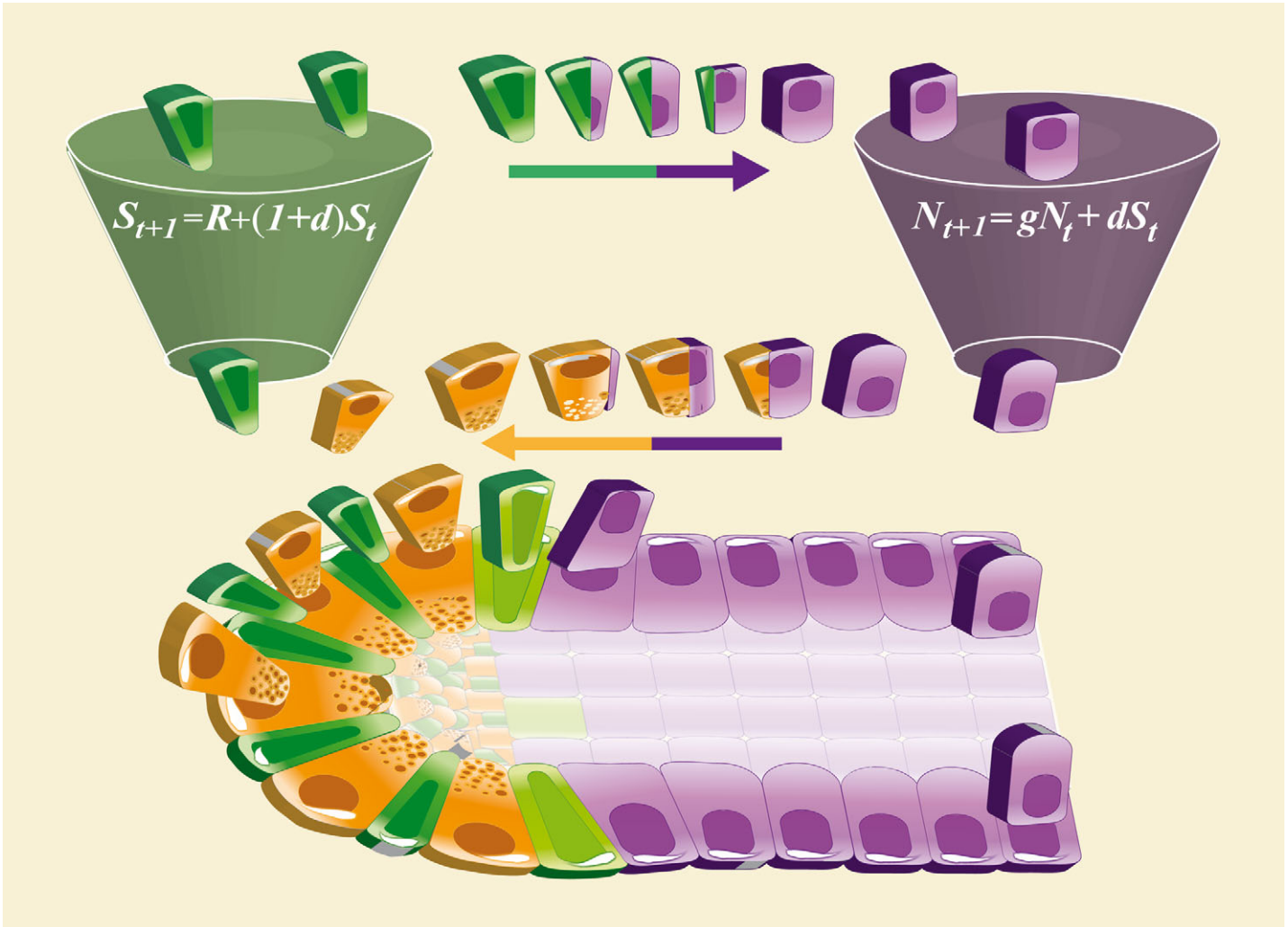

Featuring a review from Alexis J. Carulli, Linda C. Samuelson and Santiago Schnell at the University of Michigan, Ann Arbor, Michigan, USA.

Title: Unraveling intestinal stem cell behavior with models of crypt dynamics

Carulli, Samuelson and Schnell present a critical view of intestinal stem cell biology coupled with a focused discussion of mathematical modeling approaches used to probe stem cell dynamics. They discuss how compartmental modeling of the crypt combined with *in vivo* approaches can answer important unsolved questions in the field.

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Unraveling intestinal stem cell behavior with models of crypt dynamics

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The definition, regulation and function of intestinal stem cells (ISCs) has been hotly debated. Recent discoveries have started to clarify the nature of ISCs, but many questions remain. This review discusses the current advances and controversies of ISC biology as well as theoretical compartmental models that have been coupled with *in vivo* experimentation to investigate the mechanisms of ISC dynamics during homeostasis, tumorigenesis, repair and development. We conclude our review by discussing the key lingering questions in the field and proposing how many of these questions can be addressed using both compartmental models and experimental techniques.

Insight, innovation, integration

This review features a discussion of both the historical and current view of intestinal stem cells (ISCs), drawing *insights* into the main questions and controversies in the field. The paper focuses on *innovative* applications of mathematical modeling techniques, highlighting how a similar compartmental modeling framework can be applied to address very different biological questions. The core of this article stresses *integration* of modeling and *in vivo* experimentation to make advances in ISC biology. Importantly, we return to the key lingering questions in the field and directly propose how many of these questions can be addressed by compartmental population modeling.

Introduction

Adult stem cells are crucial for maintaining proper function and repair of gastrointestinal tissues. Because the intestine is one of the most rapidly regenerated tissues in the body, the intestinal crypt has provided an informative system for studying stem cell biology. In addition to advancing our understanding of stem cell physiology, intestinal stem cell (ISC) research aims to provide insight into intestinal pathologies. ISCs are thought to drive intestinal and colorectal cancers,[1,2] therefore understanding how aberrant stem cell regulation initiates such processes is a major interest in the field. Additionally, ISCs are critical for epithelial repair after intestinal damage, such as exposure to irradiation and chemical mutagens.[3–7] Understanding the repair response is important for managing radiation therapies and environmental exposures as well as developing treatments for intestinal disease. Finally, ISC tissue engineering provides hope for regenerative therapies that can treat lost or damaged intestinal tissue.[8–10] For all of these reasons, the impetus to unravel this cell's identity, function, and regulation remains a priority.

Mathematical and computational models are immensely powerful tools that can be used to probe biological systems in ways that may be very difficult to address experimentally. First, models can be used to test several parallel hypotheses to help narrow down the most likely biological explanation, which can be validated by *in vivo* analysis. New experimental findings can then be implemented into the model, and reiterations can relay new questions. Repeated refining of the model through coupled experimentation can lead to the identification of the key mechanisms underlying the behavior of the system as a whole.

Modeling has long been used as a method to understand intestinal crypt homeostasis, tumorigenesis, and injury. The full potential of these models was not realized, however, due to the limited availability of stem cell markers to identify the location and numbers of ISCs as well functional assays to validate the models *in vivo*. Much progress has been made on these fronts, resulting in a resurgence in modeling efforts to study ISC function and crypt dynamics. Apart from the specific mathematical analyses used in these models, there are two broad modeling approaches that have been applied in this field: spatial models and compartmental models. Spatial models use a geometric lattice, algorithm or boundary conditions to organize individual cells in space. These models typically consider both crypt physical forces and

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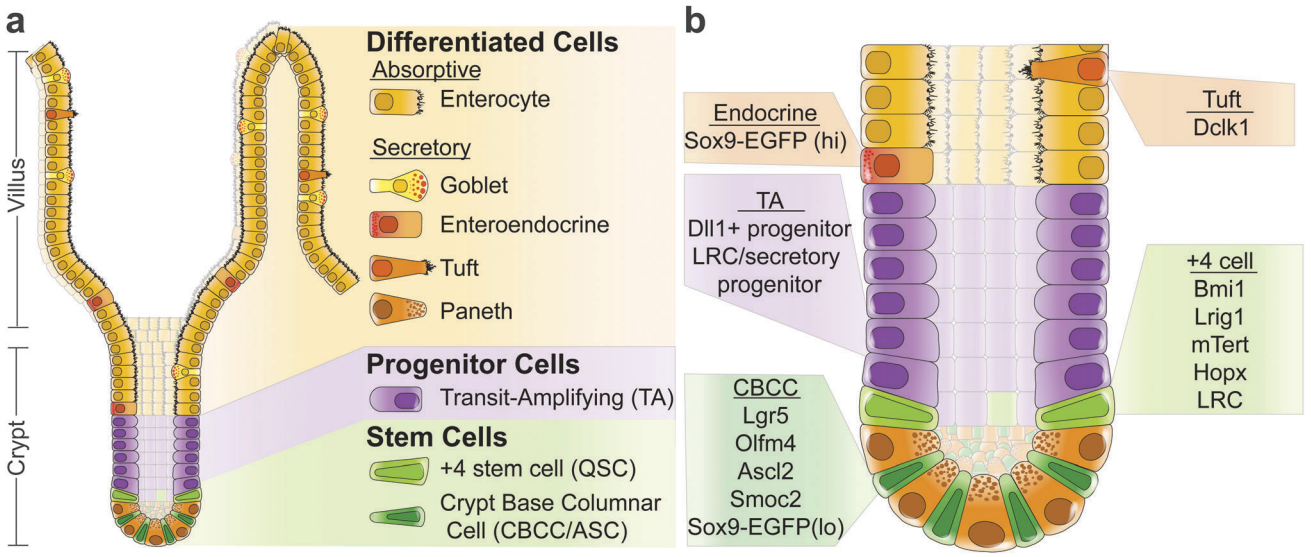

