

**Stem Cell Reports, Volume 18**

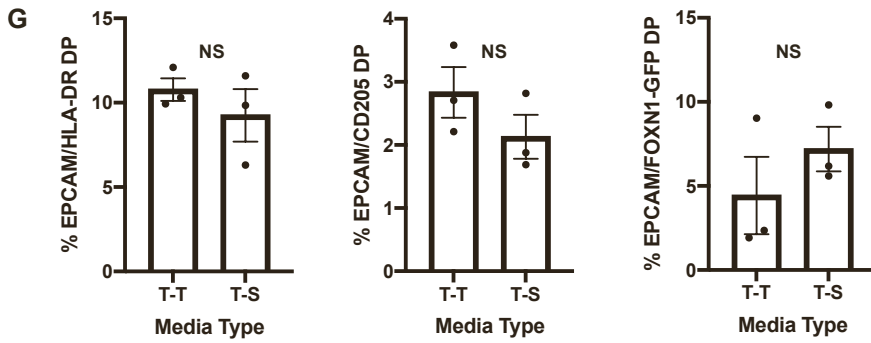
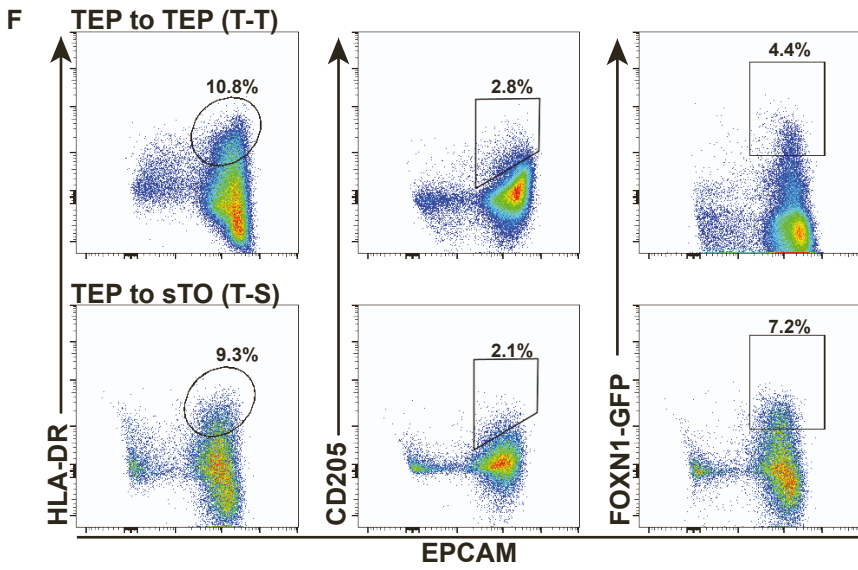
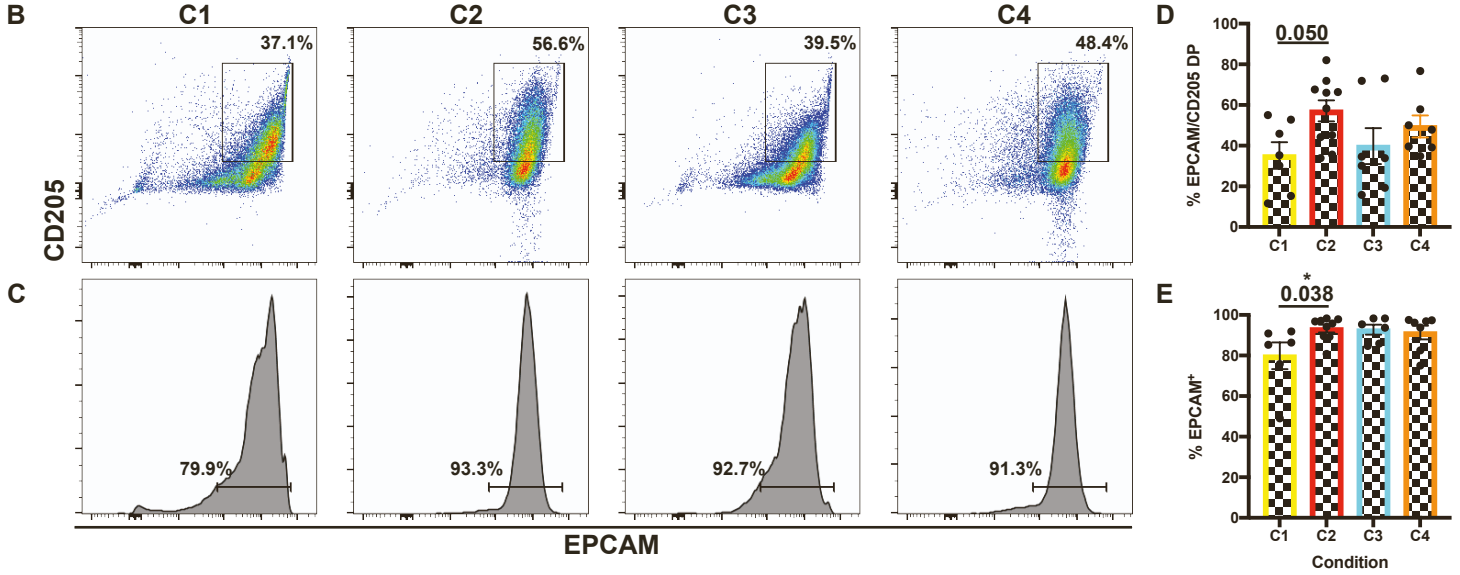
**Supplemental Information**

