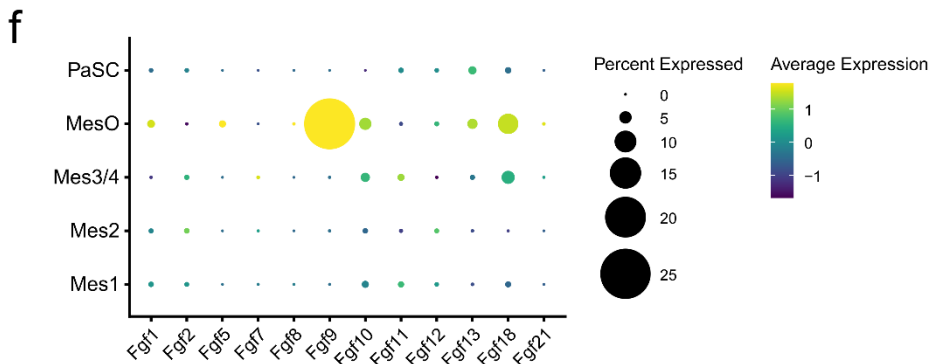
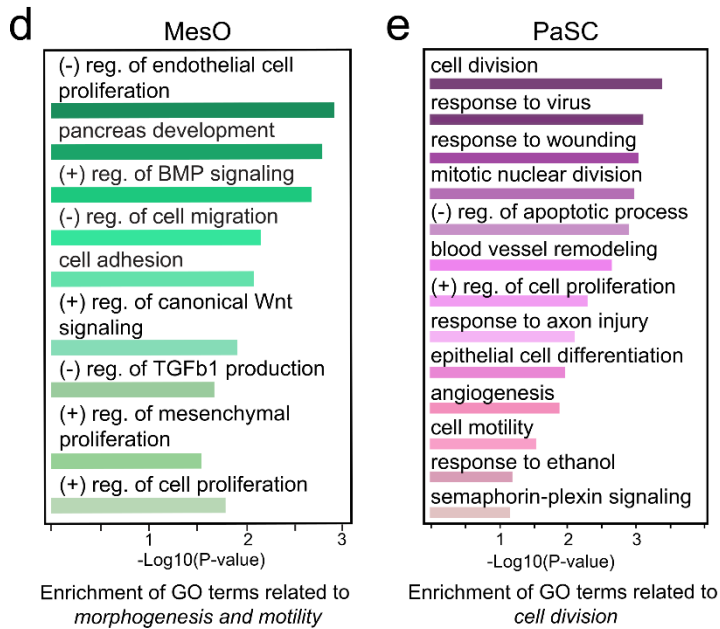
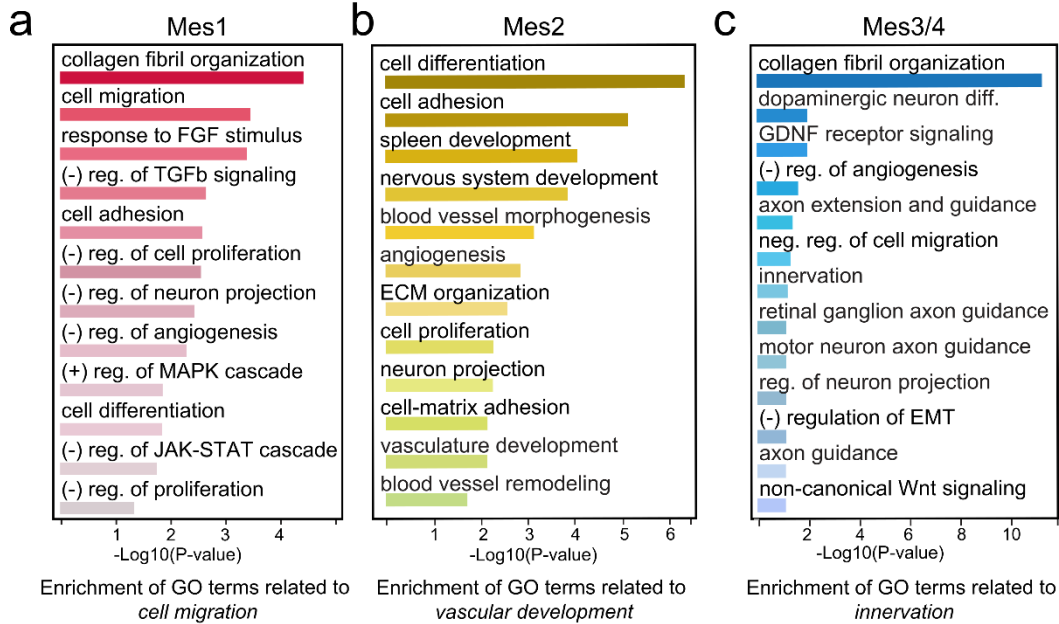


Supplementary Figure 1. Subtype specific marker validation.

- a. Quantification based on single-cell RNA-sequencing data of percentage of cells in each mesenchyme subtype expressing genes shown on the x-axis.
- b. Flow cytometry analysis of the e14.5 mouse tissue for candidate markers Dcn, Epha4, Sfrp2, Ntm, Ace2, and Nkx2-5-cre;Rosa-mTmG (Nkx2-5-eGFP). In black text shows the percentage of all pancreatic cells expressing candidate markers as found through single-cell RNA-sequencing; in green text shows the percentage of all pancreatic cells expressing candidate markers through flow cytometry readout by GFP signal. On the left, negative controls are shown, i.e. unstained, wildtype cells (top) and cells stained only with secondary antibodies (bottom).
- c-f. Representative immunostainings of Wt1+ (c; MesO marker), Ntm+ (d; Mes2 marker), Dcn (e; Mes1, Mes3/4, MesO marker), and Ace2+ mesenchyme (f) cells, in white, in mouse e14.5 pancreata in relation to epithelium (Cdh1+, in magenta) and mesenchyme cells (Vim+, in green). Scale bars = 50 μ m (left), 25 μ m (middle and right magnifications of squared regions of left images).