Fig. 1 Intestinal epithelial organization and markers. (a) The intestinal epithelium is organized into crypt and villus regions, with the stem and progenitor zone localized in the crypt. Current models favor the existence of two stem cell populations, the +4 stem cell and the crypt base columnar cell (CBCC), which are thought to be quiescent and active stem cells, respectively. Transit-amplifying (TA) progenitors arise from the stem cell compartment and differentiate into absorptive enterocytes or secretory goblet, enteroendocrine, tuft, or Paneth cells. Most of the differentiated cell populations migrate up the villi, but, uniquely, the Paneth cells move downward and reside between the CBCCs. (b) Molecular and functional markers that have been described for various proposed stem cell and potential stem cell populations. Of note, both TA cells and +4 cells have been shown to be Label Retaining Cells (LRCs). *Sox9-EGFP* has been shown to mark both CBCCs and clonogenic enteroendocrine cells, depending on the level of EGFP expression. The gene *Dclk1* has been proposed to be a stem cell marker, but it has also been shown to be a specific marker of differentiated tuft cells. It is possible that there is an independent +4 cell population that is also marked with *Dclk1*, but this has not been verified by lineage tracing.

cell–cell interactions and have recently been reviewed by De Matteis.[11] Compartment models, on the other hand, utilize the unique cellular organization of the intestine, with proliferating stem and progenitor cells at the base of the crypts and most differentiated cells migrating up the villi (Fig. 1A), to group cell lineages into discrete cell population compartments for analysis.

In this paper we integrate the discussion of *in vivo* and *in silico* advancements in ISC biology. We first review the biology of ISCs and introduce the major controversies and questions in the field noting the most important areas that modeling has influenced. Next, we review several compartmental population models in the literature and highlight their strengths, weaknesses and utility in the context of other modeling approaches. Finally, we discuss how compartmental modeling can be used to address some of the key questions that remain in the field of ISC biology.

Intestinal stem cells

There has been much debate over the location and identity of the ISC. Early studies suggested that the ISC was located approximately 4 cell positions from the base of the crypt, commonly referred to as the "+4 cell".[3,12,13] Alternatively, it was proposed that crypt base columnar cells (CBCCs), small undifferentiated cells intercalated between the Paneth cells at the base of the crypt, were the true ISCs.[14,15] The prevailing theory today suggests that there are two stem cell populations in the intestine: an active stem cell (ASC) that is responsible for the bulk of proliferation and crypt maintenance, and a quiescent or reserve stem cell (QSC) that divides more slowly and is important for replenishing ASCs during crypt recovery after injury.[6,16,17] Recent findings, however, have called this two stem cell system into question, and thus a definitive catalog of ISC populations remains an active area of investigation.[7,18–20]

Stem cell markers

Clearly, the way to reconcile the +4/CBCC cell debate was to identify a reliable marker that would allow for visualization, isolation and genetic manipulation of ISCs. The first method that allowed visualization of putative stem cells was retention of a radioactive tritiated thymidine label.[14] These "label retaining cells" (LRCs) localized to the +4 position of the crypt and were thought to be stem cells due to their long-lived nature, although no functional data was obtained to validate this hypothesis.[3]

The development of *Vil1* promoter constructs capable of expression in all intestinal epithelial cells, including stem and progenitor cells, allowed the genetic manipulation of ISCs in transgenic mice for the first time.[21,22] The capability to manipulate ISCs continues to be widely utilized to probe gene function for intestinal development or disease; however, the widespread *Vil1*-promoted transgene expression did not allow specific identification or manipulation of ISCs.

The first more specific molecular markers proposed for ISCs were the RNA-binding protein MSI1[23] and the WIP1 phosphatase.[1] Both were shown to be expressed in the same location as LRCs, however, labeling of both CBCCs and lower TA cells limited the usefulness of these markers.[1,24]

In a landmark paper published in 2007, *Lgr5*, the G-protein coupled receptor for RSPO1, was found to be a specific marker

of CBCCs.[25] The Clevers group functionally demonstrated that LGR5$^+$ cells were stem cells capable of producing all of the mature cell types of the intestine.[25] This conclusion was achieved through lineage tracing, a technique that allows permanent activation of a reporter gene in a cell and all of its progeny and is the gold standard for defining a stem cell *in vivo*.[26] In addition, isolated LGR5$^+$ cells were subsequently shown to produce intestinal enteroids, intestine-like tissue grown in perpetuity *in vitro*, another indication of this cell's stem-like function.[27] Importantly, activation of Wnt signaling in the LGR5$^+$ cell population showed progressive formation of intestinal adenomas, a feature expected of aberrantly regulated stem cells.[2] Thorough quantitative studies have demonstrated that LGR5$^+$ cells are highly proliferative, cycling approximately every 24 hours.[25,28] This rate of proliferation confirms that if the LGR5$^+$ CBCC is not the only stem cell population in the gut, it certainly is doing the bulk of the work, and thus has been indisputably considered the ASC. Notable additional markers subsequently identified for the ASC population include *Ascl2*,[29] *Olfm4*,[30] *Smoc2*,[18] and *Sox9*[5,31] (Fig. 1B) although a multi-scale stem cell signature analysis identified countless others.[18]

The +4 cell as a quiescent stem cell

Although the LGR5$^+$ CBCC had been established as the ASC, a surge of additional studies surfaced that continued to support the idea of a stem cell population that resides approximately in the +4 position. Immunostaining and lineage tracing studies identified a number of putative markers of this population including *Bmi1*,[32,33] *Lrig1*,[34] *mTert*[35] and *Hopx*[36] (Fig. 1B). Additionally, the gene *Dclk1* has been cited numerous times in the literature as a putative +4 stem cell marker;[17,37] however other studies reported that *Dclk1* marks tuft cells or tumor stem-like cells rather than actual stem cells.[38]

Similar to the *Lgr5* studies, the +4 lineage tracing experiments demonstrated that these markers were present in a stem cell population that was able to produce all of the differentiated intestinal cell types. Additionally, Wnt-activated LRIG1$^+$ cells showed even more aggressive adenoma formation than in the comparable LGR5 studies, again suggesting that these cells harbored stem-like function.[34] As opposed to ASCs, however, many of these cells were shown to cycle more slowly, furnishing the idea that these markers were identifying a QSC population.[32,34–36]

It is important to note that Potten's original studies did not suggest that the +4 cell was a quiescent cell population. Rather, it was thought that, like ASCs, this cell cycled approximately once per day and that the property of label retention was due to retention of an "immortal strand" of DNA that protected stem cells from accumulating mutations during DNA replication.[16] This hypothesis is highly controversial and has been challenged by several groups.[39,40] In particular, Escobar *et al.*[39] combined mathematical modeling with careful pulse-chase labeling experiments to show that stem cells randomly sort their chromosomes. These findings further bolster the idea that the label retaining property of the +4 population is due to the cell being a long-lived, slower-cycling stem cell.

One predicted function of a QSC population is to act as a reserve stem cell compartment. This feature was demonstrated in a number of studies that showed activation of QSCs in the post-irradiation injury setting.[5,6,36] Additionally, specific ASC ablation with diphtheria toxin led to activation of BMI1$^+$ cells to generate differentiated intestinal epithelial cells. These QSCs appear to replace LGR5$^+$ cells, thus repopulating the depleted ASC pool.[6,33] Similarly, isolated BMI1$^+$ cells were shown to create enteroids *in vitro* that ultimately contained LGR5$^+$ ASCs.[6] Interestingly, ablation of BMI1$^+$ cells with diphtheria toxin results in complete epithelial collapse, suggesting that, unlike ASCs, these cells are indispensable for epithelial homeostasis.[32]

Together, these findings support a two stem cell paradigm in the gut: the LGR5$^+$ cell is the ASC that divides every day and supports homeostasis under normal conditions and the +4 cell is the QSC that usually divides slowly and only occasionally contributes to homeostasis at baseline. In an injury setting the QSCs are activated and expanded and allow for crypt repopulation and repair of the ASC pool.

