Figure S1

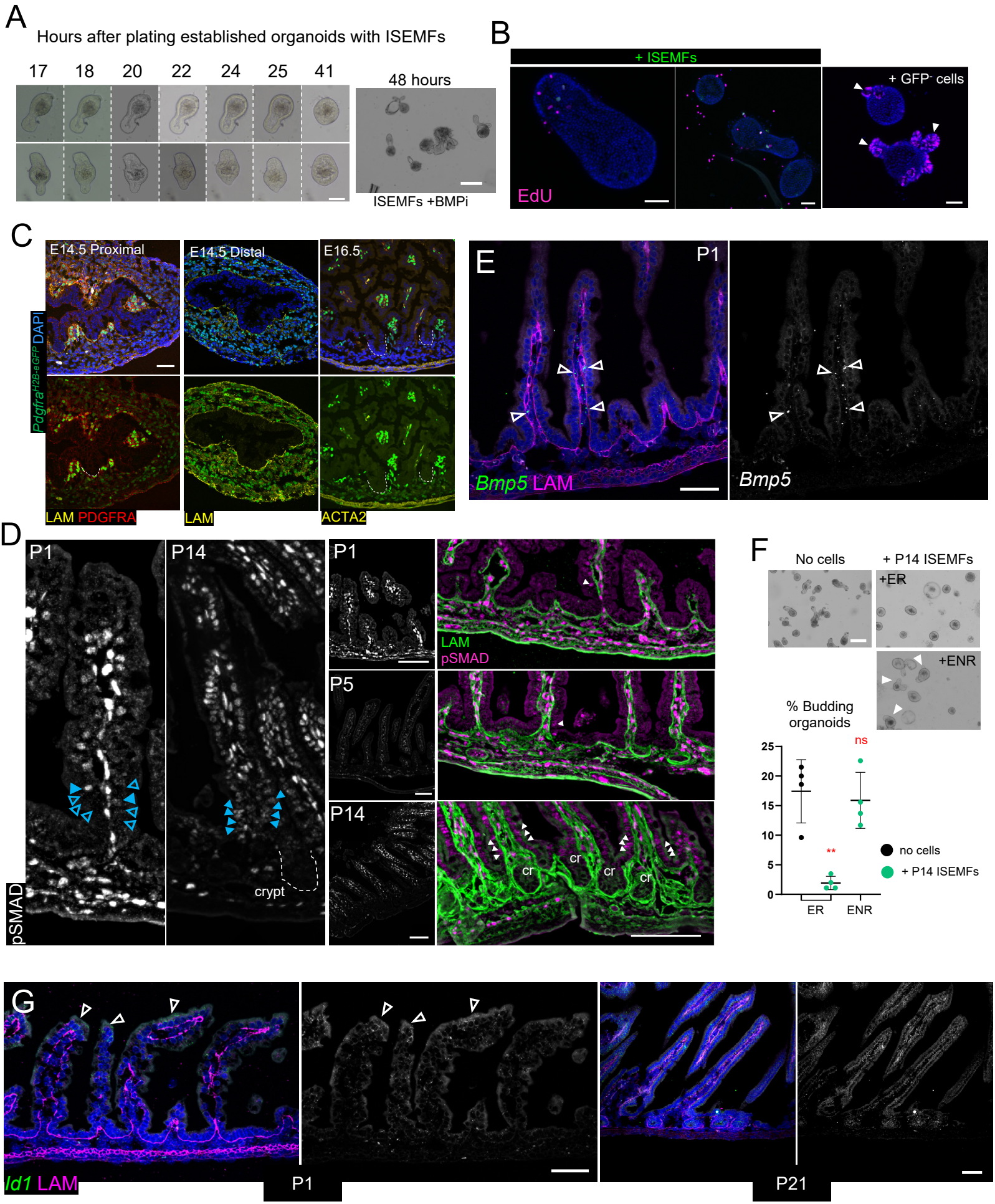

A
Hours after plating established organoids with ISEMFs
17
18
20
22
24
25
41
48 hours
ISEMFs +BMPi
B
+ ISEMFs
+ GFP- cells
EdU
C
E14.5 Proximal
E14.5 Distal
E16.5
Pdgfra H2B-eGFP DAPI
LAM PDGFRA
LAM
ACTA2
E
P1
Bmp5 LAM
Bmp5
D
P1
P14
P1
P5
P14
LAM
pSMAD
crypt
cr
pSMAD
F
No cells
+ P14 ISEMFs
+ER
+ENR
% Budding organoids
ns
**
no cells
+ P14 ISEMFs
ER
ENR
G
Id1 LAM
P1
P21

Figure S1. Organoid co-culture features, ISEMF and pSMAD1/5/9 distributions during pre- and post-natal development, and postnatal BMP pathway activation. Related to Figures 1 and 2.

A) Left: Single organoids imaged at the indicated times after ISEMF co-culture show retraction of budding structures. Scale bar 50 μm. Right: Organoids co-cultured with both ISEMFs and the BMPi rNOG, imaged 48 h after plating. Scale bar 100 μm.

B) Additional examples of EdU uptake by established organoids co-cultured with ISEMFs or GFP^- ($PDGFRA^-$) cells. Organoids grown with GFP^- cells show substantially more EdU^+ buds. EdU signal outside organoids is in the co-cultured mesenchymal cells. Scale bars 50 μm.

C) Representative immunostaining with the indicated antibodies in *Pdgfra*$^{H2B\text{-}eGFP}$ embryos 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: E16.5 inter-villus epithelium.

D) Left: Greyscale images of pSMAD1/5/9 immunostaining from Figure 1C (note absence of signal in P14 crypts). Right: Epithelial nuclear pSMAD (arrowheads) increases over time at villus bottoms. Magenta: pSMAD1/5/9, green: Laminin (basement membrane), blue: DAPI, cr: crypt. Scale bars: 100 μm.

E) RNAscope ISH for *Bmp5* at P1, showing expression in mesenchymal ISEMFs (arrowheads). Left, LAMININ (magenta) and *Bmp5* (green); right: *Bmp5* in greyscale. Scale bar 50 μm.

F) 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 budding failure in crypts co-cultured with P14 ISEMFs (arrowheads). Graph shows 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, $^{**}p < 0.01$. Scale bar 50 μm.

G) RNAscope ISH for *Id1* at P1 and P21 showing increasing expression in the epithelial compartment with age, Arrowheads point to villus tip expression at P1; this extends throughout the villus length at P21). Left: 2-color fluorescence, right: *Id1* ISH isolated in greyscale. Scale bars 50 μm.

