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EPITHELIAL STEM CELLS, SELF-RENEWAL AND DIFFERENTIATION IN THE INTESTINE

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SUMMARY

The inner lining of the gut is a simple epithelium that completely self-renews every five days because of the high mechanical and chemical stress. At the basis of the epithelial homeostasis are intestinal stem cells that are located at the bottom of crypts. Progeny is generated on a daily basis to compensate for the loss of differentiated cells that cover the villi. Under normal conditions, active cycling stem cells produce daughter cells that compete with each other for residency in the stem cell niche. Upon niche displacement, a daughter cell will lose stem cell characteristics, migrates out of the crypts and matures into a terminally specialized differentiated cell type. Here, we describe and summarize the recent developments in the identification and characterization of intestinal stem cells.

INTRODUCTION

The primary function of the intestinal tract is the digestion and absorption of food. The gut is anatomically divided into the small intestine and the colon. The inner wall of the gut is covered with a simple columnar epithelium, which performs the primary functions of (i) digestion via the secretion of enzymes, (ii) water and nutrient absorption and (iii) forms a barrier against gut pathogens. In the small intestine, the surface area is enlarged through epithelial protrusions called villi (Figure 1), while the colon has a flat surface epithelium. Proliferative cells reside in the crypts of Lieberkühn, epithelial invaginations into the underlying connective tissue. These crypts harbour stem cells and transit amplifying cells that are direct descendants of the stem cells. The majority of cells that are produced on a daily basis migrate out of the crypts and terminally differentiate into one of the major three specialized cell types of the intestinal epithelium. These are the absorptive enterocytes, mucous-secreting goblet cells and hormone-secreting entero-endocrine cells. Approximately three days after their terminal differentiation, the cells reach the tip of the villus, undergo spontaneous apoptosis and are shed into the lumen of the gut. Paneth cells are the fourth abundant cell type in the intestine and the only differentiated cell type that escapes the upward migration and instead settle at crypt bottoms (*van der Flier* and *Clevers*, 2009). Paneth cells have a function in innate immunity and antibacterial defence (*Clevers*

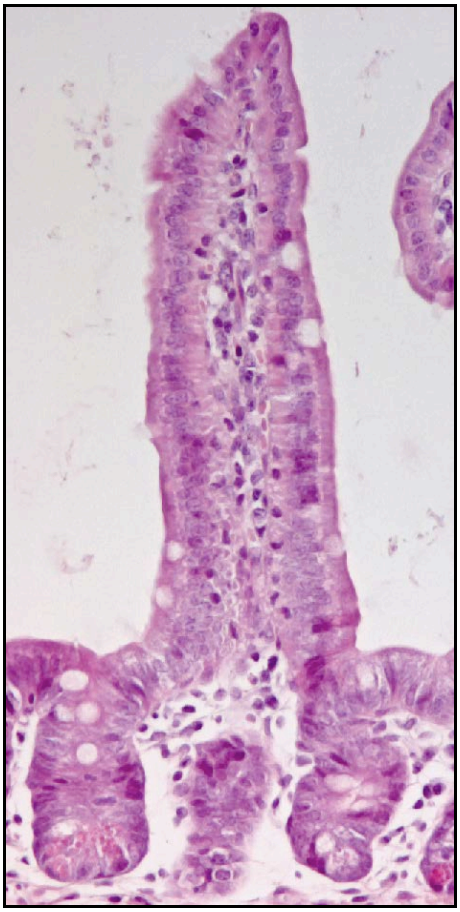

Figure 1: The intestine is lined with a single layer of epithelial cells. The H&E staining is showing the organization of the mouse small intestine in protruding villi and adjacent crypts. Crypts contain stem cells, progenitor cells and Paneth cells while the villus is covered with goblet cells, entero-endocrine cells and enterocytes.

and *Bevins*, 2013). Recently, it was shown that they also play an important role in constituting the intestinal stem cell niche (*Sato* et al., 2011b).

PROLIFERATING INTESTINAL CELLS

The intestinal epithelium is the most vigorously self-renewing tissue present in adult mammals. With the exception of stem cells and Paneth cells, the inner lining of the gut is completely renewed every 5 days. It has been known for decades that multipotent stem cells fuel the proliferative activity of the intestinal epithelium. However, the exact location of these cells was unknown for a long time since molecular markers were missing (*Barker* et al., 2008). In principal, there are only two functional requirements that cells must fulfil in order to define them as stem cells, commonly referred to as stemness.

