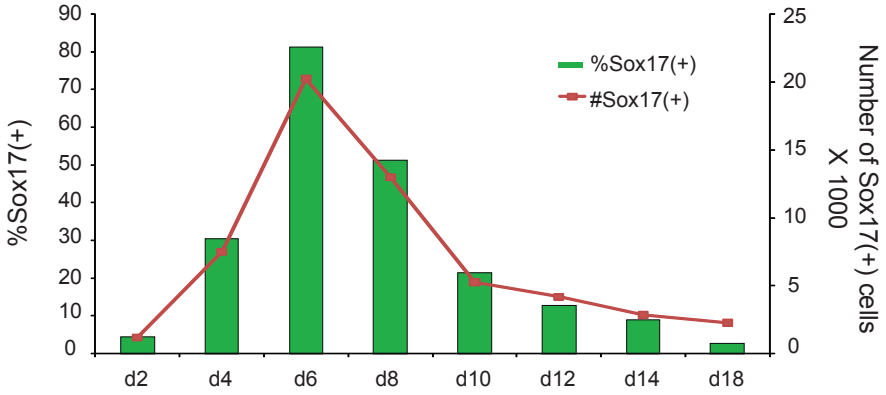
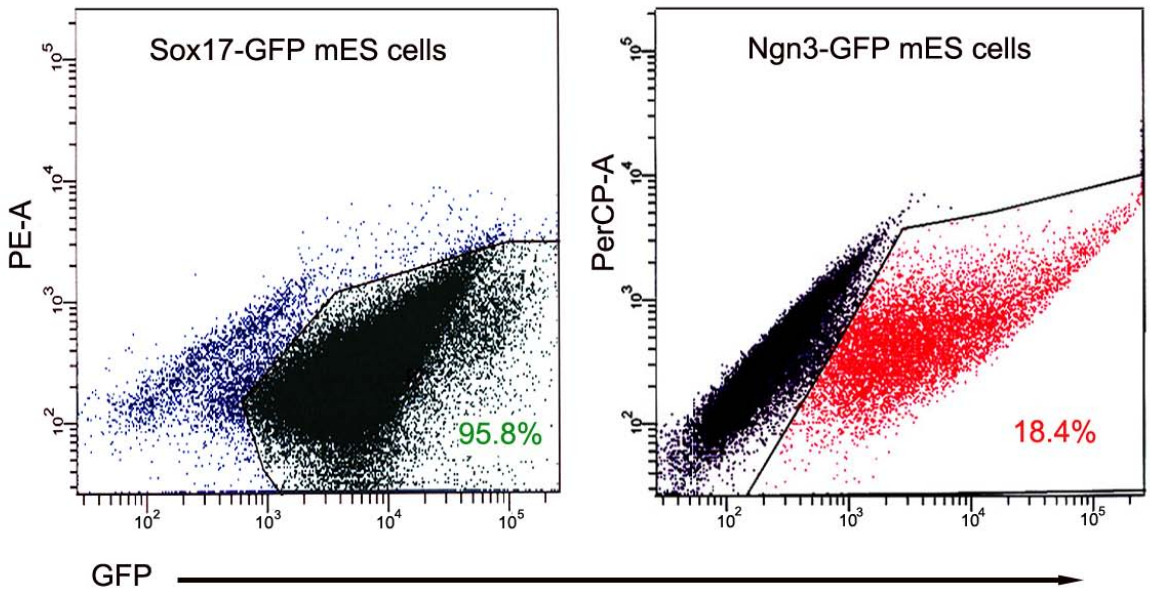
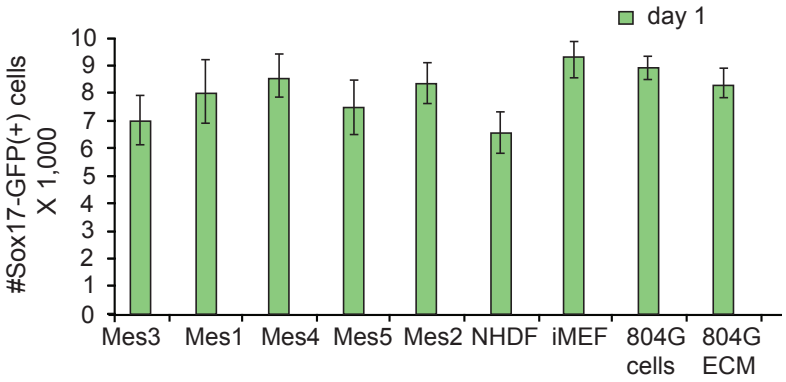
doi:10.1038/nature11463



Supplementary Figure 1: Expression of Sox17-GFP is transient during directed differentiation, with the peak at day six. Definitive endoderm (Sox17(+) cells) was generated from mES cells containing a Sox17-GFP reporter using growth factors (ActivinA and Wnt3A) for six days. Progenitors are the definitive endoderm stage can thus be captured using GFP expression. Data are shown for cells differentiated on gelatin.

