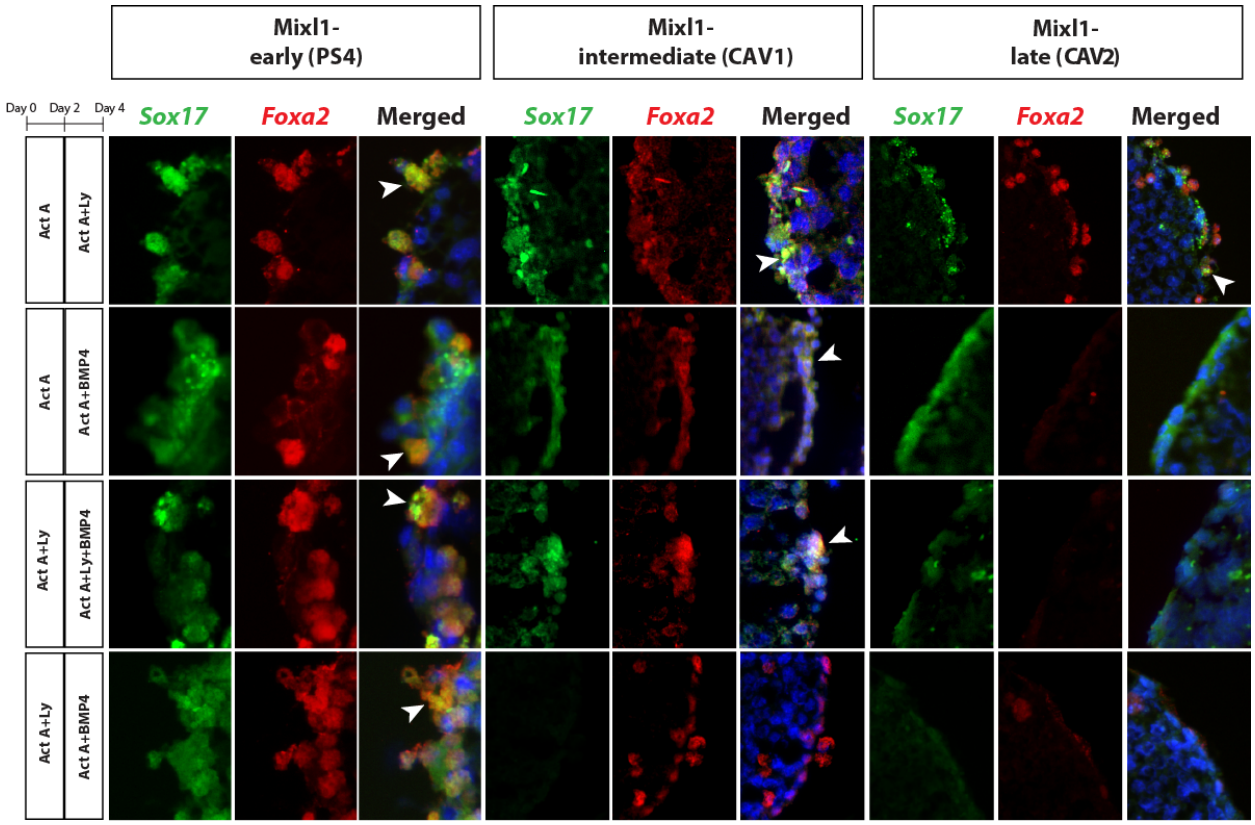
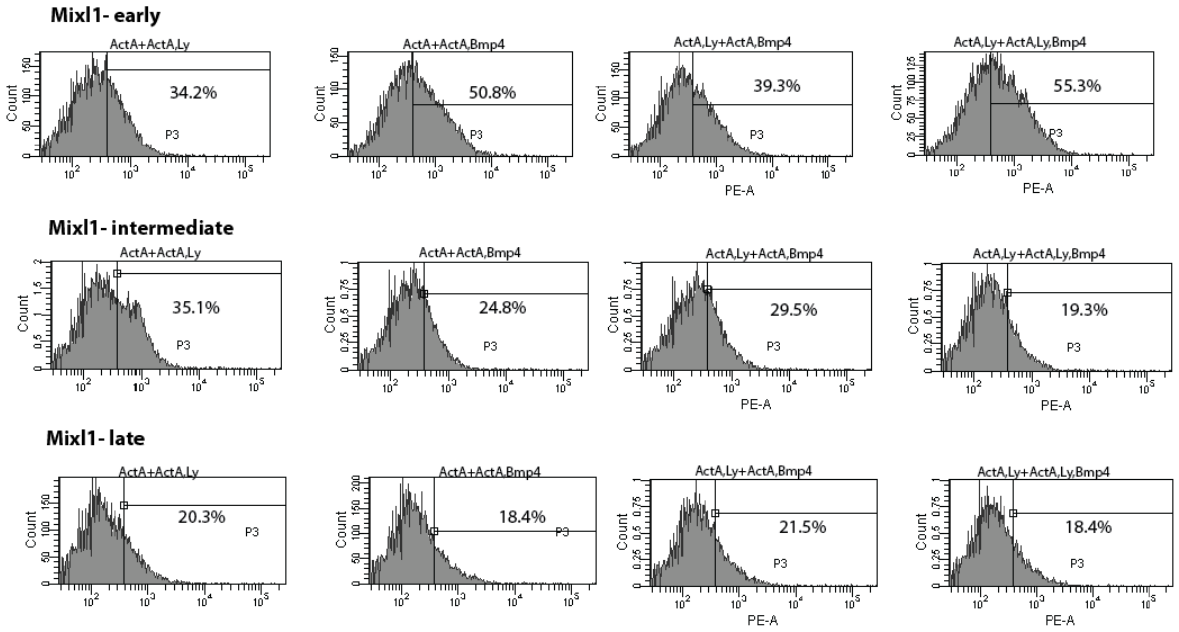
Differential response of Epiblast stem cells to Nodal and Activin signalling: a paradigm of early endoderm development in the embryo. Kaufman-Francis *et al.*, (2014)



