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REVIEW

Update on small intestinal stem cells

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Abstract

Among somatic stem cells, those residing in the intestine represent a fascinating and poorly explored research field. Particularly, somatic stem cells reside in the small intestine at the level of the crypt base, in a constant balance between self-renewal and differentiation. Aim of the present review is to delve into the mechanisms that regulate the delicate equilibrium through which intestinal stem cells orchestrate intestinal architecture. To this aim, special focus will be addressed to identify the integrating signals from the surrounding niche, supporting a model whereby distinct cell populations facilitate homeostatic *vs* injury-induced regeneration.

Key words: Intestinal stem cells; Organoids; Intestinal regeneration; Lgr5; Niche

Core tip: Among somatic stem cells, those residing in the intestine represent a fascinating and poorly explored research field. Aim of the present review is to delve into the mechanisms that regulate the delicate equilibrium through which intestinal stem cells orchestrate intestinal architecture, integrating signals from the surrounding niche and supports a model whereby distinct cell populations facilitate homeostatic *vs* injury-induced regeneration.

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INTRODUCTION

In an adult organism, stem cells are characterized by their ability to generate multiple differentiated cell types while maintaining their capacity for long-term self-renewal[1,2]. These are generally known as "adult" or "somatic" stem cells, including all stem cells residing in adult organs, regardless of the age of the individual. These include mesenchymal stem cells[3-7], residing in the connective stroma of most organs, and haematopoietic stem cells[8,9] among the best known and characterized, that are already being tested in clinical trials[10-14].

The amazing renewal capacity of the intestinal epithelium[1] has made this organ an attractive site to study stem-cell regulation. The intestinal tract is anatomically subdivided into the small intestine and large intestine. The inner mucosal surface, composed by an absorptive and secretory epithelium, is folded into repeated units comprising finger-like invaginations (called crypts of Lieberkühn) associated with numerous protrusions (villi), which increase the surface area, allowing efficient absorption of nutrients from the bowel lumen[2].

In normal homeostasis, the specialized differentiated cell types that orchestrate the uptake of nutrients into the body, are routinely and rapidly turned over. In fact, the intestinal epithelium is the most rapidly self-renewing tissue in the human body, with a 3-5 d turnover rate[2]. It is widely accepted that this complex process is regulated, *via* a highly regulated process of self-renewal, by a population of multipotent stem cells, residing within the bottom of the crypt namely the intestinal stem cells (ISCs)[15-19].

The number and location of these cells are still debated. Clonal analysis has demonstrated the existence of multiple stem cells in each crypt[20], with an estimated number in the 4-6 cells per crypt range[21]. ISCs have the properties of self-renewing and generating rapidly dividing transit-amplifying (TA) daughter cells, *via* asymmetric cell division[22]. TA cells undergo rapid cell division and migrate upwards into the villus. During their migration, TA cells start differentiating and finally localize at the surface of the villus epithelium as either mature absorptive enterocytes, which represent the main cell type, or mucous secreting goblet cells, or hormone-producing enteroendocrine cells[22]. Upon completing their life cycle, TA die and are discarded into the lumen[23,24].

A distinct cell type, the Paneth cell, evades this upward migration program, completing the differentiation at the base of the crypt, where it start producing lysozyme, maintaining the sterile environment of the crypt, and regulating the stem cell compartment[25-27].

Converging evidence suggests the existence of two distinct populations of intestinal stem cells: one that remains quiescent for a long time and one that actively proliferate[28]. The actively dividing ISCs provide to the baseline regeneration, whereas quiescent stem cells represent a reserve subpopulation that copes to injuries. These two subpopulations are located in adjacent sites within the crypt and are probably maintained by specific signals from the surrounding niche. Nonetheless, the precise identity of the ISCs is still a matter of debate. Two alternative models are currently proposed in the literature: the label-retaining cells (LRC) + 4 model, which identifies the quiescent stem cells, and the crypt base columnar (CBC) cells model, which identifies the actively cycling stem cells.

