Delineation and birth of a multi-layer intestinal stem cell niche

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Abstract

Wnt and Rspondin (RSPO) signaling triggers proliferation, and bone morphogenetic protein inhibitors (BMPi) impede differentiation, of intestinal stem cells (ISCs). Here we report that the adult mesenchymal ISC niche is a complex, multi-layered structure that encompasses distinct smooth muscle populations and describe how that niche organizes early in mouse life. Diverse sub-cryptal cells provide ISCs with redundant supportive factors; selected BMPi and the most potent Wnt co-factor, RSPO2, are restricted to single cell types. Niche functions arise during a critical period of postnatal crypt morphogenesis, in part to oppose aggregated BMP-expressing sub-epithelial myofibroblasts that promote epithelial differentiation, and a specialized muscle layer, the muscularis mucosae, is generated *de novo* to supplement other RSPO and BMPi sources. In vivo ablation of smooth muscle raises crypt BMP activity and potently limits crypt expansion. Thus, distinct and progressively refined mesenchymal components together create the milieu required to propagate crypts during rapid organ growth and to sustain adult ISCs.

Introduction

Multipotent *Lgr5*$^{+}$ intestinal stem cells (ISCs) proliferate at the base of adult small intestine (SI) crypts[1], relinquishing stem-cell properties as they leave that region[2]. Epithelial homeostasis balances Wnt/RSPO-dependent ISC self-renewal[3] against bone morphogenetic protein (BMP)-mediated differentiation[4]. Hence, mutations in human BMP signaling genes *SMAD4*, *BMPR1A* or *GREM1* expand ISCs[5-7] and forced BMPi expression in mice induces ectopic crypts[8-10]. The

crypt base is a zone of high Wnt/RSPO and low BMP activity, the same milieu required to expand ISCs *ex vivo*[11]. Sub-epithelial mesenchyme is the principal source of these signals[12,13].

Mouse villus morphogenesis has been elegantly deconstructed[14] and fetal colon cell types were recently catalogued[15]. At birth, however, the mouse SI lacks crypts. Over the ensuing ~2 weeks[16,17], crypts progressively lengthen[18], sequester ISCs at the bottom alongside Paneth cells, and later undergo fission as a dominant mode of epithelial expansion[19-22]. Crypt cell numbers[23], Paneth cells[24], bifurcation[19,21], and monoclonality[25] increase dramatically in the third week of mouse life. This dynamic period of epithelial and ISC expansion between birth and weaning (3 weeks) is likely associated with adjacent mesenchymal sculpting.

This mesenchyme, historically regarded as a collection of myofibroblasts[26], has come into sharp focus, first through identification of overlapping $CD34^{+}Gp38^{+}$ and $PDGFRA^{+}$ cell sources of crucial niche factors[27-30]. $PDGFRA^{hi}$ cells abutting the epithelium resemble telocytes, diverse cells with a distinctive morphology found in many organs[31,32], and are proposed as a key source of canonical Wnt ligands[33]. These cells are the same as intestinal sub-epithelial myofibroblasts (ISEMFs) long described in the literature[34,35], and because telocytes lack a defined molecular or functional identity across tissues, here we use the traditional ISEMF nomenclature. Sub-cryptal $PDGFRA^{lo}$ cells that co-express the BMPi *Grem1* and *Rspo3* support ISC expansion *ex vivo* in the absence of exogenous trophic factors and are therefore called trophocytes[36]. However, the totality of mesenchymal cells that serve niche functions or how the niche arises remain unclear; smooth muscle (SM) near the crypt base, for example, also expresses *Grem1*[37,38].

Here we identify adult SM contributions toward the ISC niche, showing that distinct SM populations substitute in part for selected trophic factors in *ex vivo* ISC cultures. The niche matures in parallel with postnatal murine crypts, coincident with increasing ISEMF density at the villus base. Niche elements positioned to counteract the resulting concentrated BMP source include the muscularis mucosae (MM) and previously uncharacterized superficial circular SM cells in the muscularis propria (MP). MM is molecularly distinct from physically contiguous lamina propria SM, expresses high *Rspo3* and *Grem2*, and arises *de novo* from resident stromal cells. It delimits a distinct anatomic compartment for trophocytes, which alone provide the most potent RSPO ligand, RSPO2. SM ablation *in vivo* during the pre-weaning peak in crypt fission augments crypt BMP activity and markedly attenuates crypt bifurcation. Thus, a partially redundant multi-layer ISC niche develops step-wise, as distinct mesenchymal cell types express overlapping trophic factors at defined distances from ISCs at the crypt base.

RESULTS

Formation and activity of the isthmus ISEMF shelf, a potent BMP source

BMP signaling, which induces intestinal epithelial maturation[4,39], is restricted to adult mouse villi; accordingly, phospho-SMAD1/5 (pSMAD) is absent in crypt cells[10]. Epithelial pSMAD staining at postnatal days (P) 1 and 5 is confined to the top halves of emerging and established villi, becoming steadily more pronounced at villus bottoms by P14 (Fig. 1a and Extended data Fig 1a, note: pSMAD signal is generally stronger in the stroma). Because this base-directed progression of epithelial BMP signaling could reflect increased ligand exposure or increased epithelial responsiveness, we tracked the appearance of ISEMFs, which are the dominant adult source of BMP transcripts and concentrate at villus tips and bases[15,36,40]. In *Pdgfra*$^{H2B\text{-}eGFP}$ mice[41] (see Supplementary Table 1), PDGFRA^{hi} fetal ISEMF precursors constitute the leading edges of new villi[14,42] (Extended data Fig. 1b) and GFP^{hi} cells underlie the villus epithelium after birth (Fig. 1b). Through at least the first week of life, however, high ISEMF density is evident only at villus tips; quantified relative to the trunk, aggregation at the villus base was negligible at P1 and increased progressively by P21 (Fig. 1c). Thus, villus epithelial BMP response strengthens in parallel with ISEMF density at a prominent isthmus shelf (Fig. 1d).

To test whether adult ISEMFs are a functional BMP source, as mRNA profiles indicate[15,36], we cultured adult mouse duodenal crypts in media containing recombinant (r) epidermal growth factor (EGF), rRSPO1, and the BMPi rNOG (together abbreviated ENR). After organoids had established, we replaced rNOG with various factors or with stromal cell types isolated by flow cytometry (Extended data Fig. 1c). Without exogenous cells or in the presence of GFP^{-} cells from *Pdgfra*$^{H2B\text{-}eGFP}$ mice (PDGFRA^{-} cells), organoids enlarged into budding structures that carry ISCs[11], whereas exposure to rBMPs or to GFP^{hi} ISEMFs from adult or P14 *Pdgfra*$^{H2B\text{-}eGFP}$ mice arrested organoid growth. Surviving organoids retracted the classic buds (Extended data Fig. 1d) that carry ISCs[11] and take up the S-phase marker 5-ethynyl-2'-deoxyuridine (EdU), becoming unbranched or spheroidal, and either BMPi rNOG or rGREM1 restored budding morphology (Fig. 2a-b and Extended data Fig. 1e). Organoids co-cultured with ISEMFs also showed reduced expression of ISC molecular markers and elevated levels of BMP target genes and differentiation markers (Fig. 2c). Thus, ISEMFs tilt the balance from ISC self-renewal to differentiation, in line with their concentration at the villus isthmus; subtle morphologic differences between organoids exposed to rBMPs or ISEMFs may reflect disparate BMP levels or other ISEMF factors, e.g., non-canonical Wnts.

Delineation of a multi-component sub-cryptal signaling center

Newborn mouse ISCs, which reside in inter-villus troughs before crypt morphogenesis[17,43], and the organoids they generate differ from adult ISCs[44]; a watershed period between P10 and P16 is marked by crypt deepening prior to emergence of adult ISC properties and extensive crypt fission[18-23]. To ask if trophocytes, a principal BMPi source in adult mice[27,28,30,36], are active in this period, we co-cultured various PDGFRA^{+} cell types from adult (>8 wk) or P14 *Pdgfra*$^{H2B-eGFP}$ mice with SI crypts. Adult and P14 crypts formed organoids in reduced rRSPO conditions, which allowed fine evaluation of the Bmpi component, or with adult trophocytes as a substitute (Fig. 2d and Extended data Fig. 1f). In contrast, P14 trophocytes alone did not support organoid growth and required supplemental rEGF plus rBMPi or rRSPO to reveal modest activity, even toward P14 crypts (Fig. 2e). Thus, young trophocytes lack crucial adult features and other stromal cells likely contribute ISC support, at least before weaning and possibly also in adults.

To identify such cells, we extracted SI mesenchymal cells between P1 and P14 for single-cell (sc) RNA-seq. We profiled >61,000 cells, encompassing unfractionated wild-type mesenchyme and GFP^{+} cells from *Pdgfra*$^{H2B-eGFP}$ mice (Supplementary Methods and Extended data Fig. 2a). CD45^{+} (PTPRC^{+}) leukocytes in some samples included macrophages, mast cells, and B and T lymphocytes, and early after birth we detected proliferating neural crest-derived cells, Purkinje neurons, and trophocytes (Extended data Fig. 2b-c). After excluding leukocytes, replicating cells and Peyer's patch-associated follicular cells[45], >51,000 informative cells from all stages included all known adult populations (Fig. 3a and Extended data Fig. 2d-e).

Because adult SM, broadly defined, also expresses BMPi[37,38] and *Nog* is present in fetal SM fibers[46], we first delineated specific BMPi sources within that compartment. Graph-based cell clustering from scRNA data revealed molecular distinction among SM cells historically classified by their locations: lamina propria myocytes (LPM), muscularis mucosae (MM), and the external muscularis propria (MP) containing inner circular and outer longitudinal SM layers (Fig. 3b, note: pericytes also express high *Acta2* and *Myh11*). Whereas longitudinal and circular MP are largely similar, a novel MP-like population uniquely expresses the BMPi *Nog* and *Chrdl1* and low levels of *Acta2, Actg2,* and *Myh11* compared to other SM cells (Fig. 3c and Extended data Table 1). In situ hybridization (ISH) at P14 with scRNA-derived markers enriched in different MP cells –*Pcp4* and *Osr1*– localized these unique *Nog*$^{+}$*Chrdl1*$^{+}$ cells to the superficial MP surface, closest to crypts and distinct from deeper *Pcp4*^{hi} circular or *Osr1*$^{+}$ longitudinal MP cells; marker specificity in scRNA data mirrored ISH distributions (Fig. 3d, ISH quantitation and negative control shown in Extended data Fig. 3a-b).

Unlike MP, both LPM and MM express *Hhip*, as we verified by ISH (Extended data Fig. 3c). Although villus LPM is physically contiguous with sub-cryptal MM (Fig. 3b), the populations are molecularly distinct: while LPM lacks any BMPi and all other SM cells express *Grem1*, MM uniquely expresses high *Grem2* (Fig. 3c-e and Extended data Fig. 3c-d). Double ISH also distinguished MM from *Nog*$^+$ superficial MP (Extended data Fig. 3e). Altogether, this previously unappreciated SM cell and BMPi diversity near crypts reveals that, beyond trophocytes, postnatal ISCs lie near tissue layers rich in key trophic factors.

Dynamic postnatal gene activity and de novo MM genesis culminate in adult niche structure

Each sub-cryptal population expresses at least two BMPi and one RSPO, with many SM cell types, particularly the MM, strongly *Grem1*$^+$ *Rspo3*$^+$ (Fig. 4a). Other factors pertinent to niche functions include secreted Wnt antagonists in multiple cell types, canonical *Wnt2b* in trophocytes and MM and *Wnt2* in some lymphatic endothelial cells, and prominent BMP enrichment in ISEMFs, similar to adults[36]; various mesenchymal cells also express non-canonical *Wnt4* and *Wnt5a* (Extended data Fig. 4a). Trophic factor levels increase in diverse sub-cryptal cells from birth to P14 (Fig. 4b), together with those of tissue remodeling genes for example (Extended data Fig. 4b). CellPhone DB analysis[47] suggests abundant inter-cellular signaling, with comparatively few potential ligand-receptor pairs among SM cell types and the largest number identified between ISEMFs and other populations soon after birth (Extended data Fig. 4c). These postnatal mesenchymal dynamics may reflect in ISC supportive functions such as emergence of trophocyte activity only after P14 (Fig. 2d-e).

