Supplemental Information

Controlled clustering enhances PDX1 and NKX6.1 expression in pancreatic endoderm cells derived from pluripotent stem cells

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Table S1: Significance testing summary for one-way ANOVA with Tukey post-hoc multiple comparison on qPCR data presented in Figure 1B. n.s.=p>0.05, *=p<0.05, **=p<0.01, ****=p<0.001.

	OCT3/4	**SOX17**	**FOXA2**	**HNF6**	**PDX1**	**NKX2.2**	**NKX6.1**
Overall Significance	********	*****	**n.s.**	******	******	******	**n.s.**
iPSCs vs DE	****	*	n.s.	n.s.	n.s.	n.s.	n.s.
iPSCs vs PF	****	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
iPSCs vs PE	****	n.s.	n.s.	**	*	**	n.s.
DE vs PF	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
DE vs PE	n.s.	*	n.s.	**	*	*	n.s.
PF vs PE	n.s.	n.s.	n.s.	*	n.s.	**	n.s.

Table S2: Significance testing summary for one-way ANOVA with Tukey post-hoc multiple comparison on cell density data presented in Figure 2F. n.s.=p>0.05, **=p<0.01, ****=p<0.001.

	Microwell Diameter (μm)		
	150	*300*	*500*
Overall Significance	********	********	********
Region 1 vs Region 2	**	n.s.	**
Region 1 vs Region 3	****	****	n.s.
Region 1 vs Region 4	****	****	****
Region 2 vs Region 3	****	****	****
Region 2 vs Region 4	****	****	****
Region 3 vs Region 4	****	****	****

Table S3: Detailed composition of differentiation medium used in each stage to produce PF cells.

Days	**Medium**	**Basal Medium**	**Soluble Factors**
1	S1A	MCDB131 + 10mM glucose + 1.5 g/L $NaHCO_3$ + 0.5% fatty acid free bovine serum albumin (FAF-BSA) + 1x GlutaMAX + 1% Pen/Strep	+100 ng/mL Activin A +3 µM CHIR99021
2-3	S1B		+100 ng/mL Activin A
4-5	S2		+0.25 mM ascorbic acid +50 ng/mL keratinocyte growth factor (KGF)
6-7	S3	MCDB131 + 10mM glucose + 2.5 g/L $NaHCO_3$ + 2% FAF-BSA + 1x GlutaMAX + 1% Pen/Strep	+0.25 mM ascorbic acid + 1:200 insulin-transferrin-selenium-ethanolamine (ITS-X) +50 ng/mL KGF + 0.25 µM SANT-1 + 1 µM retinoic acid + 100 nM LDN193189 + 200 nM TPB (PKC activator)
8-10	S4		+0.25 mM ascorbic acid + 1:200 ITS-X +2 ng/mL KGF + 0.25 µM SANT-1 + 0.1 µM retinoic acid + 200 nM LDN193189 + 100 nM TPB

Table S4: Primers used for qPCR

Gene	**Forward / Reverse Primer**	**Sequence (5'-3')**
GAPDH	Forward	CCCATCACCATCTTCCAAGGAG
	Reverse	CTTCTCCATGGTGGTGAAGACG
OCT 3/4	Forward	TGGGCTCGAGAAGGATGTG
	Reverse	GCATAGTCGCTGCTTGATCG
SOX2	Forward	CACAACTCGGAGATCAGCAA
	Reverse	TCCGGGAAGCGTGTACTTA
SOX17	Forward	GGCGCAGCAGAATCCAGA
	Reverse	CCACGACTTGCCCAGCAT
FOXA2	Forward	GGGAGCGGTGAAGATGGA
	Reverse	TCATGTTGCTCACGGAGGAGTA
HNF1B	Forward	TCACAGATACCAGCAGCATCAGT
	Reverse	GGGCATCACCAGGCTTGTA
HNF4A	Forward	CATGGCCAAGATTGACAACCT
	Reverse	TTCCCATATGTTCCTGCATCAG
HNF6	Forward	CGCTCCGCTTAGCAGCAT
	Reverse	GTGTTGCCTCTATCCTTCCCAT
PDX1	Forward	AAGTCTACCAAAGCTCACGCG
	Reverse	GTAGGCGCCGCCTGC
NKX2.2	Forward	CCGAGGGCCTTCAGTACTCC
	Reverse	CGGGGTCTCCTTGTCATTGT
NKX6.1	Forward	TTCGCCCTGGAGAAGACTTT
	Reverse	GCGTGCTTCTTCCTCCACTT

