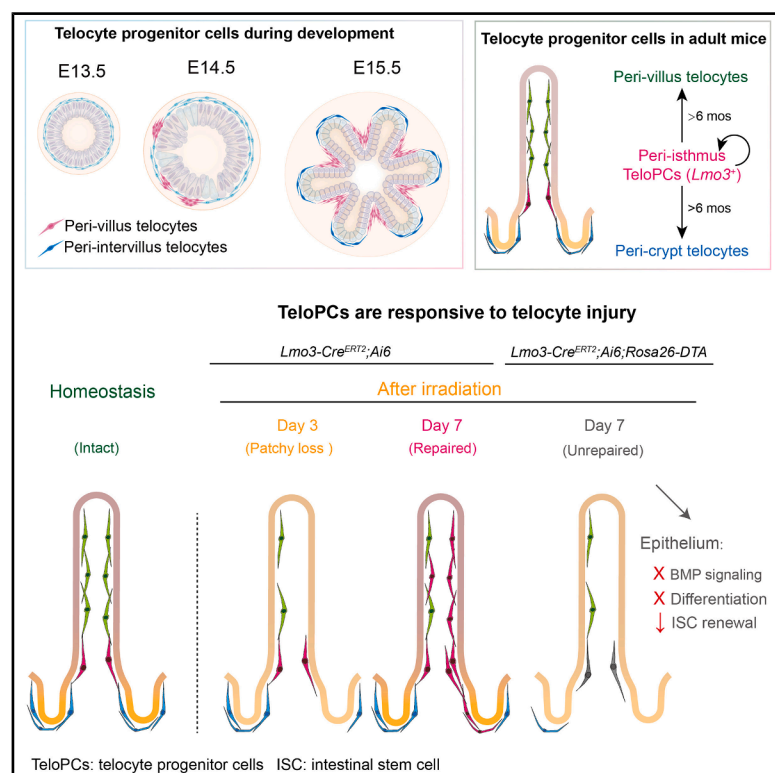


Developmental Cell

Lmo3-expressing peri-isthmus progenitor cells sustain renewal and repair of the mammalian intestinal telocyte niche

Graphical abstract



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In brief

Through single-cell analysis of mouse small intestine development, Jiang et al. identify a population of mesenchymal progenitor cells as the developmental origin of intestinal telocytes. These cells gradually become confined to the isthmus region, wherein they become long-lived progenitor cells responsible for telocyte renewal and repair in adults.

Highlights

- *Lmo3*⁺ telocytes that emerge at E13.5 are the cellular origin of post-natal telocytes
- *Lmo3*⁺ telocytes are progressively confined to the isthmus region after birth
- These cells become long-lived progenitor cells (TeloPCs) that renew telocytes in adults
- *Lmo3*⁺ TeloPCs are activated to repair the telocyte niche following damage



Article

Lmo3-expressing peri-isthmus progenitor cells sustain renewal and repair of the mammalian intestinal telocyte niche

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SUMMARY

Intestinal telocytes that reside immediately beneath the intestinal epithelium exert niche-supporting roles for intestinal stem cells and their progenies. They are heterogeneous cells compartmentalized along the crypt-villus axis, but the mechanisms governing the maintenance of this telocyte population remain unclear. Here, we identify a distinct population of subepithelial mesenchymal cells in the developing mouse embryo, marked by *LIM Domain Only 3* (*Lmo3*), as the cellular origin of post-natal intestinal telocytes. The *Lmo3*⁺ cells emerge prior to villus formation at embryonic day 13.5, and after birth, they progressively acquire a spatial confinement to the intestinal isthmus region, where they persist as long-lived, slow-cycling cells, supplying both peri-villus and peri-crypt telocytes. Further, we show that *Lmo3*⁺ cells respond rapidly to tissue damage, becoming activated to promote repair of the telocyte niche. Therefore, a quiescent and damage-responsive progenitor cell population marked by *Lmo3* maintains the intestinal telocyte niche.

INTRODUCTION

The maintenance and regeneration of the mammalian intestinal epithelium, which lines the lumen and is organized into numerous crypt-villus structures, rely on the presence of intestinal stem cells (ISCs) located at the base of the crypts,¹ where Paneth cells in the epithelial compartment and multiple cell types in the subepithelial mesenchymal compartments, collectively constitute the niche environment for ISC self-renewal.^{2–12} Among these cellular constituents, the telocytes emerged as a significant component, exhibiting distinctive characteristics as *Pdgfra*^{high} and *Foxl1*⁺ mesenchymal cells with elongated protrusions that envelop the entire intestinal epithelium.³

The telocyte population is notable for its heterogeneity, with peri-villus telocytes secreting BMPs (bone morphogenic proteins), while peri-crypt telocytes contribute Wnts (wingless-related integration site proteins).^{4,13–16} This spatial distribution of Wnt and BMP signaling forms opposing gradients along the crypt-villus axis, regulating the delicate balance between ISC self-renewal and differentiation.¹⁷ Despite the paramount importance of telocytes within the ISC niche, the developmental origin of telocyte, the lineage relationships between peri-villus and peri-crypt telocytes, and the mechanisms governing the maintenance of the telocyte population, remain elusive.

The developing gut epithelium is constantly surrounded by *Pdgfra*^{high} mesenchymal cells, the presumptive telocyte progenitor cells.¹⁸ Prior to the embryonic day 13.5 (E13.5), the intestinal progenitor cells form a flat layer of pseudostratified epithelium inner lining the primitive midgut tub.¹⁹ From E13.5 onward, these mesenchymal cells start to form patterned clusters that drive the initiation and outgrowth of villi.^{20–23} During this period, *Lgr5*⁺ ISCs emerge at the intervillus region,²⁴ an event presumably driven by the activation of Wnt signaling.^{1,25} However, relatively little is known about the development and formation of the mesenchymal cell niches as ISCs emerge.

Telocytes in adult small intestine are quiescent cells characterized by possessing very long and thin protrusions.²⁶ We reasoned that we may be able to get a better understanding of the telocyte population by tracing their developmental origin and studying the process during which the telocyte niche is established as the villus forms. We therefore conducted a high-resolution scRNA-seq analysis encompassing various developmental time points of the mouse small intestine during which the villus morphogenesis occurs. This allowed us to identify a peri-villus telocyte population that is emerged at the onset of villus morphogenesis. Intriguingly, these cells progressively acquire a spatial confinement to the intestinal isthmus region

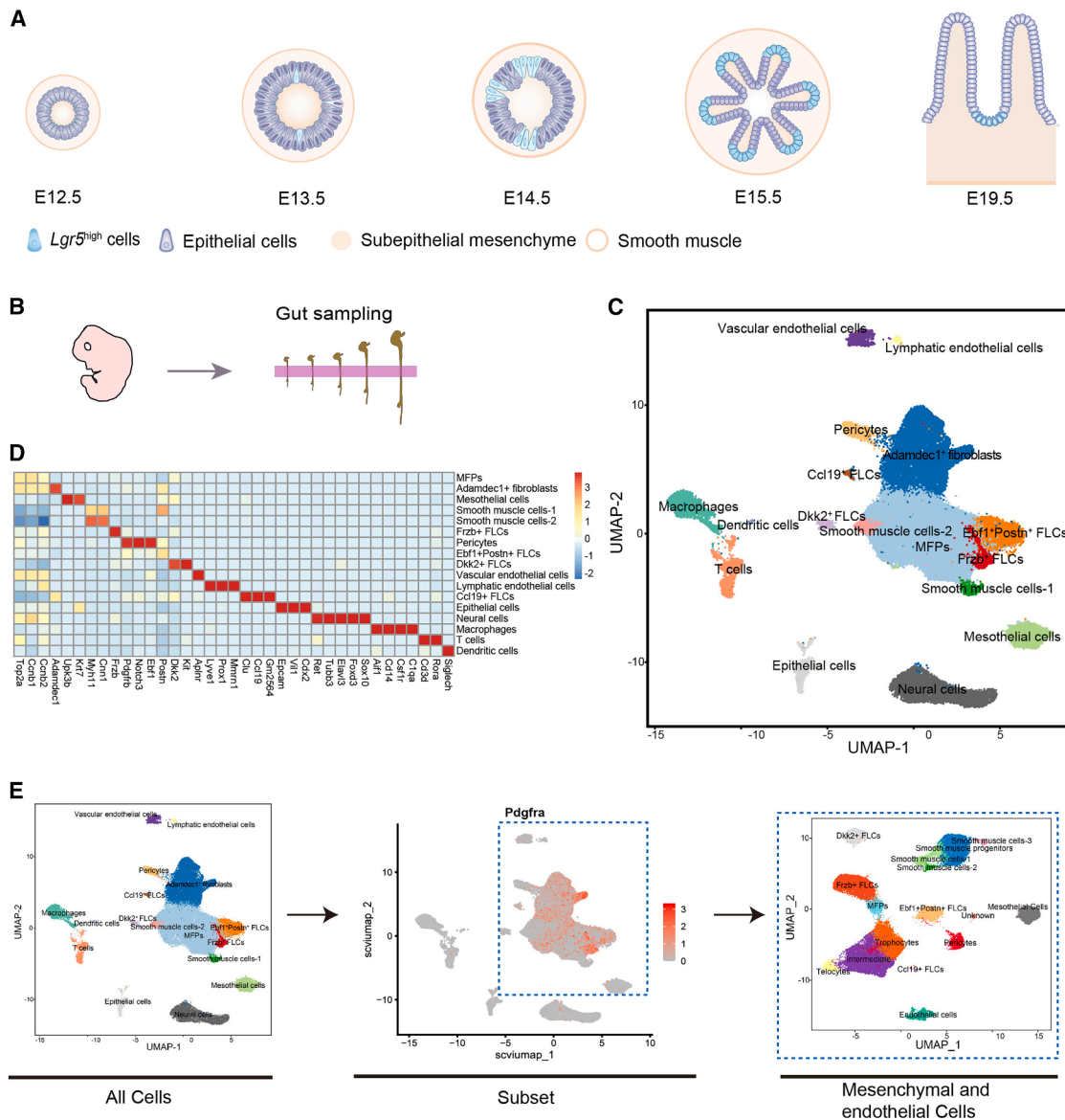


Figure 1. A High-resolution single-cell analysis of small intestine development from E12.5 to E19.5

(A) A brief diagram shows the development of mouse small intestines from E12.5 to E19.5. (B) Overview of the experimental workflow. Single cells were isolated from C57/B6j mouse embryonic SI. (C) UMAP plots with each cell color-coded for its associated cell types. (D) Cells covering five time points in the developing small intestines were annotated based on the expression of known marker genes, as shown with heatmap. (E) A diagram of how cells were extracted from scRNA-seq data of 5 developmental stages. Cells were extracted containing both *Pdgfra*⁺ mesenchymal clusters, mesothelial cells, and endothelial clusters, reanalyzed, and displayed as UMAP.

following birth, wherein they assume the role of long-lived progenitor cells responsible for telocyte renewal and repair.

RESULTS

Tracing the development of intestinal mesenchymal cells at single-cell resolution

Single cells from the middle region of full-thickness mouse small intestine at five developmental time points (E12.5, E13.5, E14.5, E15.5, and E19.5) were captured and barcoded using the 10×

Chromium platform (Figures 1A and 1B). After quality control and filtration, 70,792 individual cells with averaged 8,970 unique molecular identifiers (UMIs) for each cell were utilized for subsequent analysis. After dimensional reduction and visualization with uniform manifold approximation and projection (UMAP), these cells were grouped into 17 distinct clusters (Figures 1C and 1D).

We next extracted and analyzed the mesenchymal, mesothelial, and endothelial clusters from the single-cell data, partitioning them into 16 distinct cell clusters based on their unique

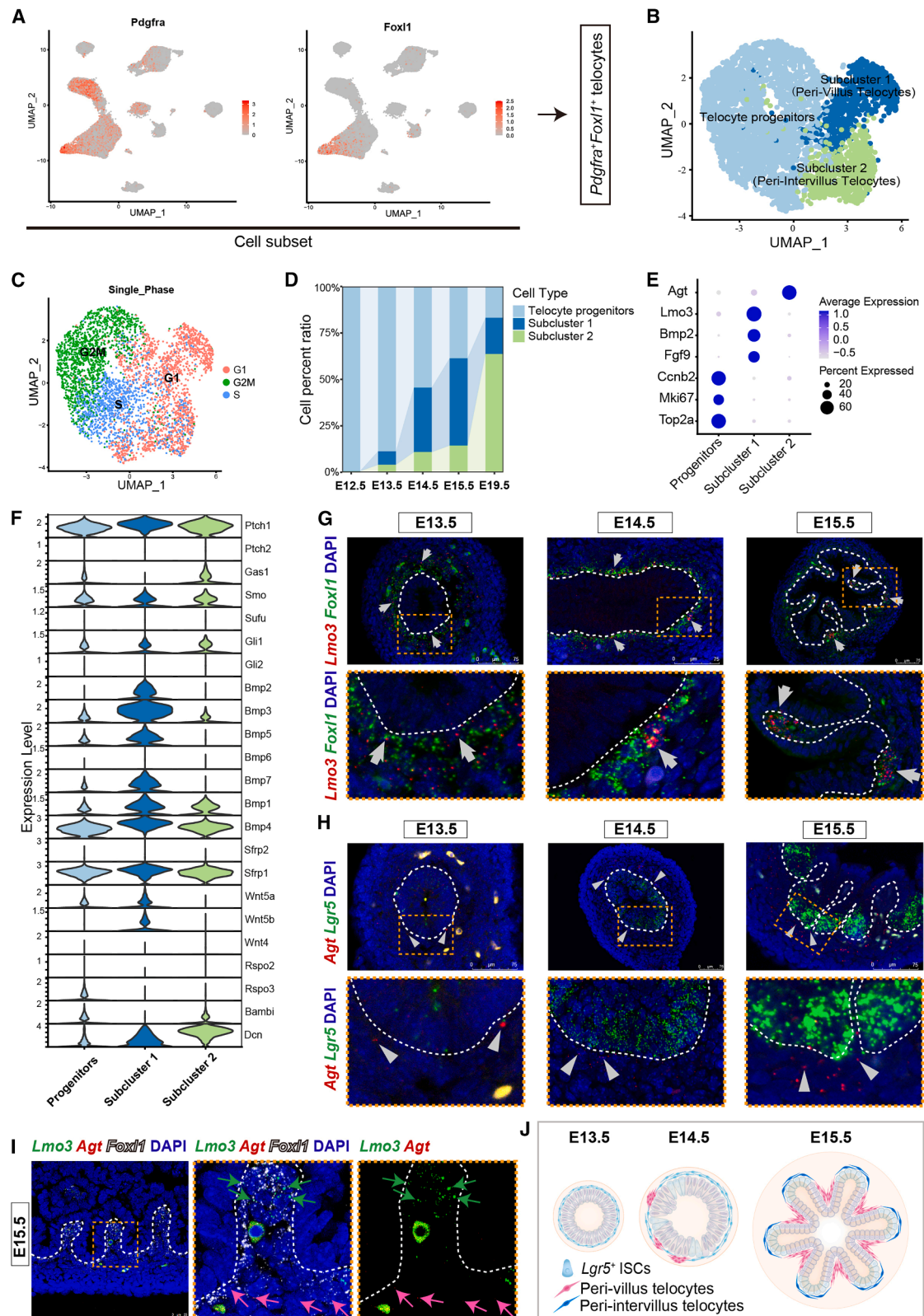


Figure 2. A rudimentary telocyte niche architecture is formed alongside the emergence of ISCs

(A) *Pdgfra*⁺*Foxl1*⁺ telocytes were extracted and reanalyzed.