According to the LRC+4 model, the ISCs should be located specifically at the +4 position from the bottom of the intestinal crypt region, precisely at the origin of the migratory epithelial cell column[29]. This prediction was supported by Potten *et al*[30], who showed that cells most commonly found in this position, are characteristically label-retaining and extremely sensitive to X- and γ-radiation, two features ascribed to stem cells. Furthermore, the expression of Bmi1, a gene thought to be involved in stem cell maintenance, was shown to be elevated in the +4 cells[31]. Alternatively, the CBC cell model is based on a series of electron microscopy studies on the crypts of the small intestine, showing slender, immature, cycling cells interspersed between Paneth cells at positions 1-4, hence termed crypt base columnar cells. To support the hypothesis of CBC as the ISCs, mutagenesis studies demonstrated that 90% of the crypts, that contained a mixed population of mutant cells of different epithelial lineages, also contained mutant CBC cells, indicating the CBC cells as the common source of these different lineages[32]. Further studies, based on targeted lineage tracing strategies, have definitively identified the CBC as the intestinal stem cells, and have revealed the strategy by which the balance between proliferation and differentiation is maintained[33]. Taken together, these studies suggested that ISCs operate within a complex and dynamic environment, in which stochastic cell loss is compensated by the proliferation of neighboring stem cells.

INTESTINAL STEM CELLS MARKERS

The crypt stem cells responsible for the renewal capacity of the intestinal epithelium, represent a minority of the whole intestinal population, therefore, their identification is extremely troublesome[14]. Indeed, until relatively recently, ISCs could be identified only by indirect measurements. The recent discovery of specific ISC markers has allowed their isolation and paved the way towards a clearer understanding of their biology and role in tissue homeostasis, repair, and cancer[14].

Among the various ISCs markers, the best characterized one is the leucine rich-repeat containing G-protein coupled receptor (Lgr5), a Wnt-target gene that expressed by the cycling crypt base columnar cells, interspersed between Paneth cells[22]. *Lgr5* encodes an orphan G-protein-coupled receptor, characterized by a large leucine-rich extracellular domain[34].

Barker and co-workers demonstrated that CBC cells are capable of long-term maintenance and support the epithelium self-renewal, using the lineage tracing technique (*i.e.*, introducing permanent genetic marker into candidate stem cell genes *in situ*, thus allowing the visualization of the modified stem cells and their progeny over time)[34]. One of the major advantages of *in vivo* lineage tracing, compared to transplantation-based methods, is the lack of a physical manipulation of the candidate stem cell, so that ISCs are studied in their physiological niche[34]. In particular, the visualization and isolation of putative ISCs was obtained by targeting the *Lgr5/Gpr49* gene locus by knock-in of a dual expression cassette encoding enhanced green fluorescent protein (EGFP) and CreERT2. This resulted in the tamoxifen-induced expression of EGFP in the Lgr5+ fraction, which allowed the *in vivo* lineage tracing when combined with inducible reporter strains such as R26RLacZ[34]. This study hence showed that Lgr5 expression was confined to CBCs, and that these cells give rise to the variety of epithelial cells present in crypts, proving that CBCs function as ISCs as well[34].

In addition, it has been demonstrated that Lgr5+ cells form self-renewing epithelial organoids in *ex vivo* culture assays, resembling the *in vivo* structure and composition

of crypt/villus epithelial units. It is also worth noticing that cells expressing low, if any, Lgr5 were unable to produce organoids[35]. Gene expression analysis of purified Lgr5+ stem cells, indicated that they express additional specific markers, such as *Olfm4* and the Achaete scutelike 2 (*Ascl2*)[36]. Ascl2 is a basic helix-loop-helix transcription factor[19]; its expression in the intestinal epithelium is regulated by the Wnt pathway and is restricted to Lgr5+ stem cells. Ascl2 deletion results in the complete loss of Lgr5+ ISCs, whereas transgenic *Ascl2* expression induces crypt hyperplasia[36].