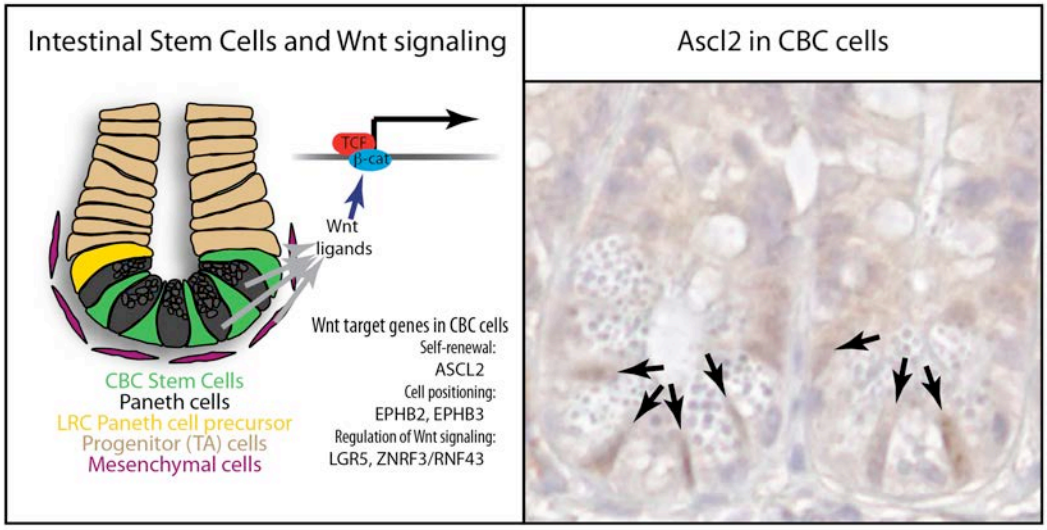

Figure 2: Left panel; schematic representation of small intestinal crypt. Wnt signalling is the driving force behind proliferation and stem cell identity. Right panel; immunohistochemical staining for transcription factor Ascl2 in mouse small intestinal crypts. Ascl2 is specifically expressed in CBC cells (arrows).

First, stem cells are long-lived, preferably throughout the life of an individual. Secondly, stem cells are mulipotent, which means that descendants of stem cells are able to differentiate into all the specialized cell type(s) that are present in a tissue.

WNT PATHWAY

The primary force that drives intestinal epithelial proliferation is the Wnt signalling pathway. Mice that are mutant for the intestine-specific Tcf4 transcription factor fail to establish proliferative crypts (*Korinek* et al., 1998; *van Es* et al., 2012a), while conditional deletion of ß-catenin (*Fevr* et al., 2007; *Ireland* et al., 2004) as well as transgenic expressing of the secreted Wnt inhibitor Dickkopf-1 (*Kuhnert* et al., 2004; *Pinto* et al., 2003) leads to disappearance of proliferative crypts in adult mice. Moreover, malignant transformation of intestinal epithelium is almost invariably initiated by activating Wnt pathway mutations (*Korinek* et al., 1997; *Morin* et al., 1997). Because of this intimate connection between Wnt signalling and intestinal biology, we and others have attempted to unravel the Wnt/Tcf4 target gene program activated in intestinal crypts and colorectal tumours (*van de Wetering* et al., 2002; *van der Flier* et al., 2007; *van Es* et al., 2005). Wnt target gene expression has been identified in three different crypt compartments. Most of the Wnt targets are expressed by the rapidly dividing progenitor cells within the transit-amplifying compartment that consist approximately the top two-third of the crypt. A second group of genes is expressed in post-mitotic Paneth cells that are located at the entire bottom of the crypt. The third

group contains only a very limited number of genes (including *Lgr5*, Ascl2, Rnf43 and EphB2) that are highly expressed in crypt base columnar (CBC) cells (Figure 2). CBC cells are small, undifferentiated cycling cells that are squeezed in between the Paneth cells at crypt bottoms. Already in the early '70, they have been suggested, based on their morphology and location, to be intestinal stem cells (*Cheng* and *Leblond*, 1974).

