculiar pattern,[114] often leading to the late prometaphase spindle being aligned "vertically" along the apico-basal axis. Once in metaphase (when all the chromosomes are aligned at the spindle equator), the spindle aligns planarly, parallel with the apical surface, also in crypt cells. During telophase, cleavage occurs perpendicularly to the apical surface.[113] In some tissues, spindles in addition align with one of the tissue axes, for example as in kidney tubules, where they align with the longitudinal axis.[115] Preferential planar and longitudinal orientation of the spindle, together, defines OCD for "Oriented Cell Division".[116-118] OCD has also been claimed to be important for shaping tissues, but this is only so when it determines

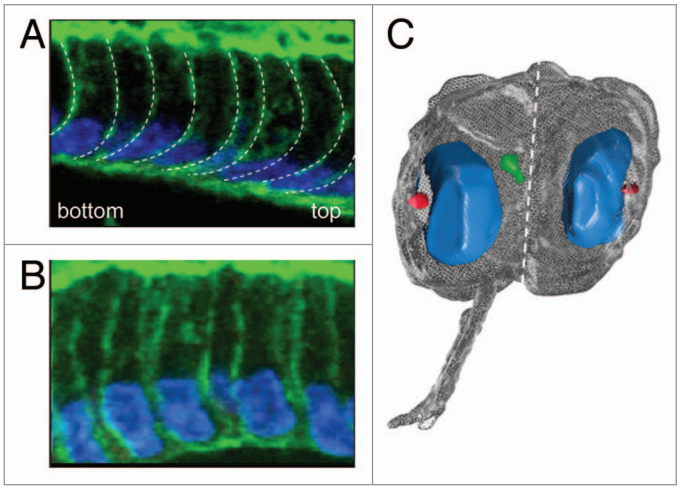

Figure 3. Topological properties of intestinal epithelial cells. (**A**) Bent shape of the interphase cells lining the crypts. (**B**) The bent cell shape is lost in the apparently normal intestinal epithelium of Apc+/Δ14 mice. (**C**) Computer-based representation of a dividing cell in telophase in the SC compartment. The dotted line denotes the cleavage plane between the two daughter cells. In this cell, the BP that connects the cell body to the basal lamina asymmetrically segregates in one daughter cell (left daughter) and this daughter also exhibits asymmetric distribution of mNumb (green spot). The nuclei are in blue and the centrosomes labeled for γ-Tubulin in red.

the ultimate position of the daughter cells as shown for the gastrulating Zebra fish embryo, the fly wing and the mouse renal tube.[117,119,120]

Using different approaches, measuring spindle orientation in SI crypts has given different results.[113,121,122] One study in particular observed statistically significant orientation biases of several sorts, in particular a tendency toward lengthwise orientations.[121] However many mitoses were observed that would result in increased radius and thickness of the crypt if the spindle orientation determined the ultimate position of the resulting daughter cells. Since this was not observed, these authors concluded that the daughter cells must rearrange after mitosis. Consequently, it was concluded that mitotic orientation probably has only a weak effect in determining tissue form. Studying spindle orientation in crypts of the descending colon the De Mey and Freund labs confirmed that like in the SI[113] all the spindles in the SC/GLC and TA zones were aligning their spindles largely planarly and found in addition that in 80% of the cells there was a preferential longitudinal orientation of the spindle parallel to the long axis of the crypt, the others taking a wide variety of orientations.[78]

These authors also reported that while leaving the semi-spherical bottom of the crypts, interphase cells adopt a bent shape in which the cell base is uniformly oriented toward the bottom of the crypt[78] (**Fig. 3A**). This observation represents a novel expression of planar cell polarity that was designed as LOBA for "Longitudinally Oriented Basal Asymmetry." As reported before for the small intestine,[113] they confirmed that the base of dividing cells is compressed and that the cell body remains connected to the basal lamina by an actin-rich Basal Process (BP). However, since LOBA persisted in dividing cells,

their BP was bent (**Fig. 3C**). The authors further reported that the combination of LOBA with OCD orchestrate anisotropic movement of one of the daughters in those cells (80%) displaying OCD (**Fig. 1A and B**). OCD/LOBA thus was proposed to form a new functional unit, that offers a mechanism for mitotic pressure on surrounding epithelial cells, previously discussed as driving cell migration,[98,123] but later dismissed as irrelevant.[23] Mitotic pressure on the other hand has been shown to contribute for up to 25% of cell migration in other settings. As pointed out in the case of the elongating renal tube, cell movement and daughter cell placement will influence each other,[124] and this is likely also the case for intestinal crypts.