Our scRNA study recovered many more MM and LPM cells at P14 than earlier (Extended data Fig. 2e) and ACTA staining revealed especially sparse MM at P1, modest increase from P5 to P10, and a consistently uniform structure only by P14 (Fig. 4c-d). Curiously, whereas adult SM cells lack *Pdgfra* (or GFP in *Pdgfra*$^{H2B\text{-}eGFP}$ mice)[36], large fractions of MM and LPM cells at P10 and P14 carried GFP$^+$ nuclei (Fig. 4c-d and Extended data Fig. 5a). Moreover, the 8,030 *Pdgfra*$^+$ (GFP$^+$) cells from P14 *Pdgfra*$^{H2B\text{-}eGFP}$ mice in our temporal scRNA survey included distinct pools of *Acta2*$^+$ *Myh11*$^+$ cells corresponding to *Hhip*$^+$ LPM and *Grem2*$^+$ *Grem1*$^+$ *Rspo3*$^+$ MM (Fig. 4e and Extended data Fig. 5b). These findings suggest that MM and LPM derive from resident PDGFRA$^+$ precursors, retaining H2B-eGFP (which is stable[48]) for some duration. To test that hypothesis, we generated *Pdgfra*$^{Cre(ER\text{-}T2)}$;*R26R*$^{L\text{-}S\text{-}L\text{-}TdTom}$ mice[49,50] (Supplementary Table 1), induced Cre activity at P0, and followed the resulting TdTom label into *Pdgfra*$^+$ cell derivatives. At P10, when PDGFRA^{lo} stroma and PDGFRA^{hi} ISEMFs were marked as expected,

LPM and MM also expressed TdTom (Fig. 4f and Extended data Fig. 5c). Thus, these SM cell types continuously arise *de novo* from resident PDGFRA$^+$ stromal cells well after birth.

MM remains anatomically distinct from the MP in adult mice (Extended data Fig. 5d) and its *Grem2*$^+$ cells lie directly beneath *Olfm4*^{hi} ISCs at the crypt base (Fig.5a), compatible with niche activity. To determine if MM retains its key early postnatal products, we generated *Myh11*$^{Cre(ER-T2)}$;*Pdgfra*$^{H2B-eGFP}$;*R26R*^{TdTom} mice, where SM cells label red and PDGFRA$^+$ cells green (Fig. 5b and Supplementary Table 1). scRNA analysis of TdTom$^+$GFP$^-$ cells from the SI, where the MP was manually removed, yielded few *Grem2*$^+$ MM cells (Extended data Fig. 5e) and integration with previous data from MP-depleted adult SI mesenchyme[36] revealed *Grem2*$^+$ *Rspo3*$^+$ MM distinct from LPM (Extended data Fig. 5f). Anticipating a higher MM yield from the adult colon, where it is thicker than in the SI (Fig. 5c), we manually removed colonic MP (including superficial *Nog*$^+$*Chrdl1*$^+$ cells) followed by scRNA-seq of TdTom$^+$GFP$^-$ cells. A distinct *Grem1*$^+$*Grem2*$^+$*Rspo3*$^+$ population corresponds to MM, separate from abundant *Hhip*$^+$ LPM (Fig. 5c), and ISH for these markers in adult colon (Fig. 5d) and SI (Extended data Fig. 6a-c) verified the expression domains that scRNA-seq had revealed in sub-cryptal MM, trophocytes, and superficial MP.

In these studies *Pdgfra*^{lo}*Grem1*$^+$ trophocytes were consistently located between *Grem1*$^+$ *Grem2*$^+$ MM and superficial MP. To resolve closely apposed mesenchymal cell layers finely, we generated *Etv1*$^{Cre(ER-T2)}$;*R26R*^{TdTom};*Pdgfra*$^{H2B-eGFP}$ mice, where Cre activity marks axons projecting from ETV1$^+$ interstitial cells of Cajal (ICC)[51] (Supplementary Table 1). PDGFRA^{lo} trophocytes are indeed sandwiched between the MM and superficial MP (Fig. 5e, intra-muscular PDGFRA^{hi} cells are Purkinje neurons[52]). Altogether, these findings reveal diverse SM populations that flank trophocytes, mature in concert with organ and crypt growth, and by virtue of BMPi and RSPO expression likely constitute a layered signaling hub.

SM requirements for ISC support in vivo

Duplicative and wide expression of niche factors in distinctive sub-cryptal cells hints at their likely joint and redundant contributions to an ISC signaling center. Ablation of adult *Grem1*$^+$ cells triggers epithelial BMP activity and rapid ISC attrition[36] but because many mesenchymal cells express *Grem1* (Fig. 4a), this finding does not implicate any single defined niche component. To isolate SM effects *in vivo*, we generated *Myh11*$^{Cre-ER(T2)}$;*R26R*$^{L-S-L-DTA}$;*Pdgfra*$^{H2B-eGFP}$ mice (*Myh11;DTA*, Supplementary Table 1), noting that Cre activity in *Myh11*$^{Cre-ER(T2)}$;*R26R*$^{L-S-L-tDtomato}$ mice mirrors ACTA2 immunostaining (Extended data Fig. 6d). Adult *Myh11;DTA* mice became

moribund within 2 days of tamoxifen (TAM) exposure, precluding assessment of intestinal niche functions, but administering TAM at P14, by when the MM has formed, allowed us to follow animals for 1 week. Weight loss and proportionally reduced SI length indicated muscle attrition (Extended data Fig. 7a); ACTA2^{+} MM was substantially depleted at P16 and P21 (Fig. 6a); circular MP was modestly reduced at P21 (Extended data Fig. 7b); PDGFRA^{hi} ISEMFs were preserved; and PDGFRA^{lo} cells were slightly reduced, likely reflecting loss of PDGFRA^{+} SM precursors (Extended data Fig. 7c). Thus, SM ablation was incomplete in the study timeframe, as reported with *R26R^{DTA}* and other Cre drivers[53] or owing to limited TAM activity in intestinal sub-epithelium[54]. Nevertheless, MM was especially vulnerable and crypt and ISC numbers were notably compromised. Crypt cell proliferation –which occurs largely in TA cells– was intact (Extended data Fig. 7d) but crypt fission –which peaks in the 3rd week of mouse life[23]– and ISC markers *Olfm4* and *Axin2* were all reduced at P21 (Fig. 6b-c). Crypt bifurcation diminished as early as P16 (Fig. 6b and Extended data Fig. 7e) and crypts with pSMAD^{+} cells were increased ~5-fold (Fig. 6d), pointing to BMPi deficiency as a basis for these defects. Thus, SM deficiency compromises ISC function, owing at least in part to unopposed BMP signaling.

Complementary ISC support from SM and trophocytes

Myh11;DTA mice implicate SM, but not a discrete SM cell type, in pre-weaning ISC support and they do not address relative trophocyte and SM contributions. Therefore, we assessed SM niche activity *in vitro*. Noting that MM is an exclusive source of *Grem2*, we found that rGREM2 supported organoid growth as potently as rGREM1 (Fig. 7a). Because a lack of selective surface markers precludes purification of MM or *Nog*$^{+}$ superficial MP by flow cytometry, first we cultured unfractionated P14 or adult MP with P14 or adult SI crypts. Neither young nor adult MP substituted for ENR medium in supporting organoid formation; however, in the presence of low doses of rEGF and rRSPO1, which were insufficient for organoid growth, MP co-cultures consistently spawned organoids (Fig. 7b and Extended data Fig. 7f). MM isolated physically as a membrane from adult *Pdgfra$^{H2B\text{-}eGFP}$* colon supported organoid growth from adult SI crypts in the absence of additional factors (Extended data Fig. 7g). Because that effect could reflect trophocyte contamination, we digested the membrane, sorted GFP^{-} (MM) from GFP^{+} cells by flow cytometry (Fig. 7c), and verified that GFP^{+} cells are CD81^{+} trophocytes (Extended data Fig. 7h). Low cell yields allowed us to co-culture only 10,000 cells from each fraction with SI crypts in small Matrigel droplets, with limited flexibility in factor supplementation. The trophocyte fraction promoted organoid growth and although MM was inert by itself, small amounts of rEGF and rRSPO1, which approximated trophocyte activity in this assay, endowed MM with supportive

activity far exceeding that of trophocytes alone (Fig. 7c). Thus, MP and MM reveal separate and intrinsic niche activity in the presence of sub-optimal factor levels, and organoid growth in the absence of rNOG suggests that they represent functional sources of BMPi.

Nevertheless, adult trophocytes alone are sufficient to expand ISCs robustly *in vitro*[36] (see Figs. 2d and 7c), which implies that these cells harbor some distinctive activity, possibly other than BMPi. *Rspo4* is absent from any cell type, serosal cells express *Rspo1*, and several cell types express *Rspo3*, but only trophocytes express *Rspo2* (Fig. 4a), as we verified by ISH (Fig. 7d). RSPO3 is superior to RSPO1 in sustaining organoids[28] and ISC compromise *in vivo* requires both RSPO2 and RSPO3 antibodies[55], but RSPO2 supportive activity has not been examined in isolation. Adult mouse SI crypts required 10 ng/mL murine rRSPO3 to generate organoids at top efficiency and 2.5 ng/mL was ineffective, but as little as 2.5 ng/mL and 1 ng/mL rRSPO2 achieved near-peak efficiency (Fig. 7e). Human RSPO2 also was more potent than RSPO1 or RSPO3 (Extended data Fig. 7i). Thus, redundant BMPi coupled with the potency and unique expression of RSPO2 likely explains organoid support from trophocytes alone.

DISCUSSION

Immature ISC precursors located in flat inter-villus epithelium first generate shallow troughs, which deepen into the earliest crypts and later undergo extensive fission to expand crypt and epithelial mass[17,18,23,25,43]. Classical[19,21,23,24] and recent[18] studies highlight the P10-P15 interval as a watershed, when crypts and ISCs acquire adult form and cell composition. Our study integrates mesenchymal scRNA profiles with high-resolution anatomic clarity and functional assessment of the emerging ISC niche during this watershed period. ISEMF aggregation at the villus isthmus coincides with elevated BMP signaling in adjacent epithelial cells and, presumably in part to counter that increase, sub-epithelial niche components develop concomitantly. One previously unknown *Acta2*^{lo}*Myh11*^{lo} population within superficial circular MP fibers uniquely expresses the BMPi *Nog* and *Chrdl1*, and mRNA expression of multiple trophic factors increases postnatally in these cells and in trophocytes. Sub-cryptal MM, a previously unappreciated niche expressing RSPO and BMPi factors, is distinct in this respect from perpendicularly oriented and contiguous LPM fibers and it arises *de novo* after birth. Together, these findings identify a finely layered hub of partially redundant RSPO- and BMPi-expressing cells that position strategically near the crypt base in preparation to fulfill adult epithelial needs.

Previous ablation of *Grem1*$^{+}$ cells, likely including SM in addition to trophocytes, affected ISCs severely[36]. Selective depletion of the *Myh11*$^{+}$ SM compartment in the present study also precipitated ISC dysfunction reflected in markedly reduced crypt fission, which normally peaks as mice approach weaning. Together with the inability of P14 trophocytes to support organoids without added BMPi, these findings imply that overlapping activities of cells with overlapping expression of trophic factors help drive postnatal crypt expansion and subsequent ISC support. For example, the MM may arise after birth specifically to counteract ISEMF aggregation at the villus base. Both adult MM and MP harbor discrete niche activities, providing BMPi but possibly insufficient RSPO for organoid growth. Although our findings in *Myh11*^{Cre}*;DTA* mice may reflect indirect consequences of SM depletion and we cannot say definitively that BMPi or other SM products enable proper crypt fission, we note that crypt fission particularly requires BMP opposition[56]. In the future, molecular markers that allow further cell fractionation and precise *in vivo* ablation will complete the emerging understanding of individual niche elements.

Our findings add developmental and fine spatial context to prior work showing BMPi activity in driving ISC properties[8-10,56], polarized BMP and BMPi expression in human colonic mucosa[29], and that BMP signaling restrains both ISC pool size[4] and crypt fission[57] independent of Wnt signaling. Furthermore, biochemical[58], functional[55], and structural studies[59-62] point to sequential Wnt and RSPO activity within the same signaling pathway, with canonical Wnts priming ISCs to express RSPO receptors. In addition to one or more BMPi, every sub-cryptal mouse niche component expresses at least one *Rspo* gene and trophocytes' unique expression of the most potent member, *Rspo2*, may explain their particularly robust support of organoids[36].

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METHODS

Animals. *Pdgfra*$^{H2B\text{-}eGFP}$ (Jackson Laboratories (JAX) strain 007669)[41], *Rosa26R*$^{LSL\text{-}TdTomato}$ (JAX strain 007909), *Myh11*$^{Cre(ER\text{-}T2)}$ (JAX strain 019079), *Pdgfra*$^{Cre(ER\text{-}T2)}$ (JAX strain 032770), *Etv1*$^{Cre(ER\text{-}T2)}$ (JAX strain 013048) and *Rosa26R*$^{LSL\text{-}DTA}$ (JAX strain 009669) mouse lines were purchased from Jackson Laboratories (Supplementary Table 1). Mice were considered adults after 8 weeks of age at the time of treatments or cell isolations. All experiments used mice of both sexes and littermates as controls. All animal procedures and experiments were approved and monitored by the Animal Care and Use Committee at the Dana-Farber Cancer Institute.

