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Lgr proteins in epithelial stem cell biology

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Summary

The ultimate success of global efforts to exploit adult stem cells for regenerative medicine will depend heavily on the availability of robust, highly selective stem cell surface markers that facilitate the isolation of stem cells from human tissues. Any subsequent expansion or manipulation of isolated stem cells will also require an intimate knowledge of the mechanisms that regulate these cells, to ensure maintenance of their regenerative capacities and to minimize the risk of introducing undesirable growth traits that could pose health risks for patients. A subclass of leucine-rich repeat-containing G-protein-coupled receptor (Lgr) proteins has recently gained prominence as adult stem cell markers with crucial roles in maintaining stem cell functions. Here, we discuss the major impact that their discovery has had on our understanding of adult stem cell biology in various self-renewing tissues and in accelerating progress towards the development of effective stem cell therapies.

Key words: Lgr5, R-Spondin, Stem, Wnt

Introduction

The leucine-rich repeat (LRR)-containing G-protein-coupled receptor 4/5/6 (Lgr4/5/6) gene family was originally identified via *in silico* screens for cDNAs that encode proteins with homology to the glycoprotein hormone receptor class of G-protein-coupled receptors (GPCRs) (Hsu et al., 2000; Hsu et al., 1998; McDonald et al., 1998). Despite intensive efforts to de-orphanize these receptors in order to decipher their *in vivo* functions, their endogenous ligands remained elusive for more than a decade. Standard gene knockout approaches were therefore adopted to investigate the functions of Lgr4/5/6 in mice. These studies revealed multiple roles for Lgr4/5/6 during embryonic development and adult tissue homeostasis (Barker and Clevers, 2010). However, the recent explosion of interest in these receptors has primarily resulted from their identification as adult stem cell markers with essential roles in regulating stem cell activity in various adult tissues (Barker et al., 2010; Barker et al., 2007; Jaks et al., 2008; Leushacke and Barker, 2012; Snippert et al., 2010a). This seminal finding has greatly improved our understanding of epithelial stem cell biology in a variety of self-renewing tissues and has also facilitated the development of novel culture methods and transplantation technologies that should accelerate progress towards stem cell-based therapies and personalized medicine in the clinic. Here, we summarize the discovery of the Lgr gene family as adult stem cell markers and highlight the resulting impact that this discovery has had on our understanding of adult stem cell biology in a range of rapidly renewing tissues.

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Lgr proteins: structure and evolution

Lgr proteins are a unique class of evolutionarily conserved seven-transmembrane (7TM) receptors characterized by a large extracellular region (ectodomain) that harbors multiple imperfect copies of a leucine-rich repeat protein interaction domain (Fig. 1A). The ectodomain mediates ligand binding as a prerequisite to modulation of downstream intracellular signaling pathways via heterotrimeric G-proteins.

The Lgr family proteins can be subdivided into three main groups (A, B and C), according to the relative abundance of LRRs within their ectodomain, together with the presence or absence of a low-density lipoprotein receptor class A domain (LDLa) and the structure of a hinge region connecting the 7TM region to the LRR domain (Kajava, 1998; Van Hiel et al., 2012) (Fig. 1B). Type A receptors, with long hinge regions and seven to nine LRRs in their ectodomain, include the glycoprotein hormone receptors follicle-stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR) and thyroid-stimulating hormone receptor (TSHR). Type C receptors have similar numbers of LRRs, but are distinguishable by the presence of a short hinge region and an LDLa motif. This subgroup includes the relaxin hormone receptors Lgr7 and Lgr8 (Hsu et al., 2000; Kumagai et al., 2002). The mechanism of signal transduction via Type A and C receptors is relatively well understood. Hormone binding to the ectodomain triggers conformational changes within the rhodopsin-like serpentine region, resulting in the activation of heterotrimeric G-proteins that are bound to the intracellular loop and the subsequent elevation of cAMP levels, with concomitant activation of intracellular signaling pathways (Vassart et al., 2004).

The type B receptor family, including Lgr4, Lgr5 and Lgr6, is characterized by the presence of 16-18 LRRs within the ectodomain. It was first identified as a highly related subclass of the glycoprotein hormone receptor GPCR family during EST database homology searches in the late 1990s (Hsu et al., 2000; Hsu et al., 1998; McDonald et al., 1998). Subsequent phylogenetic analyses revealed the existence of Lgr4-6 homologs throughout the entire animal kingdom, including ancient life forms such as Cnidaria and Placozoa (Luo and Hsueh, 2006; Van Hiel et al., 2012). This reflects both their common ancient origins and their likely functional importance. The fly ortholog Lgr2 was identified as a receptor for the neurohormone Bursicon, a cysteine-knot protein involved in cuticle hardening (Luo et al., 2005; Mendive et al., 2005). However, efforts to assign *in vivo* functions to vertebrate Lgr4-6 were hampered for more than a decade by a failure to identify either the endogenous ligand(s) or the signal transduction cascades they control. In 2012, however, the secreted Wnt agonists R-spondins (RSpo1-4) were identified as endogenous ligands of this receptor class, revealing the crucial role of Lgr proteins in stem cell homeostasis in the gastrointestinal tract (Carmon et al., 2011; Carmon et al., 2012; de Lau et al., 2011; Glinka et al., 2011; Ruffner et al., 2012). Surprisingly, this role in potentiating Wnt signaling was found to be independent of GPCR activation, further highlighting the functional divergence of the Lgr4-6 family from the Type A and C receptors (de Lau et al., 2011).

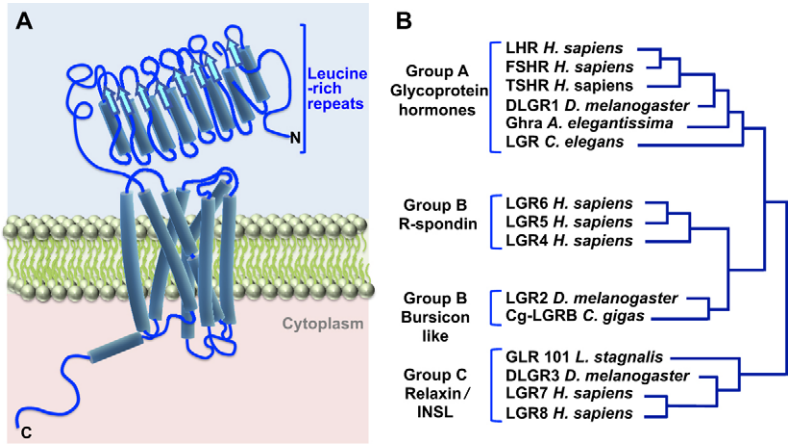

Fig. 1. Structure and phylogeny of Lgr protein families. (**A**) Leucine-rich repeat-containing G-protein-coupled receptor (Lgr) proteins are predicted to form membrane-spanning structures with large extracellular domains, which contain the leucine-rich-repeats (LRRs) and mediate ligand binding, and short cytoplasmic domains, which bind intracellular accessory molecules. (**B**) Evolutionary relationship between group A, B and C Lgr proteins. DLGR, *Drosophila* leucine-rich repeat-containing G-protein-coupled receptor; FSHR, follicle-simulating hormone receptor; GHRA, glycoprotein hormone receptor anthopleura; GLR, glutamate-like receptor; INSL, insulin like; LGR, leucine-rich repeat-containing G-protein-coupled receptor; LHR, luteinizing hormone receptor; TSHR, thryoid-stimulating hormone receptor.