OCD is generally considered as a functional readout of Planar Cell Polarity signaling, widely used in insects and vertebrates to orient spindles along a tissue axis and to align them with polarity cues able to conduct the asymmetric distribution of cell fate determinants.[116-118,120,125]

Lineage Specification

Another hotly disputed topic in the field is at what level in the SC/progeny hierarchy lineage specification is initiated and to what extent and how cell fate decisions are timed in relation to the pattern of cell divisions. A first group of workers proposes that symmetric divisions of CBC cells in the CBC/PC zone are followed by divisions asymmetric with regard to cell fate in the COD whereas another proposes that the CBC cells and their progeny arise stochastically at a population-based level and virtually total absence of divisions asymmetric with regard to cell fate.

The production of daughter cells with different fates from a common mother cell defines the concept of asymmetric division with regard to cell fate.[35,126,127] In the gut, it has long been held as the most likely hypothesis that such divisions occur at each mitosis of the SCs, so that the stemness status would be characterized by invariant asymmetric division producing one new SC and one cell loosing stemness properties to become committed into a progenitor cell and differentiate.[27] In this view, invariant asymmetric divisions occurred both within the three tier SC hierarchy and for specification of committed progenitors.

In their initial clonal analysis Bjerknes and Cheng identified Dab+ clones which persisted over months and contained both columnar cells and mucus cells. The observation that the lowest positioned cells were in cp 5–7 provided evidence for the presence of unipotent and multipotent progenitors above the CBC/PC zone, which they called MIX progenitors, namely CBC cells that have committed to differentiation after entering the COD. Because they did not consider a SC hierarchy, Bjerknes and Cheng concluded that CBC SCs divide predominantly symmetrically, also with respect to cell fate and that divisions asymmetric with regard to cell fate were a property of the Mix progenitors. According to their model, Mix contribute to lineage specification[16,61] by setting up Delta-Notch lateral inhibition between their immediate daughters, called daughter of Mix or DOM progenitors (see below). Subsequent studies using contemporary cell tracing approaches have since confirmed, extended and refined this model (see below).[18,79,128]

Recent studies from the Clevers and Winton labs combining lineage tracing in the SI with mathematic modeling have been interpreted as showing that the production of CBC cells together with progenitor cells occurs stochastically at a population-base level instead of in each CBC, ruling therefore out that invariant asymmetry is the general mode of division of CBC cells in the gut. This class of population asymmetry indeed involves neutral drift dynamics leading to characteristic signatures in long-term clone size distributions, which were found experimentally in two independent studies.[71,129] Simons and Clevers have generated a model in which in the SI, lineage specification at the cellular level does not rely on any form of asymmetric division (**Fig. 2A**), whereby instead (1) CBC cells undergo neutral competition for contact with niche-supporting cells providing most of the short-range signals for SC competence (see above), (2) differentiation occurs stochastically when a CBC cell loses contact with the signals coming from the niche and (3) resulting CBC cell loss is compensated by symmetric self-renewal of a neighboring CBC cell (Fig. 1D in 33), implying that longevity is an attribute of the CBC population, but not of individual CBC cells.[8] This model also rejects the existence of the Mix and DOM progenitors identified by Bjerknes and Chen, as formulated in.[74]

Of note, the Bjerknes and Cheng model is also compatible with the population-based mode of asymmetric division, in which originally, dividing SCs were said to produce either two new SCs or two committed progenitor cells leaving stemness properties or one SC and one progenitor cell.[127] Interestingly, this scheme effectively takes place in adult and embryonic epidermis.[130-133]

The model of Simons and Clevers proposes that chance displacement and loss of a SC from the niche is compensated for 100% by the symmetrical division of a neighboring SC (see Fig. 1D in 33). De Mey and Freund on the other hand proposed an alternative model in which in the colon SC/GLC zone, the anisotropic movement of SC sister cells generated by LOBA/OCD (see above) promotes escape of one of the daughters away from the GLC its mother was contacting[78] (dividing dark green cells in **Fig. 1 and 2**). In addition, the daughter staying in contact with the GLC cell and its sister breaking away from it can be considered as a breakage of symmetry. This type of anisotropic placement of one sister could also be used by CBC cells in SI crypts at the border between the CBC/PC zone and the COD.

