Cell

Leading Edge
Review

The Intestinal Crypt, A Prototype Stem Cell Compartment

Hans Clevers[1,2,*]
[1]Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences, 3584 CT Utrecht, the Netherlands
[2]University Medical Center, 3584 CX Utrecht, the Netherlands
*Correspondence: h.clevers@hubrecht.eu
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Due to its intense self-renewal kinetics and its simple repetitive architecture, the intestinal epithelium has become a prime model for studying adult stem cells in health and disease. Transgenic mouse models allow in vivo visualization and genetic lineage tracing of individual intestinal stem cells and their offspring. Fluorescently marked stem cells can be isolated for molecular analyses or can be cultured to build ever-expanding "mini-guts" in vitro. These studies are filling in the outlines of a robust homeostatic self-renewal process that defies some of the classical definitions of stem cell behavior, such as asymmetric division, quiescence, and exhaustion.

The epithelium of the small intestine is organized into large numbers of self-renewing crypt-villus units. Villi are finger-like protrusions of the gut wall that project into the gut lumen to maximize available absorptive surface area. A villus is covered by a simple postmitotic epithelium, underneath which capillaries and lymph vessels mediate transport of absorbed nutrients into the body. The base of each villus is surrounded by multiple epithelial invaginations, termed crypts of Lieberkühn after their discoverer Jonathan Nathanael Lieberkühn (1711–1756), who used wax injections to reveal anatomical structures (Figure 1; Lieberkühn, 1745). It has long been known that crypts are home to a population of vigorously proliferating epithelial cells, which fuel the active self-renewal of the epithelium.

Six differentiated epithelial cell types are distinguished (Figure 2) (van der Flier and Clevers, 2009). The most populous cell on the villus is the absorptive enterocyte, a highly polarized columnar cell, characterized by an elaborate lumenal brush border. Goblet cells and enteroendocrine cells secrete mucus and a variety of hormones, respectively, and occur both on villi and in crypts. Tuft cells also occur anywhere along the crypt-villus axis and may serve to sense lumenal contents. Paneth cells occupy the bottom positions in the crypt and have long been known to secrete bactericidal products such as lysozyme and defensins. And finally, microfold (M) cells reside in the specialized epithelium that overlies the Peyer's patches, lymphoid accumulations that play a key role in mucosal immunity. M cells are believed to serve as portals for lumenal antigens.

Early Studies on Self-Renewal

The Austrian physician Joseph Paneth (1857–1890) was the first to propose that the epithelium of crypts and villi derives from the same embryological origin (Paneth, 1887). Bizzozero proposed a functional connection between the two compartments in the adult when he noticed that mitoses only occur in the crypts and that daughter cells must therefore be extruded from the crypts to contribute to the surface epithelium (Bizzozero, 1893). In 1947, Leblond and Stevens published a landmark study on the rate and mechanism of self-renewal of the epithelium, using a clever strategy involving the spindle poison colchicine and a histological scoring method for mitotic cells (Leblond and Stevens, 1948; Stevens and Leblond, 1947).

They concluded that full-grown rats continue to produce large numbers of cells in crypts throughout life and that the life cycle of an individual cell is in the order of days, a statement that was met with disbelief at the time. Also, they realized that this high birth rate of crypt cells should be balanced by a graveyard located elsewhere. They concluded, "…the cells formed in the crypts of Lieberkuhn move upward along the side of the villi to be ejected when they reach the villi tips." Others had deduced a similar flow of cells from crypts to villi (Friedman, 1945). Ten years later, this conveyor belt mechanism was confirmed by injection of radioactive forms of the DNA precursors adenine and thymidine, followed over time by autoradiography (Leblond and Messier, 1958; Quastler and Sherman, 1959; Walker and Leblond, 1958).

From these observations, it followed logically that stem cells fueling this rapid self-renewal process should reside somewhere near the crypt bottoms. Such stem cells should display two basic characteristics: self-renewal and multipotency. In other words, they should persist for the lifetime of the mouse while producing all other cell types of the epithelium.

The Crypt Base Columnar Stem Cell

It was again the Leblond lab that was the first to investigate the identity of the crypt stem cell. Cheng and Leblond noted that the crypt base is not exclusively populated by Paneth cells. Wedged between these prominent postmitotic cells, electron microscopy revealed the presence of diminutive cells that are continuously cycling, the so-called crypt base columnar (CBC) cells (Figure 3B) (Cheng and Leblond, 1974a). Interestingly, I recently noted two CBC cells in one of Paneth's drawings of almost a century earlier (Figure 3A; Paneth, 1887). Following

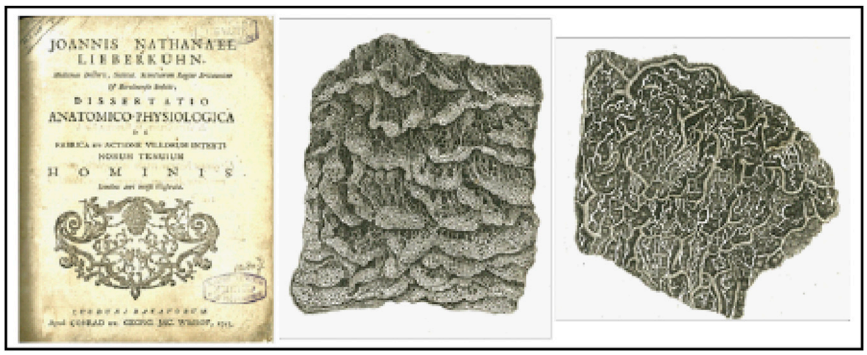

Figure 1. First Description of Crypts by Lieberkühn

(Left) Cover of Lieberkühn's thesis (Lieberkühn, 1745). (Middle) Lumenal face of a wax model showing the vessels within villi. (Right) External view of mucosa, showing blood vessels; small, round indentations represent the base of crypts. Courtesy of Museum Boerhaave, Leiden, the Netherlands.

