Very Long-term Self-renewal of Small Intestine, Colon, and Hair Follicles from Cycling *Lgr5*⁺ Stem Cells

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The intestinal epithelium and the hair follicle represent examples of rapidly self-renewing tissue in adult mammals. We have recently identified a novel stem cell gene *Lgr5* expressed in multiple adult tissues. At the bottoms of crypts in small intestine and colon as well as in hair follicles, *Lgr5* marks cycling cells with stem cell properties (Barker et al. 2007; Jaks et al. 2008). Using an inducible *Lgr5*-Cre knockin allele in conjunction with the *Rosa26-LacZ* Cre reporter strain, long-term lineage-tracing experiments were performed in adult mice. The *Lgr5*⁺ crypt-based cell generated all epithelial lineages during a 14-month period, implying that it represents the stem cell of the small intestine and colon. Similarly, lineage tracing during a 14-month period revealed that *Lgr5*⁺ cells located in the bulge of the hair follicle sustained multiple rounds of hair growth. These observations support the counterintuitive notion that *Lgr5*⁺ cells are actively cycling, yet represent long-term stem cells of these adult, self-renewing tissues.

LGR5⁺ STEM CELLS IN SMALL INTESTINE

The self-renewing epithelium of the murine small intestine is organized into crypts and villi (Gregorieff and Clevers 2005). In the mouse, it turns over every 3–5 days. A massive rate of cell production occurs in the crypts and is balanced by apoptosis at the tips of the villi. The analysis of chimeric mice and of mutagen-induced somatic clones (Winton and Ponder 1990; Bjerknes and Cheng 1999) and the study of regeneration upon injury have allowed an operational definition of stem cell characteristics. Self-renewing stem cells cycle steadily to produce the rapidly proliferating transit-amplifying (TA) cells capable of differentiating toward all lineages. The estimated number of stem cells is between four and six per crypt (Bjerknes and Cheng 1999). Three differentiated cell types (enterocytes, goblet cells, and enteroendocrine cells) form from TA cells at the crypt–villus junction. These cells continue their migration in coherent bands along the crypt–villus axis. Each villus receives cells from multiple different crypts. The fourth major differentiated cell type, the Paneth cell, resides at the bottom of the crypt.

We have previously described the *Wnt/Tcf4* target gene program in colorectal cancer cells. This program is physiologically expressed in intestinal crypts (van de Wetering et al. 2002; van der Flier et al. 2007). Because *Wnt* signals thus constitute the major driving force behind the biology of the crypt (Korinek et al. 1998), we hypothesized that some *Wnt/Tcf4(Tcf712)* target genes may be specifically expressed in the stem cells. Indeed, the *Lgr5/Gpr49* gene was expressed in a unique fashion at crypt bottoms. This *Wnt* target gene was expressed in the crypts, but not the villi, of mouse small intestine. In situ hybridization revealed expression in a limited number of cells located at all crypt bottoms as well as in adenomas in the small intestine of an *APC*^{min} mouse. This expression pattern clearly differed from that obtained with other *Wnt* target genes.

More than 30 years ago, Leblond and Cheng (1974) noted the presence of cycling cells between the Paneth cells and coined the name “Crypt Base Columnar” (CBC) cells. Cheng and Bjerknes (Bjerknes and Cheng 1981; Barker et al. 2007) and Gordon and colleagues (Stappenbeck et al. 2003) have proposed that these cells may harbor stem cell activity. The *Lgr5* gene appeared to mark these cycling CBC cells, interspersed between Paneth cells (Barker et al. 2007). The *Lgr5* gene encodes an orphan G-protein-coupled receptor (GPCR), characterized by a large leucine-rich extracellular domain (Hsu et al. 1998).

Heterozygous *Lgr5-LacZ* knockin mice allowed us to detail the expression of *Lgr5*. Before birth, a dynamic and broad expression pattern was observed (N. Barker et al., in prep.). Around birth, *Lgr5* expression was extinguished in most tissues. Expression in adult mice was restricted to rare, scattered cells in the eye, brain, hair follicle, mammary gland, stomach, adrenal gland, and intestinal tract. *Lgr5-LacZ* expression was observed in slender CBC cells at the bottom of small intestinal and colon crypts. CBC cells typically expressed the Ki67 cell cycle marker. Bromodeoxyuridine (BrdU) labeling allowed us to define the average cycling time of CBC cells to be on the order of 1 day (Barker et al. 2007).

