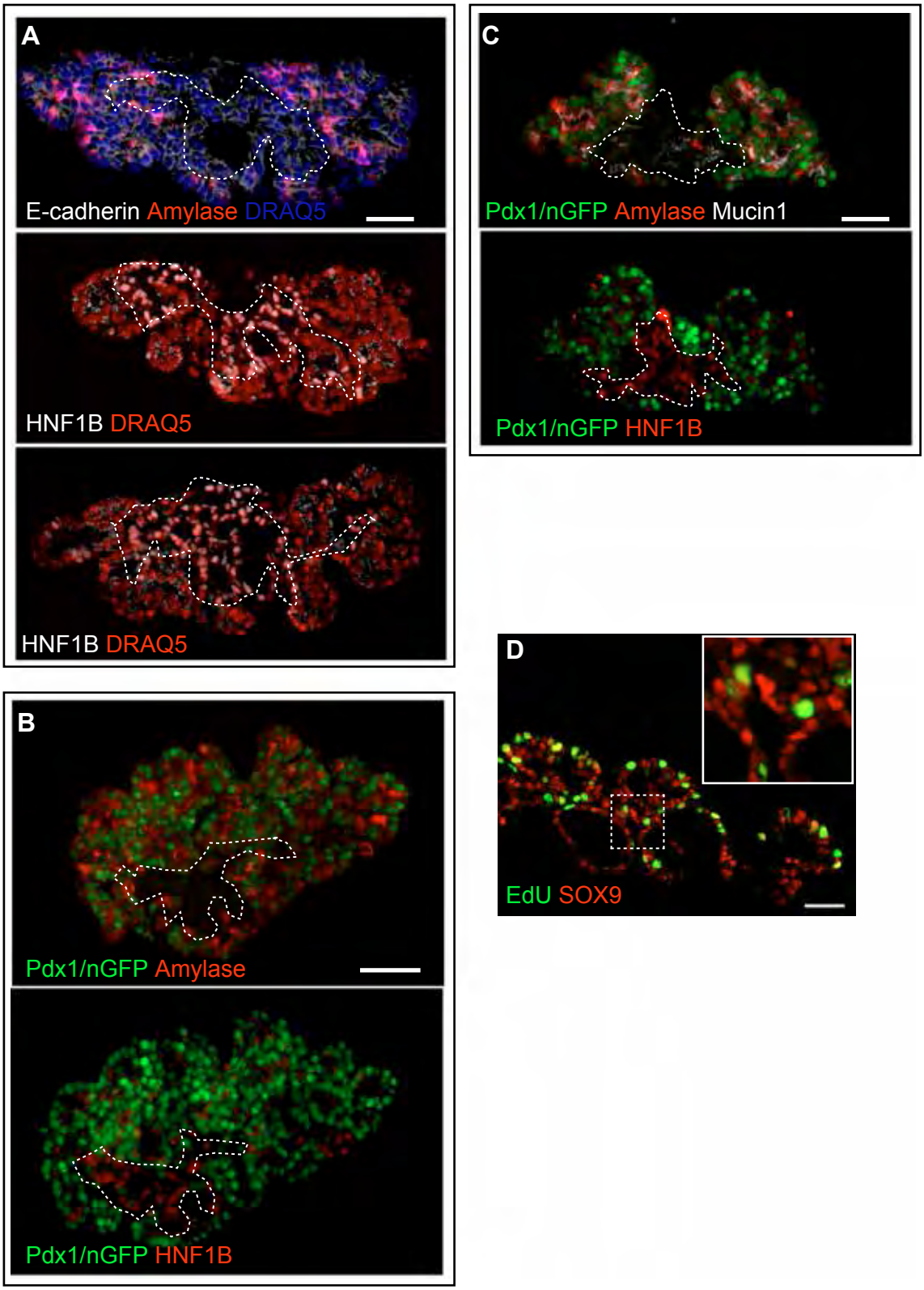

Fig. S1. Organoid expansion. (A-C) Adjacent sections showing the mostly central progenitors and more or less continuous crown of exocrine cells in 3 representative organoids. The dashed outline marks the central progenitors. Although PDX1 is expressed in central cells, the expression of the transgene reporter is lower and largely below threshold here. (D) Section of a 7-day organoid immunostained for SOX9 (red) and EdU (green), which was added in the last two hours of culture at day 7.