^{3}H-thymidine injection, some CBC cells died and were phagocytosed by surviving CBC cells, yielding radioactive (and therefore traceable) phagosomes. Such "hot" phagosomes were initially only observed within surviving CBC cells but at later time points also appeared within more differentiated cells. This rudimentary lineage-tracing experiment supported the notion that all four main differentiated lineages derive from CBC cells (Cheng and Leblond, 1974b).

Much later, Winton and Ponder exploited a clonal labeling strategy (Winton et al., 1988) based on chemical mutagenesis. They demonstrated that crypts become clonal over months and were the first to directly visualize the flow of cells from crypt bottoms to villus tips as "ribbons" (Figure 4A). Using a similar chemical mutagen approach, Bjerknes and Cheng extended these observations (Bjerknes and Cheng, 1999). Randomly marked crypt cells yielded long-lived as well as short-lived clones, consistent with the existence of a stem cell compartment and a transit-amplifying (TA) compartment. The long-lived clones comprised all major cell lineages and presented as ribbons. These ribbons consistently included at least one marked CBC cell, lending further support to it being the self-renewing, multipotent stem cell.

The "stem cell zone" model takes as its central premise that CBC stem cells and Paneth cells reside in a stem-cell-permissive environment that is restricted to the crypt bottom (Bjerknes and Cheng, 1981a, 1981b). Stem cell daughters exit the stem cell zone and pass through position 5 (one cell diameter away from the uppermost Paneth cell), the "common origin of differentiation." At this position (where direct contact with mature Paneth cells is lost), the daughters commit toward the various individual lineages. Maturation occurs during the upward migration toward the villus. As the exception, maturing Paneth cell progenitors migrate downward from position 5, such that the oldest Paneth cells reside closest to the crypt base (Bjerknes and Cheng 1981a).

The Position 4 Stem Cell

Chris Potten and colleagues reported that rare DNA-label-retaining cells (LRCs) reside directly above the Paneth cells, also known as "position 4," or "+4" (Potten et al., 1978; Potten et al., 2002). DNA label retention reveals mitotic quiescence and is widely used as a surrogate stem cell marker. It has gone unnoticed by many, however, that Potten's position 4 LRCs cycle every day. Thus, the LRC trait of position 4 cells does not reflect quiescence but, rather, is proposed to result from asymmetric segregation of old (labeled) and new (unlabeled) DNA strands into stem cells and their daughters respectively (Marshman et al., 2002; Potten et al., 2002). The "immortal strand" hypothesis was originally postulated as a mechanism to protect the stem cell genome from mutation (Cairns, 1975); these observations for the cycling position 4 LRC have not been independently confirmed since. On the contrary, cycling stem cells at the crypt base segregate their chromosomes randomly (Escobar et al., 2011; Schepers et al., 2011; Steinhauser et al., 2012).

Wnt Signals Fuel the Stem Cell Compartment

Crypt research accelerated with the almost simultaneous discovery of deregulated Wnt signaling as the primary driver of colon cancer and of physiological Wnt signaling as the driver of crypt proliferation. For a detailed overview of the Wnt pathway, the reader is referred elsewhere (Clevers and Nusse, 2012). In the absence of a Wnt stimulus, the key effector of Wnt signaling, free cytoplasmic β-catenin, displays an exceedingly short half-life due to the action of the APC destruction complex. When Wnt proteins occupy their Frizzled-Lrp5/6 receptors, β-catenin is stabilized, accumulates, and travels to the nucleus. It then engages Tcf transcription factors to activate transcription of Wnt/Tcf target genes.

In the early 1990s, the APC tumor suppressor gene was found to be mutant in most forms of colorectal cancer (CRC) (Groden et al., 1991; Kinzler et al., 1991). Soon thereafter, the APC protein was found to occur in complex with β-catenin (Rubinfeld et al., 1993; Su et al., 1993). Loss of APC in CRC cells and the consequent accumulation of β-catenin were subsequently found to induce transcriptional activation of target genes of Tcf4 (a.k.a., Tcf7l2) (Korinek et al., 1997). In rare cases of CRC, mutations of other negative regulators of the Wnt pathway, Axin2 (Liu et al., 2000) and Rnf43 (Koo et al., 2012), have been reported. Alternatively, rare oncogenic point mutants can occur in β-catenin (Morin et al., 1997), and gene fusions involving Tcf4/Tcf7l2 (Bass et al., 2011) and the secreted Wnt agonists called Rspondins (Seshagiri et al., 2012) have very recently been observed in colon cancer.

Neonatal Tcf4 knockout mice lack proliferative crypts, implying that Wnt signals are required for the establishment of the stem cell compartment (Korinek et al., 1998). Maintenance of adult crypt proliferation continues to be dependent on Wnt, as demonstrated upon transgenic expression of the Wnt receptor antagonist Dkk1 (Pinto et al., 2003) and upon conditional

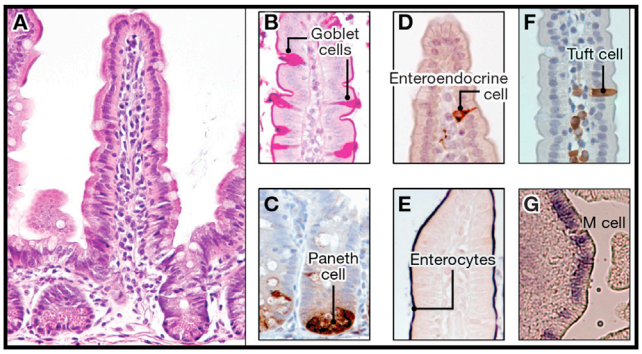

Figure 2. Epithelial Cell Types of the Small Intestine
Images adapted from van der Flier and Clevers, 2009.
(A) Hematoxylin and eosin staining of the intenstinal epithelium.
(B) Periodic acid-Schiff-stained (purple) goblet cells on villus.
(C) Lysozyme (brown)-stained Paneth cells at crypt bottoms.
(D) Chromogranin-stained (brown) enteroendocrine cell.
(E) Alkaline phosphatase-stained (blue, at lumenal brush borders) villus enterocytes.
(F) DCAMKL1-stained tuft cell (courtesy of P. Jay).
(G) Spi-B expression in microfold (M) cells.