Table S5: Antibodies and reagents used for immunocytochemistry

Antibody	**Supplier**	**Catalog #**	**Dilution**
Rabbit anti-human mAb HNF4α	Abcam	ab92378	1:400
Mouse anti-human mAb PDX1	BD Pharmigen	562160	1:200
Rabbit anti-human pAb NKX6.1	Novus Biologicals	NBP149672	1:200
Goat anti-mouse AlexaFluor 488	Life Technologies	A11001	Primary
Goat anti-rabbit AlexaFluor 568	Life Technologies	A11011	Primary
4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Life Technologies	D9542	1:1000
TRITC-conjugated phalloidin	Life Technologies	P1951	1:200

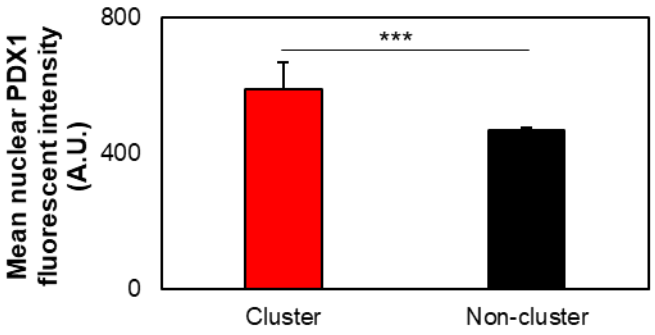

Figure S1*:* Clustered (n = 7) pancreatic endoderm cells have elevated nuclear PDX1 fluorescent intensity than surrounding non-clustered cells. ***=p<0.005 for a Student's t-test.

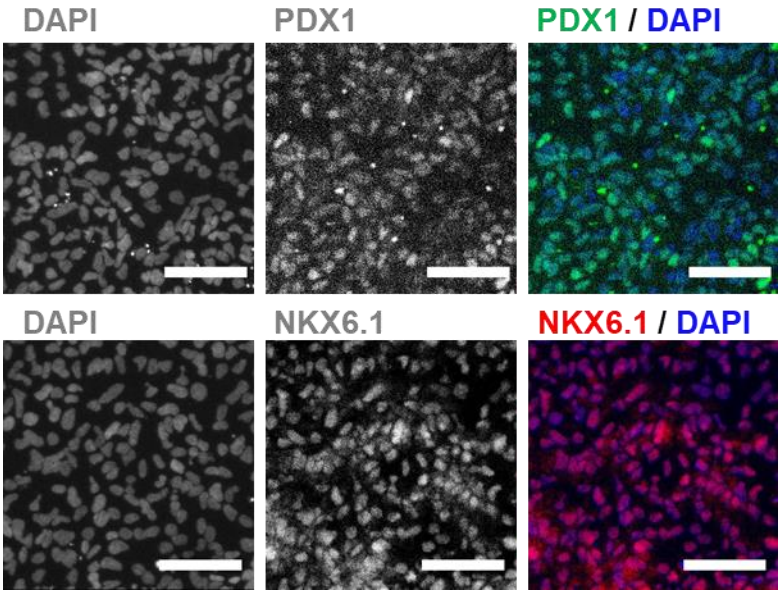

Figure S2: Unconfined pancreatic endoderm cultures show positive staining for pancreatic transcription factors PDX1 and NKX6.1. Brightness and contrast have been increased to show positive staining. Scale bars: 100 µm.