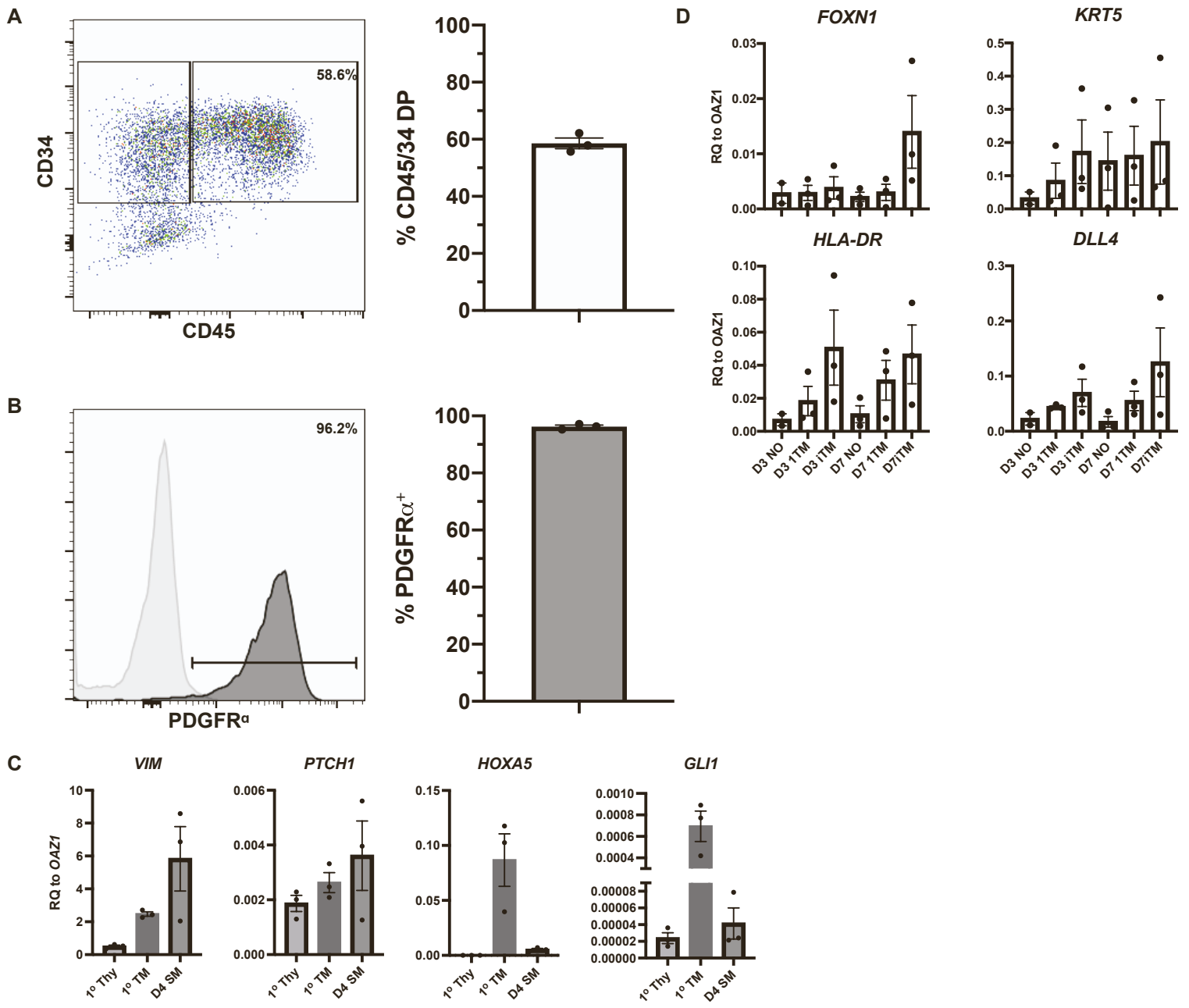
**Generation of functional thymic organoids from human pluripotent stem cells**