(B) UMAP plot showing three major subclusters of *Pdgfra*⁺*Foxl1*⁺ telocytes collected from E12.5–E19.5.

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expression patterns and specific markers (Figures 1E and S1A–S1D). The *Adamdec1*⁺ fibroblasts were further subdivided into three distinct clusters: trophocytes, intermediate cells (presumptive adult *Pdgfra*^{low}*Cd34*⁺*Cd81*[−] stroma), and telocytes. The smooth muscle cells (SMC-1 and SMC-2), along with a part of myofibroblast progenitors (MFPs), were also subdivided into four clusters (Figures S1A–S1C). These likely represent embryonic progenitors for longitudinal, circumferential, superficial, LP (lamina propria), and/or MM (muscularis mucosa) SMCs, as characterized in the small intestine of post-natal mice.⁷ However, their exact identity remains uncertain due to the lack of unique markers (Figure 1D). Notably, the telocyte, intermediate, and trophocyte clusters represent three distinct cell clusters throughout the E12.5 to E19.5 period, suggesting that these mesenchymal cell lineages had already diverged early in development, prior to E12.5 (Figure S1F). Interestingly, while most mesenchymal cell types, including muscle cells, trophocytes, intermediate cells, pericytes, and mesothelial cells, expressed various types of niche signals (such as WNTs, BMPs, *Rspo2*, *Rspo3*, *Nog*, and *Dcn*), the expression levels of these niche factors remained relatively constant from E12.5 to E19.5, with the exception of the telocyte cluster. In telocytes, BMP expression began to upregulate at E13.5 and continued to increase thereafter (Figures S1 and S2).

Grem1 (*Grem1*) encodes a BMP antagonist. In adult mice, its expression in trophocytes is crucial for ISC self-renewal and for establishing the BMP signaling gradient along the crypt-villus axis.⁵ Interestingly, the expression of *Grem1* was not evident in the developing trophocyte cell cluster (*Cd34*⁺*Postn*⁺*Rspo2*⁺) until E19.5 (Figures S1E and S2C). This observation aligns with findings that *Grem1* expression in *Cd34*⁺ mesenchymal cells is not prominent until 2 weeks after birth.¹⁶

Re-clustering of the developing endothelial cells, including vascular endothelial cells (VECs) and lymphatic endothelial cells (LECs), revealed very limited expression of niche factors from E12.5 to E19.5 (Figures S2J and S2K). This contrasts with LECs in the adult ISC niche, where they are considered as a significant source of *Rspo3*.^{8,9} *Prox1*, which is required for LEC formation,²⁷ and the LEC marker *Lyve1*, were not detectable in these developing endothelial cells until after E15.5 (Figures S2L and S2M), consistent with a previous observation.²⁸ Collectively, these observations suggest that, while various cell types within the mesenchymal compartment express a range of niche signals, the telocyte cluster is unique in exhibiting prominent changes in its niche production profile around the time of ISC emergence. This indicates a potential role for developing telocytes in regulating ISC emergence.

The telocyte progenitor population diversifies prior to villus morphogenesis

We then extracted and analyzed the developing telocyte clusters (*Pdgfra*^{high}*Foxl1*⁺), which represent a single mitotically active cell cluster until E13.5, when these cells started to diverge into two distinct subclusters (Figures 2A–2D). The subcluster 1 showed specific expression of *LIM Domain Only 3* (*Lmo3*), increased expression of BMPs (*BMP1*, 2, 3, 5, and 7), and non-canonical Wnts (*Wnt5a*, 5b), whereas the subcluster 2 showed specific expression of *Angiotensinogen* (*Agt*) and increased expression of Wnt agonist *Bambi*²⁹ and BMP antagonist *Decorin* (*Dcn*)³⁰ (Figures 2E and 2F).

In situ hybridization by RNAscope revealed the initial appearance of *Lmo3*⁺ cells at E13.5–E14.5 in a subset *Foxl1*⁺ cells (Figure 2G). These cells first appeared in small clusters beneath the stratified epithelium at E14.5, they then migrated along with the tip of villus as villigenesis occurs at E15.5 (Figure 2G). Therefore, *Lmo3*⁺ peri-villus telocytes likely represent the patterned *Pdgfra*⁺ cell clusters that drive the initiation and outgrowth of villi.^{20–23} In contrast, *Agt* was detected specifically (although weakly) in the mesenchymal cells at the intervillus regions beneath the newly emerged *Lgr5*⁺ ISCs^{23,24} (Figure 2H).

Interestingly, these peri-intervillus telocytes (subcluster 2) did not show significant Wnt or *Rspo* signals. Instead, they have an expression profile largely similar to telocyte progenitors (Figure 2F). This indicates that the divergence of telocyte population may have a role in establishing villus versus intervillus epithelial domains via providing differential BMP signaling activities. Therefore, a rudimentary telocyte cell niche structure has been formed in accompany with the emergence of ISCs during E14.5–E15.5 (Figures 2I and 2J).

The *Lmo3*⁺ peri-villus telocytes give rise to peri-intervillus telocytes

To determine the lineage relationships between the peri-villus telocytes and peri-intervillus telocytes, we generated *Lmo3*-tdTomato-*P2A*-Cre^{ERT2} (*Lmo3*-Cre^{ERT2}) knockin mice, in which *Lmo3*-tdTomato fusion protein and Cre^{ERT2} are co-expressed from a single bicistronic message (Figure 3A). Transient labeling of *Lmo3*-Cre^{ERT2}; Ai6 (*Rosa*-CAG-LSL-*ZsGreen1*) mice showed consistent results as revealed by *Lmo3* *in situ* hybridization: the *ZsGreen1*⁺ cells appeared first at E13.5–E14.5 in small clusters and these cells migrated along with the tip of villus as villus morphogenesis occurs (Figure 3B). Possibly due to abnormal protein folding, the direct fluorescence signal of LMO3-tdTomato fusion protein was invisible. However, it could be visualized by immunostaining with anti-RFP (red fluorescent protein) antibody. With this approach, we found that LMO3-tdTomato

(C) Cell phase of telocytes was determined based on a set of cell phase markers using Seurat at single-cell level.

(D) Cell percentage alteration of telocyte subpopulations during development.

(E) Selected marker genes representing different telocyte subpopulations.

(F) The subcluster 1 (peri-villus telocytes) express multiple BMPs and noncanonical *Wnt5a*, while the subcluster 2 (peri-intervillus telocytes) express Wnt agonist *Bambi* and TGFβ antagonist *Dcn*.

(G) *In situ* hybridization of *Lmo3* (red) and *Foxl1* (green) at E13.5–E15.5 by RNAscope.

(H) *In situ* hybridization of *Agt* (red) and *Lgr5* (green) at E13.5–E15.5 by RNAscope.

(I) *In situ* hybridization of *Lmo3* (green), *Agt* (red), and *Foxl1* (white) at E15.5 by RNAscope.

(J) Schematic showing the developmental process of the rudimentary ISC niche formation. From E13.5 to E15.5, the telocyte progenitors gradually diverged into peri-villus and peri-intervillus telocytes. The newly formed rudimentary niche is composed of two domains of the telocyte niche cells: peri-villus and peri-intervillus telocytes.

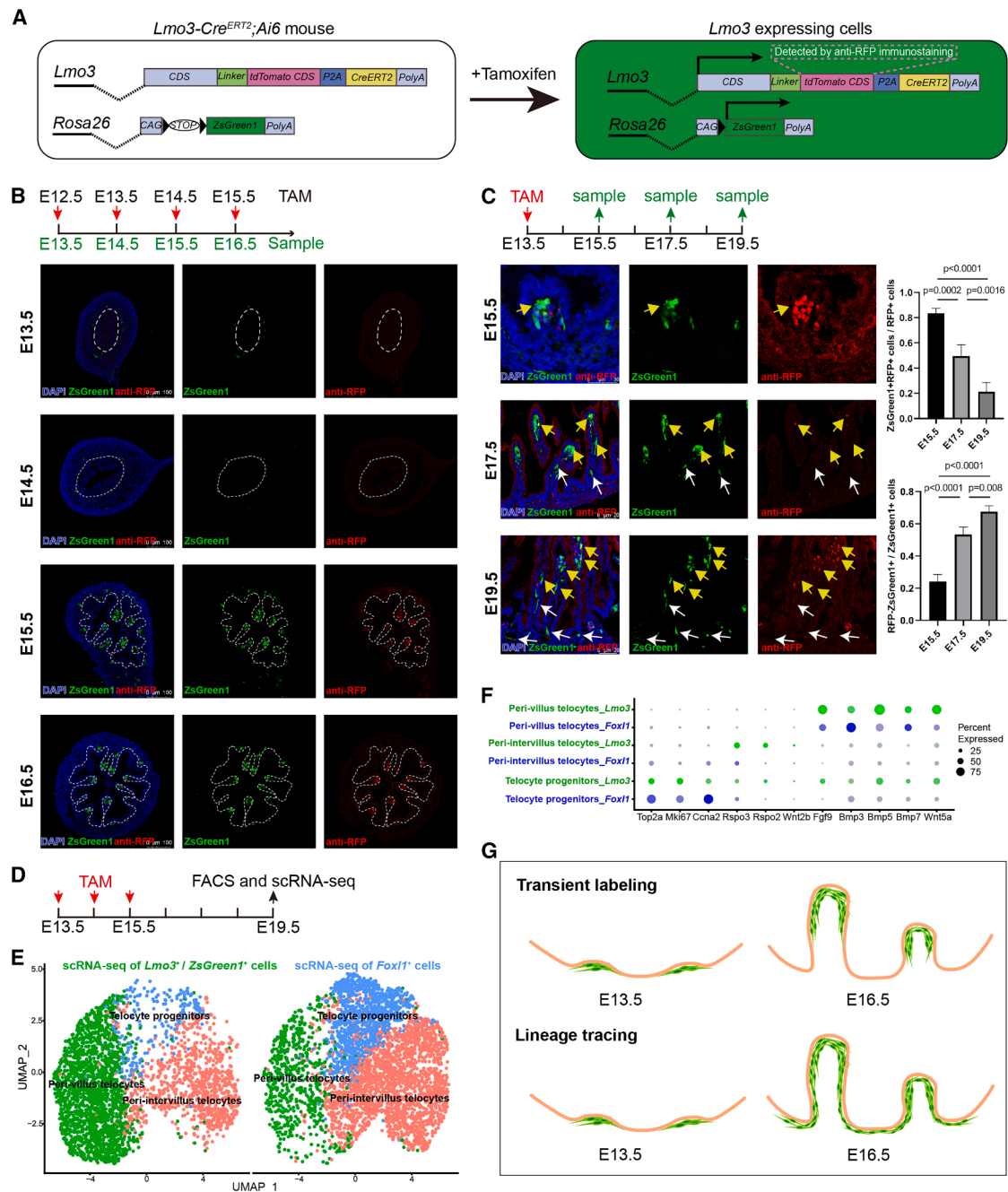


Figure 3. *Lmo3* marks peri-villus telocyte that can give rise to peri-intervillus telocyte as villus morphogenesis proceeds

(A) Generation of mice expressing *Lmo3*-tdTomato fusion protein and Cre^{ERT2} from a single bicistronic message by gene knockin into the end of the last exon of *Lmo3*. Note that *Lmo3*-tdTomato can only be detected by immunostaining using anti-RFP antibody.

(B) TAM was injected intraperitoneally 1 day before sampling during embryonic stages. *Lmo3*-tdTomato was detected using anti-RFP antibody.

(C) *Lmo3*⁺ peri-villus telocytes were lineage traced after TAM induction at E13.5. ZsGreen1⁺ progenies were detected in 2/4/6 days PTI. Yellow arrow, ZsGreen1⁺RFP⁺ cells. White arrow, progenies from *Lmo3*⁺ telocytes lack of *Lmo3*-tdTomato expression but retain ZsGreen1 lineage marker. *Lmo3*-tdTomato was stained by anti-RFP antibody. The p values were calculated by ordinary one-way ANOVA multiple comparisons. ±SD, n = 3 images from 3 embryonic mice.

(D) *Lmo3*⁺ cells were labeled by three doses of TAM induction from E13.5 to E15.5. At E19.5, cells with the expression of lineage marker ZsGreen1 were collected by FACS (fluorescence-activated cell sorting), and analyzed by scRNA-seq.

(E) UMAP plot showing the scRNA-seq data from the *Lmo3-Cre^{ERT2};Ai6* traced cells (left) and the *Foxl1*⁺ telocytes characterized in Figure 2B (right).

(F) The expression profile of selected Wnts or BMPs, representing peri-intervillus telocytes or peri-villus telocytes, in identified clusters from *Lmo3-Cre^{ERT2};Ai6* traced cells (green) or *Foxl1*⁺ telocytes (blue).

(G) Schematic showing that *Lmo3*⁺ telocytes in villus-tip can contribute to both peri-villus and peri-intervillus telocytes as villigenesis initiated.

was barely detectable until E15.5, when it was found to be specifically expressed in the nuclei of mesenchymal cells at the villus tip (Figure 3B). The relatively late expression of the tdTomato reporter might be due to the low expression level of *Lmo3* prior to E15.5 or delayed protein maturation.

