SYMPOSIUM REVIEW

GI stem cells – new insights into roles in physiology and pathophysiology

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Abstract   This overview gives a brief historical summary of key discoveries regarding stem cells of the small intestine. The current concept is that there are two pools of intestinal stem cells (ISCs): an actively cycling pool that is marked by Lgr5, is relatively homogeneous and is responsible for daily turnover of the epithelium; and a slowly cycling or quiescent pool that functions as reserve ISCs. The latter pool appears to be quite heterogeneous and may include partially differentiated epithelial lineages that can reacquire stem cell characteristics following injury to the intestine. Markers and methods of isolation for active and quiescent ISC populations are described as well as the numerous important advances that have been made in approaches to the *in vitro* culture of ISCs and crypts. Factors regulating ISC biology are briefly summarized and both known and unknown aspects of the ISC niche are discussed. Although most of our current knowledge regarding ISC physiology and pathophysiology has come from studies with mice, recent work with human tissue highlights the potential translational applications arising from this field of research. Many of these topics are further elaborated in the following articles.

Abbreviations   CBC, crypt-base columnar; EE, enteroendocrine; iHIOs, induced human intestinal organoids; ISCs, intestinal stem cells; LRC, label-retaining cell; NEC, necrotizing enterocolitis; PC, Paneth cell; TA, transit amplifying.

Introduction

The following articles summarize a selection of topics on GI stem cells that were presented and discussed at the FASEB Science Research Conference entitled 'Gastrointestinal Tract XVI: GI homeostasis, the microbiome and the barrier, development and disease', held 2–7 August 2015 in Steamboat Springs, CO, USA. These reviews illustrate the complexity of our current knowledge regarding physiology and pathophysiology of GI stem cells and point to remaining controversies and to gaps that still need to be explored.

This overview lays the scene for the following articles by presenting a historical context and by touching briefly on the various topics that are covered in detail in the subsequent reviews. While several of these focus solely on the stem cells of the small intestine, as does this overview, others extend to stem cells of diverse regions of the GI tract, including stomach, colon and liver,

highlighting the fact that there are both similarities and differences.

Historical perspective of stem cells of the intestinal epithelium

The intestinal epithelium is the most rapidly proliferating tissue in the mammalian body, being entirely replaced every 3–5 days. This turnover is critical to maintaining a healthy epithelium with dual roles of facilitating digestion and absorption as well as maintaining the barrier between the internal and external milieu. The unique crypt–villus architecture with proliferating cells confined to the lower third of the crypt (Fig. 1) has long attracted physiologists, cell biologists and molecular biologists to the challenge of understanding the behaviour of this epithelium. Pioneering studies by Cheng and Leblond in 1974 led to the 'unitarian hypothesis' that undifferentiated cells (termed 'crypt-base columnar' (CBC) cells) located in the intestinal crypts just above and between the Paneth cells may serve as multipotent stem cells responsible for the generation of all differentiated lineages of the small intestinal epithelium. These studies relied on the observation that at early times after administration of [3H]thymidine, cells damaged by the local radiation were phagocytosed only by the CBC cells, but subsequently, phagocytic fragments could be detected in cells of all four differentiated lineages (Cheng & Leblond, 1974). While the small numbers of enteroendocrine cells observed in the original study led to some debate as to a separate origin for this lineage, subsequent work confirmed the 'unitarian hypothesis' by showing that enteroendocrine cells share a common stem cell with other epithelial lineages (Bjerknes & Cheng, 1981; Thompson *et al.* 1990; Bjerknes & Cheng, 1999).