To document the potential “stemness” of *Lgr5*⁺ CBC cells, we generated another knockin allele. An enhanced green fluorescent protein–internal ribosomal entry site (EGFP-IRES)-CreERT2 cassette was integrated at the first ATG codon of the *Lgr5* gene (Fig. 1). GFP expression was specifically seen in CBC cells at crypt bottoms of the small intestine of these mice (Fig. 2, left panel). Typically, the GFP⁺ CBC cells were relatively broad at their base and contained a flat, wedge-shaped nucleus. In our original experiments (Barker et al. 2007), we crossed the *EGFP-IRES-CreERT2* knockin allele with the Cre-activatable *Rosa26-LacZ* reporter (Fig. 1) (Soriano 1999) and followed these mice during a 2-month period. Injection of

Figure 1. Expression of EGFP and CreERT2 from a single bicistronic message by gene knockin into the first exon of *Lgr5*. The resulting allele has been named *EGFP-IRES-CreERT2* and was crossed with *Rosa26-LacZ* mice (Soriano 1999). Injection of tamoxifen activates the Cre fusion protein, resulting in activation of the *LacZ* reporter (right).

tamoxifen activates the CreERT2 fusion enzyme in *Lgr5*-expressing cells. Cre-mediated excision of the roadblock sequence in the *Rosa26-LacZ* reporter should then irreversibly mark *Lgr5*⁺ cells. Moreover, although potential progeny of these cells will no longer express GFP, the activated *LacZ* reporter should act as a genetic mark, facilitating lineage tracing. *LacZ* expression was not observed in noninduced mice (not shown). When adult mice were subjected to a very low-dose tamoxifen pulse and were sacrificed at 1, 5, 12, 35, and 60 days postinduction, the following observations were made. One day postinduction, occasional CBC cells in the small intestine and colon expressed *LacZ*. Parallel ribbons of cells emanated from the crypt bottoms and ran up the side of adjacent villi at later time points. The numbers of these ribbons did not significantly change during a 2-month period (Barker et al. 2007). Importantly, double-labeling of 60-day-induced intestine revealed the presence of goblet cells, Paneth cells, and enteroendocrine cells in the *LacZ*-stained clones, underscoring the multipotency of the stem cells.

To determine whether the *Lgr5*⁺ CBC cells constitute a stem cell pool that sustains self-renewal over the life span of a mouse, we decided to follow induced *EGFP-IRES-CreERT2* × *Rosa26-LacZ* mice for 14 months. *LacZ*

staining of small intestines of these mice revealed that the numbers of clonal blue ribbons had remained essentially unchanged (Fig. 3). Histological analysis revealed that—like the situation at earlier time points—all cell types of the epithelium were represented in the blue clones, corroborating the multipotency of the cells. These observations implied that the *Lgr5*⁺ CBC cells represent long-term stem cells of the small intestine.

LGR5⁺ STEM CELLS IN THE COLON

The colon epithelium contains crypts, but it has a flat surface rather than carrying villi. The epithelium comprises two major differentiated cell types: the absorptive colonocytes and the goblet cells (Gregorieff and Clevers 2005). Before the discovery of the *Lgr5* marker, no stem cells had been identified in the colon. Analysis of the colon yielded essentially identical observations to what was seen in the small intestine. *Lgr5*⁺ cells with an appearance very similar to that of the CBC cells of the small intestine were observed in the *EGFP-IRES-CreERT2* mice (Fig. 2, middle panel) (Barker et al. 2007). When we crossed the *EGFP-IRES-CreERT2* knockin allele with the Cre-activatable *Rosa26-LacZ* reporter (see

Figure 2. EGFP expression in an *LGR5-EGFP-IRES-CreERT2* knockin mouse in the intestinal tract and hair follicle. Confocal GFP imaging was counterstained with the red DNA dye ToPro-3. (Left) *Lgr5* expression is restricted to the six to eight slender cells sandwiched between the Paneth cells at the crypt base of the small intestine. (Middle) Confocal imaging of EGFP expression in the colon confirms that *Lgr5* expression is restricted to a few cells located at the crypt base. (Right) Confocal imaging of EGFP expression in the skin reveals *Lgr5* expression in the bulge and the secondary germ of the telogen hair follicle, as well as in the lower outer root sheath (ORS) of the anagen hair follicle.