deletion of β-catenin (Fevr et al., 2007) or Tcf4 (van Es et al., 2012a) in adult mice. Among the first intestinal Wnt target genes to be discovered were cyclin D1 (Tetsu and McCormick, 1999) and cMyc (He et al., 1998), well-known drivers of proliferation of undifferentiated cells. Indeed, gene knockout of cMyc gradually halted crypt self-renewal (Muncan et al., 2006), whereas adenoma formation by APC deletion in crypts was blocked by simultaneous cMyc deletion (Sansom et al., 2007).

Lgr5 as a Marker for CBC Cells

The first microarray experiment on Wnt-pathway-controlled genes in a human colon cell line unveiled a genetic program shared between colon cancers and crypts (van de Wetering et al., 2002). One of the prominent genes on this list is *Lgr5*, which later turned out to be an exquisite marker for the CBC cell. An *Lgr5*$^{EGFP\text{-}ires\text{-}CreERT2}$ allele was then generated and crossed to the *R26R-lacZ* Cre reporter (Barker et al., 2007). Each crypt harbors around 15 Lgr5^{GFP+} cells, which are invariably in contact with Paneth cells and divide each day (Figure 3C). Tamoxifen-induced lineage tracing resulted in the tell-tale ribbons within 5 days, extending from crypt base to villus tip (Figure 4B). Many of these clonal ribbons persisted life-long and contained all epithelial cell lineages.

From fluorescence-activated cell-sorted (FACS) Lgr5^{GFP} cells, a gene expression signature has been determined (Muñoz et al., 2012; Van der Flier et al., 2007), which allowed functional analysis of additional stem cell genes. Thus, the transcription factor *Ascl2* was identified as a master regulator of the Lgr5 stem cell (van der Flier et al., 2009b). *OlfM4* represents a robust marker for Lgr5 stem cells (van der Flier et al., 2009a). Musashi-1 (Potten et al., 2003) and Prominin1/CD133 (Zhu et al., 2009) also mark Lgr5 stem cells, but their expression may extend into the lower TA compartment (Itzkovitz et al., 2012; Muñoz et al., 2012; Snippert et al., 2009).

Lgr5 encodes a serpentine receptor and is a facultative component of the Wnt receptor complex (de Lau et al., 2011).

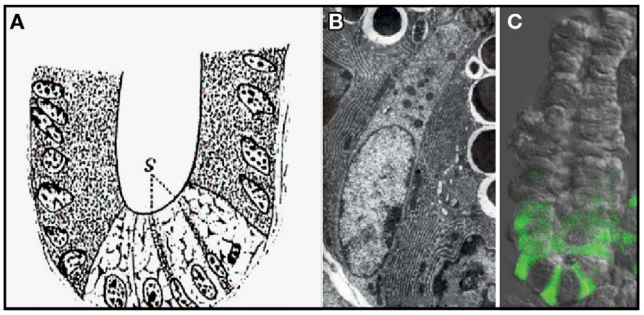

Figure 3. Paneth Cells and Crypt Base Columnar Cells
(A) Hand-drawn crypt (Paneth, 1887). The large white cells are Paneth cells. s, "schmale Zellen" (small/narrow cells).
(B) First electro-microscopic image of a crypt base columnar (CBC) cell, flanked by two Paneth cells with large black granules (from Cheng and Leblond, 1974a).
(C) Confocal image of Lgr5-GFP CBC cells in green, separated by dark, large Paneth cells.

Like its homolog Lgr4, it acts as the receptor for a small family of secreted Wnt pathway agonists called Rspondins (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011). Rspondins do not initiate Wnt signals but potently enhance such signals. Indeed, exogenous Rspondin1 induces dramatic crypt hyperplasia (Kim et al., 2005). Although deletion of the *Lgr5* gene in the intestine has little effect, mutation of *Lgr4* (which is expressed by all crypt cells) severely decreases crypt proliferation (Mustata et al., 2011). Double-*Lgr4*/*Lgr5* knockout completely abolishes proliferation (de Lau et al., 2011), in agreement with the notion that Rspondins are major drivers of crypt self-renewal.

Markers of +4 Stem Cells

Multiple recent studies have focused on the identification of markers for cells located at the +4 position that are slow-cycling/quiescent (and would thus be different from Potten's original +4 cells that cycle every 24 hr).

Bmi1 was the first +4 stem cell marker investigated by lineage tracing (Sangiorgi and Capecchi, 2008). Bmi1 was reported to mark rare, slowly cycling cells at the +4 cell position, uniquely in the proximal small intestine. In vivo lineage tracing yielded ribbons under noninjury conditions that kinetically and morphologically resembled those obtained in the Lgr5 model. A follow-up study proposed a model in which Lgr5 stem cells mediate homeostatic self-renewal, whereas Bmi1^{+} stem cells mediate injury-induced regeneration (Yan et al., 2012).

Breault and colleagues reported the occurrence of mTert-GFP^{+} cells in 1 per 150 crypts. A subfraction of these Tert-GFP+ cells were LRCs (Breault et al., 2008). In a follow-up study, it was shown that mTert expression marks a radiation-resistant pool of stem cells, distinct from Lgr5^{+} cells (Montgomery et al., 2011).