We next performed pulse-chase labeling of *Lmo3-Cre^{ERT2}*; *Ai6* mice labeled at E13.5 and examined at 2-, 4-, and 6-day post tamoxifen (TAM) induction (PTI) (Figure 3C). As expected, the initially ZsGreen1-labeled cells were mesenchymal cells at the villus tip and were RFP⁺ (Figure 3C). Interestingly, as the development proceeds from E15.5 to E19.5, the ZsGreen1-labeled mesenchymal cells gradually cover both villus and intervillus regions and become RFP⁺ cells (Figure 3C). These observations suggest that the *Lmo3*⁺ cell clusters initially appeared at E13.5–E14.5 can give rise to both peri-villus and peri-intervillus telocytes as villus morphogenesis proceeds (Figure 3G).

We also sorted the *Lmo3*⁺ telocytes and the lineage-traced progenies from E19.5 fetus and analyzed them by scRNA-seq (Figure 3D). Comparing the scRNA-seq data from lineage-traced *Lmo3*⁺ or *ZsGreen1*⁺ cells with the data from *Foxl1*⁺ cells extracted from E12.5–E19.5, we found that the telocytes characterized in these two datasets were highly similar, containing both peri-villus and peri-intervillus telocytes (Figures 3E and 3F), which again suggests that *Lmo3*⁺ peri-villus telocytes, once emerged, contribute to the entire telocyte population in small intestine before birth (Figure 3G). Therefore, the *Lmo3*⁺ telocytes localized at the villus tip serve as the origin of the telocyte population prior to birth.

***Lmo3*⁺ telocytes are gradually confined to the isthmus region after birth**

As *Lmo3*⁺ cells serve as the cellular origin for the telocyte population prior to birth, we asked whether the *Lmo3*⁺ cells persist after birth. Initially, the *LMO3*-tdTomato (visualized by immunostaining with anti-RFP antibody) was found to label all peri-villus telocyte of newborn mice, but its expression became scattered at around post-natal day 10 (P10D) and barely detectable in adult mice (Figure S3A). This dynamic pattern was virtually identical to the pattern of *Lmo3* transcripts detected by RNA-seq (Figure S3B) and is likely due to the decreased level of *Lmo3* expression after birth. Thus, *Lmo3* expression was examined using *Lmo3-Cre^{ERT2}*; *Ai6* mice in subsequent investigations.

Pulsed labeling of *Lmo3*⁺ cells using *Lmo3-Cre^{ERT2}*; *Ai6* mice revealed that, at birth, *Lmo3* marked telocytes at villus tip as well as the telocyte clusters where new villi were about to sprout (Figure S3A). Strikingly, these cells quickly changed their spatial patterns and exhibited a tendency to be confined to the isthmus region (the region connecting the villus base and the crypt neck) starting from around P10D (Figure 4A), coincident with the time point when the crypt structure is formed.³¹ Moreover, the population of these cells was declined progressively, with only a few cells remaining at the isthmus region by around P20D and in adult mice (Figure 4A). To evaluate the persistence of these cells, we examined their presence as mice age. Remarkably, these cells appeared to be long-lived, as their number remained static at adult stages and these cells could still be observed in aged mice, even in mice of 2 years old (Figures 4A and 4B).

The isthmus-specific localization of *Lmo3*⁺ cells was typical along the length of small intestine but showed more variations in the duodenum and ileum regions, as sometimes sparsely labeled

Lmo3⁺ cells could also be found in villus regions as well. To exclude potential confounding effects attributed to the *Ai6* reporter system, we employed two additional individual reporter systems by crossing *Lmo3-Cre^{ERT2}* mice with *Ai14* (*Rosa-CAG-LSL-tdTomato*) and *Rosa26-H2B-mCherry* lines, generating *Lmo3-Cre^{ERT2}*; *Ai14* and *Lmo3-Cre^{ERT2}*; *Rosa26-H2B-mCherry* mice, respectively. *Lmo3*⁺ telocytes labeled by these two additional reporter systems also showed their specific residence at the isthmus region in adult jejunum (Figures S3C and S3D).

Given the earlier observation that the embryonic *Lmo3*⁺ cells can give rise to both peri-villus and peri-intervillus telocytes, we wondered whether these *Lmo3*⁺ cells in the isthmus region remain as multipotent cells. The P10D mice (*Lmo3-Cre^{ERT2}*; *Ai6*) were labeled transiently, and intestines were then sampled and analyzed at 2, 4, and 8 days later (Figure 4C). As expected, the initially labeled *Lmo3*⁺ telocytes around the isthmus region gradually expanded and migrated upward and reached the villus tip after about 8 days. Meanwhile, the labeled telocytes also migrated downward to reach the crypt bottom (Figure 4C). These observations indicate that the telocytes at isthmus region retain high proliferative activity in neonatal mice, and they can generate progenies that migrate and differentiate into both peri-villus and peri-crypt telocytes.

To test whether the peri-isthmus telocytes specifically retain high proliferative activity, a single dosage of EdU (5-ethynyl 2'-deoxyuridine) was injected intraperitoneally for pulse-chase analysis using *Lmo3-Cre^{ERT2}*; *Ai6* mice of 31 days old that had been lineage-traced for 3 weeks (Figures 4D and 4E). By characterizing the distribution of ZsGreen1⁺EdU⁺ telocytes, we observed a predominant enrichment of proliferative telocytes at isthmus, and these EdU-labeled cells gradually migrated bidirectionally toward villus or crypt regions after 7 days (Figures 4D–4G). We next introduced the expansion index to quantify the spatial dispersion of EdU⁺ telocytes away from the isthmus region, which showed a significant increase in 7 days after EdU treatment (Figure 4H). Thus, the *Lmo3*⁺ telocytes clustered at the isthmus region after birth specifically retain high proliferative activity, and similar to the embryonic *Lmo3*⁺ telocytes, these cells can generate progenies that spread out and wrap around the entire epithelium.

The isthmus *Lmo3*⁺ telocytes in adult small intestine are slow-cycling progenitor cells

The above observations pointed to a possibility that the *Lmo3*⁺ isthmus telocyte in adult small intestine may represent a population of long-lived progenitor cells for telocytes. To test this possibility, adult *Lmo3-Cre^{ERT2}*; *Ai6* or *Lmo3-Cre^{ERT2}*; *Ai14* mice at post-natal 2 months (P2M) were injected intraperitoneally with a single dose of TAM, lineage traced, and sampled at various ages (Figure 5A). *Lmo3*⁺ telocytes, which were initially enriched in the isthmus region in adult mice, were indeed able to give rise to progenies covering the entire crypt-villus axis, but this occurred in an extremely slow speed (Figures 5A–5C). Significant cell expansion and spreading away from the isthmus region was not evident until after 2 months of lineage tracing, when some clones reached the middle villus region. It took approximately 6 months for some clones (approximately 10%) to reach the tip region of villus, and the majority of clones were able to reach the tip of villus after a year. Significant expansion and spreading to the bottom of the crypt also occurred, but it was not evident

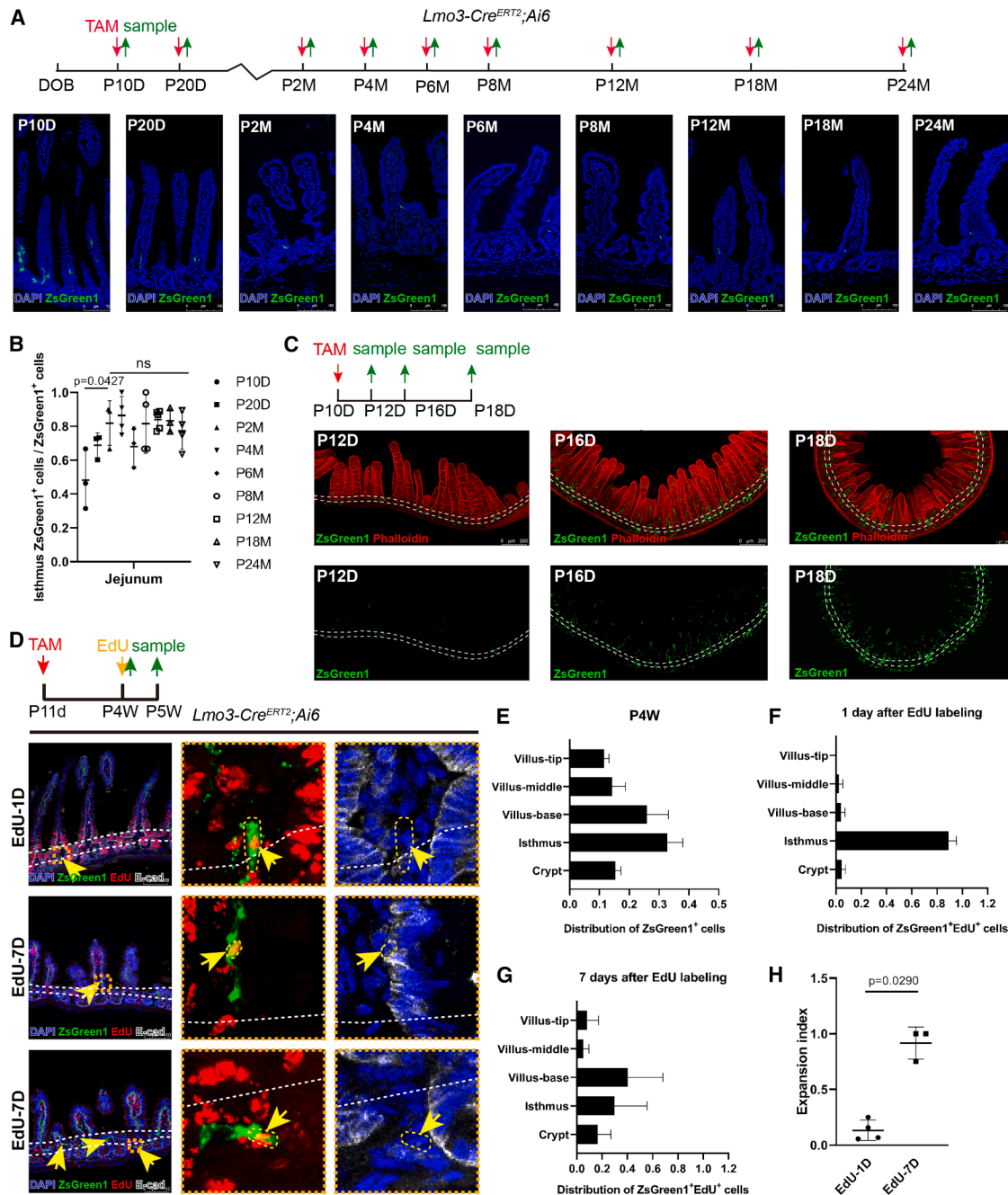


Figure 4. *Lmo3*⁺ telocytes are gradually confined to the isthmus region after birth

(A) *Lmo3-Cre^{ERT2};Ai6* mice were examined at multiple time points after birth through transient TAM labeling. Mice of different age were sampled 1 day PTI.

(B) Calculated cell ratio of isthmus-located ZsGreen1⁺ cells versus all ZsGreen1⁺ cells. At each time point, jejunum from at least three mice, with more than 30 intact villi each mouse, were analyzed. *P* values were calculated by ordinary one-way ANOVA multiple comparisons, $\pm$ SD, $n \geq 3$ mice for each time point.

(C) *Lmo3-Cre^{ERT2};Ai6* mice were labeled at P10D and sampled in 2/4/8 days PTI. The SI were whole-mounted and stained with phalloidin-568 (red).

(D) Telocytes along the crypt-villus axis were labeled through TAM injection at P10D and traced for 3 weeks, and the proliferative ability of telocytes was detected at P4W young mice through EdU pulse-chase labeling. Yellow arrow and dashed circle: ZsGreen1⁺EdU⁺ telocytes.

(E) The distribution of labeled telocytes in about 3 weeks PTI at P10D. Jejunum from at least three mice, with more than 30 intact villi in each mouse, were analyzed, $\pm$ SD.

(F and G) The distribution of ZsGreen1⁺EdU⁺ telocytes along the crypt-villus axis in 1 day (F) or 7 days (G) post EdU labeling. Jejunum from at least three mice, with more than 30 intact villi in each mouse, were analyzed, $\pm$ SD.

(H) The expansion index of ZsGreen1⁺EdU⁺ telocytes in 1 day or 7 days post EdU labeling, $\pm$ SD. The *p* value was calculated by paired *t* test, SD = 0.2288, SEM = 0.1321.

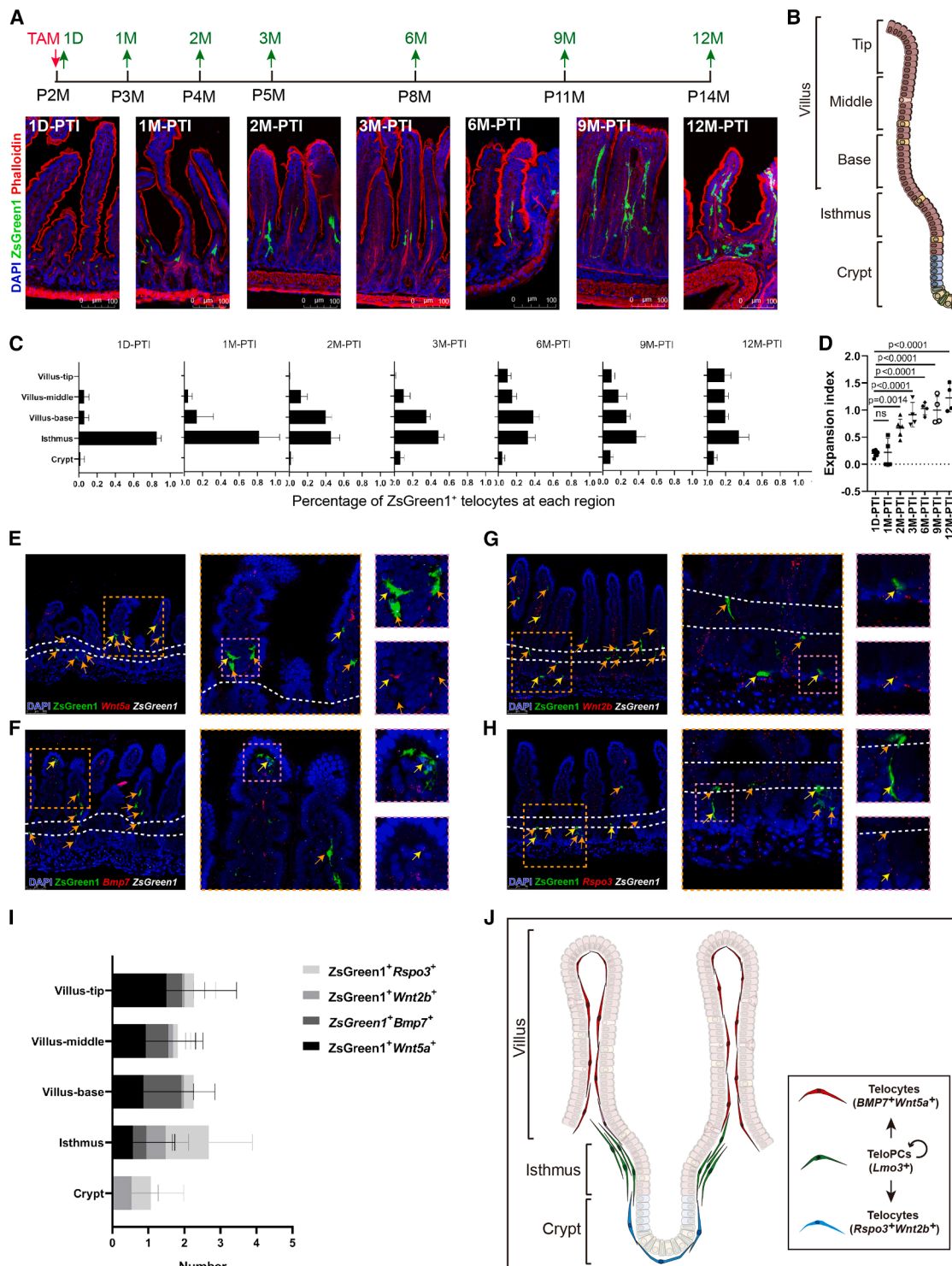


Figure 5. The peri-isthmus *Lmo3*⁺ telocytes in adult small intestine are slow-cycling, multipotent cells

(A) Mice from different time points were sampled after a single dose of TAM injection at P2M intraperitoneally.