The molecular mechanism of the specification of the Mix progenitors and their derivation from CBC cells are not understood, but counteracting canonical Wnt signaling is a likely one (see below). There are numerous strategies for accomplishing binary fate decisions,[43,46,126] but very few of them have been explored in intestinal crypts.

Several works had shown that β-Catenin-dependent Wnt activity is required for the maintenance of the SC niche in vivo,[63,64] and in vitro, to produce organoids from isolated CBC cells.[6,59] In the crypt base, it had also been shown to act in concert with Delta-Notch signaling, widely used in lineage specification (reviewed in 134). High signaling activity of the Notch

receptor keeps an undifferentiated state by activating the expression of the Hes transcription factor which subsequently acts as a transcriptional repressor of both the bHLH transcription factor Atoh1/Math1 involved in secretory lineage commitment and the production of the Notch Delta-like ligands Dll1 and Dll2 at the cell surface, thus limiting the activation of the Notch pathway in adjacent cells.[88,134] The usual assumption for establishing binary Notch-dependent specification is that it occurs upon lateral inhibitory signaling among a general pool of interacting cells that are not necessarily sister pairs of cytokinesis (**Fig. 2A**).

In both invertebrates and vertebrates, however, cell fate decisions are often timed with cell division. When this is the case, very often, the offspring of bipotential progenitor mitoses are initially identical but continue to directly interact with each other to set up lateral inhibitory Notch signaling, breaking this symmetry, and inducing a binary switch for lineage specification[133,135,136] (**Fig. 2B**). The Zoghbi lab[137] was the first to suggest this could apply to intestinal SC divisions or the divisions of their immediate progenitors.

In order to decide which model to adopt, Bjerknes and Cheng argued that under this latter assumption, only one of the sister cells commits to a secretory lineage while the other commits to the columnar one. They advanced that under the former usual assumption, this is not necessarily the case because the sister cells may frequently interact with unrelated neighbors, rather than with each other, and hence both may become committed to secretory lineages and at least initially occur as pairs. Moreover, depending on the specific signals received, they may even become committed to different secretory lineages. They therefore looked for clones containing two types of secretory cells, stemming from those instances when sister pairs commit to different secretory lineages, but never saw these, although their technique allowed to quickly screen thousands of crypts.[61] In addition, in mixed DAB^+ clones, having an origin in the COD, secretory cells were often separated by numerous columnar cells. They therefore concluded that it is more likely that the sister pairs produced by mitosis of a Mix progenitor usually interact with each other rather than with other neighbors and adopted that in their model.[16,61] Their current model, based on their original findings and additional studies[18,79,128] defines the $Mix_{M,E\ and\ P}$ progenitors as CBC progeny which have left the CBC/PC zone and initiated a differentiation program. It introduces the term Daughters of Mix (DOM) progenitors for the equivalent sister cells from mitosis of Mix progenitors whose initial symmetry is broken by lateral inhibitory Notch signaling, resulting in DOM_{Notch} and DOM_{Delta} and making these divisions asymmetric with respect to cell fate (**Fig. 2B**). The model further proposes that a gene network operates within the intestinal epithelium to define the various epithelial lineages. We will limit ourselves here to the specification of mucous and columnar cells (see Fig. 22 in 18). They showed that initially equivalent DOM_M progenitors each express low levels of Hes1 and Atoh1. Lateral inhibitory Notch signaling between the two equivalent DOM_M progenitors breaks their symmetry leading to DOM_{MNotch} and DOM_{MDelta} states which usually give rise to Hes1- and Atoh1-expressing cells, respectively. The former become the columnar cell lineage progenitors, C1. The latter commence a secretory lineage program M1 and immediately commit to one of the secretory lineages through the interaction of various downstream factors including Neurog3 and Gfi1, leading to formation of a mucous, enteroendocrine or a Paneth progenitor (M1, E1 or P1).[79] From counts of the number of PCs in a large number of crypts and mathematic modeling, it was concluded that they likely are derived from their P1 precursor (not a SC as originally thought) by symmetric divisions whereby two Paneth cells are generated[138] (**Fig. 2B**).

