Editorial

Stem Cell INVESTIGATION

# Enterocyte progenitors can dedifferentiate to replace lost Lgr5$^+$ intestinal stem cells revealing that many different progenitor populations can regain stemness

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The epithelium lining the gastrointestinal tract serves many functions, including aiding in digestion, nutrient absorption, barrier function, and immunity. In the small intestine, the intestinal epithelium is comprised of repeating crypt-villus units which undergo constant renewal to maintain intestinal homeostasis and tissue integrity (1). Multipotent intestinal stem cells (ISCs) located in the base of the crypt are responsible for constant renewal and rapid replenishment of cells lining the crypt-villus axis. ISCs give rise to rapidly proliferating transit-amplifying (TA) cell population which, as they exit crypt, differentiate into the four major mature cell types: enteroendocrine cells, goblet cells, and Paneth cells belong to the secretory lineage, while enterocytes comprise the absorptive lineage. Although it is agreed upon that each crypt contains ISCs, the exact identity of the stem cell population has been debated for many years (2,3). However, over the last decade, the identification of specific stem cell markers and creation of genetic lineage tracing mouse models has improved our understanding of hierarchal relationships among the cells that comprise the stem cell niche (4-6). One such marker is *Lgr5* (Leu-rich repeat-containing G protein-coupled receptor 5-expressing), which is specifically expressed in crypt base columnar stem cells (CBCs) (1). Lgr5$^+$ CBCs are required for normal day-to-day intestinal homeostasis, but other reserve stem cell populations identified within the crypt are capable of repopulating the stem cell niche and restoring the Lgr5$^+$ stem cell pool after injury. Directly above the CBC/Paneth cell zone, at the +4 position from the crypt base, is a population of quiescent, slow-cycling stem cells. Numerous markers have been identified for this population including *Bmi1*, *Lrig1*, *Hopx*, and *mTert* (1). For example, the Lgr5$^{DTR}$ model of intestinal stress, where treatment with diphtheria toxin (DT) is used to specifically ablate Lgr5$^+$ CBCs, has revealed that quiescent Bmi1$^+$ cells that often reside at the +4 position, proliferate to repopulate the Lgr5$^+$ population in the crypt (7). Many of these quiescent/+4 stem cell markers are also expressed in Lgr5$^+$ CBCs, further highlighting the concept of plasticity within the stem cell zone but also raising the question as to whether these +4 stem cells are distinct the Lgr5$^+$ stem cell population (5).

Much of what is known about stem cell plasticity in the intestine is largely due to models of regeneration following intestinal injury. In addition to +4 quiescent stem cells, several different secretory progenitors can act as facultative stem cell populations capable of replacing Lgr5$^+$ CBCs including label-retaining cells expressing Paneth and enteroendocrine cell markers, and delta-like ligand 1$^+$ (Dll1$^+$) TA cells (8-11). Ionizing radiation is the classic model of intestinal injury, and studies have revealed that the Lgr5$^+$ CBCs are sensitive to radiation; however, the label-retaining Paneth progenitor cell population is resistant and can contribute to the stem cell niche following radiation (3,8). Additionally, Dll1$^+$ secretory progenitors are shown to revert to the stem cell state following radiation damage to the crypt (10). Interestingly, recent studies indicate that limited changes in chromatin remodeling between Lgr5$^+$ stem cells and committed progenitor populations likely provide the permissive conditions for secretory progenitors to dedifferentiate to a stem cell state (12,13).

Because many reserve stem cell populations have been identified as secretory progenitors, the manuscript by Tetteh *et al*. asked whether absorptive enterocyte progenitors are also capable of exhibiting plasticity when the Lgr5$^{+}$ stem cell population is lost (14). To address this question, they initially used microarray analysis of FACS-sorted cell populations to identify alkaline phosphatase (*Alpi*) as a candidate enterocyte specific transcript. *Alpi* is a functional protein primarily expressed in Lgr5$^{-}$ villus cells, but is also expressed at a low level in the Lgr5$^{low}$ cell fraction. Additionally, single molecule fluorescence in situ hybridization (smFISH) results revealed that *Alpi* does not co-localize with *Lgr5* at the crypt base, and is largely found at position +6 or higher. smFISH results also showed that *Alpi* transcripts do not co-localize with *Dll1* transcripts. Their initial results imply that *Alpi* expression is distinct from *Lgr5*$^{+}$ stem cells and *Dll1*$^{+}$ secretory progenitors, and Alpi$^{+}$ enterocyte progenitors are absent from the base of the crypt and confined to the TA zone. To understand more about *Alpi* expression in the intestine Tetteh *et al.* generated a tamoxifen inducible enterocyte-specific *Cre* line, $Alpi^{CreER}$. Crossing the $Alpi^{CreER}$ mice to a $R26R^{LacZ}$ reporter mouse and inducing with tamoxifen (TAM) revealed expression solely in the enterocytes cells of the villus, remaining absent from enteroendocrine cells, goblet cells, and Paneth cells. Even at the earliest time point (15 h), expression remained at the upper crypt. Expression was exclusive to the villus by 3 days post-TAM injection and virtually all labeled cells were absent by day 6, signifying cells were displaying typical upward migration and turnover. These data confirmed that the $Alpi^{CreER}$ driver only marked enterocyte progenitors within the upper crypt and these cells were rapidly lost during normal crypt homeostasis.