(B) A diagram shows that SI is further defined into 5 regions, where the villus is divided into three equidistant regions: villus-tip, villus-middle, and villus-base.

(C) The distributions of labeled ZsGreen1⁺ telocytes along the crypt-villus axis in mice 1 day, 1 month, 2 months, 3 months, 6 months, 9 months, and 12 months PTI. For each time point, jejunum from at least three mice, with more than 30 intact villi each mouse, were sampled and analyzed, $\pm$ SD.

(D) The expansion index of labeled ZsGreen1⁺ telocytes in multiple time points PTI at P2M. The *p* values were calculated through ordinary one-way ANOVA multiple comparisons, $\pm$ SD, *n* $\geq$ 3 mice.

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until 3 months later (Figures 5A–5D and S4A). Therefore, these *Lmo3*⁺ cells are slow-cycling cells and able to generate both peri-villus and peri-crypt telocytes.

To test whether the activity of these *Lmo3*⁺ cells declines with age, 12-month-old mice with appropriate genotype were lineage labeled and examined at 3, 6, and 9 months, respectively, after TAM injection (Figures S4B–S4D). We found that in these aged mice, the initially labeled peri-isthmus telocytes could similarly give rise to new cells that migrated toward villus tip or crypt bottom, albeit at a slightly reduced speed: the expansion index at 9M-PTI was 0.8 compared with 1.0 in young mice (Figures S4B–S4D). Therefore, the peri-isthmus *Lmo3*⁺ telocytes are long-lived and slow-cycling progenitor cells.

This cell migration model predicts that if peri-villus telocytes are specifically labeled, these cells would migrate toward the villus tip but not in the opposite direction. To label and trace peri-villus telocytes, we generated *Foxl1-Cre^{ERT2}* mice, in which the tdTomato-P2A-Cre^{ERT2} cassette was inserted immediately before the stop codon of the *Foxl1* coding sequence. Although FOXL1-tdTomato signal was undetectable in the small intestine—likely due to low *Foxl1* expression—the *Foxl1-Cre^{ERT2}; Ai6* system robustly labeled peri-villus and peri-isthmus telocytes following full-dose TAM induction. Peri-crypt telocytes, however, were only sporadically labeled (Figure S5A). This is consistent with the previous observation that peri-villus telocytes have higher expression levels of *Foxl1* than peri-crypt telocytes.³ Interestingly, we found that one time injection of TAM to *Foxl1-Cre^{ERT2}; Ai6* mice was able to make specific labeling to peri-villus telocytes in some villi, with peri-isthmus telocytes being spared (Figures S4E and S4F). In these cases, we observed a gradual and persistent migration of these labeled telocytes toward the villus tip, until the labeled cells were localized only at the tip region (Figures S4E and S4F). In contrast, for villi with peri-isthmus telocytes simultaneously labeled, the labeled telocytes were constantly found along the base-tip axis of the villus (Figures S4E and S4F). These observations further support the notion that in adult mice, the *Lmo3*⁺ peri-isthmus telocytes, but not peri-villus telocytes, can self-renew and continuously give rise to daughter cells that migrate along the basement membrane toward the villus tip.

Telocytes in adult small intestine are known to be heterogeneous, each secreting different sets of niche factors along the crypt-villus axis.³² For instance, *BMPs* and *Wnt5a* (noncanonical WNT) are expressed in peri-villus telocytes, whereas *Wnt2b* (canonical WNT) and *Rspo3* are expressed in peri-crypt telocytes.¹³ To determine whether the daughter cells derived from *Lmo3*⁺ telocytes are able to differentiate into these two major types of telocytes, we performed RNA *in situ* analysis by RNAscope of the lineage-traced progenies derived from the isthmus *Lmo3*⁺ telocytes. We found that *Wnt5a* and *Bmp7* were preferentially detected in lineage-labeled cells that are peri-villus, whereas *Wnt2b* and *Rspo3* were preferentially detected in lineage-labeled cells that are peri-crypt (Figures 5E–5I). Thus, the peri-crypt and peri-villus telocytes are not strictly two distinct types

of cells but rather groups of heterogeneous cells that show gradient transition of WNT/BMP signal production ratios.

Taken together, we conclude that the *Lmo3*⁺ telocytes at the isthmus region are long-lived and slow-cycling progenitor cells that sustain the renewal of the telocyte niche in adult small intestine. We here after name them as telocyte progenitor cells (TeloPCs) (Figure 5J).

Irradiation induces damage and repair of the telocyte niche

One distinctive feature for stem cells is their ability to regenerate tissue following damage, especially for stem cells that are normally dormant. Before evaluating this ability for TeloPCs, we need to establish a method to induce damage to the telocyte niche. We used *Foxl1-Cre^{ERT2}; Ai6* mice to broadly label the subepithelial telocytes and explored a number of methods that could induce robust damage to telocyte. We found that the application of 8 gray of ionizing radiation (IR) treatment was able to induce rapid damage to telocytes (Figures S5A–S5H). Normally, the *Foxl1*⁺ telocytes forms a continuous layer of cells surrounding the intestinal epithelium (Figures S5B and S5E). However, 3 days following radiation, this continuous layer of cells was broken into interrupted patches, indicating partial loss of telocytes (Figures S5C, S5F, and S5H).

As telocytes provide BMP signals for intestinal epithelial cell differentiation, we examined whether this partial loss of telocytes could affect the BMP signaling activity of the intestinal epithelial cells. In the control epithelium, the BMP signaling activity exhibited a gradient pattern along the crypt-villus axis, as indicated by pSmad1/5 staining (Figure S5I). However, following radiation damage, the epithelial pSmad1/5 signal became scattered patches (Figure S5J). In addition, the locations where complete loss of pSmad1/5 signal occurred were associated with the loss of telocytes in those areas (Figure S5J). The damage appears to be repaired quickly, as indicated by the recovery of epithelial pSmad1/5 signals at day 7 following radiation (Figures S5K and S5O). Interestingly, the telocyte layer was also largely restored by day 7 (Figures S5D and S5G). Collectively, these observations provide direct evidence for the peri-villus telocyte in regulating BMP signaling activity in the villus epithelial cells.

We further examined whether the partial loss of telocytes affect the differentiation of the villus epithelial cells. It is known that the villus is functionally zonated and the newly formed enterocytes transverse a serial of cell states during their migration along the crypt-villus axis: the villus-base enterocytes show relatively high expression of multiple anti-microbial genes, whereas the villus-tip enterocytes show relatively high expression of purine catabolic genes (*Enpp3*, *Nt5e*, *Slc28a2*, and *Ada*).³³ A previous study reported that this villus tip program is regulated by BMP2 from the mesenchymal cells.³⁴ We found that the mosaic loss of telocyte caused by radiation dramatically disrupted the expression of villus tip protein NT5E, and some villi showed partial or mosaic loss of NT5E at the villus tip

(E–H) *In vivo* expression of *Wnt5a* (E), *Bmp7* (F), *Wnt2b* (G), and *Rspo3* (H) were detected through RNAscope in 12M-PTI samples (yellow arrows). Brown arrows: ZsGreen1⁺ cells.

(I) The distribution of ZsGreen1⁺ cells, containing *Rspo3*, *Wnt2b*, *BMP7*, or *Wnt5a* signals, along the crypt-villus axis. The spatial localizations of double positive cells from at least fifteen 600 μ m square images from two mice were analyzed, $\pm$ SD.

(J) Schematic showing peri-isthmus TeloPCs (green, *Lmo3*⁺) can self-renewal and give rise to both peri-villus (red) and peri-crypt (blue) telocytes in adult mice.

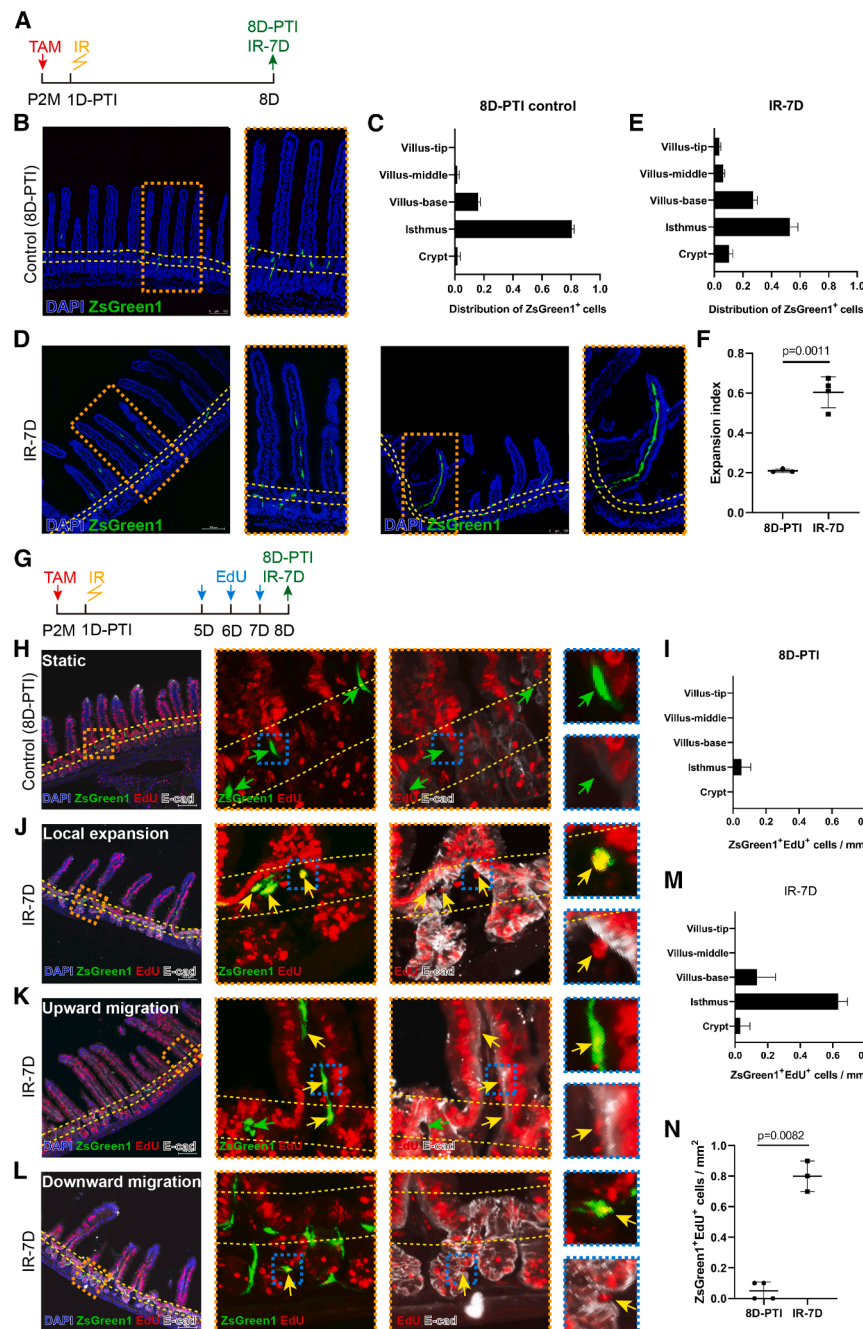


Figure 6. TeloPCs are rapidly activated following irradiation damage

(A) Transiently labeled *Lmo3-Cre^{ERT2}; Ai6* mice were irradiated through 8 Gy ionizing radiation (IR) and examined in 7 days later (IR-7D). Control mice without IR were also sampled in 8 days PTI (8D-PTI).

(B) Labeled TeloPCs in the control mice without irradiation.

(C) The distribution of ZsGreen1⁺ cells along the crypt-villus axis in 8D-PTI control mice. Jejunum from three mice, with more than 30 intact villi in each mouse, were sampled and analyzed, $\pm$ SD, $n = 3$ mice.

(D) In mice with IR, labeled TeloPCs were detected at both villus tip and crypt bottom.

(E) The distribution of ZsGreen1⁺ cells along the crypt-villus axis in 7 days after IR. Jejunum from four mice, with more than 30 intact villi in each mouse, were analyzed, $\pm$ SD, $n = 4$ mice.

(F) The expansion index of ZsGreen1⁺ cells in mice with or without irradiation, $\pm$ SD. The p value was calculated by paired t test, SD = 0.02453, SEM = 0.01416.

(G) For the control group, mice were sampled in 8D-PTI. For the radiated group, *Lmo3-Cre^{ERT2}; Ai6* mice were irradiated at 1D-PTI and sampled after 7 days (IR-7D). EdU were injected intraperitoneally from 5D-PTI to 7D-PTI each day at 50 mg/kg.