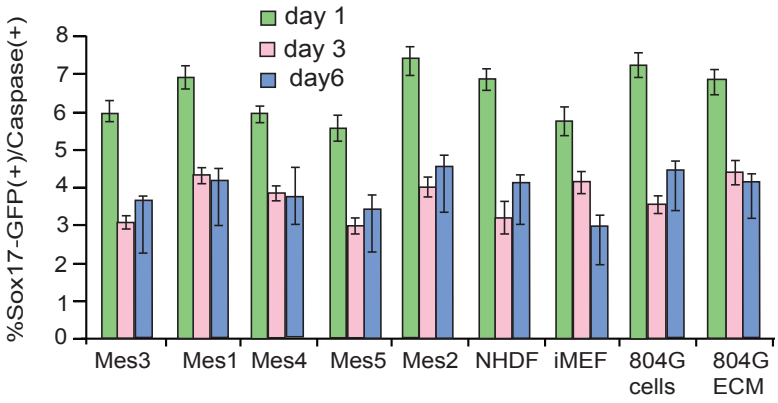


Supplementary Figure 2: Flow cytometric quantification and purification of mES derived Sox17-GFP(+) and Ngn3GFP(+) populations.

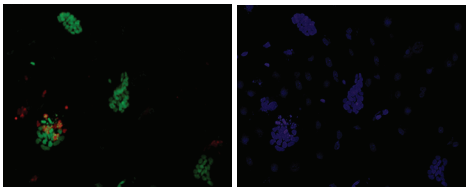


Supplementary Figure 3: No significant differences are found in the initial attachment of endoderm to mesenchyme versus controls. FACS-purified Sox17-GFP(+) cells were plated onto various surfaces and then their numbers were quantitated one day later.

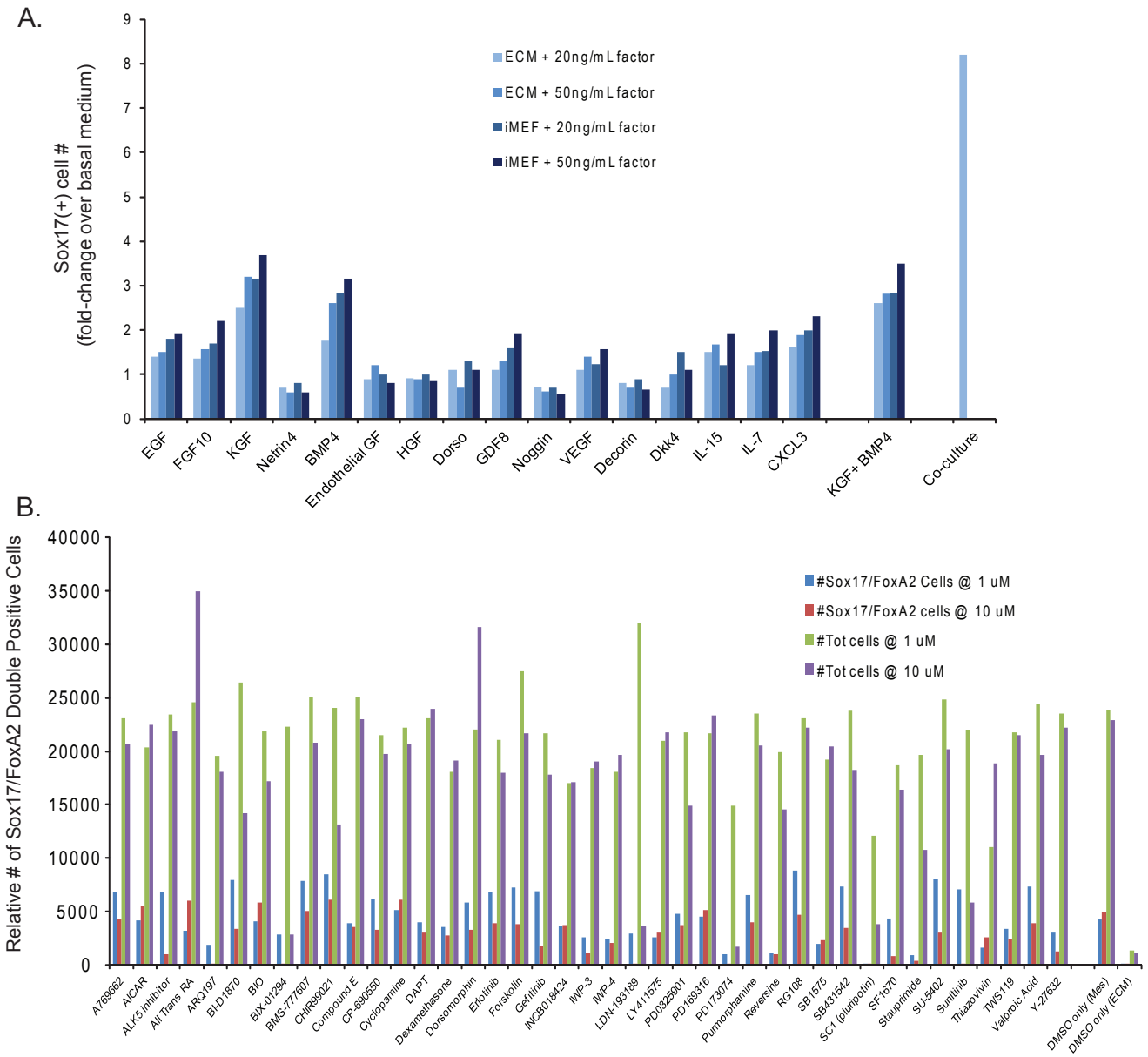
A.