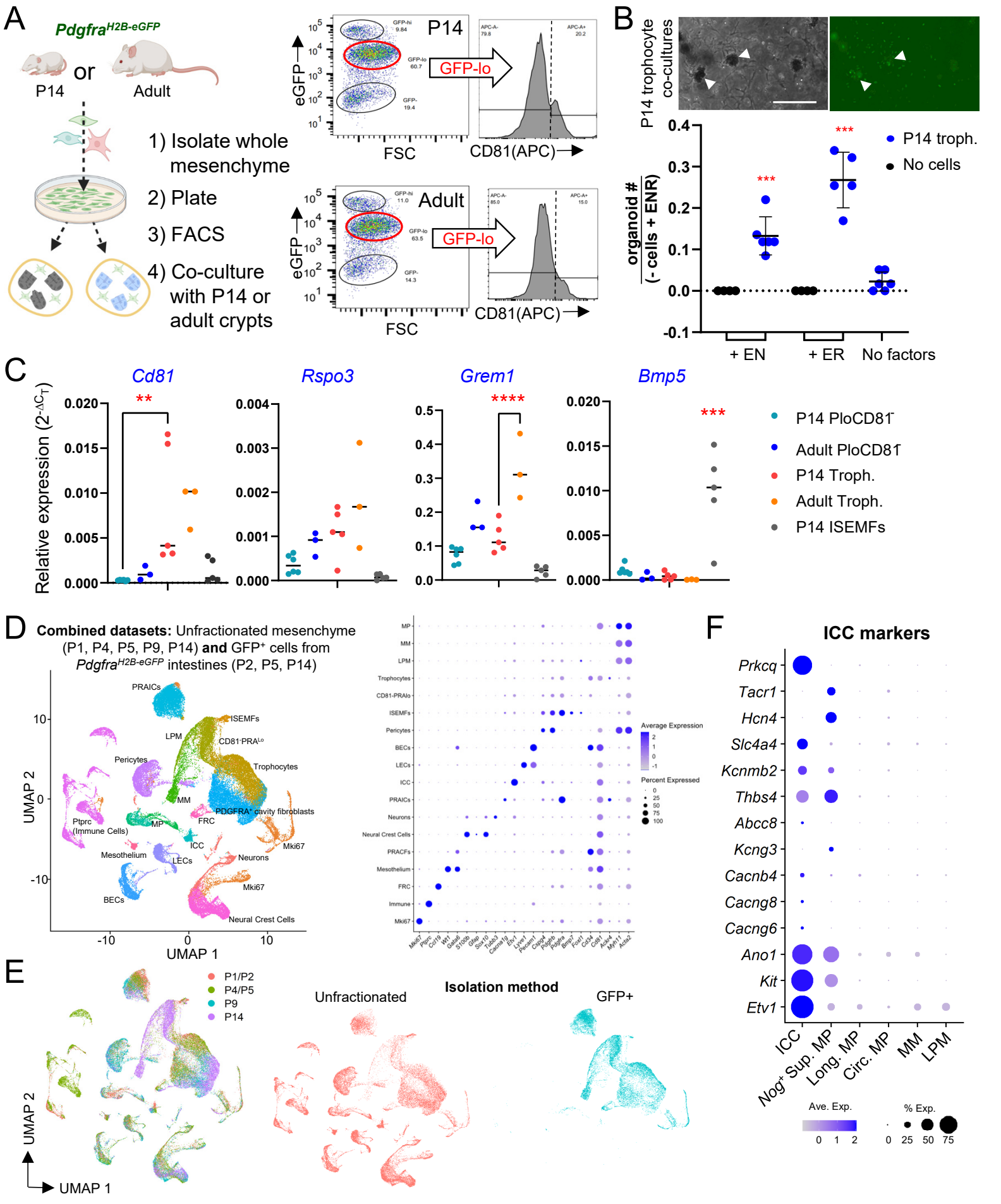

A
Pdgfra H2B-eGFP
P14
or
Adult
1) Isolate whole mesenchyme
2) Plate
3) FACS
4) Co-culture with P14 or adult crypts
P14
Adult
eGFP
FSC
GFP-lo
CD81(APC)
B
P14 trophocyte co-cultures
organoid # (- cells + ENR)
P14 troph.
No cells
+ EN
+ ER
No factors
C
Cd81
Rspo3
Grem1
Bmp5
Relative expression (2^-ΔCT)
P14 PloCD81-
Adult PloCD81-
P14 Troph.
Adult Troph.
P14 ISEMFs
D
Combined datasets: Unfractionated mesenchyme (P1, P4, P5, P9, P14) and GFP+ cells from Pdgfra H2B-eGFP intestines (P2, P5, P14)
PRAICs
ISEMFs
LPM
CD81-PRALo
Pericytes
Trophocytes
MM
PDGFRA+ cavity fibroblasts
Ptprc (Immune Cells)
MP
FRC
ICC
Mki67
Mesothelium
LECs
Neurons
BECs
Neural Crest Cells
UMAP 1
UMAP 2
Average Expression
Percent Expressed
E
P1/P2
P4/P5
P9
P14
Isolation method
Unfractionated
GFP+
F
ICC markers
Prkcq
Tacr1
Hcn4
Slc4a4
Kcnmb2
Thbs4
Abcc8
Kcng3
Cacnb4
Cacng8
Cacng6
Ano1
Kit
Etv1
ICC
Nog+ Sup. MP
Long. MP
Circ. MP
MM
LPM
Ave. Exp.
% Exp.

Figure S2. Organoid co-culture features and merged scRNA datasets from postnatal mouse SI mesenchyme. Related to Figures 2 and 3.

A) 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.

B) P14 trophocytes are viable during co-culture (arrowheads point to dying organoids surrounded by GFP^{+} trophocytes; left: bright field, right: GFP fluorescence) and promote organoid growth in the presence of low concentrations of rEGF plus rNOG (EN medium) or rRSPO1 (ER medium) that alone are insufficient for organoid growth. Organoid counts are represented relative to parallel control crypt cultures in ENR medium without added cells (59 $\pm$12.1 organoids/well, n=4). Statistical differences determined by Student's t-test at ***p <0.001 (n=4-6 biological replicates). Scale bar 250 μm.

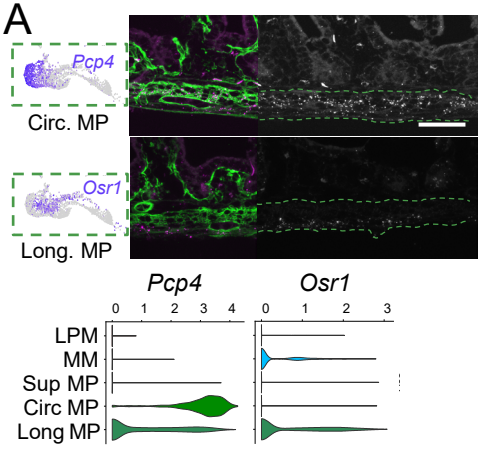
C) qRT-PCR analysis of marker genes from indicated P14 or adult cells before crypt co-cultures. Statistical differences were assessed by one-way ANOVA followed by Tukey's multiple comparisons test. **p <0.01, ***p <0.001, ****p <0.0001.

D) Clustering of cell populations by uniform manifold approximation and projection (UMAP, left) in merged postnatal scRNA-seq datasets and markers signifying distinct cell types (right). BECs, blood endothelial cells; FRC, follicle reticular cells; ICC, interstitial cells of Cajal; ISEMFs, intestinal subepithelial myofibroblasts; LECs, lymphatic endothelial cells; LPM, lamina propria myocytes; MM, muscularis mucosae, MP, muscularis propria; PRAICs, PDGFRA^{+} interstitial cells.

E) Cell distributions within the global UMAP plot with respect to postnatal age (left) or method of mesenchymal cell isolation (right, whole mesenchyme vs. GFP^{+} fraction isolated by FACS).

F) scRNA-seq data showing expression of ICC markers in the indicated cell populations.