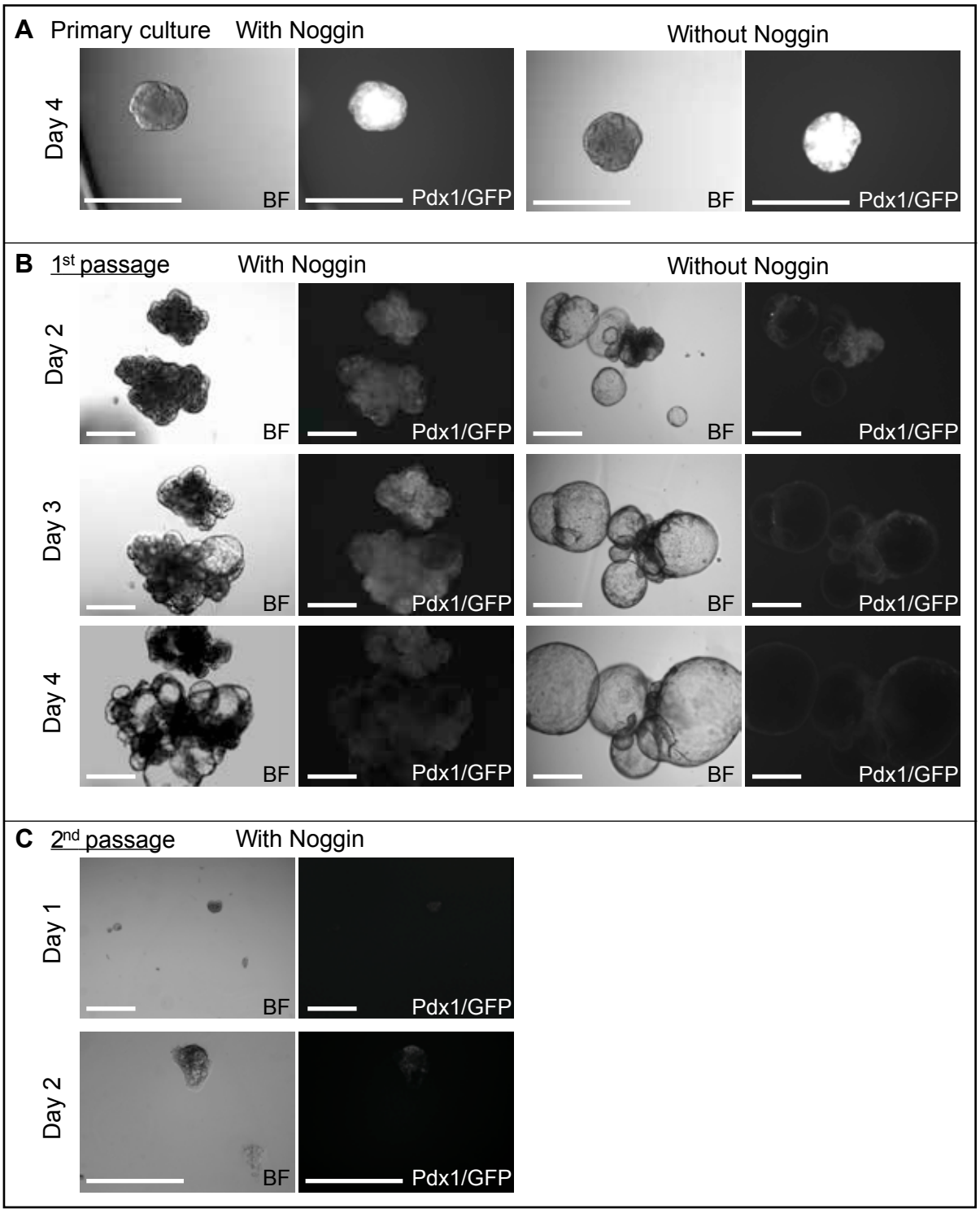

Fig. S2. Organoid passaging. (A) Organoids were generated from E10.5 dissociated pancreatic progenitors and cultured in the absence or continuous presence of 50 ng/ml Noggin. (B) After 6 days of primary culture, pancreatic organoids were mechanically dissociated into small pieces and re-seeded in Matrigel with or without Noggin. (C) 4 days after re-seeding, organoids grown in the presence of Noggin were again re-seeded but failed to maintain *Pdx1* or expand while in the absence of Noggin second passaging led to a prompt loss of *Pdx1*/GFP and viability. Scale bar 200 μm.

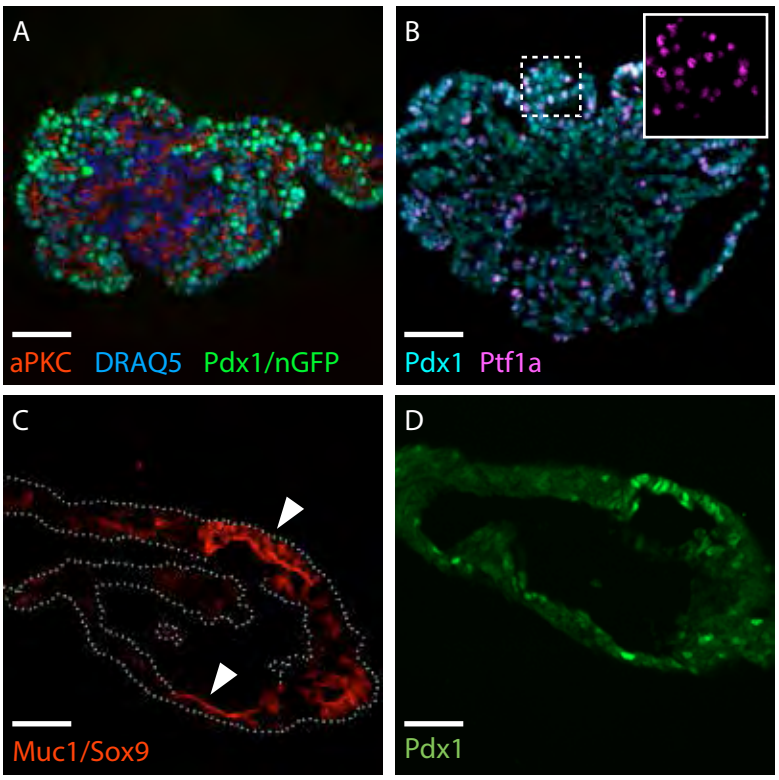

Fig. S3. Polarity of cells in organoids. (A) Histological section of a day 7-organoid showing that they are made of compact monolayers of cells expressing different levels of *Pdx1*/nGFP (green), lower in the center, their apical side expressing atypical PKC (red) lining ducts. DRAQ5 (blue) marks nuclei. *Pdx1*/nGFP in a E14.5 pancreas is also more lowly expressed in the center where ductal endocrine bipotent progenitors reside. (B) Histological section of a day 7-organoid showing PTF1a expression (purple) at the periphery (exocrine) and PDX1 (blue) in all cells (C) Cystic organoids at day 7 express heterogeneous levels of SOX9 (red nuclei). The $SOX9^{+}$ cells also express polarized Mucin (red, membrane, arrowheads) whereas the negative cells lose apical polarity. (D) An adjacent section shows that PDX1 (green) is retained only in the $SOX9^{+}$ polarized areas shown in (C). Scale bar 50 µm (C,D).