Hopx encodes an atypical homeobox protein. A *Hopx-LacZ* knockin allele was predominantly expressed by LRCs located at the +4 position along the entire intestinal tract. Lineage tracing from the *Hopx* locus resulted in long-lived ribbons. Additional evidence indicated that the Hopx^{+} and Lgr5^{+} populations represent slow-cycling and fast-cycling stem cell populations that can interconvert (Takeda et al., 2011).

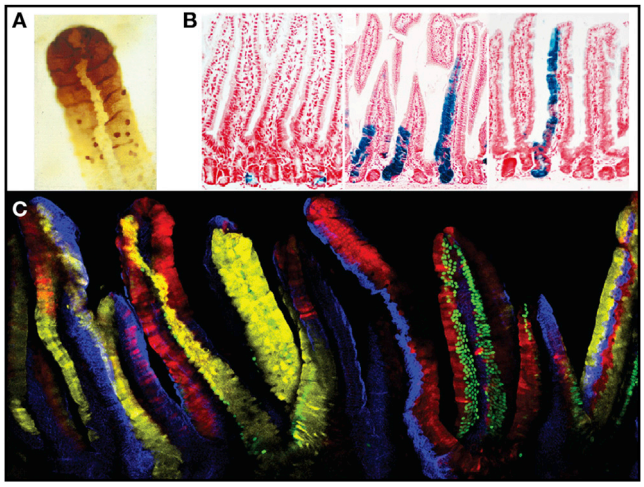

Figure 4. Ribbons and Linage Tracing

(A) The first visualization of a "stem cell ribbon." *Dlb1* mutant stem cells produce an unstained ribbon, running up the flank of a brown-stained villus toward its tip. Brown staining of wild-type cells is strongest at the villus tip (courtesy of D. Winton) (Winton et al., 1988).

(B) Rosa-LacZ tracing from an Lgr5 stem cell over time. Ribbons eventually run from crypt bottom to villius tip (Barker et al., 2007).

(C) Multiple ribbons generated from the multicolor ("confetti") Cre-reporter (Snippert et al., 2010).

Lastly, *Lrig1* encodes a transmembrane ErbB inhibitor. Lineage tracing from an *Lrig1-CreERT2* allele (Powell et al., 2012) initiated at crypt bottoms along the entire length of the intestinal tract and yielded ribbons by 7 days. Around 20% of the Lrig1$^+$ cells were LRCs. Comparative microarray profiling revealed that sorted Lgr5$^+$ cells display a proliferation signature, whereas the Lrig1$^+$ population from colon showed signs of downregulation of the cell cycle. In a simultaneous study, Jensen and coworkers (using a different antibody) reported that approximately one-third of all small intestinal crypt cells express Lrig1, with the highest levels in Lgr5$^+$ stem cells (Wong et al., 2012).

Two cautionary notes on the interpretation of these +4 studies: First, we have argued elsewhere (Barker et al., 2012) that a head-to-head comparison reveals major differences between the +4 populations as described by the different markers. Second, a series of independent studies report that the +4 markers Bmi1, Tert, Hopx, and Lrig1 are all expressed rather broadly and are most abundant in the Lgr5 stem cells, (see Muñoz et al., 2012; Wang et al., 2013). This situation complicates the interpretation of the lineage-tracing experiments in terms of identifying non-Lgr5 cells as ribbon-generating stem cells.

Stem Cell Plasticity: Reconciliation of the CBC and the +4 Stem Cell Models

The intestine has the capacity to survive the acute loss of its active stem cell pool. This may relate to the existence of quiescent "reserve" stem cells (Li and Clevers, 2010) and/or to general plasticity of the TA progenitor compartment. The classical papers from the 1970s already proposed that the earliest TA cell generations may fall back into the stem cell niche to regain stemness (Cheng and Leblond, 1974b; Potten, 1977). Indeed, the Lgr5$^+$ stem cell phenotype appears to be by no means hard-wired. As an example of plasticity, Dll1 was shown to mark an early daughter of Lgr5$^+$ stem cells residing around position +5 (van Es et al., 2012b). Lineage tracing using CreERT2 expressed from the *Dll1* locus showed that these Dll1$^+$ cells represent short-lived progenitors that—under physiological conditions—produce small, mixed clones of secretory cells. However, when Lgr5$^+$ cells are killed by radiation, these Dll1$^+$ secretory progenitors readily revert to Lgr5$^+$ stem cells during the regeneration process.

De Sauvage and colleagues applied an elegant strategy to inducibly kill Lgr5$^+$ cells, i.e., through transgenic expression of the receptor for diphtheria toxin from the *Lgr5* locus (Tian et al., 2011). Upon injection of diphtheria toxin, the Lgr5$^+$ cells died, yet crypts remained intact for at least a week (after which the animals succumbed to liver-related pathology), implying that the self-renewal process can be maintained in the absence of Lgr5$^+$ cells. As soon as the toxin injections were stopped, Lgr5$^+$ cells reappeared. Using lineage tracing from the *Bmi1* locus, it was shown that these new CBC cells derive from Bmi1$^+$ cells (Tian et al., 2011).

A very recent study has unveiled additional plasticity in the crypt niche, reconciling many of the paradoxical observations. Winton and colleagues reassessed the nature of crypt LRCs by briefly expressing the stable chromatin marker histone 2B yellow fluorescent protein (YFP) throughout the crypt (Buczacki et al., 2013). In addition to the expected label retention by Paneth cells, non-Paneth-cell LRCs remained evident in the first 2–3 weeks after the pulse. These quiescent cells surprisingly coexpressed Lgr5, Paneth markers, and +4 markers. It was concluded that this second (Lgr5$^+$) LRC type represents a nondividing Paneth/enteroendocrine precursor that persists for some weeks before its terminal differentiation. To test the properties of these cells further, the authors devised an ingenious strategy that directly exploits the quiescent state to genetically mark the Lgr5$^+$ LRCs. In healthy mice, the marked Lgr5$^+$ LRCs failed to divide and disappeared over time, presumably because of their terminal differentiation. When crypts were damaged, however, the Lgr5$^+$ LRCs generated the tell-tale stem cell ribbons. Winton's LRCs likely represent the +4 cells seen in previous studies. Because individual Lgr5$^+$ LRCs are relatively short-lived, they cannot be considered stem cells in sensu stricto. However, new Lgr5$^+$ LRCs are constantly being generated by the cycling Lgr5$^+$ stem cells. As has also been proposed elsewhere (Roth et al., 2012), a population of LRCs is thus always available as a "reserve stem cell" reservoir to be called into action upon tissue damage.

