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REVIEW

The Intestinal Stem Cell Niche: A Central Role for Foxl1-Expressing Subepithelial Telocytes

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SUMMARY

The nature of the intestinal stem cell niche has long been controversial. Recent evidence from multiple laboratories demonstrates convincingly that subepithelial are a critical source of growth factors that maintain stem and progenitor cell proliferation.

The columnar epithelium of the alimentary tract, extending from stomach to colon, is constantly renewed by proliferation of stem and progenitor cells, which give rise to the various differentiated cell types as required by the regional specification of the gut tube. Proliferation occurs in specific zones, which in the intestine form crypts that reach into the underlying stroma. Cellular replication in the crypt is supported by an intestinal stem cell niche, the identity of which has long been controversial. Multiple recent studies have identified subepithelial telocytes, marked by expression of the winged helix transcription factor Foxl1 and the hedgehog signaling mediator Gli1, as the critical source of pro-proliferative Wnt signals to the stem/progenitor cell compartment. This review attempts to summarize and integrate these findings. *(Cell Mol Gastroenterol Hepatol 2019;8:111–117; https://doi.org/10.1016/j.jcmgh.2019.04.001)*

Keywords: Stem Cell Niche; Telocyte; Foxl1; Winged Helix Transcription Factors.

The adult mammalian intestinal epithelium has the highest turnover rate of any tissue in the body and is replaced within 3–5 days, depending on the species and the anterior-posterior location within the gut tube.[1] This rapid cellular turnover is dependent on stem and progenitor cells, which rely on signals and growth factors provided by local niche cells to support their function and self-renewal. At least 2 types of intestinal stem cells have been identified. The first are the crypt base columnar (CBC) stem cells, marked by Lgr5 and Olfm4 expression, and through genetic lineage tracing they were shown to be able to give rise to all cell types in the intestinal epithelium.[2,3] CBCs are considered the active population of intestinal stem cells and cycle about once per day. Normally quiescent stem cells residing above the crypt base around the "+4" region, ie, 4 cell diameters distal to the crypt base, are marked by reporter alleles driven by *Bmi1*, *Tert*, and *Hopx* promoters[4–6] and appear to constitute a reserve cell population.[7] When the CBC population is ablated in mice, reserve stem cells produce new CBC stem cells and repopulate the intestinal epithelium.[8]

Intestinal stem cells, in turn, are dependent on a complex and only partially defined stem cell niche. This is most clearly illustrated by the expansion and growth of Lgr5-positive stem cells into organoids containing proliferating and differentiated epithelial cells in vitro for both mouse and human cells.[9] Although originally presented as building crypt-villus structures in vitro "without a mesenchymal niche,"[9] this culture system does in fact support growth and expansion of stem cells only if factors such as R-spondin, Wnt3a, Noggin, epidermal growth factor (EGF) (factors provided in vivo in part by the mesenchymal niche), and Matrigel are provided. Matrigel is an undefined mixture of extracellular matrix proteins and growth factors that provides mechanical and adhesion cues to the organoid system, without which organoid formation does not occur. Thus, the original organoid culture systems did not establish that intestinal stem cells self-renew and differentiate by using epithelial cell-autonomous or paracrine signaling in the absence of support by signaling and structural molecules provided by mesenchymal cells; it simply provided these key molecules in the culture system.

