Single-cell analysis reveals lineage segregation in early post-implantation mouse embryos

Jing Wen[1,a] , Yanwu Zeng[2,a] , Zhuoqing Fang[1], Junjie Gu[2], Laixiang Ge[2], Fan Tang[2], Zepeng Qu[2], Jing Hu[2], Yaru Cui[3], Kushan Zhang[3], Junbang Wang[3], Siguang Li[3], Yi Sun[3] , and Ying Jin[1,2,b]

Supplemental Figures and Legends
Supplemental Tables
Supplemental Experimental Procedures
Supplemental References

Supplemental Figures and Legends

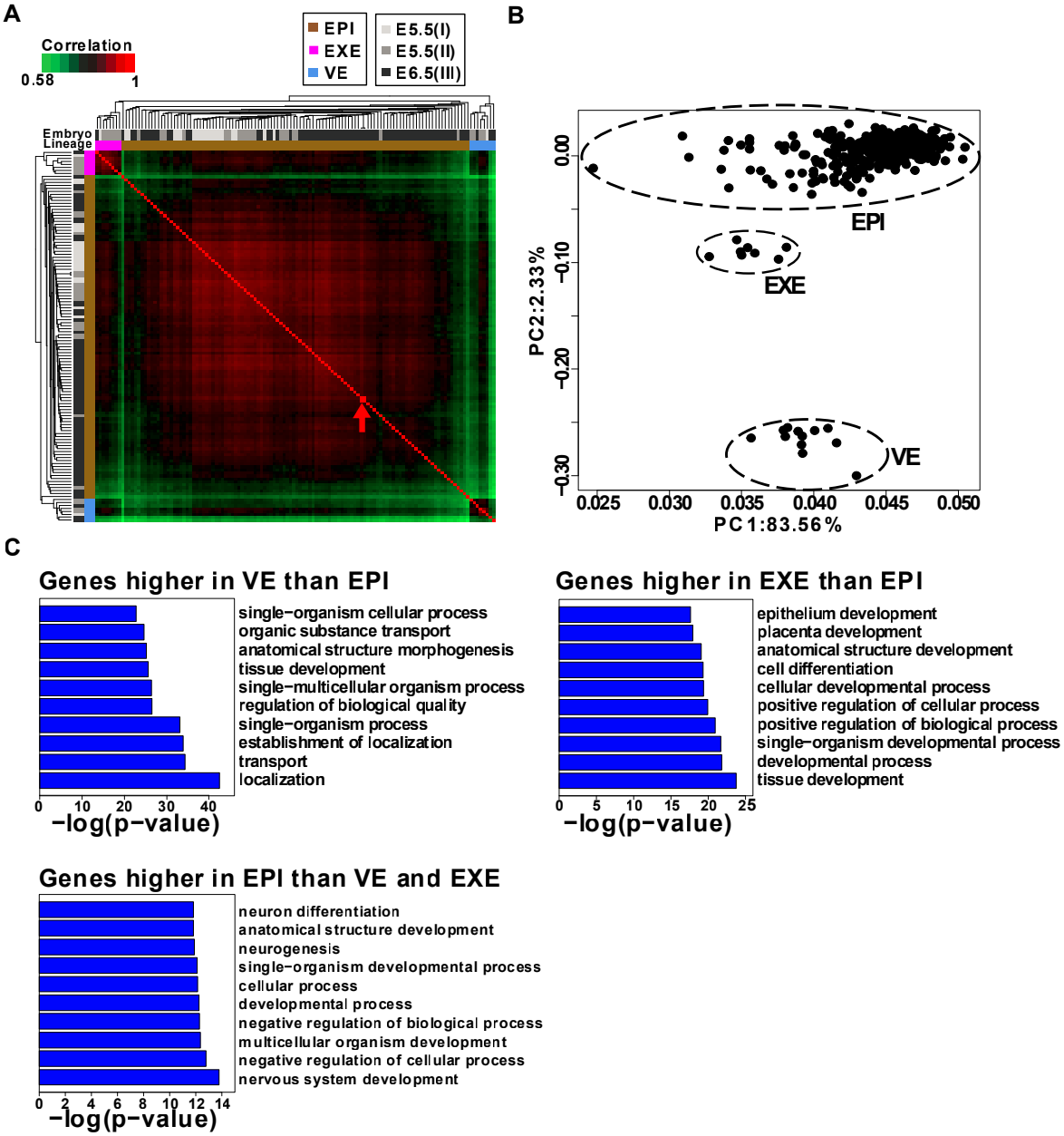

Figure S1 Gene expression patterns of EPI, VE and EXE cells revealed by single-cell RNA-Seq.