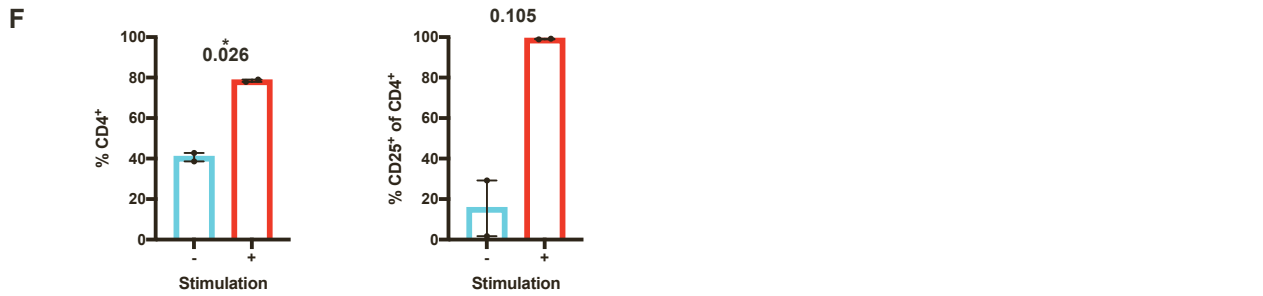
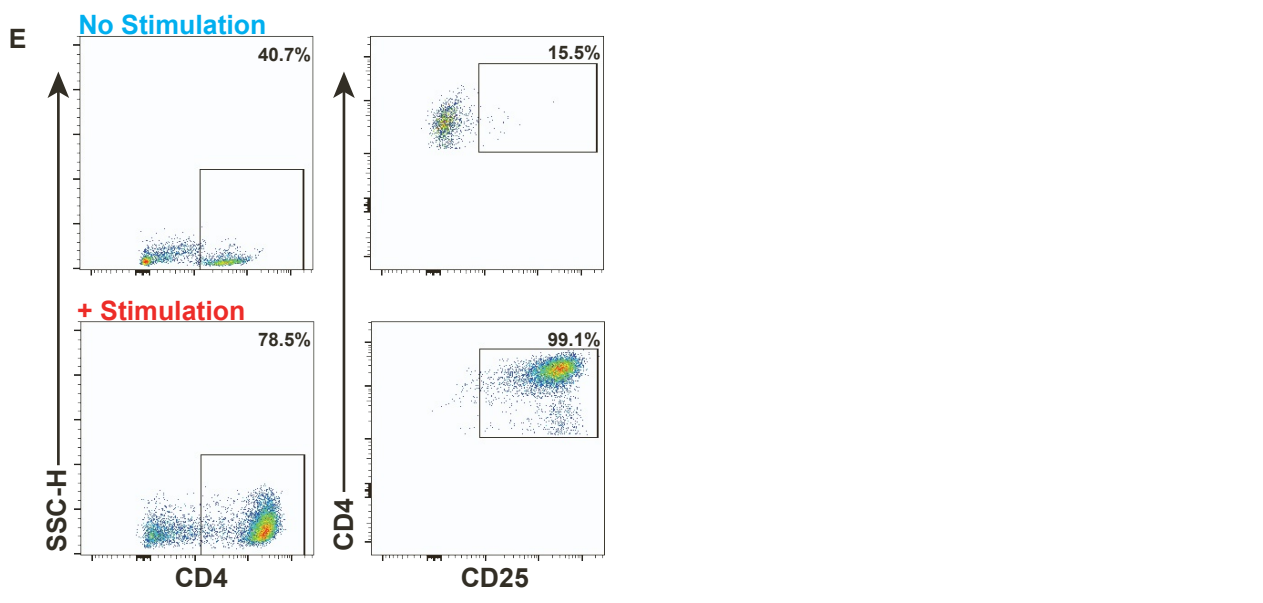
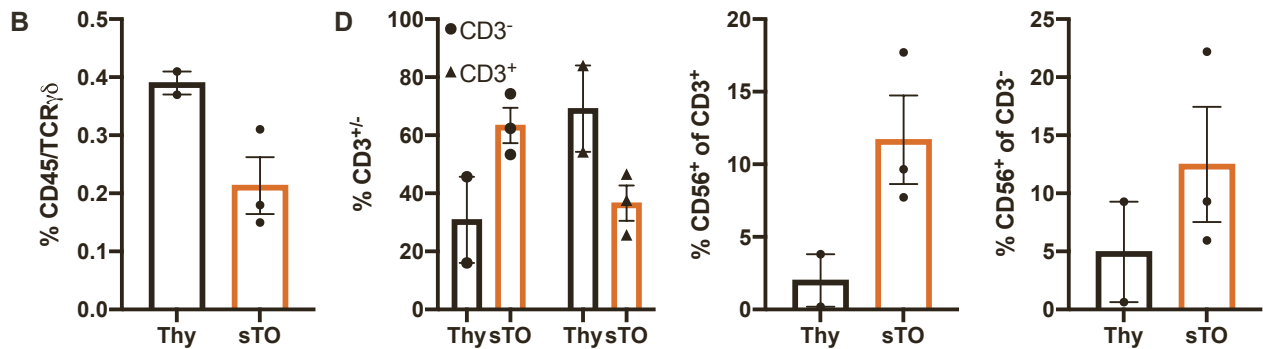