Supplementary Figure S1. Sox17+ and Foxa2+ cells in embryoid bodies derived from Mixl1-early, Mixl1-intermediate and Mixl1-late EpiSCs visualized by immunostaining.

EpiSCs of the three Mixl1 categories were differentiated as embryoid bodies for 4 days in culture conditions (left column) to induce the formation of definitive endoderm. Immunostaining was performed on cryosections of the embryoid bodies to reveal the presence of Sox17+ (green), Foxa2+ (red) and double Foxa2+Sox17+ (arrowhead) definitive endoderm-like cells.



Supplementary Figure S2. Quantitation of CXCR4+ cells in Day-4 embryoid bodies cultured in different conditions. Mixl1-early, Mixl1-intermediate and Mixl1-late EpiSCs were differentiated as embryoid bodies in 4 different conditions to induce the formation of definitive endoderm. The proportion (%) of CXCR4+ cells (the presumptive definitive endoderm) was assessed by FACS of the disaggregated embryoid bodies.

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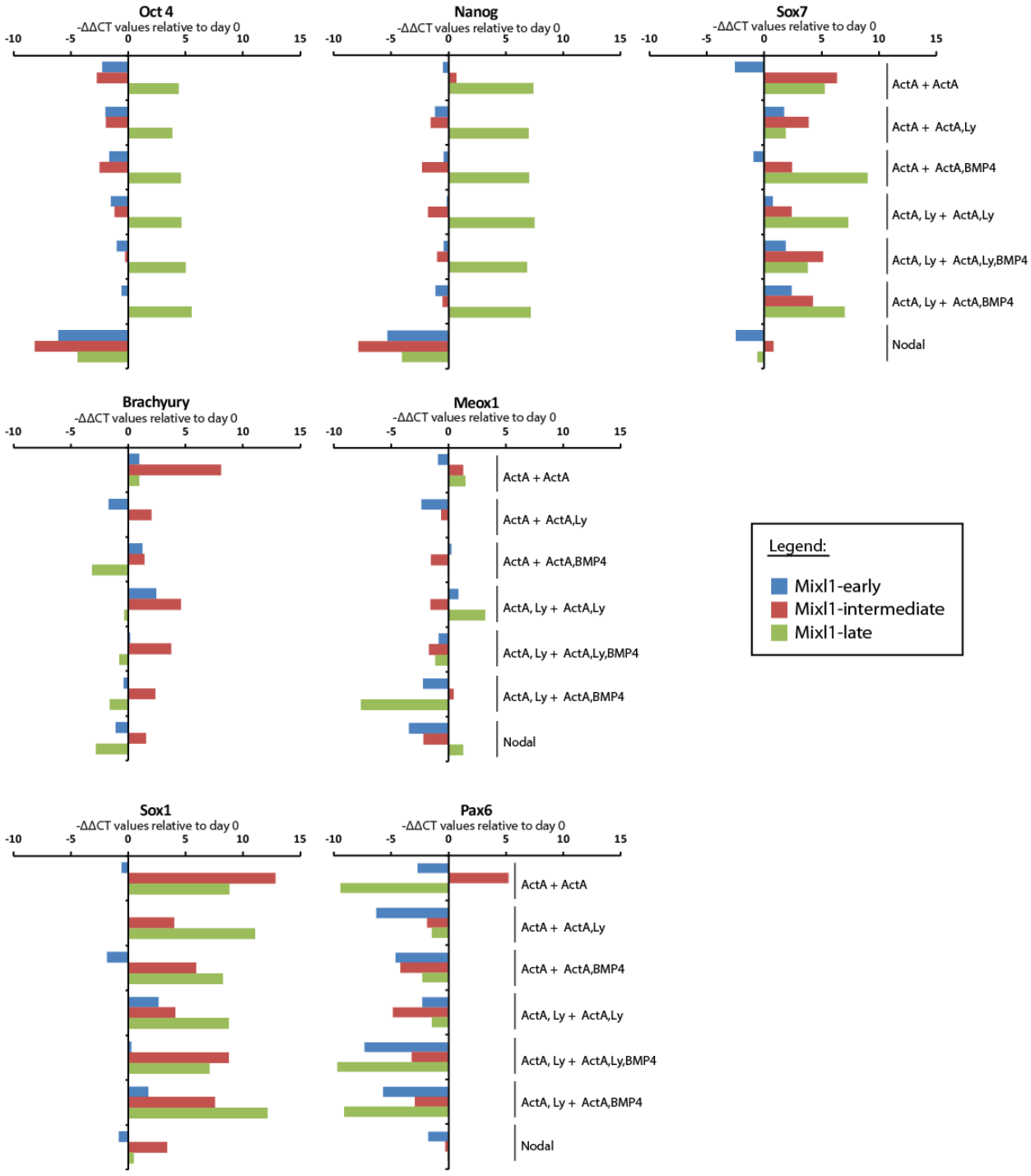
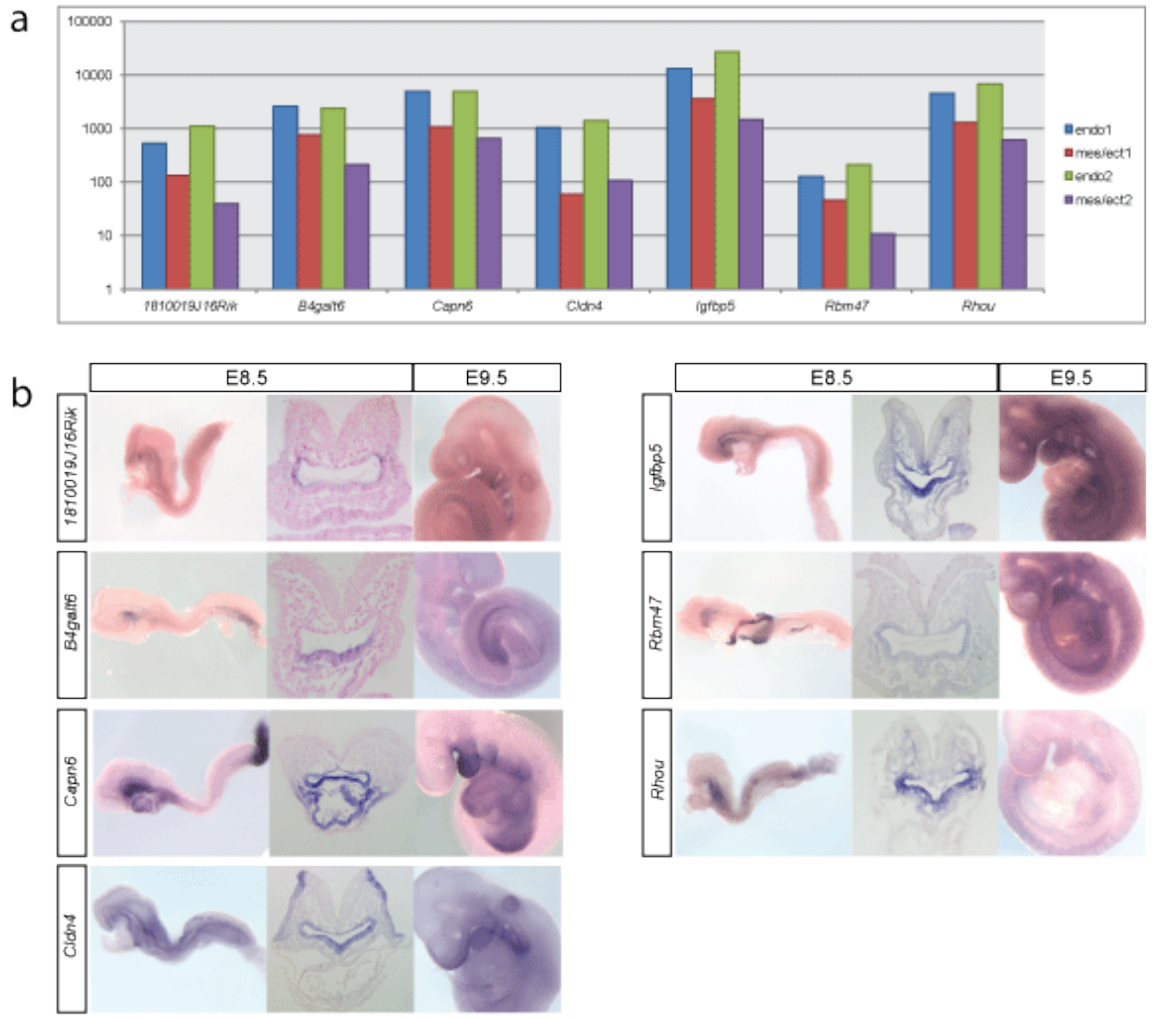