Mouse treatments. Postnatal *Pdgfra*$^{Cre(ER\text{-}T2)}$;*R26R*$^{LSL\text{-}TdTom}$ and *Myh11*$^{Cre(ERT2)}$;*Rosa26*$^{LSL\text{-}DTA}$ mice received 1 dose of 4-OH tamoxifen (Sigma-Aldrich, 1 mg per 25 g body weight) by gastric gavage at P1 or intra-peritoneal (IP) injection at P14. *Myh11*$^{Cre(ER\text{-}T2)}$;*R26R*$^{LSL\text{-}TdTom}$;*Pdgfra*$^{H2B\text{-}eGFP}$ adults received 4-OH tamoxifen (1 mg) by IP injection on 2 consecutive days to allow recombination at *LoxP* sites and were harvested at the indicated times, usually 5 days later. BrDU (10 μg/g body weight) was administered by IP injection 1 h before euthanasia. Postnatal mice were harvested at the time points indicated in the figure legends.

Immunohistochemistry and quantitation. Whole-mount tissue immunohistochemistry was performed as described[36,63]. Briefly, proximal small intestines were harvested, pinned onto agarose plates, and fixed overnight in 4% paraformaldehyde (PFA); in this and all subsequent steps, the tissue was rocked gently. After rinsing in phosphate-buffered saline (PBS), the tissue was placed in 10%, then 20% sucrose over the course of 1 day, followed by blocking buffer (PBS containing 0.125% bovine serum albumin, 0.003% Triton X-100, 0.05% donkey serum, and 0.0005% NaN_3) for 6 h and overnight in blocking buffer containing 4′,6-diamidino-2-phenylindole (DAPI). Tissue was rinsed with PBS, cut into 1-mm fragments, placed on glass slides with spacers (Grace Bio-Labs, 654002), and cleared using FocusClear (CelExplorer Labs, FC-101) for 30 min, before applying VectaShield mounting medium (Vector Laboratories) and a coverslip. To generate representative and comprehensive anatomic resolution, images were taken from at least 3 independent animals.

Proximal small intestine epithelial immunochemistry was performed as described[64]. Fresh tissue was incubated in 5 mM EDTA in Hank's Balanced Salt Solution (HBSS) at room temperature, rocked gently for 5 min, and washed briefly in PBS before separating the epithelium gently from underlying mesenchyme. Tissue was fixed in 4% PFA at 4°C overnight,

with subsequent PBS washing and incubation with DAPI to stain nuclei and Phalloidin (Invitrogen, A12381) to visualize filamentous actin. Crypt bifurcation was quantified in 3D-rendered images of whole-mount phalloidin stained intestines, reported as a fraction of >100 intact crypts.

Routine immunohistochemistry was performed on tissues fixed as described above and placed in OCT compound (Tissue-Tek, 4583). 7 μm sections were prepared using a Leica CM3050 cryostat. The following antibodies (Ab, all at 1:1000 dilution unless indicated) were used: Laminin (Sigma, L9393); GFP (Abcam, ab6662); CD31 (BD Biosciences, 557355); bromo-deoxyuridine (BrDU, Sigma, B5002); Alpha-smooth muscle actin (ACTA2; Abcam, ab5694); PDGFRA (R&D Systems, AF1062, 1:100); and Alexa Fluor-conjugated secondary goat anti-rat, goat anti-rabbit, or donkey anti-goat IgG (Invitrogen, A11081, A21071, A11058). Signals were amplified using biotin/streptavidin HRP-conjugated secondary Ab (Jackson Immuno, 111-065-003 and 016-030-084) and detected using Tyramide Signal Amplification Plus kit (Akoya Biosciences, NEL744001KT, 1:100). Images were taken using a Leica SP5X laser scanning confocal microscope and further processed using ImageJ Fiji software[65].

pSMAD1/5 was immunostained as described[66]. Briefly, antigens were retrieved by boiling slides in citrate buffer (pH 6.0) for 1 min and pSMAD1/5 Ab (Cell Signaling, 41D10) was added overnight at 4°C. pSMAD1/5^{+} crypts were quantified on images acquired and processed as above. Epithelial compartments were delineated with respect to LAM Ab-stained basement membrane, and quantified by representing the lowest and highest 5 villus epithelial cells (10 cells per villus) as the fraction of pSMAD1/5^{+} cells in >25 villi per sample (Fig. 1a) or crypts carrying $\geq$1 pSMAD1/5^{+} cell as a fraction of >80 crypts per sample (Fig. 6d). To quantify *Pdgfra*$^{H2B\text{-}eGFP}$ ISEMFs, 25 μm sections were co-stained with LAM Ab to demarcate the epithelium. Mesenchymal GFP^{hi} cells abutting the bottom 5 villus epithelial cells were counted and are reported with respect to GFP^{hi} cells abutting the next 5 epithelial cells along the villus trunk (Fig. 1c). More than 25 villi were counted per sample.

To quantify SM populations (Fig. 6a and Extended Data Fig. 7b), ACTA2^{+} cells in the MM and in the circular and longitudinal layers of the MP were counted per length of proximal SI examined (average 5 mm/sample). Co-labeled ACTA2^{+}*Pdgfra*^{lo} cells are reported as a ratio of the total number of ACTA2^{+} MM and LPM (Fig. 4c and Extended Data Fig. 5a). *Pdgfra*$^{Cre(ER\text{-}T2)}$*;Rosa26*^{DTA} intestines exposed to TAM at P0 were harvested at P10 and stained with ACTA2 Ab to quantify various SM compartments. For quantitation of *Pdgfra*^{lo} cells in *Myh11*^{Cre}*;Pdgfra*$^{H2B\text{-}eGFP}$*;DTA* experiments (Extended Data Fig. 7c), cells found in the

submucosa, between crypts and MP, are reported as numbers/length of intestine (>4mm/sample). BrdU+ cells/crypt are reported as numbers per crypt (≥25 crypts/sample).

In situ RNA hybridization and quantitation. mRNAs were localized by the RNAscope (Advanced Cell Diagnostics) method[67] on intestines collected from at least 3 different animals per treatment. Probe sets were designed by Advanced Cell Diagnostics for *Chrdl1*, *Grem1*, *Grem2*, *Hhip*, *Noggin*, *Olfm4*, *Axin2*, *Osr1*, *Pcp4*, *Rspo1*, *Rspo2*, and *Rspo3*. After hybridization according to the manufacturer's protocols, tissue sections were washed for 5 min in PBS containing 0.1% Tween-20, blocked for 1 h at room temperature in PBS containing 5% normal goat serum, and exposed overnight at 4°C to Laminin (Sigma, L9393, 1:1,000) or GFP (Abcam, ab6556, 1:100) Ab. After multiple 5-min washes in PBS and 90-min incubation with AlexaFluor-conjugated secondary Ab as above (Invitrogen) at room temperature, DAPI was applied and slides were mounted according to the RNAscope protocol. Images were taken using a Leica SP5X laser scanning confocal or a Leica Thunder Imager microscope and processed using ImageJ Fiji software[65]. For quantitation in SM layers delineated by laminin (Extended Data Fig. 3b and d, 2 samples), each cell with at least 1 fluorescent ISH dot was counted as one and reported as a fraction of all SM cells present in the respective sub-compartment. Every cell with at least one *Olfm4*+ or *Axin2*+ ISH dot was reported per crypt (Fig. 6c, >40 crypts per sample).

Schematic illustrations were generated with BioRender.

Mesenchymal cell isolation and flow cytometry. Mesenchymal cells were isolated from the pooled proximal halves of the small intestine from 3-6 wildtype or *Pdgfra*$^{H2B\text{-}eGFP}$ pups. Timepoints harvested from wildtype tissue include P1, P4, P5, P9, and P14, and from GFP+ isolated *Pdgfra*$^{H2B\text{-}eGFP}$ tissue include P2, P5, and P14. After manual stripping of external muscles (MP) and serosa, whole adult *Myh11*$^{Cre(ER\text{-}T2)}$;*R26R*$^{L\text{-}L\text{-}TdTom}$; *Pdgfra*$^{H2B\text{-}eGFP}$ SI or colon was processed as described[36]. Epithelium was denuded by shaking the tissue for 20 min at 37°C in pre-warmed HBSS (Life Technologies) containing 10 mM EDTA. The remaining tissue was rinsed with HBSS, minced using a scalpel, and digested with gentle rocking for 1 h at 37°C in 3 mg/mL collagenase II (Worthington, LS004176) diluted in HBSS containing 5% fetal bovine serum (FBS). Extracted cells were centrifuged at 300 *g* for 5 min, washed with FACS buffer (PBS containing 0.1% BSA), and leukocytes were depleted in ACK Lysis buffer (Gibco) for 3 min. Washed cells were suspended in FACS buffer and, to deplete epithelial and immune populations, stained with conjugated EPCAM (BioLegends, 118214, 1:100) and CD45

(eBiosciences, 17-0451-82, 1:100) Ab for 20 min at 4°C. Cells were sorted on a FACSAria IIII flow cytometer, with gating against DAPI (BD Pharmingen) to identify live cells. Graphs of isolated cell fractions were generated using FlowJo software v10.

scRNA-seq library preparation, sequencing, alignment, quality control, and data analysis. 5,000 to 10,000 cells isolated by flow cytometry were loaded onto a Chromium Controller (10X Genomics), followed by library preparation according to the manufacturer's recommendations (Single Cell 3' V3 assay) and sequencing on a HiSeq4000 instrument (Illumina). Libraries were de-multiplexed, aligned to the mm10 mouse transcriptome, and unique molecular identifiers (UMIs) were counted using Cell Ranger (10X Genomics) v3.1.1. Data were analyzed using the Seurat package v4.0.3 in R. Cells with $\geq$1,000 and $\leq$ 4,000 detected genes, >2,500 and <15,000 total transcripts, and <10% mitochondrial transcripts were retained. Merged datasets utilized the "merge" function and data were normalized and log-transformed using the "SCTransform" function in Seurat, regressing out mitochondrial read fractions and other confounding variables. Datasets integrated after normalization used the "integrate" function in Seurat. Differential markers were identified in clusters using the "FindAllMarkers" function in Seurat, with parameters: min.pct 0.25 and logfc.threshold 0.25. To identify cell types in each collection, the data were queried for known mesenchymal cell-specific genes[36,68]. UMAP plots for gene expression were generated using the "FeaturePlot" function.

For merged datasets (Fig. 3a and Extended Data Fig. 2a), the top 15 principal components were selected using the "FindNeighbors" function, followed by identifying clusters using the "FindClusters" (resolution: 2) function in Seurat. The "RunUMAP" function was used to reduce the top 15 principal components using the "uwot" method. Based on marker expression, clusters were merged to compile 20 mesenchymal cell populations; among these, we removed *Mki67*$^+$ (proliferating), *Ptprc*$^+$ (leukocytes), and *Ccl19*$^+$ (follicle reticular, FRC) cells (Extended Data Fig. 2a) to generate the final merged dataset (Fig. 3a). Separately, *Ptprc*$^+$ and *Mki67*$^+$ cell subsets were annotated on the basis of published markers[69] and those listed in Extended Data Fig. 2a, respectively. To generate correlation heatmaps (Fig. 3c) and gene lists (Extended Data Table 1), we separated SM cell types using the "FindAllMarkers" function in Seurat and plotted the top 10 and top 50 marker genes for each. Genes differentially expressed between P14 and P1/P2 (Fig. 4b) were identified for each cell type using the "FindMarkers" function and dot plots depicting $\log_2$ fold-differences were generated using code based on the "DotPlot" function within the ggplot2 package in R. Heatmaps show average cell expression of the top 50 differentially expressed genes at each harvest between P1 or P2 and P14 (Extended Data Fig. 4b).

Integrated analysis (Extended Data Fig. 5f) included published adult scRNA-seq data[36] (Gene Expression Omnibus, GSE130681).

For CellPhoneDB analysis, RNA counts normalized in Seurat were filtered for *Mki67* non-expressing cells and mouse genes were converted to their human orthologs using bioMart[70] and removing genes that had no ortholog. The resulting table and cell type classifications were used as input into the CellPhoneDB statistical analysis function[47], using the parameters counts-data hgnc_symbol --threshold 0.1. Using the CellPhoneDB pvalues output table, putative interactions were inferred between cell types if the p-value was <0.05 in either direction (e.g., ISEMF to CD81$^-$ stroma or vice versa, Extended Data Fig. 4c). To generate Venn diagrams, this information was used as input into ggVennDiagram[71] in R and heatmaps were generated with default CellPhoneDB parameters (Extended Data Fig. 4c).

