Supplemental Table 1. Complete gene lists and GO terms from Figure 3C.

Path 1 Genes: RP11-34P13.15, RP4-758J18.10, VWA1, CHD5, AZIN2, FOXO6, RP11-403I13.8, ARHGAP30, RGS4, LRRN2, RASSF5, SERTAD4, GJC2, RHOU, REEP1, FOXI3, SH3RF3, COL4A4, ZDHHC23, FGFR3, PPP2R2C, CTD-2031P19.4, RNF182, GRM4, PRR15, DGKI, CHMP4C, CALB1, SPAG1, KLF4, ENG, RET, GDF10, ADAMTS14, SPOCK2, MBL1P, ADAM8, LRP4-AS1, CARNS1, DGAT2, CRYAB, AP000783.1, OPCML, PLEKHG6, GDF3, EMP1, RASSF9, FAM101A, STON2, GREM1, ACTC1, CORO2B, FURIN, WFIKKN1, BAIAP3, TMC5, HS3ST4, ZFHX3, NLRP1, RASD1, CACNG4, EMILIN2, L3MBTL4, KLHL14, HMSD, RP11-849I19.1, SALL3, GADD45B, KANK3, CTC-526N19.1, ZNF888, MMP9, BMP7, PIK3IP1, MCHR1, SYTL5, CAMK2N1, PINK1, ID3, PTPRU, MANEAL, MCOLN3, LRRC8C, NTNG1, KCNC4, RP11, 430C7.5, C1orf95, ID2-AS1, ID2, GDF7, KCNG3, RGPD8, PSD4, CCDC74B, BMPR2, KAT2B, LINC00693, ZNF654, FILIP1L, SH3TC1, CPEB2, NPFFR2, TRPC3, RP11-752L20.3, FAM198B, TLL1, CDH9, PDZD2, CHSY3, GALNT10, FOXQ1, ATXN1, ID4, COL11A2, CNR1, GTF2IP4, FZD1, PAX5, RP11-35N6.1, UNC5B, NKX1-2, FAM196A, EBF3, PRRG4, LRP4, SYT7, PLBD1, GRASP, ALX1, HIP1R, LPAR6, SLITRK6, C16orf89, RP11-491F9.1, MMP2, B3GNT9, NXPH3, TNRC6C-AS1, LDLRAD4, NOL4, SMAD7, HCN2, PDE4A, KANK2, SAMD1, EXOC3L2, IL11, EMILIN3, KCNB1, DOK5, EEF1A2, A4GALT, ADGRG2, ELF4, ABCD1

