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RESEARCH HIGHLIGHT

Hierarchy and plasticity in the crypt: back to the drawing board

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The prevailing concept in the field of stem cell research is that of a multipotent self-renewing cell, positioned at the origin of a hierarchical tree of branching specificities, increasing maturity and decreasing self-renewal ability. In the epithelium of the small intestine, until very recently, the supra-Paneth crypt base columnar (CBC) cell position +4 (cp4) (counting from the bottom of the crypt) was widely assumed to be the preferred position of multipotent stem cells [1, 2]. Yet electron microscopy, as well as autoradiography and lineage tracing studies, supported the presence of undifferentiated [3], actively cycling [4], multipotent CBC stem cells located between Paneth cells in the crypt [5-7]. Based on the results of expression and lineage studies with *Lgr5-EGFP-IRES-CreERT2* knock-in mice and *Rosa26-LacZ* reporter mice, it was possible to show that multipotent CBC cells expressing the Lgr5 orphan receptor are present throughout the gastro-intestinal tract [7]. But they are not alone. Another recent lineage study revealed the existence of multipotent, self-renewing *Lgr5*$^{-}$ CBC cells expressing the *Bmi1* proto-oncogene and preferentially located above the highest Paneth cell [8]. This discovery brought the cp4 model back under the spotlight, and subsequent expression studies revealed a partial overlap between the *Lgr5*$^{+}$ and *Bmi1*$^{+}$ CBC cell populations [9]. In a recent issue of *Nature*, Huan Tian and colleagues now tackle the issue of their contribution to the turnover of the intestinal epithelium [10].

Tian and colleagues generated reporter mouse lines allowing (1) visualization of the cells expressing *Lgr5* or *Bmi1* using a fluorescent reporter (EGFP), (2) the elimination of *Lgr5*$^{+}$ cells through expression of the diphtheria toxin receptor (DTR) under the control of the *Lgr5* promoter, (3) tracing of the progeny of *Lgr5*$^{+}$ or *Bmi1*$^{+}$ stem cells through specific expression of an inducible Cre recombinase controlled by the *Lgr5* or the *Bmi1* promoter, and a LacZ reporter (R26R) that is irreversibly activated following Cre activity. Using such expression and fate-mapping genetic tools for each of the stem cell populations, Tian and colleagues reached the unexpected conclusion that *Lgr5*$^{+}$ stem cells are dispensable. A 10-day exposure to diphtheria toxin (DT) of their knock-in mouse expressing the DTR in *Lgr5*$^{+}$ cells (*Lgr5*$^{\text{DTR-EGFP/+}}$) resulted in the complete elimination of the *Lgr5*$^{+}$ cell population with no detectable effect on intestinal epithelial homeostasis. The same result was obtained after keeping intestinal epithelium-derived organoids in the presence of DT for up to two months in culture.

Could rare *Lgr5*$^{+}$ cells that survive the DT regimen be responsible for this result? To answer this question, Tian and colleagues created a hybrid knock-in mouse line in which one *Lgr5* allele enables DT-mediated *Lgr5*$^{+}$ cell ablation, and the other *Lgr5* allele allows tracing of the *LacZ*$^{+}$ progeny of any remaining *Lgr5*$^{+}$ cell. Since newborn mice do not survive inactivation of both *Lgr5* alleles, the only option was to remove pieces of small intestine before birth, graft them into immuno-compromised mice, wait for the epithelium to reach its fully mature state, and proceed with *Lgr5*$^{+}$ cell ablation combined with induction of *LacZ* expression by tamoxifen (TAM). As in the *Lgr5*$^{\text{DTR-EGFP/+}}$ knock-in mouse, homeostasis was maintained in the grafted tissue from *Lgr5*$^{\text{DTR-EGFP/Cre-ERT2}}$;*R26R* mice, with all epithelial lineages represented, but not a single *Lgr5*-derived *LacZ*$^{+}$ cell in sight.

Against all expectations, *Lgr5*$^{+}$ cells appear to be dispensable. Since *Bmi1*$^{+}$ cells had been characterized as multipotent stem cells, the next obvious question was: could *Bmi1*$^{+}$ cells substitute for *Lgr5*$^{+}$ cells? Answering this question required the production of a new mouse line, in which the progeny of *Bmi1*$^{+}$ cells could be traced after destruction of *Lgr5*$^{+}$ cells. As a result of *Lgr5*$^{+}$ cell depletion in *Bmi1-Cre*$^{\text{ER}}$;*R26R*;*Lgr5*$^{\text{DTR-}}$

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$^{EGFP/+}$ mice, the population of *Bmi1*$^{+}$ cells was greatly expanded, resulting in a higher proportion of fully *Bmi1-LacZ*$^{+}$ crypts, in which all newly produced *Lgr5*$^{+}$ cells (after 3 days of recovery without DT) were *LacZ*$^{+}$. These new and important observations led the authors to conclude that there is a hierarchy of stem cells, with *Bmi1*$^{+}$ cells taking on the role of "workhorse" stem cells that was previously attributed to *Lgr5*$^{+}$ cells [11].