Mitogens for the Transit-Amplifying Compartment

Whereas Lgr5 stem cells divide every 24 hr, TA cells take half as long for each of their four to five cell cycles. Thus, crypt output is largely determined by the proliferative activity of TA cells. It appears that the signals that maintain the proliferative state in stem cells also drive the vigorous proliferation of TA cells.

Wnt Signals

Wnt signals are crucial for the maintenance of crypts, as outlined above. In particular, the hyperplastic effects of exogenous Rspondin1 and the opposite phenotype seen in *Lgr4/5* knockout experiments (discussed above) indicate that Wnt signal amplification by Rspondins is crucial to maintain TA proliferation.

Tyrosine Kinase Receptor Signaling

Keratinocyte growth factor was among the first mitogens described for the small intestine (Estívariz et al., 1998). Yet, EGF family members appear to be more potent mitogens. Lumenally applied EGF is trophic to the small intestine of rats (Marchbank et al., 1995). Lrig1 is a negative-feedback regulator of the ErbB receptor family. It is highly expressed by most proliferative crypt cells, and its removal leads to a rapid expansion of the proliferative compartment (Wong et al., 2012). Tyrosine kinase receptors of the EphB family are also proposed to support crypt proliferation (Holmberg et al., 2006). The BMP-signaling pathway acts as a negative regulator of crypts, although an exact mechanism remains unknown. BMP-2 and -4 ligands are expressed in the mesenchyme of villi (Haramis et al., 2004), whereas BMP inhibitors are expressed in the mesenchyme around crypts (Kosinski et al., 2007). Inhibition of BMP signaling in the villus by transgenic overexpression of the BMP inhibitor Noggin results in ectopic crypt formation (Haramis et al., 2004). Similarly, conditional deletion of Bmp Receptor 1A results in hyperproliferative crypts (He et al., 2004). De novo crypt formation also occurs in juvenile polyposis patients, the majority of whom carry germline mutations in one of the various components of the BMP-signaling pathway (Howe et al., 2001; Howe et al., 1998; Zhou et al., 2001).

Notch Controls the Secretory versus Enterocyte Fate in Early TA Cells

Interaction of a Notch receptor with its cell-bound ligands (such as Dll1 or Dll4) results in proteolytic release of the Notch intracellular domain (NICD) through the actions of the γ-secretase protease. NICD translocates to the nucleus, where it binds the transcription factor CSL, thus activating transcription of target genes. Inhibition of Notch signaling in the intestinal epithelium (either genetically by conditional deletion of CSL or pharmacologically by γ-secretase inhibitors) results in the conversion of all proliferative cells into goblet cells (Milano et al., 2004; van Es et al., 2005b). The opposite occurs upon transgenic expression of NICD (Fre et al., 2005). Simultaneous deletion of Notch1 and Notch2 (coexpressed on stem cells and TA cells) has revealed that the two receptors act redundantly in this process (Riccio et al., 2008). A similar approach revealed that Dll1 and Dll4, expressed among others by Paneth cells, act redundantly as Notch ligands in the crypt (Pellegrinet et al., 2011).

A large body of evidence outlines a surprisingly simple molecular circuit downstream of the Notch receptors. It consists of the two helix-loop-helix transcription factors Hes1 and Math1 (or Atoh1). Notch signaling activates expression of Hes1, which in turn transcriptionally represses the *Math1* gene. Math1 acts as the gatekeeper of entry into the secretory lineage. What is the evidence for this? Deletion of Hes1 results in increased numbers of cells of all secretory lineages and decreased numbers of enterocytes (Jensen et al., 2000). This phenotype is more pronounced upon combined deletion of Hes1, Hes3, and Hes5 (Ueo et al., 2012). Conversely, Math1 deletion results in a complete loss of all secretory lineages (Shroyer et al., 2007; Yang et al., 2001), whereas Math1 overexpression is sufficient to direct progenitors into the secretory lineage (VanDussen and Samuelson, 2010). Indeed, Hes1 is expressed in most proliferative crypt cells, whereas Math1 is only seen in secretory cells; inhibition of Notch signaling rapidly leads to loss of Hes1 expression and to the consequent induction of Math1 expression in all crypt cells (van Es et al., 2005b). Of note, stem cells are exquisitely sensitive to Notch inhibition and instantly convert into Goblet cells. Deletion of Math1 renders TA cells and stem cells insensitive to Notch inhibition (van Es et al., 2010).

As mentioned above, *Dll1*$^+$ cells occupy position +5. (Stamataki et al., 2011; van Es et al., 2012b). These Dll1^{high} cells are immediate descendants of Lgr5 stem cells. Lineage tracing of Dll1^{high} cells resulted in small, short-lived clones that uniquely consist of all cell types of the secretory lineage. It thus appears that the secretory versus enterocyte fate is set immediately upon exit of stem cell daughters from the Paneth/stem cell zone at the "common origin of differentiation," as originally proposed by Bjerknes and Cheng (Bjerknes and Cheng, 1981a, 1981b).