Term	Count	%	PValue	Genes
regulation of pathway-restricted SMAD protein phosphorylation	9	6.34	1.31E-08	GDF3, SMAD7, GDF7, BMPR2, GDF10, GREM1, BMP7, LDLRAD4, ENG
pathway-restricted SMAD protein phosphorylation	9	6.34	1.50E-08	GDF3, SMAD7, GDF7, BMPR2, GDF10, GREM1, BMP7, LDLRAD4, ENG
BMP signaling pathway	10	7.04	6.88E-07	GDF3, SMAD7, GDF7, BMPR2, FZD1, GDF10, GREM1, BMP7, ENG, LRP4
response to BMP	10	7.04	1.24E-06	GDF3, SMAD7, GDF7, BMPR2, FZD1, GDF10, GREM1, BMP7, ENG, LRP4
cellular response to BMP stimulus	10	7.04	1.24E-06	GDF3, SMAD7, GDF7, BMPR2, FZD1, GDF10, GREM1, BMP7, ENG, LRP4
regulation of cell communication	43	30.28	9.74E-06	GDF3, KCNC4, FGFR3, HIP1R, GDF7, MMP9, BMPR2, PINK1, SYT7, GREM1, RHOU, CALB1, KANK2, IL11, UNC5B, PDE4A, PLEKHG6, CNR1, NPFFR2, ADAM8, CHD5, RET, SMAD7, KCNB1, FZD1, PSD4, CACNG4, DGKI, PIK3IP1, LDLRAD4, FURIN, ARHGAP30, SALL3, GRM4, DOK5, LPAR6, RGS4, GDF10, GADD45B, BMP7, ENG, KLF4, LRP4
ossification	13	9.15	1.19E-05	FGFR3, MMP9, BMPR2, FZD1, GREM1, MMP2, ID2, GDF10, ID4, ID3, BMP7, COL11A2, LRP4
regulation of signaling	43	30.28	1.48E-05	GDF3, KCNC4, FGFR3, HIP1R, GDF7, MMP9, BMPR2, PINK1, SYT7, GREM1, RHOU, CALB1, KANK2, IL11, UNC5B, PDE4A, PLEKHG6, CNR1, NPFFR2, ADAM8, CHD5, RET, SMAD7, KCNB1, FZD1, PSD4, CACNG4, DGKI, PIK3IP1, LDLRAD4, FURIN, ARHGAP30, SALL3, GRM4, DOK5, LPAR6, RGS4, GDF10, GADD45B, BMP7, ENG, KLF4, LRP4
embryonic morphogenesis	16	11.27	1.68E-05	GDF3, RET, GDF7, MMP9, BMPR2, FZD1, PAX5, GREM1, MMP2, ID2, BMP7, SLITRK6, ENG, LRP4, KLF4, ALX1
regulation of phosphorylation	26	18.31	1.90E-05	GDF3, FGFR3, GDF7, MMP9, BMPR2, PINK1, GREM1, IL11, NPFFR2, ADAM8, RET, KAT2B, SMAD7, EEF1A2, FZD1, PIK3IP1, LDLRAD4, CAMK2N1, GRM4, RGS4, GDF10, GADD45B, BMP7, ENG, LRP4, KLF4
transmembrane receptor protein serine/threonine kinase signaling pathway	12	8.45	2.30E-05	GDF3, SMAD7, GDF7, BMPR2, FZD1, GDF10, GREM1, BMP7, LDLRAD4, FURIN, ENG, LRP4
regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	10	7.04	2.45E-05	GDF3, SMAD7, GDF7, BMPR2, FZD1, GDF10, GREM1, BMP7, LDLRAD4, ENG
positive regulation of pathway-restricted SMAD protein phosphorylation	6	4.23	2.49E-05	GDF3, GDF7, BMPR2, GDF10, BMP7, ENG
extracellular matrix organization	12	8.45	3.21E-05	COL4A4, ADAMTS14, SPOCK2, MMP9, COL11A2, GREM1, VWA1, ADAM8, FURIN, ENG, MMP2, TLL1
extracellular structure organization	12	8.45	3.30E-05	COL4A4, ADAMTS14, SPOCK2, MMP9, COL11A2, GREM1, VWA1, ADAM8, FURIN, ENG, MMP2, TLL1

Path 2 Genes: RP11-54O7.3, SAMD11, GLIS1, MIXL1, GREM2, SP5, EOMES, PTH1R, HAND1, MSX2, T, EVX1, BHLHE22, TBX3, NOTUM, GATA6, PCAT14

Term	Count	%	PValue	Genes
regulation of transcription from RNA polymerase II promoter	11	68.75	5.40E-08	MSX2, T, BHLHE22, HAND1, TBX3, EVX1, GATA6, GLIS1, EOMES, GREM2, MIXL1
embryonic morphogenesis	8	50	7.71E-08	MSX2, T, HAND1, TBX3, GATA6, EOMES, GREM2, MIXL1
embryo development	9	56.25	1.12E-07	MSX2, T, HAND1, TBX3, EVX1, GATA6, EOMES, GREM2, MIXL1
pattern specification process	7	43.75	4.57E-07	MSX2, T, HAND1, TBX3, EVX1, EOMES, GREM2

transcription from RNA polymerase II promoter	10	62.5	1.04E-06	MSX2, T, HAND1, TBX3, EVX1, GATA6, GLIS1, EOMES, GREM2, MIXL1
heart development	7	43.75	1.16E-06	MSX2, T, HAND1, TBX3, GATA6, EOMES, MIXL1
formation of primary germ layer	5	31.25	1.63E-06	T, HAND1, GATA6, EOMES, MIXL1
mesoderm development	5	31.25	2.19E-06	T, HAND1, TBX3, EOMES, MIXL1
regulation of transcription, DNA-templated	12	75	2.42E-06	MSX2, T, BHLHE22, HAND1, TBX3, EVX1, GATA6, GLIS1, EOMES, SP5, GREM2, MIXL1
transcription, DNA-templated	12	75	2.43E-06	MSX2, T, BHLHE22, HAND1, TBX3, EVX1, GATA6, GLIS1, EOMES, SP5, GREM2, MIXL1
regulation of nucleic acid-templated transcription	12	75	2.58E-06	MSX2, T, BHLHE22, HAND1, TBX3, EVX1, GATA6, GLIS1, EOMES, SP5, GREM2, MIXL1
regulation of RNA biosynthetic process	12	75	2.72E-06	MSX2, T, BHLHE22, HAND1, TBX3, EVX1, GATA6, GLIS1, EOMES, SP5, GREM2, MIXL1
regionalization	6	37.5	3.30E-06	MSX2, T, TBX3, EVX1, EOMES, GREM2
regulation of RNA metabolic process	12	75	3.81E-06	MSX2, T, BHLHE22, HAND1, TBX3, EVX1, GATA6, GLIS1, EOMES, SP5, GREM2, MIXL1
nucleic acid-templated transcription	12	75	3.90E-06	MSX2, T, BHLHE22, HAND1, TBX3, EVX1, GATA6, GLIS1, EOMES, SP5, GREM2, MIXL1