Supplementary Figure S3. Quantitative gene expression analysis of pluripotency and cell lineage markers in Day 4 embryoid bodies derived from Mixl1-early, Mixl1-intermediate and Mixl1-late EpiSCs in response to 7 culture conditions. Expression levels of *Oct4* and *Nanog* (pluripotency markers), *Sox7* (extraembryonic endoderm marker), *Brachyury* and *Meox1* (mesoderm markers), and *Sox1* and *Pax6* (ectoderm markers) were determined by qPCR. Expression level of each gene was normalised to *β-actin* and presented as the negative value of the relative difference in normalised threshold cycles (-ΔΔCT). X-axis: positive = up-regulation, negative = down-regulation.

Supplementary Figure S4



Supplementary Figure S4. **Validation by *in situ* hybridization of the expression of foregut endoderm-enriched genes identified by microarray-based analysis of gene expression profile of early-somite stage (E8.5) embryo.**

(a) Normalized expression values derived from Affymetrix array analysis of foregut endoderm samples (endo1, endo2) and combined mesoderm and ectoderm samples (mesect1, mesect2) of two independent experiments.

(b) Whole-mount *in situ* hybridization confirmed the expression of enriched genes in the gut endoderm of E8.5 (whole mount and sectioned specimens) and E9.5 embryos (whole mount specimens). Riboprobes were generated from the inserts of MGC cDNA clones (Supplementary Table S1). Expression patterns of these genes in E8.5 and E9.5 embryos are:

1810019J16Rik E8.5: throughout the foregut endoderm, strongest in the lateral endoderm. E9.5: the pharyngeal region.

B4galt6 E8.5: foregut (ventral endoderm) and hindgut. E9.5: the hindgut.