However, it remains a possibility that other endogenous ligands, which can mediate their effects via a more conventional GPCR activation route, might exist.

The emergence of Lgr5 as a Wnt-regulated stem cell marker candidate

Canonical Wnt signaling is well established as being a master regulator of epithelial renewal in many adult organs that exhibit high turnover rates and is also implicated in driving the development and growth of multiple human cancers following its dysregulation (Clevers and Nusse, 2012). In the intestine, this is highlighted by the rapid ablation of epithelial renewal observed following abrogation of Wnt signaling activity (Korinek et al., 1998; van Es et al., 2012a) and, conversely, by the robust hyper-proliferation response caused by systemic administration of Wnt agonists such as Rspo1 (Kim et al., 2005).

Lgr5 was first identified as a Wnt target gene in human colon cancer cell lines harboring Wnt-activating mutations (van de Wetering et al., 2002). Artificial abrogation of this aberrant Wnt signaling activity *in vitro* resulted in the rapid quenching of *Lgr5* expression, and that of ~150 other genes. The majority of this Wnt target gene 'program' was later found to be expressed in healthy crypts of the small intestine, consistent with the central role of canonical Wnt signaling in regulating epithelial homeostasis in this rapidly self-renewing tissue (Muñoz et al., 2012; van der Flier et al., 2007). Each intestinal crypt comprises ~300 epithelial cells, arranged into a U-shaped structure that harbors a mixture of stem cells and Paneth cells at its base and an upper column of highly proliferative committed progenitor cells, referred to as the transit-amplifying compartment (Fig. 2A). Many of the Wnt target genes, including those encoding various cell-cycle regulators, are broadly expressed throughout the proliferative transit-amplifying compartment of the crypt. A second set of target genes is exclusively expressed on the terminally differentiated Paneth cells residing at the crypt base, reflecting the role of Wnt signaling in directing maturation of this cell lineage *in vivo* (Bastide et al., 2007; van Es et al., 2005). A small minority of genes, including *Lgr5*, display a much more restricted expression pattern at the crypt base, prompting speculation that they could mark the hitherto elusive intestinal stem cells (Fig. 2A). *In situ* hybridization reveals *Lgr5* to be selectively expressed on a population of 10-14 proliferating wedge-shaped cells intercalated with the Paneth cells at the crypt base in the small intestine (Barker et al., 2007). These cells, known as crypt base columnar (CBC) cells, were originally identified and morphologically characterized using electron microscopy in the early 1970s by Hazel Cheng (Cheng and Leblond, 1974). Although largely ignored by the broader gut research community, these morphologically immature cells later gained prominence as a candidate stem cell population following the publication of the 'stem cell zone' model by Hazel Cheng and Matthew Bjerknes (Bjerknes and Cheng, 1981). This model describes a stem cell zone comprising CBC stem cells and differentiated Paneth cells at the crypt base. The immediate descendants of the CBC stem cells occupy a 'mix' zone just above the Paneth cell compartment, with this progenitor population being responsible for generating rapidly proliferating precursors of the differentiated secretory and absorptive epithelial populations that line the crypt/villus axis. Further examination of *Lgr5* expression patterns in the mouse revealed the existence of discrete populations of Lgr5$^+$ cells in several organs, including skin, colon, stomach, mammary gland, tongue and kidney (Barker et al., 2010; Barker et al., 2012; Barker et al., 2007; de Visser et al., 2012; Jaks et al., 2008; Plaks et al., 2013; Yee et al., 2013) (Fig. 3).

Validating Lgr5$^+$ populations as adult epithelial stem cells

Adult stem cell populations are operationally defined by an ability to generate all differentiated cell types of their resident tissue (multipotency) and a long-term self-renewal capacity (population maintenance). The local niche environment has a major role in defining such stem cell characteristics of resident populations, highlighting the need to evaluate 'stemness' of candidate populations in their native environment whenever possible. *In vivo* lineage tracing, a genetic marking technique that facilitates such an 'in situ' evaluation of stemness (see Box 1), has consequently been adopted to assess the stem cell characteristics of the *Lgr5*-expressing CBC cells in the mouse small intestine. Targeted activation of a heritable *lacZ* reporter gene within *Lgr5*-expressing cells initially resulted in the appearance of *lacZ*$^+$ cells within the CBC compartment at the crypt base (Barker et al., 2007). Over the course of the following week, progressively expanding ribbons of *lacZ*$^+$ progeny extending from the crypt base towards the villus tips were generated (Fig. 2B). Similar observations were made in the colon, although the rate at which the *lacZ*$^+$ progeny migrated to the surface epithelium was lower than that observed in the small intestine (reflecting the lower epithelial turnover rate in this region of the gut). Individual *lacZ*$^+$ tracing units are present in all epithelial cell lineages and persist throughout life, thus identifying the Lgr5$^+$ cells as a truly multipotent, self-renewing population of adult intestinal stem cells. *In vitro*, single Lgr5$^+$ CBC cells are