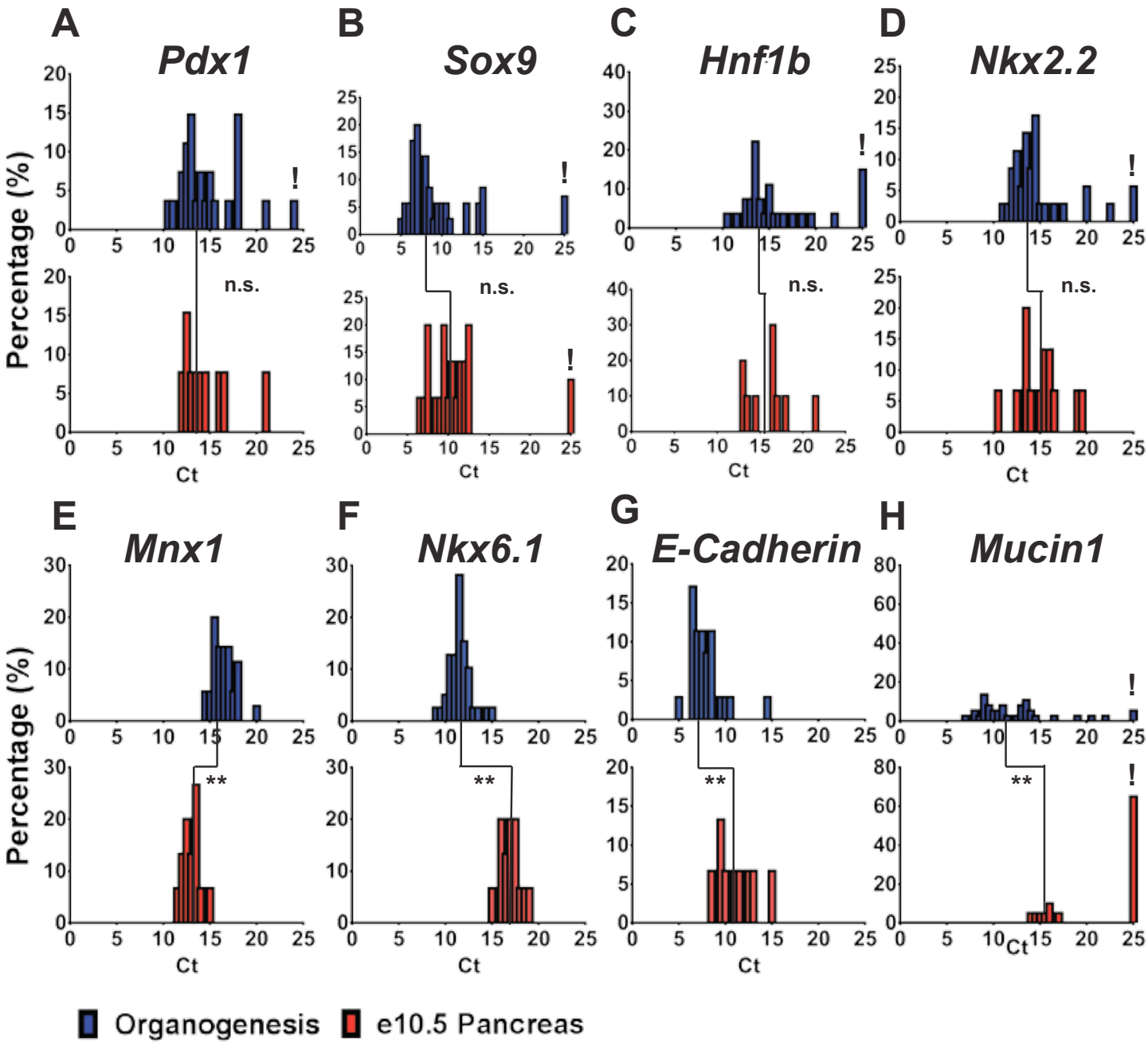

Fig. S4. Single cell q-PCR reveals similarities between cells before and after organoid culture. Cumulative frequencies (%) of cells expressing various gene expression levels (Ct value) in the starting population of E10.5 pancreas epithelium cells (red) and those grown in organogenesis conditions for 6 days (blue). A Ct >25 is considered as characterizing a non-expressing cell (!). The medians of expressing cells are shown by a black line in each histogram. The differences in median expression were statistically tested using a Mann–Whitney test (Statistical differences of $P<0.001$** were considered significant). There were no statistical differences (n.s.) between the single cell expression patterns of neither (a) *Pdx1* ($P=0.73$) (b) *Sox9* ($P=0.07$), (c) *Hnf1β* ($P=0.077$), (d) *Nkx2.2* ($P=0.21$), whereas statistical differences were observed for (e) *Mnx1* ($P<0.001$), (f) *Nkx6.1* ($P<0.001$), (g) *E-cadherin* ($P<0.001$) and (h) *Muc1* ($P<0.001$). All conditions passed KS, Shapiro-Wilkins and D′Agustino and Pearson omnibus normality tests (alpha=0.05) except for *Muc1* at E10.5 (both organoids and E10.5) as well as *Nkx2.2* and *Sox9* in organoids.