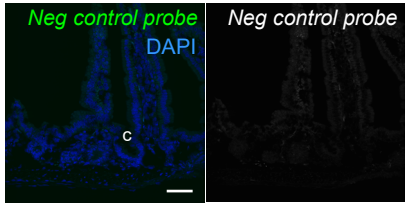
A



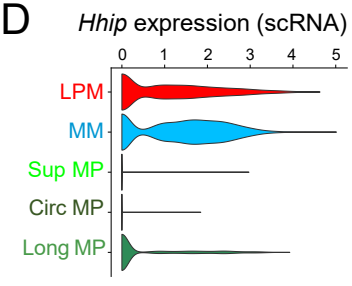
B

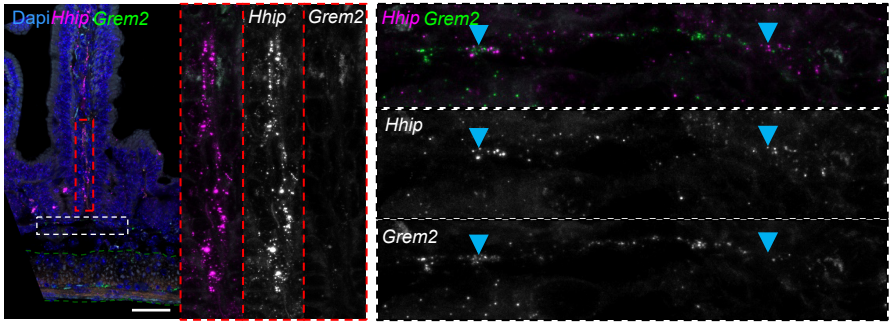
		Nog		Pcp4		Osr1	
		rep 1	rep 2	rep 1	rep 2	rep 1	rep 2
	MM	0% (15)	5% (18)	9% (11)	0% (19)	5% (17)	10% (19)
	Submucosa	3% (116)	2% (93)	6% (179)	1% (163)	2% (146)	12% (126)
Circular	Superficial	**41% (107)**	**61% (77)**	17% (75)	8% (82)	3% (94)	13% (60)
Circular	Deep	2% (276)	1% (266)	**77% (299)**	**65% (263)**	4% (245)	4% (295)
	Plexus	29% (17)	27% (22)	11% (17)	21% (14)	0% (8)	15% (13)
	Long	2% (110)	1% (122)	**47% (151)**	**38% (145)**	**87% (115)**	**70% (136)**

C

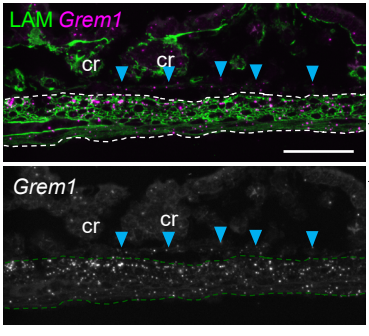


D





E



		Grem1		Grem2	
		rep 1	rep 2	rep 1	rep 2
	MM	**80% (15)**	**83% (18)**	**91% (22)**	**81% (22)**
	Submucosa	41% (101)	26% (182)	12% (196)	4% (162)
Circular	Superficial	**97% (78)**	**90% (142)**	24% (103)	38% (100)
Circular	Deep	**83% (243)**	**76% (129)**	10% (277)	8% (331)
	Plexus	0% (6)	11% (27)	**77% (22)**	**48% (31)**
	Long	**61% (170)**	**73% (152)**	15% (118)	11% (20)

F

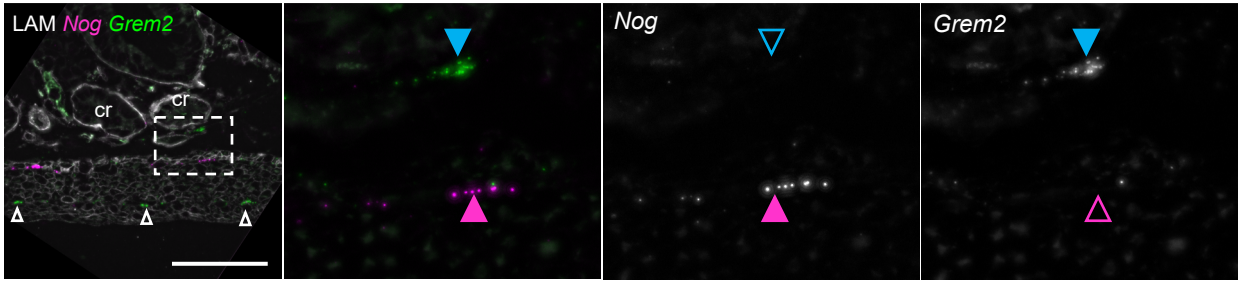


Figure S3: ISH images and quantitation of smooth muscle cell products. Related to Figure 3.

A) *Pcp4* and *Osr1* preferentially mark circumferential and longitudinal MP, respectively, as determined by ISH (top: RNAscope images) and scRNA-seq (top: projections onto the region of the scRNA UMAP boxed in Figure 3B, bottom: violin plots).

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

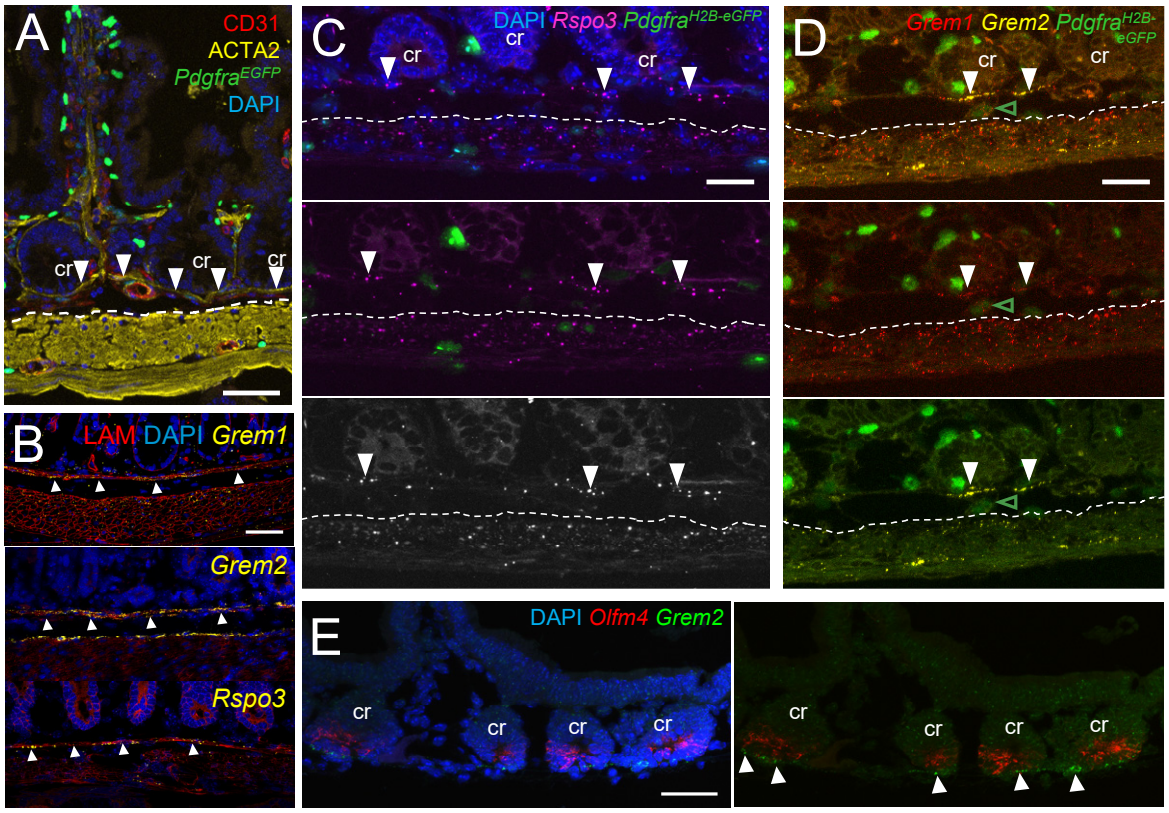
C) Negative control ISH probe (RNAscope universal control: *B. subtilis DapB* gene) hybridized to P14 SI. Left, color image; right, greyscale version of the same image.