(A) Heatmap displaying the correlation coefficient between each pair of samples. Samples were clustered by Pearson distance and Complete linkage. The colored bars beside the heatmap indicate lineage and embryo membership. The red arrow indicates a pair of highly similar cells (the correlation coefficient was 0.993).

(B) PCA of Scialdone et al.(1)'s 501 E6.5 cells using Scialdone's signature genes. The RNA-Seq data were mapped by our methods and the values of $\log_2$(RPKM+1) were used to perform PCA. Cells formed 3 major clusters, designated as EPI, VE and EXE.

(C) Gene Ontology (GO) of specific genes for VE, EXE and EPI. Scialdone et al. 's EPI, VE and EXE cells were compared in the same way as were our EPI, VE and EXE cells. The differentially-expressed genes were selected from the common list of the two datasets and subjected to GO analyses. For each panel, the top ranked 10 GO terms are displayed.

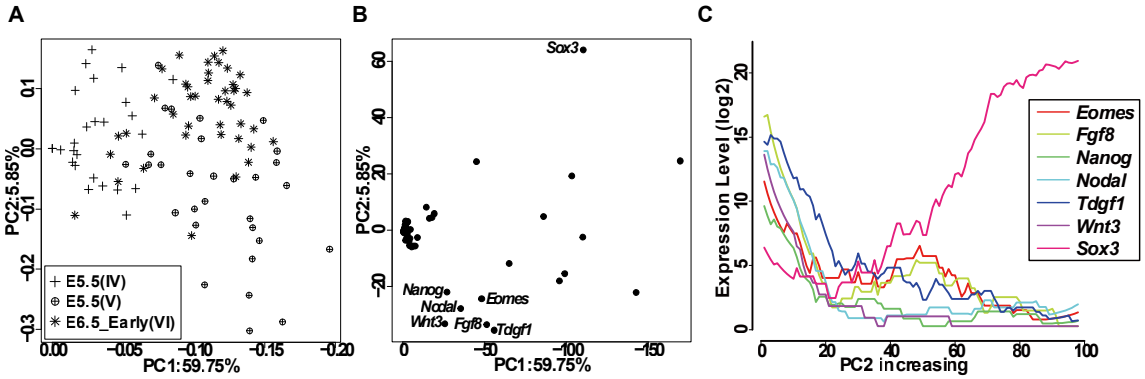

Figure S2 Verification of preMEN cells by single-cell high-throughput qRT-PCR using 45 successfully detected germ-layer markers.

(A) PC projections of 98 cells from embryos E5.5 (IV), E5.5 (V) and E6.5 (VI). 104 *Oct4*$^{+}$ cells were obtained from these 3 embryos, of which 98 cells were negative for both *Gata6* and *Hand1* (*Oct4*$^{+}$*Gata6*$^{-}$*Hand1*$^{-}$) and used for the analyses here. The 90 germ-layer markers were all examined by single-cell high-throughput qRT-PCR. However, only 45 markers were detected (Ct<40) in at least 1 of these cells. The relative expression levels (30-ΔCt) of the 45 germ-layer markers were used as the input.

(B) PC loadings of genes. There were mainly MEN markers (*Nanog*, *Nodal*, *Eomes*, *Fgf8*, *Wnt3* and *Tdgf1*) in the bottom region of the PC2 axis, while NE marker *Sox3* is in the top region of the PC2 axis.

(C) The negative correlation between *Sox3* and MEN markers in 98 cells. For clarity, only some of the genes at the two ends of the PC2 axis in (B) are shown. The cells on the x axis are sorted according to their projection scores for the PC2 so that preMEN cells below the dashed line in (B) are on the left and the rest of EPI cells are on the right. The traces represent moving averages of the given gene's expression level in overlapping windows of 20 cells.