Overlapping markers: the QSC dispute

Despite the abundant lineage tracing data that supports the idea that *Bmi1* and other +4 genes mark QSCs, there continues to be doubt that these cells are truly an independent stem cell population. Much of the argument originates from studies that find putative QSC markers to be expressed in ASCs. Sorted ASCs were shown to express high levels of *Bmi1* mRNA[29] and an independent study showed that ASCs expressed the highest levels of *Lrig1* than any other cell in the epithelium.[41] A robust transcriptomic and proteomic approach that aimed to elucidate a definitive stem cell signature for the ASC showed that many QSC markers, including *Bmi1*, *mTert*, *Hopx*, and *Lrig1*, are not only expressed in the ASC, but single molecule transcript counting showed mRNA expression was located throughout the crypt rather than in a localized +4 cell population.[18,19] To make matters more complex, Munoz *et al.*[18] was unable to replicate the lineage tracing data of Capecchi and colleagues[32] which showed that BMI1$^+$ cells were predominately located in the +4 position. Additionally, they observed that lineage tracing from BMI1$^+$ cells occurred with similar kinetics as the ASC lineage tracing, calling into question the quiescent nature of this cell population.[18] Consequently, a molecular marker that uniformly and specifically marks +4 cells remains to be identified.

Neutral drift dynamics and stem cell number

One important aspect of crypt biology that has been extensively modeled is crypt monoclonality, the process by which heterogeneous crypts, presumably fed by many stem cells, become derived from a single stem cell over time.[28,42,43] The Winton and Clevers groups have independently investigated this process by modeling the rate it takes for a lineage trace to encompass an entire crypt.[28,42] These studies conclude that this occurs through neutral competition of stem cell progeny for niche space, or neutral drift.[28,42] This finding has important implications on crypt homeostasis as it implies that stem cells are not permanently tethered to the crypt base, but rather the stem cell niche is constantly being evacuated and refilled at random. If a certain cell garners a mutation that results in a

competitive advantage, however, this mutation can quickly propagate throughout the crypt, which is very important for understanding tumor-forming mutations.

In light of the continued ASC/QSC dispute, there is no agreed upon number of total stem cells in the crypt. Interestingly, even the number of ASCs continues to be debated. Snippert *et al.*[28] calculated the number of stem cells/crypt to be 14 ± 2 cells. This was based on counting the number of GFP-labeled LGR5$^+$ cells intercalated between Paneth cells at the base of the crypt. More recent studies have challenged the idea that mere expression of *Lgr5* defines an ASC. The Winton lab has taken a stem cell marker-independent functional approach to define stem cells and has found that the number of stem cells per crypt is closer to six.[44] Additionally, this method identified that the rate of functional stem cell turnover was 0.2 cells per day as opposed to every 24 hours as previously predicted.[44] With these new data, Kozar *et al.*[44] re-modeled the neutral drift dynamics from the Clevers data set as well as their own experimental data. They found that their new parameters fit the both sets of data better than the previously tested values.[44] This study provided further evidence for neutral drift dynamics in the crypt while also challenging the accepted values for stem cell number and cell cycle rates. This example demonstrates one of the most important strengths of modeling approaches: the ability to test publicly available data sets and possibly draw new conclusions as more information from biological study is discovered and subsequently implemented in the modeling process.

Transit-amplifying cells

Like most adult tissue stem cells, ISCs do not directly form the differentiated cell types of the intestine; rather, they contribute to an intermediate progenitor pool. These cells are referred to as transit- or transiently-amplifying (TA) cells because they divide approximately every 12–18 hours, 4–6 times prior to fully differentiating into the various epithelial lineages, fundamentally amplifying the population in the crypt.[13] As these cells divide it is assumed that they become committed to specific lineages and cell types, finally leading to mitotically-inactive fully mature absorptive or secretory cells as they migrate out of the crypt. The specific timing and nature of these differentiation events and how they might affect TA clonogenicity is largely unknown.

Progenitor cell fate decisions

Early mutagenic marking studies showed that multipotent progenitors exist as well as progenitors committed to a single differentiated cell type.[26] It is well established that a binary decision occurs between absorptive and secretory cell fates, which is largely controlled by the Notch signaling pathway, however it is unclear exactly when and how this occurs.[45] Some studies suggest that this is the first decision of TA cells.[46] Other studies suggest that the specific type of secretory lineage is first determined, but that this differentiation trajectory can be aborted if the cell is later specified to be an absorptive cell.[47] Some studies have identified an intermediate cell with both Paneth and goblet cell features, which might suggest that these cells share a common progenitor,[46,48] although other studies describe a common Paneth/endocrine precursor.[7] Clearly, a definitive lineage fate map in the gut is still forthcoming. Additionally, it is unknown during which round of TA cell division that these decisions take place. A paucity of specific markers or functional assays for different TA progenitors cells has been a stumbling block for progress on these fronts. Some markers like *Msi1*[23] and *Prom1*[49,50] have been proposed, but these label both stem and progenitor cells, and it is uncertain whether they differentially label TA subpopulations.

TA cells are facultative stem cells

Studies by Potten[13,60] indicate that TA cells possess potential stem cell capabilities in the event that the actual stem cells are lost or damaged. Irradiation studies suggest that the first two rounds of TA cell division possess some regenerative capacity.[13] Later TA divisions, however, were shown to have lost this capability, suggesting that this property is either cell age- or crypt location-dependent.[13]

Recently, a cell expressing the Notch ligand *Dll1* was identified as a multi-potent progenitor cell that was definitively not a stem cell, as evidenced by its lack of robust lineage tracing and inability to form enteroids *in vitro*.[20] Interestingly, this cell population was shown to gain stem-like function by Wnt stimulation *in vitro* and crypt damage *in vivo*.[20] This study further supports the idea that early TA progenitors possess plasticity and can act as potential stem cells. Interestingly, other studies have shown a subpopulation of entero-endocrine cells in the crypt that co-express stem cell markers and seem to function as stem cells *in vitro* and *in vivo*.[5,51,52] This raises the possibility that committed TA cells or even fully differentiated cells may possess stem-like potential.

Recently, Winton and colleagues[7] returned to the approach of label retention to isolate and manipulate QSCs. In this study, LRCs were defined as non-Paneth cells that retained a YFP label for 10+ days.[7] Isolation of these cells by fluorescence-activated cell sorting followed by transcriptome profiling showed that these LRCs were a distinct subpopulation of LGR5$^+$ cells that expressed both secretory cell and stem cell markers. Using a clever split Cre construct and dimerization agent, Buczacki *et al.*[7] was able to lineage trace from LRCs and found that these cells contributed exclusively to differentiated Paneth and endocrine cell populations, a property consistent with a secretory cell progenitor. Interestingly, with ASC injury the LRCs gained full clonogenic capacity and were shown to lineage trace into all differentiated cell populations.[7] This study supports the idea that there is not a dedicated population of QSCs, but rather a population of semi-differentiated progenitor cells that can act as a reserve stem cell population in the event of ASC loss.

The stem cell niche

Many believe that stem cell identity is not cell-intrinsic, but rather a consequence of the local signaling environment, or niche, such that any cell within the niche will have stem-like properties (Fig. 2). Several signaling pathways are known to be

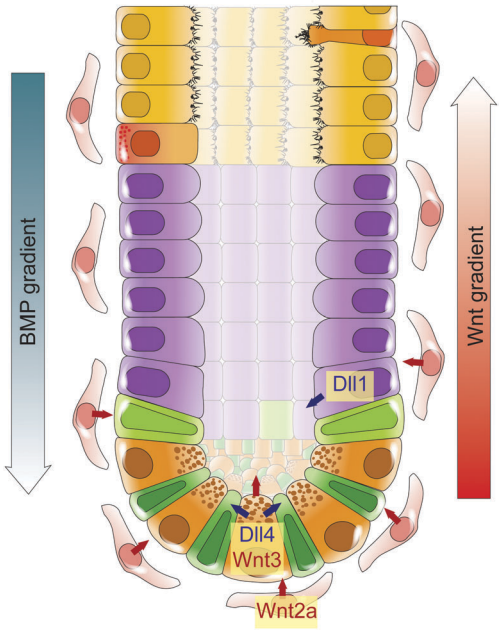

Fig. 2 The stem cell niche is defined by several molecular signals. Activation of the Bone Morphogenetic Pathway (BMP) occurs as a gradient that is higher in the villi and lower in the crypts. Conversely, Wnt activity is highest in the crypts. The Wnt gradient is established by secretion of Wnt ligands both from the mesenchymal myofibroblasts (WNT2a) as well as from epithelial cells. WNT3, in particular, is expressed in Paneth cells. The Notch signaling pathway is also critical for niche specification. Notch ligand presentation must occur from adjacent cells, and there is evidence that Paneth cells present DLL4, and that a subset of secretory progenitors express DLL1. It is unclear if other TA cell populations can present Notch ligand to stem cells.

important for intestinal epithelial homeostasis, and many of these have been implicated in forming and sustaining the stem cell niche.