After validating the specificity of the $Alpi^{CreER}$ line, Tetteh *et al.* examined whether *Alpi*$^{+}$ enterocyte progenitors are capable of dedifferentiating back to crypt stem cells when the *Lgr5*$^{+}$ stem cell population is depleted. To answer this question, they used the Lgr5$^{DTR}$ system described previously to ablate the Lgr5$^{+}$ stem cells. Specifically, they crossed their $Alpi^{CreER+/+}$; $R26R^{LacZ+/-}$ mice to the $Lgr5^{DTR\text{-}GFP+/-}$ mice and used TAM and DT to induce Cre expression and delete Lgr5 cells, respectively. After 14 days of chase, Tetteh *et al.* observed X-GAL+ lineage tracing events that extended from the base of the crypt to the top of the villi, implying that Alpi$^{+}$ progenitors are able to dedifferentiate to multipotent ISCs that fill the stem cell niche. These results were further validated when they used the *tdTomato* fluorescent reporter to lineage trace Alpi$^{+}$ cells following *Lgr5*$^{+}$ cell ablation induced by DT. Importantly, *tdTomato* expression co-localized with *GFP* expression at the base of the crypts, suggesting that the Alpi$^{+}$ enterocyte progenitors are capable of dedifferentiating to an Lgr5$^{+}$ stem-like state. In addition, the lineage tracing ribbons were also positive for enteroendocrine, goblet, and Paneth cell markers, indicating that Alpi$^{+}$ enterocyte progenitors had dedifferentiated to multipotent cells—an essential characteristic of stem cells.

After probing the potential for Alpi$^{+}$ enterocytes to dedifferentiate *in vivo*, Tetteh *et al.* asked whether this same phenomenon occurred *in vitro* following ablation of Lgr5$^{+}$ stem cells. First, enteroid cultures generated from of $Alpi^{CreER+/-}$, $R26R^{LacZ+/-}$, $Lgr5^{DTR\text{-}GFP+/-}$ crypts were treated with 4-hydroxytamoxifen and DT prior to analysis. X-GAL staining revealed that only the Lgr5$^{+}$ stem cell ablated enteroids had X-GAL staining present in the budding structures, which are analogous to crypt base. Further analysis showed that once Alpi$^{+}$ enterocytes migrate out of budding structures, which takes about 3 days, they no longer possess the ability to dedifferentiate to replace lost stem cells. These data indicate that the ability of Alpi$^{+}$ enterocyte progenitors to dedifferentiate in enteroid cultures recapitulates their behavior *in vivo*.

Tetteh *et al*. went on to characterize the transcriptome of *Alpi*$^{+}$ cells by utilizing single-cell sequencing during both homeostasis and regeneration (14). Single cells from control crypts and Lgr5$^{-}$ crypts were FACS sorted, sequenced, and analyzed by RACE ID, revealing one cluster from control crypts and four clusters from regenerating crypts. Both control and Lgr5-depleted (regenerating) groups had a cluster representing enterocyte-specific transcripts but, importantly, they were distinct clusters. The other three clusters from the regenerative crypts were enriched for ribosomal proteins and intestinal stem cell genes, Paneth-cell transcripts, and rare secretory cells. Taken together, this data suggests that the process of dedifferentiation observed by Alpi$^{+}$ enterocyte progenitors may be more complex than dedifferentiation directly to Lgr5$^{+}$ stem cells and warrants further investigation.

The minimal differences in DNA methylation and histone marks between Lgr5$^{+}$ stem cells and committed progenitor populations have provided a plausible mechanism for how crypt progenitors can dedifferentiate and repopulate the ISC niche upon injury or stress (12,13). A key question is whether the same mechanisms of cellular plasticity are conserved during tumor initiation. Two principal models for the cellular origin of human colorectal neoplasia have been described: the 'bottom up' model proposes that

 

tumor initiation originates within the crypt stem cell niche whereas the 'top down' model proposes that early adenoma formation occurs at the luminal surface; an event physically independent of the crypt stem cell niche (15). Both models have been tested in mice by the introduction of Wnt-activating mutations or by perturbations of other signaling pathways (e.g., K-Ras and BMP) within specific intestinal cell types along the crypt-villus axis. The 'bottom up' model is supported by the observation that biallelic loss of APC in Lgr5$^+$ stem cells generated progressively growing adenomas, whereas APC inactivation in short-lived transit-amplifying cells using the *Ah*$^{Cre}$ driver only produced proliferative foci that were lost or failed to progress to adenomas (16). Wnt-activating mutations introduced specifically into +4 reserve stem cells (e.g., Prom1$^+$, Bmi1$^+$ or Lrig1$^+$) can also produce rapidly growing adenomas (6,17,18). However, because gene expression of these +4 stem cell markers overlaps with Lgr5$^+$ cells it is unclear at a cellular level whether adenoma formation in +4 stem cells represents a distinct transforming event from that occurring in Lgr5$^+$ cells. By contrast, elegant studies combining DTR-mediated ablation of Lgr5$^+$ cells with APC deletion have clearly demonstrated that adenoma initiation can occur in the Lgr5$^-$ cell population (19).