***In vitro* co-cultures, imaging, quantitation, and analysis.** Unfractionated mesenchyme extracted from P14 or adult *Pdgfra*$^{H2B\text{-}eGFP}$ mice was plated on non-pyrogenic, gas plasma surface-treated polystyrene tissue culture plates (Falcon) in Dulbecco's Modified Eagle and F12 media (Gibco, 12634-010) supplemented with penicillin, streptomycin, Glutamax, HEPES buffer, and 10% FBS (Basal media + FBS, see Ref 35 [36] for extraction methods, Extended Data Fig. 1c). Using forceps, we carefully peeled off SI muscularis propria (MP), and in the colon, the muscularis mucosae from *Pdgfra*$^{H2B\text{-}eGFP}$ mice. SM was disaggregated for 10 min in HBSS containing 5%FBS and 3 mg/mL collagenase II, then plated as described above. The medium was replaced 24 h after plating; 2 to 3 days later, cells were removed using 0.25% Trypsin/EDTA (Corning), washed in FACS buffer, and GFP^{hi} (ISEMFs), CD81$^+$Pdgfra^{lo} (trophocytes), CD81$^-$Pdgfra^{lo} stroma, and GFP$^-$ cell fractions were harvested by flow cytometry (Extended Data Fig. 1c) as described previously[36] as unfractionated mesenchyme (Fig. 2a) or SM populations (Fig. 7c). MP was disaggregated using 0.25% trypsin/EDTA for 5 min, washed with PBS/0.1%BSA, and placed directly into co-cultures with isolated crypts.

For organoid co-cultures (Fig. 2a and Extended Data Fig. 1e), crypts were plated for 2 days in complete basal media supplemented with N2, B27 and N-acetylcysteine as described[72], EGF (Thermo Fisher, 50 ng/mL), RSPO1 (10% culture supernatant from 293T-HA-RspoI-Fc cells), and rNOG (Peprotech, 100 ng/mL), then removed from Matrigel in Cell Recovery Solution (Corning) for 10 min at 4°C, and recovered by centrifugation at 200 *g* for 15 min. 50-100 crypts were replated in 20 μl Matrigel drops in 24-well tissue culture plates, together with 2x10^4 mesenchymal cells harvested, cultured, and purified as described above. Complete Basal

Media was replaced containing EGF, RSPO1 (10% culture supernatant from 293T-HA-RspoI-Fc cells), and rNOG, rGREM1 (Thermo Fisher, 100 ng/ml), or BMP2 (Peprotech #120-02, 50 ng/mL) and BMP7 (R&D#5666-BP, 50 ng/mL). Bright field and fluorescent organoid images were captured using an Olympus CKX53 or Nikon Eclipse T/2 microscope, respectively. Organoid features were scored from 3 or 4 experiments with 2 or 3 technical replicates per group per experiment. RNA was harvested at 48 h and qRT-PCR was performed on 3 or 4 replicates from each treatment group; mRNA expression values are represented relative to Gapdh ($2^{-\Delta CT}$) and relative to organoids cultured without additional cells.

For culture experiments with BMPi (Fig. 7a), ~100 isolated crypts were plated in Matrigel drops in media containing a sub-optimal concentration of RSPO1 (0.5% culture supernatant from 293T-HA-Rspo1-Fc cells), EGF (50 ng/mL), and indicated concentrations of rNOG, rGREM1, or rGREM2 (R&D#2069-PR). For culture experiments with RSPOs (Fig. 7e and Extended Data Fig. 7i), ~100 isolated crypts were plated in Matrigel drops in basal condition media containing rNOG (100 ng/mL), EGF (50 ng/mL), and the indicated concentrations of mRSPO2, mRSPO3 (R&D, 6946-RS and 4120-RS), hRSPO1, hRSPO2, or hRSPO3 (R&D, 4645-RS, 3266-RS, and 3500-RS). Organoid structures were counted with a Nikon Eclipse TS100 microscope 5 days after plating. Three biological replicates are reported per condition, each including 3 technical replicates. Extended Data Fig. 6c and 7g were compiled as a composite using Microsoft 365 ProPlus Office PowerPoint.

Statistics and reproducibility. Statistical analyses were performed using Prism software package v7.03 (GraphPad). The number of independent replicates, statistical methods, and P values are given in the respective figure legends. No sample size estimations and no blinding were performed.

Data and code availability. Data are deposited in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE184158). Reviewers may access these data using the token apqtymkwpnwnlmx. No new software package was developed for this study.

FIGURE LEGENDS

Figure 1. Postnatal epithelial BMP signaling coincides with isthmus ISEMF aggregation.
a, Representative pSMAD1/5 (magenta) immunostaining (green: Laminin/basement membrane, blue: DAPI) in mouse proximal SI on indicated postnatal days. cr, crypts; scale bars 50 μm; boxed regions are magnified in adjoining images; extended images are shown in Extended data Fig. 1a. Nuclear pSMAD^{+} epithelial cells (filled arrowheads; pSMAD^{-} cells indicated by empty arrowheads) are present at villus tops from birth, while pSMAD^{+} cells at villus bottoms increase over the ensuing 2 weeks. Graph displays pSMAD1/5^{+} epithelial cell fractions at the bottoms (blue dots) and tops (grey dots) of >25 villi/sample (n = 3 mice at each age). Statistical differences are reported relative to P1 villus bottoms after two-way ANOVA followed by Tukey's multiple comparisons test. ns: not significant, *p<0.0001.
b-c, GFP^{hi} ISEMFs are present in *Pdgfra*$^{H2B\text{-}eGFP}$ mice on established and emerging (arrowhead) villi at P1 and concentrate progressively at the crypt-villus isthmus shelf (white brackets). From representative images of proximal SI at the indicated ages (**b**, scale bars 50 μm), we quantified GFP^{hi} ISEMF ratios (**c**) at the isthmus (white brackets, 5 lowest villus epithelial cells) vs. villus trunks (red brackets, next 5 cells toward villus tip); graph (**c**) displays data from >25 villi/sample (n = 3 animals at each age). Statistical differences are reported relative to P1 after two-way ANOVA followed by Tukey's multiple comparisons test. ns: not significant, *p<0.0001.
d, Increased epithelial pSMAD1/5 is concomitant with increasing isthmus ISEMF density.

Figure 2. ISEMFs promote differentiation and early postnatal trophocytes fail to support organoid growth.

a, 2 day-old organoids cultured with recombinant (r) RSPO1, EGF, and indicated cell types from *Pdgfra*$^{H2B\text{-}eGFP}$ mouse SI. Representative organoids are shown 48 h later (scale bar 100 μm). Co-cultures without cells or with GFP^{-}(i.e. PDGFRA-) cells produced budding structures, whereas crypts co-cultured with ISEMFs or rBMPs retracted buds, a process that BMP inhibitor rNOG or rGREM1 reversed. Representative images of EdU-stained organoids (scale bar 50 μm) demonstrate markedly reduced cell replication in organoids co-cultured with ISEMFs or rBMPs.

b, Fractions of budding (white), unbranched (non-budding, grey), fully spheroidal (stippled), and non-viable (black) organoids after co-culture with the indicated cells or factors. Statistics were determined for budding fractions by one-way ANOVA followed by Dunnett's posttest. *p<0.05, ****p<0.0001 (n=3-10 independent organoid cultures as indicated above each bar).

c, qRT-PCR analysis of organoids after 48 h co-culture (n=3 or 4 biological replicates). BMP- or ISEMF-exposed organoids reduced expression of ISC markers and increased markers of BMP activation and epithelial differentiation. Transcript levels are represented relative to control crypt cultures with no added cells. Statistical comparisons use one-way ANOVA followed by Dunnett's posttest. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.

d, Co-cultures of P14 SI crypts with isolated adult or P14 mesenchymal cells in factor-free medium. Unlike adult trophocytes, which support robust organoid growth, P14 trophocytes are inactive. Graph depicts organoids per sample relative to control wells (- cells + **E**GF, **N**OG, and **R**SPO1 (ENR); 94.5 ±46.8 organoids/well; n=6). Statistical significance determined by Student's t-test at **p<0.01, ***p<0.001 (n=3-6 biological replicates).

e, P14 trophocytes support organoid growth in the presence of low concentrations of rEGF plus rNOG (EN medium) or rRSPO1 (ER medium) that are by themselves insufficient for organoid growth. Organoid counts are represented relative to parallel control crypt cultures in ENR medium without additional cells (59 ±12.1 organoids/well, n=4). Statistical differences determined by Student's t-test at ***p<0.001 (n=4-6 biological replicates).

Figure 3. Diverse postnatal intestinal smooth muscle (SM) cell populations.

a, Uniform manifold approximation and projection (UMAP) of scRNA-seq data from whole mesenchyme and GFP$^+$ cells from *Pdgfra*$^{H2B\text{-}eGFP}$ mice, representing 51,084 cells from P1/P2, P4/P5, P9, and P14. MP, muscularis propria; LPM, lamina propria myocytes; MM, muscularis mucosae; ISEMFs, intestinal subepithelial myofibroblasts; ICC, interstitial cells of Cajal; LECs, lymphatic endothelial cells; BECs, blood endothelial cells.

b, Overlay of aggregate *Myh11+Acta2* expression (red) on the UMAP plot, showing expression of these SM markers in each SM population and pericytes. ACTA2-immunostained P14 *Pdgfra*$^{H2B\text{-}eGFP}$ proximal SI (*Pdgfra*$^{H2B\text{-}eGFP}$ green, ACTA2 magenta, DAPI grey) highlights key anatomic structures magnified in the adjoining image (right – ACTA2 only): LPM (dashed red outline), MM (dotted blue outlines), and MP (dashed green outlines; epithelial surface in white dashed outline). Scale bar 50 μm.

c, Top genes identified as discriminating markers for each indicated SM cell population. MP divides into *Nog*$^+$*Chrdl1*$^+$, *Pcp4*$^+$,and *Osr1*$^+$ subpopulations. Red arrowheads point to genes of particular interest; additional differences are listed in Extended data Table 1.

d, RNAscope in situ hybridization (ISH) with the indicated probes (magenta dots on left, grey in right images, LAMININ=green). Three distinct MP layers (within dashed white or green outlines) correspond to scRNA-identified subpopulations (UMAP plot from the dashed green box in **b**): superficial *Nog*$^+$ *Chrdl1*$^+$ cells, inner circular *Pcp4*$^+$ SM, and outer longitudinal *Osr1*$^+$ SM. Violin plots depict SM marker expression across indicated cell types. Sup, superficial; Circ, circular SM; Long, longitudinal SM. Scale bar 50 μm.

e, RNAscope ISH for *Grem2* at P14 showing expression in MM (dotted blue outline) and MP (dashed green outline). Top, 2-color fluorescence; bottom, *Grem2* ISH in grey. Scale bar 50 μm.

Figure 4: Sub-cryptal mesenchyme constitutes an ISC signaling center and the MM arises postnatally from native PDGFRA$^+$ precursor cells.

a, Average expression of selected BMPi and Rspo genes across all cell types represented in the postnatal mesenchymal scRNA survey (Fig. 3a). Right, aggregate *Grem1* and *Rspo3* levels projected on the global UMAP show high dual expression in trophocytes (Troph.), MM, and MP.

b, Top: schema for expression of key trophic factors in each distinct SM cell fraction in SI at P14. Bottom: $\log_2$ fold increase between P1/2 and P14 in average mRNA expression per cell of the indicated trophic factor genes. BMPi and Rspo gene expression increases significantly (p <0.05, black outline) in diverse postnatal sub-cryptal cells.

c, Representative ACTA2 immunostaining of proximal *Pdgfra*$^{H2B\text{-}eGFP}$ SI at P1, P5, and P10. Boxed areas are magnified in insets. While LPM is present at P1 (arrow), MM arises between P5 and P10 (arrowheads). Scale bar 50 μm. Graph depicts MM cells/mm tissue at indicated ages. Statistical differences with respect to P1 were determined by one-way ANOVA followed by Dunnet's multiple comparisons test. **p<0.0001, *p<0.05, n=3 animals at each age.

d, GFP$^+$ nuclei are evident in LPM (arrow) and MM (arrowheads) in *Pdgfra*$^{H2B\text{-}eGFP}$ proximal SI at P14, in agreement with scRNA data on purified GFP$^+$ cells. Areas outlined with white dashes are shown to the right or below with GFP signals only in grey. Scale bar 50 μm.

e, Aggregate *Acta2* and *Myh11* expression (red) overlaid on a composite UMAP plot of GFP$^+$ cells isolated from *Pdgfra*$^{H2B\text{-}eGFP}$ mice at P14. FRC, fibroblastic reticular cells. Inset shows *Grem2* expression projected on the cells boxed in the composite UMAP plot.

f, *Pdgfra*$^{Cre(ER\text{-}T2)}$;*R26R*^{TdTom} pups were treated with tamoxifen at P0 and their intestines were examined at P10, when LPM (arrow) and MM (arrowheads) showed *Pdgfra* lineage tracing. White dashes outline the epithelial-mesenchymal boundary. Scale bar 50 μm. tdtomato$^+$ MM and LPM cell fractions are shown (n: number of cells counted) from >4 mm of proximal SI in each of 3 independent animals.