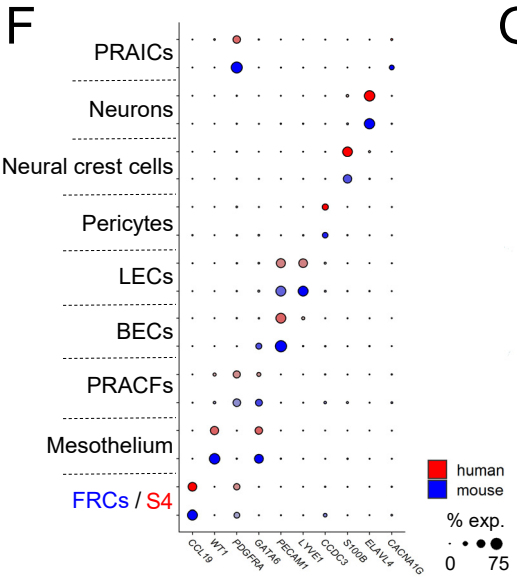
D) *Hhip* expression across P14 smooth muscle populations determined by scRNA-seq (violin plot) and ISH (RNAscope images). *Hhip*, absent in MP layers, is present in *Grem2*$^{-}$ LPM (dashed red box) and *Grem2*$^{+}$ MM (dashed white box – MM and LPM orient perpendicular to each other), both magnified 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.

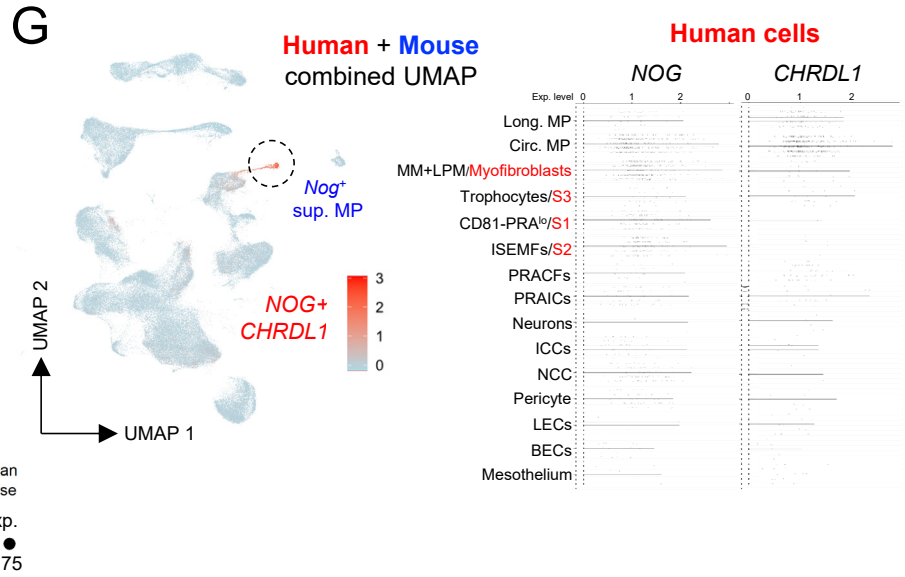
E) *Grem1* ISH at P14 reveals expression in MM and/or select MP layers, as quantified to the right in SI from 2 mice (parentheses in the table indicate cell numbers counted). Green: LAMININ, magenta: *Grem1* (greyscale version of the same image is shown below), white/green dashed lines: MP, arrowheads: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

F) 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. White arrowheads: *Grem2*-expressing ICC. All scale bars 50 μm.

Figure S4


A
CD31
ACTA2
Pdgfra^EGFP
DAPI
cr
B
LAM DAPI Grem1
Grem2
Rspo3
C
DAPI Rspo3 Pdgfra^H2B-eGFP
D
Grem1 Grem2 Pdgfra^H2B-eGFP
E
DAPI Olfm4 Grem2


F
PRAICs
Neurons
Neural crest cells
Pericytes
LECs
BECs
PRACFs
Mesothelium
FRCs / S4
CCL19
WT1
PDGFRA
GATA6
PECAM1
LYVE1
CCDC3
S100β
ELAVL4
CACNA1G
human
mouse
% exp.
0
75


G
Human + Mouse
combined UMAP
Nog+
sup. MP
NOG+
CHRDL1
UMAP 2
UMAP 1
Human cells
NOG
CHRDL1
Exp. level
Long. MP
Circ. MP
MM+LPM/Myofibroblasts
Trophocytes/S3
CD81-PRA^lo/S1
ISEMFs/S2
PRACFs
PRAICs
Neurons
ICCs
NCC
Pericyte
LECs
BECs
Mesothelium

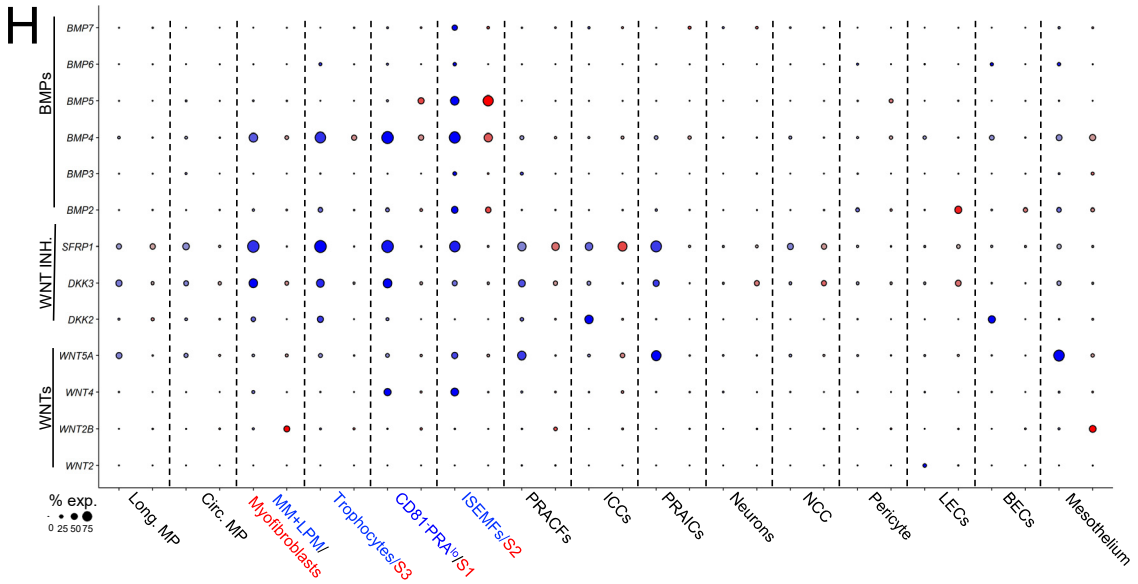

H
BMPs
BMP7
BMP6
BMP5
BMP4
BMP3
BMP2
WNT INH.
SFRP1
DKK3
DKK2
WNTs
WNT5A
WNT4
WNT2B
WNT2
% exp.
0 25 50 75
Long. MP
Circ. MP
MM+LPM/Myofibroblasts
Trophocytes/S3
CD81-PRA^lo/S1
ISEMFs/S2
PRACFs
ICCs
PRAICs
Neurons
NCC
Pericyte
LECs
BECs
Mesothelium

Figure S4. Adult ISH and human-mouse integrated dataset analysis. Related to Figure 4**.**

A) 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.