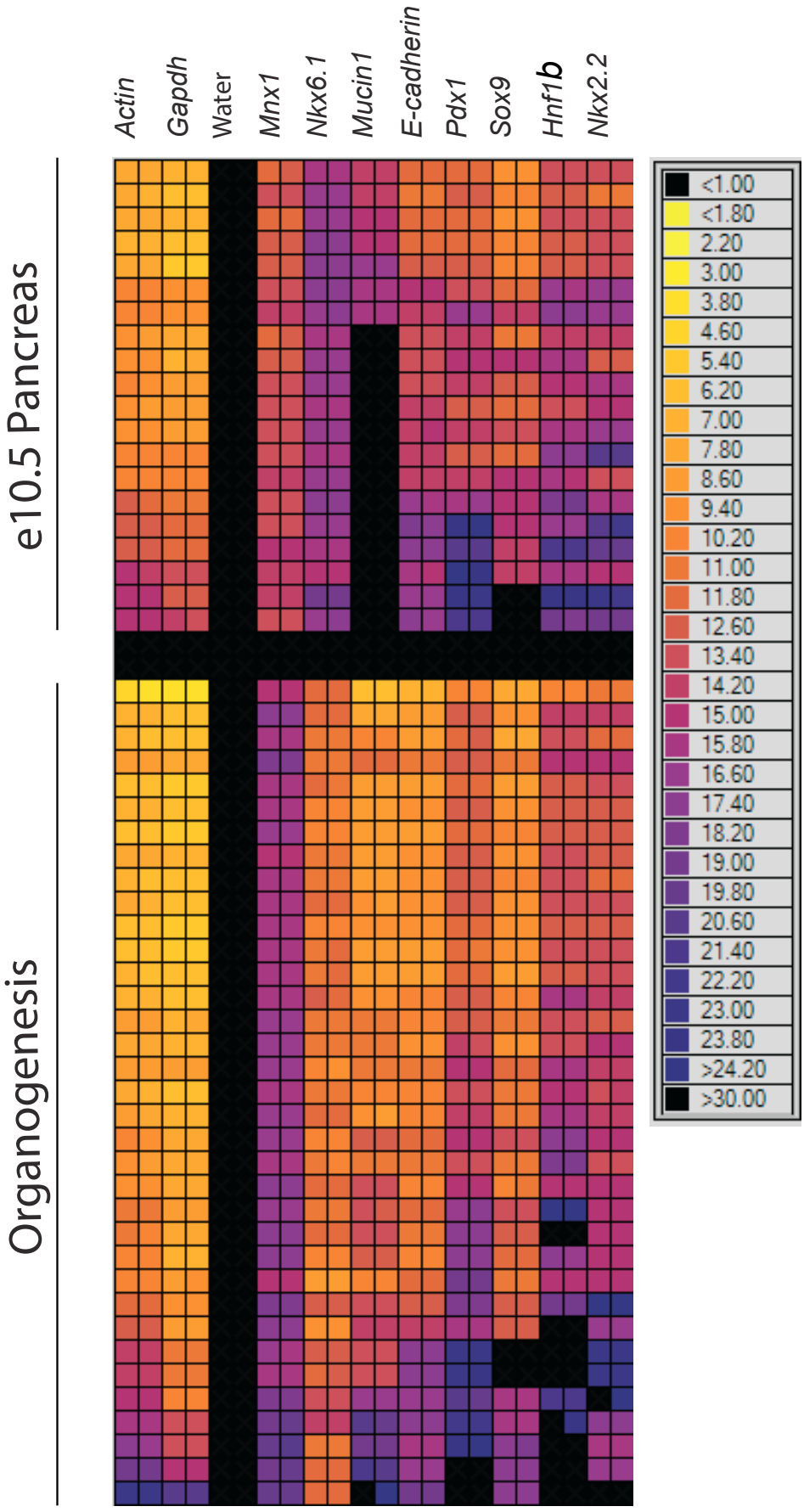

Fig. S5. Single cell q-PCR expression heat maps showing combined gene expression per cell. Heat map for each condition, one cell per row, showing the expression of genes with reliable primer melting curves. Adjacent squares show technical replicates for each cell. The colors encode *Ct* values specified on the right. *Mnx1*, *Nkx6.1, E-cadherin* and *Mucin1* expression levels discriminate E10.5 pancreatic cells before and after culture.