Figure 5. Sub-epithelial cells together constitute an ISC signaling center in adult mice.

a, *Grem2* expression revealed by RNAscope ISH (green) in adult duodenal MM, directly beneath *Olfm4*$^{+}$ (red) ISCs (arrowheads); cr, crypts. MP was removed manually before staining and the bottom image is the same as the top, with DAPI signal excluded. Scale bar 50 μm.

b, Adult *Myh11*$^{Cre(ER\text{-}T2)}$*;R26R*^{TdTom}*;Pdgfra*$^{H2B\text{-}eGFP}$ SI showing GFP^{+}Tom^{+} ISEMFs and Tom^{+} SM cells. The dashed box is magnified in the right images, where the bracket outlines MM and the arrowhead points to a GFP^{-} cell characteristic of adult MM. Scale bar 50 μm.

c, Adult *Myh11*$^{Cre(ER\text{-}T2)}$*;R26R*^{TdTom}*;Pdgfra*$^{H2B\text{-}eGFP}$ colon showing thick MM (arrowheads). The MP was removed prior to FACS sorting and a representative FACS plot shows colonic TdTom^{+}GFP^{-} cells (dashed red box), which were isolated for scRNA analysis. A UMAP plot of 3,592 cells shows aggregate *Grem1*, *Grem2*, and *Rspo3* expression in the small MM fraction, distinct from LPM, endothelial cells or pericytes. Scale bar 50 μm.

d, RNAscope ISH for *Grem1*, *Grem2*, and *Rspo3* (all signals in yellow dots, red: LAMININ) in adult mouse colon, showing predominant expression in MM (arrowheads) and superficial MP.

e, Representative whole-mount image from *Etv1*$^{Cre(ER\text{-}T2)}$*;R26R*^{TdTom}*;Pdgfra*$^{H2B\text{-}eGFP}$ mouse ileum, showing the tissue beneath crypts (dashed white outlines) in relation to axonal projections (red) from *Etv1*$^{+}$ interstitial cells of Cajal that demarcate SM layers. *Pdgfra*^{lo} trophocytes lie between sub-cryptal MM (unstained) and *Nog*$^{+}$*Chrdl1*$^{+}$ superficial MP (also unstained; neural projections separate these cells from circular *Pcp*$^{+}$ SM). GFP^{hi} nuclei far from peri-cryptal ISEMFs are Purkinje neurons[73], distinct from *Etv1*^{Cre}-labeled TdTom^{+} projections. Scale bar 50 μm.

Figure 6. BMP-associated crypt attrition in mice with intestinal SM deficiency.

a, *Myh11*$^{Cre(ER-T2)}$*;R26R*^{DTA} mice were treated with tamoxifen at P14 and intestines harvested at P16 and P21 reveal reduced ACTA2-stained MM. Representative images at P21 are shown and boxed areas are magnified in the insets, where the bracket demarcates control MM and dashed white lines mark the superficial MP border; cr: crypts. Scale bar 50 μm. To the right, MM cells are quantified from >4 mm tissue in each of 3 or 4 independent animals. Statistical significance was determined using unpaired Student's t-test. **p<0.01, ***p<0.001.

b, F-actin (phalloidin)- stained epithelial whole-mount images showing considerably fewer bifid crypts (arrowheads) in *Myh11*$^{Cre(ER-T2)}$*;R26R*^{DTA} compared to control pups injected with tamoxifen (TAM) at P14 and examined at P16 (Extended data Fig. 7a) and P21. To the right, bifid crypts are quantified from >100 crypts in each of 3-6 mice from each age. Statistical significance was determined using unpaired Student's t-test. *p <0.05, ***p<0.001.

c, Crypt base cells expressing RNA for ISC markers *Olfm4* (RNAscope ISH signal in magenta) and *Axin2* (FITC) are decreased in TAM-treated *Myh11;DTA* animals at P21. n=4 mice per condition, >40 crypts per sample, statistics by unpaired Student's t-test. *p<0.05, ***p<0.001.

d, Increased pSMAD1/5 immunostaining in TAM-treated *Myh11*$^{Cre(ER-T2)}$*;R26R*^{DTA} crypts (arrowheads), which lack pSMAD1/5 in controls (see also Fig. 1a). pSMAD1/5^{+} crypts were quantified in >80 crypts from 3 mice for each condition and statistical significance determined using unpaired Student's t-test. **p<0.01.

Figure 7. Adult MM and MP complement trophocyte activity *in vitro* by substituting for BMPi.

a, Organoids arising from SI crypts after 5 days of culture in a sub-optimal concentration of rRSPO1 (see Methods) and 50 ng/mL or 100 ng/mL of the indicated BMP inhibitors. n=3 biological replicates. One-way ANOVA with Dunnett's test shows significance of all conditions to at least $p<0.01$ compared to controls with no added BMPi.

b, Co-culture of adult SI crypts with unfractionated P14 or adult MP did not generate organoids (arrowheads) by itself but did so in the presence of sub-optimal concentrations of rEGF and rRSPO. Organoids are represented in the graph relative to the numbers generated in ENR medium (no added cells, 66 $\pm$10.4 organoids, n=3). Significance determined by Student's t-test, ****$p<0.0001$. -GF, no recombinant trophic factors.

c, After MP removal, MM stripped from *Pdgfra*$^{H2B\text{-}eGFP}$ colon retained GFP$^+$ trophocytes on the undersurface (green arrowheads in inset) and flow cytometry separated GFP$^+$ trophocytes from GFP$^-$ MM (see Extended data Fig. 7g-h). Scale bars 50 μm. In adult SI crypt co-cultures, GFP$^+$ cells supported organoid growth without added factors, while GFP$^-$ MM provided support only in the presence of inactive amounts of rEGF and rRSPO1 (ER medium). Significance determined with respect to ENR-only controls (no cells, 31.7 $\pm$5.5 organoids/well, n=5) using Student's t-test. ***$p<0.001$, ****$p<0.0001$.

d, Top: *Rspo2* (green) ISH and LAMININ (red) immunostaining in adult SI. Dashed lines indicate boundaries marked by the MM and superficial MP, between which lie submucosal trophocytes (cr: crypts). Greyscale version of the *Rspo2* signal is shown below the color image without LAM. Bottom: *Rspo1* (red) ISH confirms high expression in the serosa as indicated by scRNA-seq. Dashed line marks superficial MP. Scale bars 50 μm.

e, Crypts cultured with as little as 1 ng/mL rRSPO2 form organoids at an efficiency that requires at least 10 ng/mL rRSPO3. Graph depicts organoid-forming efficiency at 5 days relative to 100 ng/ml rRSPO3 (107.3 $\pm$19.6 organoids/well, n=3). Statistical comparisons used one-way ANOVA followed by Tukey's posttest. ****$P<0.0001$, scale bar 50 μm.

EXTENDED DATA FIGURE LEGENDS

Extended Data Figure 1. pSMAD1/5 and ISEMF distributions during pre- and post-natal development, organoid co-culture features, and isolation of mesenchymal populations.

a, Epithelial nuclear pSMAD1/5 (arrowheads) increases over time at villus bottoms. Magenta: pSMAD1/5, green: Laminin (basement membrane), blue: DAPI, cr: crypt, scale bars: 100 μm.

b, Representative immunostaining with the indicated antibodies in *Pdgfra*$^{H2B\text{-}eGFP}$ fetuses at embryonic days (E) 14.5 and E16.5. GFP^{hi} ISEMFs initially concentrate at emerging villus tips, while GFP^{lo} cells are present throughout the stroma. Top and bottom rows show the same fields with (top) or without (bottom) DAPI signals. n = 3 embryos per stage, scale bar 50 μm, dashed white lines: inter-villus epithelium.

c, Schema for mesenchyme isolation and co-culture. P14 or adult (>8 weeks) *Pdgfra*$^{H2B\text{-}eGFP}$ whole mesenchyme was plated, sorted 2-3 days later by flow cytometry (FACS), and different fractions were placed in co-culture with isolated crypt epithelium. FACS plots show GFP^{hi}, GFP^{lo}, and GFP^{-} fractions; histograms show GFP^{lo} cell staining with CD81 antibody.

d, Organoids imaged on consecutive days after ISEMF co-culture show retraction of budding structures (arrowheads).

e, Two-day old organoids cultured with recombinant RSPO1 (R), EGF (E), and either NOG (N) or GFP^{hi} ISEMFs from P14 *Pdgfra*$^{H2B\text{-}eGFP}$ mouse SI. Representative organoids are shown 48 h later (scale bar 100 μm). NOG rescues failure to bud in crypts co-cultured with P14 ISEMFs (arrowheads). Graph displays data from 4 biological replicates. Statistical differences, determined by one-way ANOVA followed by Tukey's multiple comparisons test, are reported relative to +ER (no cells). ns: not significant, **$p<0.01$.

f, Organoid numbers per sample relative to control wells (-cells +ENR, 64.5 $\pm$20.03 organoids/ well, n=6) of P14 vs. adult mesenchymal populations co-cultured with adult crypts in media without recombinant factors. Significance determined by Student's t-test; **$p<0.01$, ***$p<0.001$.

Extended Data Figure 2. Merged postnatal scRNA datasets from SI mesenchyme.

a, Clustering of cell populations by uniform manifold approximation and projection (UMAP, left) and markers signifying distinct cell types (right) in merged postnatal scRNA-seq datasets.

b, *Ptprc*$^{+}$ cells isolated from the combined dataset (UMAP, left) annotated for distinct immune cells and leukocytes based on marker expression (right).

c, Relative fractions of proliferative (*Mki67*$^{+}$) cell clusters at different postnatal ages.

d, Cell distributions within the global UMAP plot with respect to postnatal age (left) or method of mesenchymal cell isolation (right, unfractionated vs. GFP^{+} cells).

e, Cell types at each postnatal age. Compared to other populations, MM and LPM cells (bold) increase substantially between P1 and P14.

Extended Data Figure 3: ISH images and quantitation of SM cell-type marker expression.

a, Negative control ISH probe (RNAscope universal control: *B. subtilis DapB* gene) hybridized to P14 SI. Left, color image; right, greyscale version of the same image. All scale bars 50 μm.

b, Fractions of SI sub-epithelial SM cell types showing *Nog*, *Pcp4* or *Osr1* expression at P14. Parentheses indicate cell numbers counted (denominators for the fractions) in SI from 2 mice ("submucosa" refers to the trophocyte zone between MM and MP; "plexus" refers to intermuscular neurons).

c, *Hhip* expression across P14 SM populations determined by scRNA-seq (violin plot) and ISH (RNAscope images). *Hhip*, absent in MP layers, is present in *Grem2*$^-$ LPM (dashed red box) and *Grem2*$^+$ MM (dashed white box – MM and LPM are oriented perpendicular to each other), both magnified on the right. Greyscale versions of the same images are shown to the right of (LPM) or below (MM) color ISH images. Arrowheads: *Hhip* and *Grem2* co-expressing MM.

d, *Grem1* ISH at P14 reveals expression in MM and/or select MP layers, as quantified to the right in SI from 2 mice (parentheses in the table indicate cell numbers counted). Green: LAMININ, magenta: *Grem1* (greyscale version of the same image is shown below), white/green dashed lines: MP, arrowheads: MM, cr: crypts.

e, Double ISH for *Nog* and *Grem2* highlights that *Grem2*$^+$ MM is distinct from *Nog*$^+$ superficial MP. Grey: LAMININ, magenta: *Nog*, green: *Grem2* (greyscale versions without LAM signal shown to the right), arrowheads: expressing (filled) and non-expressing (blank) cells.