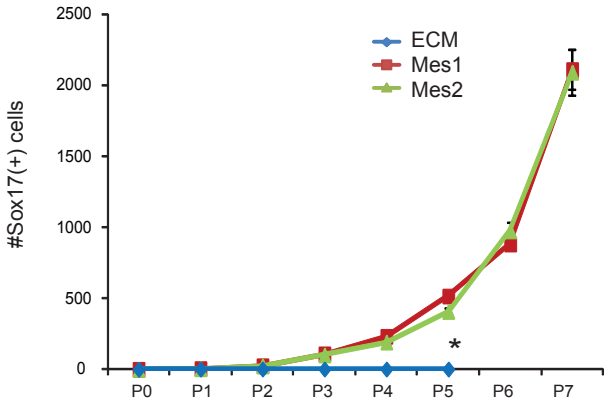
B.



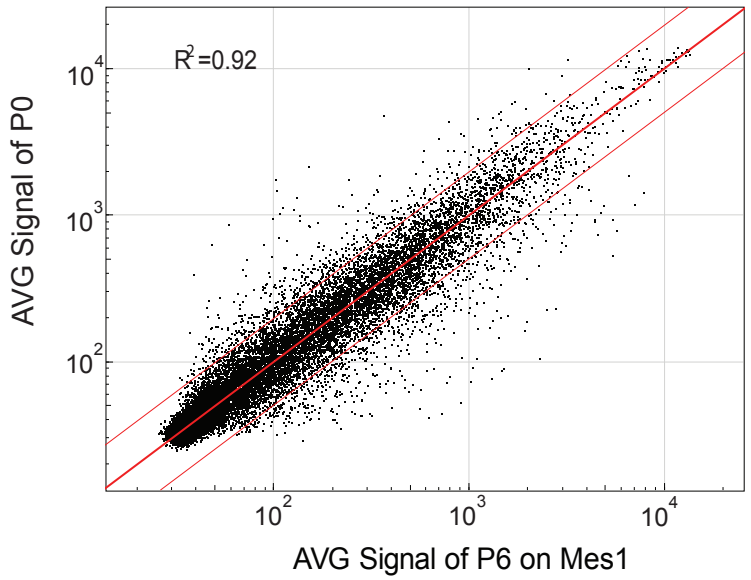
Supplementary Figure 4: Mes1 and Mes2 do not prevent apoptosis. A. Quantitation of immunofluorescence staining for cleaved caspase 3, a marker of cells undergoing apoptosis, revealed that Sox17-GFP(+) cells expanded on mesenchyme do not undergo increased apoptosis. Error bars were calculated using standard deviation. B. Representative images showing Sox17-GFP/cleaved caspase 3 immunofluorescence.

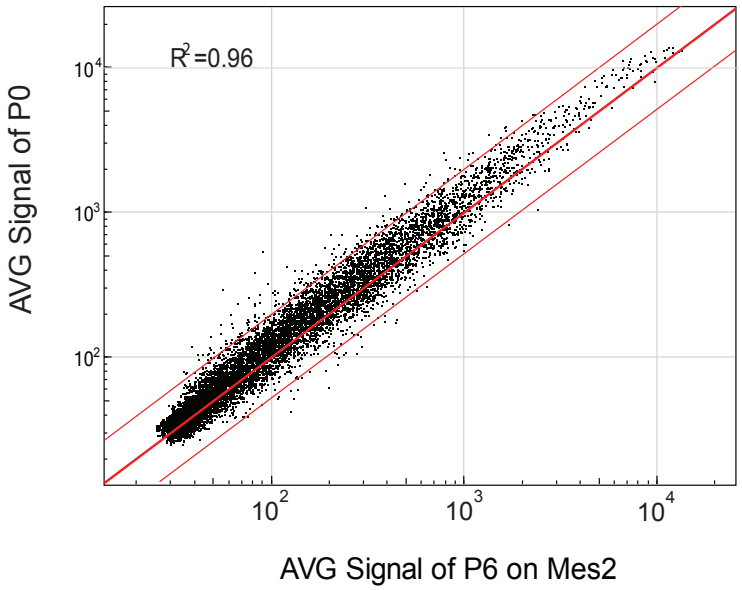


Supplementary Figure 5: Growth factor and chemical inhibitor screens. A. Growth factor screen. FACS-purified, mouse Sox17-GFP(+) cells were cultured on either ECM or on iMEF (to provide a cellular context). A panel of candidate signaling factors were screened for the ability to expand endoderm at two different concentrations. The number of Sox17-GFP(+) cells was quantitated by FACS after 6 days of treatment, and is represented as fold-change over basal medium alone. Some factors had a marginal effect, 1.5- to 2-fold more Sox17-GFP(+) cells compared to basal medium alone. Even after 6 days of treatment with Keratinocyte Growth Factor (KGF) or Bone Morphogenetic Protein 4 (BMP4), only a 3-fold increase was seen (about 40% of the effect provided by co-culture mediated expansion), with no additive effect observed when used in combination. B. Chemical compound screen. Human endoderm was cultured with Mes2, and starting the next day a panel of 41 chemical inhibitors were added to see if the co-culture- mediated expansion could be abrogated. Chemicals were added at 1 uM and 10 uM over the course of 6 days. At day 6, cells were fixed, stained for Sox17 and FoxA2, and imaged and analyzed using high-throughput imaging machinery and software. Since some compounds may be toxic to endoderm, to mesenchyme, or to both, we quantitated both the total cell number as well as the number of cells that were positive for both Sox17 and FoxA2. As negative and positive controls, cells were cultured on ECM in the presence of DMSO, and on mesenchyme in the presence of DMSO, respectively.