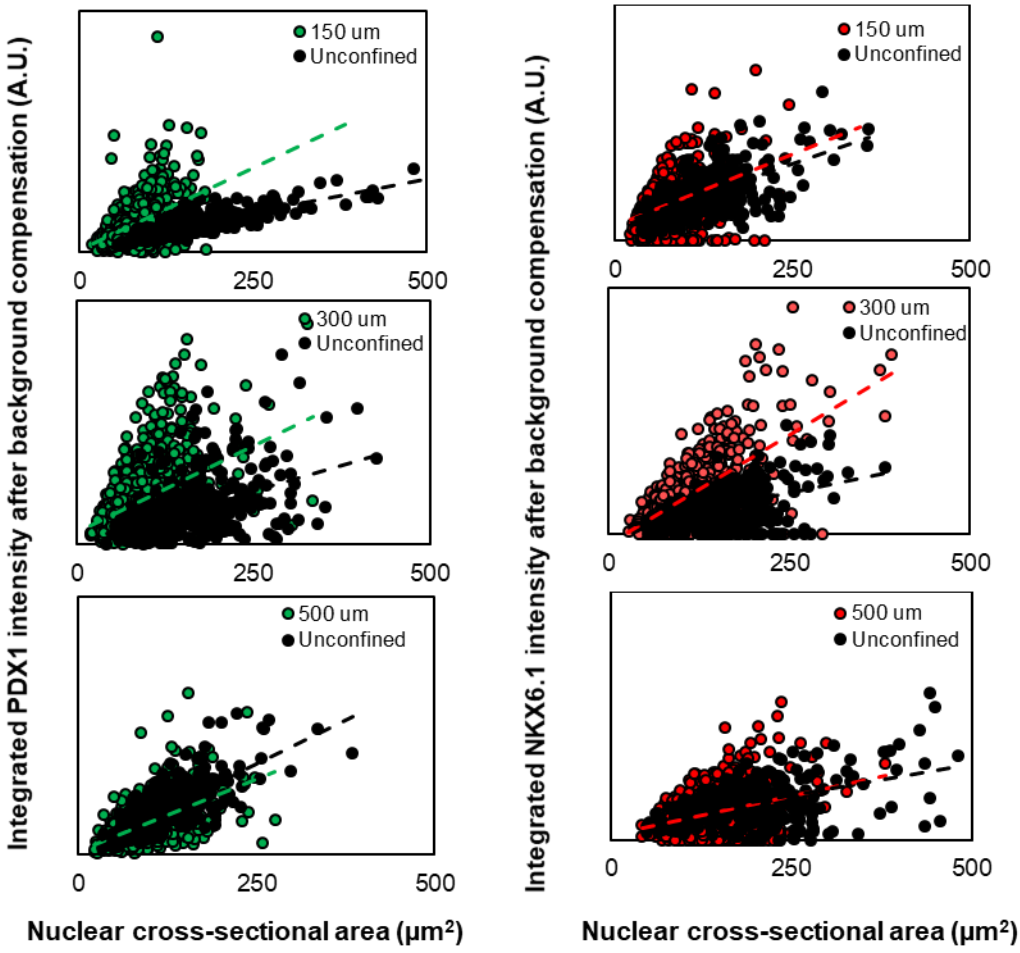

Figure S3: Representative plots of total nuclear fluorescence intensity. Each point represents a single nucleus within each culture condition. The effects of nuclear area and confined culture were determined by applying the following linear model: $y = \beta_0 + \beta_1 x_1 + \beta_2 x_2$, where y is the total nuclear fluorescence intensity, x_1 is the culture system and x_2 is the nuclear cross-sectional area. The nuclear area had a statistically significant positive correlation with the integrated fluorescence intensity of PDX1 or NKX6.1 staining in all conditions ($p<0.0001$). Confined culture had a significant positive effect on PDX1 and NKX6.1 integrated intensity in the 150 μm and the 300 μm diameter microwells, while a small but significant ($p<0.02$) negative effect was observed for 500 μm microwell confined culture compared to unconfined controls.