The explosion of interest in the field of intestinal stem cells (ISCs) is illustrated in Fig. 2, which also highlights several landmark studies. In addition to the work from Hazel Cheng and her colleagues in Toronto, the other major groups in the early years were these of Bruce Ponder and Chris Potten, both in the UK. The Ponder laboratory creatively used chimeric mice to study the clonal behaviour of crypt cells and observed that in adult mice individual crypts were always composed of only one parental type (Ponder *et al.* 1985). As discussed in a later section, crypt monoclonality has recently been reinvestigated and is
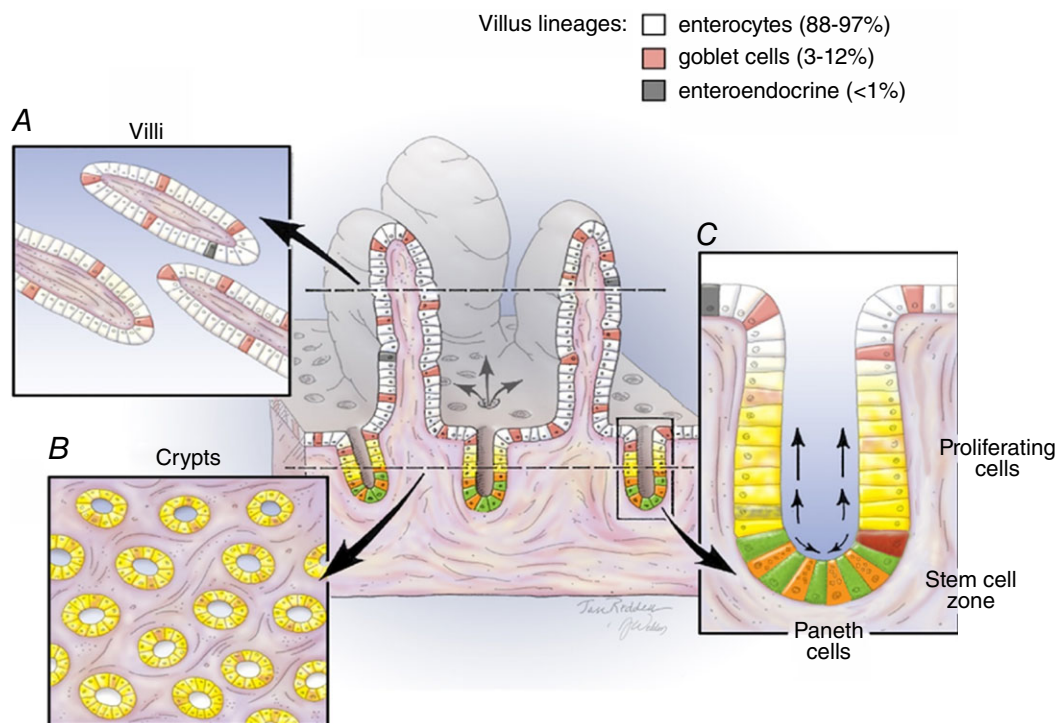
Figure 1. Architecture of the small intestinal epithelium
Centre, 3D depiction and typical 2D view as seen in a cross section showing crypt–villus units. Differentiated lineages found on the villi are colour coded as indicated. Left, structures of villi and crypts as seen in sections made along the longitudinal axis of the intestine and illustrates the fact that the villus is surrounded by multiple crypts. Right, an enlargement of the crypt area showing the proliferative transit amplifying (TA) cells (yellow), the stem cell zone and the Paneth cell lineage (orange). Within the stem cell zone, current nomenclature describes the stem cells that are intercalated between the Paneth cells at the crypt base as 'crypt base columnar' (CBC) cells (green) and those located immediately above the Paneth cells as '+4 cells' (red).

now understood at a dynamic level. Potten was a radio-biologist and the exquisite studies from his laboratory on the response of the intestinal epithelium to radiation laid the groundwork of our current understanding of the regenerative capacities of the intestinal epithelium. The microcolony assay originally described by Withers & Elkind (1970) and refined by Potten and his colleagues in the 1980s (Hendry *et al.* 1984) represented the first functional measure of ISC numbers and is still used today. Likewise, the demonstration that cells in the supra-Paneth zone, i.e. the so called '+4' position, are capable of long term retention of labelled thymidine analogues (Potten *et al.* 2002), remains a hallmark of cells in that region. These historical considerations are further discussed in the following review by Smith *et al.* (2016).

Markers for ISC populations

Until 2007 the study of ISCs was greatly hampered by a lack of markers. That year a landmark paper by Barker *et al.* reported the first definitive marker of ISCs, namely the G protein coupled receptor *Lgr5*, which is expressed specifically in the CBC cells located between Paneth cells at the base of the crypt (Barker *et al.* 2007). Lineage tracing experiments demonstrated that *Lgr5*-labelled actively cycling cells are multipotent for all mature intestinal epithelial lineages, undergo self-renewal and persist at least 60 days. Additional markers that have subsequently been identified for the Lgr5 positive

population include Ascl2, Olfm4 and Sox9 (Gracz & Magness, 2014; Tan & Barker, 2014; Tetteh *et al.* 2015). There is now a general agreement that these cells are the population normally responsible for daily homeostatic turnover of the epithelium.