Path 3 Genes: CSF3R, PTGER3, IFI16, PRRX1, PLXNA2, DUSP10, AMER3, LINC01124, ADAMTS9, TRH, COL6A6, PLSCR4, JAKMIP1, ARHGAP24, LEF1, CTD-2035E11.4, ANXA2R, AC025171.1, ANKRD55, HAPLN1, GABRB2, FOXC1, SERPINB9, HLA-DQA1, HLA-DQB1, BMPER, TBX20, RELN, KEL, DLC1, DKK4, PSKH2, HAS2, CCDC3, ST8SIA6, COL13A1, FGF8, PPAPDC1A, WNT5B, ATP12A, PCDH17, GSC, SYNE3, CA12, MESP1, ANPEP, NKD1, KRT16P2, MFAP4, AC004448.5, KRT16P3, RP11-445F12.1, TBX4, FADS6, CYGB, SLC16A3, APOBEC3B-AS1, APOBEC3G, NFAM1, MAGEB3, SLITRK2

Term	Count	%	PValue	Genes
anatomical structure formation involved in morphogenesis	16	28.57	4.49E-07	DLC1, NKD1, FGF8, GSC, PLXNA2, TBX20, TBX4, LEF1, ANPEP, ARHGAP24, DKK4, BMPER, CSF3R, RELN, FOXC1, MESP1
regionalization	9	16.07	4.05E-06	NKD1, FGF8, GSC, PLXNA2, TBX20, LEF1, RELN, FOXC1, MESP1
blood vessel development	11	19.64	4.54E-06	FGF8, BMPER, TBX20, TBX4, PRRX1, LEF1, HAS2, FOXC1, ANPEP, ARHGAP24, MESP1
vasculature development	11	19.64	7.52E-06	FGF8, BMPER, TBX20, TBX4, PRRX1, LEF1, HAS2, FOXC1, ANPEP, ARHGAP24, MESP1
blood vessel morphogenesis	10	17.86	9.44E-06	FGF8, BMPER, TBX20, TBX4, PRRX1, LEF1, HAS2, FOXC1, ANPEP, ARHGAP24
localization of cell	15	26.79	1.46E-05	DLC1, NKD1, FGF8, WNT5B, PLXNA2, TBX20, LEF1, SLC16A3, BMPER, CSF3R, RELN, CYGB, FOXC1, HAS2, MESP1
cell motility	15	26.79	1.46E-05	DLC1, NKD1, FGF8, WNT5B, PLXNA2, TBX20, LEF1, SLC16A3, BMPER, CSF3R, RELN, CYGB, FOXC1, HAS2, MESP1
organ morphogenesis	13	23.21	1.77E-05	DLC1, FGF8, NKD1, GSC, COL13A1, TBX20, TBX4, PRRX1, LEF1, CSF3R, FOXC1, HAS2, MESP1
cell migration	14	25.00	2.05E-05	DLC1, FGF8, WNT5B, PLXNA2, TBX20, LEF1, SLC16A3, BMPER, CSF3R, RELN, FOXC1, HAS2, CYGB, MESP1
pattern specification process	9	16.07	3.12E-05	NKD1, FGF8, GSC, PLXNA2, TBX20, LEF1, RELN, FOXC1, MESP1
epithelium development	13	23.21	3.42E-05	DLC1, NKD1, FGF8, WNT5B, GSC, PLXNA2, TBX20, TBX4, LEF1, DKK4, BMPER, FOXC1, MESP1
tube development	10	17.86	3.53E-05	DLC1, FGF8, BMPER, GSC, PLXNA2, TBX20, TBX4, LEF1, FOXC1, MESP1
mesenchyme development	7	12.50	5.41E-05	FGF8, GSC, TBX20, LEF1, HAS2, FOXC1, MESP1
circulatory system development	12	21.43	6.04E-05	DLC1, FGF8, BMPER, TBX20, TBX4, PRRX1, LEF1, HAS2, FOXC1, ANPEP, ARHGAP24, MESP1
cardiovascular system development	12	21.43	6.04E-05	DLC1, FGF8, BMPER, TBX20, TBX4, PRRX1, LEF1, HAS2, FOXC1, ANPEP, ARHGAP24, MESP1