(H) EdU signals could barely be detected in homeostatic control mice. Green arrows indicating ZsGreen1⁺EdU⁺ telocytes.

(I) The distribution of ZsGreen1⁺EdU⁺ telocytes along the crypt-villus axis in the 8D-PTI control group was calculated using ten 1 mm² images from each mouse, $\pm$ SD, $n = 4$ mice.

(J–L) TeloPCs were activated rapidly after IR showing local expansion (J), upward migration (K), and downward migration (L).

(M) The distribution of ZsGreen1⁺EdU⁺ telocytes along the crypt-villus axis in the IR-7D radiated group was calculated using ten 1 mm² images from each mouse, $n = 3$ mice, $\pm$ SD.

(N) Number of detected ZsGreen1⁺EdU⁺ telocytes per 1 mm² image in 8D-PTI control and IR-7D mice, $\pm$ SD. The p value was calculated by paired t test, SD = 0.1155, SEM = 0.06667.

(Figures S5L, S5M, and S5P). Similarly, the expression of NT5E were restored by day 7 following radiation, when the telocyte layer was also repaired (Figures S5N and S5P).

With this damage-and-repair model established for the telocyte niche, we then asked whether the repair of the telocyte niche is dependent on TeloPCs in this model.

***Lmo3*⁺ TeloPCs are required for irradiation-induced damage response**

The TeloPCs were transiently labeled in *Lmo3-Cre^{ERT2}; Ai6* adult mice, and these mice were subjected to the radiation

(Figures 6B and 6C). Strikingly, in radiation-treated mice, these lineage-labeled telocytes were dramatically expanded, migrated upward to the villus domain, and in some cases, these cells formed a continuous pathway and migrated all the way to the villus tip. The lineage-labeled cells also migrated downward to cover the bottom of the crypt (Figures 6D–6F). As mentioned earlier, this level of expansion and migration typically requires at least 6 months under normal homeostatic condition. Therefore, TeloPCs are able to quickly respond to telocyte damage by rapid activation, migration, and differentiation to restore the telocyte layer along the length of crypt-villus axis.

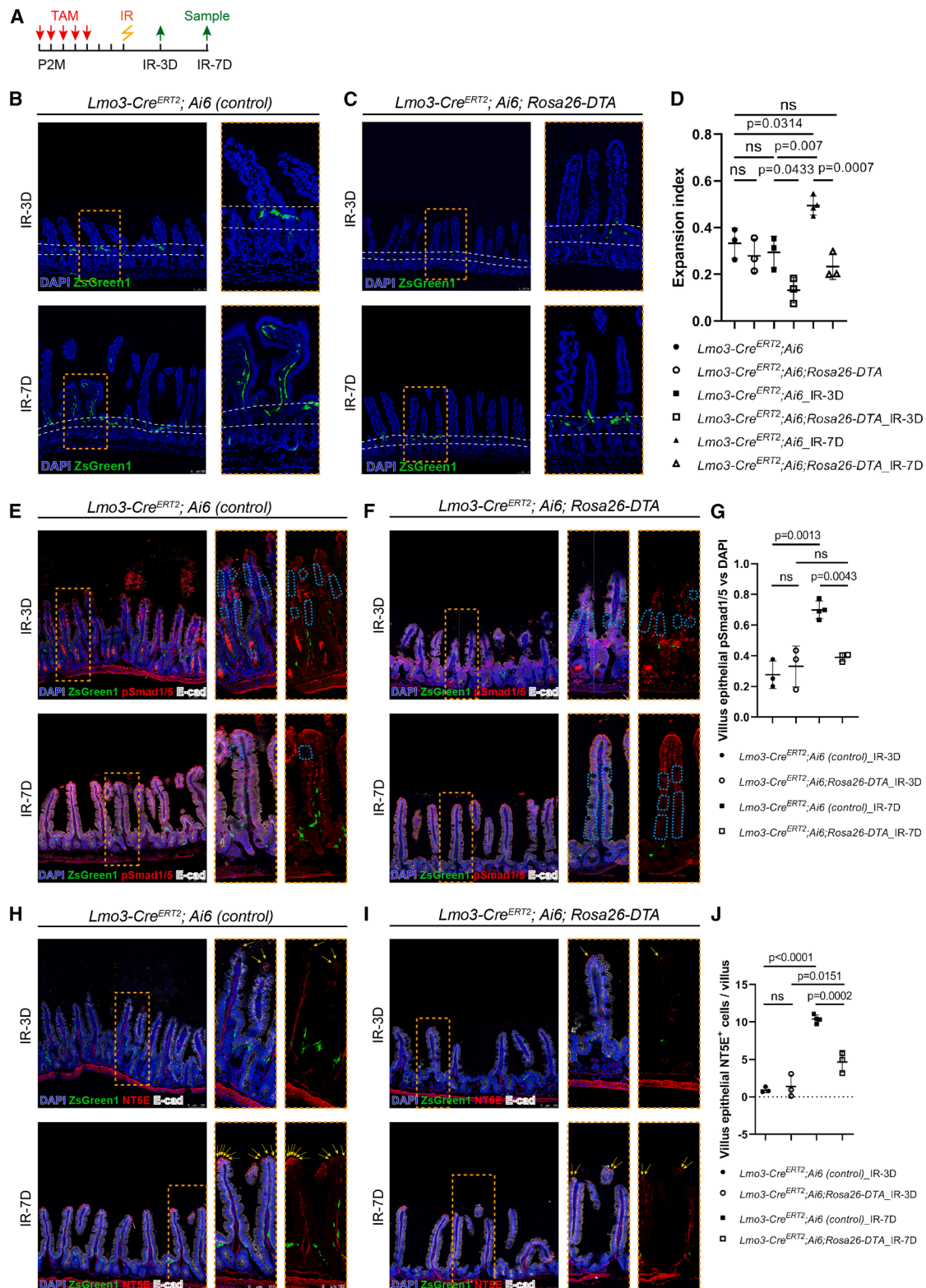


Figure 7. TeloPCs are required for telocyte repair following irradiation damage

(A) 5 dosages of TAM were injected to ablate TeloPCs, followed by 8 Gy of irradiation in 3 days after the last dose of TAM injection. Mice were sampled and analyzed in 3 and 7 days after IR.

(B and C) The distribution of ZsGreen1⁺ cells in mice without (B) or with (C) DTA ablation in 3 and 7 days after irradiation.

(legend continued on next page)

We next performed EdU labeling assay to assess whether the rapid expansion and recovery of the telocyte population is dependent on the division of TeloPCs but not differentiated telocytes. In control mice, virtually all lineage-labeled cells were found to be EdU negative (Figures 6G–6I). In irradiated mice, however, EdU positive cells were found in lineage-labeled cells localized specifically at the isthmus, villus base, or crypt bottom regions (Figures 6J–6N). These observations indicate that the recovery of the telocyte population following damage is dependent on the proliferation of TeloPCs.

To directly determine the requirement of TeloPCs in the irradiation-induced damage repair, we performed cell ablation experiment by genetically introducing diphtheria toxin (DTA) using *Lmo3-Cre^{ERT2}; Ai6; Rosa26-DTA* mice and analyzed the intestine in 3 and 7 days after irradiation (Figure 7A). Consistent with previous observations that the intestinal mesenchymal cells are somewhat resistant to TAM, ^{35,36} we found that the DTA approach did not completely deplete TeloPCs (Figures S6A and S6B). However, this approach was able to ablate approximately 41% of all genetically labeled *Lmo3*⁺ cells in the jejunum, and the remaining labeled cells, although still alive, seemed functionally impaired, as most of these cells appeared to remain quiescent and failed to supply new migratory telocytes by 7 days after irradiation (Figures 7B–7D and S6C–S6E). Consequently, the intestinal epithelial cells failed to re-establish the BMP signaling gradient along the crypt-villus axis by day 7 (Figures 7E–7G), and enterocyte differentiation was not restored back to normal (Figures 7H–7J).

To further assess the role of regenerated telocytes as a BMP source for epithelial repair after irradiation, we performed RNA *in situ* hybridization for *Bmp7*, a BMP ligand highly expressed in telocytes (Figure S1E). In control mice (*Lmo3-Cre^{ERT2}; Ai6*) at day 3 post-irradiation, *Bmp7* signal was mainly found in remaining ZsGreen1-labeled telocytes. By day 7, *Bmp7* signal was restored in newly formed telocytes migrating toward the villus tip (Figure S6F). However, in TeloPC-impaired mice (*Lmo3-Cre^{ERT2}; Ai6; Rosa26-DTA*) at day 7 after irradiation, subepithelial *Bmp7* signal was also decreased and remained only in ZsGreen1-labeled TeloPCs at the isthmus region (Figure S6G), indicating impaired ability for niche repair. Similar results were seen for *Wnt5a*, another telocyte-specific niche signal (Figures S6F and S6G). For comparison, we also examined *Rspo3* expression. Unlike *Bmp7*, *Rspo3* is only weakly expressed in telocytes but its expression is relatively higher in *Pdgfra^{low}* and other mesenchymal cells (Figure S1E). Therefore, while *Rspo3* expression was detected in regions beneath the epithelial layer, its expression levels in telocytes remained largely unchanged following telocyte ablation and regeneration (Figures S6F and S6G).

These data demonstrate that the peri-isthmus TeloPCs, characterized by slow cell cycle, can be activated in response to irradiation-induced damage. Subsequently, these activated TeloPCs undergo rapid expansion and migration and quickly repair the telocyte layer along the crypt-villus axis, thereby re-establishing the normal function of the telocyte niche.

TeloPC-derived peri-crypt telocytes contribute to ISC self-renewal

Previous studies have demonstrated an essential role for the *Foxl1*⁺ mesenchymal cells in sustaining ISCs. Genetic ablation of *Foxl1*⁺ cells, or knockout of the *Porcupine* (*Porcn*) gene (required for functional WNT signal production) specifically in *Foxl1*⁺ cells, leads to immediate ISC loss and crypt recession.^{3,4} However, *Foxl1* expression does not appear to be strictly limited to telocytes.³⁷ Gene expression profiles and *in vitro* organoid growth assays suggest that trophocytes, rather than telocytes, may be critical for ISC self-renewal.⁵ It is possible that non-specific or leaky expression of *Foxl1*-based genetic tools used in previous studies targeted other mesenchymal cell types, such as trophocytes, leading to the observed strong ISC loss phenotype following functional ablation of *Foxl1*⁺ cells.

Our identification of TeloPCs as the cellular origin of both peri-villus and peri-crypt telocytes provides an opportunity to re-examine the role of peri-crypt telocytes in ISC maintenance and intestinal homeostasis. To enable efficient labeling and ablation of *Lmo3*⁺ TeloPC-derived peri-crypt telocytes, we generated *Lmo3-Cre^{ERT2}; Ai6; Rosa-iDTR* mice. We initiated cell lineage tracing at 10 days after birth and induced targeted cell ablation after 50 days (when the mice were 2 months old) by injecting diphtheria toxin (Figure S7A). Given that TeloPCs are more proliferative in young mice, this strategy allows for near-complete labeling and ablation of all peri-crypt telocytes derived from TeloPCs (Figures S7B and S7C). Interestingly, we found that immediately following the ablation of these peri-crypt telocytes, the expression of *Olfm4*, an ISC marker, was significantly downregulated in the ISC niche at the crypt base (Figure S7D). However, crypt morphology and the number of Ki67⁺ proliferative cells within the crypt remained largely unchanged (Figure S7E). This contrasts with previous observations that ablation of *Foxl1*⁺ cells leads to rapid ISC depletion, the loss of proliferative cells, and crypt regression. Thus, peri-crypt telocytes contribute to, but are not essential for, ISC maintenance and proliferation. These results support the notion that peri-crypt telocytes represent one component of a mesenchymal cell niche that functions in concert

(D) The expansion index of ZsGreen1⁺ cells in mice with or without DTA ablation were calculated in 3 and 7 days after IR, respectively. The expansion index in mice without irradiation but with TAM induction were also analyzed. For each group, jejunum from at least three mice, with more than 30 intact villi from each mouse, were used and analyzed, $\pm$ SD, $n \geq 3$ mice. The *p* values were calculated using ordinary one-way ANOVA multiple comparisons.

(E and F) The BMP signaling within the intestinal epithelium was detected through pSmad1/5 staining in control mice without cell ablation (E) or mice with DTA (F) in 3 and 7 days after IR.

(G) The ratio of villus epithelial pSmad1/5⁺ cells in mice with or without DTA-induced TeloPC ablation. For each group, jejunum from at least three mice were sampled. For each mouse, the DAPI (4',6-diamidino-2-phenylindole)+pSmad1/5⁺ nucleus and DAPI⁺ nucleus from at least five intact villus epithelial regions were annotated using *imaris*, data were then collected to calculate *p* values using ordinary one-way ANOVA multiple comparisons, $\pm$ SD.

(H and I) The terminal differentiation of enterocytes was detected through NT5E staining in mice without (H) or with (I) cell ablation in 3 and 7 days after IR.

(J) The ratio of villus epithelial NT5E⁺ cells in mice with or without DTA-induced TeloPC ablation. For each group, jejunum from at least three mice were sampled. For each mouse, epithelial NT5E signals from more than 15 intact villi were annotated to calculate *p* values using ordinary one-way ANOVA multiple comparisons, $\pm$ SD.

with other mesenchymal and endothelial cells to support ISC self-renewal.

DISCUSSION

Our single-cell survey of mouse small intestine development has identified a population of telocyte progenitor cells that emerges during villus morphogenesis, maturing into adult stem cells that sustain the lifelong renewal and regeneration of the subepithelial telocyte niche. Furthermore, our single-cell data provide a valuable resource for advancing our understanding of the development of other subepithelial cells, including trophocytes, presumptive *Pdgfra*^{low}*Cd34*⁺ stromal cells, myofibroblasts, smooth muscle cells, and endothelial cells, as well as their signaling interactions with developing epithelial cells.

The discovery of TeloPC as a population of long-lived stem cells in adult small intestine adds another member to the category of quiescent cells that are promptly activated upon tissue damage.³⁸ TeloPCs are multipotent, capable of generating both peri-villus and peri-crypt telocytes. On the one hand, the peri-villus telocytes express a relatively high abundance of BMPs and noncanonical Wnts and are required for the differentiation of zonated enterocyte along the crypt-villus axis. On the other hand, the peri-crypt telocytes express Wnts and have relatively low production of BMPs, thereby promoting ISC self-renewal.^{5,7} However, the peri-crypt and peri-villus telocytes are not strictly two distinct types of telocytes but more likely two heterogeneous subpopulations of telocytes with gradient WNT/BMP signal production ratios. This property of telocytes indicates that their differentiation might be in turn regulated by the zonated epithelial cells and/or other types of mesenchymal cells along the length of the crypt-villus axis.