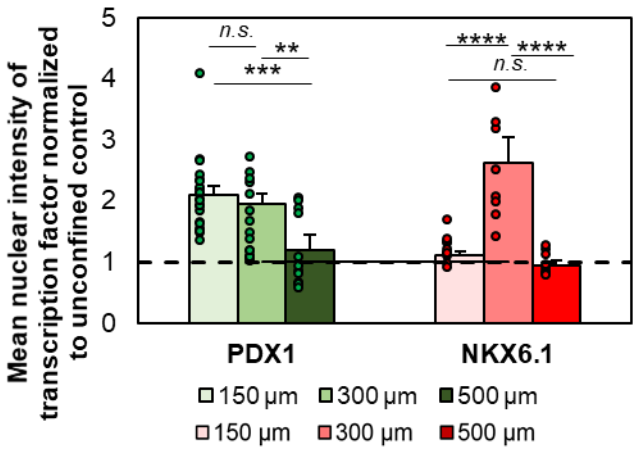

Figure S4: PDX1 (n = 21, 13, 9 for 150, 300, and 500 µm microwells) and NKX6.1 (n = 12, 8, 8 for 150, 300, and 500 µm microwells) nuclear intensity is increased when presented with sufficient geometric confinement. Each point represents a data point from a single microwell. n.s = $p>0.05$, **=$p<0.01$, ***=$p<0.005$, ****=$p<0.001$.

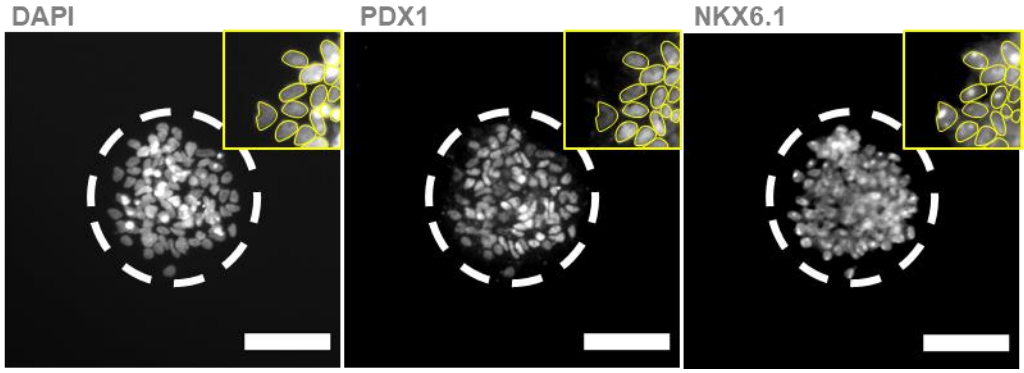

Figure S5: Double stained 150 µm microwells show nuclear colocalization of PDX1 and NKX6.1. Scale bars: 100 µm.