While the above results argue that tumor initiation can originate outside the Lgr5$^+$ stem cell niche, direct evidence supporting the 'top down' model for tumor initiation in Lgr5$^-$ cells has required constitutive Wnt activation to be combined with additional mutations, tissue injury, or changes in the microenvironment. Schwitalla *et al*. showed that mice with constitutive Wnt signaling in Lgr5$^-$ villus cells and Paneth cells driven by the *Xbp1*$^{CreER}$ driver failed to produce adenomas, but when combined with a concomitant loss of IκB or simultaneous activation of oncogenic K-Ras, these same Lgr5$^-$ cells could dedifferentiate and undergo tumor initiation (20). Similarly, Wnt-dependent tumor initiation in radioresistant Lgr5$^-$ Keratin19$^+$ cells only occurred only after radiation-induced injury and loss of radiosensitive Lgr5$^+$ cells (21). Biallelic loss of APC in doublecortin-like kinase 1 protein (DCLK1)+ tuft cells was also not sufficient to drive colon tumorigenesis but upon induction of colitis these same APC-deficient DCLK1$^+$ cells readily developed colonic adenocarcinomas (22). In addition, Davis *et al*. showed that overexpression of the BMP antagonist Gremlin 1 lead to an expansion of Lgr5$^-$ progenitor cells in ectopic crypts which proliferate but must accumulate other somatic mutations to drive intestinal neoplasia (23).

In the current manuscript, Tetteh *et al*. revisited this question and asked whether adenoma formation can occur within Alpi$^+$ enterocyte progenitors upon biallelic loss of APC (14). Interestingly, no adenoma formation was detected upon APC inactivation or when APC deletion was combined with an activating K-Ras mutation. Consistent with these *in vivo* findings, villus cells from APC-mutated or APC/K-Ras mutated mice failed to generate spheroid enteroids *in vitro* indicating a lack of transforming potential in these cells. Surprisingly, however, crypt cells from APC/K-Ras mutated mice but not from mice lacking APC alone grew as spheroid enteroids independently of R-spondin and EGF, which represent growth characteristics of transformed enteroids. Taken together, Tetteh *et al*. demonstrate that APC and APC/K-Ras mutations in Alpi$^+$ enterocyte progenitors cannot initiate adenoma formation *in vivo*. The authors suggest that the mutated enterocyte progenitors cannot form adenomas *in vivo* because these cells rapidly migrate along the crypt-villus axis and are shed from villus tips. In enteroid cultures, however, the same APC/K-Ras mutated cells are retained providing the appropriate conditions for these cells to generate transformed spheroids. In light of these findings, it would be interesting to determine if sustained tamoxifen exposure (by adding tamoxifen to chow) would increase susceptibility of APC deleted enterocyte progenitors to adenoma formation, particularly in Alpi$^+$ enterocyte progenitor cells expressing low levels of Lgr5$^+$ that would be expected to be proliferative and be less mature. It should also be noted that unlike secretory progenitors that rapidly exit the cell cycle, short-lived enterocyte progenitors undergo several rounds of proliferation before differentiating into post-mitotic enterocytes. These differences in proliferative capacity suggest that the dedifferentiation of secretory and enterocyte progenitors may occur in response to different forms of ISC injury/stress (e.g., radiation, anti-proliferative agents, or inflammation). For example, secretory progenitors and label-retaining cells are radioresistant whereas proliferating enterocyte progenitors are more radiosensitive which might hinder their regenerative capacity upon radiation-induced injury (8,19,24). Thus, by analogy, there may be a hierarchy for the dedifferentiation of different secretory and enterocyte progenitor cell populations and their involvement in the development of different intestinal tumors. Because previous studies have indicated that Lgr5$^-$ cells require other mutations or environmental stresses to undergo tumor initiation, it will also be important to test whether the APC-deficient Alpi$^+$ enterocyte progenitors reported by Tetteh *et al*. are susceptible to transformation in

 

combination of other forms of intestinal stress (e.g., Lgr5$^+$ cell ablation, IκBα inactivation, or intestinal inflammation) and whether Gremlin overexpression in Alpi$^+$ enterocyte progenitors can produce ectopic tumors.

In summary, the work by Tetteh *et al.* demonstrates that Alpi$^+$ enterocyte progenitors can dedifferentiate and act as facultative reserve stem cells to replenish the intestinal stem cell compartment upon Lgr5 cell ablation (14). Thus, multiple different intestinal progenitor populations can function as reserve stem cells highlighting the extensive cellular plasticity involved in protecting the intestinal stem cell compartment against injury.

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