

Searching for adult stem cells in the intestine

The epithelium of the small intestine is the most rapidly self-renewing tissue of mammals. Vigorous proliferation of progenitor cells occurs in crypts, while their differentiated offspring leaves the crypts and travels up the flanks of the villi to suffer death by apoptosis at villus tips 3 days later (Fig 1). The crypts have long been assumed to harbour functional stem cells. Yet, the absence of unique molecular markers has hampered their definitive identification. Although there has been agreement that crypts contain 4–6 independent stem cells, two hypotheses exist regarding their exact identity and location. The small intestinal crypt is pictured as a tube of proliferating cells bounded from below by Paneth cells (Fig 1A) (Bjerknes & Cheng, 2005). Since the late

1950s, the prevailing hypothesis has proposed that the stem cells reside at position +4 relative to the crypt bottom, while the first three positions are occupied by the terminally differentiated Paneth cells. Potten et al have provided experimental support for this model, by demonstrating that label-retaining, radiation-sensitive cells reside specifically at the +4 position (Potten, 1977; Potten et al, 1974, confirm 12 and 13). The second, more recent hypothesis is based on the identification of the crypt base columnar (CBC) cells, small cycling cells inconspicuously hidden between the Paneth cells (Cheng & Leblond, 1974a) (Fig 1A). The proposal was initially based on morphological features of these cells (Cheng & Leblond, 1974b), but clonal marking techniques have later led

Leblond, Cheng and Bjerknes to propose that the CBC cells represent the true stem cells (Bjerknes & Cheng, 1981a, 1981b, 1999).

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Neither of these two hypotheses on stem cell identity were supported by direct definitive evidence for ‘stemness’. So, what constitutes such ‘definitive’ experimental proof? A minimal definition of stemness states that a stem cell population should be maintained over long periods of time (‘longevity’), while generating all differentiated cell types of the pertinent organ (‘multipotency’). Two strategies can unambiguously demonstrate these two characteristics. The first and most widely applied strategy utilizes the physical isolation of candidate stem cells, followed by *in vitro* culture and/or transplantation into recipient animals. This approach has been championed in studies defining the haematopoietic (‘bone marrow’) stem cell (Spangrude et al, 1988), and—more recently—cancer stem cells in leukaemia (Bonnet & Dick, 1997) and in solid tumours (Dalerba et al, 2007; O’Brien et al, 2007; Prince et al, 2007; Singh et al, 2004). In an elegant application, Shackleton et al (2006) have shown that a single mammary gland stem cell can regenerate an entire mammary gland. In the second strategy, candidate stem cells are genetically marked *in situ*, to allow tracking of the modified stem cell and its offspring over time. As an example, Morris et al marked hair follicle stem cells in transgenic mice using a progesterone-activatable version of the

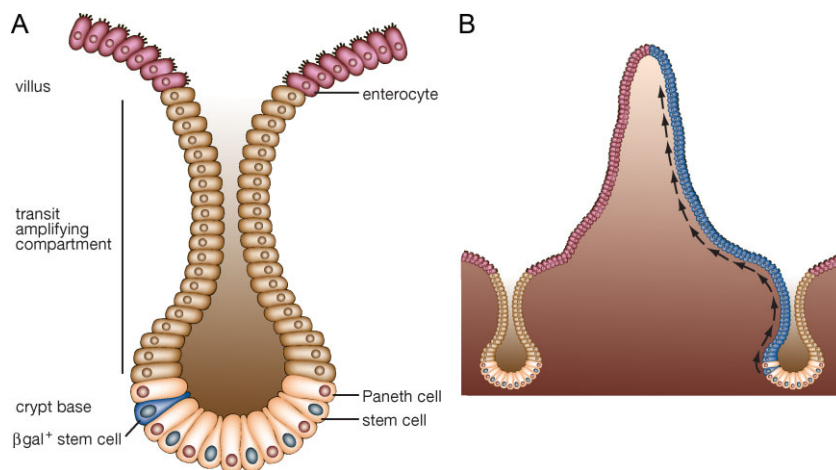


Figure 1. Stem cells of the small intestine.

- A.** A single crypt stem cell is labeled blue when a tamoxifen injection activates a LacZ-Cre reporter in an Lgr5 stem cell. Paneth cells are orange, all transit amplifying cells in brown. Differentiated cells (pink) first appear when cells exit the crypt.
- B.** The offspring of the labeled stem cell is recognizable by the blue stain. It moves up the villus and straight towards the tip of the adjacent villus, which is reached by about 5 days. This continuously renewing ‘blue ribbon’ remains present for the life time of the mouse, implying that the initial blue cell is indeed a stem cell.