As shown in Fig. 2, between 2008 and 2012 there was a flurry of reports of markers for the +4 cells, which represent slowly cycling or quiescent populations of ISCs, specifically: Bmi1, mTert, Dclk1, Sox9hi and Lrig1 (Montgomery & Breault, 2008; Gracz & Magness, 2014; Tan & Barker, 2014). All of these populations appear to function as reserve ISCs in the sense that they can become activated following intestinal damage (such as via radiation or chemotherapy) and play a critical role in the regeneration of the epithelium. HopX also marks slowly cycling cells which function as ISCs; however, the extent to which these cells are activated by damage has not yet been reported (Takeda *et al.* 2011). The question of whether the various markers of these reserve ISCs identify distinct or overlapping populations remains a matter of debate. Understanding the biology of cells that function as reserve ISCs has become even more complex in view of the current evidence (summarized in Fig. 3 and discussed further in several of the following reviews) indicating that in response to damage to the active ISC population, not only are the various quiescent ISC populations activated, but in addition more differentiated cells such as transit amplifying (TA) cells, secretory progenitors, Paneth cell (PC) precursors and enteroendocrine (EE)
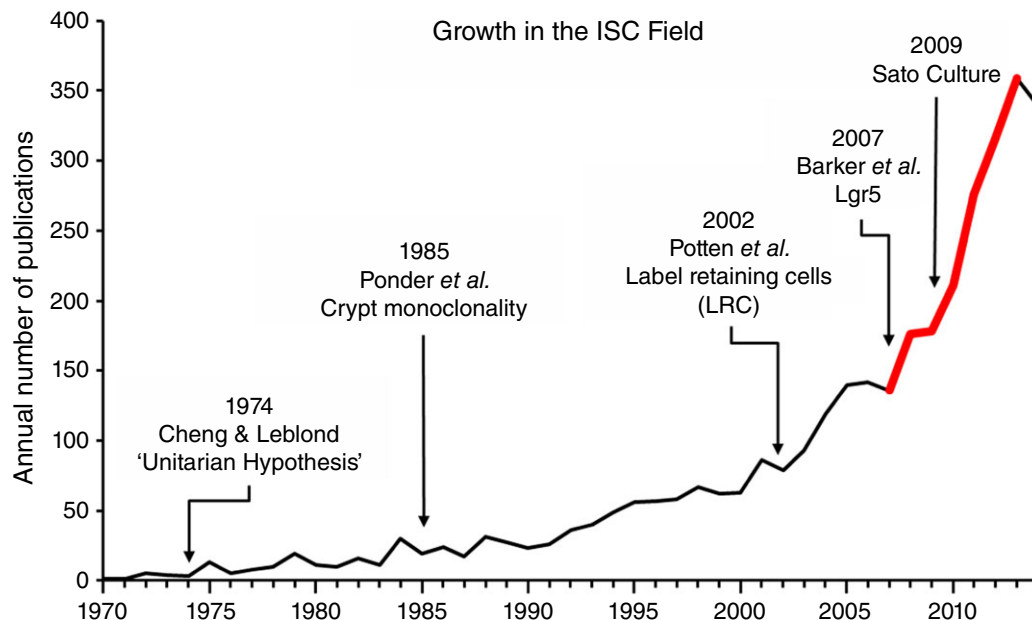

Figure 2. Historical data for publications on intestinal stem cells (ISCs)
The graph shows the number of papers per year as found in PubMed using 'stem cell intestine' as the search term. Arrows show a selection of key papers and the red portion of the line shows the period of identification of various markers for +4 cells.

precursors appear capable of reverting to an ISC state and regenerating the epithelium (Gracz & Magness, 2014; Philpott & Winton, 2014; Tetteh *et al.* 2015).

The knowledge summarized above has been generated in mouse models in which lineage tracing with genetically engineered animals has become the 'gold standard' for demonstrating stemness. While identification of specific markers for human ISCs has lagged, various investigators have made serendipitous use of naturally occurring mutations, mosaicisms and heterozygous alleles to study the clonal behaviour of cells from the base of the intestinal crypts both at homeostasis and following radiation. A thoughtful and insightful summary of this important translational work can be found in a recent review (Wright, 2012).