Capn6 E8.5: the foregut (ventral endoderm), cardiac mesoderm and allantois. E9.5: the foregut and heart (also in limb bud).

Cldn4 E8.5: throughout the gut endoderm (also in the surface ectoderm adjacent to the neurectoderm in the head. E9.5: the foregut.
Igfbp5 E8.5: ventral endoderm of the foregut. E9.5: the foregut and the midgut
Rbm47 E8.5-9.5: foregut endoderm and visceral yolk sac endoderm
Rhou E8.5-9.5: ventral foregut endoderm, somites, allantois

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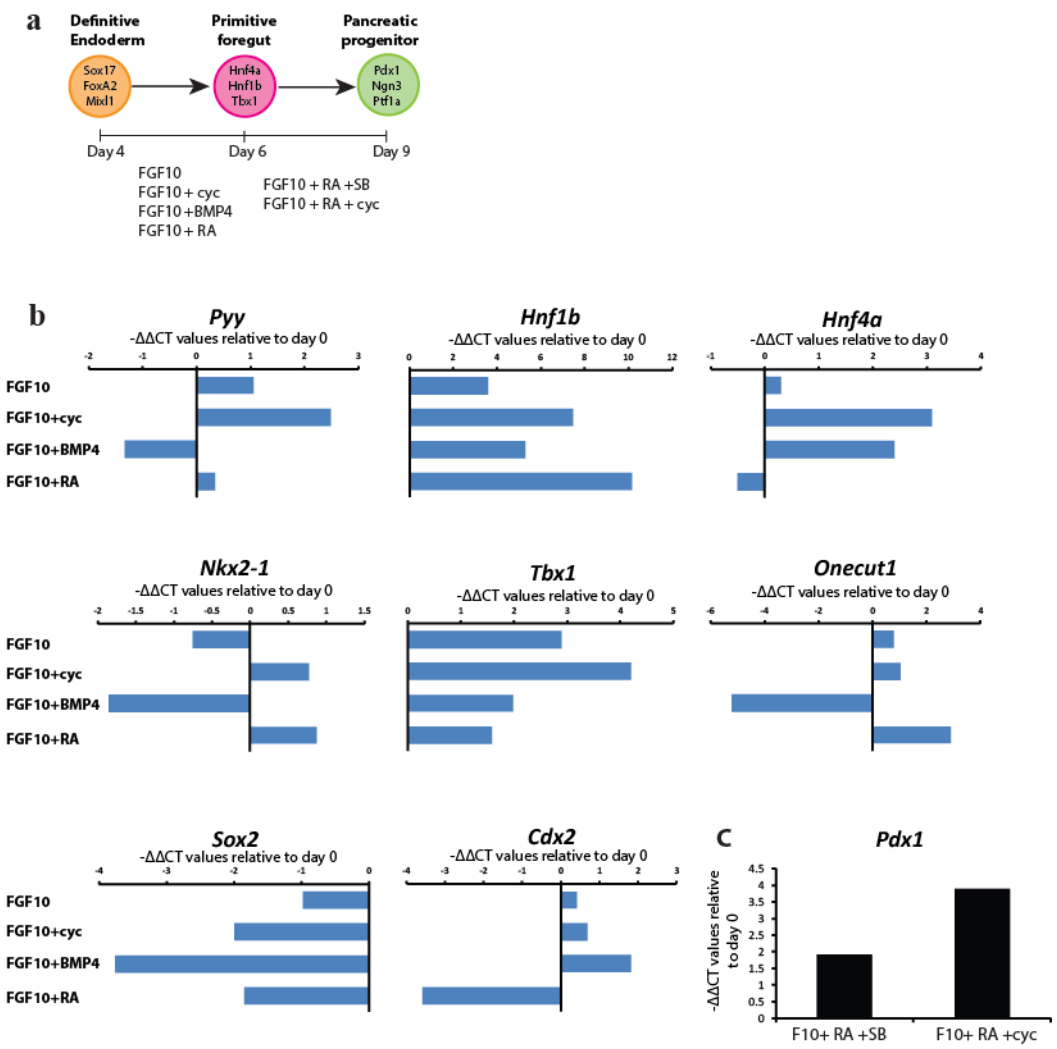

Supplementary Figure S5. Optimisation of the culture protocol for the differentiation of primitive foregut endoderm to pancreatic progenitor in Mixl1-intermediate (CAV1) EpiSCs.