Moreover, several lines of evidence have demonstrated the existence of another putative intestinal stem cells marker, the Polycomb family member Bmi1[31]. The Polycomb family plays a key role in the development and in the regulation of progenitor self-renewal in several tissues, including the nervous system[37], the retina[38] and hematopoietic organs[39]. *In vivo* lineage tracing technique, showed that Bmi1+ cells are mainly located at the +4 position in the crypts of the small intestine, contributing to the long-term maintenance of all its epithelial cell types[31]. In addition, the selective ablation of the Bmi1+ population led to a disorganization of the intestinal mucosa, resulting in the absence of the crypts[31]. Unexpectedly, Bmi1 transgenic expression was restricted only to a minority of the crypts in the proximal small intestine, while being completely absent in the distal tract[31]. This could be possibly due to the existence of Bmi1-negative ISC populations in other regions of the intestine, or, perhaps, to an inaccurate reporting of endogenous Bmi1 expression, as a result of the variegated transgene activity, frequently observed in the intestine. Interestingly, microarray analysis showed that sorted Lgr5+ cells express Bmi1, raising the question whether the two markers really characterize independent stem cells populations. Yan *et al*[40] clarified this issue by demonstrating that Bmi1 and Lgr5 mark two functionally distinct ISCs *in vivo*. Lgr5 identifies actively cycling ISCs that are sensitive to Wnt modulation, involved in homeostatic regeneration and markedly ablated by irradiation, *i.e.*, the CBC cells. Conversely, Bmi1 is expressed by quiescent ISCs insensitive to Wnt modulations, that contribute partly to homeostatic regeneration, and are resistant to radiation injury, namely the LRC stem cells[40].

Another interesting molecule in the scenario of ISCs putative markers, is the CD133/Prominin 1(Prom1), originally discovered as novel glycoprotein expressed on neural[41] and hematopoietic stem cells[42,43]. More recently, CD133 has been described as marker of epithelial stem/progenitor cells in human kidney tubules[44] and in the prostate[45]. In the study by Zhu *et al*[46], a knock-in allele was used that integrated a CreERT2-IRES-nLacZ cassette at the first ATG codon of Prom1 (Prom1C-L). This allowed demonstrating a wide expression pattern for Prom1 in the colon; on the other hand, the expression in the small intestine, appeared to be restricted to the crypt base, overlapping with the Lgr5+ CBC cells[46] The Prom1+ cells were self-renewing, multipotent adult stem cells. By contrast with these findings, Snippert *et al*[47] reported that Prom 1 expression occurred in Lgr5+ stem cells as well as in their TA progenitors. A possible explanation for this discrepancy may reside in the different sensitivity of detection methods used in the two studies.

The RNA-binding protein Musashi 1 (Msi1), a regulator of asymmetric cell division[48], is also involved in stem cell maintenance[49,50]. Particularly, in neural stem cells Msi1 is able to maintain stemness properties through Notch pathway activation[51]. Independent immunoistochemical and *in situ* hybridization analyses, demonstrated that, Msi1 is expressed in the CBC cells immediately above the Paneth cells[52-54].

Moreover, Msi1 overexpression in the intestine increases both Wnt and Notch pathways, and induces the upregulation of Lgr5 and Bmi1[55]. Interestingly, although Msi1 is expressed in putative ISCs, in knockout mice lacking this marker, no defects in the development of the intestine are detected[56]. Taken together, these observations demonstrated that Msi1 is not a specific ISCs marker, but is expressed in both ISCs and in their early progeny[57].

To sum up, different markers point to distinct stem cells within the crypt: the marker Lgr5 points to the crypt base columnar cells located in between the Paneth cells at the crypt bottom[22], whereas the markers BMI1 identify the +4 position in the crypt, just above the Paneth cells[31]. The existence and interdependency of these different types of ISCs remain a matter of debate.