Cre recombinase enzyme, expressed from the keratin-15 promoter (Morris et al, 2004). Activation of Cre by progesterone irreversibly activated the genetic marker R26R-LacZ and the stem cells as well as their offspring were then easily visualized by LacZ staining.

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In most of the tissues, no markers are available to isolate or track adult stem cells. Researchers often rely on surrogate markers of stemness such as long-term retention of DNA labels. This is, however, not without problems (e.g. differentiated non-proliferative cells are likely to equally retain the label) and reliable and specific markers of stemness are urgently sought.

Searching for intestinal candidate stem cell markers: Wnt as a clue

In an effort to uncover unique 'stemness' markers, my laboratory has pursued the characterization of target genes of the Wnt cascade in the intestine.

The Wnt pathway exerts a central role in the physiology and pathology of the intestine: it is the dominant inducer of proliferation in intestinal crypts (Hoffman et al, 2004; Korinek et al, 1998; Pinto et al, 2003), while its mutational activation represents the initiating event in colon cancer (Korinek et al, 1997; Morin et al, 1997). By microarray analysis, we have unveiled a set of target genes that are inappropriately activated in human colon cancer cells upon loss of the Wnt regulatory APC gene. Interestingly, the same genetic program of about 80 Wnt target genes is physiologically active in crypts (van de Wetering et al, 2002; Van der Flier et al, 2007). We hypothesized that some of these 80 genes could represent unique crypt stem cell markers and therefore subjected all genes to histological expression studies.

The overwhelming majority of the genes were expressed throughout the proliferative crypt compartment. However, one of the genes, the *Lgr5/Gpr49* gene, appeared to be expressed in a highly restricted fashion. The *Lgr5* gene encodes an orphan G protein-coupled receptor with a large leucine-rich extracellular N-terminal domain.

» ...*Lgr5* expression was confined to the CBC cells. ... «

We obtained a knock-in null allele that integrates LacZ directly N-terminal to the first transmembrane domain of *Lgr5*. Meanwhile, Morita et al generated an *Lgr5*^{-/-} mouse and reported early lethality due to a malformation of the tongue and lower jaw that makes newborns swallow large amounts of air (Morita et al, 2004). In our adult heterozygous *Lgr5-LacZ* mice, we could observe the expression of the transgene in rare, scattered cells in the eye, brain, hair follicle, mammary gland, reproductive organs, stomach and intestinal tract. In the small intestine, *Lgr5* expression was confined to the CBC cells, as described by Cheng and Leblond (1974b). CBC cells are never quiescent; they invariably express the Ki67 cell-cycle marker, and BrdU labelling revealed that the average cycling time of CBC cells is in the order of 24 h. At the bottom of colon crypts, *Lgr5* expression was observed in cells of similar number and shape.

To allow genetic tracing, we generated another knock-in allele, in which we integrated a cassette into the first exon of *Lgr5* encoding green fluorescent protein (GFP) and a tamoxifen-inducible version of the Cre recombinase enzyme (CreERT2). Confocal imaging of GFP expression reiterated the observations made with the *Lgr5-LacZ* knockin allele and immunoelectron microscopy demonstrated that, indeed, the GFP⁺ cells are morphologically identical to CBC cells. We crossed these mice with the Cre-activatable R26R-LacZ reporter strain (Soriano, 1999), predicting that tamoxifen should activate the CreERT2 fusion recombinase uniquely in the CBC cells.

Cre-mediated excision of the roadblock sequence in the R26R-LacZ reporter should then irreversibly mark these cells. Potential progeny of the CBC cells would not express GFP, but the activated LacZ reporter would function as a lineage tracer. As adult mice were injected with a low-dose tamoxifen pulse to activate the R26R-LacZ reporter stochastically and at low frequency in CBC cells, tracing was easily analysed right from day 1 till 14 months after injection. On day 1, occasional LacZ-positive CBC cells were observed in the crypts of the small intestine and colon (Barker et al, 2007). At later time points, parallel ribbons of blue cells emanated from crypt bottoms, running to the tips of adjacent villi (Fig 1B). The ribbons first reached the villus tips 5 days after injection. All cell types of the intestinal epithelium appeared in these clonal ribbons: enterocytes, goblet cells, Paneth cells and enteroendocrine cells were produced at normal ratios, implying that CBC cells are multipotent. The CBC cells were also found to be very long-lived. They supported maintenance of the epithelium over at least 14 months, when the frequency of blue crypts and ribbons was essentially identical to that seen in the first week after injection (Barker et al, 2008). Similar observations were made in the colon. We concluded that the *Lgr5*⁺ cells in small intestine and colon fulfilled the minimal definition of a stem cell in displaying longevity and multipotency and also support Leblond and Cheng's hypothesis on the identity of the intestinal stem cells.