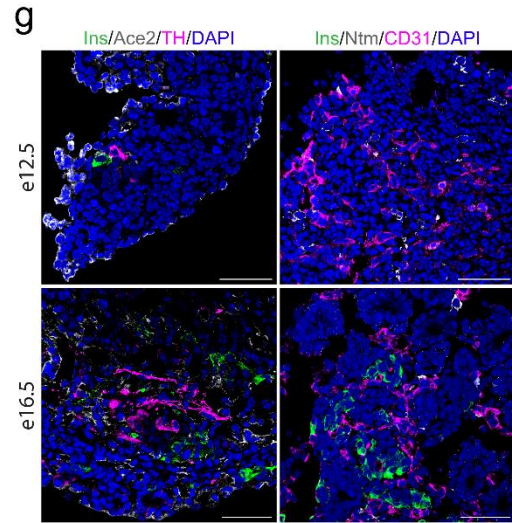
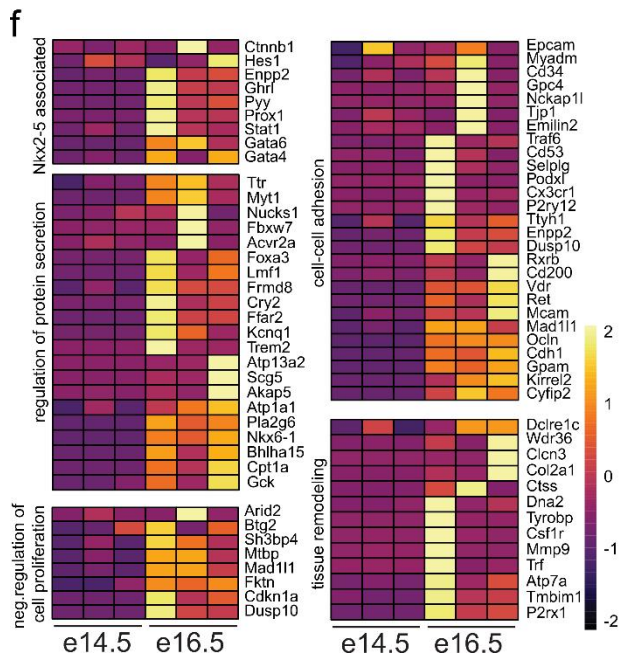
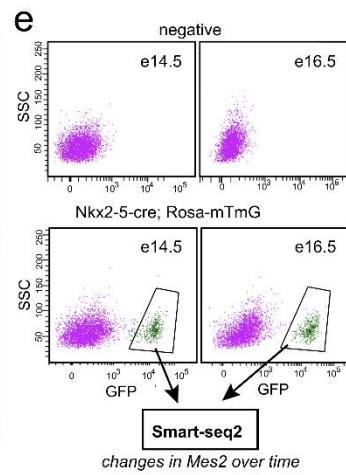
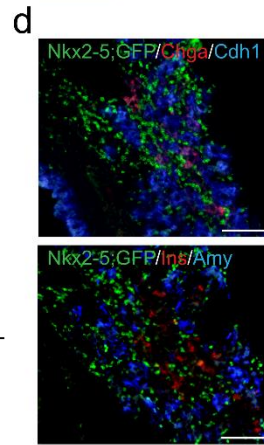
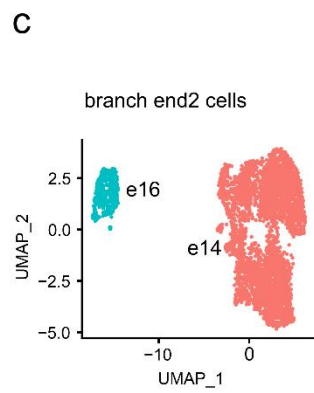
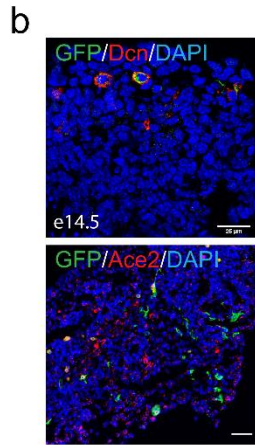
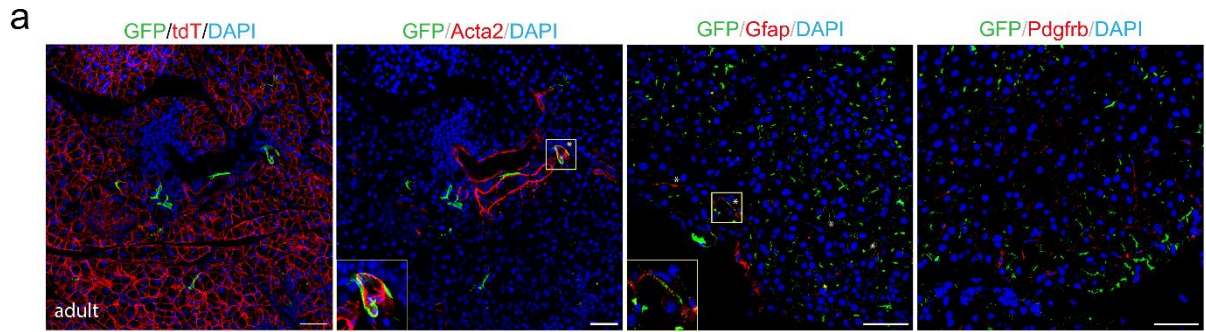
Gene ontology analysis, TOP100 genes



Supplementary Figure 2. Enrichment analysis of mesenchyme subtypes revealed by single-cell RNA-sequencing.

a-e. GO Biological process enrichment analysis of (a) Mes1, (b) Mes2, (c) Mes3/4, (d) MesO, and (e) PaSC for top100 genes enriched in each cluster.