Wnt signaling is important for stem cell establishment in the developing intestine, as well as crypt development during the postnatal period.[53] In the adult intestine, Wnt responsive cells are stimulated by soluble ligands that are released from both the surrounding mesenchymal cells as well as crypt epithelial cells, leading to a Wnt activity gradient from crypt to villus (Fig. 2).[35,53] The Wnt signal is required for stem and TA progenitor cell proliferation and has been implicated in regulating aspects of cell differentiation, likely through cross-talk with the Notch signaling pathway.[53] Wnt signaling was shown to be required for ASCs, but $BMI1^+$ QSCs appear to be insensitive to Wnt.[6] Of note, aberrant Wnt signaling is observed in almost all cases of colorectal and intestinal cancers.[54] Interestingly, a computational model of the crypt by Pin *et al.*[55] defines QSCs as the same population as ASCs, but located higher up the crypt in a different Wnt gradient. While this is an interesting hypothesis, current data, summarized above, cannot determine if QSCs are an independent population, a subset of $LGR5^+$ cells, or progenitor cells. Additionally, recent findings suggest that $BMI1^+$ QSCs are not responsive to Wnt signaling,[6] which would suggest that there are alternate niche pathways that define and regulate this cell population.

The Notch signaling pathway plays a critical role in controlling lineage specification of differentiated cells in the intestinal epithelium; *i.e.* active Notch signaling leads to the formation of the absorptive lineage while absence of Notch results in secretory cell types.[45] Notch regulates intestinal proliferation, as blocking Notch obliterates proliferation[56,57] and Notch activation has been shown to increase proliferating cell number.[58,59] In addition, Notch was recently shown to be essential for maintenance of ASC number and function.[46] Together these studies suggest Notch may be distinctly required for ASC maintenance and for TA cell fate.

Other signaling pathways shown to be involved in intestinal homeostasis and development include Bone Morphogenetic Protein (BMP), Hedgehog, Hippo, Eph/Ephrin, and EGF/ErbB. Many of these pathways play important roles in stem cell function and are likely contributing to the niche. These pathways have been reviewed elsewhere.[53,60]

It is believed that many of these essential signals are coming from the myofibroblasts in the mesenchyme underlying the epithelial basement membrane.[61] Recent studies, however, have challenged this mesenchyme-centric hypothesis. Evidence in a number of different tissues supports a model where stem cell progeny may also play an important role in defining the stem cell niche.[62]

The Paneth cell as the niche?

Paneth cells secrete antimicrobial peptides and are thought to have a role in regulating host–microbial interactions.[63] Unlike other differentiated cells, which migrate up the villi and are sloughed off the tip on the order of 3–5 days, Paneth cells migrate down to the base of the crypt, where they persist for approximately 3 weeks.[64] Modeling approaches have been used to understand the cell–cell adhesion, flow, and migration within the crypt,[65,66] which is important for understanding how stem cell progeny migrate out of the crypts as well as how Paneth cells travel to the base. In this position Paneth cells are in close association with CBCCs and thus have recently been implicated in specifying the stem cell niche. Over 80% of the CBCC surface area is in contact with neighboring Paneth cells.[67] As some niche signals, like Notch pathway components, are dependent on cell–cell interaction, the Paneth cell is the ideal candidate for ligand presentation. Indeed, expression-profiling studies suggest that Paneth cells express Notch, Wnt, and EGF ligands.[67] Additionally, the formation of epithelial-only enteroids supports the idea that mesenchymal signals may not be essential for niche formation.[27] In fact, $LGR5^+$ cell/Paneth cell doublets increased enteroid formation efficiency over 10-fold higher than $LGR5^+$ cells alone.[67]

Opponents of this theory cite that these *in vitro* culturing techniques rely on a large number of growth factors for successful enteroid formation, including a BMP antagonist, Notch ligand, EGF, WNT3a and the Wnt potentiator RSPO1, as well as a synthetic basement membrane-like matrix; all factors that could be provided by the mesenchyme or other adjacent epithelial cells *in vivo*.[27] Additionally, several studies have shown that genetic deletion of Paneth cells does not have

deleterious effects on the intestine.[68–70] Furthermore, colonic stem cells appear to function similarly to small intestinal ASCs but the colon does not contain Paneth cells, although a recent study by Rothenberg *et al.*[71] identified cells that may function like Paneth cells to support stem cells in the colon.

A recent study by the Clevers group[72] indicated that mesenchymal Wnt signals may in fact play an essential role. The study showed that the Wnt ligand secreted from the Paneth cell, WNT3, is essential for *in vitro* enteroid growth but deletion of *Wnt3 in vivo* does not affect homeostasis.[72] They identified WNT2B as a mesenchymal Wnt signal that could compensate for the lost WNT3 signal.[72] These results may explain why genetic models that lack Paneth cells may form a normal stem cell compartment. It is therefore very likely that a combination of epithelial and mesenchymal signals determine the stem cell niche.

Lingering questions

Although the body of knowledge of intestinal stem cells, the crypt proliferative compartment, and the stem cell niche has been building for decades, there is still controversy regarding the exact cells that are present and how they are regulated. Key questions that need to be addressed include: (1) How is stem cell number regulated and are all stem cells equal? (2) Are ISCs completely defined by their niche or is there some level of intrinsic stemness? If so, how is regional identity maintained by stem cells? (3) Is there a dedicated QSC population? (4) What is the nature of the TA cell compartment? Are the number of rounds of TA divisions limited by cell-intrinsic properties? If TA cells can de-differentiate and become clonogenic when stem cells are lost, what regulates this transition? (Fig. 3).

Elucidating crypt regulatory mechanisms with compartmental modeling

As discussed above, intestinal modeling approaches have aided in a number of important discoveries, ranging from exploring normal physiology to modeling tumorigenesis and improving clinical care. A recent trend in these models is to try to incorporate everything that is known about the crypt, including crypt geometry, migration, stem cell division, niche signals, differentiation, and other factors into one comprehensive model.[55,73] While these models have been able to seemingly replicate many

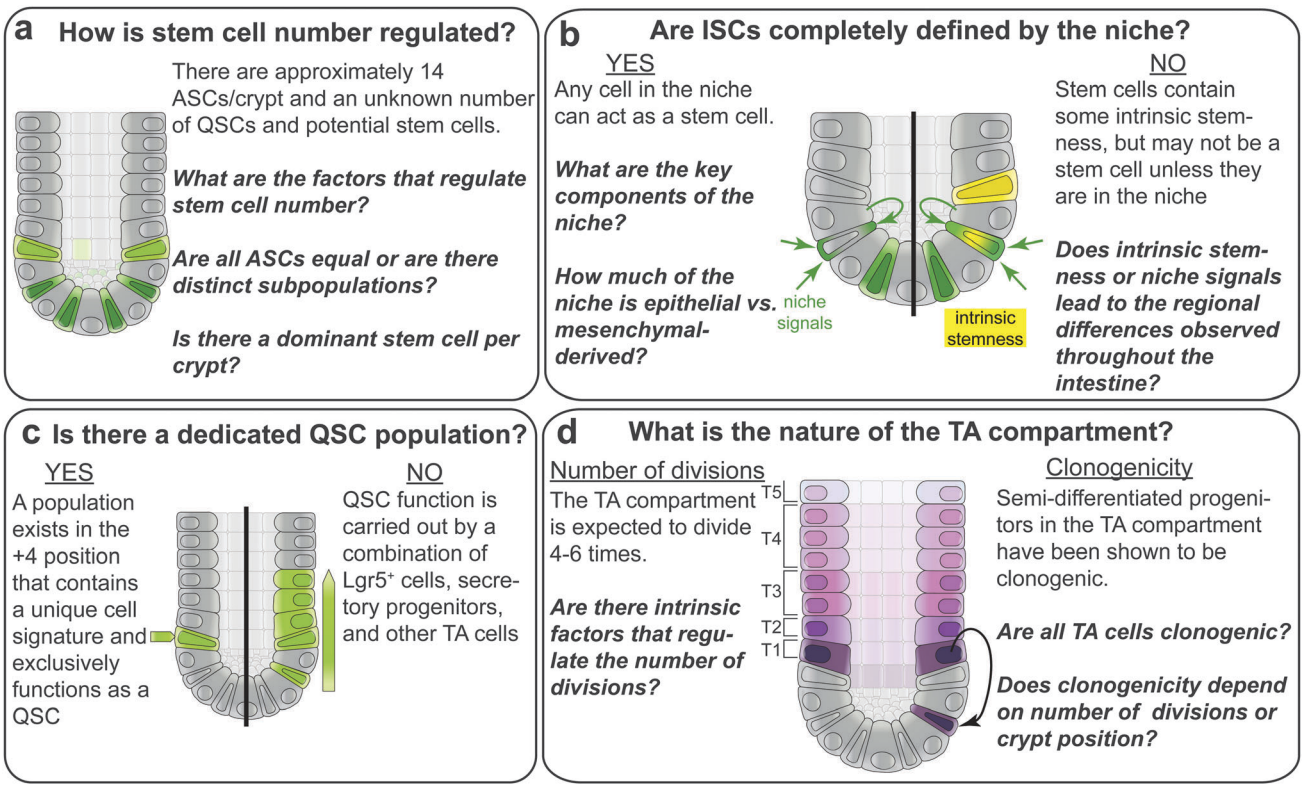

Fig. 3 Key questions in the field of intestinal stem cell biology. (a) Schematic illustration of the base of an intestinal crypt with stem cells designated in green. The mechanisms regulating stem cell number are not known. (b) An illustration of two opposing theories regarding the role of the stem cell niche. Left: the niche (green arrows) completely specifies the stem cell. Right: the niche partially specifies a cell that possesses certain features of intrinsic stemness (yellow). Only cells that acquire both extrinsic and intrinsic signals become stem cells. (c) Diagram of two possible QSC populations. Left: a single QSC that possesses a unique molecular signature (horizontal arrow) is shown. Right: QSC markers are expressed in a gradient in the crypt (vertical arrow) and any cell in this zone possesses the potential to act like a QSC. (d) Schematic of the TA cell compartment. Left: several rounds of cell divisions are shown (T1–T5). The exact number and regulation of TA cell divisions is not known. Right: a TA cell is shown to de-differentiate and replace a lost CBCC (curved arrow). Exactly which TA cells possess clonogenicity is unknown.