In studying the developmental emergence of *Lmo3*⁺ telocytes, our study brings insights into the cellular mechanisms underlying villus morphogenesis. Previous studies have established that at E14.5, the *Pdgfra*^{high} cells begin to form small cell clusters, which creates a basal curve of the associated epithelium, a process understood as an early sign of villus formation.³⁹ We find that these cluster-forming *Pdgfra*^{high} cells are distinctive from the rest of *Pdgfra*^{high} cells, in terms of their specific expression of *Lmo3* and their ability to generate both peri-villus and peri-intervillus telocytes. The *Lmo3*⁺ telocytes also exhibit enriched expression of cell migration, cytoskeleton organization, and extracellular matrix organization genes (Spreadsheet S1). In addition, the peri-villus telocytes had increased production of BMPs and noncanonical Wnts, which may help explain previous observations that villus epithelial cells are relatively less proliferative compared with the intervillus epithelial cells.⁴⁰ Collectively, these observations indicate that *Lmo3*⁺ telocytes, which emerge at E13.5–E14.5, may exert an instructive role for the initiation of villus morphogenesis that is, their clustering and migration initiates the aforementioned basal epithelial curve, and the secreted signals result in reduced proliferation of adjacent epithelial cells, which together leading to epithelial sprouting.

Telocytes are characterized by their long protrusions and are found in many tissues and organs, such as pancreas and lung.¹⁷ It would be interesting to investigate whether there are stem cells that sustain telocyte homeostasis in these organs. There are many different types of mesenchymal cells, and it is

tempting to speculate that there might be additional progenitor cell types that sustain different kinds of subepithelial mesenchymal cells. Notably, a population of *Grem1*⁺ reticular stem cells is known to reside in the isthmus as well, and it has been shown to slowly generate mesenchymal cells that migrate toward the villus and crypt regions.⁴¹ As *Grem1* is not expressed in telocytes or TeloPCs, the identity of the *Grem1*⁺ stem cells and the lineage relationships between *Grem1*⁺ stem cells and TeloPCs remain to be determined. A study examining the telocyte cluster based on single-cell data for human fetus development also reported that human telocyte progenitors also specifically express *Lmo3*,⁴² indicating that TeloPCs might also exist in humans. Finally, given studies linking subepithelial mesenchymal cells to intestinal inflammation and tumorigenesis,^{43–47} and the discovery of TeloPCs will improve our understanding of how mesenchymal cells influence intestinal development and diseases.

Limitations of the study

Telocytes in the adult small intestine are difficult to dissociate from the tissue, which hampers single-cell analysis of the complete telocyte lineage during adulthood. Although *Lmo3* can robustly label all telocyte progenitor cells during development, it is not an ideal marker for identifying all TeloPCs in adult mice. While *Lmo3*⁺ TeloPCs are activated to support repair of the damaged telocyte niche, it remains unclear whether additional sources of telocytes exist or if other mesenchymal cell types can transdifferentiate to contribute to telocyte niche repair. Moreover, *Lmo3* is expressed in multiple tissues and organs, and sustained ablation of *Lmo3*⁺ cells often result in lethality. Identifying a more specific and robust marker for this stem cell population would greatly facilitate the investigation of its biology and functional roles in both homeostasis and disease.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rongwen Xi (xirongwen@nibs.ac.cn).

Materials availability

This study did not generate new unique reagents. All mice generated in this study are available from the lead contact with a completed materials transfer agreement.

Data and code availability

- All sequencing data have been deposited at GEO and the National Genomics Data Center (NGDC) as GEO: GSE252975 and NGDC: CRA014306, respectively, and are publicly available as of the date of publication.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

The scRNA-seq data for five developmental stages was generated by G.Z., Z. J., and N.Q. The experimental design was conceived by D.J., G.Z., and R.X. Experiments were carried out by D.J. and G.Z., with assistance from Q.S., H.Y., and K.T. Bioinformatic analysis was performed by Y.Z. and J.W., with guidance from T.C. Mice *in vitro* fertilization was conducted by F.W. The manuscript was written by D.J., G.Z., and R.X.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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REFERENCES

1. Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haeghebarth, A., Korving, J., Begthel, H., Peters, P.J., et al. (2007). Identification of stem cells in small intestine and colon by marker gene *Lgr5*. *Nature* 449, 1003–1007. <https://doi.org/10.1038/nature06196>.
2. Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Shroyer, N.F., van de Wetering, M., and Clevers, H. (2011). Paneth cells constitute the niche for *Lgr5* stem cells in intestinal crypts. *Nature* 469, 415–418. <https://doi.org/10.1038/nature09637>.
3. Aoki, R., Shoshkes-Carmel, M., Gao, N., Shin, S., May, C.L., Golson, M.L., Zahm, A.M., Ray, M., Wiser, C.L., Wright, C.V.E., et al. (2016). *Foxl1*-expressing mesenchymal cells constitute the intestinal stem cell niche. *Cell. Mol. Gastroenterol. Hepatol.* 2, 175–188. <https://doi.org/10.1016/j.jcmgh.2015.12.004>.
4. Shoshkes-Carmel, M., Wang, Y.J., Wangenstein, K.J., Tóth, B., Kondo, A., Massasa, E.E., Itzkovitz, S., and Kaestner, K.H. (2018). Subepithelial telocytes are an important source of Wnts that supports intestinal crypts. *Nature* 557, 242–246. <https://doi.org/10.1038/s41586-018-0084-4>.
5. McCarthy, N., Manieri, E., Storm, E.E., Saadatpour, A., Luoma, A.M., Kapoor, V.N., Madha, S., Gaynor, L.T., Cox, C., Keerthivasan, S., et al. (2020). Distinct Mesenchymal Cell Populations Generate the Essential Intestinal BMP Signaling Gradient. *Cell Stem Cell* 26, 391–402.e5. <https://doi.org/10.1016/j.stem.2020.01.008>.
6. Krawczyk, J., McCarthy, N., Malagola, E., Tie, G., Madha, S., Boffelli, D., Wagner, D.E., Wang, T.C., and Shivdasani, R.A. (2023). Graded BMP signaling within intestinal crypt architecture directs self-organization of the Wnt-secreting stem cell niche. *Cell Stem Cell* 30, 433–449.e8. <https://doi.org/10.1016/j.stem.2023.03.004>.
7. McCarthy, N., Tie, G., Madha, S., He, R., Krawczyk, J., Maglieri, A., and Shivdasani, R.A. (2023). Smooth muscle contributes to the development and function of a layered intestinal stem cell niche. *Dev. Cell* 58, 550–564.e6. <https://doi.org/10.1016/j.devcel.2023.02.012>.
8. Goto, N., Goto, S., Imada, S., Hosseini, S., Deshpande, V., and Yilmaz, Ö. H. (2022). Lymphatics and fibroblasts support intestinal stem cells in homeostasis and injury. *Cell Stem Cell* 29, 1246–1261.e6. <https://doi.org/10.1016/j.stem.2022.06.013>.
9. Niec, R.E., Chu, T., Scherthanner, M., Gur-Cohen, S., Hidalgo, L., Pasolli, H.A., Luckett, K.A., Wang, Z., Bhalla, S.R., Cambuli, F., et al. (2022). Lymphatics act as a signaling hub to regulate intestinal stem cell activity. *Cell Stem Cell* 29, 1067–1082.e18. <https://doi.org/10.1016/j.stem.2022.05.007>.
10. Palikuqi, B., Rispal, J., Reyes, E.A., Vaka, D., Boffelli, D., and Klein, O. (2022). Lymphangiocrine signals are required for proper intestinal repair after cytotoxic injury. *Cell Stem Cell* 29, 1262–1272.e5. <https://doi.org/10.1016/j.stem.2022.07.007>.
11. McCarthy, N., Krawczyk, J., and Shivdasani, R.A. (2020). Cellular and molecular architecture of the intestinal stem cell niche. *Nat. Cell Biol.* 22, 1033–1041. <https://doi.org/10.1038/s41556-020-0567-z>.
12. Zhu, G., Hu, J., and Xi, R. (2021). The cellular niche for intestinal stem cells: a team effort. *Cell Regen.* 10, 1. <https://doi.org/10.1186/s13619-020-00061-5>.
13. Bahar Halpern, K., Massalha, H., Zwick, R.K., Moor, A.E., Castillo-Azofeifa, D., Rozenberg, M., Farack, L., Egozi, A., Miller, D.R., Averbukh, I., et al. (2020). *Lgr5*⁺ telocytes are a signaling source at the intestinal villus tip. *Nat. Commun.* 11, 1936. <https://doi.org/10.1038/s41467-020-15714-x>.
14. Brügger, M.D., and Basler, K. (2023). The diverse nature of intestinal fibroblasts in development, homeostasis, and disease. *Trends Cell Biol.* 33, 834–849. <https://doi.org/10.1016/j.tcb.2023.03.007>.
15. Degirmenci, B., Valenta, T., Dimitrova, S., Hausmann, G., and Basler, K. (2018). *GLI1*-expressing mesenchymal cells form the essential Wnt-secreting niche for colon stem cells. *Nature* 558, 449–453. <https://doi.org/10.1038/s41586-018-0190-3>.
16. Stzpouriginski, I., Nigro, G., Jacob, J.M., Dulauroy, S., Sansonetti, P.J., Eberl, G., and Peduto, L. (2017). CD34⁺ mesenchymal cells are a major component of the intestinal stem cells niche at homeostasis and after injury. *Proc. Natl. Acad. Sci. USA* 114, E506–E513. <https://doi.org/10.1073/pnas.1620059114>.
17. Kondo, A., and Kaestner, K.H. (2019). Emerging diverse roles of telocytes. *Development* 146, dev175018. <https://doi.org/10.1242/dev.175018>.
18. Walton, K.D., Kolterud, A., Czerwinski, M.J., Bell, M.J., Prakash, A., Kushwaha, J., Grosse, A.S., Schnell, S., and Gumucio, D.L. (2012). Hedgehog-responsive mesenchymal clusters direct patterning and emergence of intestinal villi. *Proc. Natl. Acad. Sci. USA* 109, 15817–15822. <https://doi.org/10.1073/pnas.1205669109>.
19. Grosse, A.S., Pressprich, M.F., Curley, L.B., Hamilton, K.L., Margolis, B., Hildebrand, J.D., and Gumucio, D.L. (2011). Cell dynamics in fetal