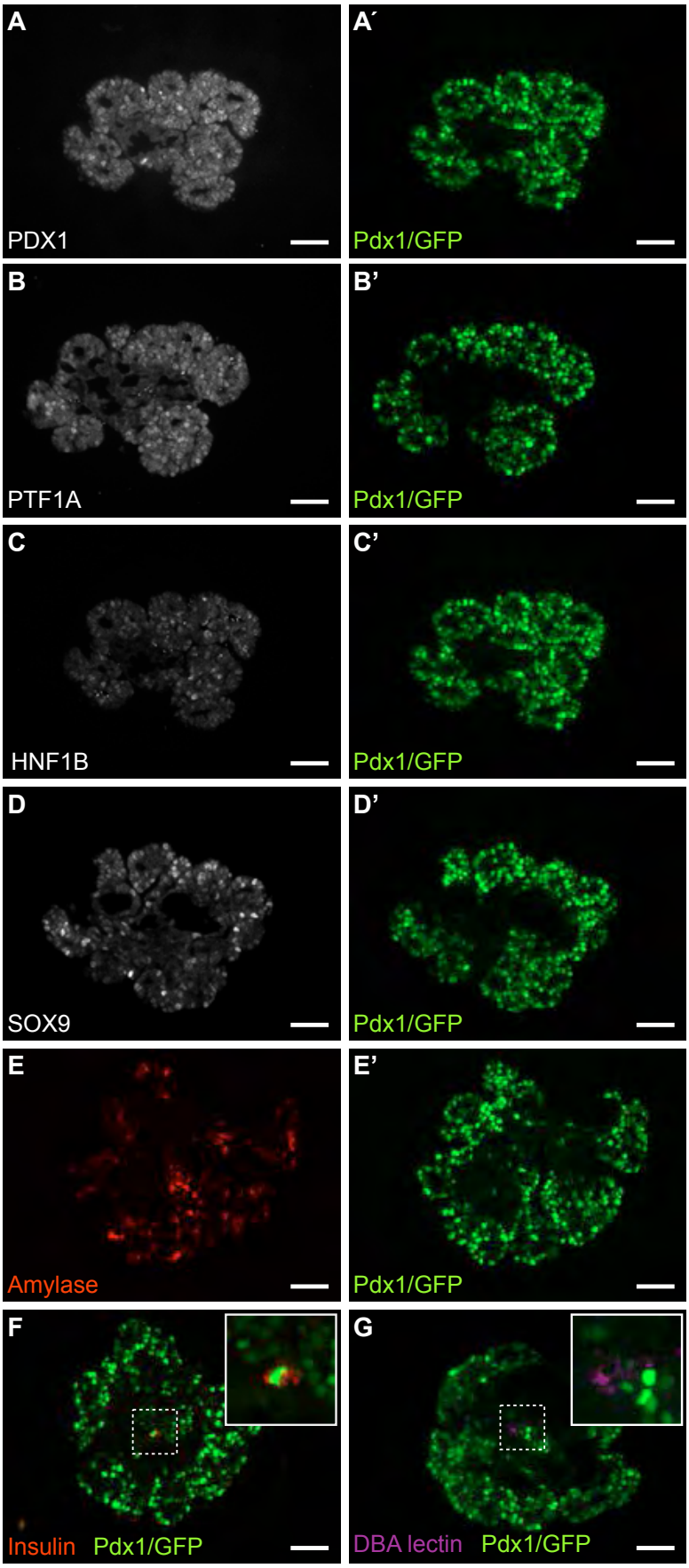

Fig. S6. R-spondin1 is not necessary for organoid development. Representative sections of day 7 organoids grown in the absence of R-spondin1 and immunolabelled with antibodies against the antigens indicated in the corner of each picture. (A-D) Pancreatic progenitor markers. (E-F) Pancreatic differentiation markers. In the small insets, a magnification of the dashed squared area in the corresponding picture. (A´,B´,C´,D´,E´) show *Pdx1*/nGFP reporter for (A,B,C,D,E) respectively. Scale bar: 50 µm.