STEM CELL NICHE: HOMEOSTASIS AND MORPHOGENESIS SIGNALS

A key role in the dynamics of ISCs is ascribed to the niche, a complex and dynamic setting, that adapts in response to environmental stimuli and provides the cells essential signals, including the morphogenetic pathways, such as Wnt, Notch, bone morphogenetic proteins (BMPs) and Hedgehog[21,58-63]. The microenvironment of the niche surrounding ISCs features extracellular matrix, neural cells, lymphocytes, macrophages, endothelial cells, fibroblasts, smooth muscle cells, and myofibroblasts, that generate signals able to regulate stem cells properties and behavior[64-67].

A wide range of evidence indicates that the Wnt pathway has a crucial role in intestinal proliferation and ISC maintenance[68-74].

The Wnt pathway molecules are evolutionary conserved intracellular signaling molecules which regulate cellular fate in the crypt-villus axis in normal gut epithelium, and are implicated in stem cells self-renewal[75]. Indeed, loss of Wnt signaling *in vivo* effectively blocks cell proliferation in the intestinal crypts, destroying the epithelium[76]. Moreover, when the Wnt secretion inhibitor (IWP1) was added to organoids, the LacZ signal derived from Lgr5+ cells, was completely lost, and the proliferation was inhibited; this inhibition could be overcome administrating exogenous Wnt3A[76]. Recent evidences have

also demonstrated that Paneth cells residing next to ISCs are crucial for their maintenance and serve as the stem cell niche[77]. Paneth cells are known to secrete a variety of bactericidal products, such as cryptidins/defensins and lysozyme, epidermal growth factor (EGF), transforming growth factor β (TGF-β), and represent the main sources of Wnt3a. Indeed, the ablation of Paneth cells, decreases the number of ISCs in the crypt[78] confirming that an active Wnt signal is crucial for ISC maintenance[79].

A Wnt signaling gradient exists along the crypt-villus axis. When cells migrate away from the Wnt source at the base of the crypt, they progressively lose their proliferative capacity and differentiate. The activity of the Wnt pathway, in conjunction with other pathways such as Notch and bone morphogenetic protein (BMP), is vital for the proper organization of the colic epithelium.

In the small intestine, Notch activity determines lineage differentiation between enterocytes and secretory cell differentiation; indeed, Notch inhibition results in an increase of goblet cells, while its activation results in goblet cells depletion[78]. Recent data support the idea that Notch promotes proliferation when Wnt activity is high, while induces enterocyte differentiation when Wnt activity decreases at the top of the crypt[29]. Given that Notch receptors are membrane-bound, it would appear that only the neighboring Paneth cells can maintain active Notch signaling in Lgr5 stem cells.

BMP belongs to a family of ligands which comprises BMP and TGF-β family members, acting through the SMAD intracellular signaling cascade[80-82].

In the intestine, BMP2 and BMP3, are expressed by mesenchymal cells and are able to arrest proliferation at the crypt-villus edge, rather than promoting differentiation[29].

In fact, both mice lacking the BMP receptor (Bmpr1a), and mice overexpressing the BMP inhibitor noggin, present hyperproliferation and crypt fission[80].

Under physiological conditions, the amount of stem cells within the niche remains constant, thus these processes need to be highly regulated, probably through negative feedback mechanisms[40]. In fact, stem cells may divide: (1) asymmetrically, giving rise to another stem cell, which remains in the niche, and to a daughter cell which form a progenitor cell, that migrates upwards in the crypt and differentiate into a mature element; and (2) symmetrically, giving rise to two daughter stem cells, or two daughter non-stem progenitor cells, the latter phenomenon leading to stem cells exhaustion[75].

Overall, the current scenario indicates a niche organized into a complex network of morphogenetic signals, each crucial for ISCs and crypt maintenance[28].

STEM CELLS DERIVED ORGANOIDS: THERAPEUTIC APPLICATIONS FOR GASTROINTESTINAL DISEASES

Rapid progress in the field of intestinal stem cell biology now lends promise to the application of adult stem cell therapy in gastroenterology. The apparently unlimited scale at which these stem cells can be expanded *in vitro* offers particularly exciting therapeutic possibilities[83-85].