Path 4 Genes: FAM46B, ACTA1, NXPH2, LRP2, CCDC141, MAP2, UNC80, IGFBP5, STAC, EPHA3, FOXL2NB, TM4SF18, P2RY1, PTX3, SLITRK3, LIMCH1, CXCL5, LRAT, IRX2, GCNT4, FAM65B, C6orf141, HECW1, IGFBP3, VWC2, COBL, SFRP1, NPM1P21, PRDM14, MAMDC2, EIF2S2P3, ANO1, MAP6, ADAMTS8, A2M, PAPLN, GABRA5, GPR176, GRIN2A, SEZ6, CH17-431G21.1, PRKCA, DCC, SLC24A3, KIAA1644, APLN

Term	Count	%	PValue	Genes
neurogenesis	12	26.67	1.60E-04	DCC, COBL, SLITRK3, SFRP1, P2RY1, MAP2, GABRA5, GRIN2A, VWC2, MAP6, SEZ6, EPHA3
type B pancreatic cell proliferation	3	6.67	3.49E-04	SFRP1, IGFBP3, IGFBP5
nervous system development	14	31.11	3.76E-04	DCC, COBL, GABRA5, GRIN2A, EPHA3, CCDC141, SLITRK3, SFRP1, P2RY1, MAP2, VWC2, MAP6, LRP2, SEZ6
regulation of hormone levels	7	15.56	4.92E-04	PRKCA, GCNT4, LRAT, SFRP1, P2RY1, ANO1, APLN

cell development	13	28.89	5.88E-04	DCC, COBL, ACTA1, GABRA5, EPHA3, FAM65B, PRDM14, SLITRK3, SFRP1, MAP2, VWC2, MAP6, SEZ6
dendrite development	5	11.11	7.27E-04	DCC, COBL, MAP2, MAP6, SEZ6
neuron differentiation	10	22.22	9.44E-04	DCC, COBL, SLITRK3, SFRP1, MAP2, GABRA5, VWC2, MAP6, SEZ6, EPHA3
cell-cell signaling	11	24.44	1.07E-03	PRKCA, HECW1, GPR176, CXCL5, SFRP1, P2RY1, ANO1, GABRA5, GRIN2A, SEZ6, APLN
locomotion	11	24.44	1.14E-03	PRKCA, DCC, CCDC141, PRDM14, CXCL5, SFRP1, P2RY1, GRIN2A, IGFBP3, EPHA3, IGFBP5
localization of cell	10	22.22	1.68E-03	PRKCA, DCC, CCDC141, PRDM14, CXCL5, SFRP1, P2RY1, IGFBP3, EPHA3, IGFBP5
cell motility	10	22.22	1.68E-03	PRKCA, DCC, CCDC141, PRDM14, CXCL5, SFRP1, P2RY1, IGFBP3, EPHA3, IGFBP5
generation of neurons	10	22.22	1.93E-03	DCC, COBL, SLITRK3, SFRP1, MAP2, GABRA5, VWC2, MAP6, SEZ6, EPHA3
regulation of hormone secretion	5	11.11	2.12E-03	PRKCA, SFRP1, P2RY1, ANO1, APLN
cell migration	9	20.00	3.22E-03	PRKCA, DCC, CCDC141, CXCL5, SFRP1, P2RY1, IGFBP3, EPHA3, IGFBP5
hormone secretion	5	11.11	3.64E-03	PRKCA, SFRP1, P2RY1, ANO1, APLN

Path 5 Genes: TNFRSF8, PADI2, AIM1L, FABP3, LCK, LPAR3, TSPAN2, TXNIP, FAM110C, APOB, DPYSL5, ACTG2, TCF7L1, ACOXL, B3GALT1, ITGA6, SLC16A14, SLC6A11, DLEC1, HESX1, CP, VEPH1, PEX5L, MUC4, DNAH5, RASGRF2, POLR3G, KCNN2, DPYSL3, RNF144B, TTBK1, SLC29A1, FILIP1, AIM1, AKAP7, ICA1, PNMA2, VN1R51P, ZNF483, PAPPA, HMCN2, LIPA, SLC16A12, TLL2, JAKMIP3, ADM, BDNF, GYLTL1B, SYTL2, RND1, METTL7A, CHGA, LINGO1, ARRDC4, NECAB2, PIPOX, ARHGAP23, ERBB2, POLR3GP2, PLA2G4C, PTPRT, D21S2088E, MAP7D2, CHST7