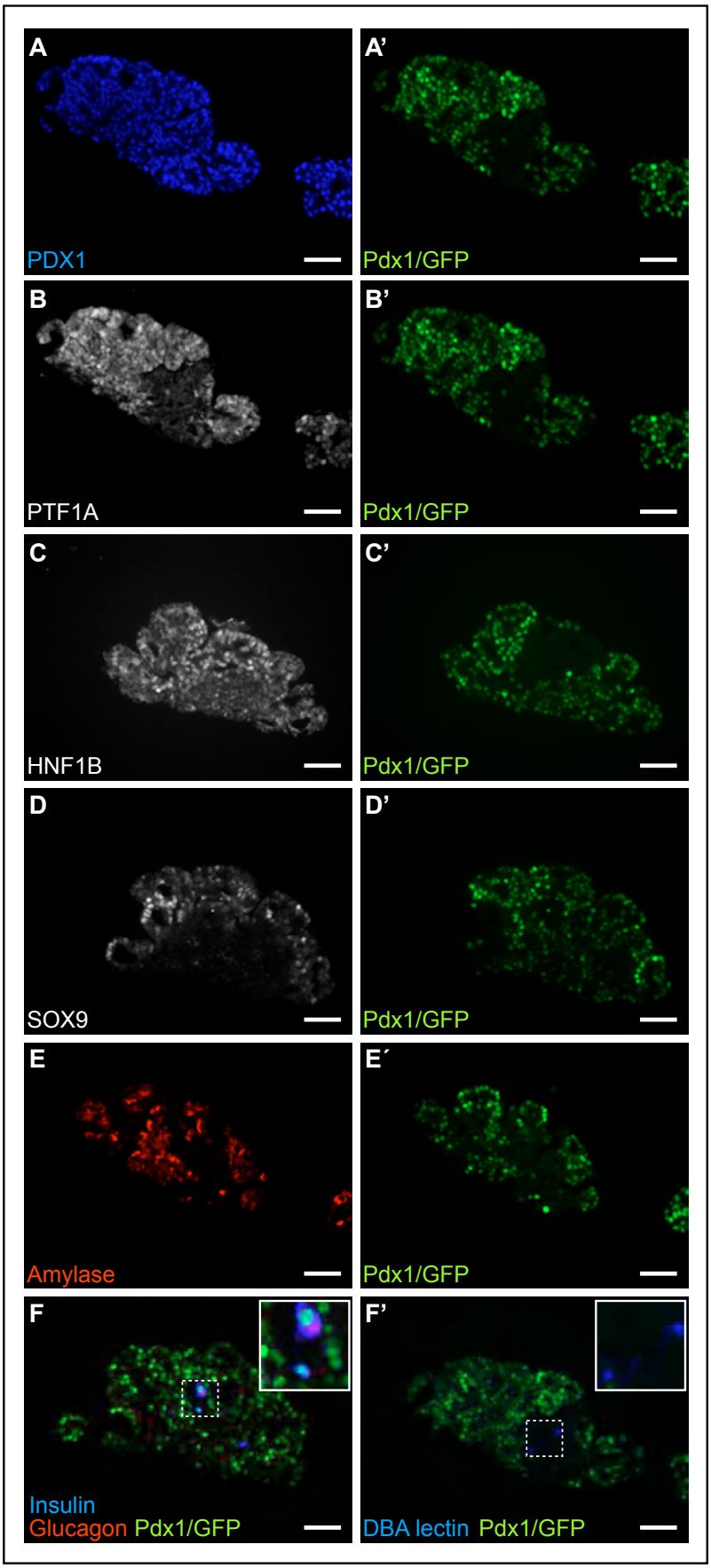

Fig. S7. EGF is not necessary for organoid development. Representative sections of day 7 organoids grown in the absence of EGF and immunolabelled with antibodies against the antigens indicated in the corner of each picture. (A-D) Pancreatic progenitor markers. (E-F) Pancreatic differentiation markers. In the small insets (F,F′), a magnification of the dashed squared area in the corresponding picture. (A´,B´,C´,D´,E´) show *Pdx1*/nGFP reporter for (A,B,C,D,E) respectively. Scale bar: 50 µm.

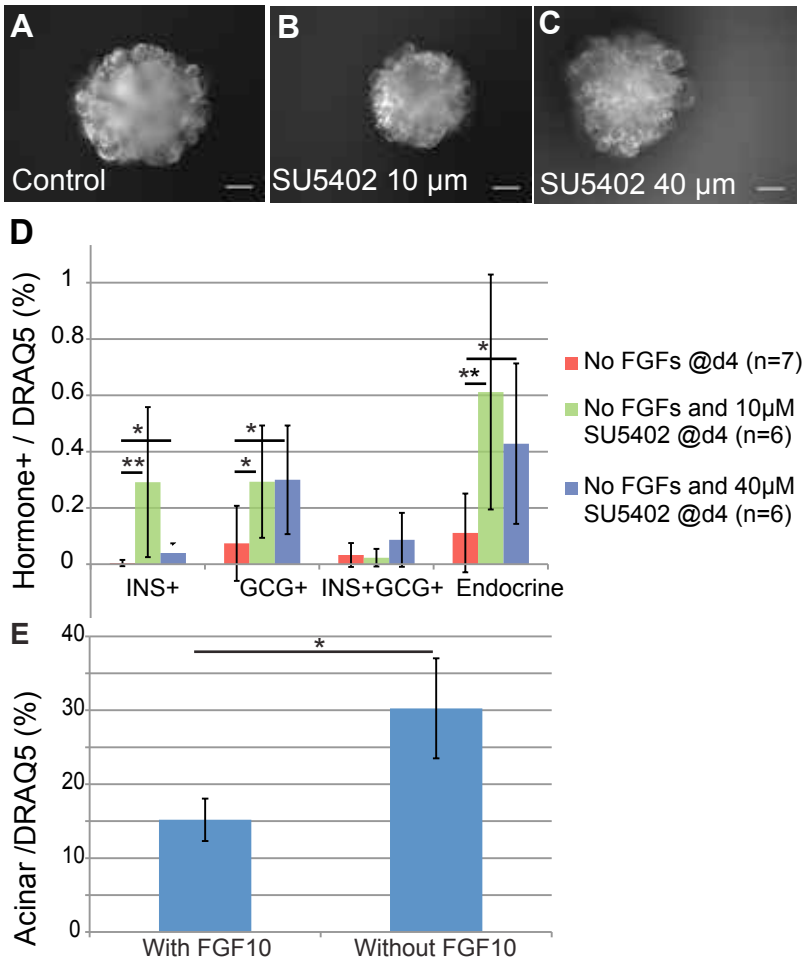

Fig. S8. FGF inhibition after expansion increases endocrine differentiation. (A) *Pdx1*/nGFP expression of a 7-day organoid grown in organogenesis medium, (B) after FGF inhibition with 10 µm SU5402, or (C) 40 µm SU5402 provided at day 4. Expansion and *Pdx1*/nGFP expression are not affected by FGF inhibition when compared to the control (A). (D) Quantification of the number of Insulin$^+$, Glucagon$^+$, cells co-expressing the two hormones (INS$^+$GCG$^+$) and cumulated endocrine cells (INS$^+$ and GCG$^+$) in the 3 conditions shows increased endocrine differentiation. (E) Quantification of the number of acinar cells (amylase) in the presence or absence of FGF10. * indicates a p-value <0.05 by Mann–Whitney non-parametric test and ** indicates a p-value <0.01. Scale bar: 50 µm.