Lgr5, Bmi1 and CD133 are intestinal stem cell markers

Sangiorgi and Capecchi have described a comparable intestinal lineage-tracing experiment utilizing a Bmi-Cre-ER knock-in allele (Sangiorgi & Capecchi, 2008). The Bmi1 gene has a role in the self-renewal of neuronal, haematopoietic, and leukaemic cells (Lessard & Sauvageau 2003; Leung et al, 2004; Molofsky et al, 2003). The first cells in which the activated Cre reporter was observed, 20 h after induction, were predominantly located at the presumed

position +4 directly above the Paneth cells. Over the next few days, the marked cells produced offspring with similar kinetics to what is seen in Lgr5-based tracing experiments and the rapid appearance of labelled offspring suggests that these are non-quiescent. CBC cells and Bmi-1 cells share crucial functional characteristics. They produce offspring within days, yet persist for at least a year, and are multipotent. Despite some differences reported in both populations, we favour the notion that Bmi cells and CBC cells represent identical populations of stem cells. In favour of this, we have performed Bmi1 expression analysis on sorted cells derived from different segments of the small intestine and colon and consistently observed the highest Bmi1 expression levels in sorted Lgr5 stem cells for all segments of the small intestine and colon (van der Flier et al, 2009a).

Zhu et al have used CD133-knockin mice to show that CD133 cells at the crypt bottoms co-express Lgr5 (Zhu et al, 2009). Like Lgr5 cells, CD133⁺ cells can generate the entire intestinal epithelium, establishing CD133 as an additional marker for CBC-type intestinal stem cells.

Acceleration of intestinal stem cell research

The anatomy of the intestinal crypt and its fast cellular turnover make it an exceptional model to study adult stem cells in their niche. With the Lgr5-, Bmi- and CD133-based genetic tools in hand, it has now become possible to visualize, isolate and genetically modify stem cells of the adult intestine at will. In addition, we have recently defined long-term culture conditions for crypts: a 3D-matrigel based growth medium containing EGF, Noggin and the Wnt-agonist R-spondin1 as growth stimuli (Sato et al, 2009). Under these culture conditions, single crypts undergo multiple crypt fission events, while simultaneously generating villus-like epithelial domains in which all differentiated cell types are present. Even single sorted Lgr5 stem cells can initiate these crypt-villus organoids and tracing experiments indicate that the Lgr5 stem-cell hierarchy is

maintained in the organoids. Very surprisingly, the cultures do not require the presence of non-epithelial niche cells.

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These findings and the available *in vivo* and *in vitro* model systems will prompt rapid progress in our understanding of the biology of the intestinal stem cell niche. We have already started to unravel the properties of Lgr5 stem cells in the intestine and also in other organs.

Genetic wiring of intestinal Lgr5 stem cells

Starting from the Lgr5-GFP knockin mice, we have been able to sort Lgr5 stem cells essentially to purity (van der Flier et al, 2009a). A single mouse yields approximately 100–200,000 cells from its small intestine. By microarray expression analysis, we have determined a gene signature for these Lgr5 stem cells. One of the genes within this stem cell signature encodes the *Achaete scute-like 2* (*Ascl2*) transcription factor. *Ascl2*-like *Lgr5*— is a Wnt target gene. Transgenic expression of the *Ascl2* transcription factor throughout the intestinal epithelium induced crypt hyperplasia and the presence of ectopic crypts on villi. In the reverse experiment, induced deletion of the *Ascl2* gene in adult small intestine led to disappearance of the Lgr5 stem cells within days. Many of the genes controlled by *Ascl2* are themselves specifically expressed or enriched in Lgr5 stem cells (van der Flier et al, 2009a). The combined results from these gain- and loss-of-function experiments imply that *Ascl2* controls the fate of intestinal stem cell. Several other genes within the signature promise to be of similar importance to the biology of the Lgr5 stem cells. One of these, the *Olfm4* gene, has already proven to be a highly robust marker for intestinal stem cells in mouse and man (van der Flier et al, 2009b).

Lgr5 stem cells in other organs

As stated above, Lgr5 expression can be visualized in rare cells at multiple sites outside the intestine of the Lgr5-knockin mice. In collaboration with Rune Toftgard's laboratory, we have studied Lgr5 cells located within the hair follicle by lineage tracing (Jaks et al, 2008; please see also Watt & Jensen, 2009, this issue). Like in the intestine, we found that the Lgr5⁺ cells constitute an actively proliferating and multipotent stem cell population capable of giving rise to new hair follicles and maintain all cell lineages of the hair follicle over long periods of time (Barker et al, 2008; Jaks et al, 2008). Ongoing studies are providing similar evidence for Lgr5 cells located at the base of glands in the pyloric region of the stomach epithelium (Barker et al, submitted) and for Lgr5⁺ basal cells in the mammary epithelium.