Among the factors provided by the niche to the intestinal stem and progenitor compartment, the Wnt/R-spondin system is the major mitogen. Wnt signaling is the key driver for proliferation of intestinal stem and progenitor cells, as shown, for instance, by the rapid loss of proliferation when the Wnt inhibitor Dickkopf-1 is overexpressed in the gut epithelium.[10,11] The Wnt/β-catenin pathway is activated by binding of palmitoylated Wnt ligands to Frizzled-LRP5/6 receptor complexes, which initiates a signaling cascade leading to stabilization of cytoplasmic β-catenin, its translocation into the nucleus, and action as a transcriptional co-activator with the Tcf/Lef transcription

Abbreviations used in this paper: **BMP, bone morphogenetic protein; CBC, crypt base columnar cell; EGF, epidermal growth factor; SMA, smooth muscle actin.**

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factors.[12] The medical relevance of perturbations in this system was demonstrated more than 25 years ago through the identification of the gene mutated in patients with familial adenomatous polyposis, an inherited cancer predisposition syndrome.[13,14] The gene, termed *APC*, encodes a large protein that functions in the control of nuclear β-catenin activity as part of the cytoplasmic "destruction complex".[15] Thus, unbridled β-catenin activity leads to uncontrolled cell division in the stem cell compartment and, eventually, development of colonic adenomas and carcinomas. Likewise, activating mutations in β-catenin itself, although less frequent, can also lead to colorectal cancer.[16] Multiple genetic studies in mice have shown the key role for intracellular mediators of the Wnt signaling in stem and progenitor proliferation and stem cell maintenance.[10,17] In addition, Valenta et al[18] showed recently that when Wnt production is blocked globally through the conditional ablation of Wntless, a transmembrane protein required for the secretion of mature Wnt proteins, using a ubiquitously expressed Cre driver, homeostatic renewal of the intestinal epithelium was greatly impaired. However, the critical source of the Wnt ligands remained controversial until recently.

In 2011, Sato et al[19] proposed that postmitotic, differentiated Paneth cells intercalated between the CBC cells at the crypt base constitute the niche for $Lgr5^+$ stem cells in intestinal crypts. This conclusion was based on the observation that Paneth cells express signaling molecules such as Wnt3 and EGF. Furthermore, they found that co-culture with a Paneth cell-enriched population stimulated in vitro organoid formation by $Lgr5^+$ CBC stem cells. This model was quickly challenged by the findings in 2 independent studies showing that loss of Paneth cells in mice lacking the transcription factor Math1 (Atoh1) had no effect on intestinal stem cell maintenance or proliferation or in the regenerative response of the epithelium to injury.[20,21] Furthermore, loss of all Wnt production in the epithelium using cell type-specific gene ablation of the obligate Wnt processing enzyme Porcupine (Porcn),[22,23] or the deletion of Wnt3 in Paneth cells, had no effect on crypt health.[24] The latter study was consistent with earlier work by Gordon and colleagues who used 2 independent methods to ablate mature Paneth cells and found no effect on stem cell function in intestinal crypts.[25] Together, these studies suggested the existence of an alternate or additional source of Wnt ligands. Whether this second source provides the primary niche Wnt signal to CBC stem cells or acts redundantly with Paneth cell-derived Wnt signals was uncertain until recently.

One possible and obvious alternative source of mitogenic Wnt signals is the mesenchymal compartment or stroma, which contains myofibroblasts and other cell types that are located in close proximity to intestinal epithelial stem cells.[26] San Roman et al[23] investigated the possibility that myofibroblasts represent the alternate niche Wnt source through inhibition of all Wnt ligand production via ablation of *Porcn* in Myh11-expressing myofibroblasts and smooth muscle cells. Somewhat surprisingly at the time, ablating Wnt signaling in the *Myh11-Cre* model had no effect on intestinal morphogenesis and stem cell function. Initially, these studies seemed to favor the notion that redundant Wnt signaling from both epithelial and mesenchymal compartments is responsible for crypt maintenance.