Extended Data Figure 4. Differential postnatal gene expression and CellPhone DB analysis.

a, Average expression of Wnt, secreted Wnt antagonist, and BMP genes in combined postnatal intestinal mesenchyme dataset (Fig. 3a).

b, Average expression of the top 50 genes differentially expressed between P1/2 (aggregated) and P14, shown at those ages, P4+P5 (aggregated), and P9 in trophocytes (left) and *Nog*$^{+}$ superficial MP (right).

c, CellPhone DB analysis of putative cell-cell interactions inferred from reciprocal expression of ligands and receptors in different postnatal SI mesenchymal cell populations. Heatmaps show total number of potential interactions (ligand-receptor pairs) between cell types, with ISEMFs accounting for the largest number. Venn Diagrams below show fewer interactions at P9 and P14 than from P1 to P5 between ISEMFs and Pdgfra^{lo}CD81^{-} stroma (left) or LPM (right).

Extended Data Figure 5. Analysis of postnatal *Pdgfra*$^{H2B\text{-}eGFP}$ and *Pdgfra*^{CreERT2};*Rosa26*^{tdTom} and adult MM populations.

a, Fractions of GFP$^+$ MM or LPM cells in *Pdgfra*$^{H2B\text{-}eGFP}$ intestines at the indicated ages. GFP$^+$ cells are in a majority until at least P14, but both adult populations are GFP$^-$. 3 independent animals per timepoint, n: total cells counted in each replicate (rep).

b, Markers and graph-based UMAP clustering in GFP$^+$ cell fractions from P14 (top) and average BMPi and Rspo gene expression at P14 (bottom).

c, *Pdgfra*$^{Cre(ER\text{-}T2)}$;*Rosa26*^{TdTom} pups were treated with tamoxifen (TAM) at P0 and intestines were examined at P1. In the representative image, both PDGFRA$^+$ ISEMFs (PDGFRA antibody labels ISEMFs green, arrowhead) and undefined stroma (arrow, red PDGFRA^{lo} cells above ACTA2+ yellow cells) express TdTom after TAM-mediated Cre activation. Scale bar, 50 μm.

d, Delineation of mesenchymal cell populations in adult *Pdgfra*$^{H2B\text{-}eGFP}$ duodenum. ACTA2 (smooth muscle) and CD31 (blood vessels) Ab stains identify thin MM (arrowheads) and LPM, distinct from the thick MP. Dashed white line: superficial MP, cr: crypts.

e, scRNA analysis of 2,224 adult *Myh11*$^+$ SI cells and graph-based UMAP clustering of cell populations. *Hhip* (red) expression principally marks LPM; *Grem2* (blue) marks the substantially fewer recoverable MM cells.

f, scRNA data from *Myh11*$^+$ SI cells (**e**) were combined with scRNA data from unfractionated adult SI mesenchyme[36] (GEO series GSE130681). Molecular markers identified each cell type (see Extended Data Fig. 2a). Average BMPi and Rspo gene expression and cell numbers in each cluster are shown to the right.

Extended Data Fig. 6. ISH in Adult SI and SM ablation in Myh11;DTA mice.

a-b, Representative *Rspo3* (**a**, magenta and below greyscale), *Grem1* and *Grem2* (**b**, red and yellow respectively) ISH of adult *Pdgfra*$^{H2B\text{-}eGFP}$ SI. In line with scRNA data (Fig. 4a), *Rspo3* is expressed in MM (arrowheads), MP (dotted white lines demarcate superficial MP), and scattered submucosal cells. In addition to these cells, *Grem1* is expressed in GFP^{+} sub-cryptal trophocytes (green arrowheads in **b**). *Grem2* is expressed in MM, superficial MP, and interstitial cells of Cajal (ICC; cr: crypts).

c, Composite *Grem1* ISH overlayed onto IHC-stained *Pdgfra*$^{H2B\text{-}eGFP}$ adult mouse duodenum (sections 10 μm removed from each other), showing high expression in both MM (arrowhead) and superficial MP (between brackets; PDGFRA in red, *Pdgfra*$^{H2B\text{-}eGFP}$ in green, DAPI in blue, LAMININ in yellow).

d, Representative image of a *Myh11*$^{Cre(ER\text{-}T2)}$*; Pdgfra*$^{H2B\text{-}eGFP}$;*R26R*^{TdTom} mouse injected at P14, harvested at P21, and stained for ACTA2 (blue). Cre activity (TdTom, magenta) and ACTA2 expression coincide (n= 3 mice). All scale bars: 50 μm.

Extended Data Figure 7. Investigation of SM functions and potency of human RSPO.

a, Reduced body weight and SI length at P16 and P21 after TAM treatment of *Myh11*$^{Cre(ER-T2)}$*;R26R*^{DTA} pups at P14. Images show examples at P21. n= 7 or 8 animals, ***p <0.001, ****p <0.0001 using unpaired Student's t-test.

b, Quantitation of circular and longitudinal MP cells at P16 and P21 in control and *Myh11*$^{Cre(ER-T2)}$*;R26R*^{DTA} mice after TAM treatment at P14. n=3 or 4 animals, >4 mm examined per SI. *p <0.05 using unpaired Student's t-test.

c, Crosses with *Pdgfra*$^{H2B-eGFP}$ mice show a small decrease in sub-cryptal *Pdgfra*^{lo} cells (arrowheads), likely reflecting loss of *Myh11*$^{+}$ MM and LPM precursors. n=3 animals, >4 mm examined in each, *p <0.05 using unpaired Student's t-test.

d, Crypt cell proliferation, assessed by BrdU injection 1 h before euthanasia, is similar in TAM-injected control and *Myh11;DTA* mice. n=3 animals in each cohort, ≥25 SI crypts per animal, differences are non-significant by unpaired Student's t-test. Scale bar 50 μm.

e, F-actin (phalloidin)-stained SI epithelium whole-mounts show fewer bifid crypts at P16 in *Myh11*$^{Cre(ER-T2)}$*;Rosa26*^{DTA} pups injected with TAM at P14. Scale bar 50 μm.

f, Neither P14 nor adult MP co-cultures with P14 SI crypts elicit organoid growth but both yield robust organoids in the presence of sub-optimal rEGF (E) and rRSPO (R) concentrations. Organoid numbers are represented relative 76.7 ±12.9 organoids in ENR medium (n=3). ****p <0.0001 using Student's t-test.

g, SI crypts cultured over manually isolated colonic MM (dashed white line) develop within 1 day into spheroidal structures (n=3). The image is a collage assembled from multiple microscopic fields (scale bar 200 μm). Inset magnifies one such field (scale bar 100 μm).

h, Representative FACS histogram of GFP^{+} cells isolated from manually stripped colonic MM and examined for CD81 expression. -Ab: control flow cytometry without CD81 Ab.

i, Adult SI crypts cultured in media containing murine rEGF, rNOG, and different concentrations of the indicated human RSPO (n=3 biological replicates each). Organoid numbers are graphed with respect to 146.3 ±20.6 organoids formed in 100 ng/ml hRSPO3. Statistical significance was determined using one-way ANOVA followed by Tukey's posttest. ***P< 0.001, *P <0.05.

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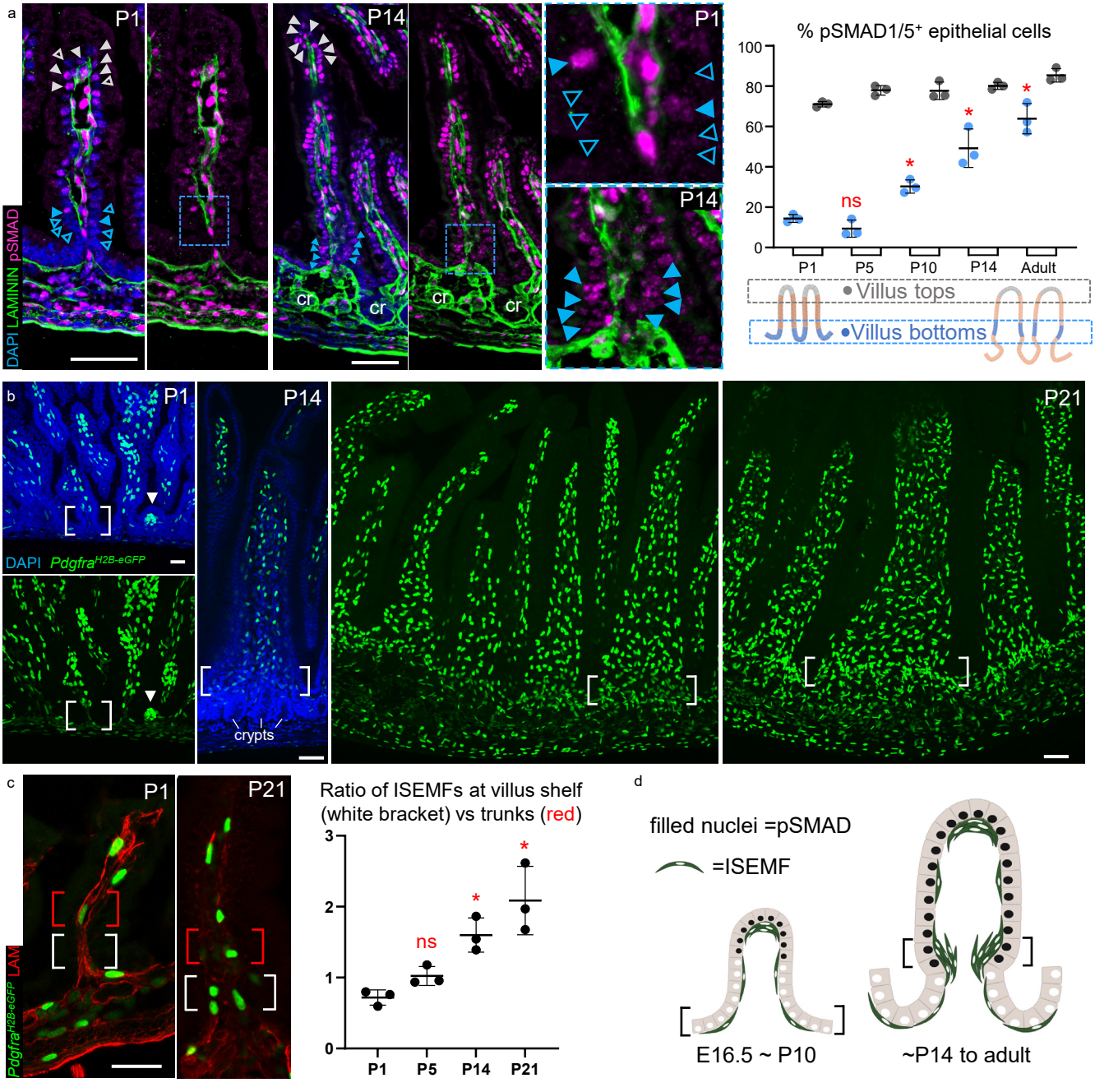
Fig. 1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P14
Adult
Villus tops
Villus bottoms
b
P1
P14
P21
DAPI Pdgfra H2B-eGFP
crypts
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P21
Pdgfra H2B-eGFP LAM
Ratio of ISEMFs at villus shelf
(white bracket) vs trunks (red)
3
2
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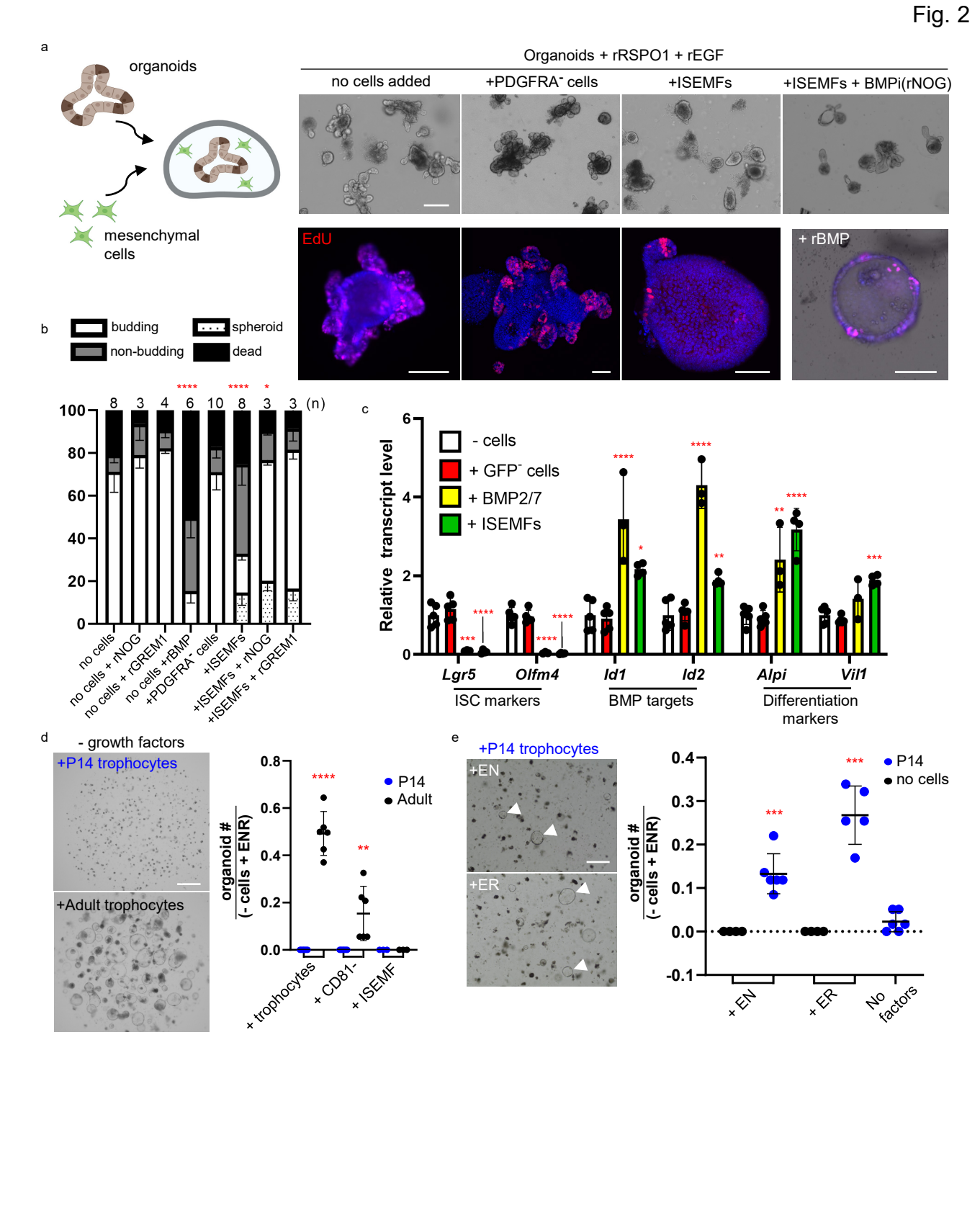
~P14 to adult