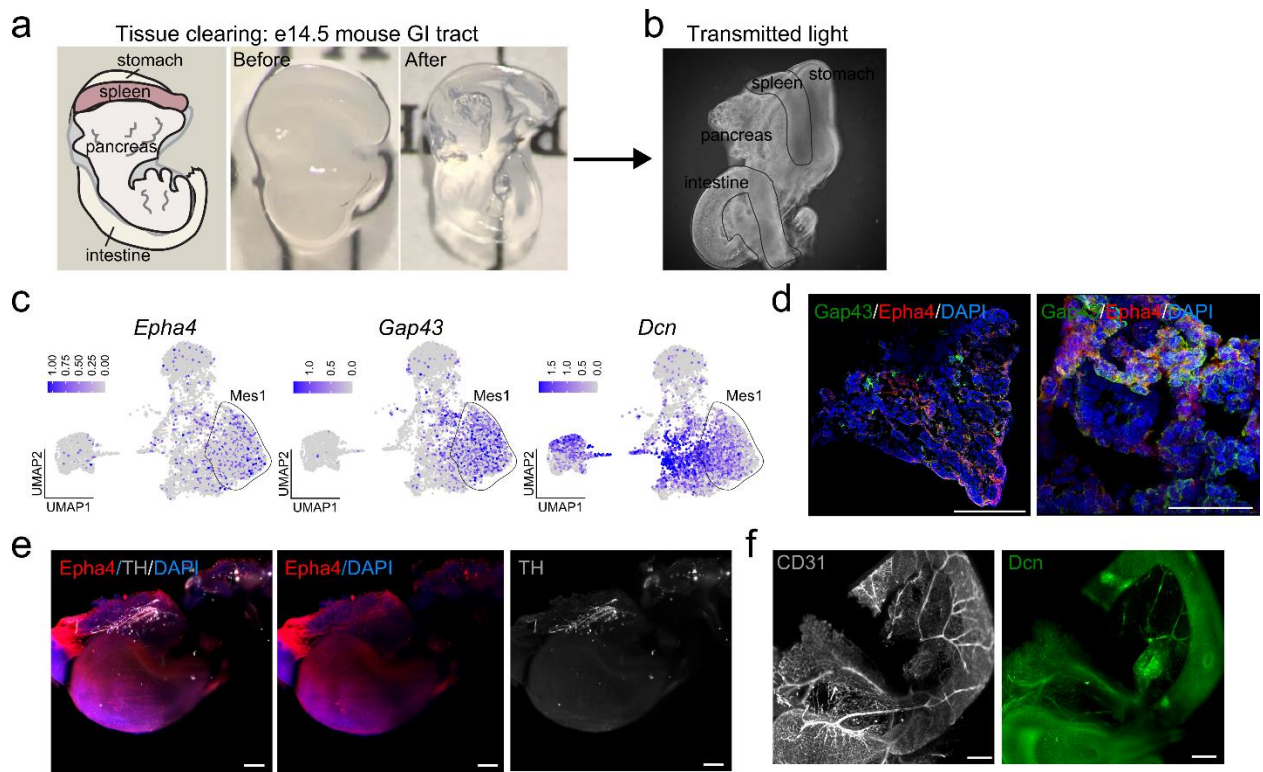
f. Fibroblast growth factor (Fgf) family members RNA expression in mesenchyme subtypes.



Supplementary Figure 3. Timelapse dynamics of pancreatic mesenchyme.