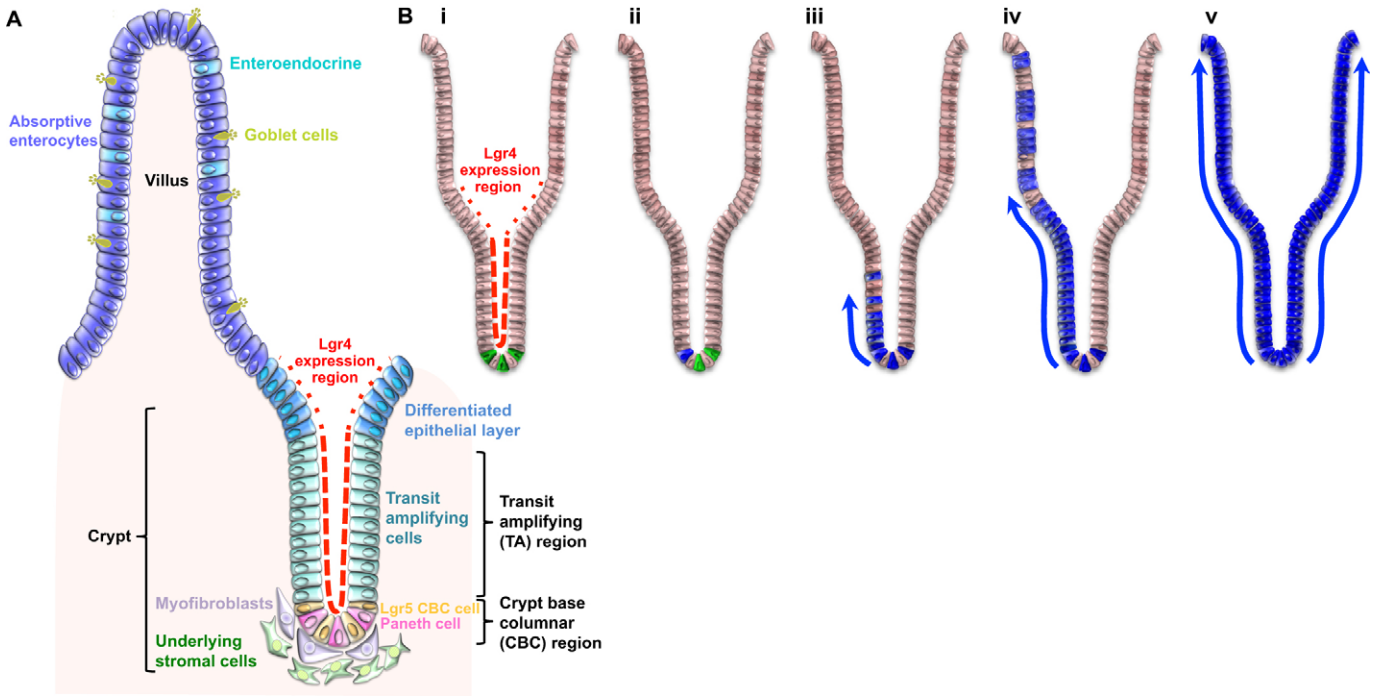

Fig. 2. Validation of Lgr5 as a marker of adult stem cells in the intestine. (**A**) Leucine-rich repeat-containing G-protein-coupled receptor 5 (*Lgr5*) expression (yellow) is restricted to 12-14 crypt base columnar (CBC) cells at the intestinal crypt base. *Lgr4* expression is found throughout the crypt (dotted red line). *Lgr6* is not expressed in the intestine in mice. (**B**) *In vivo* lineage tracing using *Lgr5-EGFP-ires-CreERT2/RosalacZ* mice. (**i**) Lgr5$^+$ stem cells expressing *GFP* and *CreERT2* (green) are present at the crypt base. (**ii**) Stochastic activation of the *lacZ* reporter gene is evident in individual Lgr5$^+$ cells (blue) at the crypt base 1 day post-induction. (**iii-v**) This *lacZ*-expressing Lgr5$^+$ cell generates *lacZ*$^+$ progeny of all differentiated lineages that rapidly expand to encompass the entire crypt-villus epithelium. The *lacZ*-expressing Lgr5$^+$ cell continues to supply *lacZ*-expressing progeny over the entire lifetime of the mouse, identifying it as a multipotent, self-renewing epithelial stem cell.

capable of generating self-organizing, self-renewing epithelial organoids with an architecture and composition that are remarkably similar to that of *in vivo* crypt/villus units (Sato et al., 2009). Collectively, these independent observations identify the Lgr5$^+$ cells in the mouse intestine as proliferating stem cells that are responsible for the daily self-renewal capacity of the epithelial lining. Somewhat counter-intuitively, the intestinal epithelium survives conditional ablation of the Lgr5$^+$ stem cell compartment *in vivo*, leading to speculation that a dedicated 'reserve' Lgr5$^-$ stem cell population may exist (Kim et al., 2012). However, an alternative explanation was recently provided when it was shown that secretory progenitor cells expressing the *Dll1* marker gene, which encodes the Notch ligand Delta-like 1, re-acquire a multipotent stem cell identity following damage-induced Lgr5$^+$ stem cell ablation (van Es et al., 2012b).

Cells with the characteristic wedge-shaped morphology of the mouse Lgr5$^+$ crypt-based columnar cells are also found intermingled with Paneth cells in the human small intestine. However, the current lack of specific Lgr5 antibodies has hampered efforts to document whether Lgr5 also selectively marks this human population *in vivo*. This has also precluded the isolation of human Lgr5$^+$ cells for direct evaluation of their stem cell identity via transplantation or *ex vivo* organoid culture. However, Batlle and colleagues have successfully isolated human colonic stem cells using the surface-expressed Wnt target gene *EPHB2* as a marker and have shown that these stem cells express the highest levels of *lgr5* in the crypt (Jung et al., 2011).

In vivo lineage tracing has also been used to characterize *Lgr5*-expressing populations in the adult hair follicle, adult distal stomach, mammary gland, taste buds and embryonic kidney as epithelial stem cells (Barker et al., 2010; Barker et al., 2012; de Visser et al., 2012; Jaks et al., 2008; Plaks et al., 2013; Yee et al., 2013) (Fig. 3). Other Lgr5-deficient adult tissues displaying much lower turnover rates, such as the liver, respond to acute damage by activating Wnt signaling to generate a population of Lgr5$^+$ stem cells that subsequently drive efficient tissue regeneration (Huch et al., 2013). Given the central role of the Wnt signaling pathway in tissue regeneration, damage-induced Lgr5$^+$ stem cells may well be found to exist in other adult organs.

The mechanics of Lgr5$^+$ stem cell-driven epithelial homeostasis in the intestine

Stem cell-driven homeostasis of the constantly renewing intestinal epithelial lining requires the balanced daily production of both committed progeny and new stem cells throughout our lifetime. Understanding how the adult stem cell compartment achieves this delicate balance *in vivo* is crucial if we are to harness safely its *ex vivo* regenerative medicine potential clinically and for deciphering how perturbations to this balance contribute to disorders such as gastrointestinal cancers. In solid tissues, adult stem cells are generally considered to achieve this balance via obligate asymmetric division to generate one stem cell daughter and one committed progenitor cell. Indeed, the stem cells of the *Drosophila* intestine do appear to undergo such asymmetric divisions routinely *in vivo* (Goulas et al., 2012). Although limited evidence has been published in support of a similar mode of strict asymmetric stem cell division in the mouse intestine (Quyn et al., 2010), more recent studies have provided

Box 1. *In vivo* lineage tracing: a brief introduction

In vivo lineage tracing is a powerful fate-mapping technique that relies on the targeted introduction of heritable genetic marks into candidate stem cell populations *in situ* within living tissues. Any descendants of these marked stem cell candidates will inherit the same genetic mark, thus facilitating their *in situ* visualization. If all differentiated cell lineages can be traced back to a single marked candidate stem cell candidate, one can conclude that this cell is multipotent. The long-term production of marked cell lineages in a given tissue demonstrates the self-renewal capacity of the stem cell candidate. A candidate cell demonstrating both multipotency and self-renewal capacity in this system fulfils the operational definition of an adult stem cell.