a
organoids
mesenchymal cells
Organoids + rRSPO1 + rEGF
no cells added
+PDGFRA⁻ cells
+ISEMFs
+ISEMFs + BMPi(rNOG)
EdU
+ rBMP
b
budding
spheroid
non-budding
dead
8 3 4 6 10 8 3 3 (n)
no cells
no cells + rNOG
no cells + rGREM1
no cells +rBMP
+PDGFRA⁻ cells
+ISEMFs
+ISEMFs + rNOG
+ISEMFs + rGREM1
c
- cells
+ GFP⁻ cells
+ BMP2/7
+ ISEMFs
Relative transcript level
Lgr5
Olfm4
Id1
Id2
Alpi
Vil1
ISC markers
BMP targets
Differentiation markers
d
- growth factors
+P14 trophocytes
+Adult trophocytes
organoid # / (- cells + ENR)
P14
Adult
+ trophocytes
+ CD81-
+ ISEMF
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+P14 trophocytes
+EN
+ER
organoid # / (- cells + ENR)
P14
no cells
+ EN
+ ER
No factors

Fig. 3

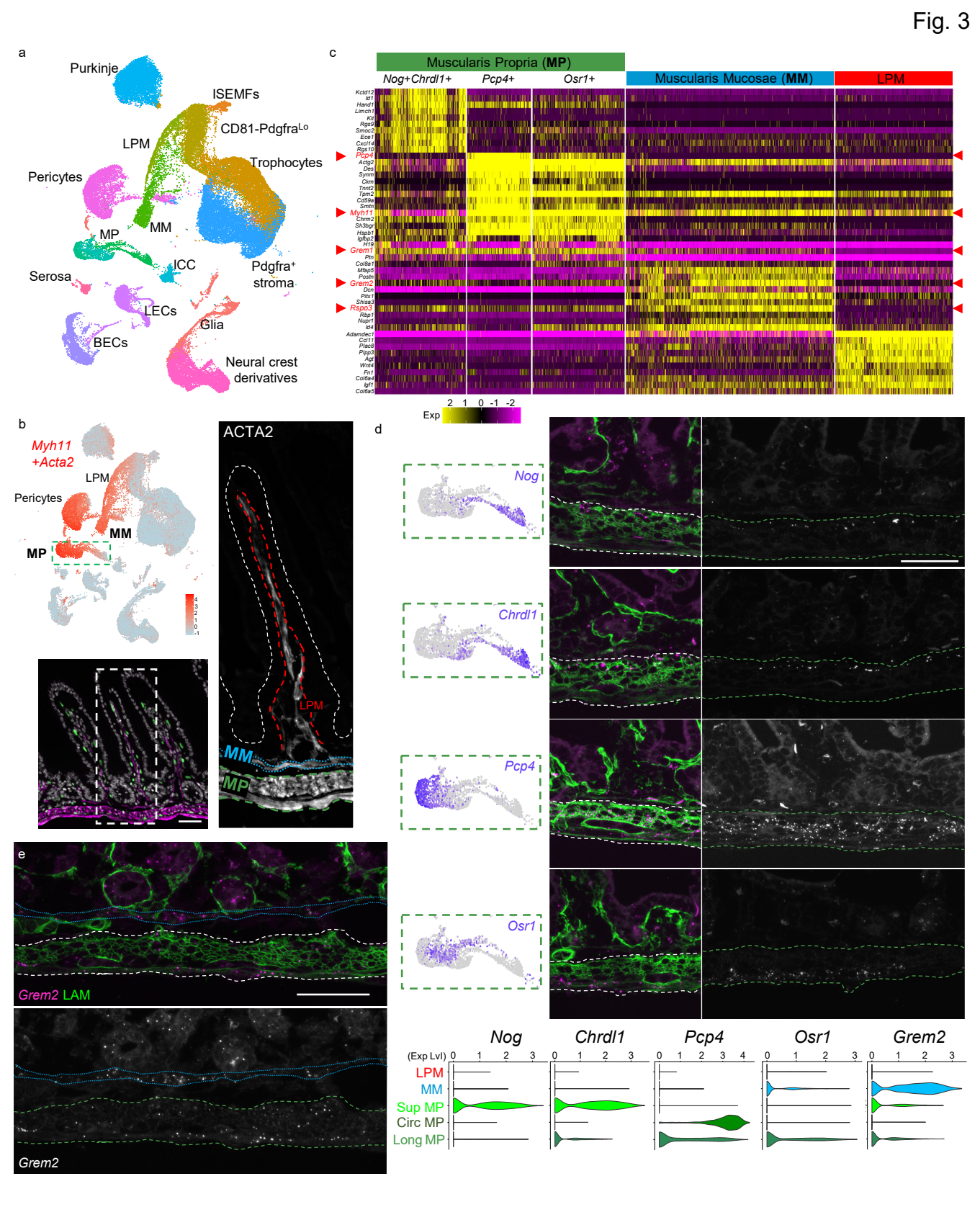
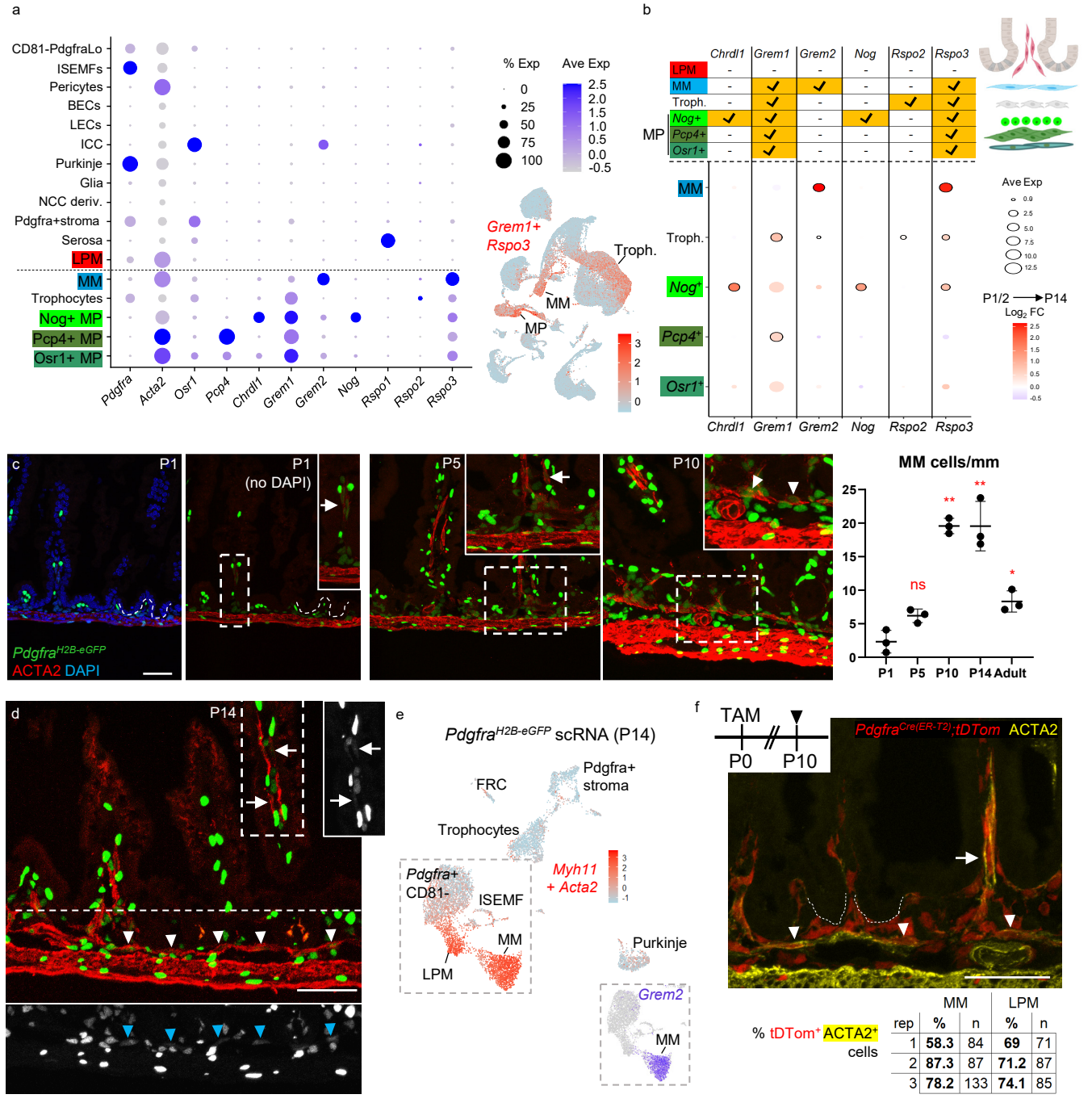

a
Purkinje
ISEMFs
CD81-PdgfraLo
LPM
Trophocytes
Pericytes
MM
MP
ICC
Pdgfra+ stroma
Serosa
LECs
Glia
BECs
Neural crest derivatives
c
Muscularis Propria (MP)
Nog+Chrdl1+
Pcp4+
Osr1+
Muscularis Mucosae (MM)
LPM
Kctd12
Id1
Hand1
Limch1
Kit
Rgs9
Smoc2
Ece1
Cxcl14
Rgs10
Pcp4
Actg2
Des
Synm
Ckm
Tnnt2
Tpm2
Col5a2
Smtn
Myh11
Chrnm2
Sh3bgr
Hspb1
Igfbp2
H19
Grem1
Ptn
Col8a1
Mfap5
Postn
Grem2
Dcn
Pitx1
Shisa3
Rspo3
Rbp1
Nupr1
Id4
Adamdec1
Ccl11
Plac9
Plpp3
Agt
Wnt4
Fn1
Col6a4
Igf1
Col6a5
2 1 0 -1 -2
Exp
b
Myh11 +Acta2
LPM
Pericytes
MM
MP
ACTA2
LPM
MM
MP
d
Nog
Chrdl1
Pcp4
Osr1
e
Grem2 LAM
Grem2
Nog
Chrdl1
Pcp4
Osr1
Grem2
(Exp Lvl)
LPM
MM
Sup MP
Circ MP
Long MP

Fig. 4



rep	MM %	MM n	LPM %	LPM n
1	**58.3**	84	**69**	71
2	**87.3**	87	**71.2**	87
3	**78.2**	133	**74.1**	85