Recently, we discovered that a rare population of submucosal cells expressing the transcription factor Foxl1 is required for crypt maintenance.[27,28] Foxl1 is a DNA-binding transcription factor of the forkhead class that is characterized by an evolutionarily conserved 100 amino acid winged helix DNA binding domain.[29,30] In the gut, Foxl1-expressing cells appear first during midgestation (embryonic day 12.5 in the mouse),[31] just before the epithelial transition. The epithelial transition, or formation of villi, refers to the transformation of the pseudo-stratified epithelium of the primitive gut tube, which is radially surrounded by mesenchymal cells, to the columnar epithelium structured into villi with mesenchymal cores that are characteristic of the mature intestine.[32] This epithelial transition, which occurs on embryonic day 14.5 in mice and during gestational weeks 8–10 in humans, coincides with the first appearance of bone morphogenetic protein (BMP)–expressing mesenchymal clusters that accompany the formation of nascent villi.[32] In fact, Walton et al[32] showed that multiple BMPs (2, 4, 5, and 7) are expressed in these clusters, and furthermore, that inhibiting BMP signaling caused the merging of clusters and the formation of fewer villi.[32] The relationship between Foxl1 activity, BMP signaling, and villus formation is supported by the finding that villus formation is delayed in Foxl1 null embryos, and that expression of Bmp 2 and 4 is reduced.[33] Foxl1, in turn, is activated by hedgehog signaling from the epithelium via the binding of Gli transcription factors to multiple ultra-conserved Gli binding sites in the *Foxl1/Foxf1* locus.[34]

In adulthood, Foxl1-expressing cells remain intimately associated with the basolateral membranes of all alimentary columnar epithelial cells (Figure 1) and expand through the elaboration of long, thin cytoplasmic extensions, reaching several hundred micrometers in size, compared with the approximately 10-μm width of a columnar epithelial cell.[28] Cells with these properties were described as early as 1964, when Deane[35] published the first ultrastructural analysis of the lamina propria and called these cells fibrocytes. Kaye and colleagues used transmission electron microscopy to determine that these cells form a "pericryptal fibroblast sheath" and are distinct from other mesenchymal cells, including the previously studied myofibroblasts.[36,37] They showed that these cells are closely appositioned to the epithelial cells of the colonic crypt, with only the 60-nm basal lamina separating the 2 cell types, and engage all epithelial cells in the proliferative region of the crypt. They estimated that each pericryptal fibroblast contacts 50–70 epithelial cells, depending on the position along the colonic crypt. Using autoradiography of ^{3}H-thymidine pulse-labeled colon, they demonstrated further that pericryptal fibroblasts are 35 times less likely to be in the cell cycle than crypt epithelial cells.[37] Very recently, genetic pulse-labeling using *Gremlin1-CreER::Lox-Stop-Lox-GFP* mice confirmed the slow turnover of the pericryptal fibroblast, because it took about 12 months after a tamoxifen pulse until all sheath cells had been replaced by cells expressing the GFP lineage label.[38]