Unlike traditional transplantation methods, which require the removal of candidate stem cells from their instructive niche environment, *in vivo* lineage tracing facilitates a direct evaluation of 'stemness' in a completely physiological setting. However, this technique can only be applied to stem cell candidates for which there are single, highly specific markers available in mice.

compelling proof of a prevailing stochastic, symmetrical cell division mode within the Lgr5$^+$ stem cell compartment. The strongest evidence of this is provided by multicolor lineage tracing, which facilitates fate mapping of individual Lgr5$^+$ stem cells within a crypt population (Snippert et al., 2010b). Short-term tracing data demonstrate that symmetrical divisions predominate within the Lgr5$^+$ stem cell compartment – that is, Lgr5$^+$ stem cells rarely generate daughter cells that adopt divergent fates (stem cell versus committed progenitor cell). Longer-term tracing analyses reveal a gradual shift towards clonality (evidenced by conversion of a multicolor stem cell pool to a single-color population over time). Taken together, these findings indicate that Lgr5$^+$ stem cells double their numbers daily and stochastically adopt either stem cell or committed progenitor cell fates. As long as the number of symmetrical divisions resulting in either stem cells or committed progeny is balanced, epithelial homeostasis is maintained. Schepers et al. have independently demonstrated that Lgr5$^+$ intestinal stem cells segregate their chromosomes randomly during mitosis, consistent with the idea that cell fate choices following stem cell division are stochastic, rather then being pre-ordained by selective segregation of genetic material (Schepers et al., 2011). Similar conclusions have been reached by an independent study employing a predominantly mathematical modeling approach to study intestinal stem cell-driven homeostasis (Lopez-Garcia et al., 2010).

The underlying molecular mechanisms that direct Lgr5$^+$ intestinal stem cell division still remain to be deciphered. Both local biomechanical and niche influences are likely to be instrumental in directing cell fate choices during stem cell division, but it is technically challenging to evaluate this accurately *in vivo*. Recently developed computer models (Buske et al., 2011; Buske et al., 2012) may be a viable alternative here, although the validity of any resulting predictions would still need to be proven in a physiological setting. Other important issues that remain to be addressed include the influence of genetic and epigenetic mutations on stem cell division outcomes as a prelude to understanding how rogue stem cells cause cancer. Ultimately, it will also be important to determine whether Lgr5$^+$ adult stem cells in other tissues employ similar mechanisms to achieve tissue homeostasis *in vivo*.

Mining the Lgr5$^+$ stem cell expression signature for mechanistic insight and novel markers

The discovery of the Wnt target gene *Lgr5* as a common surface-expressed marker of adult stem cell populations in a range of tissues with distinct developmental origins and highly divergent functions was somewhat unexpected. In-depth analyses of their global gene expression profiles via microarray indicate that this shared expression of *Lgr5* reflects a common dependence of the various stem cell pools on canonical Wnt signaling. However, besides this overlap in Wnt target gene expression, the individual gene expression 'signatures' of the skin, stomach and intestine stem cell pools are highly distinct, probably reflecting the diversity of the local instructive niche environments in which they reside (Barker et al., 2010; Jaks et al., 2008; Muñoz et al., 2012; van der Flier et al., 2007).

Probably the best-characterized population is the Lgr5$^+$ intestinal stem cell, which has been extensively profiled via independent transcriptomic and proteomic approaches (Muñoz et al., 2012; van der Flier et al., 2007). In the absence of well-validated Lgr5 antibodies capable of facilitating the isolation of *Lgr5*-expressing epithelial populations *ex vivo*, *Lgr5-EGFP* reporter mice were used to purify Lgr5-EGFP^{hi} stem cells and their Lgr5-EGFP^{lo} progeny from small intestine. Comparative expression profiling of these stem and daughter cell populations identified ~500 genes as being enriched within the stem cell compartment. Of these, a handful (besides *Lgr5*) are selectively expressed on the intestinal stem cells, including *Ascl2*, *Olfm4* (olfactomedin 4), *Smoc2*, *RNF43* and *Troy*. *Ascl2* encodes Achaete scute-like 2, a member of the basic helix-loop-helix (BHLH) family of transcription factors that plays a vital role in maintaining stem cell viability *in vivo* (van der Flier et al., 2009). Smoc2 (SPARC-related modular calcium binding 2) is a matricellular protein thought to mediate the selective inhibition of BMP signaling on the stem cells (Muñoz et al., 2012), a process known to be essential for conferring stemness on epithelial cell populations (Haramis et al., 2004). RNF43, an E3 ubiquitin ligase, and Troy (Tnfrsf19 – Mouse Genome Informatics), a tumor necrosis factor receptor family member, are Wnt target genes thought to be involved in negative-feedback loops that fine-tune Wnt signal levels on the stem cell compartment (Fafilek et al., 2012; Hao et al., 2012; Koo et al., 2012a). Of interest, three additional genes, *Bmi1* (a polycomb ring finger oncogene), *Lrig1* (leucine-rich repeats and immunoglobulin-like domain 1) and *Hopx* (HOP homeobox) were robustly expressed on the Lgr5$^+$ stem cell compartment, in apparent contradiction to their proposed identity as markers of an Lgr5-independent stem cell pool considered to exist at position +4 above the crypt base (Powell et al., 2012; Sangiorgi and Capecchi, 2008; Takeda et al., 2011).

Exploiting the potential of Lgr5$^+$ stem cells for regenerative medicine

Our ability to identify and purify populations of live Lgr5$^+$ stem cells from a range of adult tissues opens up exciting opportunities to exploit their regenerative medicine potential clinically. *Ex vivo* culture systems that are capable of maintaining the stem cell properties of purified populations during their subsequent expansion or manipulation (e.g. via gene therapy), or systems that sustain their ability to produce new functional epithelia for use in orthotopic transplantation, are essential for realizing this clinical potential. However, it is far from easy to develop *ex vivo* culture systems that accurately recapitulate the highly complex