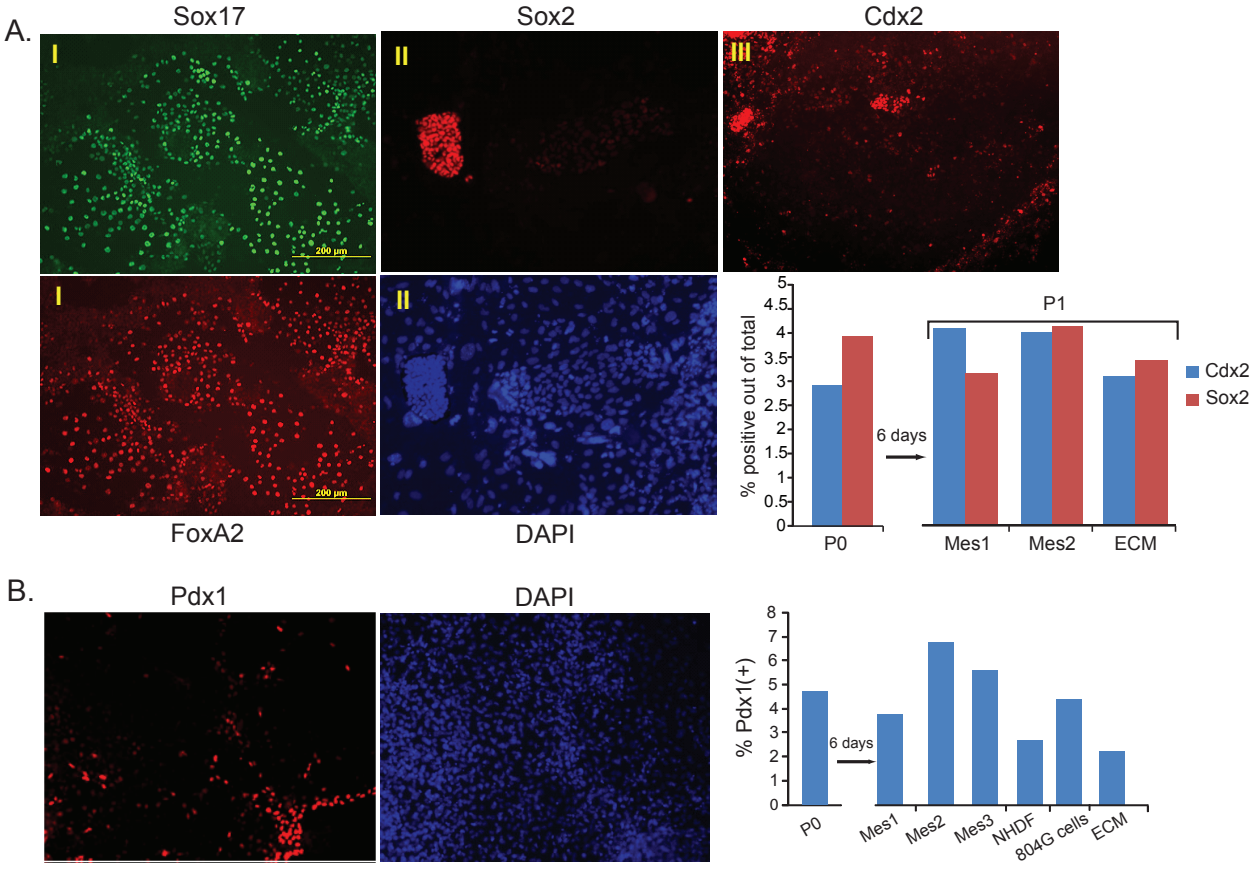


Supplementary Figure 6: Long-term expansion of mouse endoderm with successive FACS sorting yields expansion of approximately 2,000-fold more cells on Mes1 and Mes2. After seven passages, lasting 42 days, we observed a 2,000-fold expansion of purified mouse endoderm progenitors when the Sox17(+) cells were purified by FACS at each successive passage. The percentage of Sox17(+) cells at each stage remained steady (between 77% and 87%, data not shown.) In contrast to Sox17(+) cells passaged on mesenchyme, cells cultured on ECM alone were progressively lost within 5 passages (asterisk). As FACS sorting can be harsh to cells, we observed significant loss at each passage.