The Clevers group identified and characterized a subset of immediate CBC daughters strongly expressing the Notch ligand Dll1 but lacking typical CBC markers (author's note: and therefore different from the immature Paneth cell precursors and LRCs,[75] see above). Lineage tracing in Dll1(GFP-ires-CreERT2) knock-in mice revealed that single $Dll1_{(high)}$ cells generate small, short-lived clones containing all four secretory cell types, proving that lineage specification occurs in the COD in cells derived from CBC cells within 1–2 cell divisions. They also identified immediate progenitors of CBC cells (or +4 SCs) expressing the ets-domain transcription factor Spdef, which they showed is acting downstream of Atoh1/Math1 to promote terminal differentiation of a progenitor pool of PC and Goblet cells.[100] Both data sets are in agreement with the model of Bjerknes and Cheng.[30,58,61] However, in line with their model refuting any contribution of divisions asymmetric with regard to cell fate,[139] Clevers and collaborators opted for stochastic loss of Notch expression on CBC cells, when they lose direct access to Delta ligands on PCs when entering the COD and strongly and stochastically upregulate Dll1 expression, thereby setting their own secretory fate (**Fig. 2A**). In fact, this corresponds to the usual assumption for establishing binary Notch-dependent specification by which lateral inhibitory signaling between one Dll1 expressing cell and 6–8 neighboring Notch-expressing transit-amplifying cells occurs.[88] The latter will maintain an active Notch pathway and keep their commitment toward the columnar lineage.[74]

Recently, the De Mey and Freund labs reported the occurrence of sister cell doublets of cells in the SC/GLC zone, in which Atoh1/Math1 was asymmetrically expressed in the sister nuclei.[78] This finding is highly suggestive of cell divisions asymmetric with respect to cell fate. They are likely the result of the well-established mechanism of braking symmetry in order to set up lateral inhibitory Notch signaling between Delta and Notch sister pairs. This mechanism is widely used in lineage specification in invertebrate and vertebrate tissues.[136,140,141] It also strongly supports the model proposed by Bjerknes and Cheng[18,61] since it is compatible with their proposal that Mix_M progenitors yield DOM_{MNotch} and DOM_{MDelta} progenitors by this process. The advantage of Mix progenitors is that the actively cycling SCs can undergo controlled expansive proliferation, while Mix progenitors, by dividing immediately or after one additional symmetric division, in the form of a division asymmetric with regard to cell fate, initiate the differentiation process of their respective progeny.

At the sub-cellular level asymmetric divisions are mostly displaying a cortical asymmetric distribution of several proteins in the mitotic mother cell prior to cytokinesis. For several of these

this is coordinated with a reorientation of the spindle axis resulting in their asymmetric distribution, thus leading to differential gene expression in daughter cells.[142] The midgut of adult insects is also maintained by intestinal SCs, which generate both self-renewing and differentiating daughter cells, and until recently, the control of SC identity and maintenance was poorly understood.[143] Knoblich and colleagues now have demonstrated that this asymmetry involves a widely used mode of asymmetric division preceded by setting up Dll-intrinsic asymmetry, coordinated spindle orientation and asymmetric segregation of cell fate determinants (see below).[144] Previously, the Näthke lab had reported a vertical reorientation of the spindle axis, linked to an asymmetric segregation of the polarity complex Par3 and DNA strands in the vast majority of the dividing CBC cells of the CBC/PC zone.[145] Because these are hallmarks of invariant asymmetric division, they proposed this was evidence for this process to be predominant in this zone. As discussed above, two main groups in the field now agree that the CBC cells within the CBC/PC zone divide symmetrically to produce two new CBC cells and do not receive signals to commit into a differentiation path.[18,74,139] This interpretation is also at odds with the model of stochastic cell population-based asymmetry championed by the Clevers group, who subsequently attributed the spindle reorientation merely to the slender shape of the CBC cells.[71] More importantly, for cells in the COD, where divisions asymmetric with respect to cell fate are thought to occur at least by some,[18,137] there is evidence favoring the view that all the cells set up spindles that are oriented in the plane of the monolayer.[113] With respect to DNA strand segregation, these data were not confirmed by others.[72,146] Assessing the presence of cell divisions involving a spindle reorientation along the apico-basal polarity axis in the SC/GLC zone of descending colon crypts, it was found that all the cells set up their cleavage furrow starting from the basal side up toward the apical pole,[78] ruling out that the derivation of Notch and Delta sister cells involves any differential spindle re-orientation in cycling SCs. It cannot be ruled out, however, that asymmetric divisions comprising spindle reorientation and/or asymmetric DNA strand segregation are used by some of the rare, quiescent SCs, upon their activation by signals alerting that more frequently cycling SCs are needed. Performing targeted 3D analysis of anaphases marked by a specific marker such as Lrig1[80]could be worthwhile.