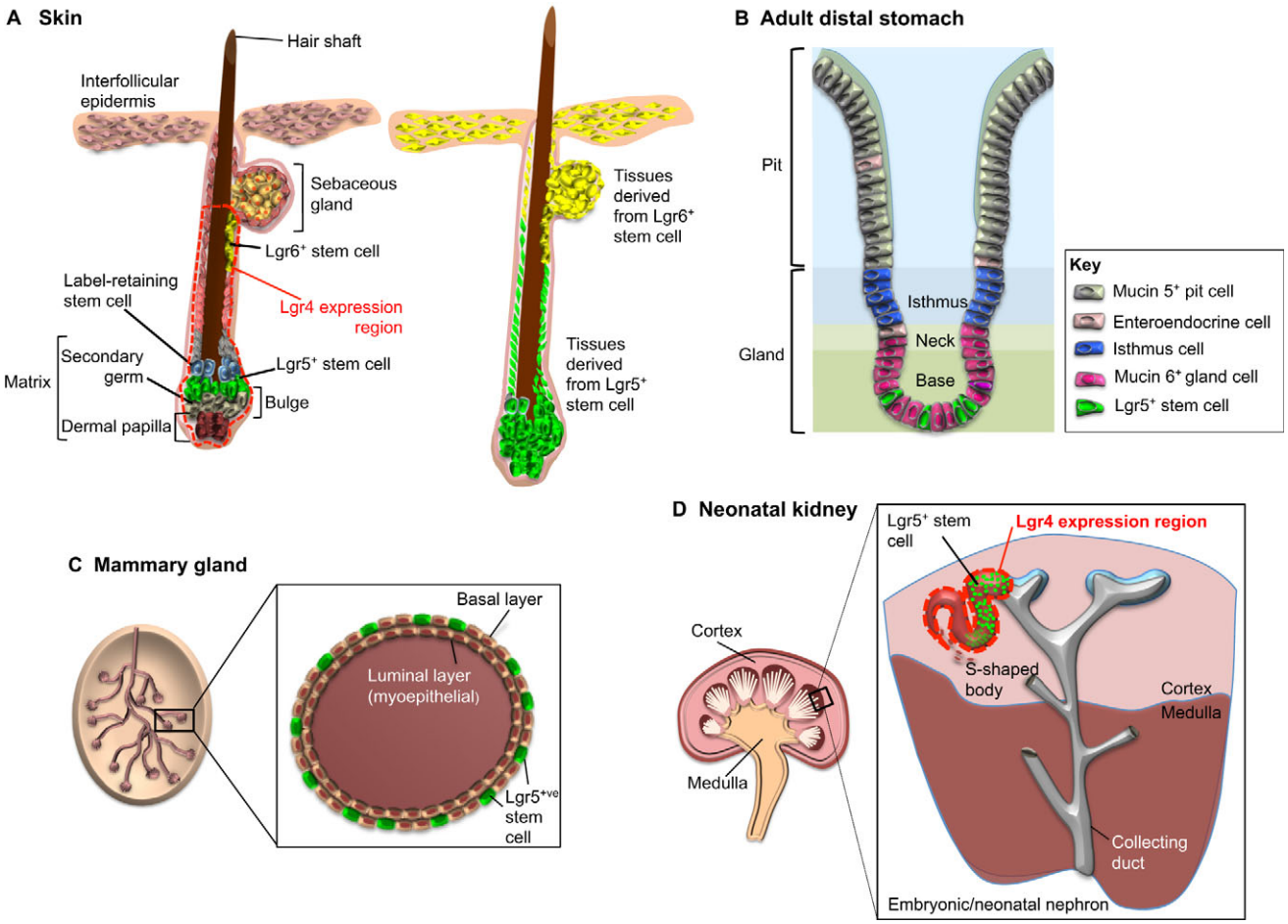

Fig. 3. Location of Lgr$^+$ stem cells within other epithelia. (**A**) Leucine-rich repeat-containing G-protein-coupled receptor 5 (*Lgr5*) and *Lgr6* exhibit restricted, yet non-overlapping, expression patterns in resting adult hair follicles (left panel). *Lgr4* expression (outlined with a red dotted line) encompasses both the Lgr5$^+$ (green) and Lgr6$^+$ (yellow) populations. Lineage-tracing studies (right panel) have shown that Lgr5$^+$ cells in the lower bulge/hair germ contribute progeny (labeled green) to all components of the hair follicle (excluding the sebaceous gland) during subsequent cycles of growth and regression over the entire lifetime of the mouse. Lgr6$^+$ cells exclusively contribute differentiated progeny (labeled yellow) to the sebaceous gland and interfollicular epidermis over the entire lifetime of the mouse. (**B**) In the adult distal stomach, 4-10 Lgr5$^+$ stem cells (green) are exclusively located at the gland base. *Lgr6* is not expressed in the mouse stomach, and *Lgr4* expression is currently unknown. (**C**) In adult mammary glands, Lgr5$^+$ stem cells (green) that are responsible for maintaining the basal epithelium are exclusively located within the basal layer. *Lgr4* and *Lgr6* expression has not been reported. (**D**) In neonatal kidney, Lgr5$^+$ stem cells (green) residing within the S-shaped body contribute to the formation of nephron filtration units. *Lgr4* expression is present throughout the S-shaped body (outlined with red dotted line). *Lgr6* expression has not been reported.

biochemical and biomechanical features of the endogenous stem cell niche. There is currently no system available for maintaining and expanding pure populations of Lgr5$^+$ stem cells *ex vivo*. However, Sato and colleagues have successfully developed a Matrigel-based culture system for growing self-renewing pieces of intestinal epithelium from purified Lgr5$^+$ stem cells (Sato et al., 2009). Using their extensive knowledge of the signaling pathways that regulate stem cell-driven intestinal epithelial homeostasis *in vivo*, they developed a 3D culture system incorporating laminin-rich Matrigel and a defined cocktail of factors, including the Wnt agonist R-spondin1, a Bmp inhibitor Noggin, epidermal growth factor (Egf), the Notch ligand jagged 1 and a small-molecule inhibitor of Rock1 (Y27632). This system supported the growth of intestinal 'organoids' from single Lgr5$^+$ cells isolated from mouse small intestine, further underscoring their stem cell identity. Quite remarkably, the basic architecture and composition of these epithelial organoids was highly reminiscent of the intestinal lining *in vivo*; the organoids contain multiple crypt structures comprising intercalated stem cell/Paneth cell populations at their base and adjacent TA progenitor cells linked by villus-like regions comprising differentiated enterocytes, goblet cells, enteroendocrine cells and tuft cells. After mechanical dissociation, crypt fragments quickly grow into new organoids, with *de novo* crypt formation driven by a process that resembles crypt fission. Modification of this small intestinal culture system to include Wnt3A facilitated the routine growth of mouse colonic organoids from isolated colon crypts, although Wnt3A withdrawal from established organoid cultures was necessary to permit differentiation towards the mature epithelial lineages (Sato et al., 2011a). A common feature of these cultured epithelia is the absence of any associated stroma, which is commonly thought to be essential for providing a specialized niche environment to anchor and support the stem cell population. A search for a potential *in vivo* epithelial source of the niche growth factors found that Paneth cells, which are intimately associated

with the Lgr5$^+$ stem cells at the crypt base, are a vital source of Wnt3, Egf and Notch ligand (Sato et al., 2011b). Indeed, Paneth cells are absolutely essential for organoid growth *ex vivo*, although it is likely that alternative mesenchymal sources of Wnt3 and other niche factors do exist *in vivo* (Durand et al., 2012; Farin et al., 2012; Kim et al., 2012). Paneth cells are absent from the most of the colon, but evidence suggests that Kit$^+$ goblet cells perform a similar support function for the Lgr5$^+$ stem cell pool (Rothenberg et al., 2012).