Term	Count	%	PValue	Genes
nervous system development	15	23.81	6.65E-03	TSPAN2, SLC6A11, ERBB2, DPYSL5, PADI2, LPAR3, DPYSL3, DNAH5, HESX1, LINGO1, APOB, BDNF, RND1, ADM, TTBK1
cell projection organization	11	17.46	7.23E-03	LINGO1, BDNF, ITGA6, TSPAN2, ADM, FAM110C, ERBB2, DPYSL5, LPAR3, DPYSL3, DNAH5
positive regulation of apoptotic process	7	11.11	9.35E-03	TXNIP, PNMA2, ITGA6, ADM, RASGRF2, LCK, TNFRSF8
positive regulation of programmed cell death	7	11.11	9.74E-03	TXNIP, PNMA2, ITGA6, ADM, RASGRF2, LCK, TNFRSF8
neuron development	9	14.29	1.08E-02	LINGO1, BDNF, RND1, TSPAN2, ADM, ERBB2, DPYSL5, LPAR3, DPYSL3
glycerolipid catabolic process	3	4.76	1.10E-02	APOB, FABP3, PLA2G4C
positive regulation of cell death	7	11.11	1.23E-02	TXNIP, PNMA2, ITGA6, ADM, RASGRF2, LCK, TNFRSF8
axon development	6	9.52	1.34E-02	LINGO1, BDNF, TSPAN2, ERBB2, DPYSL5, LPAR3
lipid catabolic process	5	7.94	1.42E-02	APOB, LIPA, ACOXL, FABP3, PLA2G4C
neuron projection development	8	12.70	1.44E-02	LINGO1, BDNF, TSPAN2, ADM, ERBB2, DPYSL5, LPAR3, DPYSL3
cellular lipid catabolic process	4	6.35	2.02E-02	APOB, ACOXL, FABP3, PLA2G4C
locomotion	11	17.46	2.10E-02	APOB, CHGA, BDNF, ITGA6, FAM110C, ERBB2, LCK, DPYSL5, PADI2, DPYSL3, DNAH5
movement of cell or subcellular component	12	19.05	2.39E-02	APOB, CHGA, BDNF, ITGA6, FAM110C, ERBB2, LCK, KCNN2, DPYSL5, PADI2, DPYSL3, DNAH5
positive regulation of collateral sprouting	2	3.17	3.14E-02	BDNF, LPAR3
leukocyte migration	5	7.94	3.15E-02	APOB, CHGA, ITGA6, LCK, PADI2

Path 6 Genes: HES3, KLHDC7A, FOXD3, PAX8, GBX2, SORBS2, HTR1A, CTB-180C19.1, CTC-286N12.1, COL12A1, YWHAZP4, VGF, RP11-132A1.3, ARC, ZNF322P1, TRIM22, LINC00678, SIX6, ARHGAP36, SOX3

Term	Count	%	PValue	Genes
regionalization	4	23.53	1.89E-03	ARC, HES3, PAX8, GBX2
midbrain-hindbrain boundary morphogenesis	2	11.76	3.12E-03	HES3, GBX2
pattern specification process	4	23.53	4.19E-03	ARC, HES3, PAX8, GBX2
embryo development	5	29.41	5.05E-03	HES3, PAX8, GBX2, COL12A1, FOXD3
sensory organ development	4	23.53	6.39E-03	SOX3, PAX8, GBX2, SIX6
positive regulation of transcription from RNA polymerase II promoter	5	29.41	6.89E-03	HES3, PAX8, GBX2, SIX6, FOXD3
midbrain-hindbrain boundary development	2	11.76	7.01E-03	HES3, GBX2
embryonic morphogenesis	4	23.53	8.87E-03	HES3, PAX8, GBX2, COL12A1
rostrocaudal neural tube patterning	2	11.76	9.33E-03	HES3, GBX2
transcription from RNA polymerase II promoter	6	35.29	9.40E-03	SOX3, HES3, PAX8, GBX2, SIX6, FOXD3
regulation of transcription from RNA polymerase II promoter	6	35.29	9.74E-03	SOX3, HES3, PAX8, GBX2, SIX6, FOXD3
anterior/posterior pattern specification	3	17.65	1.03E-02	ARC, HES3, GBX2
positive regulation of nucleic acid-templated transcription	5	29.41	1.69E-02	HES3, PAX8, GBX2, SIX6, FOXD3
positive regulation of transcription, DNA-templated	5	29.41	1.69E-02	HES3, PAX8, GBX2, SIX6, FOXD3
positive regulation of RNA biosynthetic process	5	29.41	1.78E-02	HES3, PAX8, GBX2, SIX6, FOXD3