Lgr5 stem cells in cancer of the intestine

As discussed above, we first identified *Lgr5* as a gene expressed in colon cancer cells. It is expressed in other cancers (McClanahan et al, 2006; Yamamoto et al, 2003) and, as described in our recent study, also in premalignant mouse adenomas (Barker et al, 2009). The Lgr5-CreERT2 mouse allows us—specifically and at will—to genetically modify intestinal stem cells and we have thus been able to address the identity of the cell-of-origin of intestinal cancer. When we deleted the *Apc* gene in the Lgr5 stem cells, we observed their immediate malignant transformation (Fig. 2A,B). The transformed stem cells remained located at crypt bottoms, while fueling a growing microadenoma. These microadenomas showed unimpeded growth, and developed into macroscopic adenomas within 3–5 weeks. In contrast, when *Apc* was deleted in short-lived transit-amplifying cells using a different Cre strategy, the growth of the induced microadenomas rapidly stalled and, even after half a year, large adenomas remained rare in these mice. From this, we conclude that intestinal cancer originates in Lgr5 stem cells.

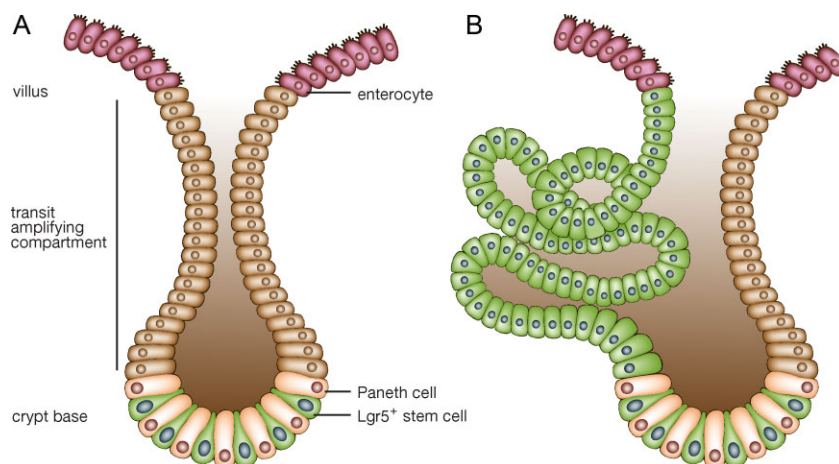


Figure 2. Lgr5 stem cells in cancer of the intestine.

- A.** A normal crypt with Lgr5 stem cells in green, Paneth cells in orange, all transit amplifying cells in brown. Differentiated cells (pink) first appear when cells exit the crypt.
- B.** When APC is deleted in one of the stem cells, the offspring of the transformed stem cell continuously proliferates and fails to leave the crypt and differentiate. As a consequence a large, green adenoma forms.

» ...intestinal cancer originates in Lgr5 stem cells. «

We have also asked whether there is any role for the transformed Lgr5 cells within established adenomas? We found that Lgr5-GFP expression was restricted to about 5% of the cells within large adenomas (Barker et al, 2009). Lgr5 may mark the limited population of tumour-initiating/propagating cells thought to exist within colon cancers—the so-called cancer stem cells. To date this has not been demonstrated, yet the available mouse models will in principle allow the genetic tracking of transformed Lgr5 cells and their offspring within established tumours. Since this strategy does not involve xeno-transplantation, such an approach would allow the first *in situ* demonstration of cells with stem cell properties within established tumours.

Perspectives

While we look forward to the data on the putative Lgr5 cancer stem cells, other questions keep popping up in our minds. Unexpectedly, we found that Lgr5 stem cells in the intestine and hair follicle are not quiescent and yet, they live as long as the mouse in which they reside. This

implies that these cells go through many hundreds of cell divisions, a logistical nightmare in terms of stem cell exhaustion, telomere collapse, loss of essential genetic information and the risk for malignant transformation. So how do Lgr5 cells deal with these challenges? How are stem cells maintained in the right numbers? What exactly makes a stem cell behave like a stem cell, and not like its transit-amplifying daughter, only one cell division away? The newly available tools allow the design of experiments aimed at the heart of these stem cell issues.

The author declares that he has no conflict of interest.



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