Figure 3. Very long-term lineage tracing in the small intestine and colon. (a) *LGR5*-EGFP-IRES-CreERT2 knockin mouse crossed with *Rosa26*-*LacZ* reporter mice 12 hours after tamoxifen injection; (b) whole-mount analysis at day 5 postinduction; (c) histological analysis of b; (d–f) same as a–c for colon epithelium; (g–i) whole-mount and histological analysis of tamoxifen-induced *LacZ* marking of small intestine after 14 months; (j–l) whole-mount and histological analysis of tamoxifen-induced *LacZ* marking of colon after 14 months.

Fig. 1) and followed these mice during a 2-month period, the *Lgr5*⁺ cells yielded blue clones emanating from the bottoms of the colon crypts (Fig. 3) (Barker et al. 2007). These clones contained colonocytes as well as goblet cells and essentially remained unchanged during the 60 days of chase. One significant difference with the situation in the small intestine involved the kinetics of clone formation. At 5 days, blue staining in most crypts was still restricted to the bottom and entirely blue crypts were only rarely observed, implying that the colon stem cells were more often quiescent than their small intestinal counterparts.

To determine whether the *Lgr5*⁺ cells of the colon epithelium constitute a stem cell pool that sustains self-renewal during the life span of a mouse, we analyzed the colon of induced *EGFP*-IRES-CreERT2 × *Rosa26*-*LacZ* mice after 14 months. *LacZ* staining revealed that the numbers of clonal blue ribbons had remained essentially unchanged (Fig. 3). Histological analysis revealed that—like the situation at earlier time points—all cell types of the epithelium were represented in the blue clones, corroborating the multipotency of the cells. This is evidenced by the fact that no cells of labeled crypts were *LacZ*-neg-

ative (Fig. 3f). These observations implied that the *Lgr5*⁺ cells represent long-term stem cells of the colon.

LGR5⁺ STEM CELLS IN HAIR FOLLICLES

The skin is the largest organ in mammals. The interfollicular epidermis (IFE) is a stratified, keratinized epithelium that is constantly self-renewing and essentially produces a single differentiated cell type, the keratinocyte. In contrast, the hair follicles undergo cycles consisting of growth, involution, and resting phases (Alonso and Fuchs 2006). The stem cells of the hair follicle are thought to reside in the hair follicle bulge and have been defined by expression of the CD34 cell surface marker, expression of cytokeratin 15 (Cotsarelis et al. 1990; Trempus et al. 2003; Blanpain et al. 2004; Morris et al. 2004), and retention of either DNA or histone labels during long time periods (Braun et al. 2003; Tumber et al. 2004). Cells with stem cell properties have been isolated from other areas of the hair follicles (Ito et al. 2004; Nijhof et al. 2006). Of particular interest, at certain stages of its cycle, the lower parts of the murine vibrissae follicle contain cells that can

reconstitute a complete hair follicle upon transplantation (Oshima et al. 2001). Thus, the bulge might not be the only reservoir of stem cells. Indeed, studying *Lgr5*^{+/ve} cells in the hair follicle, we have recently reported evidence for the notion that a *Lgr5*^{+/ve} stem cell pool exists that can sustain hair follicles for up to 6 months (Jaks et al. 2008). Specifically, we found that an actively cycling *Lgr5*^{+/ve} cell population exists in the bulge and the secondary germ of the telogen hair follicle, as well as in the lower outer root sheath (ORS) of the anagen hair follicle (see Fig. 2, right panel) (Jaks et al. 2008). Analysis of sorted cells in colony-forming and transplantation assays revealed that the *Lgr5*^{+/ve} cells comprise an actively proliferating and multipotent stem cell population able to give rise to new hair follicles. We provided histological images of lineage tracing in induced *EGFP-IRES-CreERT2* × *Rosa26-LacZ* mice to demonstrate that *Lgr5*⁺ cells maintained all cell lineages of the hair follicle during 6 months.

To determine whether the *Lgr5*^{+/ve} cells of the hair follicles constitute a stem cell pool that sustains self-renewal during the life span of a mouse, we analyzed the hair follicles of induced *EGFP-IRES-CreERT2* × *Rosa26-LacZ* mice after 14 months. *LacZ* staining revealed that the numbers of blue follicles had remained essentially unchanged (Fig. 4). Histological analysis revealed that—like the situation at earlier time points—all cell types of the follicle were represented in the blue clones, corroborating the multipotency of the cells (not shown). These observations implied that the *Lgr5*^{+/ve} cells represent long-term stem cells of the hair follicle.

