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If a Stem Cell Dies in the Crypt, and No One Is Around to See It...

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Two recent studies continue the debate regarding lineage and hierarchy in the intestinal epithelium. One reports that quiescent crypt cells are Paneth cell precursors (Buczacki et al., 2013). The second shows that tamoxifen induces apoptosis in crypt cells and that suppressing apoptosis alters lineage tracing patterns (Zhu et al., 2013).

Epithelial tissues are routinely replenished during homeostasis and require repair after damage. A small number of self-renewing stem cells are the source of new cells in many epithelia. The identification of these stem cells has been the focus of intense investigation, and the principle approach has involved tamoxifen-inducible Cre lineage-tracing systems.

One of the best-understood epithelial stem cell systems is that of the intestine, whose entire lining is replaced every few days. New epithelial cells are born at the crypt base before migrating out of the crypt and differentiating into one of several cell types. Mature cells continue to migrate along the villus and are eventually shed into the gut lumen. The life span of a typical epithelial cell in the small intestine is 3–5 days, although Paneth cells located at the crypt base persist for several weeks.

Lineage tracing and other functional studies, building on decades of earlier work, point to two populations of intestinal stem cells. Crypt base columnar cells (CBCs), interspersed between Paneth cells and marked by the gene Lgr5, generate all the cell types of the epithelial crypt in vivo (Barker et al., 2007). These cells, of which there are approximately 15 per crypt, can be isolated and grown in vitro to form organoid "miniguts" that resemble the intestinal crypt (Sato et al., 2009). Alternatively, cells at the +4 position just above the Paneth cell zone have been put forward as stem cells. These cells die from low doses of radiation, which may prevent accumulation of mutations in the stem cell pool (Barker et al., 2012). Several genes are reported to mark these rare cells, including Bmi1, mTert, Hopx, and Lrig1. These cells are also thought to be quiescent, consistent with the classical notion that stem cells do not frequently cycle.

The debate regarding intestinal stem cell identity has been driven by several conflicting arguments. Crypts are clonal (Winton and Ponder, 1990), meaning that they are derived from a single cell, a finding that appears to be at odds with the presence of a large number of Lgr5-positive stem cells per crypt. This paradox suggests the existence of a "master" stem cell hierarchically above the CBCs. In addition, the active proliferation of Lgr5-positive cells has vexed those who believe that a defining characteristic of adult tissue stem cells is quiescence. Finally, targeted killing of Lgr5-positive cells has no obvious short-term effects on crypt architecture or function, suggesting that these cells are dispensable (Tian et al., 2011).

Despite these issues, it has become accepted that Lgr5-positive cells are bona fide crypt stem cells. Models of neutral drift have countered arguments that the clonality of the crypt is inconsistent with a role for Lgr5-positive cells as stem cells (Snippert et al., 2010). Definitive analysis of +4 cells is challenging because markers of quiescent stem cells are also expressed in Lgr5-positive cells (Barker et al., 2012). Finally, cells fated for differentiation and loss can revert to a stem-cell-like state after tissue damage and stem cell death, making plasticity in the crypt one potential explanation for the dispensability of Lgr5-positive cells. For example, progenitor cells that express the Notch ligand Dll1, which are normally restricted to a secretory fate, can dedifferentiate after irradiation and contribute to multiple lineages (van Es et al., 2012).

The argument for plasticity has been strengthened by a new report that a subset of Lgr5-positive cells are quiescent and are fated to become Paneth cells (Buczacki et al., 2013). Buczacki and colleagues developed an ingenious lineage tracing strategy that marks only label-retaining cells in the intestinal epithelium. The authors fused a fragment of Cre recombinase (CreA) to Histone 2B, under the control of a β-naphthoflavone (βNF)-inducible promoter expressed in intestinal epithelial cells. After a pulse of βNF, followed by a chase of several days, only quiescent cells (whose histones had not turned over) retained CreA. Administration of a dimerization agent reunited the histone-fused CreA fragment with its ubiquitously expressed counterpart CreB. This triggered lineage tracing in label-retaining cells through recombination. The authors found that during homeostasis, label-retaining cells were Lgr5-positive and were destined to become Paneth cells (Figures 1A and 1B). Much like Dll1-positive progenitor cells, upon crypt damage these differentiated cells reverted to a stem-cell-like state (Figure 1C).