Approaches to the isolation of ISCs

The generation of enhanced green fluorescent protein (EGFP) reporter mice for Lgr5 and other markers greatly facilitated the isolation and characterization of putative ISC populations. Likewise the development of the H2B–yellow fluorescent protein (YFP) reporter mouse (Hughes *et al.* 2012; Buczacki *et al.* 2013) allowed modern approaches to the study of the label-retaining cells (LRCs) originally described by the Potten laboratory. In parallel with deployment of various reporter mice, several groups continued to pursue non-reporter methods to isolate ISCs on the philosophy that such methods are critical to the translation of knowledge generated in mouse models to the human intestinal epithelium. Early studies with side population sorting (Dekaney *et al.* 2005; Gulati *et al.* 2008) led to the identification of the membrane protein CD24 as a useful marker for antibody-based sorting (von Furstenberg *et al.* 2011). Concurrently, work from the laboratory of Melissa Wong showed that CD166 marks cells at the crypt base and that antibodies to CD166 can be used to isolate these cells (Levin *et al.* 2010). Some of the pitfalls of antibody-based sorting were carefully documented in a multicentre study using CD44 to isolate cells from the lower crypt (Magness *et al.* 2013). Although none of these CD antibodies are specific for ISCs, subsequent studies have shown that careful combinations can be used to yield distinct populations of ISCs from both mouse and human (Gracz *et al.* 2013; Wang *et al.* 2013). Interestingly although Lgr5 has an ectodomain, attempts to develop Lgr5 antibodies suitable for sorting have not met with success (Tan & Barker, 2014). At this stage Lrig1 remains the only specific ISC marker for which antibody-based sorting is possible (Powell *et al.* 2012). The recent demonstration that different antibodies to Lrig1 mark different subsets of epithelial cells illustrates the challenges of identifying antibodies suitable for genuine ISC isolation (Poulin *et al.* 2014). In this regard, side population sorting (Dekaney *et al.* 2005; Gulati *et al.* 2008) is an attractive option since it does not depend on antibodies. Moreover this approach has recently been further developed and shown to be capable of clearly distinguishing between actively cycling ISCs and quiescent ISCs (von Furstenberg *et al.* 2013). With the multiple isolation methods now available, the field is poised for cutting edge studies such as single cell transcriptome analyses (Gracz *et al.* 2015) to assess heterogeneity of ISC populations and further understand their biological properties.

Culture of ISCs and crypts

Another critical development in the ISC field (Fig. 2) was the report by Sato *et al.* in 2009, that isolated Lgr5-EGFP cells could be clonogenically grown *in vitro* in 3D cultures using Matrigel with an appropriate cocktail of factors (Sato *et al.* 2009). Such cultures have now become part of the standard repertoire of ISC investigators. Unfortunately there remains a debate about the appropriate nomenclature for the structures generated *in vitro*, namely 'organoids' (Sato & Clevers, 2013) *versus* 'enteroids' (Stelzner *et al.* 2012). Nevertheless the growth of these complex self-renewing structures from isolated ISCs is generally recognized as a useful surrogate for *in vivo* stemness. While the parent method of Matrigel culture had very low clonogenic efficiencies (less than 1%), modifications which markedly enhance the efficiency have subsequently been developed (Wang *et al.* 2013; Yin *et al.* 2014). The method can also be made more affordable by the use of serum and conditioned medium
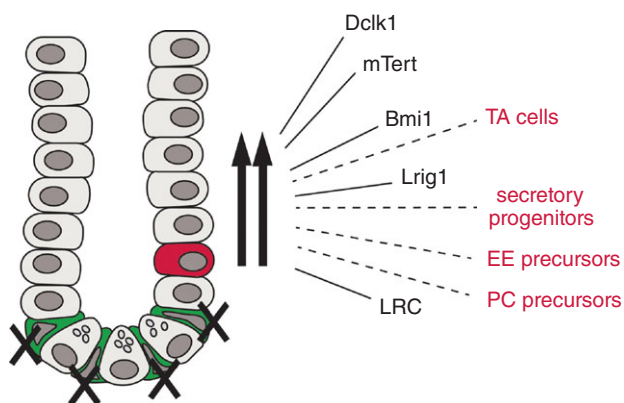
Figure 3. Schematic drawing illustrating the response of the intestine to damage
Most data come from studies of the effect of radiation, which ablates the active ISCs (green) and activates reserve ISCs (red). The latter appear to be heterogeneous with subpopulations (shown by black arrows) marked by Dclk1, mTert, Bmi1 and Lrig1, and having the property of label retention (LRC). In addition there is evidence that multiple, more differentiated cells (dashed lines and red labels) are capable of reverting to a stem cell phenotype after damage (TA, transit amplifying cells; EE, enteroendocrine; PC, Paneth cell).