INTESTINAL STEM CELLS

The exact location and identity of intestinal stem cells has been the subject of an intense scientific debate for years. In addition to the CBC cell as candidate intestinal stem cell, another popular postulation was that the cells that were physically located on top of the Paneth cell compartment, the so-called +4 location (cell position counted from the crypt bottom), functioned as the intestinal stem cells. Rational for this hypothesis was that these cells are label retaining (*Potten* et al., 1974) and extremely radiation sensitive (*Potten*, 1977). DNA label retention by the +4 cells was postulated to be the result of asymmetric segregation of old and new DNA strands (*Potten*, 1977; *Potten* et al., 2002) in order to protect their genome from damage. Asymmetric DNA segregation was suggested to be a stem cell specific phenomenon.

However, definitive prove for stemness needs to be demonstrated by experimental assays, rather than association based on morphology, location, marker expression or any other specifically assigned cell-characteristic. An experimental assay to prove stemness is a transplantation experiment. Hereby a cell population is isolated based of specific presence (or absence) of markers. Once isolated from the donor, the cells are transplanted into a recipient and tested for their ability to give rise to a complete "recovery" effect. For example in bone marrow transplantation experiments, transplanted donor haematopoietic stem cells repopulate the entire blood. A critical note by this kind of assay is that the ability of the cell population to function as stem cells is tested in a challenged/stressed system. Therefore, cell transplantations between individuals or species primarily test the ability of cells to adapt and grow in a foreign milieu, rather than testing the actual behaviour of cells prior to experimentation. Nevertheless, transplantation assays are a powerful tool to test stemness potential, but it is likely that the population of cells with stemness potential is larger than the number of cells that actually do function as stem cells in normal homeostasis.

An alternative method to test stemness is based on genetic marking and is commonly referred to as lineage tracing or fate mapping. Here, the DNA of specific (candidate) stem cells is marked at any specific moment in time. Subsequently, over time all descendants of the marked population inherit the mutation. Moreover, the genetic marking can be visualized via straightforward laboratory techniques and allows experimental testing of the two functional descriptions of stemness. First, over time the genetic marking needs to be detectable in all the cell types that are present in the tissue (multipotency). Second, the presence of the genetic marking needs to be maintained within the tissue for the entire life-time of the organism (self-renewal). The stemness of a specific cell population is tested without the pres-

ence of stress or other injury responses. Therefore, lineage tracing assays test actual stemness, i.e. it identifies cells that function as stem cells in normal homeostasis. However, also with tracing experiments care needs to be taken into account since it is possible that only a limited number of cells within the marked group are the real stem cells. It is likely that the population of cells that contain actual stemness fall within the larger population of cells that consists stemness potential.

LGR5 IS AN INTESTINAL STEM CELL MARKER

Lineage tracing was used to prove that the *Lgr5* gene is specifically expressed in intestinal stem cells and can be used as a stem cell specific marker. In situ hybridization experiments (mapping of mRNA expression patterns) as well as different knock-in alleles revealed expression of *Lgr5* in CBC cells (*Barker* et al., 2007; *Tian* et al., 2011). Most importantly, the genetic inducible activation of *LacZ* expression in *Lgr5*$^+$ CBC cells (the genetic clonal marking) was inherited over time by all the differentiated cell types in the intestinal epithelium, thereby experimentally linking *Lgr5*$^+$ CBC cells to multipotency. Moreover, once *LacZ* expression was activated in CBC cells, the marking could be found during the rest of the lifespan of the animals in the intestinal epithelium, showing that *Lgr5* expressing CBC cells represent long-lived multipotent stem cells of the intestine (*Barker* et al., 2007).

Lineage tracing has also been performed for several markers that were claimed to be +4-cell specific, including: Bmi-1 (*Sangiorgi* and *Capecchi*, 2008), Hopx (*Takeda* et al., 2011), mTert (*Montgomery* et al., 2011) and Lrig1 (*Powell* et al., 2012). However, robust expression of most of these markers was also detected in *Lgr5*$^+$ CBC cells (*Munoz* et al., 2012). A recent study shows that genetic lineage tracing of DNA label-retaining cells identified a rare, non-dividing secretory precursor that co-expresses *Lgr5* and all +4 makers. These cells are located near crypt bottoms where they undergo terminal differentiation over periods of weeks towards Paneth cell lineage. However upon tissue damage, premature secretory precursors can revert their fate back into a cycling, *Lgr5*$^+$ CBC cell (*Buczacki* et al., 2013).