Mouse colonic organoid cultures have been used successfully to repair damaged colonic epithelia *in vivo* when delivered by means of a simple enema, highlighting the therapeutic potential of such epithelial culture systems for treating a range of human gastrointestinal tract disorders such as epithelial ulcers (Fig. 4) (Yui et al., 2012). More recently, the culture system has been further adapted to grow epithelia from human small intestine and human colon (Jung et al., 2011; Sato et al., 2011a). This required the addition of small-molecule inhibitors of the anaplastic lymphoma kinase (Alk) and p38 MAPK (Mark14 – Mouse Genome Informatics) signaling pathways, plus gastrin and nicotinamide to the mouse colon culture conditions. The subsequent removal of Wnt3a, nicotinamide and the p38 inhibitor from established organoid cultures was found to be essential for driving differentiation towards the functional epithelial lineages, again underscoring the essential role of growth factor gradients within the intestinal crypts *in vivo*. In addition to their potential for growing healthy epithelia for transplantation use in the clinic, these new human culture systems are expected to be invaluable tools for investigating the regulation of intestinal stem cell self-renewal and cell differentiation in humans. One can envisage employing selective small-molecule inhibitors and/or agonists of key signaling pathways, such as Wnt and Notch, or retroviral-based methods to effect knockdown of selected genes in order to dissect their roles in regulating stem cell-driven epithelial renewal of human organoid cultures (Koo et al., 2012b). Another major application will be the expansion of matched normal and tumor epithelia from patient biopsies, both as a means of generating sufficient material for deep sequencing efforts to reveal underlying genetic mutations (aiding personal medicine) and for use in drug screens to identify therapeutics that efficiently and selectively kill the tumor epithelia.

Lgr6 as a marker of adult stem cells in the skin

In contrast to *Lgr5*, the expression of *Lgr6* appears to be independent of canonical Wnt signaling in the mouse, providing an explanation for its absence from the Wnt-dependent epithelial lining of the gastrointestinal tract (Snippert et al., 2010a). Analysis of *Lgr6-lacZ/EGFP* reporter mice reveals that, like *Lgr5*, the expression of *Lgr6* is largely restricted to minor populations of cells in multiple organs. In adult skin, *Lgr6* expression is restricted to the central isthmus region that interconnects the main hair-follicle stem cell repository (the bulge) and the sebaceous gland (Fig. 3A). Transplantation and *in vivo* lineage-tracing experiments have documented the long-term contribution of Lgr6$^+$ cells to the sebaceous gland and associated interfollicular epidermis, thus identifying them as adult stem cells for these skin compartments (Snippert et al., 2010a). No contribution to the hair follicle is evident under physiological conditions, highlighting the distinct identities of the Lgr6$^+$ sebaceous gland/epidermal stem cell and Lgr5$^+$ hair-follicle stem cell pools (Jaks et al., 2008; Snippert et al., 2010a). By contrast, both Lgr5$^+$ and Lgr6$^+$ stem cell compartments actively contribute to epidermal repair following acute wounding (Kasper et al., 2011; Snippert et al., 2010a). Lineage tracing during late embryogenesis reveals the contribution of Lgr6$^+$ cells to all skin compartments, including hair follicles, implying that the dedicated Lgr5$^+$ hair-follicle stem cell reservoir in adult skin is derived from embryonic Lgr6$^+$ cells that reside within the developing epidermis.

Lgr proteins: functions and mechanisms of action in adult stem cells

Most of what we know about the *in vivo* functions of Lgr4, Lgr5 and Lgr6 has been gleaned from detailed phenotypic analyses of mice harboring null or conditional loss-of-function mutations (Table 1). *Lgr4* is widely expressed in the proliferative

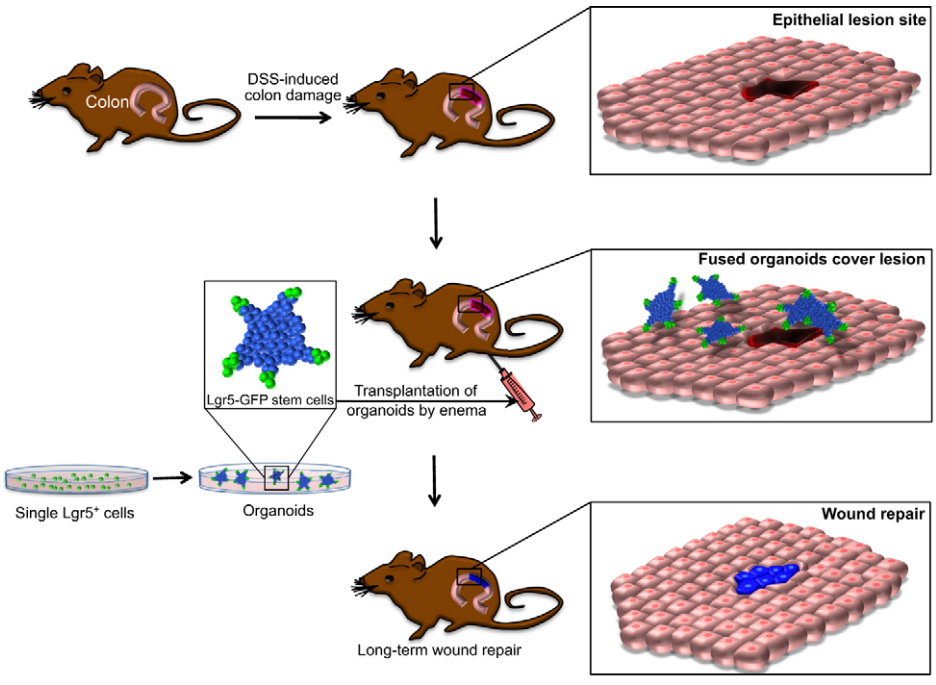

Fig. 4. Exploiting the regenerative medicine potential of Lgr5$^+$ stem cells. Labeled colonic organoids (blue) can be grown from a single leucine-rich repeat-containing G-protein-coupled receptor 5-positive (Lgr5$^+$) stem cell (green) in the laboratory. Such organoids can be effectively used to repair colonic epithelium damaged via dextran sodium sulphate (DSS)-induced inflammation when administered to live mice via an enema. The labeled (blue) transplanted organoids integrate into the damaged epithelium and contribute to the permanent repair of the epithelial wound.

Table 1. Reported Lgr tissue expression patterns and related loss-of-function phenotypes in mice