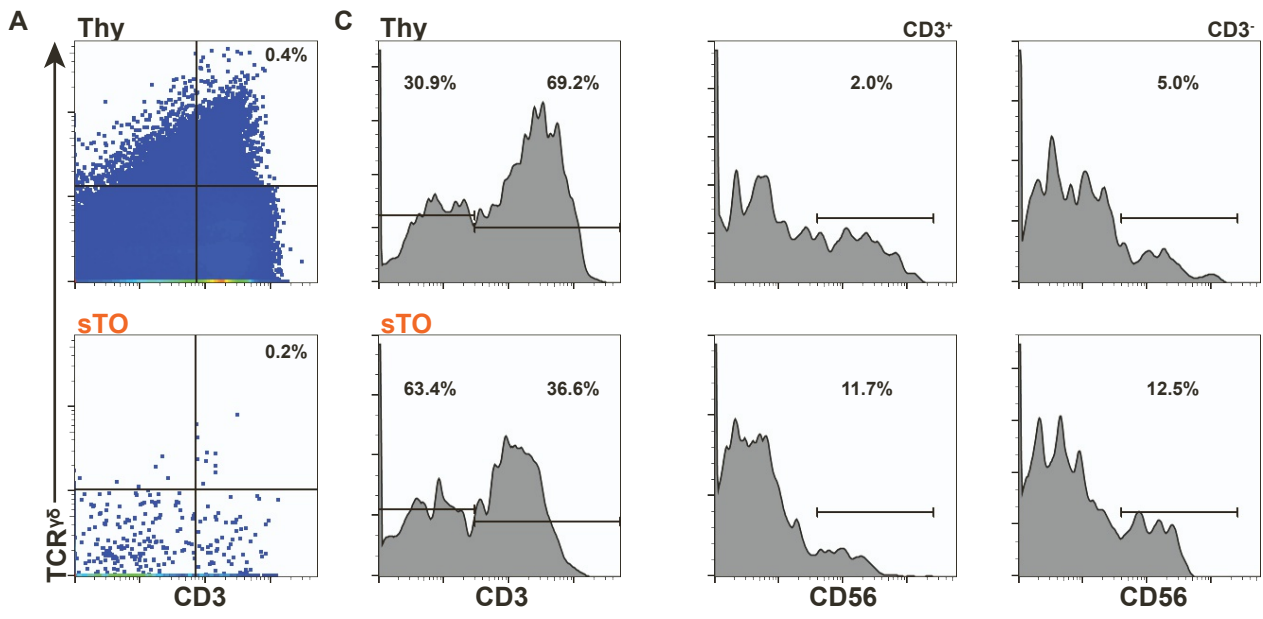
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**Supplementary Information:  
Supplementary Figures**