experimental outcomes, these efforts must be interpreted with caution, as these models are created to fill a set of known outcomes and are often filled with assumptions that cannot be validated experimentally. Models of this nature typically have rule-based algorithms that depend on cell location and identity to determine cell behavior. With all of the ambiguity surrounding the existence and function of QSCs, TA cell plasticity and required niche signals, it is premature to develop these types of comprehensive models of the crypt.

An alternate approach would be to tackle individual questions to limit the amount of assumptions in the model. Compartmental models, which look at different cell lineages in the intestine as separate independent compartments, are ideally suited to answer many of the questions that dominate the ISC field. Depending on the specific question being asked, these compartments can be made as nuanced or broad as desired. For instance, we can consider a model with a stem cell compartment that is inclusive of all types of stem cells, or we can define our compartments more specifically and designate ASC, QSC, and facultative TA cells as separate stem cell compartments. The advantage of this approach is that models can be developed to what is known at present and updated as more definitive information about stem cell populations becomes known. The benefit of working with such a model is that the simple design allows for addressing very specific questions.

For this review we will discuss compartmental models for homeostasis and tumorigenesis by Johnston *et al.*,[74] crypt recovery post-irradiation by Paulus *et al.*,[75] and a model for crypt development by Itzkovitz *et al.*[76] We present here the background, main findings, and impact of each model to inform our understanding of ISCs.

Models of homeostasis and tumorigenesis

The level of cellular proliferation and turnover in the intestine is quite remarkable. In humans, an estimated 10^{11} cells are shed and replaced every day.[77] Colon cancer remains the third most prevalent and third most deadly cancer,[78] thus, appreciating how normal proliferation is kept in check is essential for understanding when these processes go awry and lead to tumor formation. Early theories proposed that tumor initiation could be mediated by mutations that led to increased cellular proliferation of immortal stem cells.[79] Fearon and Vogelstein[80] contextualized these mutations with their genetic model for tumor initiation in the colon, characterized as a systematic acquisition of mutations: both activation of oncogenes and loss of tumor suppressors. More recently, modeling of colorectal tumors showed that as tumors grow they become more heterogeneous as new mutations are acquired.[81,82] This heterogeneity implies that more than one treatment approach is needed to eradicate the tumor. Modeling has also been used to directly determine how therapies should be applied. For example, a model of colon cancer carcinogenesis and tumor response to irradiation has been developed to better tune dosing of radiation therapy.[83]

In 1995, Tomlinson and Bodmer[84] probed the mechanisms through which mutations act to incite tumor initiation with their computational model of crypt homeostasis and tumorigenesis. This simple model divided the crypt into 3 compartments: stem cells, semi-differentiated cells, and fully-differentiated cells, with cell populations determined by the rates of death, differentiation, renewal, and removal (Fig. 4). The model was simplified to assume that all cell divisions occurred synchronously and updated at each subsequent generation. This study[84] explored normal cell division as well as the resulting effect on cellular homeostasis when changing the rates of cells undergoing death or differentiation in each compartment. The findings were striking: under normal conditions, this model found that there are very stringent parameters that must be met in order for steady-state to be reached; small perturbations in rates of death, differentiation, or renewal led to exponential growth or decay. Importantly, the model[84] suggested that alterations in stem cell number that lead to tumorigenesis might be

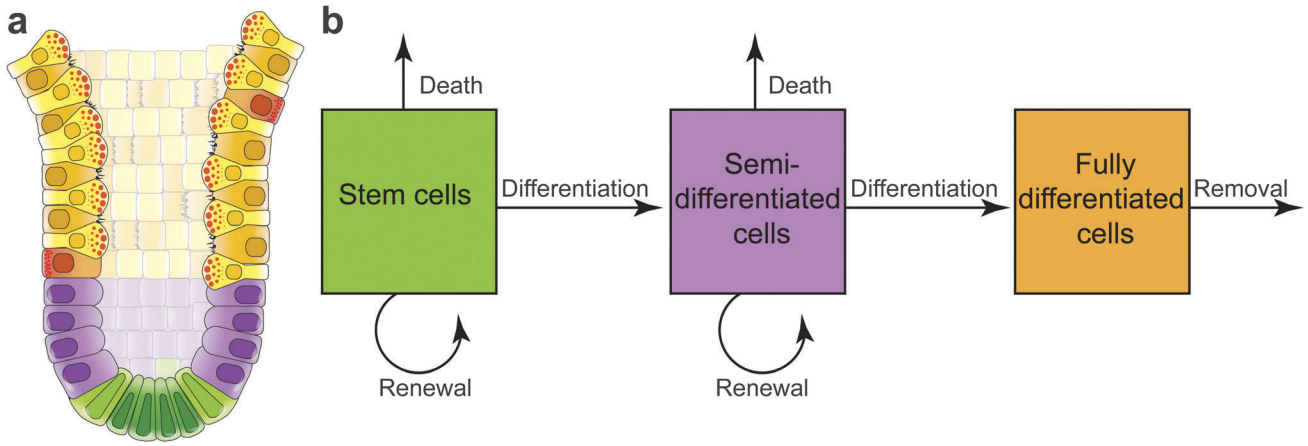

Fig. 4 Compartmental model of homeostasis and tumorigenesis. (a) An illustration of the colonic crypt as modeled by Johnston *et al.*[74] Unlike the small intestine the colon does not have villi nor traditional Paneth cells. (b) Compartmental model of homeostasis and tumorigenesis adapted from Fig. 1 of Johnston *et al.*[74] Cell populations include stem cells, semi-differentiated cells and fully differentiated cells. Cell flows into and out of the compartments are indicated by arrows and are defined by rates of death, differentiation, and renewal from the stem and semi-differentiated compartments. There is no renewal in the fully differentiated compartment and cells leave by removal.

through mechanisms other than simply increased stem cell proliferation rate, highlighting that it is not necessarily the mechanism of a tumorigenic mutation that is of key importance, but the crypt compartment that is affected.

Refining the model

Several models have been adapted from the general framework of the Tomlinson and Bodmer[84] study. In particular, Johnston and colleagues[74] aimed to improve the model by eliminating synchronous division as a simplification to more closely match crypt physiology. To do this they created two different revisions of the model: an "age-structured model" using partial differential equations that takes into account asynchronous cell divisions and a "continuous model" using ordinary differential equations (ODEs) that looks at the average cell population over time. In the age-structured model they explored the effect of cells in each compartment being in different stages of the cell cycle prior to undergoing renewal, differentiation, or death at certain time points. The resultant population of semi-differentiated cells from a crypt that started with all cells at the same point of the cycle was compared to one that started with an evenly distributed age profile. Since this resulted in similar populations, they concluded that it was unnecessary to specifically follow each cell's age, and validated the use of the continuous model to study this system.

Johnston *et al.*,[74] like the Tomlinson and Bodmer model,[84] found that both the age-structured and continuous models were "structurally unstable," that is they reach stable steady-state populations only at very precise parameter values. Any deviation from these values results in exponential growth or decay of the crypt. In the intestine, unbounded growth would be equivalent to tumorigenesis and decay would result in eventual crypt loss. Due to this complication, Johnston *et al.*[74] sought to test feedback mechanisms to model the steady-state that occurs in the actual crypt during homeostasis. Two alternative feedback models were tested, "linear feedback" and "saturating feedback". In the linear feedback model, logistic growth of the stem cell population was implemented leading to a limited population size. In this case, tuning the parameters below a certain point resulted in exponential decay, but unlike the model without feedback, no set of parameters resulted in exponential growth. Effectively, the linear feedback model creates a crypt that is incapable of initiating tumors no matter how many mutations are accumulated that change cell renewal and differentiation rates, unless the mutation compromised the feedback mechanism.