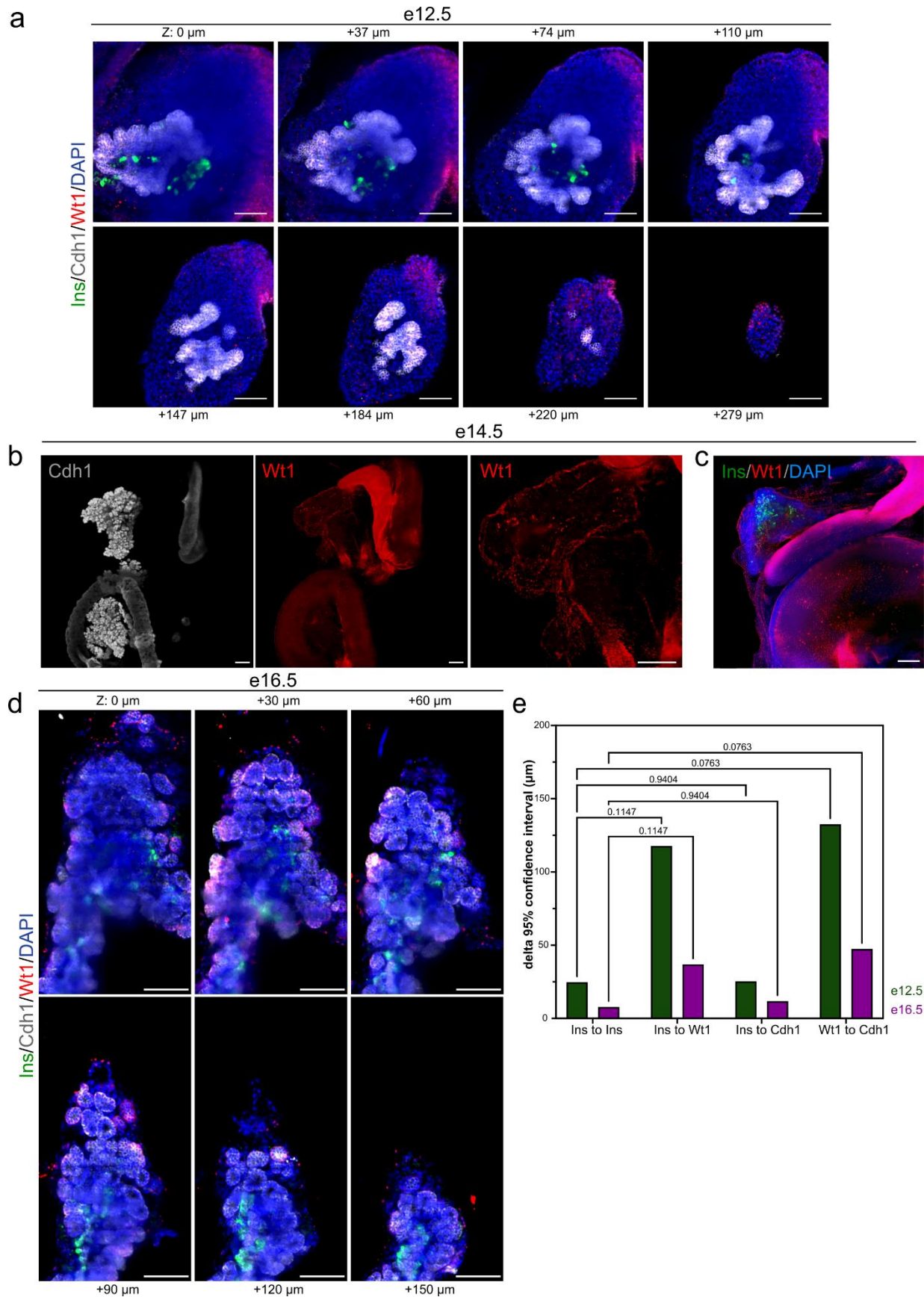
- a. Representative immunostaining of adult pancreata of *Pdgfra-CreERT;Rosa-mTmG* mice that were pulsed with tamoxifen at e12.5. On the far left image, tdT (red) stains membranes of all cells except those cells that expressed *Pdgfra* at e12.5, and progeny of these cells, which express membrane-associated GFP (green). GFP does not overlap extensively with *Acta2* (vascular smooth muscle cells marker; pseudocolored red), *Gfap* (adult PaSCs marker, pseudocolored red) or *Pdgfrb* (pericyte marker, pseudocolored red) positive cells.
- b. Representative immunostaining of e14.5 pancreata showing GFP⁺ cells (e12.5 *Pdgfra*+ progeny, in green) partially overlapping with *Dcn*⁺ (*Mes1*, *Mes3/4*, *MesO*), and *Ace2*⁺ mesenchyme cells, in red. DAPI (in blue) stains nuclei. Scale bar = 25 μ m (top), 50 μ m (bottom).
- c. UMAP reduction of single cell transcriptomes after subclustering e14.5 mesenchyme subtypes *Mes1*, *Mes3/4* and merging with e16.5 single-cell RNA-sequencing of *Mes1*, *Mes3/4*.
- d. *Top*: Immunostaining of mesenchyme *Mes2* subtype marker *Nkx2-5*;GFP (in green) using *Nkx2-5-cre;Rosa-mTmG* mice at e14.5 with endocrine cells (*Chga*, in red) and epithelium (*Cdh1*, in blue). *Bottom*: Immunostaining of mesenchyme *Mes2* subtype marker *Nkx2-5* (in green) using *Nkx2-5-cre;Rosa-mTmG* mice at e14.5 with beta cells (*Ins*, in red) and acinar cells (*Amy*, in blue). Scale bars = 100 μ m.
- e. FACS isolation of *Nkx2-5-cre; Rosa-mTmG* GFP⁺ cells for analysis by Smart-seq2. N=3 biological replicates.
- f. Heatmap showing select enriched genes in e16.5 *Mes2* compared to e14.5. Associated functions by GO analysis are annotated to the left. Expression ranges from low (dark purple) to high (yellow).

- g. Representative immunostaining of e12.5 and 16.5 pancreata for mesenchymal subtype-enriched genes *Ace2* and *Ntm*, together with beta cells (*Ins*⁺, in green), neurons/alpha cells (*TH*⁺), and blood vessels (*CD31*⁺), in magenta. Scale bars = 50 μ m.



Supplementary Figure 4. Light-sheet imaging of optically cleared e14.5 tissue reveals Mes1 subtype localization.