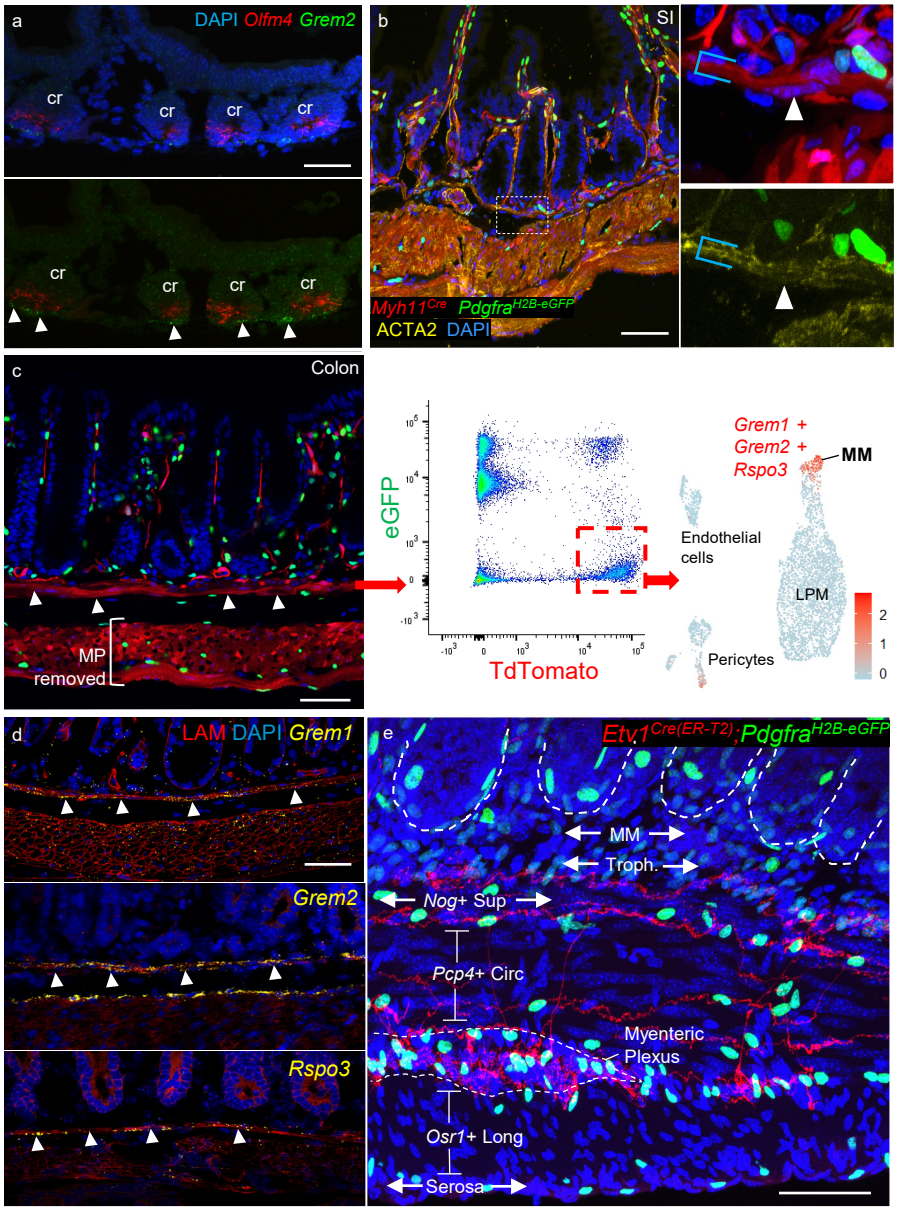
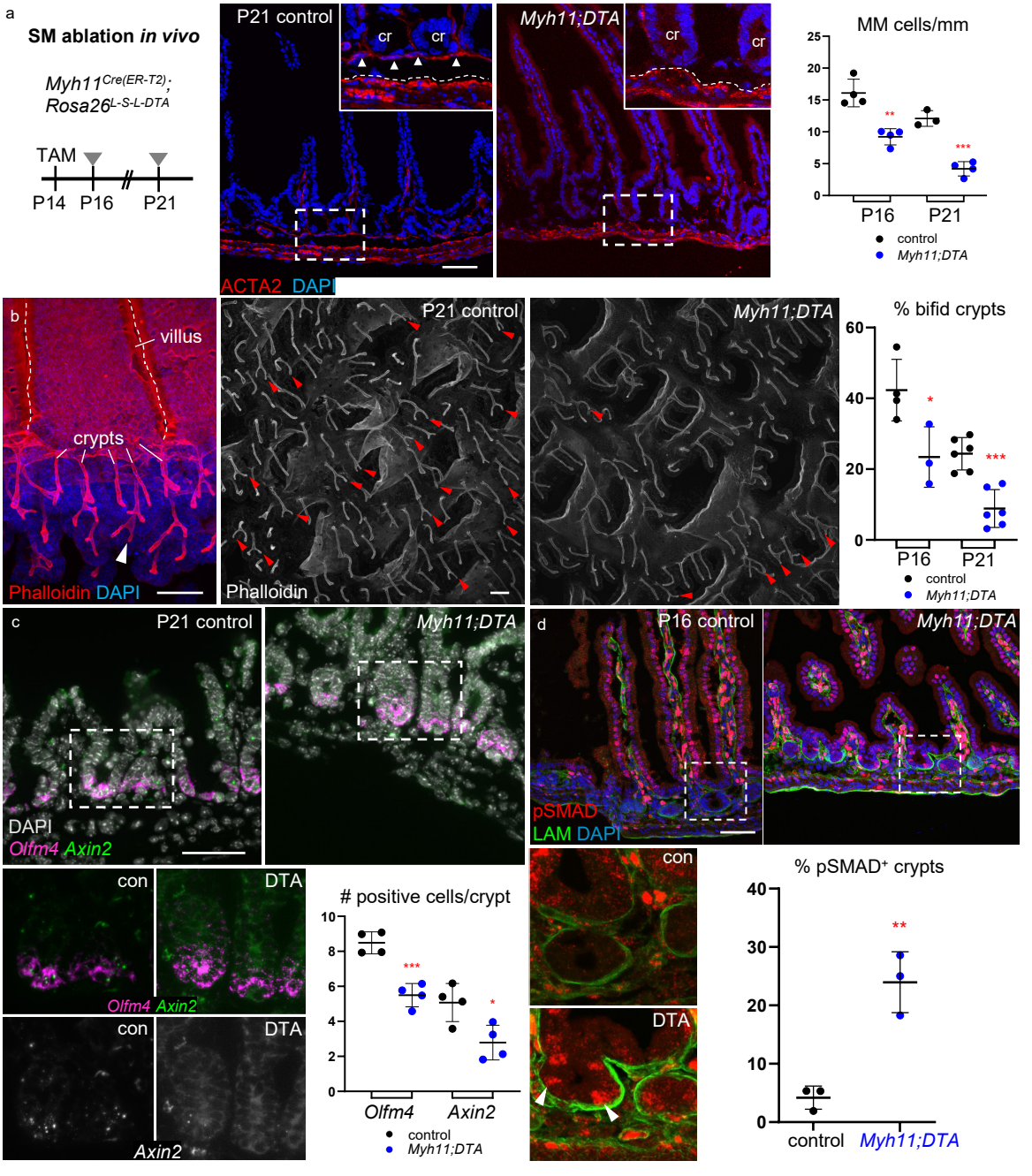
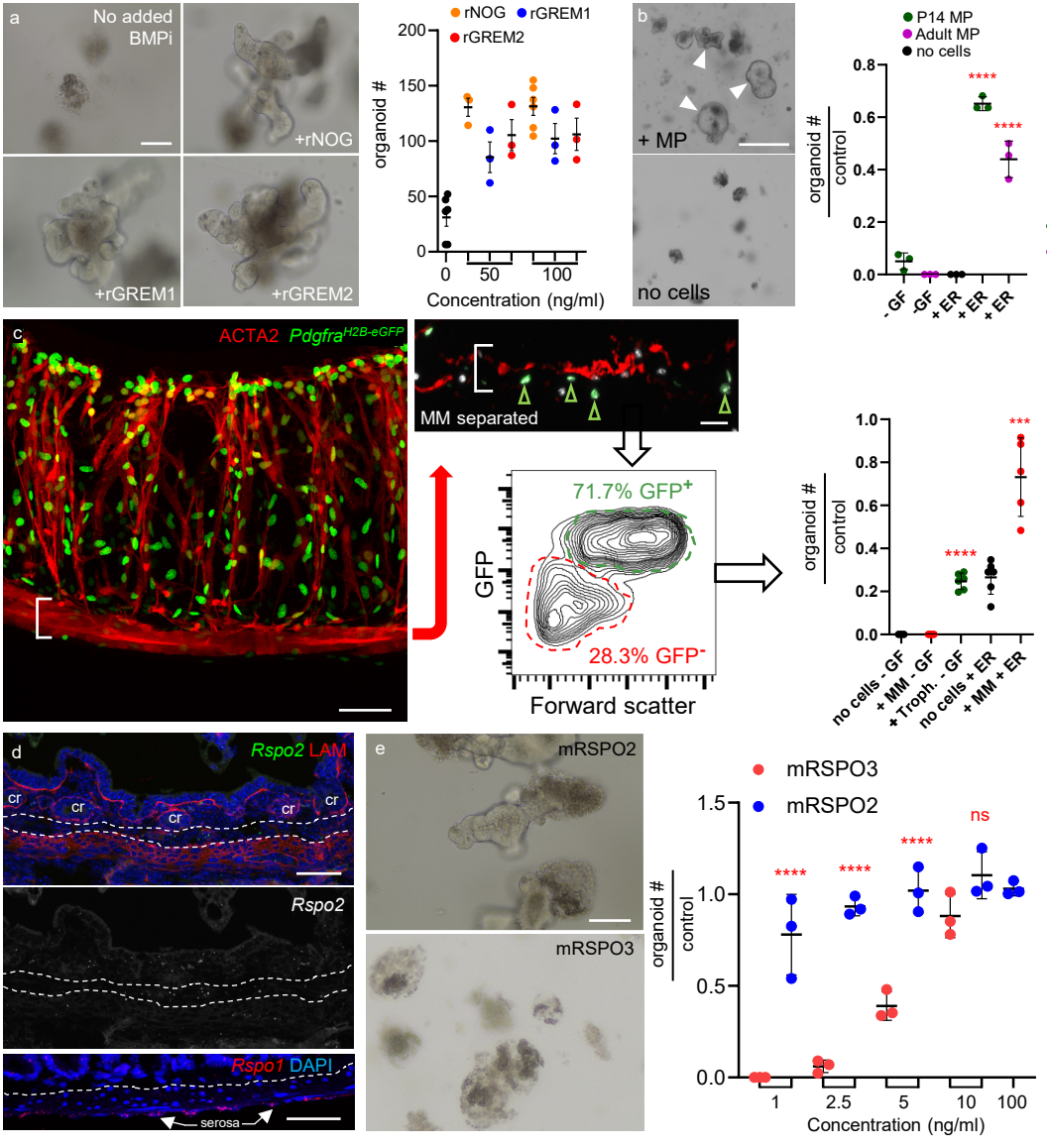

a
DAPI Olfm4 Grem2
cr
cr
cr
cr
cr
cr
cr
cr
b
SI
Myh11Cre PdgfraH2B-eGFP
ACTA2 DAPI
c
Colon
MP
removed
eGFP
TdTomato
Grem1 +
Grem2 +
Rspo3
MM
Endothelial
cells
LPM
Pericytes
2
1
0
d
LAM DAPI Grem1
Grem2
Rspo3
e
Etv1Cre(ER-T2);PdgfraH2B-eGFP
MM
Troph.
Nog+ Sup
Pcp4+ Circ
Myenteric
Plexus
Osr1+ Long
Serosa

Fig. 6


a
SM ablation in vivo
Myh11Cre(ER-T2);
Rosa26L-S-L-DTA
TAM
P14 P16 P21
P21 control
Myh11;DTA
cr
ACTA2 DAPI
MM cells/mm
P16 P21
control
Myh11;DTA
b
villus
crypts
Phalloidin DAPI
Phalloidin
% bifid crypts
c
DAPI
Olfm4 Axin2
con
DTA
positive cells/crypt
Olfm4 Axin2
d
pSMAD
LAM DAPI
% pSMAD+ crypts
control Myh11;DTA


a
No added BMPi
+rNOG
+rGREM1
+rGREM2
rNOG
rGREM1
rGREM2
organoid #
Concentration (ng/ml)
b
+ MP
no cells
P14 MP
Adult MP
no cells
organoid # / control
- GF
-GF
+ ER
+ ER
+ ER
c
ACTA2 Pdgfra H2B-eGFP
MM separated
71.7% GFP+
28.3% GFP-
GFP
Forward scatter
organoid # / control
no cells - GF
+ MM - GF
+ Troph. - GF
no cells + ER
+ MM + ER
d
Rspo2 LAM
cr
Rspo2
Rspo1 DAPI
serosa
e
mRSPO2
mRSPO3
mRSPO3
mRSPO2
ns
organoid # / control
Concentration (ng/ml)