These experiments help to reconcile competing notions of clonality, quiescence, hierarchy, and plasticity in the crypt. However, a recent study in *Cell Stem Cell* adds another perspective to the story, suggesting that the very methods used to identify intestinal stem cells may bias those results. Zhu and colleagues showed that intraperitoneal injection of tamoxifen, an agent used extensively to activate the inducible CreER molecules upon which most lineage tracing approaches rely, led to cell death in the crypt base (Zhu et al., 2013). They

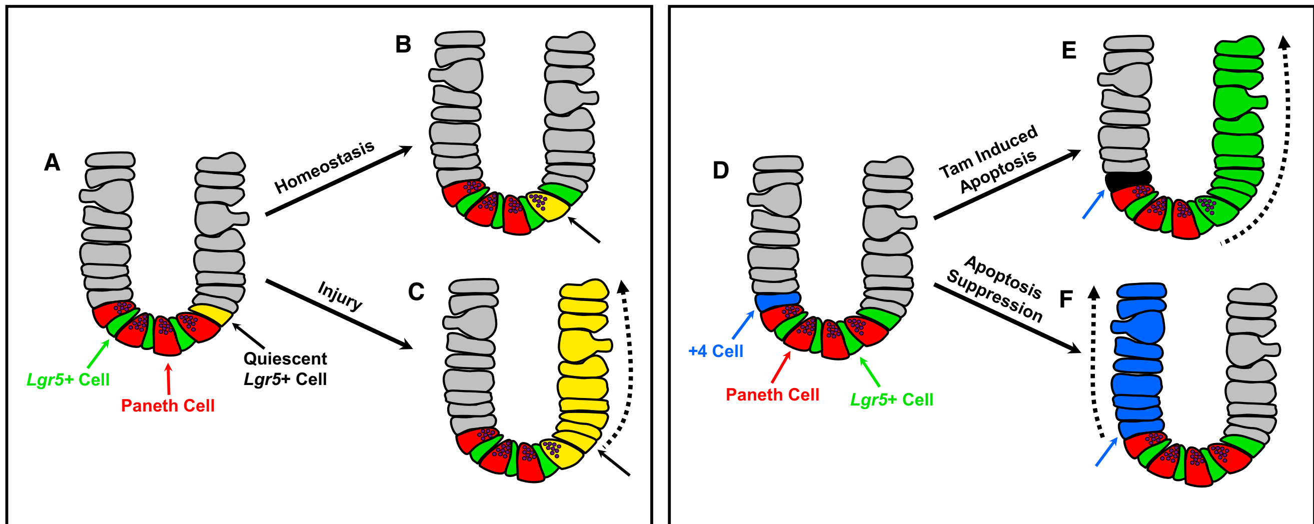

Figure 1. Quiescence and Apoptosis in the Intestinal Crypt
(A) A subset of Lgr5-positive cells are quiescent (black arrow) and function as Paneth cell precursors.
(B) Under normal conditions, quiescent Lgr5-positive cells are destined to become Paneth cells (black arrow).
(C) After damage to the crypt, quiescent Lgr5-positive Paneth cell precursors (black arrow) can revert to a stem-cell-like state and give rise to lineage tracing clones as shown by Buczacki et al. (2013).
(D) Lgr5-positive CBCs are interspersed between Paneth cells. +4 cells (blue arrow) reside just outside the CBC/Paneth cell zone.
(E) Upon administration of tamoxifen (Tam), a small number of crypt base cells die (black cell with blue arrow), including CBCs and +4 cells. Zhu and colleagues argue that tamoxifen-induced death of +4 cells enhances lineage tracing from Lgr5-expressing CBCs.
(F) Suppression of apoptosis blocks cell death in the +4 position and promotes lineage tracing from +4 cells. Lineage tracing from Lgr5-positive CBCs is severely reduced.

found that doses of tamoxifen typically administered in lineage tracing experiments induced apoptosis in both Lgr5-positive cells and Lgr5-negative cells in the +4 position (Figure 1E). Because this phenomenon could substantially affect results garnered from lineage tracing using tamoxifen-induced recombination, the authors asked whether suppression of apoptosis affects lineage tracing. They found that transgenic mice that repressed apoptosis, either through overexpression of Bcl2 or deletion of Chk2, had markedly different lineage tracing patterns compared to their wild-type counterparts. Importantly, they found that suppression of apoptosis led to a decrease in the number of lineage tracing ribbons from Lgr5-positive cells, whereas lineage tracing from Bmi1-positive cells was increased (Figures 1E and 1F). Consequently, the authors suggest that apoptosis in one stem cell population confers an advantage to another population of stem cells.

A gut reaction to this study is that many intestinal lineage-tracing experiments have been unintentionally biased due to the undetected death of progenitor cells. However, a number of issues must be addressed before far-reaching conclusions can be drawn. It is possible that the genetic strategies used in this study, such as the overexpression of Bcl2, could influence the physiology of the crypt and alter the behavior of stem cell populations. It is unclear why limited cell death has such a broad effect on lineage tracing from Lgr5-positive cells, and it is surprising that overexpression of Bcl2 thoroughly shut down lineage tracing from Lgr5-positive cells. It is also possible that environmental factors, or genetic effects such as strain background, can differentially influence progeny production by specific stem cell pools. These questions can be addressed by the development of nonnoxious lineage tracing agents.

The work by Zhu et al. raises an important point, which is that the methods used for lineage tracing should be stringently evaluated for unintended side effects and possible biases. As stated in a recent review, "For any lineage tracer, the key features are that it should not change the properties of the marked cell, its progeny, and its neighbors" (Kretzschmar and Watt, 2012). The new studies by Buczacki et al. and Zhu et al. continue the debate about the identity and location of intestinal stem cells, which does not yet appear to be over.

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