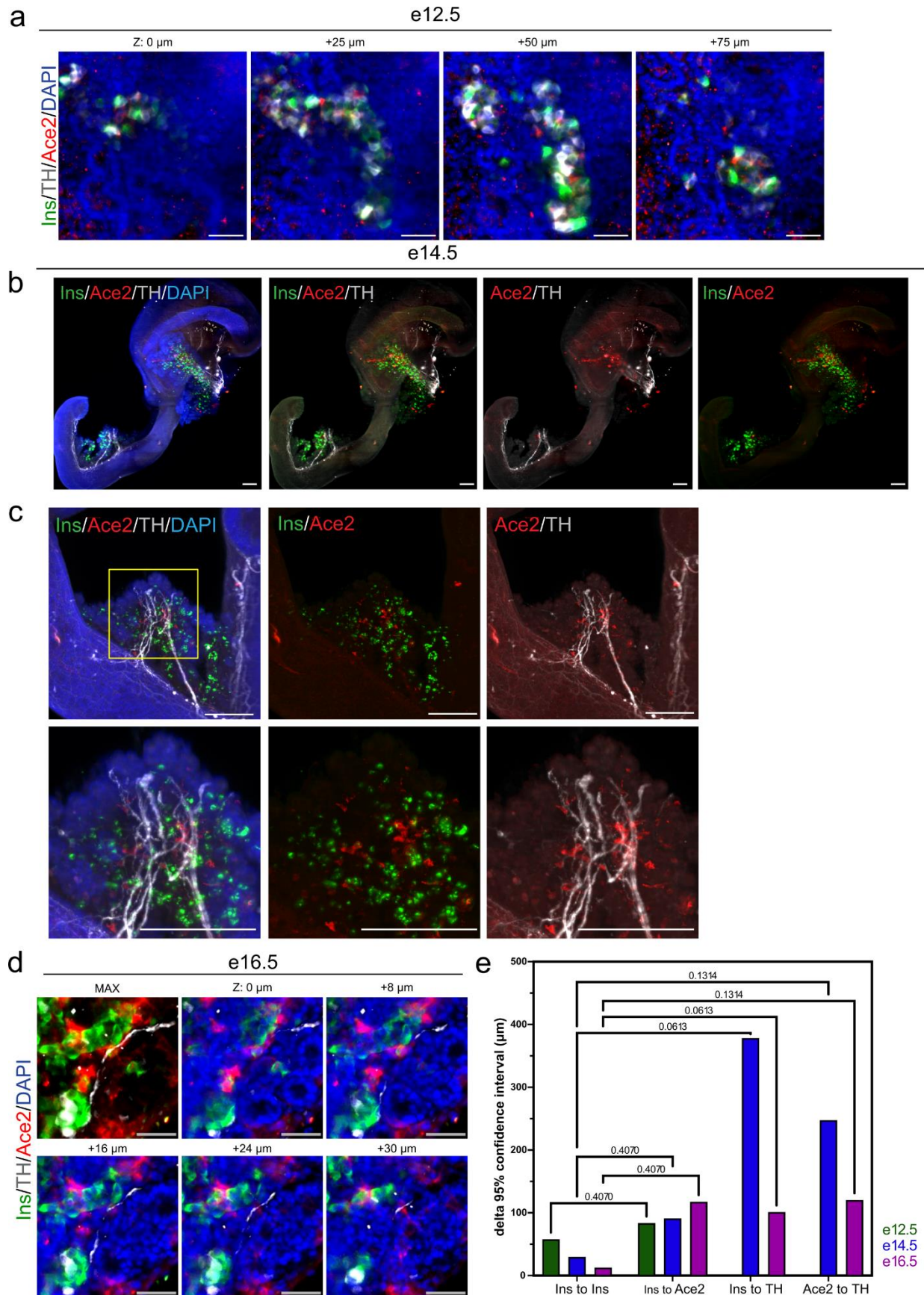
- a. Optically clearing of e14.5 GI tract. Tissues were dissected from the esophagus to the duodenum to ensure inclusion of the entire pancreatic region and surrounding niche, before hydrogel formation and breaking up of lipid bilayers through electrophoresis with detergents.
- b. Optically cleared e14.5 GI tract in light-sheet by transmitted light.
- c. Single-cell RNA-sequencing UMAP feature plots showing mesenchymal expression of *Gap43*, *Epha4* and *Dcn* in Mes1 cluster with dark blue showing high expression and gray showing no expression. The circles outline specific clusters.
- d. Immunostaining of Mes1 markers Gap43 (in green) and Epha4 (in red) in 2D sections of embryonic pancreas tissue at e14.5. Nuclei are marked by DAPI in blue. Scale bars = 50 μm .
- e. Light-sheet images of optically cleared, immunostained 3D mouse e14.5 pancreas for mesenchymal marker Epha4 (in red) with neurons (TH, in white). Nuclei are marked by DAPI in blue. Scale bars = 200 μm .
- f. Light-sheet images of optically cleared, immunostained 3D mouse e14.5 pancreas for Mes1 (*Dcn* in green) and vasculature (CD31, in white). Scale bars = 200 μm .



Supplementary Figure 5. Light-sheet imaging of optically cleared e12.5-e14.5 mouse tissue reveals MesO subtype localization in relation to pancreatic epithelium and beta cells.