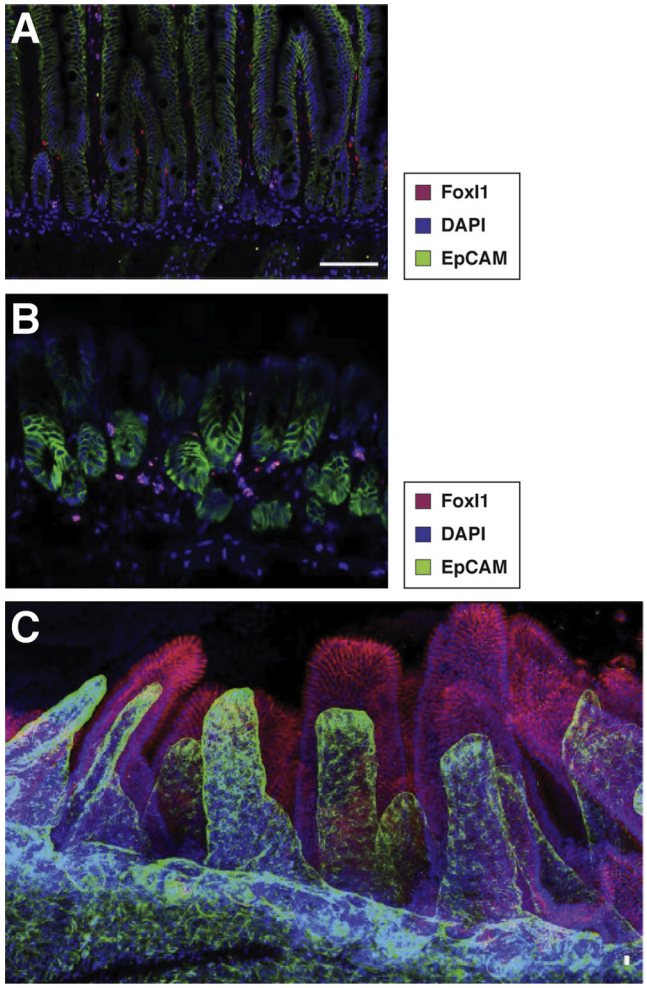

Figure 1. Foxl1$^+$ telocytes establish a subepithelial plexus immediately underneath the gastrointestinal epithelium. (*A*) Nuclei of telocytes in the mouse jejunum are labeled by Foxl1 immunostaining (*red*). EpCAM (*green*) outlines epithelial cells, and DAPI (*blue*) outlines all nuclei. Reprinted with permission from Aoki et al.[27] (*B*) Nuclei of telocytes in the mouse glandular stomach are labeled by Foxl1 immunostaining (*red*). EpCAM (*green*) outlines epithelial cells, and DAPI (*blue*) outlines all nuclei. (*C*) Confocal imaging of X-Clarity (Logos Biosystems, Anyang-si, Gyeonggi-do, South Korea) cleared mouse jejunum demonstrates the telocyte plexus, here visualized by platelet-derived growth factor receptor-alpha staining (*green*) that underlies all epithelial cells. EpCAM (*red*) was used to label epithelial cells. Reprinted with permission from Shoshkes-Carmel et al.[28]

The first immunocytochemical stain for colonic pericryptal sheath cells was developed by the Bodmer group, although their PR 2D3 antibody also reacted with vascular smooth muscle and the smooth muscle cells in the muscularis mucosae.[39] Bodmer and colleagues argued that pericryptal sheath cells belong to the smooth muscle or myofibroblast lineage, based on staining of the cells for smooth muscle actin (SMA) and myosin. We demonstrated previously that Foxl1-positive subepithelial cells are negative for α-smooth muscle actin (αSMA) and myosin heavy chain 11 (Myh11) immunoreactivity and express critical crypt signaling proteins such as Wnt2b, Wnt5a, and R-spondin3 as well as the BMP antagonists Gremlin 1 and Gremlin 2.[27] Shortly thereafter, Stzepourginski et al[40] identified Cd34$^+$gp38$^+$ mesenchymal cells as crypt stromal cells and showed that these cells support proliferation of intestinal stem cells in the organoid culture system. Although Foxl1$^+$ cells form a continuous plexus from the crypt bottom to the villus tip (Figure 1*C*), Cd34$^+$gp38$^+$ are restricted to the crypt region.[40] The notion that Foxl1$^+$ cells are distinct from myofibroblasts is supported further by the aforementioned finding from the Shivdasani group that ablating all Wnt signaling in myofibroblasts using *Myh11-CreER*;*Porcn*^{loxP} mice had no effect on epithelial health.[23] Thus, Foxl1$^+$ cells are not classic myofibroblasts.

Popescu coined the term *telocytes* for these pericryptal sheath cells, which had previously been known as interstitial Cajal-like cells but later found in 2010 to be completely distinct from Cajal cells, which are pacemaker cells involved in controlling smooth muscle contractions.[41,42] Telocytes are a distinct type of interstitial cells characterized by a small cell body and extremely long and thin extensions termed *telopodes*, and we have adopted this term for the Foxl1-positive cells for brevity and because it better distinguishes this cell type with unique morphologic features from other interstitial cells such as fibroblasts, myofibroblasts, and smooth muscle cells.