ADDITIONAL INTESTINAL STEM CELL MARKERS

Isolation of *Lgr5* expressing CBC cells using FACS allowed global gene expression analysis (*Munoz* et al., 2012; *van der Flier* et al., 2009). One of the genes enriched in the *Lgr5*$^+$ cells turned out to be the CBC restricted Wnt target Ascl2 (*Jubb* et al., 2006; *Sansom* et al., 2004; *van der Flier* et al., 2007). Ascl2 is one of the mammalian homologous of the Drosophila Achaete-scute complex genes encoding a basic helix-loop-helix transcription factor. Transgenic overexpression of Ascl2 throughout the intestinal epithelium induces crypt hyperplasia and de novo crypt formation on villi. The opposite experiment, induced deletion of Ascl2 from the intestine, results in rapid loss of CBC stem cells. The combined results from these genetic gain and loss of function studies in the intestinal epithelium show that Ascl2

controls the intestinal stem cell fate (*van der Flier* et al., 2009). Other stem cell specific Wnt target genes are Rnf43, an ubiquitin ligase for Wnt receptors (*Koo* et al., 2012) and EphB2, a cell surface marker which expression pattern was utilized to isolate human intestinal stem cells (*Jung* et al., 2011).

NEUTRAL DRIFT COMPETITION BETWEEN MULTIPLE INTESTINAL STEM CELLS

On average, there are around 14 *Lgr5*$^{+}$ CBC cells at the bottom of intestinal crypts that are sandwiched in between similar numbers of post-mitotic Paneth cells (*Snippert* et al., 2010). To be able to map the fate of neighbouring stem cells simultaneously, a multicolour reporter mouse was generated (*R26R-Confetti*). Upon activation of *R26R-Confetti*, each cell inherits one out of four possible fluorescent markers via DNA recombination, i.e. green, yellow, red or blue. This 'colour picking' is completed in a random fashion, is specific per individual cell and inherited by daughter cells. Using the *R26R-Confetti* mouse, the individual behaviour of multiple intestinal stem cells in the same crypt was followed in time. The study revealed that *Lgr5*$^{+}$ CBC cells double their numbers each day by symmetric divisions, after which the daughter cells stochastically adopt stem cell or transit amplifying cell fates based on their relative positioning towards the Paneth cells (niche cells). Due to the limited number of Paneth cells, CBC stem cells compete in a neutral fashion for limited niche space (*Snippert* et al., 2010). As long as *Lgr5*+ CBC (daughter) stem cells are in close proximity to Paneth cells they will maintain stemness. In the case that a (daughter) stem cell is displaced from Paneth cells, i.e. loss of direct contact, they will be primed for differentiation (*Sato* et al., 2011b). This stochastic stem cell model in which cell fate is determined by presence in the Paneth cell niche fits also well with recent data that demonstrated that progenitor cells that initially lost their stemness, can revert towards their stem cell fate upon stress and injury responses (*Buczacki* et al., 2013; *Tian* et al., 2011; *van Es* et al., 2012b).

INTESTINAL ORGANOIDS

Most of the above described stem cell work is based on genetic mouse models. However, it is essential to translate those scientific findings from the mouse to the human situation, for instance via transplantation assays or *in vitro* culturing assays. The culture of individual clones of cells that are derived from various types of progenitor populations has become a widely used method to define the identity and behaviour of (human) stem cell types. Most cultures are named after the tissue they were derived from, for example, neurospheres (*Reynolds* and *Weiss*, 1992), mammospheres (*Shackleton* et al., 2006; *Stingl* et al., 2006) or colonspheres. A few years ago, another *ex vivo* cell culture system has been developed for initially wild-type small intestinal epithelia. It allows long-term growth of so-called mini-guts or intestinal organoids (*Sato* et al., 2009) (Figure 3). In contrast to sphere