In the saturating feedback model,[74] rather than limiting total population size, feedback was incorporated to only limit the rate of differentiation. With this feedback, three states of stem cell population growth were possible: crypt extinction, homeostasis, and exponential growth. Thus, the saturating feedback model establishes a simple model to explore the initiation and growth kinetics in tumorigenesis associated with multiple mutation acquisition. Alterations in the rate of renewal, differentiation, and death due to genetic mutations would change the governing rate parameters, leading to altered steady-state populations.

Impact of Johnston *et al.* model on cancer research

Several studies have confirmed that the Johnston *et al.*[74] model predicts experimental findings in tumorigenesis.[85,86] Additionally, there have been adaptations of the model for colon cancer and other systems. For example, one study maintained the general framework of the model but included telomere length as a parameter that was regulated by location in the stem cell niche.[87] In another study,[88] the Johnston *et al.*[74] scaffold was used to build a model for hematopoiesis and treatment of Chronic Myeloid Leukemia (CML). This study[88] tested synchronized discrete, age-structured, and continuous models with feedback mechanisms to determine that modulating growth factor signaling through the use of tyrosine kinase inhibitors should be able to cure CML by regulating CML progenitor cell populations.

A crypt post-irradiation recovery model

The intestine is the earliest hit and most severely damaged tissue in the irradiation setting, and thus understanding both early and late injury responses has been a key interest in the field.[89] Additionally, acute irradiation damage has been used as a mechanism to study pathways involved in intestinal recovery, and, as mentioned above, has been a key tool in studying activation of QSCs. The acute irradiation response can be distilled into two stages. Initially, there is crypt apoptosis, mitotic arrest, and a decrease in both crypt and villus cell numbers.[90] Next, there is a robust rebound "overshoot" in population before homeostasis is re-established.[90] Paulus *et al.*[75] aimed to create a model that would faithfully replicate the post-irradiation recovery to test their hypothesis that the damage control response resided solely in the stem cell compartment. They engineered a compartmental model of the crypt to map the effect of the post-irradiation response on stem and TA cell populations (Fig. 5).

Prior to the publication of Paulus *et al.*,[75] several models had been designed to describe the intestinal response to irradiation. Many of these attempts were fueled by the observation that irradiation injury leads to shortened villi prior to crypt expansion and proliferative cell surge.[90] This was first investigated in the compartmental model by Sato *et al.*,[91] which posited that irradiation-induced changes to cell number and proliferation were generated from a feedback mechanism where villus damage sends signals to the crypts to regenerate. One complication with this hypothesis was the observation of subtle changes in the crypt prior to the onset of villar atrophy, suggesting that not all of the effects originated from a villus feedback mechanism.[92]

Paulus *et al.*[75] challenged the idea that the irradiation recovery response had any aspect of villus-to-crypt feedback. Rather, they hypothesized that all cellular consequences could be traced back to changes in the stem cell compartment. Their study tested whether a stem cell-centric response could replicate the experimental findings in cell number, labeling index (incorporation of a tritiated thymidine label), and mitotic index in the post-irradiation recovery. To begin their model, they drew from a comprehensive data set that included 35 time

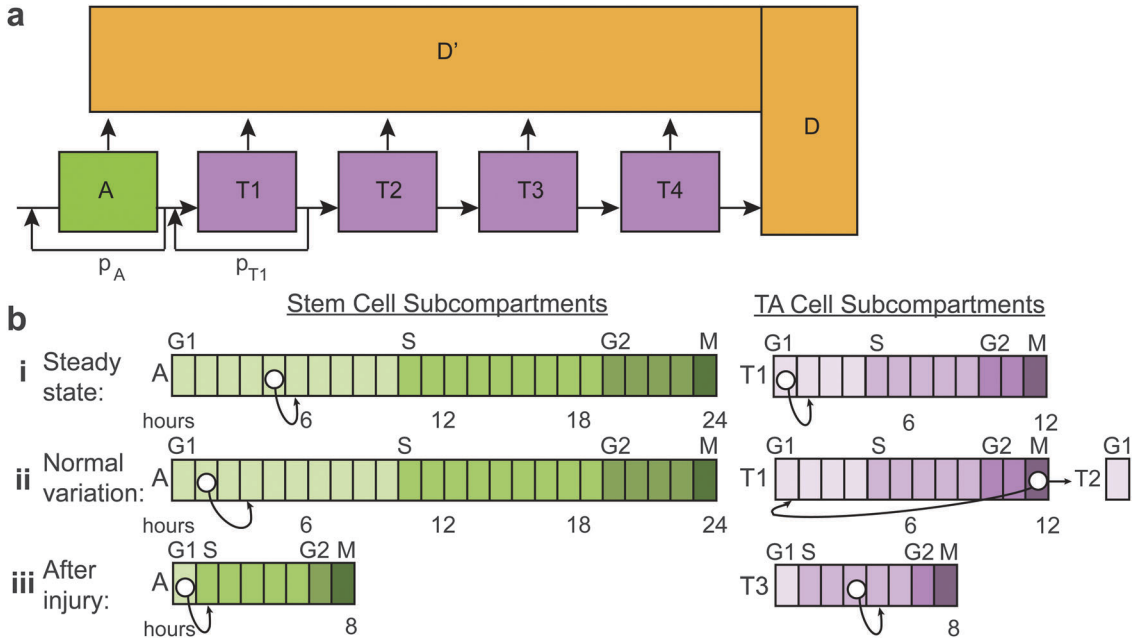

Fig. 5 Compartmental model of irradiation recovery. (a) Diagram of the cell population compartments of the crypt post-irradiation model adapted from Fig. 2 of Paulus *et al.*[75] Cell populations include stem cells (A), TA cells (T1–T4), differentiated cells (D), and previously proliferative cells that stopped cycling due to irradiation injury (D′). Cells move from one compartment to the next after completing the cell cycle. Cells in A and T1 can re-enter their compartment with the probability p_A and p_{T1}, respectively. (b) Diagram of different cell cycle subcompartments are shown. (i) Subcompartments during steady state when the cell cycle time is 24 hours for A and 12 hours for T compartments. Cells (white circles) advance to the next subcompartments every hour of the simulation. For clarity we have included G1, S, G2 and M phases of the cell cycle, but the lengths of G2 and M that were used during the Paulus *et al.*[75] simulation was not made clear in the manuscript. (ii) Normal stochasticity in the model. Cell cycle time was allowed to vary slightly for each individual cell. This variation was limited to the G1 compartment and was achieved by skipping a subcompartment. Renewal in the A and T1 compartment was accomplished by re-entering the first G1 subcompartment after completing M phase. (iii) Alteration in subcompartments after maximal irradiation injury, where cell cycle lengths are decreased to 8 hours.

points after various irradiation doses from 2.5–12Gy. In these experiments, mice were administered tritiated thymidine 40 minutes prior to sacrifice, and ileal crypts were scored for labeling index and mitotic activity on histological sections.

Paulus *et al.*[75] used an agent-based approach, a computational model where cells are modeled as autonomous decision-making entities called agents that behave according to a set of rules defined from experimental observations of the phenomenon under investigation. Unlike many agent-based models that align cells to a geometrical lattice and study spatio-temporal dynamics of the system, this model situated the cells into one of six population compartments: stem cells (A), four TA cell compartments (T1–T4) and differentiated cells (D) (Fig. 5A). Each compartment contained sub-compartments allocated to specific portions of the cell cycle (Fig. 5B). Movement of cells from one compartment to the next was based on two regulated processes during the simulation: cell cycle time and self-maintenance probability; all other parameters were held constant. During the simulations, cell cycle time was very highly regulated based on experimental findings: 24 hours for stem cells and 12 hours for TA cells during steady-state; but after irradiation injury, cell cycle for both populations was shortened, with a minimum cell cycle time of 8 hours. Additionally, the number of stem cells in the crypt influenced stem cell cycle time, while TA cells were not regulated in this fashion. The model also embraced the idea of TA cells as potential stem cells, although it is assumed that only T1 cells have self-renewal capabilities. Therefore, after completing the cell cycle, cells in the A and T1 compartments either moved to the next compartment or re-entered the same compartment with the probabilities p_A and p_{T1}.

Testing the model

Paulus *et al.*[75] used data from administration of 8Gy irradiation to fit their parameters: cell death, ''irreversible proliferative inhibition'' *i.e.* removal of previously proliferative cells to a non-proliferative compartment (D′) (Fig. 5A), mitotic delay, cell numbers, cell cycle times, and villus transit time. With these parameters, they were able to replicate the observed labeling index and cell numbers, including the expected overshoot in population, simply by regulating cell cycle time and self-maintenance probability. To validate their model, they changed the initial values to match the observed cell numbers after 2.5Gy and 12Gy irradiation, and again were able to replicate the labeling index and overshoot populations observed after these levels of irradiation damage.

Impact of crypt irradiation recovery model

The Paulus *et al.*[75] model served its goal to debunk the idea that the irradiation-response is a villus feedback mechanism.