Each Foxl1$^+$ telocyte is in close contact with dozens of epithelial cells, and together the Foxl1$^+$ cells form a subepithelial plexus that extends from the glandular stomach to the colon and thus is in contact with all columnar epithelia of the alimentary canal (Figure 1).[28] The first functional evidence that Foxl1-expressing telocytes are a critical component of the intestinal stem cell niche came from experiments in mice published in 2016 where diphtheria toxin–mediated ablation in *Foxl1-DTA* mice resulted in cessation of epithelial proliferation within a few days after administration of the toxin.[27] Remarkably, just 3 days after loss of Foxl1$^+$ telocytes, the length of both small and large bowel was shortened, jejunal villus length was cut in half, and the number of cycling cells per crypt was reduced by more than 95%. This study established an absolute requirement for Foxl1-expressing cells in crypt maintenance. Although Wnt signaling to the epithelium, as assessed by nuclear β-catenin staining, was clearly reduced, this study did not unequivocally address whether the effect of Foxl1$^+$ telocyte ablation was mediated solely by Wnt proteins or whether additional signaling or support molecules produced by these cells act to maintain CBC self-renewal and crypt-villus fidelity.

Using the genetic handle of the *Foxl1-Cre* driver, Shoshkes-Carmel et al[28] sorted intestinal telocytes from *Foxl1-Cre::Rosa26-mTmG* mice and performed transcriptome profiling. From this experiment, 2 important themes emerged. First, Foxl1$^+$ telocytes have an expression profile clearly distinct from Foxl1-negative mesenchymal cells (eg, myofibroblasts) or epithelial cells, with high expression of specific Wnt molecules such as the canonical Wnt ligand Wnt2b and the non-canonical ligand Wnt5a, as well as the key Bmp molecules 4, 5, 6 and 7, the receptors and

Table 1. Mouse Models and Their Phenotypes

Gene targeted	Timing of gene ablation	Targeted cell types	Phenotype	Reference
Porcupine (Porcn)	Adulthood	Foxl1+ telocytes, conditionally ablated in adult mice	Loss of active Wnt signaling in the crypt, loss of all crypt proliferation	28
Porcupine (Porcn)	Fetal life	Intestinal epithelial cells (VillinCre)	Normal	23
Porcupine (Porcn)	Fetal life	Myofibroblasts (Myh11-Cre)	Normal	23
Porcupine (Porcn)	Fetal life	PDGFRalpha expressing submucosal cells	Early postnatal lethality; reduced epithelial proliferation	45
R-spondin 3 (Rspo3)	Fetal life	PDGFRalpha expressing submucosal cells	Epithelial homeostasis normal, increased sensitivity to DSS colitis	45
Wntless (Wls)	Adulthood	Gli1-expresssing submucosal cells	Loss of active Wnt signaling in colonic crypts, loss of colonic crypt proliferation. Small intestinal crypts appear normal	44

DSS, dextran sodium sulfate; PDGFR, platelet-derived growth factor.

downstream mediators of the hedgehog pathway, as well as other key growth factors. Second and initially puzzling, Foxl1 telocytes express not just the Wnt ligands as expected but also multiple antagonists of the canonical Wnt pathway, such as Dkk2 and 3, and the decoy receptor Sfrp1. This apparently paradoxical observation was resolved by using single molecule RNA-FISH, which showed that telocytes partition the production of Wnt activators and inhibitors such that the former are enriched near the crypt base, whereas the latter are more abundant near the crypt-villus junction. It will be fascinating to investigate how Foxl1 cells control the localized production of these key signaling molecules to ensure that proliferation only occurs in the crypt.