In particular, organoids, derived from *in vitro* expansion of a single adult colonic stem cell, can be used to repair damaged colon tissue.

Indeed, as discussed above, intestinal organoid cultures, with a gut like structure, and containing all epithelial cell types, can be derived from single Lgr5+ sorted stem cells[86]; the resulting organoids can be expanded efficiently and over long periods of time, without losing tissue identity. To date, protocols for the efficient generation of organoids from stomach, human small bowel and colon have been developed[87-90].

Interestingly, the growth factors used to supplement the culture medium are the natural growth factors to which the stem cells are exposed *in vivo*, suggesting a high clinical-grade biocompatibility of this approach[87]. Moreover, no genetic manipulation required, making the entire procedure extremely safe.

As a first step toward the development of stem cell transplantation, it has been shown that, using the colonic organoids culture system, significant amounts of tissue can be grown *in vitro* from a single adult colon stem cell[91,92].

Colon organoids were reintroduced into superficially damaged recipient colons of immunocompromised (rag2$^{-/-}$) mice, pretreated with dextran sulphate sodium (DSS), which induces superficial mucosal lesions. The engrafted organoids RFP+, were able to readily integrate into the existing epithelium (RFP-), and generated histologically and functionally normal crypts containing all differentiated cell types, covering the area that lacked epithelium in recipient mice. At 4 wk after transplantation, the donor-derived cells constituted a single-layered epithelium, which formed self-renewing crypts that were functionally and histologically normal. In long term studies, carried out at 25 wk after transplantation, the grafts still contributed to the epithelium without any sign of adenomatous or dysplastic transformation[91,93].

Moreover, transplanted mice displayed a significant lower weight loss than control mice[91]. These data showed the feasibility of colon stem-cell therapy based on the *in vitro* expansion of a single adult colonic stem cell; graft rejection can be managed by standard approaches, *i.e.*, by leukocyte antigens matching of donor and acceptor and by immunosuppressive therapy, as currently used for organ transplantation.

Protocols have also been developed to expand human small intestine and colon organoids from small biopsies[94]. As a first application, Cleavers and his collaborators have transplanted the organoid-derived small intestinal epithelium into the bowel of patients affected by the microvillus inclusion disease[48]. This is a rare hereditary defect of the enterocyte brush border resulting in insufficient nutrients' assimilation[95-97], requiring colon transplantation as the unique therapeutic strategy. Intes-

tinal organoids technology may allow a novel venue into gene therapy approaches, that involves the introduction of DNA sequences into the genomes of cells of the pertinent patient. The achievement of a safe gene transfer represents the major hurdle, which has largely hampered the introduction of gene therapy into the clinic despite three decades of intensive efforts. On this regard, retrovirus- and lentivirus-mediated gene transfer has already been proven to be feasible in organoids systems[98,99].

These viral vectors are though associated to documented risk for insertional mutagenesis. As organoids can be grown from single sorted stem cells, one could envisage an approach in which individual stem cells are analyzed after integration of the recombinant DNA sequences. Only stem cells with safe integrations could then be expanded clonally for subsequent transplantation.

Overall, adult stem-cell therapy holds promise for the treatment of gastrointestinal diseases, using tissues "harvested" from a single living donor, overcoming the difficulties of the organ transplantation, that is still limited by the availability of donor.

Clinical application of this protocol still waits the translation of the technical procedures to the good clinical practice standards, to generate the adequate amounts of tissue to treat human subjects, and the development of efficient transplantation approaches.

CONCLUSION

ISCs could be reasonably considered the key players that orchestrate the high-rate regenerative capacity of the intestinal epithelium. The understanding of the interplay between the ISCs and their niche, led by a complex molecular network, will pave the way for the future development ISCs based therapy especially to the application in gastroenterology.

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