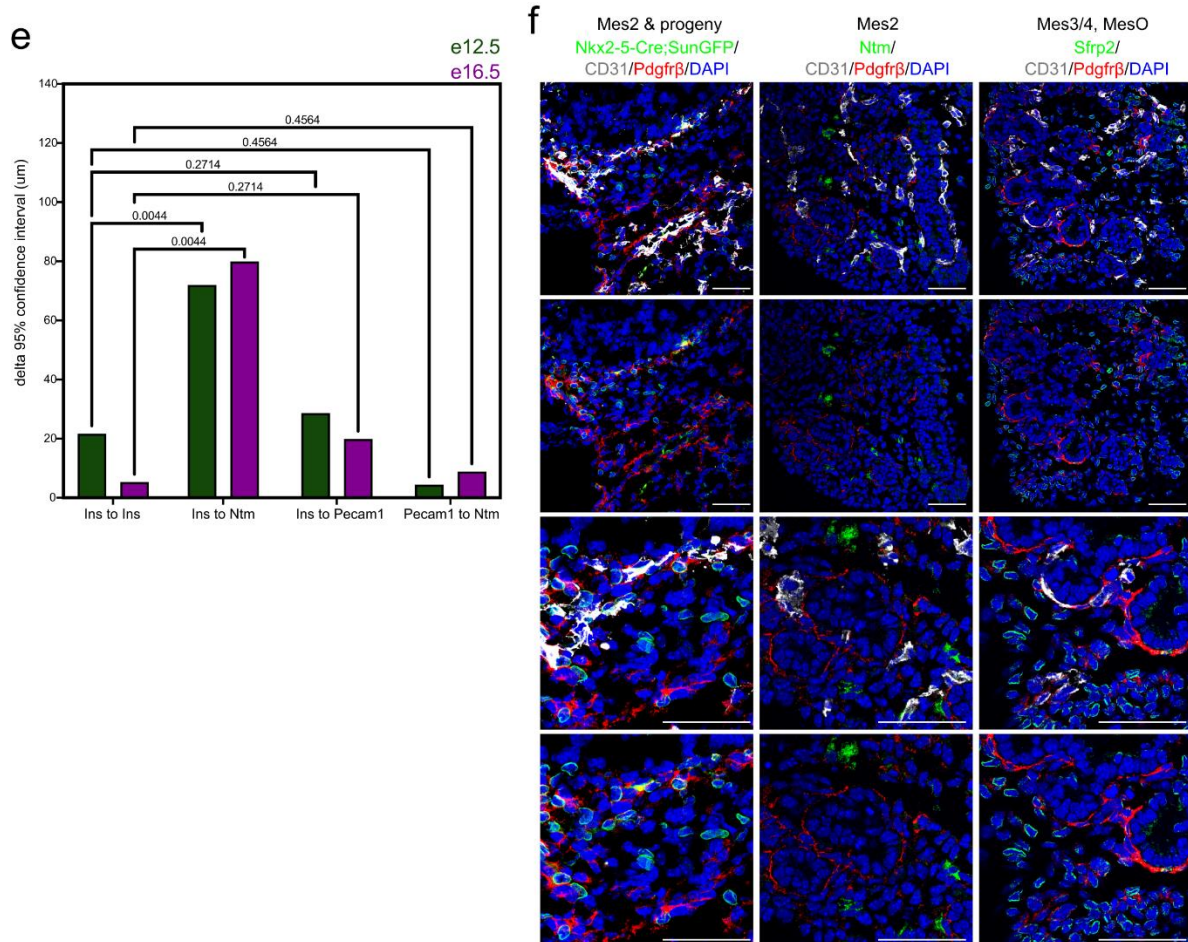
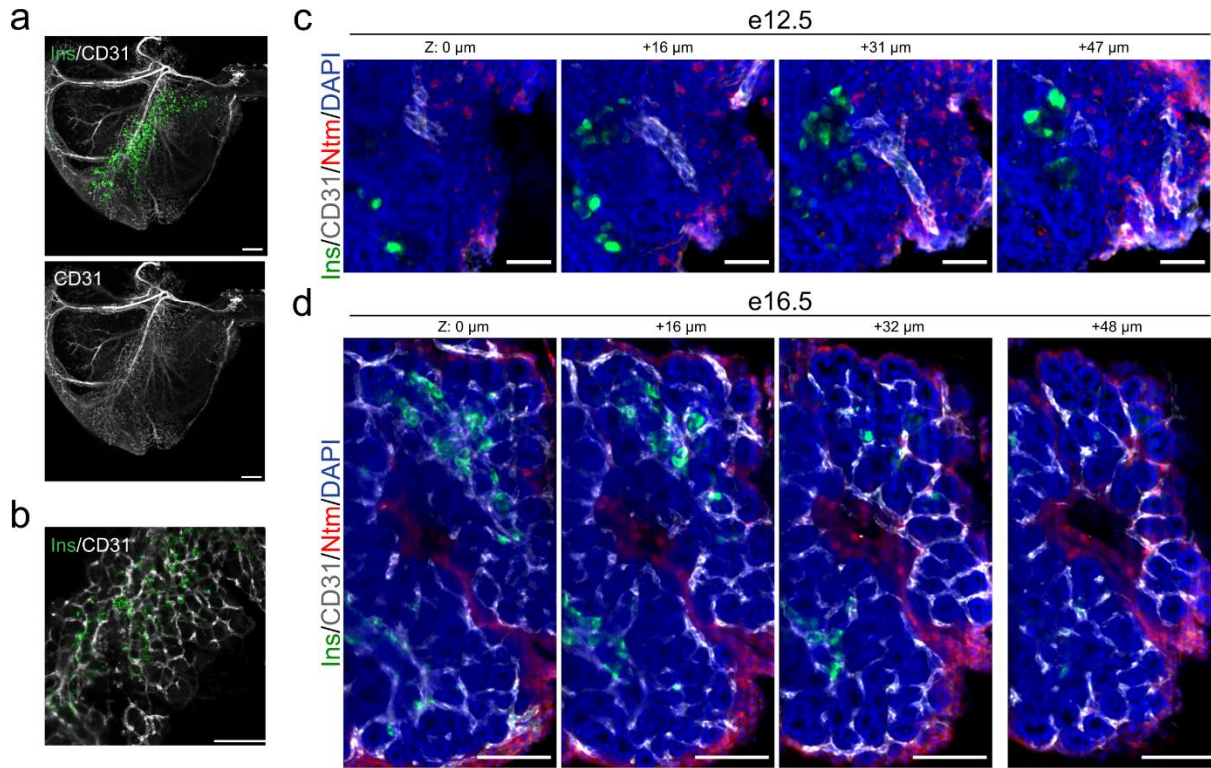
- a. Optical section images of wholemount 3D light-sheet images of mouse e12.5 pancreas stained for mesenchymal marker *Wt1* (in red), epithelium (*Cdh1*, in white) and beta cells (*Ins*, in green). Numbers above subsequent section images denote Z-dimension distance shift from the first image. DAPI marks nuclei. Scale bars = 100 μm .
- b. Single-channel maximum intensity projection images of wholemount mouse e14.5 pancreas stained for mesenchymal marker *Wt1* (in red) and epithelium (*Cdh1*, in white), corresponding to Fig. 3B. Image on the right shows higher magnification. Scale bars = 200 μm .
- c. Maximum intensity projection image of wholemount mouse e14.5 pancreas stained for mesenchymal marker *Wt1* (in red) and beta cells (*Ins*, in green), corresponding to Fig. 3C. Scale bar = 200 μm .
- d. Optical section images of wholemount 3D light-sheet images of mouse e16.5 pancreas stained as in (a). Numbers above subsequent section images denote Z-dimension distance shift from the first image. DAPI marks nuclei. Scale bars = 100 μm .
- e. Bar plot showing mean of delta 95% confidence intervals of shortest distances between indicated cell types at e12.5 and e16.5. Using the distance between *Ins*⁺ cells to other *Ins*⁺ cells as a control, we mapped the 95% confidence intervals of spatial relationships, with lower values indicating less variability within biological replicates or a higher likelihood that cells can be found within a specific distance of other cells. As anticipated, *Ins*⁺ cells had low intervals, indicating that they are faithfully observed at expected locations relative to other beta cells at both e12.5 and e16.5. In addition, *Ins*⁺ beta cells were always found at similar distances to the *Cdh1*⁺ epithelium, with no significant difference between the distance between *Ins*⁺ cells with each other and *Ins*⁺ cells with *Cdh1*⁺ cells. Confidence intervals mapped for *Wt1*⁺ mesothelial cells with *Ins*⁺ cells and the epithelium were not

significantly increased compared to Ins-Ins relationships. Delta was calculated by subtracting upper and lower confidence intervals. P values (Tukey's test post-hoc two-way ANOVA) are shown. N = 3 embryos.



Supplementary Figure 6. Light-sheet imaging and analysis of Ace2+ pancreatic mesenchyme in spatial relationship with neurons (TH) and beta cells (Ins).

