Losing the MAPK reveals the path to stem cell quiescence and the elusive enteroendocrine cell

MAP(K)ing the path to stem cell quiescence and the elusive enteroendocrine cell

Hi Sheila, I prefer your title, lets go with that.

Simon J. Leedham PhD, FRCP

Intestinal Stem Cell Biology Laboratory, Oxford Centre for Cancer Gene Research, Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford, OX3 7BN, United Kingdom

Word count: 1078

No disclosures/conflict of interests

Details for correspondence:

Phone: +44 1865 8766

Fax: +44 1865 287664

E-mail: simon.leedham@well.ox.ac.uk

Summary

The existence and interaction of proliferating and quiescent intestinal stem cells have been debated since their discovery in the 1970's. Here, using murine intestinal organoids, Basak *et al.* induce stem cell quiescence by selective inhibition of EGF/MAPK signaling, and define culture conditions that direct differentiation to the enteroendocrine lineage.

The existence of different populations of intestinal epithelial stem cells has been debated since the 1970's when actively dividing crypt based columnar (CBC) (Cheng and Leblond, 1974) and quiescent label retaining cells (LRC's) (Potten, 1977) were first identified. Forty years later, the identification of selectively expressed marker proteins permitted transgenic labeling and lineage tracing from these cell populations, demonstrating tissue appropriate multilineage differentiation (Barker et al., 2007; Sangiorgi and Capecchi, 2008). This intensified the debate about the interaction and interdependence of these apparently distinct proliferating and quiescent stem cell populations. (Li and Clevers, 2010). In 2013, an important paper from the Winton group used label

retention properties to functionally characterize, identify and segregate the quiescent cell population. This unbiased, stem cell-marker independent approach, identified a population of slowly cycling secretory progenitor cells in the +4 crypt position. These cells continue to express the CBC marker, *Lgr5*, but are committed to secretory lineage differentiation normally forming mature Paneth and enteroendocrine (EEC) cells. They do however, retain stem-cell competence, and can be recalled to clonogenic capacity in a crypt regenerative setting (Buczacki et al., 2013). Now, in this issue of *Cell Stem Cell*, the Clevers group undertake a comprehensive *in vitro* assessment of the signaling pathways that regulate proliferative quiescence in *Lgr5* expressing cells, and then go on to define the culture medium context that directs secretory cell progenitor differentiation to the elusive enteroendocrine cell fate (Basak et al., 2016).

The work is undertaken in murine intestinal organoids; an epithelial culture system that retains endogenous differentiation potential, but is dependent upon the media supplementation of critical niche-derived factors. Media manipulation allows assessment of how extrinsic intercompartmental and cell-intrinsic morphogen signalling combine in the regulation of intestinal cell fate determination. Basak *et al.* show that in the presence of sustained Wnt signaling, inhibiting Epidermal Growth Factor (EGF) or downstream Mitogen Associated Protein Kinase (MAPK) pathway activity induced a quiescent state in *Lgr5+*ve stem cells that was readily reversible with resumption of EGF signaling. RNA sequencing showed enrichment of the Buczacki *et al.* label retaining secretory progenitor signature in quiescent versus proliferative *Lgr5* expressing cells, and this included a 7.3 fold rise in chromogranin A expression - a marker of enteroendocrine cells. Given this apparent skew towards enteroendocrine cell

fate, Basak and colleagues then used combinations of Notch, Wnt and EGFR/MAPK inhibitors to meticulously define the conditions that favoured enteroendocrine differentiation. This demonstrated that coordinate inhibition of Wnt, Notch and EGFR/MAPK, massively promoted the differentiation of enteroendocrine cells with induction of an EEC cell fate in up to 50% of organoid cells. Comprehensive single-cell transcriptomic analysis showed that organoid culture was able to recapitulate the EEC diversity seen *in vivo*, with a wide range of identified hormone expression permitting a bioinformatic taxonomic classification of enteroendocrine cell type. Interestingly, organoids retained a cell-endogenous 'memory' of their intestinal regional identity, as hormone expression varied, and was dependent on the position of the tissue source. Thus, organoids derived from the duodenum expressed greater levels of Gastric inhibitory polypeptide (GIP), whilst distal small intestinal organoids consistently upregulated Neurotensin (NTS) and Glucagon (GCG).