in place of recombinant growth factors (Miyoshi & Stappenbeck, 2013). Initially developed for mouse tissue, these Matrigel-based approaches have now been applied to culture of isolated human ISCs (Gracz *et al.* 2013; Wang *et al.* 2013). Although the original culture method is labour intensive, recent work (Gracz *et al.* 2015) shows that high throughput adaptations are feasible, which will make ISC culture more amenable to detailed study of ISC biology and to translational applications such as drug screening.

While the ability to grow isolated ISCs *in vitro* has generated valuable knowledge regarding ISC behaviour, the application of the same technique to the growth of intact crypts may be even more important. The key difference is that in the 3D Matrigel system, the culture efficiency (defined as yield of enteroids *versus* number of units plated) is 10–100 times greater using crypts as the starting material than when the same conditions are used for isolated ISCs. With crypts from mouse small intestine, efficiencies are typically in the order of 60–80% on initial plating and approach 100% during passaging (Fuller *et al.* 2012). Efficiencies with human crypts are somewhat lower, but still sufficient to generate abundant cultures from endoscopic biopsies. Enteroid cultures derived from human crypts have been used to study physiological functions as well as host–pathogen interactions (Foulke-Abel *et al.* 2014). Such enteroids can be efficiently passaged and frozen, allowing valuable patient samples to be studied indefinitely (Foulke-Abel *et al.* 2014; Mahe *et al.* 2015). At least in mouse, crypts retain a high level of viability even following storage of tissue for 24–30 h at 4°C (Fuller *et al.* 2013), pointing to the possibility of a useful harvest from postmortem specimens. Exciting recent advances have shown that enteroids grown from crypts can be transfected by both viral and non-viral methods (Miyoshi & Stappenbeck, 2013; Sato & Clevers, 2013; Foulke-Abel *et al.* 2014), thus allowing genetic modification either for studies of basic biology or for therapeutic application (Schwank *et al.* 2013). As noted by Smith *et al.* in the following review (Smith *et al.* 2016), the caveat of all these advances is that the basic culture conditions (Sato *et al.* 2009) were devised specifically for Lgr5-ISCs and are probably pharmacological for those cells and possibly totally inappropriate for the reserve ISC populations.

Despite the extent to which 3D Matrigel culture of ISCs has advanced the field, investigators have recently recognized the advantages of 2D culture systems. A promising development in this arena is the report of alternative culture conditions (involving a fibroblast feeder layer) which allow long term growth of ISCs in 2D (Wang *et al.* 2015). This system, in which differentiation is induced by exposure to an air–liquid interface, may prove more amenable for large scale growth of ISCs and for certain studies of physiological functions. A caveat is that

to date the method has only been reported for isolated epithelial cells derived from 20–21 week human fetuses. However, other authors have reported that using crypts from adult intestine as the starting material, conditions can be devised to allow monolayer cultures which are highly polarized and thus suitable for functional studies (Foulke-Abel *et al.* 2014; Jabaji *et al.* 2014).

Generation of intestinal tissue for transplantation

Beyond culture of isolated ISCs and of intestinal crypts, other approaches that can be seen as critical for translational applications are the use of organoid units and of iPS cells to generate intestinal tissue suitable for transplantation. As reviewed recently (Spurrier & Grikscheit, 2013), organoid units have been the choice of investigators in the field of tissue engineering. These units are generated by mild digestion of the intestinal tissue, which allows crypts to be released along with adherent mesenchymal cells. When seeded onto appropriate scaffolds, they can be implanted in the omentum where they grow and vascularize. The resulting structures display fully developed crypts and villi with all four lineages and with functional capacity (Grant *et al.* 2015). Anastomosis of the neointestine with the native intestine has been demonstrated to yield clinical improvement in a rat model of short bowel syndrome (Spurrier & Grikscheit, 2013). Using a slightly different approach, other investigators have shown that organoid units can be used to ameliorate a rat model of bile acid malabsorption (Avansino *et al.* 2005). As tissue engineered small intestine has already been generated from human and pig tissue, the roadblocks to clinical application are rapidly being surmounted (Spurrier & Grikscheit, 2013).