B) 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.

C-D) Representative ISH of adult *Pdgfra*$^{H2B\text{-}eGFP}$ SI with probes for *Rspo3* (**C**, magenta and below greyscale), *Grem1* and *Grem2* (**D**, red and yellow respectively). *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^{+} trophocytes (green arrowheads in **D**). *Grem2* is expressed in MM, superficial MP, and interstitial cells of Cajal (ICC; cr: crypts).

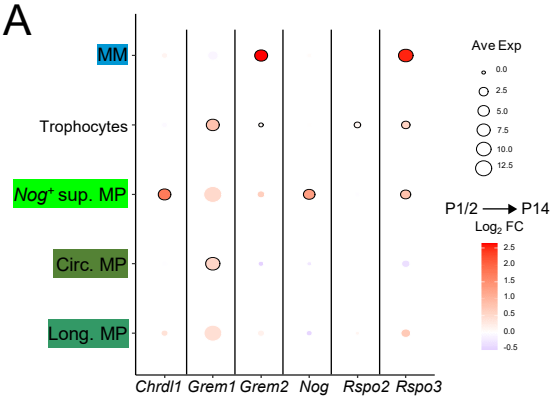
E) *Grem2* expression revealed by RNAscope ISH (green) in adult duodenal MM, directly beneath *Olfm4*$^{+}$ (red) ISCs (arrowheads); cr, crypts. MP was removed manually before staining. Left and right images are the same, with DAPI signal excluded on the right. Scale bar 50 μm.

F) Continuation of table (see Fig. 4C) of molecular markers for cell types identified separately in postnatal mouse (this study) and fetal human (Fawkner-Corbett et al., 2021) SI mesenchyme.

G) Substantive expression of *Nog* and *Chrdl1* is confined to mouse superficial MP. In human cells, scRNA-seq identifies low levels of either transcript only in cells classified as myofibroblasts (corresponding to mouse LPM + MM) and circumferential MP.

H) Average expression of BMP, secreted Wnt antagonist, and Wnt genes in cell clusters from integrated human and mouse scRNA-seq datasets.

A

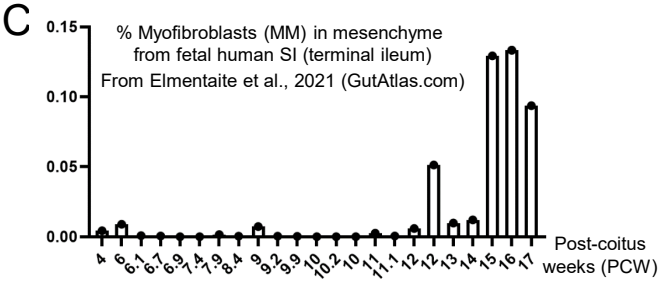


B

	Neurons	ISEMFs	Trophocytes	CD81⁻PRA^{Lo}	**LPM**	**MM**	MP	ICC
P1/P2:	281	398	2082	168	**17**	**84**	818	236
P4/P5:	1095	97	2526	254	**90**	**274**	509	276
P9:	605	41	1307	95	**19**	**89**	628	212
P14:	239	66	2092	3102	**935**	**1436**	262	61

	PRAICs	PRACFs	BECs	LECs	Pericytes	Ptprc	NCC	FRC	Mesothelia
P1/P2:	2702	1447	248	296	412	1336	370	105	134
P4/P5:	1695	2102	1123	1234	680	4164	1520	204	241
P9:	1250	2440	814	721	2652	209	1703	122	116
P14:	1059	2691	366	271	484	22	1812	188	93

C

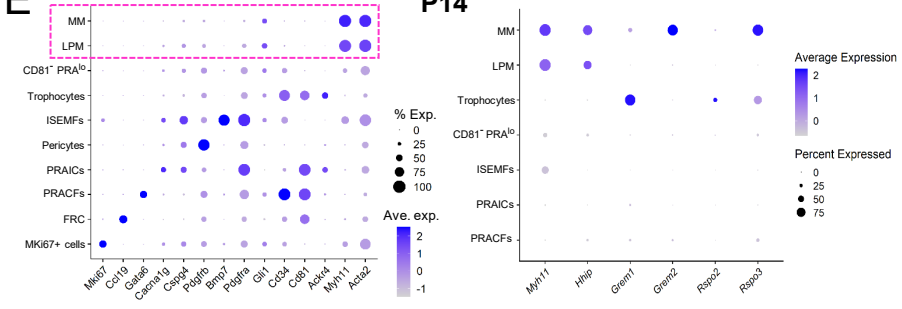


D

GFP⁺ (*Pdgfra*^{H2BeGFP}) ACTA2⁺ cells

		MM		LPM	
	rep	%	n	%	n
P1	1	**86**	35	**98.1**	52
	2	**100**	9	**87.5**	32
	3	**100**	4	**96.4**	28
P5	1	**80**	54	**96**	101
	2	**100**	47	**75.3**	69
	3	**100**	82	**82.5**	63
P10	1	**94**	130	**62.3**	69
	2	**97**	95	**65.8**	85
	3	**92**	133	**56.9**	65
P14	1	**87**	89	**80.9**	69
	2	**86**	118	**69.6**	85
	3	**71**	78	**82.2**	65
Adult	1	0	45	5.1	39
	2	2.3	42	6.3	47
	3	0	34	0	81

E



F

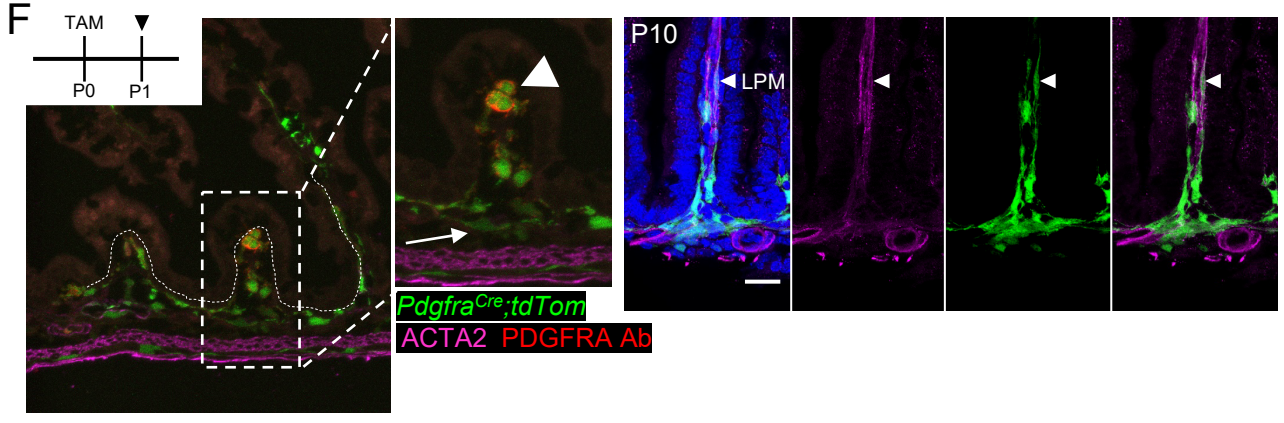


Figure S5. Analysis of postnatal *Pdgfra*$^{H2B\text{-}eGFP}$ and *Pdgfra*^{CreERT2};*Rosa26*^{tdTom} and adult MM populations. Related to Figures 4 and 5.