More recent studies have made it clear that the acute post-irradiation response is a crypt-centric process fueled by stem cell proliferation.[5,32] Interestingly, although some stem cells undergo apoptosis after irradiation, a recent study showed that surviving ASCs possess radioresistance by activating DNA-damage repair processes.[4] Additionally, a recent study investigating lineage tracing of SOX9-EGFP during post-irradiation showed a marked increase in SOX9-EGFP low cells (which are thought to be CBCCs), but also found increased numbers of SOX9-EGFP high cells (which are thought to be differentiated enteroendocrine cells.[5]) This finding suggests that more mature cells can de-differentiate to replace lost stem cells, not just the T1 compartment as proposed by Paulus *et al.*[75]

Stem cell expansion during development

In the adult intestine, stem cell divisions must result, on average, in one stem cell and one TA cell in order to maintain homeostasis. This is usually termed asymmetric stem cell division, since the two daughter cells are of different lineages. Asymmetric division is one of the defining characteristics of stem cells as it allows for self-renewal.[93,94] However, stem cells must also possess the ability to divide symmetrically to increase numbers in development and after injury.[95] This property is especially crucial as the intestine grows in length and develops crypts during postnatal development.[96]

There have been a number of mathematical models probing the question of stem cell symmetry in adult tissues. Clayton *et al.*[97] devised the first model of this kind for the mammalian epidermis, demonstrating that stem cells had flexibility in cell division symmetry. Their probabilistic model of clone labeling concluded that adult skin stem cells were undergoing asymmetric division 84% of the time and symmetric division 16% of the time.[97] In the intestine, mathematical models of stem cell symmetry have come to slightly different conclusions. As mentioned above, neutral drift studies have suggested that stem cell division results in two equipotential daughter cells, which compete for spots in the niche. Essentially this means that stem cell divisions never truly occur asymmetrically, rather that population asymmetry occurs *via* stochastic availability of niche positions.[28,42] While these studies call into question asymmetric division, they do indicate precedence for symmetric division in the intestine, the mechanism assumed to be essential for stem cell expansion.

Itzkovitz *et al.*[76] aimed to answer the question of exactly how shifts between asymmetric and symmetric stem cell division can create a mature crypt in the developing intestine in the optimal, or shortest, amount of time. The main question the group focused on was if there were multiple types of cell division occurring simultaneously or if all cells completed similar types of division during the same window of time. This compartmental model defined two cell populations: stem cells and non-stem cells. Differentiated cell populations were not directly addressed, however non-stem cell extrusion was included as a possible outcome of cell division. The system is defined by a set of stochastic ODEs where the state variables are the population of stem cells and non-stem cells. The parameters include rates of stem cell and non-stem cell division, as well as extrusion from the non-stem cell compartment. The number of cells are governed by the probabilities that each compartment will undergo symmetric or asymmetric cell division.

Since more than one type of symmetric stem cell division is addressed in the model, a shorthand nomenclature is used for this discussion: symmetric (S) or asymmetric (A), with a number indicating stem cell (1) or non-stem cell (2) progeny (Fig. 6). The authors[76] started with the assumption that development of mature intestine occurred with a certain probability of S1 and A stem cell divisions, but no S2 division. They next utilized optimal control theory[98] to determine the probabilities of each of these division events to take the initial population of cells at birth to the population of cells in the mature crypt in the least time possible. As the immature gut contains only a short supply of differentiated cells at birth,[96,99] the authors rationalized that time was the driving force for creating a mature crypt.

By solving for minimal time, they found that all stem cells would always divide the same way at the same time, either S1 or A, never mixed.[76] With this criteria, there are two options for behavior, (1) cells will always divide the same way with no transition to another type of symmetry or (2) cells can switch which type of symmetry they undergo one or multiple times until maturation is achieved. The authors found that in order to reach mature crypts in the minimal amount of time symmetry would need to switch once and only once during development. Thus if the stem cells started with S1 division they would all switch to A division and continue dividing asymmetrically until the mature crypt was established. Alternatively, stem cells could begin with A division and switch to S1. This type of control mechanism containing a single on/off switch in behavior is referred to as ''bang–bang control''.[100,101]

Next, the authors[76] embraced the idea that stem cells could divide symmetrically into two non-stem cells (S2 division) (Fig. 6). Strikingly, they found that if S1 divisions occurred for the duration of crypt development, with the last round of divisions switching to S2, they could also achieve mature crypt populations, without any A divisions. Importantly, this approach led to overshooting the mature crypt stem cell population, before attaining normal levels. Interestingly, this ''overshoot'' model resulted in mature crypt formation in less time than with bang–bang control (Fig. 6).

Validating the model

Itzkovitz *et al.*[76] then investigated *in vivo* their ''bang–bang'' *vs.* ''overshoot'' control findings. They used *Lgr5 in situ* hybridization to visualize stem cells and performed a kinetic analysis to measure proliferation rates. First, they found that the proliferation rate of stem cells and non-stem cells was maximal during crypt development, which was in accordance with their prediction for attaining mature crypts in minimal time. In fact, they measured stem cell cycle time (15.7 hours) to be essentially the same as TA cell time (16.9 hours), a marked decrease from the

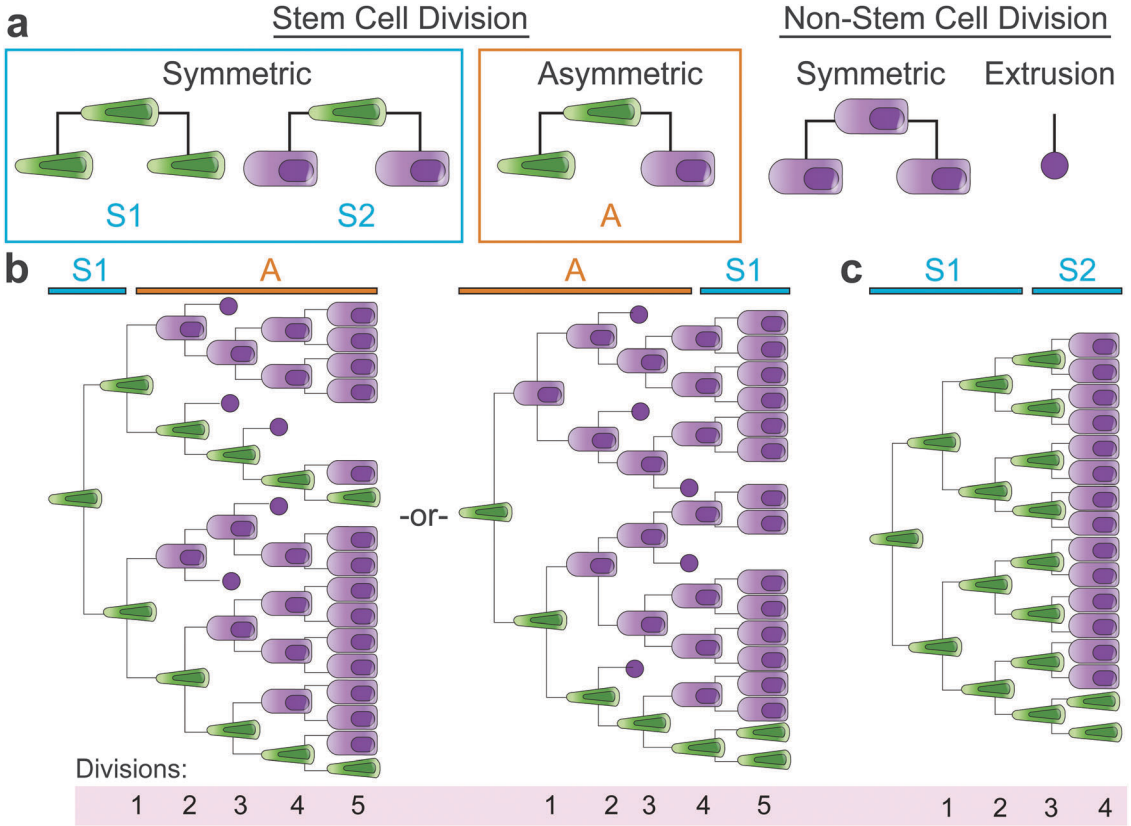

Fig. 6 Compartmental model of crypt development. This figure has been adapted from Fig. 3 of Itzkovitz *et al.*[76] (a) Definitions of types of stem and non-stem cell divisions. Stem cells can undergo two types of symmetric division, S1 and S2, or asymmetric division, A. Non-stem cells always divide symmetrically or are extruded from the crypt. (b) Depiction of the two types of "bang–bang" model outcomes. The rounds of division have been limited to 5 for clarity. The left cell lineage tree shows bang–bang division that shows a switch from S1 stem cell division to A division. The right lineage tree shows A division preceding S1 division. (c) Depiction of the "overshoot" model where stem cells undergo S1 division followed by S2 division. The final cell composition is the same as the bang–bang models, but it only takes 4 rounds of divisions instead of 5 to achieve this.

normal adult stem cell cycle time (22.4 hours). They were able to feed these proliferation rates back into their model to determine that the type of "bang–bang" control that would be favored is S1 division followed by A division. Additionally, their *Lgr5 in situ* data showed that developing crypts were initially filled, almost exclusively, with stem cells, and only later contained non-stem cell progeny, nicely corroborating this prediction. Importantly, they did observe *Lgr5*$^{-}$ non-stem cell progeny prior to the last round of division, which surprisingly favored the less efficient "bang–bang" model over the "overshoot" model. Finally, they performed lineage tracing studies that showed that they never observed S2 division, suggesting that the "overshoot" mechanism did not occur. Interestingly, this is in direct contrast to the findings in adult intestines, where S2 division is predicted to frequently occur.[28,42] This inconsistency could point to a difference in the regulation of stem cell symmetry specifically during development or could call these earlier results into question.