Conditions	Stage 1	Stage 2	Stage 3
1	Wnt3a d0; ActA d0-4; RA d4	BMP4, RA, LY, SAG d5-8	BMP4, RA, LY, Wnt3a, FGF8, SANT1
2	Wnt3a d0; ActA d0-4; RA d4	BMP4, RA, LY, SAG d5-8	LDN, RA, Wnt3a, FGF8, SANT1, ActA
3	Wnt3a d0; ActA d0-4; RA d4	BMP4, RA, LY, SAG d5-8	BMP4, RA, Wnt3a, FGF8, SANT1, ActA
4	Wnt3a d0; ActA d0-4; RA d4	BMP4, RA, LY, SAG d5-8	LDN, RA, LY, Wnt3a, FGF8, SANT1







## Supplementary Figure Legends

### **Fig. S1. Modulation of culture conditions for TEP and sTO differentiation; related to**

**figure 1 and 2.** (A) Table of TEP differentiation conditions tested. (B-C) Representative FC plots of EPCAM/CD205 (B) and EPCAM (C) expression and quantification on ~day 20 TEPs (n=7-10, 4 hPSC lines). (D-E) Quantification of EPCAM/CD205 DP (D) and EPCAM (E) expression corresponding to (B) and (C), respectively. P-values determined by t-test comparing previously published condition (1) to optimized condition (2). FC plots (F) and quantification (G) of EPCAM, HLA-DR, CD205, and GFP expression in TEPs cultured in final stage TEP media or sTO media for 2 weeks past ~d20 (n=3) without air liquid interface culture. This analysis shows no significant effect on TEC marker expression by different medias. Plots depict percent mean; data shown as mean±SEM.

### **Fig. S2. Directed differentiation of hPSCs to multiple cellular lineages, related to figure 3.**

(A) FC analysis of CD34/CD45 expression on hPSC-derived HPCs (n=3, 3 hPSC lines). (B) FC analysis of PDGFR $\alpha$  on hPSC-derived splanchnic mesenchyme (n=3, 3 hPSC lines). Light grey histogram represents unstained control. (C) qPCR analysis of splanchnic mesoderm (SM) markers in primary whole thymus and expanded mesenchyme and day 4 SM (n=3; 3 hPSC lines) (D) qPCR analysis of TEC markers in day 3 and 7 TEP-only sTOs with either no mesenchyme, 1 $^{\circ}$  mesenchyme, or hPSC-derived mesenchyme. Plots depict percent mean; bar graphs show mean±SEM.

### **Fig. S3. sTOs support development of multiple immune cell types, related to figure 4.**

(A-B) CyTOF plots (A) and quantification (B) of CD3/TCR $\gamma\delta$  expressing  $\gamma\delta$  T cells in primary thymi and sTOs (n=2 thymi, 3 sTOs). (C, D) CyTOF plots (C) and quantification (D) of CD3 $^{+/-}$  cells and corresponding CD3/CD56 DP iNKT and CD3 $^{-}$ /CD56 $^{+}$  NK cells in primary thymi and sTOs (n=2

thymi, 3 sTOs). Plots depict percent mean; data shown as mean $\pm$ SEM. **(E)** FC plots and quantification of CD4 and CD25 expression of PBMCs activated with anti-CD3/CD28 Dynabeads (n=2).