A) Plot depicting $\log_2$ fold increase between P1/2 and P14 in average mRNA expression per cell of the indicated trophic genes. BMPi and Rspo transcript levels increase significantly ($p < 0.05$, black outline) in diverse postnatal sub-cryptal cells.

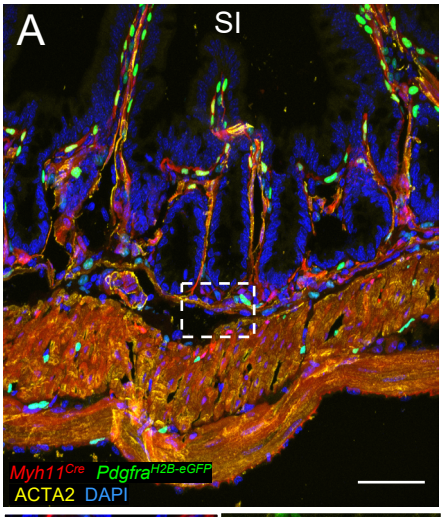
B) Numbers of each cell type harvested at each postnatal age. Compared to other populations, MM and LPM cells (bold) increase substantially between P1 and P14. BECs, blood endothelial cells; FRC, follicle reticular cells; ICC, interstitial cells of Cajal; ISEMFs, intestinal subepithelial myofibroblasts; LECs, lymphatic endothelial cells; LPM, lamina propria myocytes; MM, muscularis mucosae, MP, muscularis propria; NCC, neural crest cells; Ptprc, immune cells; PRAICs, PDGFRA$^+$ interstitial cells.

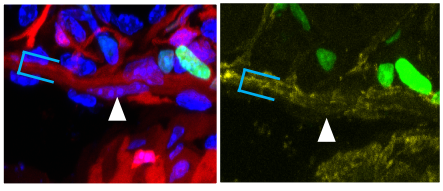
C) Percent of human SI "myofibroblasts" (corresponding to mouse MM + LPM) isolated at the indicated fetal ages, from scRNA-seq data reported in GutAtlas.com (Elmentaite et al., 2020).

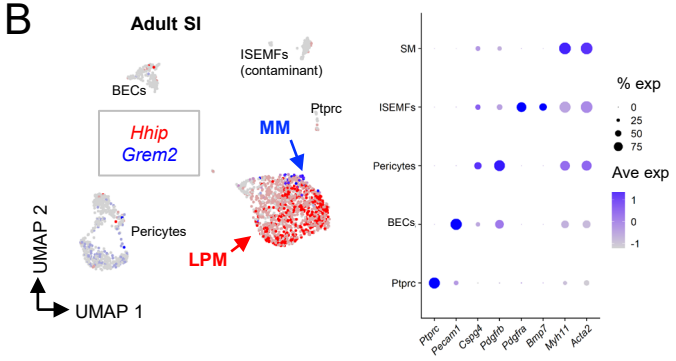
D) Fractions of GFP$^+$ MM or LPM cells in *Pdgfra*$^{H2B\text{-}eGFP}$ intestines at the indicated ages. GFP$^+$ cells are in a majority until at least P14, whereas both adult populations lack GFP. 3 animals were assessed at each of the indicated ages, n: total cells counted in each replicate (rep).

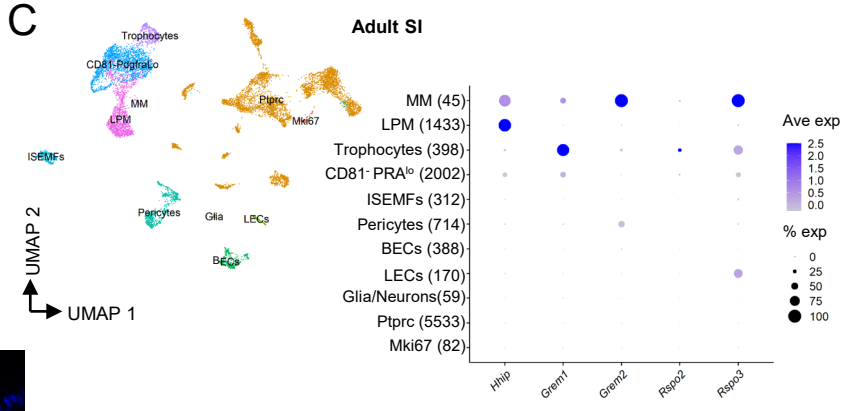
E) Markers for graph-based UMAP clustering in GFP$^+$ cell fractions from P14 (left) and average BMPi and Rspo gene expression at P14 (right).

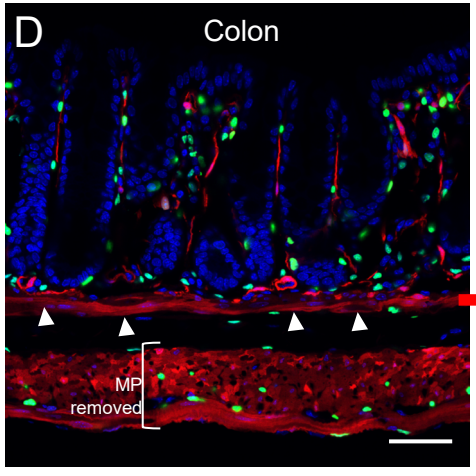
F) Left: *Pdgfra*$^{Cre(ER\text{-}T2)}$;*Rosa26*^{TdTom} pups were treated with tamoxifen (TAM) at P0 and intestines were examined at P1. In the representative image, both PDGFRA$^+$ ISEMFs (PDGFRA antibody labels ISEMFs red, arrowhead) and undefined stroma (arrow, green PDGFRA^{lo} cells above ACTA2$^+$ magenta cells) express TdTom after TAM-induced Cre activation. Right: *Pdgfra*$^{Cre(ER\text{-}T2)}$;*Rosa26*^{TdTom} pups were treated with TAM at P0 and intestines were examined at P10. Arrowhead points to double-positive *Pdgfra*$^{Cre(ER\text{-}T2)}$;*Rosa26*^{TdTom} (green) ACTA2$^+$ (magenta) cell. Scale bar, 50 μm.