It was also reported that, in about half of the telophases in the SC/GLC zone only, the cell fate determinant mNumb is asymmetrically distributed in sister cells during cytokinesis[78] (**Fig. 3C**). This was the first example of a cell fate determinant asymmetrically distributing in the two daughter cells at the level of the intestinal SC niche. The cells presenting this phenomenon were thought to be SCs, but we now consider it more likely that they are Mix progenitors. Indeed, the mNumb asymmetry likely indicates the beginning of the symmetry breaking that will lead to lateral Delta/Notch inhibition between sister cells. Of note, this asymmetry was not displayed at the level of a cortical crescent as in most model systems described so far,[142] but appeared in the form of a large vesicle cluster between one of the reassembling nuclei and the cleavage furrow of cells accomplishing cytokinesis. mNumb-labeled vesicles in the cell periphery, on the contrary, were distributed evenly between daughter cells. In addition, in the majority of these cleaving cells, the mNumb structures are present in the part of the cell that remains attached to the basal lamina (see above), further indicating this was not a fortuitous observation. No asymmetric distribution of key PCP proteins Celsr1 and Vangl2 (reviewed in 142), nor of their linker to the spindle apparatus positioning machinery, NuMA[125] was reported. Thus, according to these markers, these divisions would be symmetrical. The way the vesicle cluster enriched in mNumb is formed and what may be its role in Notch regulation awaits further investigation.

In conclusion, there is now evidence in support of divisions asymmetric with regard to cell fate in about half of the early progenitor cells of descending colon crypts. Real proof, however, awaits further analysis.

How PCP Signaling Pathways could Contribute to Crypt Morphogenesis and Homeostasis

The PCP pathway regulates uniformly polarized cellular behaviors in a field of cells. The observations discussed above are the only indicators so far that PCP is at work in intestinal crypts. In this section, we will review additional evidence that PCP signaling could be at work in intestinal crypts and reflect on its possible role.

The so-called core PCP pathway is composed of some of the members of the membrane receptor family Frizled (Fz), the cytoplasmic protein Disheveled (Dvl), the transmembrane protein Vangoghlike (Vangl1&2), the atypical cadherin Celsr1 and the cytoplasmic proteins Prickle (Pk) and Inversin (Inv).[120] Established at the level of individual cells, PCP affects polarity over an entire tissue. Within a cell, PCP results in the polarized localization of proteins, signaling platforms and organelles such as microtubule organizing centers (MTOCs) and basal bodies at the basis of non-motile primary cilia.[120,124,147] In fact, in some tissues, many of the PCP determinants are themselves asymmetrically localized within the plane of the cell and are required for the subcellular localization of other pathway determinants. It is still not clear if a polarized localization of the core determinants is organized in all vertebrate tissues manifesting PCP. In colon crypts, two proteins of the PCP pathway, Celsr1 and Vangl2, were localized along the baso-lateral plasma membrane domain, but did not reveal detectable asymmetric cortical accumulation.[78] As discussed by Zallen, this may be typical for actively moving cells[120] which is also the case of crypts cells.