- a. Optical section images of wholemount 3D light-sheet images of mouse e12.5 pancreas stained for Ace2+ Mes3/4 mesenchyme (in red), neurons (TH, in white) and beta cells (Ins, in green). At e12.5 we did not find TH+ neuronal processes. Instead, TH marked endocrine alpha-cells. Numbers above subsequent section images denote Z-dimension distance shift from the first image. DAPI marks nuclei. Scale bars = 50 μ m.
- b. Maximum intensity projection image of wholemount mouse e14.5 pancreas stained for mesenchymal marker Ace2 (in red), neurons (TH in white) and beta cells (Ins in green), corresponding to Fig. 4C. Nuclei are marked by DAPI in blue. Scale bars = 200 μ m.
- c. Maximum intensity projection image of wholemount mouse e14.5 pancreas stained for mesenchymal marker Ace2 (in red), neurons (TH in white) and beta cells (Ins in green). The yellow square denotes zoomed region shown for each image in the bottom panel. Nuclei are marked by DAPI in blue. Scale bars = 200 μ m.
- d. Optical section images of wholemount 3D light-sheet images of mouse e16.5 pancreas stained as in (a). Numbers above subsequent section images denote Z-dimension distance shift from the first image. DAPI marks nuclei. Scale bars = 50 μ m.
- e. Bar plot showing mean of delta 95% confidence intervals of shortest distances between indicated cell types at e12.5, e14.5 and e16.5. Delta was calculated by subtracting upper and lower confidence intervals. P values (Tukey's test post-hoc two-way ANOVA) are shown. N = 3 embryos.



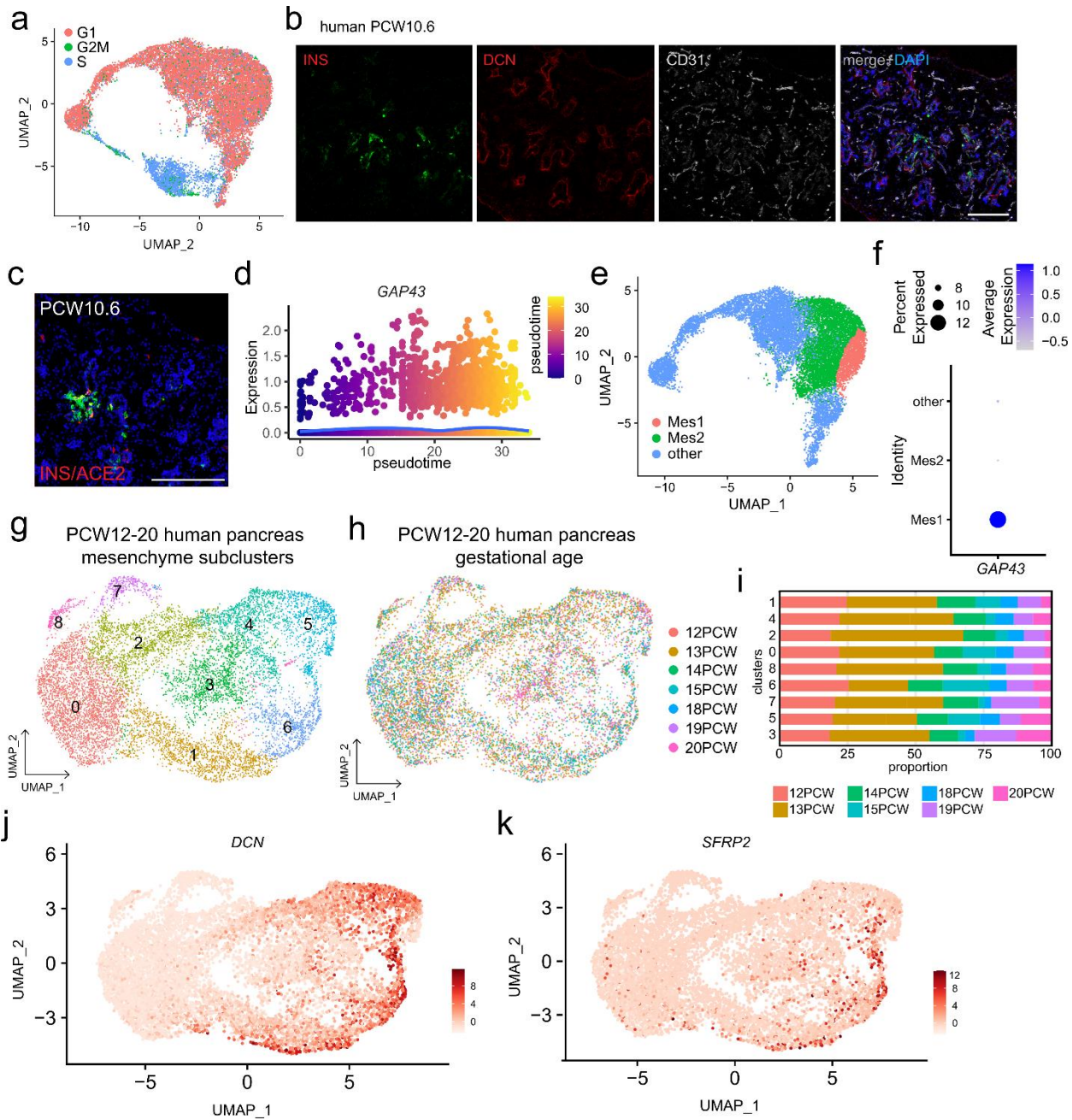
Supplementary Figure 7. Mes2 spatial relationship to pancreatic vasculature and beta cells.