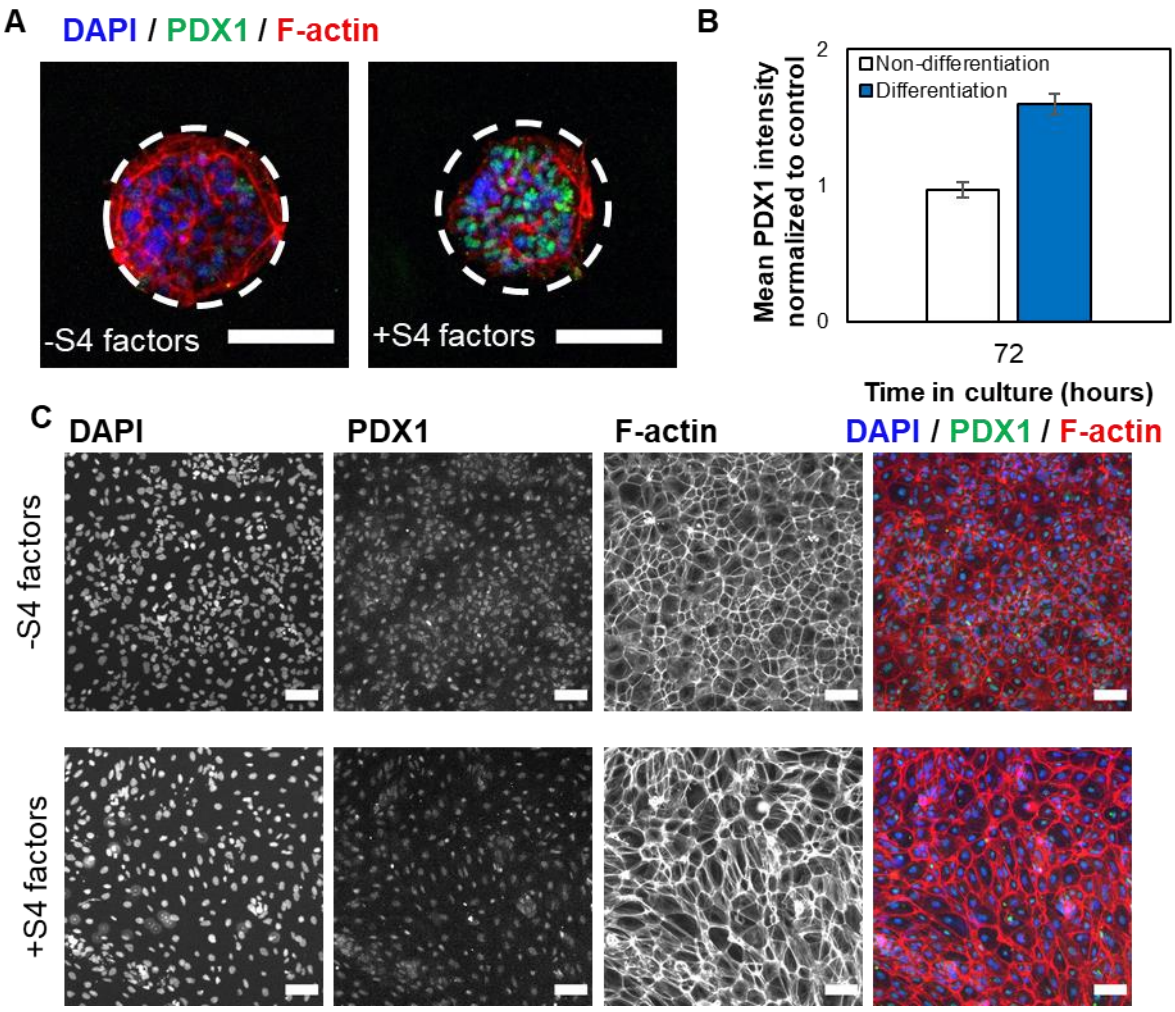

Figure S6: Culture in absence of differentiation inducing factors abrogates increased PDX1 expression, reduces cell density, and disrupts benefits of microwell culture. (A) Removal of S4 differentiation factors halts cytoskeletal reorganization and abrogates any improvements in pancreatic differentiation from microwell culture shown by immunocytochemistry of PF cells confined within 150 µm wells. (B) Confined culture in absence of soluble factors does not upregulate PDX1 expression over the unconfined control. (C) Decreased cell density is observed in unconfined controls when PE inducing differentiation factors are removed. Scale bars: 100 µm.