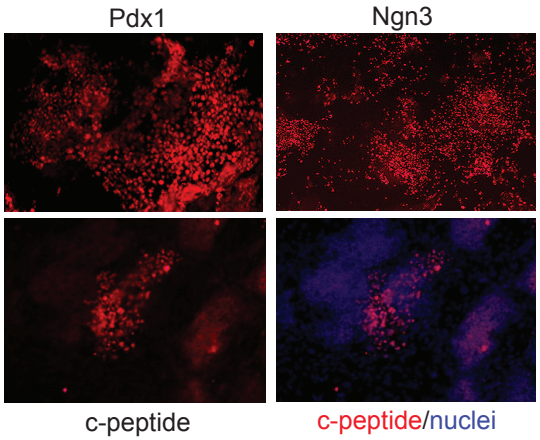




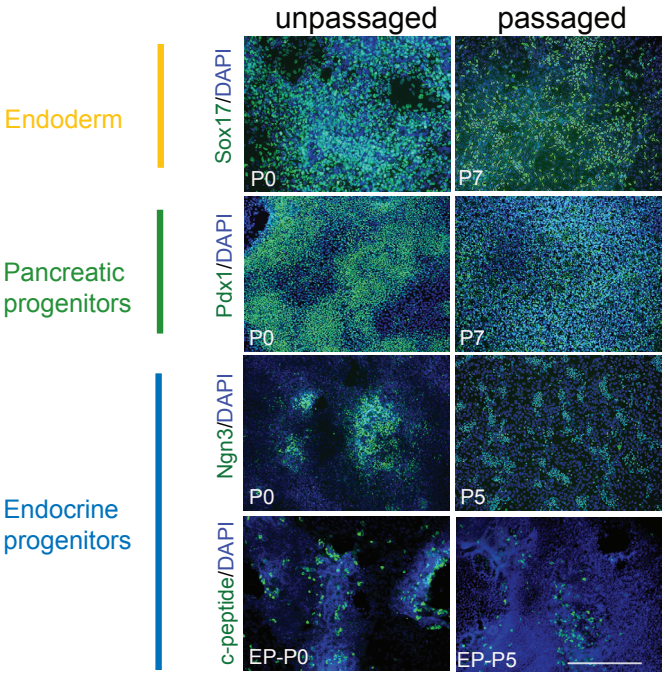
Supplementary Figure 7: A scatter plot showing the average signal of all genes expressed by unpassaged endoderm (P0, y axis) versus those expressed by endoderm after 6 passages on mesenchyme Mes1 or Mes2 (P6, x axis). Sorted mouse endoderm that was unpassaged (P0) or passaged for 6 times on Mes1 or Mes2 (P6) was compared by global gene expression analysis. The concordance is quite high between P0 endoderm and P6 endoderm on Mes1 (R2 = 0.92), and similarly between P0 and P6 on Mes2 (R2 = 0.96). Red lines indicate the level for 2-fold differences between genes. As a reference, our lab previously reported that the R2 value for FACS-purified Sox17(+) cells from E7.75 mouse embryos vs. untreated mES cells was 0.55.[1]



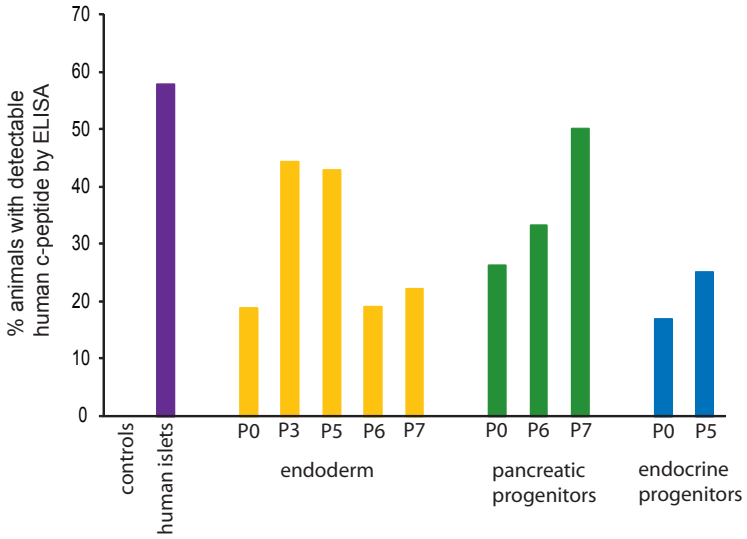
Supplementary Figure 8: Characterization of endoderm expanded by mesenchymal signals. A. Expression of endodermal markers. I. The majority (>95%) of Sox17(+) cells also express the transcription factor FoxA2. In contrast, the expression of Sox2 (which marks lung lineage commitment, panel II) or of Cdx2 (a marker of gut endoderm, panel III) are rare; the incidence is 3% and 4% of cells, respectively, and this does not markedly increase during mesenchyme-mediated expansion. The quantification of Cdx2 and Sox2 is shown as the percentage of Sox17(+) cells. B. Endoderm cultured in the presence of Mes1 or Mes2 does not spontaneously differentiate into Pdx1(+) pancreatic progenitors. After six days of expansion 3.5% (on Mes1) and 6.8% (Mes2) of all cells express Pdx1 compared to 3.1% of cells cultured on ECM The percentage of total cells is shown.



Supplementary Figure 9: Mouse endoderm passaged 6 times (P6) on Mes2 can be further differentiated to pancreatic progenitors (Pdx1(+)), endocrine progenitors (Ngn3(+)), and c-peptide-expressing cells.



Supplementary Figure 10: Immunofluorescence of unpassaged or passaged cells, at each pancreatic stage, shows the expression of expected markers before injection into the kidney capsule. Cells were differentiated and expanded in vitro, then stained to ensure proper differentiation before transplantation. Endoderm (P0 and P7) was stained for Sox17, pancreatic progenitors (P0 and P7) were stained for Pdx1, and endocrine progenitors (P0 and P5) were stained for Ngn3 and for c-peptide. Even at the latest stage (endocrine progenitors), few cells express c-peptide before injection and maturation in vivo. Images were taken at 10X magnification.