An exciting parallel development is the demonstration that intestinal tissue can be generated *in vitro* by endodermal programming of both hES cells and hIPS cells (McCracken *et al.* 2011; Spence *et al.* 2011). Interestingly, the resulting structures spontaneously generate mesenchyme as well as epithelium and thus have appropriately been termed 'induced human intestinal organoids' and abbreviated 'iHIOs' (Stelzner *et al.* 2012). When transplanted under the kidney capsule of immune deficient mice, HIOs grew 50–100 times their original size and demonstrated presence of all differentiated lineages as well as digestive and absorptive functions (Watson *et al.* 2014). In addition these transplanted HIOs were shown to be capable of responding to systemic signals following surgical resection of the host mouse (Watson *et al.* 2014). Despite these promising results, HIOs have not yet been transplanted into the intestine. Thus, application of the techniques from the tissue engineering field to HIO growth and transplantation is an obvious future avenue.

Regulation of ISC biology

As detailed in previous reviews (Clevers, 2013; Gracz & Magness, 2014; Tan & Barker, 2014) as well as in the following articles, the combination of *in vivo* lineage tracing, cell sorting and *in vitro* culture has led to extensive analysis of the biological properties of both the actively cycling CBC-ISCs and the +4 quiescent ISCs. Homeostatic balance of self-renewal and differentiation of CBC-ISCs is dependent on multiple signalling pathways, including Wnts, EGF, Notch, Bmp and others (Sato & Clevers, 2013; Gracz & Magness, 2014; Tan & Barker, 2014). As noted by Demitrack and Samuelson in a following review (Demitrack & Samuelson, 2016), given that the Lgr5 active ISC population is more numerous and more readily accessed, to date the majority of studies on regulatory pathways have focused on these cells. The populations that serve as quiescent or reserve ISCs may have some regulatory features in common with active ISCs (e.g. Notch signalling), but must also have distinct differences to account for their markedly lower rates of proliferation. Recent studies have begun to document these differences. For example, the elegant work of Yan *et al.* showed clearly that while the Lgr5 population is stimulated by Wnt signalling, this is not the case for the Bmi1 population (Yan *et al.* 2012). Intriguingly, a recent report by Roche *et al.* suggests that differing expression levels of the transcription factor Sox9 may be responsible for quiescence *versus* active cycling, with the former being maintained by high levels of Sox9 (Roche *et al.* 2015). The following review by Richmond *et al.* (Richmond *et al.* 2016) draws on literature from various tissues to predict the smorgasbord of other factors that are likely to be involved in the maintenance of reserve ISCs in the quiescent state. Since these ISC populations are activated following epithelial damage (such as radiation or chemotherapeutic agents), uncovering the interplay of factors responsible for activation is an area of clear clinical relevance. Likewise, elucidating the mechanisms that return these cells to their quiescent state (which may be distinct from the mechanisms responsible for quiescence during homeostasis) will be critical in the development of ISC based therapies. An intriguing question is whether the various quiescent ISC populations which have been identified to date are regulated via common or distinct mechanisms. This is a particularly challenging question given the evidence summarized above (Fig. 3) and in recent reviews (Clevers, 2013; Gracz & Magness, 2014; Philpott & Winton, 2014) for significant plasticity wherein epithelial cells at various stages of differentiation can reacquire a stem cell state in response to intestinal injury. Interestingly, as reviewed by Aloia *et al.* in this series of articles, similar plasticity following damage is found in both the stomach and the liver (Aloia *et al.* 2016).