This paper is important for several reasons. Firstly, it provides a very valuable addition to the *in vivo* work of Buczacki *et al.* demonstrating the inter-relationship of the 'active' and 'quiescent' stem cell populations in the intestine. Accordingly, a model arises, where actively dividing *Lgr5* CBC stem cell are responsible for daughter cell generation and maintenance of crypt homeostasis. Among the immediate progeny of CBC cell division is a population of quiescent secretory progenitors, usually located at the +4 cell position. In health, these cells are transient and are committed to Paneth or enteroendocrine cell fate, but act as a functional facultative stem cell pool, capable of reverting back to stem cell function in the event of tissue damage or CBC loss. The work presented by Basak *at al.* contributes to this model by demonstrating that the conversion of an active

Lgr5 cell to a quiescent state is regulated by sustained Wnt signaling, with a concomitant reduction in EGFR/MAPK activity. Whilst the intestinal organoid culture system cannot fully recapitulate the nuanced intercompartmental signaling microenvironment of the intestinal crypt, this is consistent with *in vivo* findings. The pan Erb-B inhibitor *Lrig1* is expressed in quiescent, label retaining cells and it is conceivable that this mediates a cell-specific reduction in EGFR activity in these cells (Powell et al., 2012).

Secondly, this paper identifies the contextual requirements necessary to direct *Lgr5* cell differentiation towards an enteroendocrine cell fate and describes an *in vitro* system capable of generating large numbers of a diverse population of these cells. Although scarce *in vivo*, these are a potent and an important intestinal cell type with known or putative roles in the regulation of satiety, intestinal motility, obesity and depression. The reproducibility of the process defined here, allows a readily accessible model for unraveling EEC cell biology; research previously hindered by cell rarity. How such coordinated signaling pathway inhibition is regulated in individual EEC cells *in vivo* is not clear, but this work illustrates the complexity of context dependent morphogen regulation and intracellular signal interpretation in the intestine. It is this complexity that allows a limited number of signaling pathways to confer such diverse cellular functions in different cell types and tissues.

Finally, this paper touches on the development of neuroendocrine tumours (NET's). Although these are the most common tumours of the small bowel, they are still rare events and research into their pathophysiology has been hindered by the absence of a useful model system. Banck et al showed that 29% of small intestinal NET's carry amplifications or activating mutations in the

PI3K/AKT/mTOR pathway whilst other tumours harbour mutations that induce *MAPK* signaling (*BRAF* mutation or *MEK2* amplification) (Banck et al., 2013). This implies that the EGF signal necessarily inhibited for physiological EEC cell differentiation is aberrantly re-activated in neuroendocrine tumour pathogenesis. The organoid culture system described by Basak et al, will be vital in determining how, and when this pathological reactivation occurs.

This paper builds upon the outstanding work of the Clevers group in this field and significantly enhances our understanding of the dynamic interaction of intestinal stem cell populations, and the morphogen signaling pathways that contribute to stem cell quiescence and cell fate determination. Furthermore this paper can be used as a framework to guide the generation of the *in vitro* tools necessary to answer some of the many questions surrounding the cell biology of the enigmatic enteroendocrine cell.

References.

Banck, M. S., Kanwar, R., Kulkarni, A. A., Boora, G. K., Metge, F., Kipp, B. R., Zhang, L., Thorland, E. C., Minn, K. T., Tentu, R.*, et al.* (2013). The genomic landscape of small intestine neuroendocrine tumors. J Clin Invest *123*, 2502-2508.

Barker, N., van Es, J., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P., and Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature *449*, 1003-1007.

Basak, O., Beumer, J., Wiebrands, K., Seno, H., van Oudenaarden, A., and Clevers, H. (2016). Induced Quiescence of Lgr5+ Stem Cells in Intestinal Organoids Enables Differentiation of Hormone-Producing Enteroendocrine Cells. Cell Stem Cell. This issue.

Buczacki, S. J., Zecchini, H. I., Nicholson, A. M., Russell, R., Vermeulen, L., Kemp, R., and Winton, D. J. (2013). Intestinal label-retaining cells are secretory precursors expressing Lgr5. Nature *495*, 65-69.

Cheng, H., and Leblond, C. P. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cell. Am J Anat *141*, 461-479.

Li, L., and Clevers, H. (2010). Coexistence of quiescent and active adult stem cells in mammals. Science *327*, 542-545.

Potten, C. S. (1977). Extreme sensitivity of some intestinal crypt cells to X and gamma irradiation. Nature *269*, 518-521.

Powell, A. E., Wang, Y., Li, Y., Poulin, E. J., Means, A. L., Washington, M. K., Higginbotham, J. N., Juchheim, A., Prasad, N., Levy, S. E.*, et al.* (2012). The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. Cell *149*, 146-158.

Sangiorgi, E., and Capecchi, M. R. (2008). Bmi1 is expressed in vivo in intestinal stem cells. Nat Genet *40*, 915-920.