- a-b. Light-sheet images of optically cleared, immunostained 3D mouse e14.5 pancreas for vasculature (CD31, in white) and beta cells (Ins, in green). Scale bars = 200 μm .
- c-d. Optical section images of wholemount 3D light-sheet images of mouse (c) e12.5 and (d) e16.5 pancreata stained for Ntm+ Mes2 mesenchyme (in red), endothelium (CD31, in white) and beta cells (Ins, in green). Numbers above subsequent section images denote Z-dimension distance shift from the first image. DAPI marks nuclei. Scale bars = (c) 50 μm and (d) 100 μm .
- e. Bar plot showing mean of delta 95% confidence intervals of shortest distances between indicated cell types at e12.5 and e16.5. Delta was calculated by subtracting upper and lower confidence intervals. P values (Tukey's test post-hoc two-way ANOVA) are shown. N = 3 embryos.
- f. Representative immunostainings of Nkx2-5cre (left; Nkx2-5-cre;SunGFP mice; marks Mes2 and their progeny), Ntm+ (middle; Mes2 marker), and Sfrp2+ (right; Mes3/4 marker), in green, in mouse e14.5 pancreata in relation to Pdgfrb+ PaSCs (in red) and CD31+ blood vessels (in white). DAPI (in blue) marks nuclei. Scale bars = 50 μm . Two bottom rows are magnifications of two top rows.

Supplementary Figure 8. Connectome analysis in e14.5 mouse pancreas

- a. Signaling networks from non-mesenchymal pancreatic cells to mesenchymal subtypes shown using dot plot visualization of centrality analysis. Selected signaling networks (x - axis) are shown with the upper outgoing showing expression of network ligands in non-mesenchymal cells subtypes and the lower incoming graphs showing corresponding network receptors in mesenchymal subtypes. Dot colors represent cell type. Edgewise fraction (y-axis) reflects weighted use of a network, with values closer to 1 indicating cell types with higher use as compared to other cell types. In-graph labels indicate cell types with highest edgewise values within each network. A dot size reflects normalized expression level of ligands (outcoming graph) or receptors (incoming graph) belonging to a network within a cell type.
- b. Connectome interaction maps showing the predicted cellular communication from mesenchymal subtypes to endothelial and EP cells via collagens. Line colors indicate ligand-expressing cell type and thickness of line ends depends on edgewise score of each particular end, i.e. a ligand or a receptor score.
- c. Dot plot showing expression of endothelial cell-expressed receptors revealed by Connectome analysis and detected in more than 20% cells.
- d. Ligand-receptor analysis scatter plot showing relative expression levels of ligands (y-axis) and their receptors (x-axis) expressed by Mes2 and endothelial cells, respectively. Dot size (weight_sc) reflects a cumulative expression of a ligand-receptor pair.
- e. Ligand-receptor analysis scatter plot showing relative expression levels of ligands (y-axis) and their receptors (x-axis) expressed by Mes3/4 and neurons, respectively. Dot size (weight_sc) reflects a cumulative expression of a ligand-receptor pair.
- f, g. Connectome interaction maps showing the predicted cellular communication from mesenchymal subtypes to (f) bipotent progenitor and (g) endocrine cells. Line colors

indicate ligand-expressing cell type and thickness of line ends depends on edgeweight score of each particular end, i.e. a ligand or a receptor score.



Supplementary Figure 9. Mesenchyme heterogeneity in human fetal pancreas

- a. UMAP feature plot of PCW7-11 human fetal pancreatic mesenchyme showing cell cycle stage of each cell. Separate clusters of G2M (green) and S-phase (blue) cells were removed from the dataset prior to analysis.
- b. Immunostaining of human w10.6 pancreas showing developing beta cells (INS, in green) and blood vessels (CD31, in white). DCN+ cells (Mes1, Mes3/4, and MesO) are shown in red. Nuclei are marked by DAPI in blue. Scale bar = 50 μm .
- c. Representative Immunostaining of human PCW10.6 pancreas showing developing beta cells (INS, in green) and ACE2+ mesenchyme, in red. Nuclei are marked by DAPI in blue. Scale bar = 200 μm .
- d. Plots showing expression changes of *GAP43* expression along pseudotime. Dots represent individual cells.
- e. UMAP feature plot of PCW7-11 human fetal pancreatic mesenchyme with identified *GAP43*+ enriched Monocle3 trajectory branch of Mes1/2 cluster, denoted as Mes1 (in red).
- f. Dotplot of *GAP43* expression in Mes1 Monocle3 trajectory branch (Supplementary Fig. 8e - in red) as compared to the rest of Mes1/2 cluster (herein called Mes2; Supplementary Fig. 8e - in green) and other mesenchymal populations (Supplementary Fig. 8d - in blue).
- g-h. Single-cell-RNA-sequencing⁴⁸ UMAP feature plots of 12 to 20 PCW human fetal pancreata (GSE197064 dataset) with colors featuring (g) mesenchymal subtypes or (h) gestational age.
- i. Plot showing proportions of pancreatic mesenchyme subtypes at different gestational ages in human fetal pancreas.
- j. UMAP plot of *DCN* relative expression levels in 12 to 20 PCW human fetal pancreatic mesenchyme.

- k. UMAP plot of *SFRP2* relative expression levels in 12 to 20 PCW human fetal pancreatic mesenchyme.

Supplementary Table 1. Primary antibody list

Antibody	Catalogue #	Vendor	Dilution
Ace2	PA5-47488	Thermo	1:50
Ace2	ab15348	Abcam	1:100
Acta2	19245	Cell Signal	1:400
Amylase	A8273	Sigma-Aldrich	1:400
aTH	AB152	Millipore	1:200
CD31 (Pecam1)	AF3628	R&D	1:200
Cdh1	AF748	R&D	1:400
Chga	AB15160	Abcam	1:100
Dcn	6D6	DSHB	1:100
Epha4	AF641	R&D	1:50
Gap43	NB3000-143ss	NovusBio	1:500
Gfap	3670	Cell Signal	1:300
GFP	ab13970	Abcam	1:1000
Ins	3014	Cell Signal	1:200
Ins	PA1-26938	Invitrogen	1:100
Ngf	ab52918	Abcam	1:300
Ntm	sc-390941	Santa Cruz	1:100
Pdgfrb	3169	Cell Signal	1:300
Sfrp2	ab137560	Abcam	1:100
Vim	AB5733	Millipore	1:400
Wt1	ab89901	Abcam	1:100

Movie 1. Three-dimensional video showing Ins, Ace2, and TH in the embryonic pancreas and surrounding tissues.

Movie 2. Three-dimensional video showing Ins, Wt1, and Cdh1 in the embryonic pancreas and surrounding tissues