Protein	Stage	Tissue	Localization	Loss-of-function phenotype	Reference(s)
Lgr4	Adult	Adrenal gland	Unknown	Unknown	(Van Schoore et al., 2005)
	Adult	Bladder	Smooth muscle	Unknown	(Van Schoore et al., 2005)
	Embryo/adult	Bone	Endosteum and periosteum	Block in oestoblast differentiation and bone remodelling	(Luo et al., 2009; Van Schoore et al., 2005)
	Embryo/adult	Eye (multiple sites)	Cornea epithelium, iris pigment cells and retina ganglia	Anterior segment dysgenesis (impaired iris/cornea development)	(Weng et al., 2008; Van Schoore et al., 2005)
	Adult	Male reproductive tract	Seminiferous tubule and epididymis	Impaired epididymis development	(Hoshii et al., 2007; Mendive et al., 2006; Lambot et al., 2009; Van Schoore et al., 2005)
	Adult	Intestine	Broad expression in crypt epithelium	Impaired epithelial proliferation and block in Paneth cell differentiation	(de Lau et al., 2011; Mustata et al., 2011)
	Adult	Colon	Broad expression in crypt epithelium	Unknown	(de Lau et al., 2011)
	Embryo/adult	Gall bladder	Epithelial (and mesenchymal in embryos)	Impaired embryonic development	(Yamashita et al., 2009; Van Schoore et al., 2005)
	Embryo/adult	Kidney	Epithelial structures in cortex	Impaired kidney morphogenesis/ glomerulus formation and premature ureteric bud differentiation	(Kato et al., 2006) (Mohri et al., 2011) (Van Schoore et al., 2005)
	Adult	Skin	Keratinocytes and hair follicle placode	Impaired keratinocyte migration (eyelid) and impaired hair follicle formation	(Kato et al., 2007; Mohri et al., 2008; Van Schoore et al., 2005)
	Adult	Stomach	Broad expression throughout gland epithelium	Unknown	(Barker et al., 2010)
	Embryo/adult	Tongue	Epithelium	Unknown	(Van Schoore et al., 2005)
	Adult	Trachea	Cartilage and smooth muscle	Unknown	(Van Schoore et al., 2005)
	Embryo/adult	Cartilage	Around ribs	Unknown	(Van Schoore et al., 2005)
	Adult	Teeth	Odontoblasts and ameloblasts	Unknown	(Van Schoore et al., 2005)
	Adult	Female reproductive tract	Epithelium of oviduct and uterus, smooth muscle of uterus and corpus luteum	Impaired uterus development/ reduced fertility	(Van Schoore et al., 2005; Mohri et al., 2010; Mazerbourg et al., 2004)
	Adult	Mammary gland	Basal epithelium	Impaired MG branching/ elongation	(Oyama et al., 2011; Van Schoore et al., 2005)
	Embryo	Liver	Hepatocytes and erythroid precursor cells	Impaired erythropoiesis	(Song et al., 2008)
Lgr5	Embryo	Various craniofacial structures, including tongue	Unknown	Neonatal lethal ankyloglossia resulting from fusion of the tongue to the jaw, preventing suckling	(Morita et al., 2004)
	Embryo/adult	Small intestine	Crypt base columnar stem cells	None (rapid loss of stem cell compartment in compound *Lgr4*/*Lgr5* knockout mice)	(de Lau et al., 2011; Barker et al., 2007)
	Embryo/adult	Colon	Crypt base stem cells	Unknown	(Barker et al., 2007)
	Embryo/adult	Distal stomach	Gland base stem cells	Unknown	(Barker et al., 2010)
	Embryo/adult	Skin (hair-follicle)	Lower bulge/secondary germ hair follicle stem cells	Unknown	(Jaks et al., 2008)
	Adult	Inner ear (cochlea)	Sensory hair cell stem/progenitors	Unknown	(Shi et al., 2012)
	Adult	Uterus (pre-puberty)	Epithelial	Unknown	(Sun et al., 2009)
	Embryo/adult	Mammary gland	Basal epithelium stem cells (adult)	Unknown	(de Visser et al., 2012)
	Embryo	Kidney	Epithelial nephron progenitors	Unknown	(Barker et al., 2012)
Lgr6	Embryo/adult	Skin (central isthmus region)	Sebaceous gland/interfollicular epidermis stem cells	None (*Lgr6*-null mice are healthy and fertile)	(Snippert et al., 2010a)

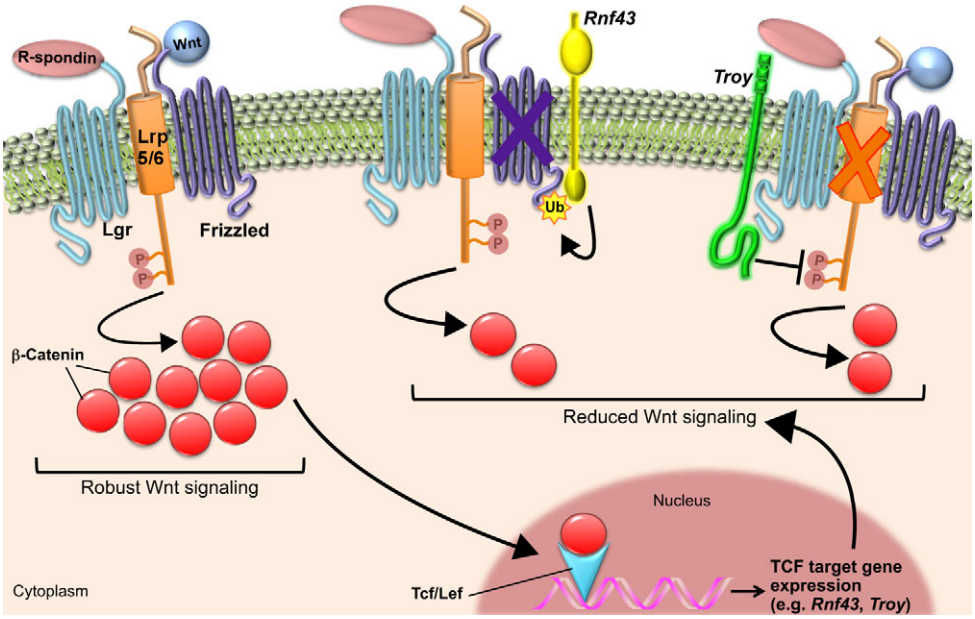

Fig. 5. Lgr proteins as regulators of Wnt signaling in epithelial stem cells. (Left) Leucine-rich repeat-containing G-protein-coupled receptor (Lgr) proteins are facultative components of the Lrp/Fzd Wnt receptor complex at the membrane that bind secreted R-spondin ligands to amplify Wnt signals on responding cells. (Middle and right) The resulting upregulation of the stem cell-specific Wnt target genes *Rnf43* and *Troy* initiates a negative-feedback loop: Rnf43 ubiquitylates frizzled receptors (purple), thereby targeting them for degradation and thus dampening Wnt signaling; Troy interacts with Lgr proteins and is recruited to the Wnt receptor complex, where it destabilizes Lrp6 and thereby reduces canonical Wnt signaling. Such intrinsic positive- and negative-feedback mechanisms ensure Wnt signal strengths are optimized for stem cell function.

compartments of many embryonic and adult tissues *in vivo* (Van Schoore et al., 2005) and it is therefore not surprising that loss-of-function mutations in this family member adversely affect the development or function of multiple organs, including the kidneys, liver, mammary glands, intestine, reproductive tract and skin (Barker and Clevers, 2010; Kato et al., 2006; Kato et al., 2007; Mohri et al., 2008; Mohri et al., 2011; Mohri et al., 2012; Mohri et al., 2010; Mustata et al., 2011; Oyama et al., 2011). As a result of such pleiotropic effects, *Lgr4*-null mutations are usually fatal. *Lgr5*-null mice invariably die within 24 hours of birth owing to a craniofacial defect reminiscent of the human condition ankyloglossia that prevents them from suckling (Morita et al., 2004). No other major phenotype is apparent at this developmental stage, with the possible exception of an increased Paneth cell frequency in the intestine (Garcia et al., 2009). By contrast, *Lgr6*-null mice are healthy and fertile, with no apparent phenotypic abnormalities (Snippert et al., 2010a).