(a) Differentiation protocols for primitive foregut and pancreatic progenitors from Day-4 definitive endoderm-like cells derived from Mixl1-intermediate EpiSCs. (b) Expression levels of foregut markers in Day-6 EpiSC-derived cells (normalised to β-actin value). Expression levels of the foregut markers were generally higher in cells treated with FGF10+cyc on days 4-6. (c) Higher level of *Pdx1* expression in Day-9 cells that were treated with FGF10+RA+cyc than FGF10+RA+SB on days 6-9. RA, retinoic acid; SB, SB431542; cyc, Cyclopamide.

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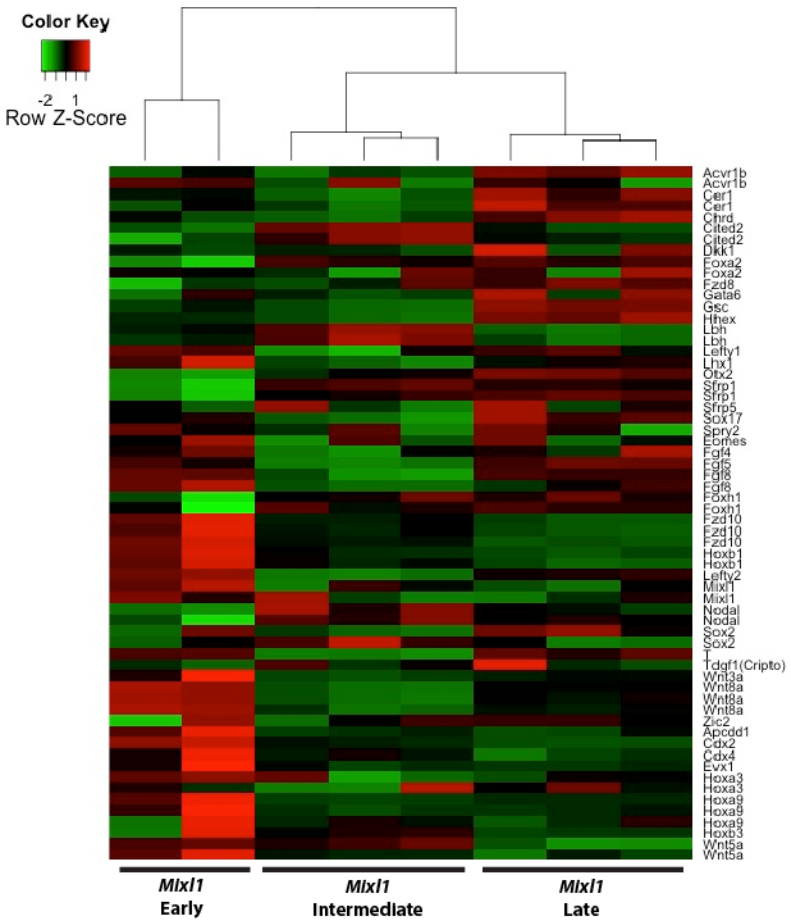
a