Supplemental Table 2. List of probes used for single-cell qPCR analysis.

ACTB	CHAT	FGF5	GBX2	ITGB4	NANOG	POU4F2	SLC2A2
ALB	COL10A1	FOXA1	GDF3	KRT10	NESTIN	POU5F1	SLC32A1
APLNR	COMP	FOXD3	GFAP	KRT14	NEUROD1	PROM1	SMTN
APOH	CPA1	FOXG1	GSC	KRT19	NEUROG2	PTCRA	SOX17
AQP1	CTSK	G6PC	HAND1	LEFTY1	NKX2-2	RCVRN	SOX2
B2M	DCN	GAD1	HAND2	MAP3K12	NKX2-5	RPLP0	SOX7
BMP4	DCX	GAD2	HES5	MIOX	NPPA	RUNX1	T
CCR5	DNMT3B	GALC	HNF4A	MIXL1	OLIG2	RYR2	TAT
CD34	DPP4	GAPDH	HPRT1	MSLN	OTX2	SFTPB	TUBB3
CD3E	ENO1	GATA1	IBSP	MYH1	PAX6	SFTPD	TYR
CD79A	EOMES	GATA2	IGF2	MYH7	PDGFRA	SLC17A6	ZFP42
CER1	FABP7	GATA6	INS	MYL3	PODXL	SLC17A7	ZIC1

Supplemental Table 3. Primer sequences used in qPCR gene expression analysis.

Gene	**Forward Sequence**	**Reverse Sequence**
OCT4	TCAGCCAAACGACCATCTGCCG	AGCAAGGGCCGCAGCTTACA
SOX2	TACAGCATGTCCTACTCGCAG	GAGGAAGAGGTAACCACAGGG
Nanog	ACGCAGAAGGCCTCAGCACCTA	AGGTTCCCAGTCGGGTTCACCA
T	ACCTGTGTCGCCACCTTCCA	ACCACTGGCTGCCACGACAA
MIXL1	TCCTCAACCACTGTGCTCCTGG	AACCCCGTTTGGTTCGGGCA
EOMES	AGGCGCAAATAACAACAACACC	ATTCAAGTCCTCCACGCCATC
GSC	CGCGGGACACTTGCCCGTATTA	AAGGCAGCGCGTGTGCAAGA
PAX6	CCAGAAAGGATGCCTCATAAA	TCTGCGCGCCCCTAGTTA
Nestin	CCGCATCCCGTCAGCTGGAAAA	GCTTGGGCACAAAAGCCAGCA
OTX2	CTTAAGCAACCGCCTTACGC	AGGGGTGCAGCAAGTCCATA
AFP	AGCTGACCTCGTCGGAGCTGAT	TCCCTCGCCACAGGCCAATAGT
KDR	ACCGTTAAGCGGGCCAATGGA	ACCACGGCCAAGAGGCTTACCT
GAPDH	TTCTTTTGCGTCGCCAGCCG	TGACCAGGCGCCCAATACGA
EF1a	GCTGGCTTCACTGCTCAGGTGATT	TGCAATGTGAGCCGTGTGGCA

Supplemental Experimental Procedures

Spatial Analysis

Spatial analysis for cells seeded as colonies at different split ratios or single-cells at different densities was performed using the SpatStat package for R (Baddeley and Turner, 2005; Baddeley et al., 2015). The spatial coordinates of each cell within a field (i.e. each microscopic image) were converted into a spatial point pattern, and each field was divided into 5x5 grids (totaling 25 quadrats). The number of points (or cells) per quadrat was quantified using the 'quadratcount' feature in Spatstat for each quadrat in each image acquired for a given well. The Coefficient of Variation (CV; standard deviation divided by mean) was then calculated for the number of cells per quadrat for each seeding condition. For each well, the total number of cells was quantified, and normalization of CV values was performed by calculating the CV of a simulated random uniform distribution of points (equal to the cell number in that well) using the 'runifpoint' function in Spatstat.