METHODS

Mice. LGR5-EGFP-IRES-CreERT2 mice were generated by homologous recombination in embryonic stem

cells targeting an EGFP-IRES-CreERT2 cassette to the ATG of LGR5. *Rosa26-LacZ* Cre reporter mice were obtained from Jackson Labs.

Tamoxifen induction. Mice of >8 weeks were injected intraperitoneally with 200 µl of tamoxifen in sunflower oil at 10 mg/ml.

BrdU injection. Mice were injected intraperitoneally at 4-hour intervals with 200 µl of a BrdU solution in phosphate-buffered saline (PBS) at 5 mg/ml.

Tissue preparation for immunohistochemistry, GFP confocal microscopy, and LacZ analysis. These were performed as described previously (Barker et al. 2007; Jaks et al. 2008).

CONCLUSIONS

Our previous observations have provided evidence for the existence of a pool of cycling *Lgr5*^{+/ve} stem cells in the epithelium of the small intestine and colon and in hair follicles. Of note, the first two organs are of endodermal origin, whereas the skin derives from ectoderm, implying that *Lgr5* represents a stem cell marker across germ layers. The *Lgr5*^{+/ve} cells are generally not quiescent, but they are rapidly cycling, as evidenced by the expression of Ki67 and phosphohistone H3, the incorporation of BrdU, and the observed kinetics of self-renewal (Barker et al. 2007; Jaks et al. 2008). *Lgr5*^{+/ve} cells of the small intestine appear to be more actively dividing than their colonic counterparts, likely reflecting differences in the rate of epithelial turnover between the two organs. We have not analyzed cell cycle kinetics of the *Lgr5*^{+/ve} cells in the hair

Figure 4. Very long-term lineage tracing in hair follicles. (a) LGR5-EGFP-IRES-CreERT2 knockin mouse. GFP expression in a telogen hair follicle to indicate where tracing starts; (b) entirely labeled hair follicles 37 days after tamoxifen induction; (c) histological analysis of b; (d–f) the same as in b and c, 14 months after tracing.

follicle. It appears somewhat counterintuitive that stem cells are actively dividing. This is, however, not unprecedented. Germ stem cells in the *Drosophila* testis and ovary of the fly, arguably the best-studied adult stem cells in animals, cycle throughout the lifetime of the adult fly (Ohlstein et al. 2004). Similarly, a recent elegant study based on a very similar tracing strategy as exploited in the current study, demonstrated that adult stem cells of mammalian skin are continuously cycling (Clayton et al. 2007). The observations in the current study imply that *Lgr5*⁺ cells in the small intestine accomplish the surprising feat of predictedly completing more than 400 cell cycles during the duration of the experiment, without deleterious effects on their telomeres, their genomic content, or their capacity to serve as stem cells to the epithelium. In essence, we believe that the *Lgr5*⁺ cells of the three organs studied here constitute stem cell pools that persist for the lifetime of an animal, given that a mouse rarely lives beyond 1.5–2 years of age. If these observations can be extrapolated to humans, our CBC cells complete an astounding number of cell cycles.

It is rather unusual that adult stem cells can be defined based on the expression of a single gene. This phenomenon may not be restricted to the intestine and hair follicle, because we observe highly restricted *Lgr5* expression in several other tissues. Although patterns of proliferation in stomach glands have indicated that the epithelial stem cells reside at the isthmus, halfway between the gland base and epithelial surface (Bjerknes and Cheng 2002), we find *Lgr5* expressed at gland bottoms. Ongoing lineage-tracing experiments imply that entire glands derive from these cells (N. Barker and H. Clevers, unpubl.). In the mammary gland, stem cells reside in the basal epithelial layer (Sleeman et al. 2007), where we observe *Lgr5* expression (not shown). *Lgr5* may thus represent a more general marker of adult stem cells. Two closely related genes exist in the mammalian genomes, *Lgr4* and *Lgr6*. Our ongoing studies using similar knockin strategies indicate that these two homologs are expressed in unique, yet overlapping, expression patterns when compared to *Lgr5*. At several sites, we believe that the two homologs mark stem cells, much like *Lgr5* (A. Haegebarth et al., unpubl.). The function of the three *Lgr* genes in stem cells remains to be determined, but it is likely redundant among the three homologs.

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