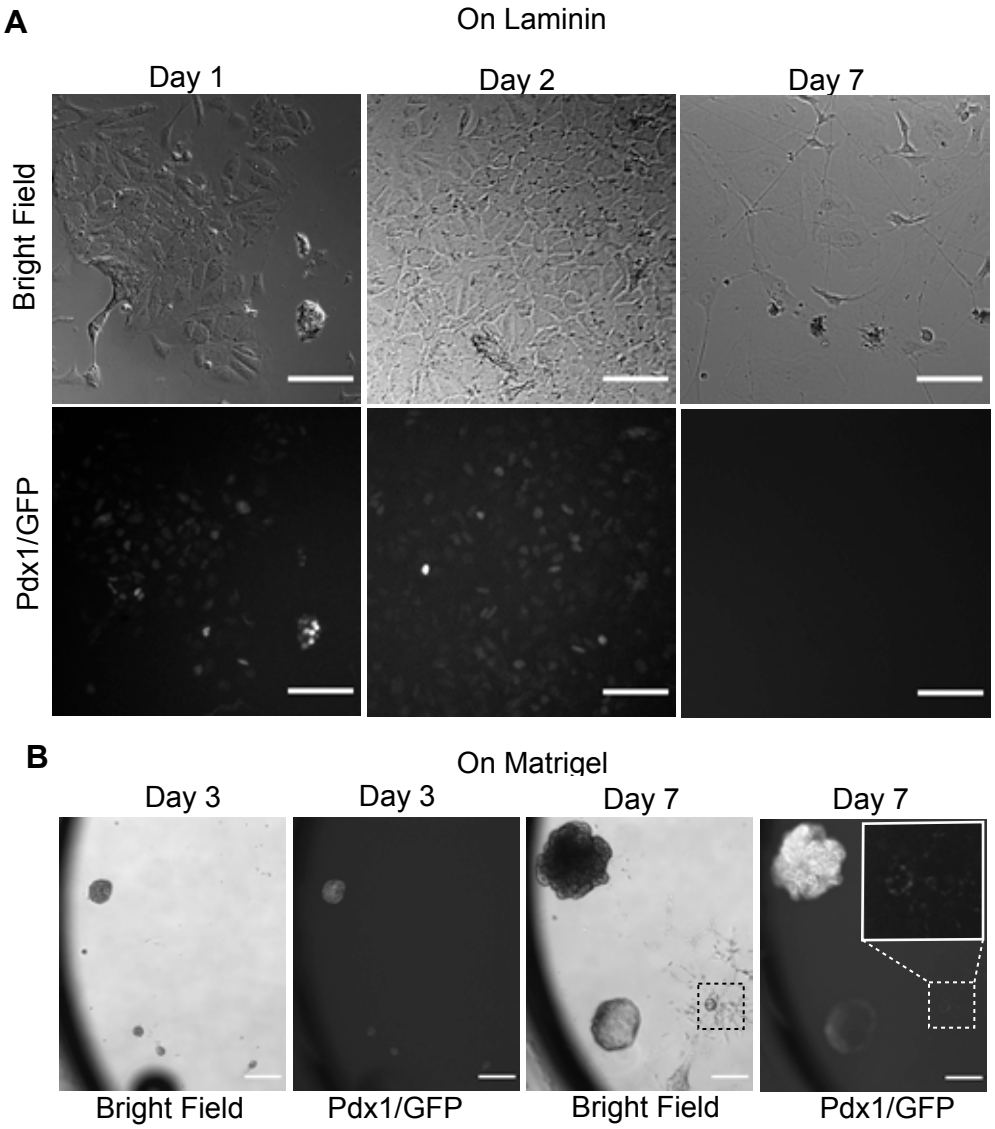

Fig. S9. Pancreatic progenitor culture in 2D on laminin or on Matrigel. (A) Flat colonies generated from E10.5 pancreatic progenitors seeded on laminin-coated plates and cultured for 7 days. The pancreatic cells spread on matrix and although they initially express *Pdx1*/nGFP at day 1 and 2, they lose it by day 5 and it is documented here at day 7. In the meantime the cells extend long filaments. (B) On Matrigel ™ most cells spread on matrix and progressively lose *Pdx1*/nGFP, as shown at day 7 (inset). However, some clusters invade the underlying Matrigel ™ and subsequently behave as if seeded in Matrigel ™. Scale bar: 100 µm.

Movie 1. 63-hour imaging of pancreas progenitor maintenance and expansion in organogenesis conditions. E10.5 pancreas progenitors from *Pdx1-Ngn3-ER^{TM}-ires-nGFP^{+}* were dispersed and seeded in Matrigel ™ in organogenesis medium at medium density. Phase contrast and GFP images were captured every hour for 63 hours. This frame (one of the 120 taken in each experiment) shows 4 initial seeds. The top seed is made of 6 cells, expands and remains homogeneously GFP^{+}. The one below has one cell which dies after 5-6 hours. The two below have 4 cells. They expand (little for the bottom one) but progressively lose GFP. Although no mesenchyme was detected around organoids by histology, rare mesenchymal cells were detected by imaging and moved in the gel, occasionally contacting expanding epithelial clusters.