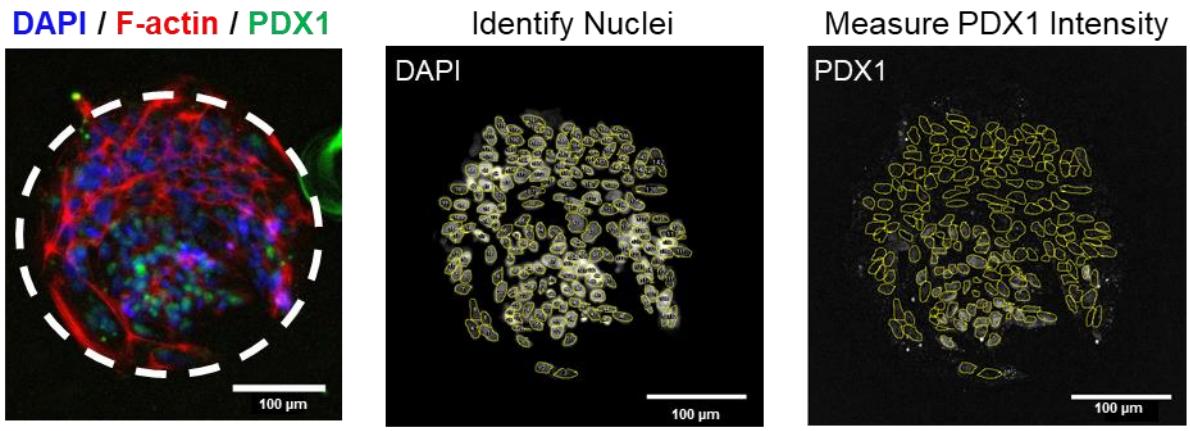

Figure S7: Image analysis work flow. First nuclei were manually selected as regions of interest using the DAPI counterstain. Next, the intensity of the PDX1 stain was measured within the selected regions of interest.

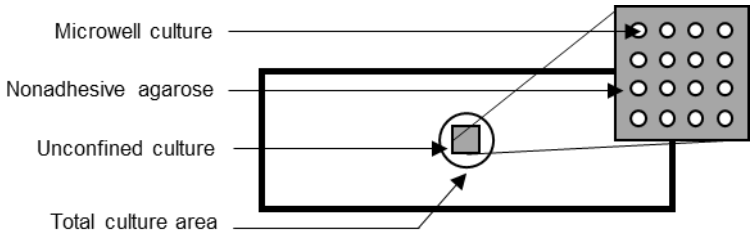

Figure S8: Layout of slide containing microwell cultures.

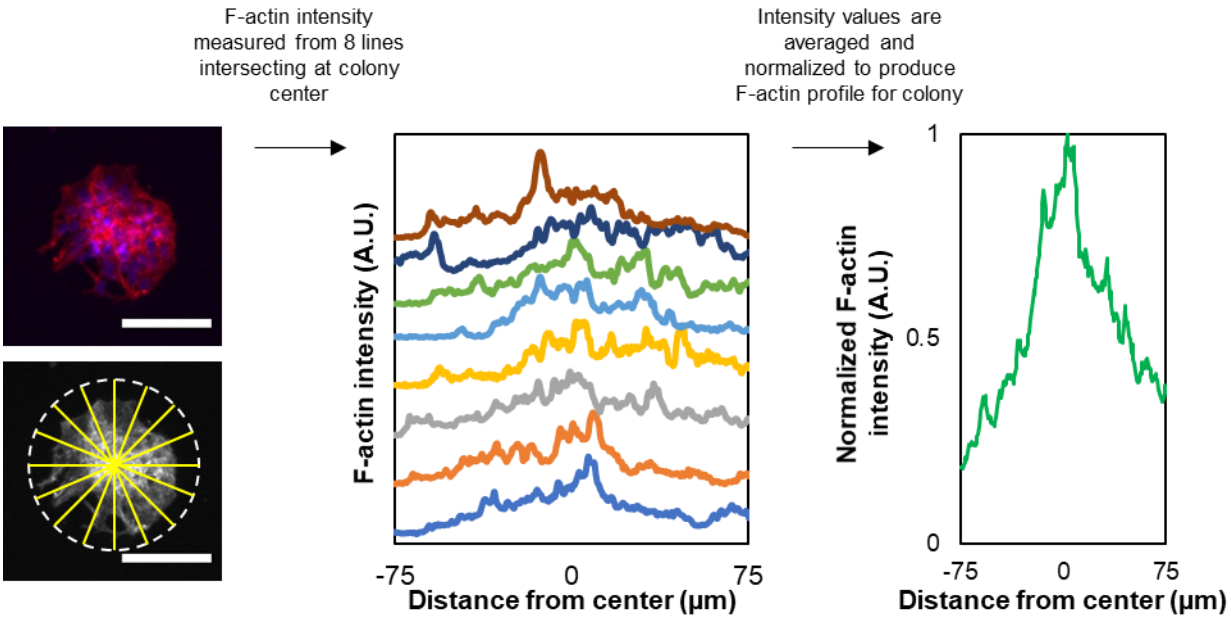

Figure S9: Process flow to obtain actin intensity profile shown in Figure 5B. The reported actin intensity profiles are the average of 8 actin profiles which intersect at the microwell colony center.