Surprisingly, conditional ablation of Lgr5 function on the intestinal epithelium in adult mice had no discernible effect on epithelial homeostasis (de Lau et al., 2011), whereas conditional deletion of *Lgr4*, which is normally expressed throughout the intestinal crypts, led to a marked reduction in cell proliferation that is consistent with impaired function of the transit-amplifying compartment, together with a reduction in Paneth cell differentiation (de Lau et al., 2011; Mustata et al., 2011). However, the strongest phenotype was observed following concomitant ablation of both *Lgr5* and *Lgr4*, which causes a rapid and robust downregulation of the Wnt target gene program, loss of the adult stem cell compartment and a consequent block in epithelial renewal (de Lau et al., 2011). Collectively, these observations indicate that the structurally related receptors Lgr5 and Lgr4 have essential, yet redundant, functions in maintaining Wnt signaling on the stem cells. This was confirmed when the secreted Wnt agonists R-spondin1-4 were revealed to be common ligands for Lgr4, Lgr5 and Lgr6 (Carmon et al., 2011; Carmon et al., 2012; de Lau et al., 2011; Glinka et al., 2011; Ruffner et al., 2012).

Reporter gene assays demonstrate that depletion of Lgr proteins from the cell surface does not impair the ability of a cell to activate intracellular canonical Wnt signals elicited by binding of Wnt to its Frizzled/low-density lipoprotein receptor-related protein (Fzd/Lrp) receptor complex. However, Lgr depletion does abrogate the potent ability of R-spondin proteins to amplify these canonical Wnt signals. The precise mechanism by which R-spondin/Lgr complex formation mediates its effect on Wnt signaling remains unclear; there is no evidence of a role for G protein-mediated activation of typical intracellular messengers such Ca^{2+} or cAMP (Carmon et al., 2011). However, the demonstration of a physical interaction between Lgr proteins and Fzd/Lrp6 at the membrane lends support to a model in which binding of R-spondin to Lgr proteins directly influences the turnover rates of the Wnt receptor components Fzd or Lrp6 at the cell surface (Carmon et al., 2011). Thus, Lgr proteins appear to be facultative components of the Wnt receptor complex that bind secreted R-spondin proteins to boost existing canonical Wnt signals selectively on responding cells (Fig. 5). It is currently unclear whether the R-spondin ligands are secreted locally in the Lgr-expressing tissues, or whether they are delivered via the circulatory system from more distant sources (de Lau et al., 2012).

The restricted expression of *Lgr5* on intestinal stem cells may therefore have evolved as a means of selectively amplifying canonical Wnt signals above a threshold compatible with ensuring stem cell homeostasis *in vivo*. As mentioned above, the E3 ubiquitin ligase *Rnf43* and the tumor suppressor *Troy* are Wnt target genes that are found at high levels in stem cells; recent studies have implicated them in fine-tuning Wnt signaling on the intestinal stem cell compartment. Rnf43 dampens Wnt signaling by ubiquitylating Fzd receptor proteins, thereby targeting them for degradation (Hao et al., 2012; Koo et al., 2012a). Troy is recruited to the Wnt receptor complex via direct interaction with Lgr proteins, where it destabilizes the Lrp6 co-receptor at the cell surface and thereby reduces canonical Wnt signaling (Fafilek et al., 2012). A picture is therefore emerging in which intestinal stem cells have evolved an intrinsic set of positive- and negative-feedback regulators to refine canonical Wnt signal strengths, thus allowing them to ensure effective stem cell homeostasis *in vivo*. It will be important to determine whether $Lgr5^{+}$ stem cell populations in other adult tissues, such as the stomach or skin, employ similar Wnt-centric homeostatic mechanisms.

Box 2. Tumor-resident Lgr5$^+$ cells: cancer stem cells?
Cancer stem cells are considered to be discrete populations of tumor-resident stem cells that are responsible for tumor growth and progression. Targeting their elimination is expected to be a highly effective cancer therapy – hence the interest in identifying and characterizing these populations in human cancers. Aberrant activation of Wnt signaling in normal leucine-rich repeat-containing G-protein-coupled receptor 5-positive (Lgr5$^+$) intestinal stem cells is considered to be the major route to colon cancer formation (Barker et al., 2009). The resulting intestinal tumors also contain a small population of Lgr5$^+$ cells, prompting speculation that these could be cancer stem cells. *In vivo* lineage tracing has recently demonstrated that these Lgr5$^+$ cells in tumors do indeed contribute to tumor growth in mice (Schepers et al., 2012). Human colorectal cancers also contain Lgr5$^+$ cells, which are reported to be the only tumor population capable of sustaining spheroid culture *in vitro* (a surrogate assay for cancer stem cell activity) when purified using antibodies (Kemper et al., 2012). The next challenge will be to develop ways of selectively killing these Lgr5$^+$ cancer stem cells without damaging the healthy Lgr5$^+$ stem cells that are essential for maintaining our intestinal epithelium.

Perspectives

The discovery of Lgr stem cell markers and their R-spondin ligands has had a major beneficial impact on our understanding of stem cell biology in a range of rapidly renewing tissues. It has also been instrumental in facilitating the development of near-physiological epithelial culture systems that provide a renewable source of biological material for studying adult stem cells and the regulation of epithelial renewal in isolation. These culture systems also support the massive expansion of *Lgr*-expressing stem cells from limited quantities of starting material, such as patient biopsies. This is expected to facilitate the development of gene therapy-based approaches for treating genetic diseases, as well as providing a source of healthy tissue type-matched epithelia for treating epithelial diseases such as chronic gastrointestinal tract ulcers. A particularly exciting application of this culture technology is the ability to grow sufficient quantities of matched healthy and tumor epithelia from patient biopsies for use in personalized drug-screening. Such deep sequencing efforts can be used as a means of designing treatment regimes that deliver maximal therapeutic benefit. The exciting discovery of damage-induced Lgr5$^+$ stem cells in the liver has potentially important regenerative medicine implications. Future studies should focus on investigating whether similar regeneration-specific Lgr5$^+$ stem cells are present in other adult organs.

Important advances have also been made in defining the roles of *Lgr*-expressing stem cells in driving the initiation and progression of gastrointestinal tract cancers (Barker et al., 2009; Schepers et al., 2012) (see Box 2). The identification of Lgr5 as a marker of cancer stem cells in intestinal tumors (Kemper et al., 2012; Schepers et al., 2012) is expected to expedite their isolation and expression profiling as an essential prerequisite to developing more effective cancer therapeutics. Future studies are needed to determine whether Lgr5 is marking cancer stem cell populations in other tissues maintained by Lgr5$^+$ stem cells, such as the stomach. A major challenge will be to selectively kill the Lgr5$^+$ cancer stem cells without damaging the healthy Lgr5$^+$ stem cell pool needed to maintain healthy epithelia.

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