Movie 2. 63-hour imaging of pancreas progenitor maintenance and expansion in organogenesis conditions. E10.5 pancreas progenitors from *Pdx1-Ngn3-ER^{TM}-ires-nGFP^{+}* were dispersed and seeded in Matrigel ™ in organogenesis medium at medium density. Phase contrast and GFP images were captured every hour for 63 hours. This frame (one of the 120 taken in each experiment) shows 6 initial seeds, 5 of which are in focus. The top seed is made of 4 cells, it expands but expression of GFP becomes heterogeneous. The one below is made of two cells. It expands slightly but loses GFP. It finally merges with the one above, lying very closely. Their migration is generally small and fusions were not observed at lower seeding density. The one on the right has one starting cell which quickly loses GFP but survives until the end of culture. The bottom one is made of 7 initial cells. It expands the most and GFP remains homogeneously expressed. Although no mesenchyme was detected around organoids by histology, rare mesenchymal cells were detected by imaging and moved in the gel, occasionally contacting expanding epithelial clusters.

Movie 3. 60-hour imaging of endocrine progenitor emergence in organoids. E10.5 pancreas progenitors from *Ngn3(EYFP)*, marking endocrine progenitors, were dispersed and seeded in Matrigel ™ in organogenesis medium without FGF1 at medium density. YFP (top) and phase contrast (bottom) images were captured every hour for 60 hours. These two frame clips show two initial seeds. The right has one YFP^+ cell at the beginning and turns down YFP by the end of the movie and 3 others turn it on during the 60 hours. The left seed has no YFP^+ cell at movie onset but one cell turns it on during movie time. Quantification shows that 7% of clusters are YFP^+ at seeding (n=8); 27% become YFP^+ during culture (n=31) and 66% remain YFP^- during movie (n=76).

Movie 4. 63-hour imaging of pancreas progenitor maintenance and expansion in sphere medium. E10.5 pancreas progenitors from *Pdx1-Ngn3-ER™-ires-nGFP*$^+$ were dispersed and seeded in Matrigel ™ in sphere medium at medium density. Phase contrast and GFP images were captured every hour for 63 hours. This frame (one of 120 taken in each experiment) shows one initial seed in focus in the center. This seed of 3 initial GFP^+ cells grows by 60% in diameter in 63 hours to reach about 8 GFP^+ cells. At the end, the lumen can be seen in the center and will subsequently enlarge.

Table S1. Primary antibodies

Epitope	Generated in:	Dilution	Provider
Amylase	Rabbit	1/400	Calbiochem
Glucagon	Rabbit	1/100	Zymed
Glucagon	Guinea pig	1/400	Linco
Carboxypeptidase A (CPA)	Rabbit	1/800	Anawa
C-Peptide 1	Rabbit	1/1500	BCBC
C-peptide 2	Rabbit	1/1500	BCBC
Dolichos biflorus agglutinin (DBA)	Biotinylated	1/500	Vector
E-cadherin	Mouse	1/50	BD Transduction Lab
HNF1B	Rabbit	1/100	Santa Cruz Biotechnology
Insulin	Guinea pig	1/100	Dako
Mucin 1	Rabbit	1/300	Abcam
Mucin 1	Armenian Hamster	1/200	Thermo Fisher Scientific
NEUROG3	Goat	1/1000	BCBC
Proconvertase (PC) 1/3	Rabbit	1/2000	Chemicon
PDX1	Goat	1/2000	BCBC
phospho Histone H3 (pHH3)	Rabbit	1/1000	Upstate
PTF1A	Rabbit	1/250	BCBC
SOX9	Rabbit	1/500	Chemicon

BCBC, Beta Cell Biology Consortium.

Table S2. Single-cell PCR primer list

Target	Forward primer	Reverse primer	Design RefSeq
Single-cell qPCR primers			
Actb	CCCTAAGGCCAACCGTGAAA	CAGCCTGGATGGCTACGTAC	NM_007393.3
Gapdh	AGACGGCCGCATCTTCTT	TTCACACCGACCTTCACCAT	NM_008084.2
Hnf1b	CGGCAAAAGAATCCCAGCAA	AGACCCCTCGTTGCAAACA	NM_009330.2
Mnx1	CAAGCGTTTTGAGGTGGCTAC	TTCATTCGGCGGTTCTGGAA	NM_019944.2
Muc1	AGTACCAAGCGTAGCCCCTA	CACCACAGCTGGGTTGGTATAA	NM_013605.1
Neurog3	GCTGCTTGACACTGACCCTA	GGATGGTGAGCGCATCCAA	NM_009719.6
Nkx2.2	CACCGAGGGCCTCCAATAC	GCCCTGGGTCTCCTTGTCA	NM_010919.2
Nkx6.1	GGCCTATTCTCTGGGGATGAC	GCTGCGTGCTTCTTTCTCC	NM_144955.2
Pdx1	TCCCTTTCCCGTGGATGAAA	TCGGGTTCCGCTGTGTAA	NM_008814.3
Sox9	AGTACCCGCATCTGCACAA	GTCTCTTCTCGCTCTCGTTCA	NM_011448.4
qPCR primers			
Hes1	TGCCAGCTGATATAATGGAGAA	CCATGATAGGCTTTGATGACTTT	NM_008235
Hprt1	GGCCAGACTTTGTTGGATTTG	TGCGCTCATCTTAGGCTTTGT	NM_013556.2
Pdx1	CCCAGTTTACAAGCTCGCTG	CTCGGGTTCCGCTGTGTAAG	NM_008814.3
Sox9	CCACGGAACAGACTCACATC	CTGCTCAGTTCACCGATGTC	NM_011448.4

To determine the amplification efficiency of each individual primer set, a calibration curve was generated using a positive sample (a mixture of embryonic pancreas from E10.5 and E13.5).