The ISC niche

Any discussion of the regulation of ISC biology needs to include consideration of the stem cell niche. As reviewed in the following article by Sailaja and Li (Sailaja & Li, 2016) and elsewhere (Shaker & Rubin, 2010; Smith *et al.* 2012; Tan & Barker, 2014), the ISC niche comprises both epithelial and stromal elements. Cells of the niche can exert their influence on ISCs via cell–cell contact, via paracrine secretions, or via changes in the composition of the extracellular matrix. With respect to the epithelial component of the ISC niche, cells that should be considered as players include Paneth cells, enteroendocrine cells, Tuft cells, goblet cells, intraepithelial lymphocytes and the ISCs themselves. Amongst these, to date by far the most attention has been given to Paneth cells (Clevers, 2013). This is not surprising in view of their close association with both the CBC-ISCs and the +4-ISCs (Fig. 1). Paneth cells have been shown to express numerous factors which stimulate ISC proliferation (including Wnt, Notch and EGF signals) and have been shown to enhance ISC growth *in vitro* (Sato *et al.* 2011), likely to be via cell–cell contact (Gracz *et al.* 2015). However, their role *in vivo* is a matter of debate in view of the reports that mice engineered to have reduced numbers of Paneth cells generally show normal intestinal architecture and normal regenerative responses to epithelial damage (Tan & Barker, 2014). This may reflect redundant sources of key factors such as Wnts (Kabiri *et al.* 2014; San Roman *et al.* 2014) as well as activation of reserve ISCs to replace reduced numbers of Lgr5 ISCs (Tan & Barker, 2014). The one aspect of ISC biology that appears to be definitely mediated by Paneth cell signalling (both *in vitro* and *in vivo*) is the increase in Lgr5 ISCs that occurs following caloric restriction (Yilmaz *et al.* 2012). Surprisingly this increase is associated with shorter rather than longer villi, suggesting that normal lineage allocation and differentiation is disrupted by caloric restriction. While this could be viewed as an adaptation to the reduced nutrient load for digestion and absorption, further studies are needed to determine whether these effects reflect luminal signalling or the altered metabolic state elicited by caloric restriction.

Stromal elements of the ISC niche include myofibroblasts, fibroblasts, endothelial cells, smooth muscle cells, neurons and immune cells (Shaker & Rubin, 2010; Smith *et al.* 2012; Tan & Barker, 2014). Of these, the majority of studies have focused on the intestinal sub-epithelial myfibroblasts. *In vitro* these cells have been shown to enhance the growth of human and mouse crypts and of isolated mouse Lgr5 ISCs (Lahar *et al.* 2011; Lei *et al.* 2014). Candidate factors responsible for this *in vitro* effect have been identified as members of the Wnt signalling pathway, including R-spondin (Lei *et al.* 2014). *In vivo* studies have pointed to elevated secretion of

the EGF family member amphiregulin as well as the Bmp antagonist chordin-like 2 in response to epithelial damage (Shao & Sheng, 2010; Seiler *et al.* 2015). The following review by Mah and Kuo (Mah & Kuo, 2016) highlights the fact that for several factors known to influence ISC behaviour (e.g. individual Wnts and R-spondins), the precise cellular sources within the ISC niche have not yet been established.

In addition to epithelial and stromal elements, the luminal environment should also be considered as part of the ISC niche. Within the lumen, while pathogens have long been recognized as having dramatic effects on the intestinal epithelium, in the last 20 years commensal microbes have been demonstrated to play numerous roles in the normal physiology of the intestinal epithelium. The presence of microbial sensing receptors (such as the TLR family) within the stem cell zone suggests the possibility that the intestinal microbiota may also influence ISC behaviour (Neal *et al.* 2012; Brown *et al.* 2014; Nigro *et al.* 2014). While *in vitro* studies with live bacteria (either commensal or pathogenic) remain a challenge, bacterial products have been shown to influence the behaviour of intestinal crypts in culture. To date, the story that has emerged for commensal organisms is somewhat confusing. Two groups have studied the effect of LPS, which acts through TLR4. One of these, using ileal crypts (Neal *et al.* 2012), reported that LPS reduces crypt proliferation and increases apoptosis, whereas the other study, using jejunal crypts (Davies *et al.* 2015), found no effect of added LPS. Regional differences are a logical explanation for this apparent discrepancy. The potential clinical significance of the adverse effect of TLR signalling in ileal ISCs was elegantly illustrated by the use of a mouse model of necrotizing enterocolitis (NEC) which showed that when the signalling was blocked, the deleterious effect of the NEC protocol was prevented (Neal *et al.* 2012). Adverse effects of the viral mimetic polyI:C, which signals via TLR3, have also been reported in crypt cultures (Davies *et al.* 2015). These authors found evidence of decreased differentiation of jejunal enteroids; however, they did not determine whether these effects reflect direct action on the ISCs. Interestingly, in contrast to the adverse effects of signalling through both TLR3 and TLR4, another microbial product, MDP, acting through Nod2, has the converse effect of eliciting cytoprotection (Nigro *et al.* 2014), both in crypt cultures and *in vivo* (using a doxorubicin model of epithelial damage). These studies suggest that overall the effects of the microbiome on ISC biology are complex. This is an area clearly needing further investigation, especially in view of reports that TLR4 receptors are elevated in ileal crypts of patients with Crohn's disease (Brown *et al.* 2014) and ileal tissue from babies with NEC (Leaphart *et al.* 2007). Further, Nod2 mutations have been reported to be associated with Crohn's disease (Hugot *et al.* 2001).