From these observations, a "classic" Notch lateral inhibition scenario can be scripted that governs enterocyte-secretory fate specification in the crypt. At the crypt base, Dll1$^+$Dll4$^+$ Paneth cells trigger Notch1 and Notch2 on stem cells, thus repressing Math1 expression and restraining the Lgr5 stem cells from terminal differentiation into the secretory lineage. Cells that exit the Paneth/stem cell zone pass through the common origin of differentiation at position +5, where they no longer see the membrane-bound Notch ligands of the Paneth cells. Stochastically, some of these cells shut off Notch expression and induce Math1 and Dll1 expression. Thereby, they establish their own secretory fate yet present Dll1 to multiple neighboring Notch$^+$ TA cells. These, in turn, will maintain an active Notch pathway, will repress Math1, and will thus stay proliferative and fated toward the enterocyte lineage.

Growing "Mini-Guts" from Single Stem Cells

It is generally assumed that adult somatic cells cannot be cultured for prolonged periods of time without undergoing senescence or transformation. Indeed, after more than four decades of bone marrow transplantation, it has remained impossible to significantly expand hematopoietic stem cells in culture. Because Lgr5 stem cells divide every day, they complete around 1,000 cell divisions in the lifetime of a laboratory mouse, thus defying the Hayflick limit in vivo. Based on the growth factor requirements observed in vivo, we have established a Matrigel-based culture system that allows the formation of ever-expanding organoids, or "mini-guts," in vitro from a single Lgr5 stem (Figure 5) (Sato et al., 2009). An essential component of these cultures is the Wnt agonist Rspondin1, the ligand of Lgr5. The other constituents are EGF and the BMP inhibitor Noggin. The mini-guts faithfully recapitulate the central features of normal gut epithelium. They consist of crypts (with resident Lgr5 cells, Paneth cells, and TA cells) that feed into a central lumen lined by mature epithelial cells of all villus lineages. Self-renewal kinetics resemble the in vivo situation: cells are born in the crypts, proliferate, differentiate, and are shed into the central lumen about 5 days later. Clonal organoids expanded from a single adult colonic Lgr5$^+$ cell have been transplanted into multiple recipient mice in which epithelial damage had been induced by

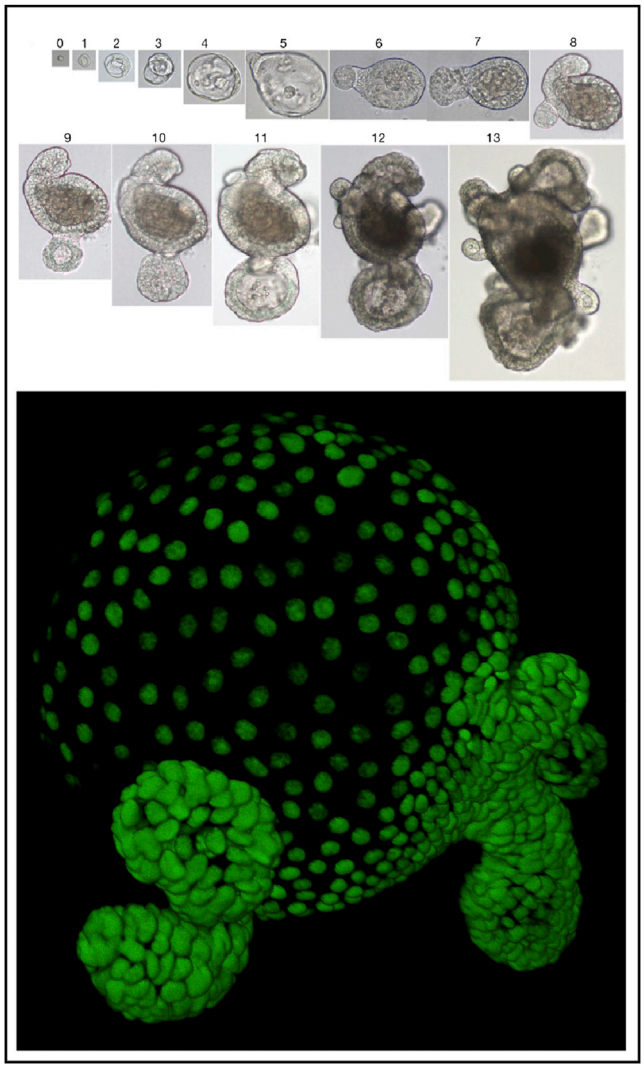

Figure 5. Growing an Organoid from a Single Cell
(Left) An organoid grown from a single sorted Lgr5-GFP cell (Sato et al., 2009). (Right) A mini-gut organoid expressing histone 2B-GFP. Five crypts emanate from a large central body that contains the main lumen (courtesy of G. Schwank).

chemical treatment. The grafted organoids remained healthy and functional for at least 6 months after transplantation (Yui et al., 2012).

The Stem Cell Niche

It was striking to observe that mini-guts are fully self-organizing in the absence of a nonepithelial niche, as this suggested the existence of an epithelial "crypt organizer" cell. Could the Paneth cell play this role? At crypt bottoms, Lgr5 cells and Paneth cells are geometrically distributed in such a fashion that individual Paneth cells are surrounded by Lgr5 stem cells and vice versa. Gordon and coworkers originally rejected the hypothesis that Paneth cells serve to supply essential stem cell niche signals. They expressed a diphtheria toxin transgene specifically in Paneth cells and observed that, although fewer than 20% of Paneth cells remained, crypt proliferation was largely normal (Garabedian et al., 1997).

We reassessed Gordon's hypothesis by using the Lgr5 stem cell marker in conjunction with the mini-gut culture system. In vitro, single sorted Lgr5 cells rarely survived, whereas doublets consisting of one stem cell and one Paneth cell robustly generated mini-guts. This "doublet assay" has meanwhile been exploited to demonstrate that Paneth cells monitor the metabolic state to fine-tune stem cell activity (Yilmaz et al., 2012). In vivo, three genetic mouse models were shown to display severe reductions in Paneth cells (Bastide et al., 2007; Garabedian et al., 1997; Mori-Akiyama et al., 2007; Shroyer et al., 2005). We observed that, in all cases, genetic removal of Paneth cells resulted in the concomitant loss of Lgr5 stem cells (Sato et al., 2011). This was confirmed more recently in a fourth mouse model of Paneth cell loss (Geiser et al., 2012).