Supplementary Figure 11: Human c-peptide is detectable in the plasma of animals that have received transplants containing both unpassaged and passaged endoderm, pancreatic progenitors, and endocrine progenitors. Graph depicts a summary of each stage of progenitors that was injected, and what percentage of animals receiving those cells showed detectable human c-peptide by ultrasensitive ELISA (where detectable was defined as greater than 15 pmol/L). No c-peptide was detected in animals that received negative controls (in which PBS alone, or mesenchyme alone were injected). As a positive control for engraftment, survival, and function, human islets were also injected.

Primary Mesenchymal Cell Lines	Developmental Stage	Species	Organ of Origin
Mes1	E18.5	Mouse	Pancreas
Mes2	Adult	Mouse	Pancreas (islet fraction)
Mes3	Adult	Human	Pancreas (islet fraction)
Mes4	E13.5	Mouse	Pancreas
Mes5	E12.5	Mouse	Pancreas
Mes6	Adult	Human	Pancreas (islet fraction)
Mes7	Adult	Human	Pancreas (islet fraction)
Mes8	E14.5	Mouse	Pancreas
Mes9	Adult	Human	Pancreas (acinar fraction)
Mes10	E15.5	Mouse	Pancreas
Mes11	E16.5	Mouse	Pancreas
Mes12	E17.5	Mouse	Pancreas
Mes13	E17.5	Mouse	Pancreas
Mes14	E17.5	Mouse	Intestine
Mes15	E17.5	Mouse	Liver
Mes16	P0	Mouse	Spleen
Control Cell Lines	**Description**	**Species**	**Organ of Origin**
C1: Endo	bEnd.3	Mouse	Brain
C2: Endo	MS1 VEGF	Mouse	Pancreas (islet)
C3: Epith	804G (rat carcinoma cell line)	Rat	Bladder
C4: Fibro	Normal Human Dermal Fibroblasts (NHDF)	Human	Skin
C5: MEF	Mouse embryonic fibroblasts (MEF)	Mouse	E12.5 embryo
Extracellular Matrices	**Description**	**Species**	**Organ of Origin**
ECM1	Conditioned medium from 804G cell line (rat carcinoma)	Rat	Bladder
ECM2	Gelatin		
ECM3	Laminin		

Supplementary Table 1: A list of cells and ECM used in the screen. 16 primary mesenchymal lines were derived from mouse and human tissue. Mesenchyme from mouse was dissected from embryonic (days 12.5 through 18.5 after fertilization), neonatal (Postnatal day 0 (P0)) or adult mouse tissues. Mesenchyme from normal human adult donors was derived from the islet or acinar fractions alone.

Supplementary Table 2: Summary of human ES-derived pancreatic cells injected into animals

Group	Internal ID#	Source	Age	Material injected	Cautery ?	vol (ul) injected	method injection	Included in GTT (Fig. 4c)?	T0 c-peptide (pmol/L) (Fig. 4d)	T45 c-peptide (pmol/L) (Fig. 4d)
Control 1	738	Charles River	~7 wks	mesenchyme only	Y	30	syringe			
2	721	Charles River	~7 wks	mesenchyme only	Y	30	syringe			
3	746	Charles River	~7 wks	mesenchyme only	Y	50	syringe			
4	762	Charles River	~7 wks	mesenchyme only	Y	50	syringe			
5	740	Charles River	~7 wks	mesenchyme only	Y	50	syringe			
6	768	Charles River	~7 wks	mesenchyme only	Y	50	syringe			
7	715	Charles River	~7 wks	mesenchyme only	Y	30	syringe			
8	716	Charles River	~7 wks	mesenchyme only	N	30	syringe	Yes	5.8	9.8
9	717	Charles River	~7 wks	mesenchyme only	Y	30	syringe			
10	718	Charles River	~7 wks	mesenchyme only	Y	30	syringe	Yes	0.7	6.8
11	719	Charles River	~7 wks	mesenchyme only	Y	30	syringe	Yes	1.6	0.4
12	724	Charles River	~7 wks	mesenchyme only	Y	30	syringe			
13	725	Charles River	~7 wks	mesenchyme only	Y	30	syringe			
14	726	Charles River	~7 wks	mesenchyme only	Y	30	syringe			
15	728	Charles River	~7 wks	mesenchyme only	Y	30	syringe			
16	736	Charles River	~7 wks	mesenchyme only	Y	30	syringe			
17	737	Charles River	~7 wks	mesenchyme only	Y	30	syringe	Yes	0.6	1.6
18	739	Charles River	~7 wks	mesenchyme only	Y	30	syringe			
19	982	Harlan	~7 wks	saline	Y	50	syringe	Yes	3.8	0.9
20	983	Harlan	~7 wks	saline	Y	50	syringe	Yes	2.5	2.0
21	991	Harlan	~7 wks	saline	Y	50	syringe	Yes	4.4	8.0
22	700	Charles River	~7 wks	saline	Y	30	pipetteman			
23	701	Charles River	~7 wks	saline	Y	30	pipetteman			
24	702	Charles River	~7 wks	saline	N	30	pipetteman			
25	703	Charles River	~7 wks	saline	N	30	pipetteman			
Endo P0 1	704	Charles River	~7 wks	hES-endoderm P0	Y	30	pipetteman			
2	705	Charles River	~7 wks	hES-endoderm P0	Y	30	pipetteman			
3	706	Charles River	~7 wks	hES-endoderm P0	Y	30	pipetteman			