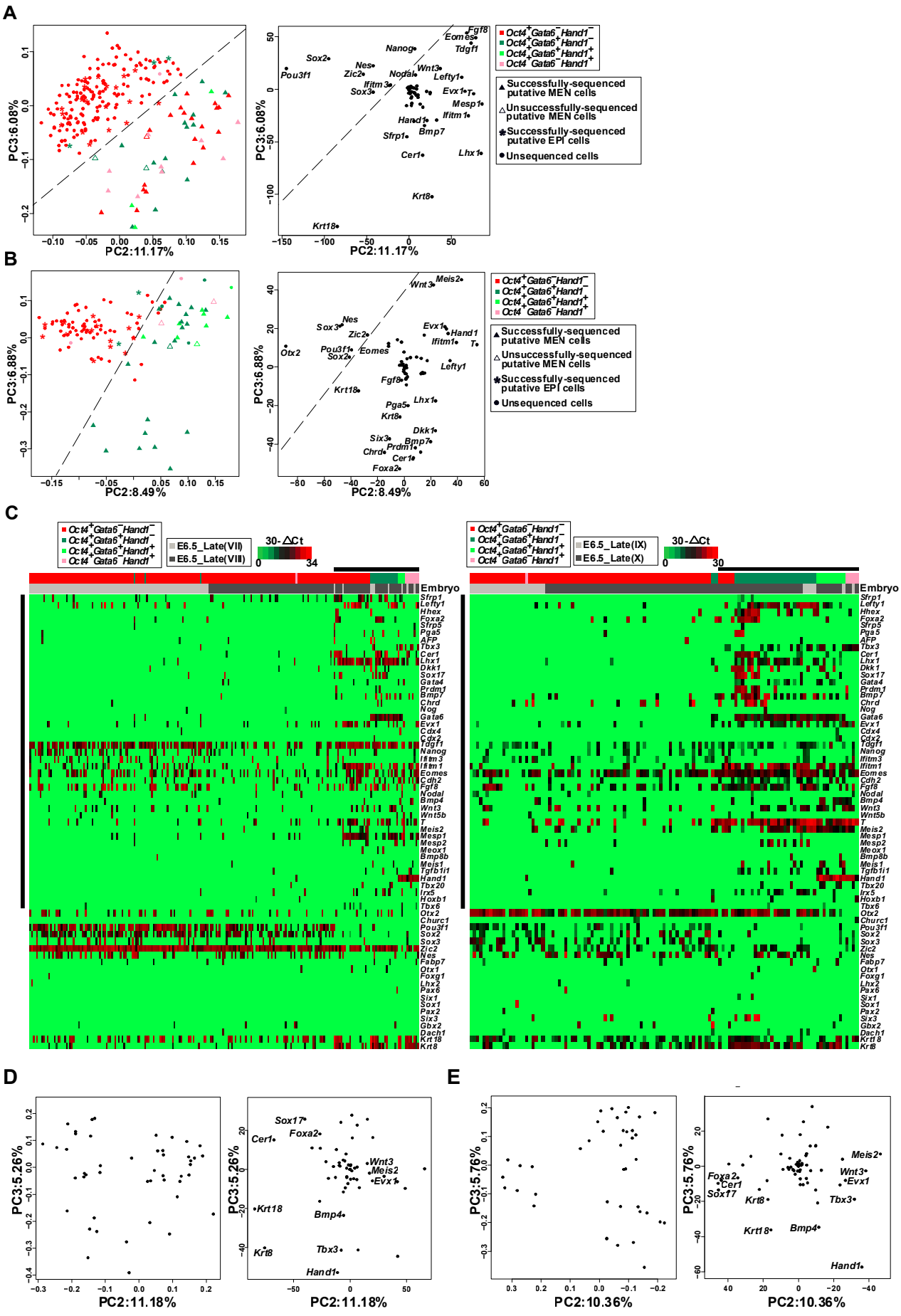
A
B
C
D
E
PC2:11.17%
PC3:6.08%
PC3:6.88%
PC2:8.49%
Oct4+Gata6−Hand1−
Oct4+Gata6+Hand1−
Oct4+Gata6+Hand1+
Oct4−Gata6−Hand1+
Successfully-sequenced putative MEN cells
Unsuccessfully-sequenced putative MEN cells
Successfully-sequenced putative EPI cells
Unsequenced cells
E6.5_Late(VII)
E6.5_Late(VIII)
E6.5_Late(IX)
E6.5_Late(X)
30-ΔCt
Embryo
PC2:11.18%
PC3:5.26%
PC2:10.36%
PC3:5.76%