The Itzkovitz *et al.*[76] model exemplifies the importance of closely linking mathematical modeling efforts with *in vivo* validation. Had they only relied on their modeling data they may have assumed that the mechanism of crypt development was the "overshoot" model since it reached maturity in less time than the "bang–bang" control model.

Impact of Itzkovitz *et al.* model

Although only recently published, the work by Itzkovitz *et al.*[76] has spurred investigation into the discrepancy it introduced regarding the absence of S2 division in the developing intestine. Hu *et al.*[102] reported both *in vivo* and modeling data suggesting that stem cell symmetry shifts from strict asymmetry (*via* A division) to population symmetry (*via* stochastic S1 and S2 division) with intestinal maturation. This report suggests that Itzkovitz *et al.*[76] and the adult modeling studies by Lopez-Garcia *et al.*[42] and Snippert *et al.*[28] could be correct and that stem cell symmetry is dependent on tissue age.

A comparative look at crypt compartmental models

While the theoretical models discussed above share the feature of analyzing the intestinal epithelia as compartmental populations, the compartments that they utilize and the mathematical/computational approach that they employ are very different: Johnston *et al.*[74] applied ODEs and partial differential equations, Paulus *et al.*[75] embraced an agent-based model, and Itzkovitz *et al.*[76] used a stochastic ODE system. These differences

emphasize the versatility of the compartment modeling approach; many different types of questions can be addressed simply by restructuring the compartments and altering the theoretical framework. Since each approach is best suited to a specific type of system, it is wise to carefully consider which method will best answer the anticipated questions. There are strengths and weaknesses for each of these approaches.

Johnston *et al.* model

The Johnston model[74] comes to the conclusion that an ODE-derived "continuous" model is the simplest and most appropriate model of crypt homeostasis, and that changes in the rate parameters can be modulated to model both homeostasis and the process of tumorigenesis (Fig. 4). Since ODE models look at averages of cells over time, they assume that the cells in the system are uniform and that cell number is very large. Additionally, neither ODE nor partial differential equation models can resolve changes in small cell numbers that occur rapidly or transiently. While the number of cells in the intestinal crypt range in the several hundred, the number of stem cells is estimated to be 6 to 14 per crypt.[28,44] This population number is too small to be well-described with a deterministic ODE model. When there are very small numbers like this, small random variations in stem cell number due to asymmetric *vs.* symmetric stem cell division can only be accurately captured with stochastic models. This does not invalidate the model, but does limit the questions that the model can answer. For example, this model would be inappropriate to probe post-irradiation recovery where the changes in stem cell number change very quickly.

Another weakness is that this model treats the TA, or semi-differentiated cell population, essentially as a stem cell population, which does not reflect crypt physiology. In order to appropriately capture the limited cell divisions in the TA compartment this model would need to use discrete equations or an agent-based system.

Paulus *et al.* model

The Paulus *et al.*[75] model of crypt post-irradiation recovery takes a unique approach. They treat cells as individual agents, but distribute the cells into compartments and follow each one through sub-compartments that reflect cell cycle time. One of the strengths of an agent-based approach is that all cells are accounted individually so the model allows for small cell numbers and rapid changes in cell populations. One of the disadvantages of agent-based models is that they have arbitrary physical units, which need to be explicitly defined *a priori* to interpret the simulation results with the experimental findings.[103] Unlike most agent-based models, however, the Paulus *et al.*[75] model is free from a geometrical lattice and cells are not influenced by the behavior of neighbors. Although individual cell interactions cannot be resolved in a model like this, population-level behavior can be inferred. For instance, the probability that a T1 cell can re-enter the T1 compartment is dependent on the population of stem cells in the A compartment. This approach could be used to ask questions about homeostasis or tumorigenesis, and would be informative to probe the interaction between different stem cell populations.

Itzkovitz *et al.* model

The Itzkovitz *et al.* model[76] used a stochastic ODE system to model the probabilities of symmetric or asymmetric stem cell division during the process of crypt development. One of the assumptions this approach makes is that the mechanisms controlling crypt development are optimized to take the shortest amount of time biologically possible. This is a stringent feature that leads to the conclusion that stem cell divisions occur *via* "bang–bang" control. If time were not the determining factor, the optimal control theory would be the wrong approach to take. A strength of this model is it was validated *in vivo*, and the model clearly helped to inform the appropriate experiments to perform in this respect. This is one of the most important aspects of these types of models: the ability to inform biological experiments to test mechanisms regulating the complex process of epithelial cell homeostasis.

Weaknesses of this model include failure to address that the developing intestine does not receive all of the mature niche signals.[104] Since the niche is changing as crypt expansion occurs, it is possible that the rates of division intrinsic to the stem cell population could change throughout the process. They also assume that the crypt starts with one stem cell and zero TA cells, which does not account for any immature proliferative cells that may be located in the intervillus zone. The timing for TA cell appearance has not been critically determined, but cells expressing differentiated cell markers are apparent in prenatal intestine and intestinal function to absorb nutrients is essential upon birth, so differentiated cell populations must occur before crypts mature.[96] Finally, Itzkovitz *et al.*[76] determined that there is a shift from stem cells dividing symmetrically to asymmetrically during crypt maturation. Notably, their *in vivo* studies determined that Paneth cells were not responsible for this shift because the timing occurred prior to Paneth cell maturation.[76] One option they did not address is whether signaling for the shift could occur through immature Paneth cell precursors, which have not been well defined in the immature intestine. Immature intestine contains cells that express Paneth-like markers, which may provide signals to the developing ISCs to regulate their behavior. If the process of ISC maturation is totally independent from Paneth-like cell development, then it will be important to identify which signal may control this process during intestinal development.

Future directions

The models discussed above utilized a compartmental framework to investigate the mechanisms of tumorigenesis, post-irradiation recovery, and development. Similar techniques can be used to tackle some of the lingering questions that remain in the field of stem cell biology (Fig. 3).

(1) How is stem cell number regulated? Modulations of several signaling pathways as well as various injury models have dramatic effects on stem cell numbers in the crypt. Compartmental modeling of stem cell populations in normal conditions compared to models of unbounded growth can provide insights into the signals that are necessary for regulation of

stem cell number. Use of ASC markers like the *Lgr5-GFP-CreERT2* mouse[25] can be used to validate stem cell numbers in different experimental conditions. Compartmental models can also help to address whether all ASCs are equal or if there are subpopulations by sub-compartmentalizing this population.

(2) Are ISCs completely defined by the niche? A compartmental model could distinguish whether all progenitors are potential stem cells if they are exposed to niche signals or if only cells possessing intrinsic stemness are competent to become stem cells. This could be achieved using a subcompartment approach, where the large compartments would be niche and not niche, and the smaller compartments would be the cell populations. Total cell population censes would be measured with inclusion of an intrinsically programmed cell compartment compared to a compartment that contained equipotential stem cells. This could be tested with an *in vitro* enteroid system where local signals can be precisely controlled by ligand-coated beads.

(3) Is there a dedicated QSC population? A compartmental model would test whether QSCs are always in existence, or whether they only arise during times of injury by de-differentiation of TA and differentiated cell populations. If QSCs are a true dedicated stem cell population then crypts containing these cells would have a slightly larger cell population than a crypt where QSCs are actually cells with other functions. Refined cell counting *in vivo* would be required to determine if this additional cell compartment exists.

(4) What is the nature of the TA compartment? A model of TA cells could investigate the stringency of the number of divisions that these cells undergo. Proliferating cell numbers during homeostasis, injury, and post-injury could be utilized to determine if TA cells have an intrinsic division limit, or if the number of divisions is externally regulated. Although there are no specific markers for TA cells, TA cell number can be approximated by subtracting the number of stem cells from the total number of cells that proliferate during a 12 hour window.

There are a number of other ways that these questions can be approached, combining *in vivo* and modeling techniques. Regardless of the specific modeling method used, the key issue is to keep the models focused and modest. By limiting the number of parameters in the system, we have the best chance to validate and refine the models experimentally, as modeling and *in vivo* experimentation work best synergistically, so that experimental hypotheses can be tested and refined more efficiently. Ideally, these smaller scale models would be useable by non-experts, which would allow for more broad utilization of the combined *in vivo*/*in silico* approach and would fuel further advancement of the field.

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