4	707	Charles River	~7 wks	hES-endoderm P0	Y	30	pipetteman			
5	709	Charles River	~7 wks	hES-endoderm P0	Y	30	pipetteman			
6	710	Charles River	~7 wks	hES-endoderm P0	Y	30	pipetteman			
7	711	Charles River	~7 wks	hES-endoderm P0	Y	30	pipetteman			
8	712	Charles River	~7 wks	hES-endoderm P0	Y	30	pipetteman			
9	713	Charles River	~7 wks	hES-endoderm P0	Y	30	pipetteman			
10	784	Harlan	~7 wks	hES-endoderm P0	N	50	syringe			
11	786	Harlan	~7 wks	hES-endoderm P0	N	50	syringe			
12	787	Harlan	~7 wks	hES-endoderm P0	N	50	syringe			
13	789	Harlan	~7 wks	hES-endoderm P0	N	50	syringe			
14	790	Harlan	~7 wks	hES-endoderm P0	N	50	syringe			
15	797	Harlan	~7 wks	hES-endoderm P0	N	50	syringe			
16	798	Harlan	~7 wks	hES-endoderm P0	N	50	syringe			
Endo P3 1	744	Charles River	~7 wks	hES-endoderm P3	N	60	syringe			
2	753	Charles River	~7 wks	hES-endoderm P3	N	60	syringe			
3	785	Harlan	~7 wks	hES-endoderm P3	N	60	syringe			
4	791	Harlan	~7 wks	hES-endoderm P3	N	60	syringe			
5	792	Harlan	~7 wks	hES-endoderm P3	N	60	syringe			
6	793	Harlan	~7 wks	hES-endoderm P3	N	60	syringe			
7	794	Harlan	~7 wks	hES-endoderm P3	N	60	syringe			
8	795	Harlan	~7 wks	hES-endoderm P3	N	60	syringe			
9	796	Harlan	~7 wks	hES-endoderm P3	N	60	syringe			
Pdx1 P5 1	807	Harlan	~7 wks	hES-endoderm P5	N	50	syringe			
2	892	Harlan	~7 wks	hES-endoderm P5	N	50	syringe			
3	894	Harlan	~7 wks	hES-endoderm P5	N	50	syringe			
4	811	Harlan	~7 wks	hES-endoderm P5	N	50	syringe			
5	812	Harlan	~7 wks	hES-endoderm P5	N	50	syringe			
6	813	Harlan	~7 wks	hES-endoderm P5	N	50	syringe			
7	814	Harlan	~ 7 wks	hES-endoderm P5	N	50	syringe			
Endo P6 1	903	Harlan	~7 wks	hES-endoderm P6	N	50	syringe			
2	904	Harlan	~7 wks	hES-endoderm P6	N	50	syringe			
3	905	Harlan	~7 wks	hES-endoderm P6	N	50	syringe			
4	906	Harlan	~7 wks	hES-endoderm P6	N	50	syringe			
5	907	Harlan	~7 wks	hES-endoderm P6	N	50	syringe			
6	908	Harlan	~7 wks	hES-endoderm P6	N	50	syringe			
7	909	Harlan	~7 wks	hES-endoderm P6	N	50	syringe			
8	808	Harlan	~ wks	hES-endoderm P6	N	50	syringe			
9	911	Harlan	~7 wks	hES-endoderm P6	N	50	syringe			
10	912	Harlan	~7 wks	hES-endoderm P6	N	50	syringe			
11	913	Harlan	~7 wks	hES-endoderm P6	N	50	syringe			
12	914	Harlan	~7 wks	hES-endoderm P6	N	50	syringe			
13	917	Charles River	~7 wks	hES-endoderm P6	N	50	syringe			
14	919	Charles River	~7 wks	hES-endoderm P6	N	50	syringe			
15	920	Charles River	~7 wks	hES-endoderm P6	N	50	syringe			
16	921	Charles River	~7 wks	hES-endoderm P6	N	50	syringe			
17	922	Charles River	~7 wks	hES-endoderm P6	N	50	syringe			
18	923	Charles River	~7 wks	hES-endoderm P6	N	50	syringe			
19	928	Charles River	~7 wks	hES-endoderm P6	N	50	syringe			
20	929	Charles River	~7 wks	hES-endoderm P6	N	50	syringe			
21	930	Charles River	~7 wks	hES-endoderm P6	N	50	syringe			
Endo P7 1	932	Harlan	~7 wks	hES-endoderm P7	N	50	syringe			
2	933	Harlan	~7 wks	hES-endoderm P7	N	50	syringe			
3	934	Harlan	~7 wks	hES-endoderm P7	N	50	syringe			
4	935	Harlan	~7 wks	hES-endoderm P7	N	50	syringe			
5	936	Harlan	~7 wks	hES-endoderm P7	N	50	syringe			
6	937	Harlan	~7 wks	hES-endoderm P7	N	50	syringe			
7	938	Harlan	~7 wks	hES-endoderm P7	N	50	syringe			
8	939	Harlan	~7 wks	hES-endoderm P7	N	50	syringe			
9	941	Harlan	~7 wks	hES-endoderm P7	N	50	syringe			