Figure S3 Mesendodermal properties of *Oct4*$^{+}$*Gata6*$^{+}$ cells and criterion for selection of cells to be sequenced.

(A) PCA of 224 *Oct4*$^{+}$ cells from embryos E6.5_Late (VII) and E6.5_Late(VIII). The 90 germ-layer markers used in Figure 2A were all examined by single-cell high-throughput qRT-PCR, and the relative expression level (30-ΔCt) of 60 sucessfully detected markers (Ct<40 in at least 1 cell) were used as the input. Colors indicate expression of *Oct4, Gata6* or *Hand1*. Judged from the PCA map, cells in the lower-right region of the left panel (on the right of the dashed line) should be putative MEN cells, because neuroectodermal markers (*Pou3f1*, *Sox2*, *Sox3*, *Nes*) are in the uppper-left region (on the left of the dashed line) of the right panel, and mesendodermal markers (*Evx1*, *T*, *Mesp1*, *Lhx1*, *Cer1*, *Sfrp1*, *Hand1*, *Bmp7*) are in the lower-right region (on the right of the dashed line) of the right panel. The *Oct4*$^{+}$*Gata6*$^{+}$ cells (including *Oct4*$^{+}$*Gata6*$^{+}$*Hand1*$^{-}$ cells which are marked in the deep green; and *Oct4*$^{+}$*Gata6*$^{+}$*Hand1*$^{+}$ cells which are marked in the light green) are mainly in the lower-right region. Some *Oct4*$^{+}$*Gata6*$^{-}$*Hand1*$^{-}$ cells (red) are also in the lower-right region. Cells on the right of the dashed line were all deemed as MEN cells and cDNAs from them were sequenced. The cells successfully sequenced were marked by solid triangles; and the cells unsuccessfully sequenced were marked by hollow triangles. A number of cells on the left of the dashed line, mainly *Oct4*$^{+}$*Gata6*$^{-}$*Hand1*$^{-}$ cells, were stochastically selected to be sequenced, of which 22 cells were successfully sequenced and marked by asterisks.

(B) PCA of 119 *Oct4*$^{+}$ cells from embryos E6.5_Late (IX) and E6.5_Late (X). The 90 germ-layer markers used in Figure 2A were all examined by single-cell high-throughput qRT-PCR, and the relative expression level (30-ΔCt) of 64 sucessfully detected markers (Ct<40 in at least 1 cell) were all used as the input. Colors indicate expression of *Oct4*, *Gata6* or *Hand1*. Judged from the PCA map, cells in the right region of the right panel (on the right of the dashed line) should be putative MEN cells, because neuroectodermal markers (*Otx2*, *Sox2*, *Pou3f1*, *Nes* and *Sox3*) are in the upper-left region and mesendodermal markers (*Wnt3*, *Evx1*, *Hand1*, *T*, *Lefty1*, *Lhx1*, *Bmp7*, *Cer1*, *Foxa2*) are in the right region of the right panel. The *Oct4*$^{+}$*Gata6*$^{+}$ cells (including *Oct4*$^{+}$*Gata6*$^{+}$*Hand1*$^{-}$ cells marked in the deep green and *Oct4*$^{+}$*Gata6*$^{+}$*Hand1*$^{+}$ cells marked in the light green) are mainly in the right region of the left panel, separating from most of the *Oct4*$^{+}$*Gata6*$^{-}$*Hand1*$^{-}$ cells (red). *Oct4*$^{+}$*Gata6*$^{+}$ cells in the right region of the left panel (on the right of the dashed line) were all sequenced, with cells successfully sequenced marked by solid triangles, and cells unsuccessfully sequenced marked by hollow triangles. A number of stochastically-selected *Oct4*$^{+}$*Gata6*$^{-}$*Hand1*$^{-}$ cells were also sequenced, of which 14 cells were successfully sequenced and marked by asterisks.