A
SI
Myh11Cre PdgfraH2B-eGFP
ACTA2 DAPI




B
Adult SI
BECs
ISEMFs
(contaminant)
Ptprc
Hhip
Grem2
MM
Pericytes
LPM
UMAP 2
UMAP 1
SM
ISEMFs
Pericytes
BECs
Ptprc
% exp
0
25
50
75
Ave exp
1
0
-1
Ptprc
Pecam1
Cspg4
Pdgfrb
Pdgfra
Bmp7
Myh11
Acta2


C
Adult SI
Trophocytes
CD81-PdgfraLo
MM
LPM
ISEMFs
Ptprc
Mki67
Pericytes
Glia
LECs
BECs
UMAP 2
UMAP 1
MM (45)
LPM (1433)
Trophocytes (398)
CD81- PRAlo (2002)
ISEMFs (312)
Pericytes (714)
BECs (388)
LECs (170)
Glia/Neurons(59)
Ptprc (5533)
Mki67 (82)
Hhip
Grem1
Grem2
Rspo2
Rspo3
Ave exp
2.5
2.0
1.5
1.0
0.5
0.0
% exp
0
25
50
75
100


D
Colon
MP
removed

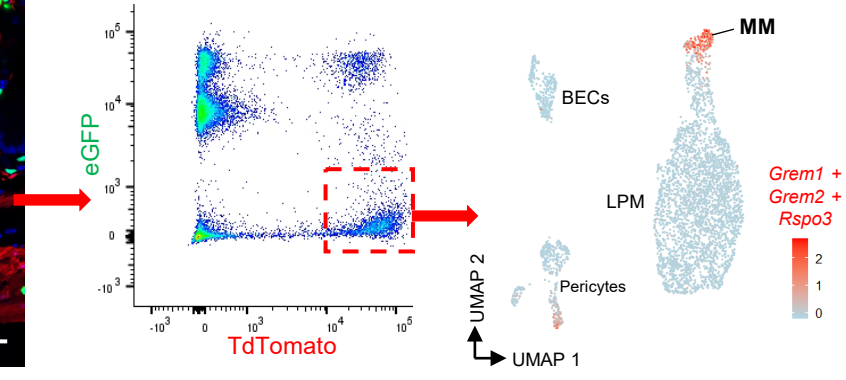

eGFP
TdTomato
MM
BECs
LPM
Pericytes
Grem1 +
Grem2 +
Rspo3
2
1
0
UMAP 2
UMAP 1

Figure S6. Resolution of MM from other smooth muscle populations. Related to Figure 5.

A) 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.

B) 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

C) Left: UMAP representation of scRNA data from *Myh11*$^{+}$ SI cells (**B**) combined with scRNA data from unfractionated adult SI mesenchyme [30] (GEO series GSE130681). Right: Molecular markers (see Figure S2D) identified discrete cell types (numbers in parentheses) and average BMPi and Rspo gene expression are shown for each cluster.

D) Adult *Myh11*$^{Cre(ER\text{-}T2)}$*;R26R*^{TdTom}*;Pdgfra*$^{H2B\text{-}eGFP}$ colon showing thick MM (arrowheads). The MP was stripped prior to FACS sorting and a representative FACS plot shows colonic Tom^{+} 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.

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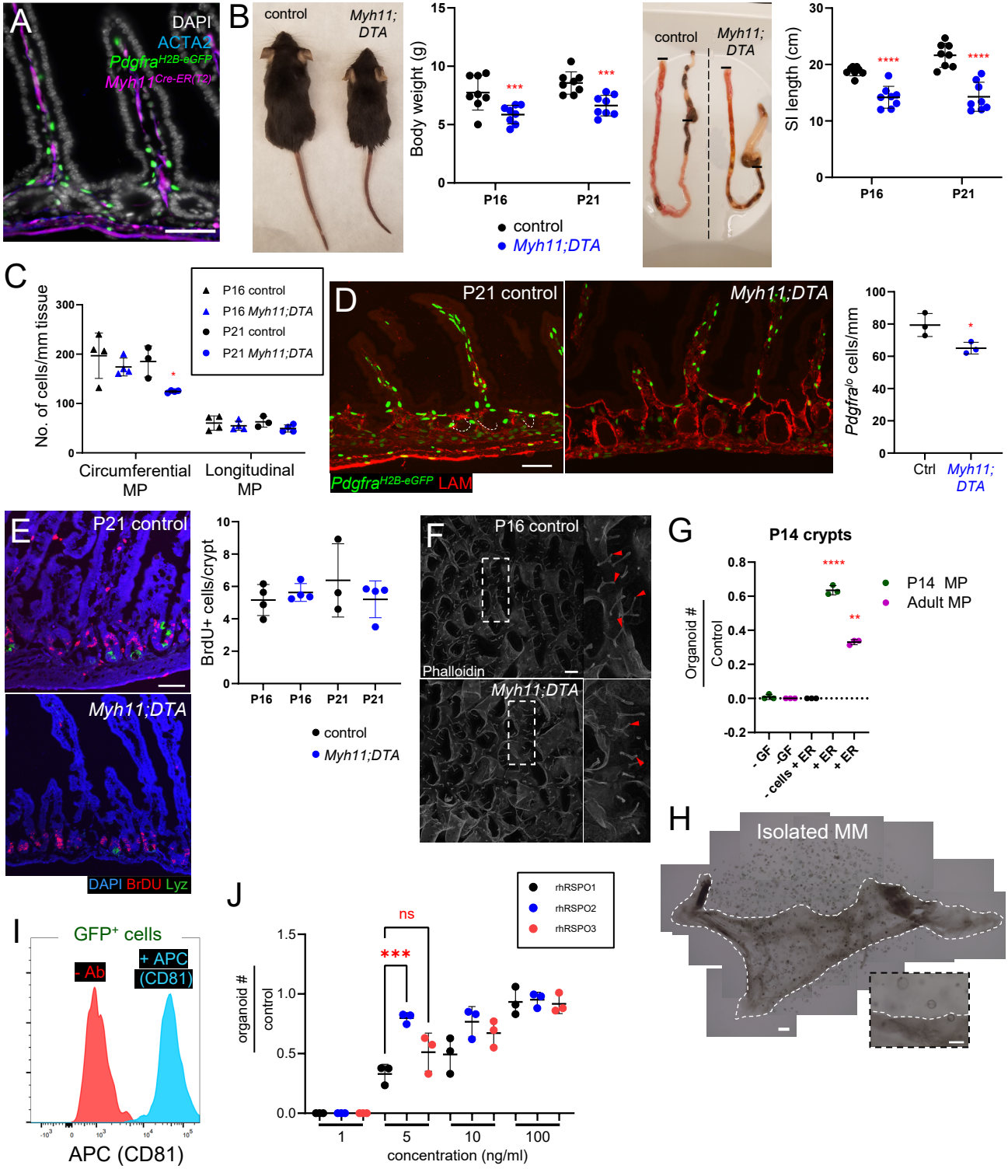
A
DAPI
ACTA2
Pdgfra H2B-eGFP
Myh11 Cre-ER(T2)
B
control
Myh11; DTA
Body weight (g)
P16
P21
control
Myh11;DTA
SI length (cm)
C
No. of cells/mm tissue
P16 control
P16 Myh11;DTA
P21 control
P21 Myh11;DTA
Circumferential MP
Longitudinal MP
D
P21 control
Myh11;DTA
Pdgfra H2B-eGFP LAM
Pdgfra lo cells/mm
Ctrl
Myh11; DTA
E
P21 control
Myh11;DTA
DAPI BrDU Lyz
BrdU+ cells/crypt
P16 P16 P21 P21
control
Myh11;DTA
F
P16 control
Phalloidin
Myh11;DTA
G
P14 crypts
Organoid # Control
P14 MP
Adult MP
- GF
- GF
+ ER
+ ER
+ ER
- cells
H
Isolated MM
I
GFP+ cells
- Ab
+ APC (CD81)
APC (CD81)
J
rhRSPO1
rhRSPO2
rhRSPO3
ns
organoid # control
concentration (ng/ml)

Figure S7. Investigation of SM functions and potency of human RSPO. Related to Figures 6 and 7**.**

A) 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.

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

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

D) Crosses with *Pdgfra*$^{H2B\text{-}eGFP}$ 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.

E) 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.

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

G) 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 to 76.7 ±12.9 organoids that grew in ENR medium (n=3). ****p <0.0001 using Student's t-test.