**Supplementary Tables:**

<b>Factor</b>	<b>Concentration</b>	<b>Day</b>	<b>Supplier</b>
A=ActA	100ng/ml	0-4	R&D Systems
Wnt3a	50 ng/ml	0	R&D Systems and Bio-Techne
ITS	1:5000	0	Thermo Electron
ITS	1:2000	1-4	Thermo Electron
R=TTNPB	6 $\mu$ M	4	R&D Systems
B=BMP4	20ng/ml	5-8	R&D Systems
LY=LY364947	5 $\mu$ M	5-8	R&D Systems
SAG	100ng/mL	5-8	R&D Systems
A=ActA	20ng/ml	9+	R&D Systems
F=FGF8b	50ng/ml	9+	PeptoTech
LDN=LDN193189	500nM	9+	Stemcell Technologies
R=TTNPB	6 $\mu$ M	9+	R&D Systems
S1=SANT1	0.25 $\mu$ M	9+	Tocris
Wnt3a	50 ng/ml	9+	R&D Systems and Bio-Techne
ITS= insulin-transferrin-selenium	1:2000	9+	Thermo Electron
Ascorbic Acid	50 $\mu$ g/ml	9+	Sigma-Aldrich
EGF	20ng/ml	9+	R&D Systems
Heparin	10 $\mu$ g/ml	9+	Sigma-Aldrich
Hydrocortisone	0.5 $\mu$ g/ml	9+	Sigma-Aldrich
ITS	1:2000	9+	Thermo Electron
Non-Essential Amino Acids	1:100	9+	Thermo Fisher Scientific
Trolox	0.1mM	9+	Millipore Sigma

**Table S1: Factors, concentrations, and days for hPSCs to TEP differentiation.**

<b>Factor</b>	<b>Concentration</b>	<b>Day</b>	<b>Supplier</b>
A=ActA	30ng/ml	0	R&D Systems
B=BMP4	40ng/ml	0	R&D Systems
C=Chir	6 $\mu$ M	0	Bio-Techne
F=FGF2	20ng/ml	0-4	R&D Systems
P=PIK90	100nM	0	Cayman Chemical Company
A83-A8301	1 $\mu$ M	1-4	MedChem Express
B=BMP4	30ng/ml	1-4	R&D Systems
C59	1 $\mu$ M	1-4	Cellagen Technologies
R=TTNPB	6 $\mu$ M	2-4	R&D Systems

**Table S2: Factors, concentrations, and days for hPSC to splanchnic mesoderm differentiation.**



<b>Factor</b>	<b>Concentration</b>	<b>Stage</b>	<b>Day</b>	<b>Supplier</b>
IMDM/F12				Thermo Fisher Scientific
Hybridoma Mix	4%	Base Media		Thermo Fisher Scientific
Human Serum	0.1%	Base Media		Gemini
Polyvinyl alcohol	0.1%	Base Media		Sigma-Aldrich
Methyl cellulose	0.1%	Base Media		Sigma-Aldrich
GlutaMAX	1x	Base Media		Thermo Fisher Scientific
Ascorbic acid-2-phosphate	50ug/ml	Base Media		Sigma-Aldrich
ITSE AF	1:1000	Base Media		Invitria
Lipid Mixture 1	1x	Base Media		Sigma-Aldrich
P/S	1x	Base Media		Thermo Fisher Scientific
BME	22uM	Base Media		Thermo Fisher Scientific
Rock Inhibitor	10uM	I	0	R&D Systems
CHIR	0.5uM	I	0	Bio-Techne
Activin A	10ng/ml	I	0	R&D Systems
BMP4	20ng/ml	I	0	R&D Systems
SCF	20ng/ml	I	0	PeproTech
VEGF	20ng/ml	I	0	PeproTech
FGF2	10ng/ml	I	0	R&D Systems
CHIR	0.5uM	II	1	Bio-Techne
Activin A	10ng/ml	II	1	R&D Systems
BMP4	20ng/ml	II	1	R&D Systems
SCF	20ng/ml	II	1	PeproTech
VEGF	20ng/ml	II	1	PeproTech
FGF2	10ng/ml	II	1	R&D Systems
CHIR	3uM	III	2-3	Bio-Techne
SB	3uM	III	2-3	STEMCELL Technologies
Activin A	10ng/ml	III	2-3	R&D Systems
BMP4	20ng/ml	III	2-3	R&D Systems
SCF	20ng/ml	III	2-3	PeproTech
VEGF	20ng/ml	III	2-3	PeproTech
FGF2	10ng/ml	III	2-3	R&D Systems
BMP4	20ng/ml	IV	4-7	R&D Systems
SCF	50ng/ml	IV	4-7	PeproTech
VEGF	50ng/ml	IV	4-7	PeproTech
IGF-II	20ng/ml	IV	4-7	PeproTech
FGF2	10ng/ml	IV	4-7	PeproTech
SCF	100ng/ml	V	8+	PeproTech