(C) A heatmap representing qPCR data of E6.5_Late cells. The left panel contains 224 cells from embryos VII and VIII (the same as in A), and the right panel contains 119 cells from embryos IX and X (the same as in B). The upper colored bar above the heatmaps denotes the expression of Oct4/Gata6/Hand1, and the lower colored bar denotes the embryonic membership of the cells. The putative MEN cells (cells on the right of the dashed lines in A and B, respectively) are indicated by black lines above the colored bars. MEN markers (belonging to the first 3 groups of markers in Figure 2A, indicated by black bars on the left of each panel) displayed co-expression patterns in the putative MEN cells.

(D) PCA of 49 putative MEN cells from embryos VII and VIII. The samples were cells on the right of the dashed line in (A). The genes were the same as in (A). The DE signature genes *Cer1*, *Sox17* and *Foxa2* clustered together on the projection map of genes (the right panel).

(E) PCA of 43 putative MEN cells from embryos IX and X. The samples were cells on the right of the dashed line in (B). The genes were the same as in (B). DE signature genes *Cer1*, *Sox17* and *Foxa2* clustered together on the projection map of genes (the right panel).

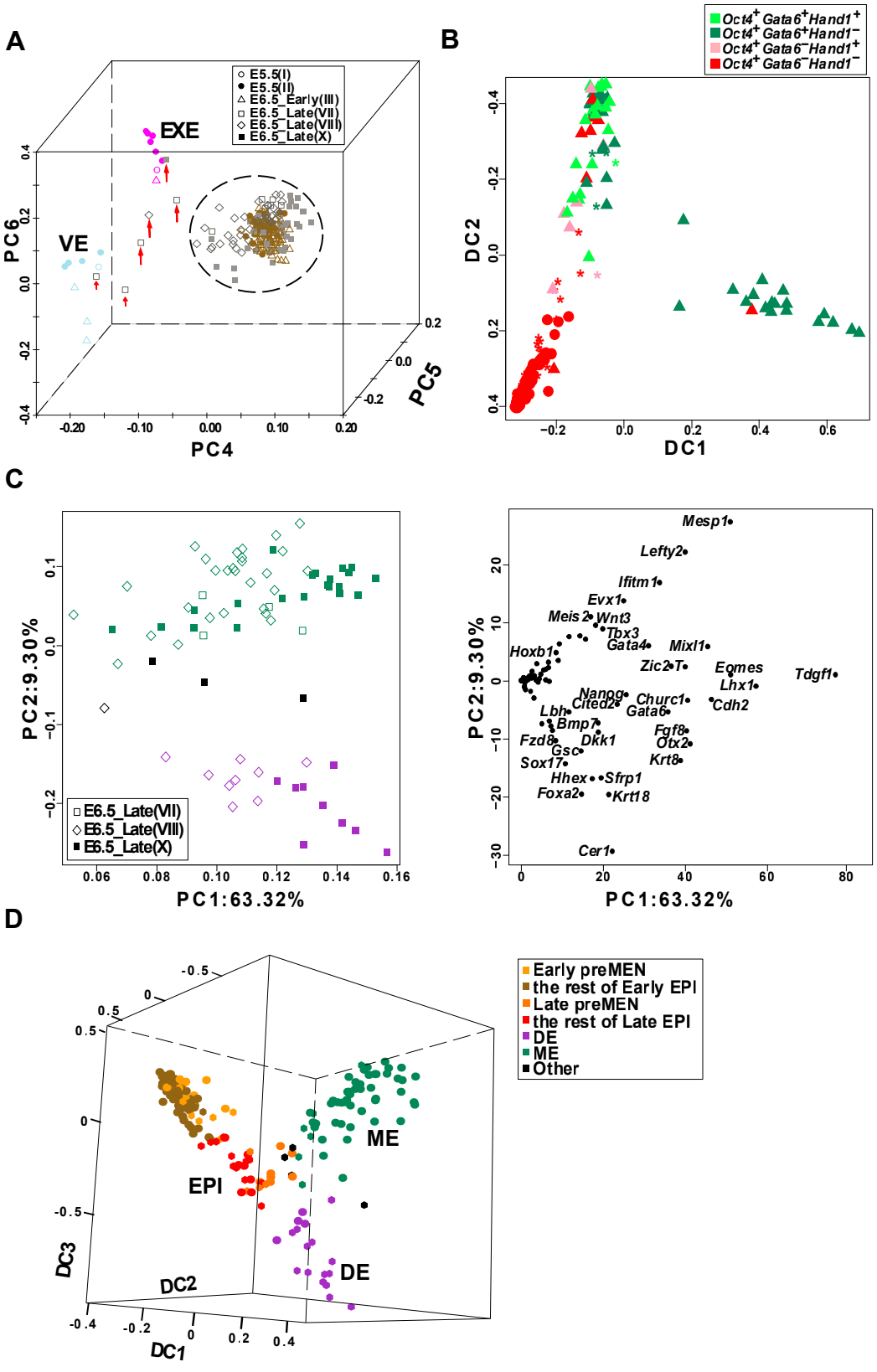

Figure S4 Analyses related to the identification of DE and ME cells in E6.5_Late embryos.

(A) PCA of all sequenced cells using transcriptome data as the input. There were 236 samples in total, including 124 cells from E5.5 and E6.5_Early embryos (including 108 EPI cells, 8 VE cells and 8 EXE cells) and 112 cells from E6.5_Late embryos (which were all *Oct4*$^{+}$). The EPI, VE and EXE cells (identified according to Figure 1B) from E5.5 and E6.5_Early embryos are colored in the brown, blue and rose, respectively. Cells from E6.5_Late embryos are colored in the grey. 6 E6.5_Late cells were excluded from downstream analyses, including 4 cells close to EXE cells from E5.5 and