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

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

J) 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.

Table S1: Mouse lines and intestinal mesenchymal populations marked therein.
Related to the STAR Methods section.

Mesenchymal cell types marked by endogenous GFP or lineage traced by *Rosa26*$^{L\text{-}S\text{-}L\text{-}tdTomato}$

Genotype	Jackson Labs Stock No.	Age tested	Cell types marked	Verified by
Pdgfra^{H2BeGFP}	7669	P1-P14	ISEMFs Trophocytes, Pdgfra^{lo}CD81^{-} stroma PDGFRA^{+} interstitial cells Pdgfra^{+} cavity fibroblasts Follicle reticular cells Newly arising MM, LPM	Fluorescence microscopy scRNA
		Adult	ISEMFs Trophocytes, Pdgfra^{lo}CD81^{-} stroma PDGFRA^{+} Interstitial cells	Fluorescence microscopy scRNA, bulk RNA (McCarthy et al., 2020b)
Pdgfra$^{Cre(ER\text{-}T2)}$	32770	P1-P10	ISEMFs Trophocytes, Pdgfra^{lo}CD81^{-} stroma PDGFRA^{+} interstitial cells Newly arising MM, LPM	Fluorescence microscopy scRNA
Myh11$^{Cre(ER\text{-}T2)}$	19079	Adult	LPM, MM, pericytes, ISEMFs	Fluorescence microscopy scRNA
Etv1$^{Cre(ER\text{-}T2)}$	32770	Adult	Interstitial cells of Cajal	Fluorescence microscopy

Table S2: Top 50 gene markers that distinguish intestinal smooth muscle cell populations and ICC in scRNA analysis. Related to Figure 3.

Blue: Genes involved in ISC differentiation/support
Bold: Contractile genes and MP markers

		Muscularis propria			
LPM	MM	Nog+	Circular	Longitudinal	ICC
Adamdec1	*Mfap5*	*Cxcl14*	***Pcp4***	*Ptn*	*Pcdh17*
Ccl11	*Dcn*	*Kctd12*	***Actg2***	*Dlk1*	*Etv1*
Plac8	*Postn*	*Id1*	***Des***	*Tnnt2*	*Kit*
Plpp3	*Pitx1*	*Rgs9*	*Synm*	*Chodl*	*H19*
Agt	***Rspo3***	*Limch1*	*Ckm*	*Sfrp2*	*Pde3a*
Wnt4	*Shisa3*	*Pln*	*Smtn*	*Vwa1*	*Prkcq*
Tmem176a	*Id4*	*Ece1*	*Tpm2*	*Sh3bgr*	*Slc12a2*
Fn1	*Nupr1*	*Chl1*	*Mylk*	*Alcam*	*Chchd10*
Igfbp3	***Grem2***	*Colec10*	***Myh11***	*Csrp2*	*Ap1s2*
Col6a4	*Rbp1*	*Hand1*	*Cd59a*	*Lgi2*	*Edn3*
Bmp4	*Fzd1*	*Tnfrsf11b*	*Hspb1*	*Slit2*	*Pcp4l1*
Hmcn1	*Ptger1*	*Chrdl1*	*Cnn1*	*Smoc2*	*Elovl6*
Rarres2	*Col1a2*	*Cidea*	*Tpm1*	*Col14a1*	*Ldhb*
Tagln2	*Calcrl*	*Tspan12*	*Tnnt2*	*Fxyd6*	*Osr1*
Col6a2	*Cxcl12*	***Nog***	*Myl9*	*Nbas*	*Car2*
Zeb2	*Lum*	*Irx5*	*Ckb*	*Chrm2*	*Kcnk3*
Pdlim1	*Enpp2*	*Irx3*	***Acta2***	*Meg3*	*Itpk1*
Nkx2-3	*Col1a1*	*Angptl6*	*Sh3bgr*	*H19*	*Ano1*
4-Sep	*Col3a1*	*Lrig1*	*Hspb6*	***Osr1***	*Atp2a3*
Mmp2	*Tgfbr3*	*Col9a1*	*Cald1*	*Plagl1*	*Col26a1*
Tpm4	*Serf1*	*Inafm2*	*Bdh1*	*Abcc9*	*Atp1a1*
Col6a1	*Tes*	*Sctr*	*Rims1*	*Itga5*	*Gpr20*
Aldh1a3	*Pde5a*	*Adamtsl3*	*Tinagl1*	*Itga8*	*Enpep*
Lgmn	*Angptl1*	*Clmn*	*Myl6*	***Myh11***	*Adgrd1*
Adgrl3	*Rps27rt*	*Efnb2*	*Chrm2*	*Csrp1*	*Dkk2*
Igf1	*Aspn*	*Cavin2*	*Dstn*	*Fbn2*	*Me2*
Vstm4	*P2ry14*	*Id3*	*Popdc2*	***Des***	*Cox7a1*
Ncam1	*Frem2*	*Cux1*	*Sorbs2*	*Dgkb*	*Ppargc1a*
Col6a5	*Sparc*	*Thbs4*	*Atp2b4*	*Ogn*	*Iqgap2*
Mfap4	*Plppr5*	*Gm26778*	*Ppp1r12a*	*Mylk*	*Rasd2*
Lamb1	*Nrxn3*	*Tmod1*	*Synpo2*	*Loxl2*	*Lbhd2*
Lama2	*Hmgcs2*	*Smoc2*	***Grem1***	*Kcnj8*	*Eif1b*
Cygb	*Zeb2*	*St3gal5*	*Csrp1*	*Cdkn1c*	*Atp2b1*

Bst2	*S100a6*	*Id2*	*Flna*	*Tm6sf1*	*Tmem158*
Tcf21	*Npnt*	*Slc6a6*	*Pdlim7*	*Ppp1r12a*	*Zfhx3*
Pla2g4a	*Eln*	*Igfbp5*	*Prss23*	*Vim*	*Cpe*
Rdh10	*Scoc*	*Foxq1*	*Tagln*	*Igf2r*	*Rgs10*
Tmem176b	*Tecr*	*Cpe*	*Psd*	*Bend5*	*Nrp2*
Gpx3	*Il33*	*Mat2a*	*Sparcl1*	*Pgm5*	*Atp1b3*
Fhl1	*Peg3*	*Gm13889*	*Hspa2*	*Ramp1*	*Slco3a1*
Pros1	*Filip1l*	***Grem1***	*Ccdc107*	*Lama4*	*Aldh1b1*
Ptch1	*Maf*	*Rgs10*	*Itga1*	*Cnn1*	*Mdfic*
Pltp	*Crip1*	*Aldh1a3*	*Gng2*	*Lox*	*Tpi1*
Pmepa1	*Fmo2*	*Plce1*	*Calm2*	*Synpo2*	*Cycs*
Fbxl7	*Gas1*	*Dusp1*	*Ssfa2*	***Myl9***	*Gja1*
Btbd3	*Pi15*	*Tmem100*	*Selenom*	*Id2*	*Isoc1*
Aspn	*Ogn*	*Cav1*	*Dmpk*	*Gm10076*	*Arhgap20*
Gng11	*Stbd1*	*Ehd4*	*Pdlim3*	*Esyt2*	*Aldh1a1*
Ifi27l2a	*Hspa1a*	*Arhgap42*	*Igfbp2*	*Mgp*	*Hes1*
Hhip	*Hspa1b*	*Fos*	*Col8a1*	*Lsp1*	*Gadd45b*