Answering the question of whether Wnt signals emanating from telocytes are indeed required for crypt function, Shoshkes-Carmel et al went on to develop an inducible *Foxl1-CreER* line and crossed these with mice carrying a loxP-flanked allele of *Porcn*.[28,43] *Porcn* encodes a Golgi-resident enzyme that is required for the maturation of all Wnt proteins, and thus its ablation eliminates all Wnt production from a $Porcn^{-/-}$ cell. Remarkably, the consequences for intestinal and organismal health were striking and immediate. Within 24 hours after tamoxifen administration, DNA replication in the crypt compartment had ceased, and by 72 hours crypts had collapsed, jejunal villus length was reduced by 90%, and epithelial integrity had been lost. In keeping with the loss of crypt function, expression of the stem cell marker Olfm4 was eliminated, and the Wnt/β-catenin pathway was shut off. This finding provided clear evidence that the Wnt proteins produced by epithelial cells including the Paneth cells are not able to compensate for the loss of Wnt signals from $Foxl1^{+}$ telocytes, and neither were myofibroblasts or other Foxl1-negative stromal cells. This study therefore established Foxl1-expressing subepithelial telocytes as the critical source of Wnt signals that are necessary to maintain the proliferation and function of intestinal stem and transit amplifying cells. Although other intestinal cell types, including epithelial Paneth cells, express multiple Wnt proteins, they are unable to compensate for the loss of Wnt ligands produced by Foxl1-positive cells. Further support for this notion comes from a study by Degirmenci et al[44] that ablated *Wntless* – like Porcupine required for the production of all Wnt proteins – in Gli1-producing cells in the mesenchyme.[44] Colonic, but not small intestinal, crypts collapsed in this model, albeit with much slower kinetics. This difference compared with the very rapid crypt collapse in the $Foxl1\text{-}CreER::Porcn^{loxP/loxP}$ model is likely the result of the very long half-life of the Wntless protein. Previously, Valenta et al[18] had ablated *Wntless* by using the ubiquitously expressed *Rosa26-CreER* driver and showed that whereas Wntless mRNA was eliminated 6 days after tamoxifen treatment as expected, Wntless protein was still detectable a full 10 days after the onset of gene deletion. *Foxl1* and the related and genetically linked gene *Foxf1* had been shown almost 10 years ago to be targets of the Gli proteins,[34] responding to hedgehog proteins secreted by the gastrointestinal epithelium; thus it fits well that ablation of Wnt secretion from Gli1-expressing cells results in a phenotype similar to its elimination from $Foxl1^{+}$ telocytes. A striking difference between the model by Degirmenci et al,[44] ie, ablation of *Wntless* using an inducible Gli1-CreER driver, and ablation of *Porcupine* using the Foxl1-CreER transgene[28] is the fact that only the latter shows a dramatic phenotype in the small intestine. *Wntless* ablation by Gli1-CreER only caused small intestinal crypt collapse when combined with a Villin-CreER driver, which thus removed any Wnt proteins emanating from the intestinal epithelium.[44] Why epithelial Wnt proteins can compensate for loss of *Wntless* in $Gli1^{+}$ stromal cells, although they cannot overcome the loss of *Porcn* in $Foxl1^{+}$ telocytes, is presently unknown, although it is possible that the differences in the kinetics of Wnt signaling removal might be at least part of the cause.