Paneth cells are commonly known as the producers of bactericidal products that protect the stem cells from microbial attack (reviewed in Clevers and Bevins, 2013). Gene expression profiling of sorted Paneth cells revealed the additional expression of EGF and the related TGFα, of Wnt3, and of the Notch ligands Dll1 and Dll4 (Sato et al., 2011). Indeed, Wnt3 mutant Paneth cells cannot support the growth of mini-guts, which can be overcome by the addition of exogenous Wnt (Farin et al., 2012). Of note, this arsenal of Paneth cell growth signals is remarkably similar to the composition of the mini-gut culture system.

When Shivdasani and colleagues conditionally deleted *Math1*, they observed the complete elimination of all secretory cells, including the Paneth cells. Paradoxically, stem cells appeared to function normally in the absence of Paneth cells (Kim et al., 2012). Durand et al. pointed out that genetic removal of Math1 relieves the in vivo dependence on Notch signals, normally provided by Dll1/4 on Paneth cells (Durand et al., 2012). They confirmed that Math1 deletion eliminates Paneth cells in vivo without obvious changes to the stem cells yet noted that Math1 mutant mini-guts did not grow in vitro. This implied a crucial in vitro dependence of the stem cells on another (non-Notch) signal provided by Paneth cells, likely Wnt3. Indeed, genetic deletion of Wnt3 has no effect in vivo but produces the same in vitro growth inhibition of stem cells (Farin et al., 2012).

From this, it can be concluded that Paneth cells play a unique role as niche cells in vitro but that redundancy exists with other sources of growth signals in vivo. Only the Notch ligands that are presented by Paneth cells to neighboring Lgr5 stem cells are essential in vivo, whereas none of the other Paneth-born stem cell signals are uniquely required. Wnts are produced in a redundant fashion by Paneth cells and by subepithelial mesenchyme (Farin et al., 2012), as are EGFs. Of note, two essential factors are not produced by Paneth cells. BMP inhibitors are produced by the mesenchyme (Kosinski et al., 2007), whereas the in vivo source of the important Rspondins is not epithelial and remains to be identified.

Homeostatic Control of the Paneth/Stem Cell Zone

It appears that "touching a Paneth cell" is necessary and sufficient to maintain an Lgr5 stem cell. Each crypt contains a surprisingly constant number of around 15 Lgr5 stem cells and 10

Paneth cells. From short- and long-term clonal tracing data of individual Lgr5^{hi} cells, it has been deduced that stem cells do not divide asymmetrically (Figure 4C) (Lopez-Garcia et al., 2010; Snippert et al., 2010). Rather, all stem cells divide symmetrically each day, after which all daughters compete for limited niche space, likely formed by the available Paneth cell surface. This leads to a model in which the stem cells in a given crypt are in constant competition, without any single stem cell having a higher a priori chance to "win." Indeed, it takes, on average, 3 months for crypts to become monoclonal. In other words, at any given time, all crypt cells derive from only 1 of the 15 stem cells that coexisted 3 months earlier. In this "neutral competition" model, the available Paneth cell surface determines the number of Lgr5^{hi} stem cells in a crypt.

Thus, Paneth cells are a key determinant of the stem cell niche, and their numbers must therefore be tightly controlled under homeostatic conditions. How is this accomplished? It is evident that strong Wnt signals, in the absence of Notch signals, drive formation of new Paneth cells (Andreu et al., 2008; Farin et al., 2012; van Es et al., 2005a). Because Paneth cells are the Wnt source that drive formation of new stem cells as well as of Paneth cells, a Wnt-driven positive-feedback loop exists that would potentially lead to ever-expanding crypts. A recent study describes a potential counterforce. Within the Wnt gene signature of colon cancer, two related Wnt target genes were observed, *Rnf43* and *Znrf3* (Hao et al., 2012). These genes encode transmembrane E3 ligases that remove Frizzleds from the cell surface and thus constitute a negative-feedback loop in the Wnt pathway. The two genes are coexpressed with Lgr5 in a stem-cell-specific fashion (Koo et al., 2012). Simultaneous conditional deletion in crypt stem cells of *Rnf43* and *Znrf3* resulted in rapidly expanding stem/Paneth compartments that ultimate grew into large adenomas. From these observations, it follows that *Rnf43* and *Znrf3* serve as negative-feedback inhibitors to control the size of the crypt niche.

What mechanism sorts the Paneth cells away from all other cells? EphB receptors and their EphrinB ligands are surface-bound molecules that exert repulsive forces between cells expressing EphBs and cells expressing EphrinBs. *EphB3* is a Wnt target gene and is expressed by Paneth cells at crypt bottoms, its expression driven by local Wnt production. EphrinB1 is expressed by differentiated cells in a reverse, villus-to-crypt gradient. In *EphB3*$^{-/-}$ mice, Paneth cells fail to home efficiently to the crypt base but tend to comigrate with all other cells toward the villus tip (Batlle et al., 2002). This implies that the producer of Wnt3, the Paneth cell, autoinduces a surface receptor that forces it to move in a direction opposite of all other cells to remain at the bottom of the crypt. When proliferating TA cells are mechanically pushed away from the crypt bottom by newly born TA cells, they will experience rapidly decreasing levels of the Wnt signal. This will drive their terminal differentiation into one of the (EphrinB^{+}) villus-epithelial cell types, with Notch lateral inhibition acting as the enterocyte-secretory fate switch in this process.