However, there may be other interpretations. The efficiency of the method used to eliminate *Lgr5*$^{+}$ cells leaves no room for speculation: in *Bmi1-Cre*^{ER};*R26R*;*Lgr5*$^{DTR-EGFP/+}$, all *LacZ*$^{+}$ cells, including *Lgr5*$^{+}$ cells (after three days in the absence of DT), are derived from *Lgr5*$^{-}$ cells. Most importantly, replenishment of *Lgr5*$^{+}$ cells is not a pre-requisite to maintain homeostasis, as shown in *Lgr5*$^{DTR-EGFP/+}$ mice treated with DT for 10 days. So, in case of severe *Lgr5*$^{+}$ cell depletion, *Bmi1*$^{+}$ cells can take over cell production, all lineages included, in a substantial (36%) proportion of the crypts. But the situation may be quite different in the intact epithelium, where a small proportion of CBC cells stains positive for both Lgr5 and Bmi1 [14]. The low frequency (2.3%) of fully *LacZ*$^{+}$ crypts observed in *Bmi1-Cre*^{ER};*R26R* control mice does not warrant the participation of *Lgr5*$^{-}$*Bmi1*$^{+}$ cells (cp4 and above), but may instead reflect that of *Lgr5*$^{+}$*Bmi1*$^{+}$ cells. Taken together, these results suggest that *Bmi1*$^{+}$*Lgr5*$^{-}$ cells probably are not heavily involved in the normal turnover of the intestinal epithelium but are actively recruited after destruction of *Lgr5*$^{+}$ cells. Therefore, coming to a decision as to whether the *Bmi1*$^{+}$*Lgr5*$^{-}$ cell sits at the apex of a hierarchy in the intact epithelium may require further evaluation of the potential of such cells. In *Bmi1-Cre*^{ER};*R26R*;*Lgr5*$^{DTR-EGFP/+}$ mice treated with DT, the majority (64%) of the crypts is only partially *LacZ*$^{+}$, suggesting that other cryptogenic cells can also substitute. The key issue is to find out whether restoration of homeostasis after a severe injury involves a dedicated reserve of CBC stem cells (hierarchy), or simply any other kind of crypt progenitor normally destined to differentiate (plasticity). In the latter case, the degree of proximity to the niche may be important in determining whether or not a crypt progenitor can revert to a stem cell state and contribute to restoring a normal intestinal epithelium architecture. The distribution of *Bmi1*$^{+}$ cells with a peak at cell positions 4-6 from the bottom of the crypt may explain their crypt regeneration capacity, but other candidates may also exist. It should be kept in mind that *Bmi1* expression is restricted to the duodenum and jejunum, which leaves wide open the question of the identity of the cells capable of replacing missing *Lgr5*$^{+}$ cells in the ileum and colon.

In future studies it will be interesting to extrapolate the *Lgr5*$^{+}$ cell ablation protocol to *Bmi1*$^{+}$ cells: can *Lgr5*$^{+}$ cells restore the *Bmi1*$^{+}$ cell pool in *Bmi1*^{DTR} mice treated with DT? Because Bmi1 is required for the postnatal maintenance of stem cells in multiple tissues [12-14], targeting the *Bmi1*^{DTR} construct specifically to intestinal epithelial cells may spare the other organs in order for the mice to survive. Depending on the results, it should be possible to determine whether or not a hierarchy of stem cells exists within the crypt, and the nature of the relationship between *Bmi1*$^{+}$ cells and *Lgr5*$^{+}$ cells. Again, however, this should not necessarily be extrapolated to the organization of the intact, unperturbed epithelium.

The surprising dispensability of *Lgr5*$^{+}$ cells makes us now wonder whether both *Bmi1*$^{+}$ CBC cells and *Lgr5*$^{+}$ CBC cells might be dispensable? Could other types of progenitors take over and rescue the damaged epithelium? Self-renewal is not a trait specific to stem cells: analysis of Dlb-1 lectin-positive clones induced by ENU in the intestinal epithelium of *Dlb-1*$^{-/-}$ mice revealed the presence of clones containing only one type of long-lived progenitor (mucous, columnar or stem) [5]. Combining *Bmi1*$^{+}$ and *Lgr5*$^{+}$ cell ablation in *Lgr5*^{DTR};*Bmi1*^{DTR} mice with a dose of abdominal irradiation sufficient to kill progenitors locating higher up in the crypt [15], could be useful to determine the limit of damage along the crypt-villus axis beyond which restoration of normal epithelial architecture and function is impossible.

Finally, as Matthew Bjerknes and Hazel Cheng said in their recent paper: "when shifts in cell type proportion are observed following perturbation, cellular reprogramming also needs to be considered as a contributing cause, either at the level of a multipotent precursor, or in their committed progeny" [16]. Recent literature incessantly pushes back the limits of cell reprogramming, allowing new conceptual frameworks to depict tissue regeneration and cellular plasticity around the stem cell phenotype. Further surprising results are expected.

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