Bioinformatics

FASTQ sequencing data for each sample was aligned to GENCODE version 23 human genome annotations (hg38), using the HISAT2 alignment tool (version 2.0.1) (Kim et al., 2015). The 'featureCounts' function from the Rsubread package (version 1.22.3) (Liao et al., 2019)was used to count reads per gene, which were passed onto DESeq2 (version 1.12.4) (Love et al., 2014)for library size normalization and detection of differentially expressed genes (FDR <= 0.05). Hierarchical clustering was done with the R 'heatmap.2' function from the gplots package (version 2.17.0) for differentially expressed genes. Principal component analysis was done using an in-house R script and the built-in R PCA function 'prcomp'. Temporal expression path clustering was generated by unsupervised hierarchical clustering a log2 fold (log2FC) change matrix using R's built-in 'hclust' function. This was done for all genes with an absolute log2FC of at least 2 in any single timepoint. Clusters which were visibly similar were then manually combined, and cluster trajectories were then plotted using an in-house R script. Gene set enrichment analyses (Subramanian et al., 2005) were conducted by comparing differentially expressed genes from RNA-seq data to custom Gene Sets produced in-house. The -log10(p-value) for each gene from RNA-seq data was used as a custom weighting when GSEA analysis was conducted (version 2.1.0). Gene ontology analysis was performed using DAVID version 6.8 (Huang et al., 2009) and the BINGO plugin for Cytoscape (Maere et al., 2005).

Single-cell Gene Expression Analysis

Cycle numbers to enrichment for each gene in each cell were normalized to housekeeping genes between cells. Normalized cycle numbers were converted to a cycle difference measurement, subtracting observed cycle number from the mean cycle number of the corresponding gene across all cells. An aggregate vector of all cycle differences was used to produce Z-scores for each gene in each cell; these Z-scores were then used to plot heatmaps, using the R 'heatmap.2' function from the gplots package (version 2.17.0). t-SNE plots were also produced from cycle differences using the Rtsne package (version 0.10) and an in-house script for plotting the results. Tissue and cell type enrichment for single cell expression data was done using Enrichr (Chen et al., 2013; Kuleshov et al., 2016).

Chondrocyte Micromass Differentiation and Analysis

Following 48-hour pre-differentiation in E8, E6 or BA, cells (H9 hESCs or iPSCs) were dissociated using TrypLE Express and resuspended in chondrogenic media supplemented with 10 μM Y-27632. Chondrogenic media consisted of high glucose DMEM (Life Technologies) containing 1% KOSR, 1% ITS+ premix (BD Biosciences), 1% Sodium Pyruvate, 1% non-essential amino acids, 1% Penicillin-Streptomycin (Life Technologies), 100 μg/mL ascorbic acid 2-phosphate, 10^{-7} M dexamethasone, and 40 μg/mL L-proline (Sigma). Cells were resuspended at a density of $2x10^7$ cells/mL and plated as 15 μL drops in Matrigel-coated 12-well plates for 2 hours, after which 1 mL of chondrogenic media containing Y-27632 was added. Fresh chondrogenic media was added daily for course of the 7-day protocol.

To assess differentiation by matrix production, 4-5 micromass cultures were pooled and digested overnight at 65°C using 40 μg/mL papain enzyme in digestion buffer and processed for quantification as described (Lee et al., 2011). Sulfated glycosaminoglycan (s-GAG) content was quantified using Dimethylmethylene blue (DMMB). Hydroxyproline (OH-Pro) content was quantified by acid hydrolysis of papain-digested samples followed by neutralization, oxidation, and addition of 4-dimethylaminobenaldehyde. Hoechst 33258 dye was used to quantify DNA content in the samples, and total s-GAG or OH-Pro content were normalized to amount of DNA. Proteoglycan production was assessed after 7 days by Alcian Blue staining. Micromass cultures were fixed in 4% PFA for 15 min, rinsed with 0.2N HCl, and stained with 0.1% Alcian Blue solution at pH1 (diluted with 0.2N HCl from 0.3% w/v Alcian blue in 70% ethanol solution; Sigma) overnight at room temperature. Cultures were rinsed thoroughly with distilled water and imaged using a Zeiss Axio Zoom V16.