E6.5_Early embryos (indicated by long arrows), and 2 cells close to the VE cells from E5.5 and E6.5_Early embryos (indicated by short arrows). The rest of E6.5_Late cells (106/112) clustered with 108 EPI cells from E5.5 and E6.5_Early embryos. These 214 cells were considered as embryonic cells.

(B) A diffusion map of 214 identified embryonic cells using RNA-Seq data of 90 germ-layer markers as the input. Samples were 214 cells in the large cluster in (A). The 90 germ-layer markers were the same markers used in Figure 2A. Colors indicate the expression of *Oct4*, *Gata6* or *Hand1* (RPKM > 1 was considered "+"). The shapes (star and triangle) used to designate E6.5_Late cells were consistent with those used in Figure S3A and S3B. The MEN cells identified by qRT-PCR results in Figure S3A and S3B (marked by triangles) are mainly in the two branches in the diffusion map, and most of the cells in the two branches are *Oct4*$^{+}$*Gata6*$^{+}$ cells (the light green or deep green).

(C) PCA of cells in the two branches in (B) using RNA-Seq data of 90 germ-layer markers as the input. Shapes indicate embryo membership of the cells. Colors indicate the lineages as identified in Figure 4A. The DE and ME cells are separated on the PC2 axis. The ME signature genes *Mesp1*, *Lefty2*, *Ifitm1* and *Evx1* are in the top region of the right panel. The DE signature genes *Cer1*, *Foxa2*, *Hhex* and *Sox17* are in the bottom region of the right panel.

(D) A diffusion map of 214 identified embryonic cells using 822 germ-layer markers. The markers are a combination of the 90 germ-layer makers with genes related to the GO terms "Ectoderm", "Endoderm" and "Mesoderm" (http://amigo.geneontology.org) (Table S6). The pattern is largely in accordance with the pattern in Figure 4A, with EPI, DE and ME cells in distinct clusters. However, Early preMEN cells mixed with the rest of Early EPI cells, and Early EPI cells as a whole were separated from Late EPI cells.