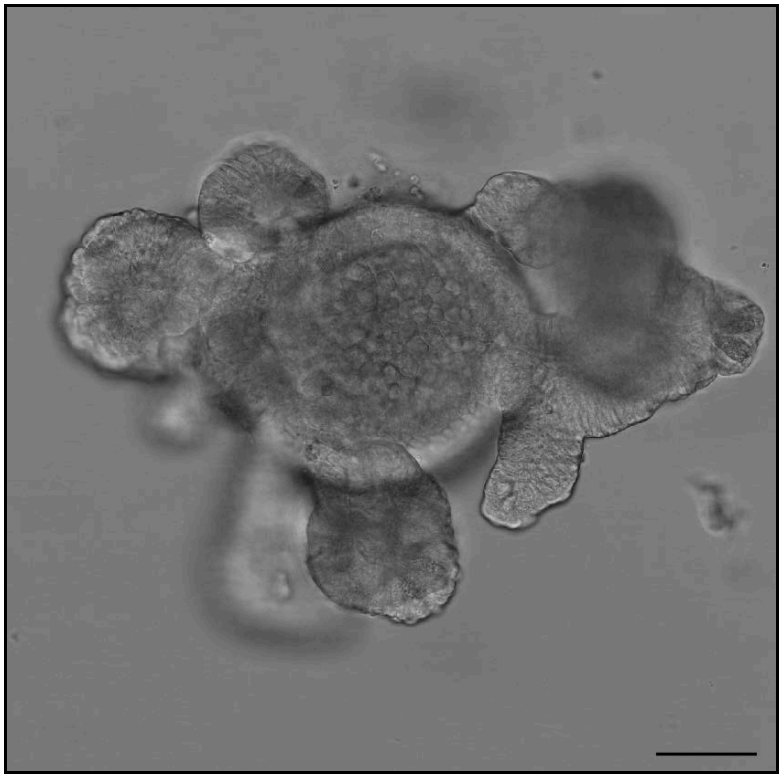

Figure 3: Bright field image of mouse small intestinal organoid. Scale bar is 50 µm.

cultures, organoids are three-dimensional asymmetric cell-cultures that can be grown from primary cells that are isolated from mouse and human intestines (*Sato* et al., 2011a). Organoids are cultured using matrixgel and a medium that contains a cocktail of growth factors such as R-spondin, EGF and Noggin. Important, these organoids share a lot of characteristics with the normal intestinal epithelium, such as the presence of all the different cell types, compartmentalization between progenitor and differentiated zones and correct positioning and migration of cell types along the crypt-villus axes. Since intestinal organoid cultures do not contain mesenchymal cells, the intestinal stem cell niche is provided by Paneth cells (secreting Wnt, EGF and Notch ligands) and the limited number of added growth factors (*Sato* et al., 2011b).

INTESTINAL STEM CELL NICHE

By using neonatal tissues, an alternative long-term multi-lineage intestinal epithelial culture has been established in which mesenchymal parts are present as well (*Ootani* et al., 2009). Mesenchymal niche architecture is also maintained once organoid cultures are initiated from human embryonic stem cells which differentiation is guided towards intestinal fate (*Spence* et al., 2011). Interesting, the mesenchymal layer develops and differentiates along

with the epithelium with the same kinetics, suggesting an intimate signalling crosstalk between mesenchyme and epithelium.

Active Wnt signalling is essential for organoid cultures. In the absence of mesenchymal tissue, Wnt ligands are either produced endogenously by the epithelium (Wnt3A in Paneth cells of mouse small intestine) or need to be added exogenously in the medium. Interestingly however, *Wnt3a* knockout mice don't have an intestinal phenotype, while organoid cultures from these mice fail to develop. To compensate for the lack of endogenous Wnt3a production, Wnt3A mutant organoids can be rescued by addition of Wnt3A or by growing them on primary mesenchymal cells that produce Wnt proteins (*Farin* et al., 2012). Above results illustrate that the mesenchyme surrounding crypts, as well as Paneth cells, function together as the niche by providing the essential signals to the intestinal epithelial stem cells.

CONCLUDING REMARKS

The intestinal epithelial (stem cell) field has developed very rapidly since the identification in 2007 of *Lgr5* as the first definitive stem cell marker. The intestinal epithelium represents a unique model to study adult stem cell biology and lineage specification. The combination of a rapid self-renewing tissue, evident compartmentalization of proliferating and differentiated cell types and a relative simple, repetitive tissue architecture, is ideal for the visualization and identification of stem cell types, cell fate specification and cellular behaviour. In the past, genetic mouse studies have created a wealth of new insights on the biology of the intestinal epithelium. The recently established long term culture conditions of intestinal epithelium, especially mini-organs from human origin, will probably boost the research field for the next generation by providing a variety of possibilities for research and therapeutic applications of intestinal biology.

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