b

Mixl1- Early > Mixl1- Intermediate (KEGG,GO)	Mixl1- Early > Mixl1- Late (KEGG,GO)
- anterior/posterior pattern formation (p=3.79E-20) - pattern specification process (p=4.65E-18) - regionalization (p=1.90E-17) - embryonic organ morphogenesis (p=3.28E-09) - embryonic organ development (p=5.25E-09) - embryonic morphogenesis (p=6.64E-09) - Wnt receptor signaling pathway (p=4.92E-06) - Pathways in cancer (p=8.70E-04) - Wnt receptor signaling pathway, calcium modulating pathway (p=3.33E-03)	- extracellular region (p=4.99E-20) - extracellular matrix (p=2.55E-16) - proteinaceous extracellular matrix (p=8.91E-16) - extracellular region part (p=2.41E-15) - growth factor activity (p=7.79E-07) - negative regulation of cell differentiation (p=1.31E-06) - collagen catabolic process (p=5.25E-06) - regulation of cell proliferation (p=1.70E-05) - collagen metabolic process (p=2.64E-05) - positive regulation of nitrogen compound metabolic process (p=3.64E-05) - extracellular space (p=9.80E-05) - regulation of ossification (p=1.53E-03) - MAPK signaling pathway (p=9.47E-03)
IFITM1, WNT3A, HOXD10, HOXC6, HOXA1, HOXC9, HOXA5, LHX1, HOXA7, HOXA9, MLLT3, EN1, HOXD9, HOXC10, HOXB4, HOXB1, HOXD8, HOXB7, MEOX1, HOXB5, HES3, HOXD3, HOXD4, HOXB6, ROR2, HOXB9, TCF15, GNA13, FGF8, CDX2, IFITM1, EFNB1, TGFBR2, FGF8, WNT7B, FBXW8, RSPO3, CSNK2A2, FZD10, LZTS2, NXN, NLK, BRD7, WIF1, WNT8A, E2F2, HSP90AA1, FGF17, SKP2, FADD, STAT3, CCNE2, WNT7B	AEBP1, FGF7, IGFBP7, VTN, VGF, MMP3, MMP2, GDNF, IL11, B2M, BDNF, WISP2, CTGF, COL12A1, CYR61, CRTAP, MGP, MCPT8, MMP13, MMP11, PLAUR, INHBB, VEGFB, MMP10, INHBA, NPC2, THBD, COL1A1, ADAMTS2, MFAP5, WNT5A, CXCL1, CCK, CCL2, TNC, LUM, CRLF1, TAC1, DCN, POMC, GREM1, TIMP2, CCL7, TIMP1, ITGBL1, ANGPTL7, LIF, LGALS3BP, COL7A1, NPTX2, COL6A3, COL6A2, COL6A1, PTX3, FIGF, ANGPTL2, OLFM1, SPP1, ANGPTL4, COL4A2, LGALS3, IL1RL1, LGALS1, EMILIN2, COL16A1, ECM1, CXCL14, SRPX2, CAPG, CMTM8, HBEGF, SST, PLAU, WNT5A, CXCL1, ZFP36, IRX3, CAV1, TBX3, NFKBIA, ISL1, NKX6-1, THY1, HOXA2, AXIN2, TWIST2, KLF4, NKX2-2, MMP10, SAT1, FOSL2, SPHK1, ANXA1, CDKN1A, OSR2, CDKN2A, NUPR1, BTG1, EGR1, KLF6, CEBPB, HTATIP2, EGR2, ONECUT2, SMAD5, WT1, FOS, CSRNP1, GPX4, KLF2, FOSL1, SERTAD1, EGR2, JUND, FOS, DUSP1, MAPK13, GADD45G, RRAS, GADD45B, DUSP8, DUSP6

Supplementary Figure S6. Expression profiles of primitive streak markers in Mixl1-early, Mixl1-intermediate and Mixl1-late EpiSC.

(a) Heat map comparing the expression levels of primitive streak markers in the three categories of Mixl1 EpiSCs: two Mixl1-early and three each of Mixl1-intermediate and Mixl1-late. Gene expression values are extracted from our published microarray analysis dataset (GEO Accession number GSE462227) [1]. (b) Lists of genes that were up-regulated in Mixl1-early EpiSCs compared to Mixl1-intermediate or Mixl1-late EpiSCs. The functional annotation of the up-regulated genes was deduced using DAVID Bioinformatics resources.

[1] Kojima Y, Kaufman-Francis K, Studdert JB, Steiner KA, Power MD, Loebel DA, et al. The transcriptional and functional properties of mouse epiblast stem cells resemble the anterior primitive streak. Cell Stem Cell. 2014;14(1):107-20.