- intestinal epithelium: implications for intestinal growth and morphogenesis. *Development* 138, 4423–4432. <https://doi.org/10.1242/dev.065789>.
20. Mao, J., Kim, B.M., Rajurkar, M., Shivdasani, R.A., and McMahon, A.P. (2010). Hedgehog signaling controls mesenchymal growth in the developing mammalian digestive tract. *Development* 137, 1721–1729. <https://doi.org/10.1242/dev.044586>.
21. Linda Karlsson, P.L., Heath, J.K., and Betsholtz, C. (2000). Abnormal gastrointestinal development in PDGF-A and PDGFR-alpha deficient mice implicates a novel mesenchymal structure with putative instructive properties in villus morphogenesis. *Development* 127, 3457–3466. <https://doi.org/10.1242/dev.127.16.3457>.
22. Walton, K.D., Whidden, M., Kolterud, Å., Shoffner, S.K., Czerwinski, M.J., Kushwaha, J., Parmar, N., Chandrasekhar, D., Fredo, A.M., Schnell, S., et al. (2016). Villification in the mouse: Bmp signals control intestinal villus patterning. *Development* 143, 427–436. <https://doi.org/10.1242/dev.130112>.
23. Huycke, T.R., Häkkinen, T.J., Miyazaki, H., Srivastava, V., Barluet, E., McGinnis, C.S., Kalantari, A., Cornwall-Scoones, J., Vaka, D., Zhu, Q., et al. (2024). Patterning and folding of intestinal villi by active mesenchymal dewetting. *Cell* 187, 3072–3089.e20. <https://doi.org/10.1016/j.cell.2024.04.039>.
24. Nigmatullina, L., Norkin, M., Dzama, M.M., Messner, B., Sayols, S., and Soshnikova, N. (2017). Id2 controls specification of Lgr5+ intestinal stem cell progenitors during gut development. *EMBO J.* 36, 869–885. <https://doi.org/10.15252/embj.201694959>.
25. Clevers, H., Loh, K.M., and Nusse, R. (2014). Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. *Science* 346, 1248012. <https://doi.org/10.1126/science.1248012>.
26. Popescu, L.M., and Faussone-Pellegrini, M.S. (2010). TELOCYTES - a case of serendipity: the winding way from Interstitial Cells of Cajal (ICC), via Interstitial Cajal-Like Cells (ICLC) to TELOCYTES. *J. Cell. Mol. Med.* 14, 729–740. <https://doi.org/10.1111/j.1582-4934.2010.01059.x>.
27. Johnson, N.C., Dillard, M.E., Baluk, P., McDonald, D.M., Harvey, N.L., Frase, S.L., and Oliver, G. (2008). Lymphatic endothelial cell identity is reversible and its maintenance requires Prox1 activity. *Genes Dev.* 22, 3282–3291. <https://doi.org/10.1101/gad.1727208>.
28. Kim, K.E., Sung, H.K., and Koh, G.Y. (2007). Lymphatic development in mouse small intestine. *Dev. Dyn.* 236, 2020–2025. <https://doi.org/10.1002/dvdy.21200>.
29. Lin, Z., Gao, C., Ning, Y., He, X., Wu, W., and Chen, Y.G. (2008). The pseudoreceptor BMP and activin membrane-bound inhibitor positively modulates Wnt/beta-catenin signaling. *J. Biol. Chem.* 283, 33053–33058. <https://doi.org/10.1074/jbc.M804039200>.
30. Yamaguchi, Y., Mann, D.M., and Ruoslahti, E. (1990). Negative regulation of transforming growth factor-beta by the proteoglycan decorin. *Nature* 346, 281–284. <https://doi.org/10.1038/346281a0>.
31. Sumigay, K.D., Terwilliger, M., and Lechler, T. (2018). Morphogenesis and Compartmentalization of the Intestinal Crypt. *Dev. Cell* 45, 183–197.e5. <https://doi.org/10.1016/j.devcel.2018.03.024>.
32. Rosa, I., Marini, M., and Manetti, M. (2021). Telocytes: An Emerging Component of Stem Cell Niche Microenvironment. *J. Histochem. Cytochem.* 69, 795–818. <https://doi.org/10.1369/00221554211025489>.
33. Moor, A.E., Harnik, Y., Ben-Moshe, S., Massasa, E.E., Rozenberg, M., Eilam, R., Bahar Halpern, K., and Itzkovitz, S. (2018). Spatial Reconstruction of Single Enterocytes Uncovers Broad Zonation along the Intestinal Villus Axis. *Cell* 175, 1156–1167.e15. <https://doi.org/10.1016/j.cell.2018.08.063>.
34. Berková, L., Fazilat, H., Yang, Q., Kubovciak, J., Stastna, M., Hrckulak, D., Vojtechova, M., Dalessi, T., Brügger, M.D., Hausmann, G., et al. (2023). Terminal differentiation of villus tip enterocytes is governed by distinct Tgfbeta superfamily members. *EMBO Rep.* 24, e56454. <https://doi.org/10.15252/embr.202256454>.
35. Chee, Y.C., Pahnke, J., Bunte, R., Adsool, V.A., Madan, B., and Virshup, D. M. (2018). Intrinsic Xenobiotic Resistance of the Intestinal Stem Cell Niche. *Dev. Cell* 46, 681–695.e5. <https://doi.org/10.1016/j.devcel.2018.07.023>.
36. Kolev, H.M., Tian, Y., Kim, M.S., Leu, N.A., Adams-Tzivelekis, S., Lengner, C.J., Li, N., and Kaestner, K.H. (2021). A FoxL1-CreERT-2A-tdTomato Mouse Labels Subepithelial Telocytes. *Cell. Mol. Gastroenterol. Hepatol.* 12, 1155–1158.e4. <https://doi.org/10.1016/j.jcmgh.2021.05.009>.
37. Kolev, H.M., and Kaestner, K.H. (2023). Mammalian Intestinal Development and Differentiation-The State of the Art. *Cell. Mol. Gastroenterol. Hepatol.* 16, 809–821. <https://doi.org/10.1016/j.jcmgh.2023.07.011>.
38. van Velthoven, C.T.J., and Rando, T.A. (2019). Stem Cell Quiescence: Dynamism, Restraint, and Cellular Idling. *Cell Stem Cell* 24, 213–225. <https://doi.org/10.1016/j.stem.2019.01.001>.
39. Walton, K.D., and Gumucio, D.L. (2021). Hedgehog Signaling in Intestinal Development and Homeostasis. *Annu. Rev. Physiol.* 83, 359–380. <https://doi.org/10.1146/annurev-physiol-031620-094324>.
40. Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. J., and Clevers, H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat. Genet.* 19, 379–383. <https://doi.org/10.1038/1270>.
41. Worthley, D.L., Churchill, M., Compton, J.T., Taylor, Y., Rao, M., Si, Y., Levin, D., Schwartz, M.G., Uygur, A., Hayakawa, Y., et al. (2015). Gremlin 1 identifies a skeletal stem cell with bone, cartilage, and reticular stromal potential. *Cell* 160, 269–284. <https://doi.org/10.1016/j.cell.2014.11.042>.
42. Holloway, E.M., Czerwinski, M., Tsai, Y.-H., Wu, J.H., Wu, A., Childs, C.J., Walton, K.D., Sweet, C.W., Yu, Q., and Glass, I. (2020). Mapping Development of the Human Intestinal Niche at Single-Cell Resolution. *Cell Stem Cell* 28, 568–580.e4. <https://doi.org/10.1016/j.stem.2020.11.008>.
43. Roulis, M., Kaklamanos, A., Scherthanner, M., Bielecki, P., Zhao, J., Kaffé, E., Frommelt, L.S., Qu, R., Knapp, M.S., Henriques, A., et al. (2020). Paracrine orchestration of intestinal tumorigenesis by a mesenchymal niche. *Nature* 580, 524–529. <https://doi.org/10.1038/s41586-020-2166-3>.
44. Katajisto, P., Vaahtomeri, K., Ekman, N., Ventelä, E., Ristimäki, A., Bardeesy, N., Feil, R., DePinho, R.A., and Mäkelä, T.P. (2008). LKB1 signaling in mesenchymal cells required for suppression of gastrointestinal polyposis. *Nat. Genet.* 40, 455–459. <https://doi.org/10.1038/ng.98>.
45. Di Carlo, S.E., Raffenne, J., Varet, H., Ode, A., Granados, D.C., Stein, M., Legendre, R., Tuckermann, J., Bousquet, C., and Peduto, L. (2023). Depletion of slow-cycling PDGFRα+ADAM12+ mesenchymal cells promotes antitumor immunity by restricting macrophage efferocytosis. *Nat. Immunol.* 24, 1867–1878. <https://doi.org/10.1038/s41590-023-01642-7>.
46. Haramis, A.P.G., Begthel, H., van den Born, M., van Es, J., Jonkhoeer, S., Offerhaus, G.J.A., and Clevers, H. (2004). De Novo Crypt Formation and Juvenile Polyposis on BMP Inhibition in Mouse Intestine. *Science* 303, 1684–1686. <https://doi.org/10.1126/science.1093587>.
47. Howe, J.R., Roth, S., Ringold, J.C., Summers, R.W., Järvinen, H.J., Sistonen, P., Tomlinson, I.P., Houlston, R.S., Bevan, S., Mitros, F.A., et al. (1998). Mutations in the SMAD4/DPC4 gene in juvenile polyposis. *Science* 280, 1086–1088. <https://doi.org/10.1126/science.280.5366.1086>.
48. Bernier-Latmani, J., and Petrova, T.V. (2016). High-resolution 3D analysis of mouse small-intestinal stroma. *Nat. Protoc.* 11, 1617–1629. <https://doi.org/10.1038/nprot.2016.092>.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-RFP	Rockland	Cat# 600-401-379; RRID: AB_2209751
Rabbit monoclonal anti-Olfm4	Cell Signaling Technology	Cat# 39141S; RRID: AB_2650511
Rat monoclonal anti-Lyve1	ThermoFisher	Cat# 14-0443-82; RRID: AB_1633414
Rabbit polyclonal anti-aSMA	Abcam	Cat# ab5694; RRID: AB_2223021
Rabbit monoclonal anti-NT5E	Cell Signaling Technology	Cat# 13160S; RRID: AB_2716625
Rabbit monoclonal anti-pSmad1/5	Cell Signaling Technology	Cat# 9516; RRID: AB_491015
Rat monoclonal anti-Ki67	ThermoFisher	Cat# 14-5698-82; RRID: AB_10854564
Rat monoclonal anti-E cadherin	Abcam	Cat# ab11512; RRID: AB_298118
Goat anti-rabbit Alexa Fluor 488	ThermoFisher	Cat# A11034; RRID: AB_2576217
Goat anti-rabbit Alexa Fluor 568	ThermoFisher	Cat# A11036; RRID: AB_10563566
Goat anti-rabbit Alexa Fluor Cy5	ThermoFisher	Cat# A10523; RRID: AB_2534032
Goat anti-rat Alexa Fluor Cy5	ThermoFisher	Cat# A10525; RRID: AB_2534034
Horseradish enzyme labeled goat anti-rabbit IgG	ZSGB-BIO	Cat# ZB-2301; RRID: AB_2747412
Chemicals, peptides, and recombinant proteins		
Alexa Fluor 568 phalloidin	ThermoFisher	Cat# A12380
Collagenase I	Gibco	Cat# 17100-017
Collagenase	Sigma-Andrich	Cat# C2139-1G
Elastase	Sigma-Andrich	Cat# E2058
Dispase II	Gibco	Cat# 17105041
DNase I	Merk	Cat# 10104159001
Y27632 (5000X)	R&D Systems	Cat# 1254/50
TrypLE	Gibco	Cat# 12605-010
FBS	Gibco	Cat# 16000-044
ACK lysis buffer	Gibco	Cat# A10492-01
HBSS	Gibco	Cat# 14170-112
DPBS	Gibco	Cat# C14190500CP
Gentamicin / Amphotericin	Gibco	Cat# 50-0640
Penicillin / Streptomycin B	Gibco	Cat# 15140-122
HEPES	Sigma-Andrich	Cat# H0887-100ML
F12 culture medium	Gibco	Cat# 11765-054
Tamoxifen	Sigma-Andrich	Cat# T5648-5G
EdU	Sigma-Andrich	Cat# 900584
Diphtheria toxin	Merk	Cat# D0564
Paraformaldehyde	Sigma-Andrich	Cat# 441244-1KG
Bovine Serum Albumin	Merck	Cat# V900933
FocusClear	CelExplorer	Cat# FC-101
Antifade mounting media	Vector Laboratories	Cat# H-1000-10
Propidium Iodide	ThermoFisher	Cat# P3566
DAPI	Sigma-Andrich	Cat# D9542-10MG
Opal 520 reagent pack	Akoya Biosciences	Cat# FP1487001KT
Opal 570 reagent pack	Akoya Biosciences	Cat# FP1488001KT
Opal 650 reagent pack	Akoya Biosciences	Cat# FP1496001KT

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Click-iT™ Plus EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 555 dye	ThermoFisher	Cat# C10638
Alexa Fluor™ 555 Tyramide SuperBoost™ Kit, streptavidin	ThermoFisher	Cat# B40933
RNAscope Multiplex Fluorescent Reagent Kit v2	ACDBio	Cat# 323100
Deposited data		
GEO: Mouse small intestine developmental scRNA-seq	This paper	GSE252975
GSA: Mouse small intestine developmental scRNA-seq	This paper	CRA014306
Experimental models: Organisms/strains		
Mouse: <i>Lmo3-tdTomato-P2A-Cre^{ERT2}</i>	This paper	N/A
Mouse: <i>Foxl1-tdTomato-P2A-Cre^{ERT2}</i>	This paper	N/A
Mouse: <i>Ai6</i>	The Jackson Laboratory	Cat# 007906
Mouse: <i>Ai14</i>	The Jackson Laboratory	Cat# 007914
Mouse: <i>Rosa26-H2B-mCherry</i>	The Jackson Laboratory	Cat# 023139
Mouse: <i>Rosa26-DTA</i>	The Jackson Laboratory	Cat# 009669
Mouse: <i>Rosa26-iDTR</i>	The Jackson Laboratory	Cat# 008040
Software and algorithms		
Seurat	Satija lab	https://satijalab.org/seurat/
scvi-tools	scvi-tools development team	https://docs.scvi-tools.org/
Harmony	immunogenomics	https://github.com/immunogenomics/harmony
SeuratExplorer	fentouxngui	https://github.com/fentouxngui/SeuratExplorer
scRNAtoolVis	junjun lab	https://github.com/junjunlab/scRNAtoolVis
Cell Ranger	10× Genomics	https://www.10xgenomics.com/cn/support/software/cell-ranger/latest
Scrublet	swolock	https://github.com/swolock/scrublet
Pheatmap	CRAN	https://cran.r-project.org/web/packages/pheatmap/
Prism 8	GraphPad Software Inc.	https://www.graphpad.com/features
ImageJ	National Institutes of Health	https://imagej.net/ij/
Imaris	Oxford Instruments	https://imaris.oxinst.com/
Other		
Mm- <i>Foxl1</i>	ACDBio	Cat# 407401
Mm- <i>Lmo3</i>	ACDBio	Cat# 497631
Mm- <i>Lmo3-C3</i>	ACDBio	Cat# 497631-C3
Mm- <i>Agt</i>	ACDBio	Cat# 426941
Mm- <i>Lgr5</i>	ACDBio	Cat# 312171
Mm- <i>Lgr5-C3</i>	ACDBio	Cat# 312171-C3
Mm- <i>Bmp7</i>	ACDBio	Cat# 407901
Mm- <i>Wnt5a</i>	ACDBio	Cat# 316791
Mm- <i>Wnt2b-C2</i>	ACDBio	Cat# 405031-C2
Mm- <i>Rspo1-C2</i>	ACDBio	Cat# 401991-C2
Mm- <i>Rspo3-C2</i>	ACDBio	Cat# 402011-C2
Mm- <i>Rspo3-C3</i>	ACDBio	Cat# 402011-C3
Mm- <i>Nog</i>	ACDBio	Cat# 467391
Mm- <i>Acta2-C2</i>	ACDBio	Cat# 319531-C2
Mm- <i>ZsGreen1-C3</i>	ACDBio	Cat# 895651-C3
Probe diluent	ACDBio	Cat# 300041
4-plex positive control probe	ACDBio	Cat# 321811

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
4-plex negative control probe	ACDBio	Cat# 321831
Falcon 70μm cell strainer	Corning	Cat# 352350
Falcon 40μm cell strainer	Corning	Cat# 352340
Falcon 5 mL polystyrene tube	Corning	Cat# 352054
FLOWMI cell strainer, 40μm	Bel-Art	Cat# 136800040
BD FACSAria III cell sorter	BD biosciences	N/A
RS2000 pro X-ray irradiation apparatus	RAD Source	N/A
Tissue-Tek O.C.T. Compound	Sakura	Cat# 4583

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

All the research performance underwent strict ethical review and was approved by the Ethics Committee of the National Institute of Biological Sciences, Beijing. The embryonic small intestines (E12.5d, E13.5d, E14.5d, E15.5d, and E19.5d) isolated from wild-type C57BL/6J mice in this project were used to study the gene expression landscapes through single-cell RNA-seq transcriptome analyses. Wild-type C57BL/6J, *Ai6* (*Rosa-CAG-LSL-ZsGreen1*, JAX007906), *Ai14* (*Rosa-CAG-LSL-tdTomato*, JAX007914), *Rosa26-DTA* (*Rosa26-LSL-DTA*, JAX009669), *Rosa26-iDTR* (*Rosa26-LSL-iDTR*, JAX008040) and *Rosa26-H2B-mCherry* (*Rosa26-CAG-LSL-H2B-mCherry*, JAX023139) mice used in this project were from the Jackson Laboratory. *Lmo3-tdTomato-Cre^{ERT2}* and *Foxl1-tdTomato-Cre^{ERT2}* mice were generated via ViewSolid Biotech. All mice mentioned in this work were housed at the animal center in the National Institute of Biological Sciences in a Specific Pathogen Free (SPF) facility within individually ventilated cages as well as the controlled temperature, humidity, and light-dark cycle, and a regular rodent laboratory chow diet.