In vertebrates, certain members of the Wnt ligands (mainly Wnt5a and Wnt11) signaling through a group of receptors and co-receptors play a crucial role in establishing PCP.[148] They signal by interacting with a Fz receptor to Dvl, both also components of the core PCP molecules. For a Wnt ligand to activate β-Catenin-independent Wnt-signaling, it needs to interact with a Fz family member and Ror1/2, a Tyrosine Receptor Kinase (Trk). The downstream cascade can lead to activation of Rho GTPases and Jnk, resulting in polarization of the cytoskeleton and PCP. The canonical and non-canonical pathways antagonize each other so that in one particular cell at one particular moment, only one pathway may be active.[148] In crypts, whereas canonical

Wnt-signaling regulates proliferation and keeps cells undifferentiated or force cells to stay at the bottom of the crypts,[6] non-canonical Wnt-signaling could promote the formation of early progenitors. Proteins involved in non-canonical Wnt-signaling are expressed in the intestinal mucosa, albeit with regional differences.[89,149] In descending colon crypts (but not in the SI) non-canonical Wnt5a is produced by mesenchymal cells neighboring the crypt base, whereas the epithelial cells of the crypt produce Fz6, an important mediator of PCP signaling and partner of Wnt5a (see Fig. 1 in 149). Wnt5a is therefore ideally placed to form a morphogen gradient that can be sensed by Fz6. In certain cases, gradients of Wnt proteins indeed seem to play a role in establishing PCP, whereas in others, they just play a permissive role.[120,124] When Wnt proteins bind a Ror the latter forms a complex with Vangl2, which recruits Prickle (PK). It has been proposed that PK and Dvl bind and antagonize each other to generate asymmetrical protein localization, a critical regulatory event of PCP.[150] Ror binding to Vangl results in the latter's Wnt dose-dependent phosphorylation. As Vangl2 phosphorylation is required for Wnt/PCP signal transduction, it has been hypothesized that a Wnt5a signaling gradient could orient the cells it signals to by regulating Vgl2 phosphorylation in each cell.[150] This could be a lead for understanding PCP signaling in crypts.

PCP signaling also promotes collective cell migration and spindle orientation, often in tandem with the Fat pathway. Fat comprises a family of large, atypical Cadherins, which genetically interact with Vgl2.[119,151,152] Fat is also a major activator in the Hippo pathway which in certain stem cell niches like that of the fly midgut SCs,[153] suppresses Yap-dependent proliferation of undifferentiated cells, possibly through Notch inhibition (reviewed in 4). Nothing is known about the expression of Fat and its partner Dachsous in the adult gut epithelium, but both play a role in its morphogenesis, which involves PCP.[152,154] With this respect, the findings with respect to Yap1's involvement in regulating Dvl signaling to the nucleus discussed above[112] are of great significance in view of its role to control proliferation of CBC cells and maybe reactivated SCs during regeneration. PCP signaling contributing to crypt homeostasis thus makes a lot of sense, and deciphering its place in the complex gene networks controlling gut epithelium may turn out to be worthwhile.

Conclusion

This review has summarized how building on the previous works of numerous labs, the last five years have produced remarkable progress in understanding intestinal SC biology, cell lineage specification and the signaling pathways at work. We have tried to illuminate ongoing debates on the nature of the "actual" SCs and the mechanisms underpinning lineage specification. The CBC cells seem to be firmly established as cells with all the attributes of stemness. The nature of the more quiescent population of SCs remains open. Are they a distinct cell population that communicates bidirectionally with CBC cells or are they progeny of CBC cells that are reprogrammed to take on stemness when needed? We also reviewed the current thinking on cell lineage specification and its relation to cell division. Whereas most workers agree that CBC cells divide symmetrically, also with regard to cell fate, no data are as yet available for the more quiescent SCs. Cells displaying asymmetric division with regard to cell fate and asymmetric sister cell distribution of mNumb have been reported recently. If confirmed as relevant to cell lineage specification, the model may at least in part rely on breaking symmetry to establish Delta/Notch signaling between sister cells. The genetic networks regulated by multiple signaling pathways governing the morphogenesis and homeostasis of intestinal crypts are only beginning to become understood. Recent data reviewed here suggest that studying the contributions of the Hippo, non-canonical Wnt- and the Fat-PCP pathways could be worthwhile. It will be necessary to further assess cellular and mechanistic details of SC divisions asymmetric with respect to cell fate and to define the exact cells displaying this mode of lineage specification. Better understanding these aspects will be invaluable for understanding gut pathologies and finding new tracks for improved therapy, regeneration or prevention.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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