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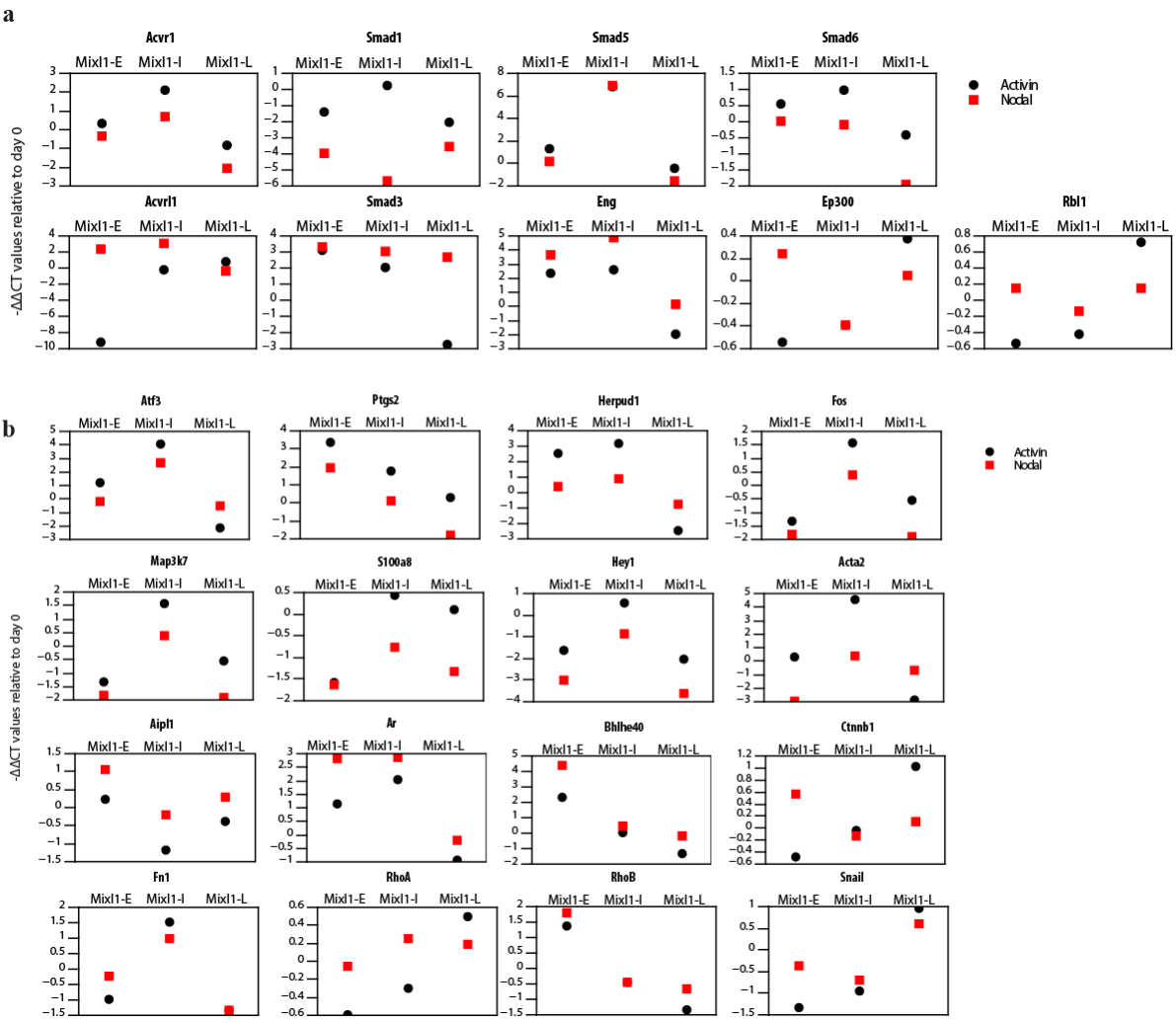

Supplementary Figure S7. Differential expression levels of Tgfβ pathway components and the response genes in Day-4 embryoid bodies differentiated in the presence of Activin A or Nodal.

qPCR analysis of the differential expression of (a) genes coding for Tgfβ signalling pathway components and (b) response genes in Day-4 embryoid bodies in response to Activin A or Nodal. Differences in the pattern of expression of a specific marker of the signalling activity were generally consistent among the EpiSCs of the Mixl1-E (Mixl1-early), Mixl1-I (Mixl1-intermediate) and the Mixl1-L (Mixl1-late) categories. Gene expression values were normalised against *Actb*, *B2m*, *Gapdh*, *Hsp90ab1* and *Gusb*.