Specification of Individual Lineages

Cell fates are set at the common origin of differentiation at the +5 position, only one division away from the stem cell. As discussed above, Notch activity serves to repress *Math1* expression and thus the secretory cell fate. When *Math1* is genetically deleted, all cells become fated toward the enterocyte lineage, the "default fate." Factors that control (or are required for) the various other fates are continuously being discovered. For a comprehensive recent review, the reader is referred to Noah et al. (2011).

Goblet Cells

Goblet cell appear to represent the default fate within the secretory lineage, as all proliferative cells convert into goblet cells upon acute Notch inhibition (see above). The transcription factor SPDEF is important for their formation (Gregorieff et al., 2009; Noah et al., 2010).

Paneth Cells

Paneth cell formation is dependent on Sox9, a transcription factor itself encoded by a Wnt target gene and expressed in all cells at crypt bottoms (Bastide et al., 2007; Mori-Akiyama et al., 2007). Indeed, active Wnt signals promote the formation of Paneth cells (Farin et al., 2012; van Es et al., 2005a). The dependence of Paneth cell formation on FGF-R3 (Vidrich et al., 2009) implies a role for one or more fibroblast growth factors (FGFs) in this process.

Enteroendocrine Cells

Enteroendocrine cells exist in several subtypes that are defined by the hormones that they produce. Lineage specification requires Neurogenin3 (Jenny et al., 2002). Indeed, ectopic expression of Neurogenin3 in the embryo directs enteroendocrine differentiation within the secretory lineage (López-Díaz et al., 2007). Genes involved in subspecification of enteroendocrine cells are discussed elsewhere (May and Kaestner, 2010).

Tuft Cells

Tufts cells are very rare cells characterized by pronounced actin bundles, probably involved in chemical sensation of lumenal contents. Although they derive from Dll-positive precursors, they do not depend on Math1 for their formation (Bjerknes et al., 2012) and should thus not be considered secretory cells. Indeed, formation of tuft cells is unaffected by deletion of transcription factors that are crucial for other secretory cell types, such as Neurog3, Sox9, or Spdef (Bjerknes et al., 2012; Gerbe et al., 2011).

M Cells

Peyer's patches are domains of specialized intestinal epithelium overlying the gut-associated lymphoid tissue. Lumenal antigens are transported through M cells toward the lymphoid cells. The Ets family transcription factor SpiB is specifically expressed in M cells, and M cells are entirely absent in *SpiB*$^{-/-}$ mice (de Lau et al., 2012; Kanaya et al., 2012). The cytokine RankL induces M cell development in vivo (Knoop et al., 2009). Its receptor, RANK, is expressed on all epithelial cells. Stimulation with RankL quantitatively transforms mini-guts into functional M cells in vitro, implying that a single signal emanating from the underlying tissue can divert differentiating cells away from the "default" villus fates toward the M cell fate (de Lau et al., 2012).

Epilogue and Outstanding Questions

Based on the fact that Lgr5 alone identifies CBC cells with exquisite specificity, transgenic mouse models have been generated that allow direct experimental access to these stem cells with

minimal manipulation in vivo. Moreover, these mouse models have been instrumental in the establishment of long-term in vitro culture systems. The combination of these two advances makes the CBC cell arguably one of the best-documented adult mammalian stem cells in terms of behavior in situ and in culture. A summary of these insights is visualized in a recent *Cell* SnapShot (Clevers and Batlle, 2013). CBC stem cells fail to display many of the classical stem cell attributes. They are not quiescent but cycle every day and, despite this, do not appear to ever be subject to stem cell exhaustion. They do not depend on an exogenous, pre-existing niche but, rather, build their own. They divide symmetrically: fates of individual daughters are determined extrinsically, i.e., by the nature of the neighboring cells rather than by an intrinsic mechanism set at mitosis.

Self-renewal (the almost magical capacity of a stem cell to produce a daughter cell while recreating a copy of itself) of CBC cells may actually be the product of a rather mundane process. Self-renewal of CBC cells occurs at the population level and reflects nothing more or less than the ability to proliferate as long as the stem cell resides in the right location: ''touching a Paneth cell.'' For lifelong proliferation, it only needs to guarantee the integrity of its genome and the length of its telomeres. Indeed, CBC cells have high levels of telomerase (Schepers et al., 2011). Interestingly, Wnt signals activate telomerase expression, which may in part explain the crucial role of this pathway in stem cell biology (Hoffmeyer et al., 2012). It appears rather straightforward to protect all nongenomic components of a CBC cell against wear and tear. Each component undergoes a doubling each day. Thus, damage to ''old'' cellular structures is automatically diluted by newly synthesized material.

Similarly precise in vivo research tools have become available for adult stem cells of other tissues, such as the testis, the mammary gland, and the skin. Comparison of the characteristics of these unrelated stem cells may lead to novel operational definitions of the central attributes of self-renewing adult stem cells.

Despite this progress there are many outstanding questions to resolve. Why does the intestinal epithelium self-renew with such an unprecedented rate? How is the integrity of the CBC stem cell genome maintained over many years despite the cells' daily cell division and their exposure to constant external insult. Do CBC stem cells entertain a specialized metabolism—and if so, why? A metabolic gradient exists along the crypt axis with the highest levels of glycolysis in the cycling stem cells (Stringari et al., 2012). What molecules mediate the heterotypic adhesion between Paneth cells and stem cells? A timer appears to control the life cycle of an epithelial cell in vivo as well as in culture, inevitably leading to apoptosis 4–5 days after birth, independent of P53 status. What is the molecular nature of the timer, and what drives the apoptosis? What signals induce the various enteroendocrine lineages such as the L cell? Manipulation of such signals may be of clinical value, given, for instance, the role of L-cell-derived glucagon-like peptides in diabetes. Upon damage, the remaining healthy crypts go into ''overdrive.'' What controls the induction of such hyperplasticity, and how—once the tissue is healed—is it reverted? Lastly, are the niche requirements of normal crypt stem cells maintained in neoplasia, and can these be exploited as therapeutic targets?

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