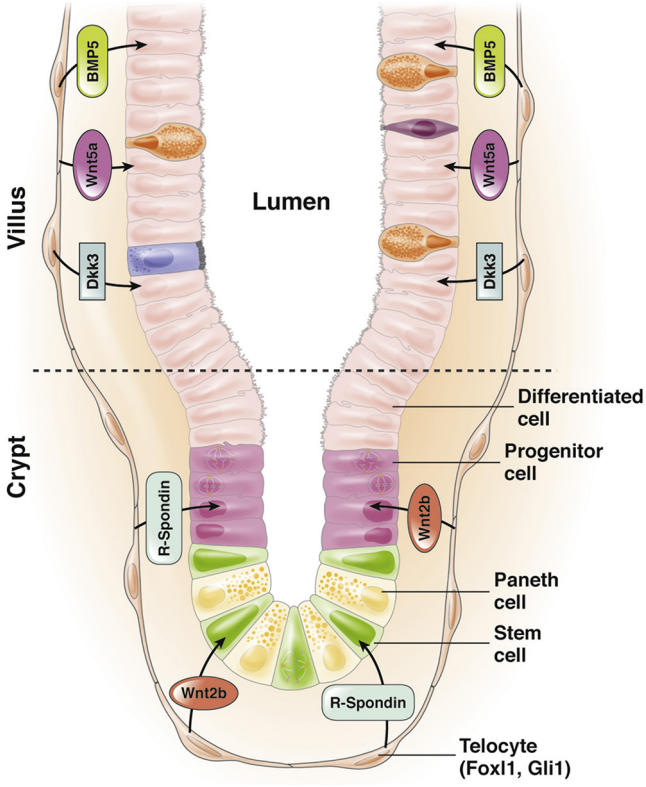

Figure 2. Foxl1$^+$ telocytes are closely appositioned to the intestinal epithelium and compartmentalize production of key signaling molecules along the crypt-villus axis. Foxl1/Gli1 positive telocytes are large, thin cells that form a continuous plexus underneath the gastric and intestinal epithelium. Telocytes compartmentalize production of signaling molecules such that expression of pro-proliferative factors such as R-Spondin and Wnt2b is highest in the crypt, and that of pro-differentiation factors such as BMP5 and Wnt5a is highest in the villi, thus supporting both intestinal regeneration as well as differentiation.

In addition, ablation of Porcupine using a *PDGFRα-Cre* line in a study by Grecius et al[45] caused a marked decrease in the number of proliferating cells in neonatal crypts, again consistent with the findings of Shoshkes-Carmel et al.[28] This study also established that R-spondin 3 is a more potent activator of the Wnt pathway than R-spondin 1 and confirmed that R-spondin 3 is expressed in pericryptal fibroblast, or intestinal telocytes. However, PDGFRα-Cre driven ablation of R-spondin 3 did not perturb normal intestinal homeostasis; effects were only seen in dextran sodium sulfate–induced colitis. Greicius et al showed further that pericryptal fibroblasts genetically labeled by a PDGFRα-Cre driver support the growth of organoids deficient for Porcupine. It should be noted that although all Foxl1$^+$ cells expressed PDGFRα, most PDGFRα cells in the gut stroma are Foxl1-negative (ie, Foxl1+ telocytes are a small subset of PDGFRα$^+$ cells),[28] making the *Foxl1-CreER* model more specific for gastrointestinal telocytes. Table 1 summarizes the observations from the relevant mouse models discussed here. Taking the findings from multiple laboratories together, it appears that the debate on the identity of the intestinal stem cell niche is settled.[46] A schematic summarizing the current view of the intestinal stem cell niche is shown in Figure 2.

Foxl1-expressing telocytes are not restricted to the mouse model but have recently been described in the human colon as well.[47] Kinchen et al[47] used single cell RNA-seq analysis of the colonic stroma from patients with ulcerative colitis and healthy controls. A population they termed *stromal 2* closely resembles the murine telocytes described above, because it is Foxl1 positive and expresses Bmp2 and Wnt5a. Interestingly, there appeared to be a decrease in the proportion of stromal 2 cells in the patients with ulcerative colitis, raising the exciting possibility that these cells might play a role in the pathogenesis of inflammatory bowel disease. Because of the unique location of telocytes at the interface between epithelium and submucosa, it is tempting to speculate that these cells could mediate the interactions of luminal antigens with the immune system after failure of the intestinal barrier. Clearly, much work remains to be done to understand the full contribution of these important players in the intestinal stem cell niche.

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Conflicts of interest
The author discloses no conflicts.