ISC clonality, dynamics and numbers

Early studies using mouse chimeras showed that during the first two postnatal weeks of mouse development, crypts are polyclonal, but by the third week they become monoclonal (Schmidt *et al.* 1988) and remain monoclonal through adulthood (Ponder *et al.* 1985). Crypt clonality was subsequently confirmed by a somatic mutation approach (Winton *et al.* 1988; Gordon *et al.* 1992). Despite these observations, in the following years the generally accepted model of ISC division was that of invariant asymmetry in which one daughter cell is maintained as a stem cell and the other progresses to the TA state and then to one of the differentiated lineages. Not until 2010 was the inconsistency between this model and crypt clonality addressed. In that year, studies in the laboratory of Doug Winton used an inducible lineage tracing approach together with statistical modelling, to study the dynamic behaviour of clonal crypt populations (Lopez-Garcia *et al.* 2010). These analyses pointed unambiguously to neutral drift dynamics in which homeostasis is achieved at the population level as a result of stem cells randomly dividing to yield either two stem cells or two TA cells. The same conclusion was simultaneously reached by studies from the laboratory of Hans Clevers using a multicolour lineage tracing approach (Snippert *et al.* 2010). As noted by Lopez-Garcia *et al.*, this model 'provides an inherently flexible assembly in which any stem cell can be deployed to differentiate into one of a number of cell types, to replace stem cells locally, and respond to changing environmental demand' (Lopez-Garcia *et al.* 2010). The following review by Mah and Kuo (Mah & Kuo, 2016) points to the regulation of ISC fate as a key area of future investigation.

Over the years, the precise number of true stem cells in each crypt has been a matter of some debate. Estimates probably depend on the methodology employed and on the likelihood that active and quiescent subpopulations of ISCs may have been differentially detected. That complication notwithstanding, most approaches put the number at five to six (see below) although some studies point to the number being as low as one stem cell per crypt (Ponder *et al.* 1985; Gordon *et al.* 1992; Cosentino *et al.* 1996) and as high as 16 (see below). Interestingly, although the early studies from Hazel Cheng's laboratory have subsequently been viewed as mainly documenting the existence of the active ISC population, whereas those from the laboratory of Chris Potten were focused on the '+4' ISCs, both of these groups concluded there were four to five ISCs per crypt (Potten, 1998; Bjerknes & Cheng, 1999). Subsequently, studies using Lgr5 as a reporter concluded there were 14–16 ISCs per crypt (Lopez-Garcia *et al.* 2010; Snippert *et al.* 2010). More recently an elegant study from the Winton laboratory using a marker-independent approach, together with mathematical modelling, showed

that the number of functional stem cells per crypt in the small intestine is in the order of six (Kozar *et al.* 2013). These authors concluded that only a subset of the Lgr5 cells behave as functional stem cells. Confirmation of this finding was rapidly forthcoming via a landmark study using multiphoton intravital microscopy, which showed that the behaviour of Lgr5 cells is heterogeneous based on location in the niche, those at the base having a survival advantage and those at the niche boundary being more likely to be lost (Ritsma *et al.* 2014). Thus, only a fraction of Lgr5 cells retain long term self-renewal. Higher resolution analysis identified the basis of this by showing that Lgr5 cells at the niche border can become passively displaced from the niche after division of a neighbour. Conversely, when Lgr5 cells were depleted, there was a sporadic transfer of cells from the TA zone into the niche border with acquisition of Lgr5 expression (Ritsma *et al.* 2014). Hopefully, future studies will use similar imaging approaches to assess the behaviour of the various subpopulations of quiescent ISCs and more differentiated cells (Fig. 3) which are involved in restitution of the epithelium after damage.

Concluding remarks

We hope that this overview together with the following articles will serve as a resource both for those who work in the area of GI stem cells and those with broader interests in the GI epithelium. Taken together the series emphasizes that despite the dramatic explosion of research on GI stem cells in the last 10 years, there are still many gaps in our knowledge and controversial issues to be resolved. Nevertheless, these reviews also point to exciting potential clinical applications and thus to the importance of continued dialogue and collaboration between basic science investigators, physician scientists, and clinicians in order to see these come to fruition.

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Additional information

Competing interests

None declared.

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