Endothelial Progenitor Cell Differentiation and Analysis

Differentiation of iPSCs derived from late-EPCs (described in Chang et al., 2013) was performed using an adapted protocol from Tatsumi *et al* (Tatsumi et al., 2011). Briefly, cells were pretreated for 48 hours with E6 supplemented with 10 μg/ml of both BMP4 and Activin A. Subsequently, cells were cultured in DMEM/F12 (Life Technologies) supplemented with B27 (Life Technologies), N2 (Life Technologies) and BIO (Sigma) for 72 hours with daily media change performed. Cells were then cultured in StemPro-34 (Life Technologies) supplemented with 50 μg/ml of $VEGF_{165}$ (R&D Systems) for a further 48 hours prior to MAC-selection with CD144

microbeads (Miltenyi Biotech). CD144 enriched cells were then further expanded and routinely passaged in StemPro-34 media supplemented with 50 µg/ml $VEGF_{165}$.

Endothelial cell differentiation was assessed by flow cytometry against a panel of endothelial cell surface markers. Briefly, following six days of differentiation, cells were harvested and dissociated into single cell suspension using TrypLE. A total of 2 $x10^5$ cells in 200 µl of flow buffer was enumerated and aliquotted into each tube. Directly conjugated antibodies (all from BD Bioscience) to CD31 (Cat. No. 340297), CD34 (Cat. No. 345802), CD45 (Cat. No. 555482), CD144 (Cat. No. 560411) and VEGFR (Cat. No. 560494) were then added at the recommended manufacturer's dilution into each tube (i.e. 20 µl per test) and incubated on ice in the dark for 30 mins. To halt the staining process, 400 µl of flow buffer was added per tube and the entire volume of 600 µl was subjected to centrifugation at 400rpm for 5 mins. Supernatant was decanted and cells resuspended in 300 µl flow buffer prior to analysis on an Attune acoustic focus cytometer (Life Technologies). To gate samples for FACS analysis, cells (20,000 events) were initially gated by FSC-A vs SCS for the exclusion of debris. Data were analyzed using FlowJo v10.0.6 software.

Primary Antibodies

OCT4A (Cell Signaling Technology C52G3; 1:400), Brachyury T (R&D Systems AF2085, 5 µg/mL), SOX2 (R&D Systems MAB2018, 8 µg/mL; EMD Millipore AB5603, 1:200), Nestin (EMD Millipore ABD69, 1:500), Desmin (Abcam ab191181, 1:200) and SOX17 (R&D Systems AF1974, 1:100).

Supplemental References

Baddeley, A., and Turner, R. (2005). **spatstat** : An *R* Package for Analyzing Spatial Point Patterns. J. Stat. Softw.

Baddeley, A., Rubak, E., and Turner, R. (2015). Spatial Point Patterns: Methodology and Applications with {R} (London: Chapman and Hall/CRC).

Chang, W.Y., Lavoie, J.R., Kwon, S.Y., Chen, Z., Manias, J.L., Behbahani, J., Ling, V., Kandel, R.A., Stewart, D.J., and Stanford, W.L. (2013). Feeder-independent derivation of induced-pluripotent stem cells from peripheral blood endothelial progenitor cells. Stem Cell Res. *10*, 195–202.

Chen, E.Y., Tan, C.M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G., Clark, N.R., and Ma'ayan, A. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics *14*, 128.

Huang, D.W., Sherman, B.T., and Lempicki, R.A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. *4*, 44–57.

Kim, D., Langmead, B., and Salzberg, S.L. (2015). HISAT: a fast spliced aligner with low memory requirements. Nat. Methods *12*, 357–360.

Kuleshov, M. V, Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M., Lachmann, A., et al. (2016). Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. *44*, W90-7.

Lee, W.D., Hurtig, M.B., Kandel, R.A., and Stanford, W.L. (2011). Membrane Culture of Bone Marrow Stromal Cells Yields Better Tissue Than Pellet Culture for Engineering Cartilage-Bone Substitute Biphasic Constructs in a Two-Step Process. Tissue Eng. Part C Methods *17*, 939–948.

Liao, Y., Smyth, G.K., and Shi, W. (2019). The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. Nucleic Acids Res.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550.

Maere, S., Heymans, K., and Kuiper, M. (2005). BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. Bioinformatics *21*, 3448–3449.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., et al. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U. S. A. *102*, 15545–15550.

Tatsumi, R., Suzuki, Y., Sumi, T., Sone, M., Suemori, H., and Nakatsuji, N. (2011). Simple and highly efficient method for production of endothelial cells from human embryonic stem cells. Cell Transplant. *20*, 1423–1430.