VEGF	50ng/ml	V	8+	PeproTech
FGF2	10ng/ml	V	8+	PeproTech
IL7	20ng/ml	V	8+	PeproTech
FLT3L	10ng/ml	V	8+	PeproTech

**Table S3: Factors, concentrations, differentiation stages, and days for HPC differentiation.**

Antigen	Conjugate	Dilution	Supplier	Cat #
CD3	PE	1:100	Biologend	317308
CD3	APC-Cy7	1:100	Biologend	344818
CD4	AF647	1:100	Biologend	300520
CD5	APC-Cy7	1:180	Biologend	364010
CD7	FITC	1:60	Biologend	343104
CD7	PE-Cy7	1:60	Biologend	343114
CD8	AF488	1:100	Biologend	300916
CD8	PE-Cy5	1:100	Biologend	300910
CD25	APC-Cy7	1:20	Biologend	302614
CD34	BV421	1:40	Biologend	343610
CD45	BV510	1:180	Biologend	304036
CD45	PerCP	1:180	Biologend	304017
CD104	FITC	1:50	Biologend	327806
CD104	PE	1:50	Biologend	327808
C127	AF700	1:50	Biologend	351344
CD205	PerCP-Cy5.5	1:50	Biologend	342210
EPCAM	AF700	1:50	eBioscience	56-9326-42
PD1	AF405	1:50	R&D Systems	1615114
HLA-DR	BV421	1:50	Biologend	307636
AIRE		1:100	eBioscience	13-9534-82
CD3		1:100	Abcam	ab5690
CD205		1:100	Thermo Fisher	MA5-34695
EPCAM		1:200	Biologend	324202
HLA Class II		1:50	Biologend	361708
KRT5		1:100	Abcam	ab52635
KRT5		1:100	Sigma Aldrich	305R-14
KRT8		1:100	Santa Cruz Biotechnology	300910
PD-1		1:50	Abcam	ab237728
PDGFR $\alpha$		1:500	Abcam	ab203491
TE-7		1:100	Novus Biologics	NBP2-50082
CD4	166Er		Miltenyi	130-122-283
CD45	89Y		Fluidigm	3089003B

CD56	161Dy		Miltenyi	130-108-016
CD117	175Lu		Biolegend	313202
CD326	141Pr		Fluidigm	3141006B
FOXP3	162Dy		Fluidigm	3162011A
HLA-DR	174Yb		Miltenyi	130-122-299
TCF1	163Dy		Cell Signaling	2203S
TCR $\gamma/\delta$	169Tm		Miltenyi	130-122-291
TdT	164Dy		Fluidigm	3164015B
KRT8	152Sm		R&D systems	MAB3165

**Table S4: Antibodies used for flow cytometry, immunofluorescence, and CyTOF analysis.**

<b>qPCR Probes/Primers</b>	
<b>Target</b>	<b>Assay ID/Sequence</b>
AIRE	Bio-Rad: qHsaCIP0029272
DLL4	Bio-Rad: qHsaCEP0051500
FOXP1	ThermoFisher: Hs00919266_m1
HLA-DRA	Bio-Rad: qHsaCEP0040019
KRT5	Bio-Rad: qHsaCEP0055058
KRT8	Bio-Rad: qHsaCEP0041467
OAZ1	ThermoFisher: Hs00427923_m1
ACTB F	CATGTACGTTGCTATCCAGGC
ACTB R	CTCCTTAATGTCACGCACGAT
Pro-Insulin F	GCAGCCTTTGTGAACCAACAC
Pro-Insulin R	CCCCGCACACTAGGTAGAGA
Islet Antigen 2 F	CGGGACACATGATTCTGGCAT
Islet Antigen 2 R	CTGCTTGGTAGGCACAGAGG
GAD1 F	GCGGACCCCAATACCACTAAC
GAD1 R	CACAAGGCGACTCTTCTCTTC
MBP F	GGCCGGACCCAAGATGAAA
MBP R	CCCAGCTAAATCTGCTCAGG
TG F	AGACACCTCCTACCTCCCTCA
TG R	TCCTTGGACATCGCTTTGGC

**Table S5: Taqman probes and qPCR primer sequences for qPCR analysis.**