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.

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 Hsieh, 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 (Carmo et al., 2011; Carmo 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).
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., 2012; Jaks et al., 2012). 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; Jakš 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
capable of generating self-organizing, self-renewing epithelial organs 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...
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 than being preordained 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.

**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 fulfills 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.

**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 isolating the population of Lgr5-expressing epithelial populations ex vivo, Lgr5-EGFP reporter mice were used to purify Lgr5-EGFPhi stem cells and their Lgr5-EGFPlo 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 (BHHL) 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 (Fafilék et al., 2012; Hao et al., 2012; Koo et al., 2012a). Of interest, three additional genes, Bmi1 (a polycomb ring finger oncoprotein), 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 Capecci, 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...
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, epithelial 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

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.
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...
<table>
<thead>
<tr>
<th>Protein</th>
<th>Stage</th>
<th>Tissue</th>
<th>Localization</th>
<th>Loss-of-function phenotype</th>
<th>Reference(s)</th>
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<tbody>
<tr>
<td>Lgr4</td>
<td>Adult</td>
<td>Adrenal gland</td>
<td>Unknown</td>
<td>Unknown</td>
<td>(Van Schoore et al., 2005)</td>
</tr>
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<td></td>
<td>Adult</td>
<td>Bladder</td>
<td>Smooth muscle</td>
<td>Unknown</td>
<td>(Van Schoore et al., 2005)</td>
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<td></td>
<td>Embryo/adult</td>
<td>Bone</td>
<td>Endosteum and periosteum</td>
<td>Block in oestoblast differentiation and bone remodelling</td>
<td>(Luo et al., 2009; Van Schoore et al., 2005)</td>
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<tr>
<td>Embryo/adult</td>
<td>Eye (multiple sites)</td>
<td>Cornea epithelium, iris pigment cells and retina ganglia</td>
<td>Anterior segment dysgenesis (impaired iris/cornea development)</td>
<td>(Weng et al., 2008; Van Schoore et al., 2005)</td>
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<td>Adult</td>
<td>Male reproductive tract</td>
<td>Seminiferous tubule and epididymis</td>
<td>Impaired epididymis development</td>
<td>(Hoshii et al., 2007; Mendive et al., 2006; Lambot et al., 2009; Van Schoore et al., 2005)</td>
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<tr>
<td>Adult</td>
<td>Intestine</td>
<td>Broad expression in crypt epithelium</td>
<td>Impaired epithelial proliferation and block in Paneth cell differentiation</td>
<td>(de Lau et al., 2011; Mustata et al., 2011)</td>
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<td>Adult</td>
<td>Colon</td>
<td>Broad expression in crypt epithelium</td>
<td>Unknown</td>
<td>(de Lau et al., 2011)</td>
<td></td>
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<td>Embryo/adult</td>
<td>Gall bladder</td>
<td>Epithelial (and mesenchymal in embryos)</td>
<td>Impaired embryonic development</td>
<td>(Yamashita et al., 2009; Van Schoore et al., 2005)</td>
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<td>Embryo/adult</td>
<td>Kidney</td>
<td>Epithelial structures in cortex</td>
<td>Impaired kidney morphogenesis/ glomerulus formation and premature ureteric bud differentiation</td>
<td>(Kato et al., 2006; Mohri et al., 2011) (Van Schoore et al., 2005)</td>
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<tr>
<td>Adult</td>
<td>Skin</td>
<td>Keratinocytes and hair follicle placode</td>
<td>Impaired keratinocyte migration (eyelid) and impaired hair follicle formation</td>
<td>(Kato et al., 2007; Mohri et al., 2008; Van Schoore et al., 2005)</td>
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<td>Adult</td>
<td>Stomach</td>
<td>Broad expression throughout gland epithelium</td>
<td>Unknown</td>
<td>(Barker et al., 2010)</td>
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<td>Tongue</td>
<td>Epithelium</td>
<td>Unknown</td>
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<td>Cartilage and smooth muscle</td>
<td>Unknown</td>
<td>(Van Schoore et al., 2005)</td>
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<td>Embryo/adult</td>
<td>Cartilage</td>
<td>Around ribs</td>
<td>Odontoblasts and ameloblasts</td>
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<td>(Van Schoore et al., 2005)</td>
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<td>Female reproductive tract</td>
<td>Epithelium of oviduct and uterus, smooth muscle of uterus and corpus luteum</td>
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<td>(Van Schoore et al., 2005; Mohri et al., 2010; Mazerboung et al., 2004)</td>
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<td>Adult</td>
<td>Mammary gland</td>
<td>Basal epithelium</td>
<td>Impaired MG branching/ elongation</td>
<td>(Oyama et al., 2011; Van Schoore et al., 2005)</td>
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<td>Embryo</td>
<td>Liver</td>
<td>Hepatocytes and erythroid precursor cells</td>
<td>Impaired erythropoiesis</td>
<td>(Song et al., 2008)</td>
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<td>Lgr5</td>
<td>Embryo</td>
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<td>(Morita et al., 2004)</td>
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<tr>
<td>Embryo/adult</td>
<td>Small intestine</td>
<td>Crypt base columnar stem cells</td>
<td>None (rapid loss of stem cell compartment in compound Lgr4/Lgr5 knockout mice)</td>
<td>(de Lau et al., 2011; Barker et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>Embryo/adult</td>
<td>Colon</td>
<td>Crypt base stem cells</td>
<td>Unknown</td>
<td>(Barker et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>Embryo/adult</td>
<td>Distal stomach</td>
<td>Gland base stem cells</td>
<td>Unknown</td>
<td>(Barker et al., 2010)</td>
<td></td>
</tr>
<tr>
<td>Embryo/adult</td>
<td>Skin (hair-follicle)</td>
<td>Lower bulge/secondary germ hair follicle stem cells</td>
<td>Unknown</td>
<td>(Jaks et al., 2008)</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>Inner ear (cochlea)</td>
<td>Sensory hair cell stem/progenitors</td>
<td>Unknown</td>
<td>(Shi et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>Uterus (pre-puberty)</td>
<td>Epithelial</td>
<td>Unknown</td>
<td>(Sun et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>Embryo/adult</td>
<td>Mammary gland</td>
<td>Basal epithelium stem cells (adult)</td>
<td>Unknown</td>
<td>(de Visser et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>Embryo</td>
<td>Kidney</td>
<td>Epithelial nephron progenitors</td>
<td>Unknown</td>
<td>(Barker et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>Lgr6</td>
<td>Embryo/adult</td>
<td>Skin (central isthmus region)</td>
<td>Sebaceous gland/interfollicular epidermis stem cells</td>
<td>None (Lgr6-null mice are healthy and fertile)</td>
<td>(Snippert et al., 2010a)</td>
</tr>
</tbody>
</table>
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-spondin 1-4 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 Ca2+ 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 facilitative components of the Wnt receptor complex that bind secreted R-spondin ligands 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 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.
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. Ablation 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 without damaging the healthy Lgr5+ stem cell pool needed to maintain healthy epithelia.

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