Ngn3 P0 1	931	Harlan	~7 wks	hES-Ngn3 P0	N	50	syringe			
2	unmarked	Harlan	~7 wks	hES-Ngn3 P0	N	50	syringe			
3	unmarked 2	Harlan	~7 wks	hES-Ngn3 P0	N	50	syringe			
4	unmarked 4	Harlan	~7 wks	hES-Ngn3 P0	N	50	syringe			
5	unmarked 6	Harlan	~7 wks	hES-Ngn3 P0	N	50	syringe			
6	unmarked 8	Harlan	~7 wks	hES-Ngn3 P0	N	50	syringe			
Ngn3 P5 1	962	Harlan	~7 wks	hES-Ngn3 P5	N	50	syringe			
2	963	Harlan	~7 wks	hES-Ngn3 P5	N	50	syringe			
3	964	Harlan	~7 wks	hES-Ngn3 P5	N	50	syringe			
4	967	Harlan	~7 wks	hES-Ngn3 P5	N	50	syringe			
5	969	Harlan	~7 wks	hES-Ngn3 P5	N	50	syringe			
6	970	Harlan	~7 wks	hES-Ngn3 P5	N	50	syringe			
7	975	Harlan	~7 wks	hES-Ngn3 P5	N	50	syringe			
8	unmarked 7	Harlan	~7 wks	hES-Ngn3 P5	N	50	syringe			
Pdx1 P0 1	984	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
2	985	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
3	986	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe	Yes		
4	987	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
5	988	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe	Yes		
6	989	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe	Yes	48.0	169.0
7	990	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
8	992	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe	Yes		
9	993	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe	Yes	51.5	249.7
10	994	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
11	995	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
12	996	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe	Yes	64.4	149.6
13	949	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
14	950	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
15	952	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
16	953	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
17	943	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
18	954	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
19	955	Harlan	~7 wks	hES-Pdx1 P0	N	50	syringe			
Pdx1 P4 1	942	Harlan	~7 wks	hES-Pdx1 P4	N	50	syringe	Yes	79.3	285.4
2	944	Harlan	~7 wks	hES-Pdx1 P4	N	50	syringe	Yes		
3	945	Harlan	~7 wks	hES-Pdx1 P4	N	50	syringe	Yes	63.3	240.0
4	946	Harlan	~7 wks	hES-Pdx1 P4	N	50	syringe			
5	947	Harlan	~7 wks	hES-Pdx1 P4	N	50	syringe	Yes		
6	948	Harlan	~7 wks	hES-Pdx1 P4	N	50	syringe	Yes		
Pdx1 P6 1	977	Harlan	~7 wks	hES-Pdx1 P6	N	50	syringe			
2	979	Harlan	~7 wks	hES-Pdx1 P6	N	50	syringe			
3	980	Harlan	~7 wks	hES-Pdx1 P6	N	50	syringe			
4	981	Harlan	~7 wks	hES-Pdx1 P6	N	50	syringe			
Pdx1 P7 1	957	Harlan	~7 wks	hES-Pdx1 P7	N	50	syringe	Yes	116.2	228.5
2	961	Harlan	~7 wks	hES-Pdx1 P7	N	50	syringe	Yes		
Islets1	741	Charles River	~7 wks	human islets from donor 1	Y	50	syringe			
2	743	Charles River	~7 wks	human islets from donor 1	Y	50	syringe			
3	745	Charles River	~7 wks	human islets from donor 1	Y	50	syringe			
4	747	Charles River	~7 wks	human islets from donor 1	Y	50	syringe			
5	751	Charles River	~7 wks	human islets from donor 1	Y	50	syringe			
6	754	Charles River	~7 wks	human islets from donor 1	Y	50	syringe	Yes		
7	756	Charles River	~7 wks	human islets from donor 1	Y	50	syringe			
8	757	Charles River	~7 wks	human islets from donor 1	Y	50	syringe			
9	764	Charles River	~7 wks	human islets from donor 1	Y	50	syringe			
10	767	Charles River	~7 wks	human islets from donor 1	Y	50	syringe			
11	800	Harlan	~7 wks	human islets from donor 2	N	60	syringe			
12	802	Harlan	~7 wks	human islets from donor 2	N	60	syringe	Yes	28.0	264.9
13	804	Harlan	~7 wks	human islets from donor 2	N	60	syringe			
14	805	Harlan	~7 wks	human islets from donor 2	N	60	syringe			
15	806	Harlan	~7 wks	human islets from donor 2	N	60	syringe			
16	815	Harlan	~7 wks	human islets from donor 3	N	50	syringe	Yes	93.4	226.8
17	820	Harlan	~7 wks	human islets from donor 3	N	50	syringe	Yes	261.2	401.6
18	972	Harlan	~ 7 wks	human islets from donor 3	N	50	syringe	Yes	198.2	544.3
19	unmarked #5	Harlan	~ 7 wks	human islets from donor 3	N	50	syringe	Yes	107.1	466.9

REFERENCES

1. Borowiak, M. et al. Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells. Cell Stem Cell 4, 348-58 (2009).