METHOD DETAILS

Antibodies and probes

Antibodies used in this study including: rab anti-RFP (Rockland, 600-401-379), rab anti-Olfm4 (CST, 39141S), rat anti-Lyve1 (Thermo Fisher, 14-0443-82), rab anti-aSMA (Abcam, ab5694), rab anti-NT5E (CST, 13160S), rab anti-pSMAD1/5 (CST, 9516), rat anti-Ki67 (Thermo Fisher, 14-5698-82), rat anti-E-cadherin (Abcam, ab11512), Alexa Fluor 568 phalloidin (A12380), and Alexa Fluor 488-, 594- and 647-conjugated secondary antibodies, obtained from Life Technologies.

Probes used for single-molecule RNAscope were purchased from ACD (Advanced Cell Diagnostics): *Foxl1* (C1), *Lmo3* (C1 & C3), *Agt* (C2), *Lgr5* (C1 & C3), *BMP7* (C1), *Wnt5a* (C1), *Wnt2b* (C2), *Rspo1* (C2), *Rspo3* (C2 & C3), *Nog* (C1), *Acta2* (C2), *ZsGreen1* (C3).

Sample collection

Embryonic mouse samples were obtained from timed mating. E0.5d was defined as the morning of the day when the copulatory plug was observed. For embryonic (E12.5d, E13.5d, E14.5d, and E15.5d) samples, several small intestine samples were mixed in tissue pooling due to the small sample size. For the E19.5d sample, small intestines were collected from the middle region of the small intestine from individual mice. For experiments shown in Figure 3D, small intestines from E19.5d mice were obtained collectively from gravid mice after euthanasia.

Cell isolation and FACS

For experiments shown in Figure 1B, isolated intestinal samples were washed in cold Dulbecco's phosphate-buffered saline (DPBS, Gibco, C14190500CP) to remove intestinal contents. The E19.5d tissues were opened longitudinally and sliced into small fragments for the following digestion. The embryonic (E12.5d, E13.5d, E14.5d, and E15.5d) tissues were directly pooled into the tube with collagenase I (Gibco, 17100-017) and elastase (Sigma, E2058) to perform digestion at room temperature to obtain cell mixture. The cell mixture was then passed through a 40-μm filter to obtain the single-cell suspension. The obtained single-cell suspension was centrifuged at 300g for 10 min, and incubated with ACK lysis buffer (Gibco, A10492-01) for 1 min on ice. Another centrifugation at 300g for 10 min was performed and the supernatant was removed. The final fraction was mixed in suitable DPBS with 0.05% BSA and Propidium Iodide.

For experiments shown in Figure 3D, small intestines were collected at E19.5, minced into small pieces, and processed in a solution composed of DNaseI (Merk, 10104159001), and Y27632 (R&D Systems, 1254/50,5000X) in TrypLE (Gibco, 12605-010) for about 15min in 37°C on 150rpm shaker. The remaining tissues were further digested in digestion buffer containing 1.2mg/ml Collagenase (Sigma, C2139-1G), 1mg/ml Dispase II (Gibco, 17105041), 0.1mg/ml DNase I, and Y27632 in Hank's balanced salt solution (HBSS, Gibco 14170-112) for 20min in 37°C, 150rpm shaker. Tissues were resuspended every 6min through pipetting. The reaction was stopped by adding 3-volume of wash buffer containing 5% FBS (Gibco, 16000-044), Gentamicin / Amphotericin (Gibco, 50-0640,

500X), Penicillin / Streptomycin B (Gibco, 15140-122, 100X), HEPES (Sigma, H0887-100ML, 100X), and Y27632 in F12 culture medium (Gibco, 11765-054). The lysates were passed through 70- μ m filters, centrifuged at 300g for 8 min, and resuspended in the wash buffer. 1ml ACK lysis buffer was added to remove the red blood cells on ice for 4min. The remaining cells were washed twice with wash buffer and filtered again with 40- μ m Millipore. The final fraction was resuspended into the wash buffer with 1 μ g/ml DAPI.

Cell sorting

A FACS machine (BD FACSAria III) was used to remove cell debris, dead cells and thus enrich the live cells. The cell concentration and viability of the enriched cells were measured with Trypan blue using a TC20 cell counter from American Bio-Rad company. For *Lmo3-Cre^{ERT2};Ai6* traced cells (Figure 3D), *Lmo3*⁺ cells and traced progenies were collected based on the expression of *ZsGreen1*⁺.

Single-cell RNA sequencing

Only the cell suspension with at least 80% cell viability were diluted to the appropriate concentration and loaded on a 10x Genomics Chromium Single Cell Controller (Pleasanton, CA) with a target of about 5000 cells per sample. Single-cell library preparation was completed using the 10x Genomics Chromium Single Cell 3' Library & Gel Bead Kit v2 strictly following the manufacturer's protocol. The quality and quantity testing of obtained short cDNA fragment libraries using an Agilent 2100 Bioanalyzer and Invitrogen Qubit Fluorometer. Then the single-cell cDNA Libraries were sequenced on an Illumina HiSeq X Ten instrument.

Data analysis

For scRNA-seq of developmental small intestine, the sequencing reads were mapped to the mouse reference genome mm10 (Version 3.0.0), downloaded from 10x Genomics support, to generate the feature-barcode matrices using Cell Ranger (Version 3.0.2) software. Next, all feature-barcode matrices were imported into R package Seurat (Version 3.2.0) to filter low-quality cells separately. The doublets score was estimated by Python package scrublet, cells with doublets score > 0.2 were removed for downstream analysis. Then all datasets were merged into one Seurat object, and integrated by scVI (Version 0.16.1) with 2 hidden layers for encoder and decoder NNs. scRNAtoolVis was used to plot the cell type composition dynamics by day. Selected cell type markers were shown on a heatmap generated by the R package pheatmap (Version 1.0.12).

For the *Lmo3*⁺ scRNAseq datasets analysis, the *ZsGreen1* sequence was added to the mm10 reference during the Cell Ranger pipeline. After being imported into Seurat, low-quality cells are removed, and only *Lmo3* or *ZsGreen1* positive cells were kept for downstream analysis. Then all 3 samples were merged by Seurat, and a small proportion of cells expressing *Epcam* or *Ptprc* were removed and integrated by Harmony (Version 1.0).

Cell subset

To extract telocytes from all cells (Figure 1E), mesenchymal, mesothelial and endothelial cells were firstly extracted, with scattered cells far away from the cell cluster on the UMAP coordinates removed for the downstream re-analysis. Cells from different datasets were integrated using Harmony (Version 1.0), top 30 harmony reductions were used for the following dimension reduction and cell clustering. Next, *Pdgfra* and *Foxl1* double positive cells (counts > 0) were extracted and integrated using scVI with 1 hidden layer.

For analysis in Figure 3E, scRNA-seq data of *Lmo3-Cre^{ERT2};Ai6* traced cells at E19.5 were merged with the scRNAseq data of *Foxl1*⁺ telocytes from all developmental stages and then integrated using scVI with 2 hidden layers.

Mice treatment

Transient labeling and lineage tracing of *Lmo3-Cre^{ERT2};Ai6* and *Foxl1-Cre^{ERT2};Ai6* mice were conducted by injecting one dose of 120mg/kg tamoxifen (Sigma, T5648-5G) intraperitoneally. For cell-specific ablation in the subepithelial telocytes, *Lmo3-Cre^{ERT2};Ai6;Rosa26-DTA* and their littermate control mice were injected with one dose of TAM (120mg/kg) every day for five continuous days. *Lmo3-Cre^{ERT2};Ai6;Rosa26-IDTR* and their littermate control mice were injected with diphtheria toxin (25mg/kg) every day for seven days.

EdU labeling assay

For EdU pulse-chase experiment shown in Figure 4D, mice were injected with one dose of EdU at 50mg/kg intraperitoneally. Samples were then collected after 1 or 7 days and stained using Alexa Fluor 555 (Invitrogen, C10638) kit to detect EdU signals. For EdU labeling experiments shown in Figure 6G, three doses of 50mg/kg EdU were injected continuously before sampling and EdU detection.

Injury models

For the irradiation model, candidate mice were irradiated by 8Gy X-ray (0.856Gy/min) using RS2000-pro animal irradiator in the Laboratory Animal Resource Center, Tsinghua University.

Immunofluorescence

The intestinal tissues were fixed overnight in 4% paraformaldehyde (PFA) at 4 °C, followed by dehydration in 30% sucrose until the intestinal tissues sank to the tube bottom completely. The intestinal tissues were embedded in OCT for quick-frozen and cut into 15 μ m-thick frozen sections. Tissue slices were fixed again in 4% PFA for 10min to remove OCT, then washed three times with DPBS, and incubated in DPBS containing 0.5% Triton X-100 (DPBST) for 30min. Samples were subsequently incubated in blocking

buffer (DPBS containing 10% NGS and 1% BSA) for 1h at room temperature, followed by primary antibodies incubation overnight at 4°C, then rinsed with DPBS three times and incubated with appropriate secondary antibodies at room temperature in the dark for 2 hours. Slides were rinsed again with DPBS twice and stained with 1ug/ml DAPI (Sigma-Aldrich, D9542), mounted, and sealed before imaging.

For Olfm4 and Nt5E staining, prepared tissue slices were fixed again in 4% PFA for 10 minutes to remove OCT, then washed twice with phosphate-buffered saline (DPBS), then washed twice with sterilized water. Tissues were deoxygenated in 3% H₂O₂ for 20min at room temperature, washed twice with sterilized water, and antigen retrieved in antigen retrieval buffer from ACD or in sodium citrate buffer (10mM sodium citrate and 0.05% Tween20 in sterilized water, pH 6.0) through boiling at 100°C for 10min, followed by blocking and immunostaining as previously described.

Immunostaining of pSMAD1/5 was conducted as previously described.⁶ Briefly, fixed frozen tissue sections were fixed again in 4% PFA for 10min to remove OCT, antigen retrieved by boiling for 10min in antigen retrieval buffer from ACD or in sodium citrate buffer (pH 0.6), and permeabilized with DPBST for 30min. Slides were incubated in 0.3% H₂O₂ for 1h to inactivate endogenous peroxidase and blocked in DPBS containing 3% BSA for 1h at room temperature. Anti-pSMAD1/5 (Cell Signaling Technologies, 9516) primary antibody was added at 1:100 in 3% BSA solution and incubated at 4°C overnight. Slides were washed 3 times with DPBS and incubated with HRP-conjugated 2nd antibody (1:300 in 3% BSA; ZSGB-BIO, ZB-2301) for 2h at room temperature, then washed 3 times with DPBS, incubated with Tyramide signal amplification (TSA) solution according to the manual (Invitrogen, B40933), and stained with 1ug/ml DAPI for 5min, washed, mounted, and sealed before imaging.

Whole mount staining

Samples were prepared and immunostained as previously described, with some modification.⁴⁸ Briefly, the intestinal tissues were fixed overnight in 4% paraformaldehyde (PFA) at 4°C, followed by dehydration in 30% sucrose until the intestinal tissues sank to the tube bottom completely. Interested regions were dissected and opened sagittally to expose the intestinal lumen, then cut horizontally into tissue strips with roughly 1 mm in thickness (about 2 villi). Around 5 tissue strips were collected into PCR tubes, washed 3 times with ice-cold DPBS to remove sucrose fully, and permeabilized with 0.5% DPBST for 1h at 4°C on the orbital shaker. Tissues were then blocked with blocking buffer (10% NGS, 1% BSA, and 0.3% Triton-X100 in DPBS) for 6h at 4°C, followed by the primary antibody incubation at 4°C overnight at orbital shaker. Tissues were washed 3 times with ice-cold DPBS for 30min each time, incubated with the secondary antibody overnight at 4°C orbital shaker (phalloidin could be added at this step), then fixed again with 4% PFA for 48h at 4°C orbital shaker. Tissue strips were washed 3 times with ice-cold DPBS for 30min each time to remove PFA, submerged into FocusClear (CellExplorer, FC-101) for 30min at room temperature to make tissues more transparent, and stained with 1ug/ml DAPI for 30min at room temperature. Remove the supernatant carefully and relocate the tissue strips onto the microscope slides with 3 layers of loose-leaf paper protector to avoid squash. Tissues were mounted with antifade mounting media (Vector Laboratories, H-1000), and sealed with neutral balsam.

RNAscope in situ hybridization and confocal imaging

Probes for RNAscope were used according to the manufacturer's manual with the RNAscope Multiplex Fluorescent Reagent Kit v2 (ACD). Slides were mounted with antifade mounting media. Slides were imaged and processed using the Leica SP8 DLS or Nikon AX microscope. All fluorescent images are presented as originally acquired, without manual stitching. It should be noted that images captured in the Cy5 channel (white) may exhibit varying degrees of background noise when overexposed, including those shown in [Figures 4, 7, S4, and S5–S7](#).

QUANTIFICATION AND STATISTICAL ANALYSIS

Intestinal jejunum was used and analyzed for all experiments without specific notation. For transient labeling or labeling-trace of *Lmo3-Cre^{ERT2}* mice, cells with reporting fluorescence colocalized with DAPI, from more than 30 villi were calculated in at least 3 mice for each time point. The expansion index used here was calculated as below according to the physiological distance (for the jejunum of adult mice, the villus length is about 300μm and the length beneath isthmus region is about 100μm) of cell nucleus away from the isthmus region, where [X] means the percentage of *Lmo3*⁺ telocytes located in region X:

$$\text{Expansion index} = [\text{Villus} - \text{Tip}] * 3 + [\text{Villus} - \text{Middle}] * 2 + [\text{Villus} - \text{Base}] * 1 + [\text{Isthmus}] * 0 + [\text{Crypt}] * 1$$

To determine the distinctive transcriptome of villus- and crypt-located telocytes in *Lmo3-Cre^{ERT2};Ai6* mice, cells with interested genes and ZsGreen1 expression were calculated in more than 15 views (600μm x 600μm) from two independent mice ([Figure 5I](#)). For the calculation of pSMAD1/5 signals, DAPI⁺ and pSMAD1/5⁺ cells were annotated separately using imaris (version 7.6.0). More than 5 intact villi were annotated per mouse with more than three mice were utilized in each group ([Figures 7G and S5O](#)). Quantitative data were presented as the mean ± standard deviation (SD). Statistical analysis to determine significant differences was performed using GraphPad Prism 8 software (GraphPad Software Inc.). The p-values were calculated using the paired t test or one-way ANOVA with Tukey's multiple comparisons test.