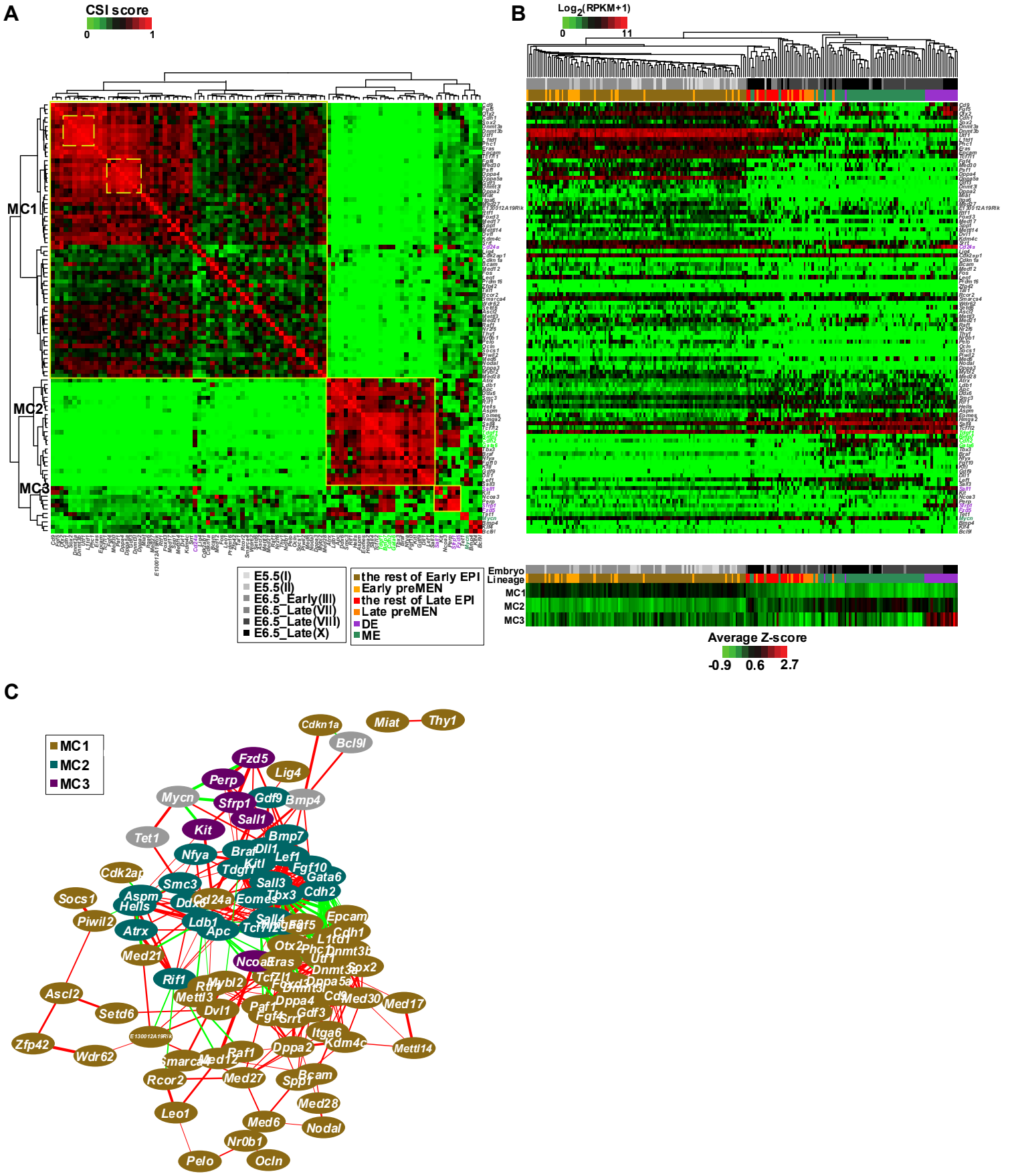
A
CSI score
0
1
MC1
MC2
MC3
E5.5(I)
E5.5(II)
E6.5_Early(III)
E6.5_Late(VII)
E6.5_Late(VIII)
E6.5_Late(X)
the rest of Early EPI
Early preMEN
the rest of Late EPI
Late preMEN
DE
ME
B
Log2(RPKM+1)
0
11
Embryo Lineage
MC1
MC2
MC3
Average Z-score
-0.9
0.6
2.7
C
MC1
MC2
MC3
Cdkn1a
Miat
Thy1
Bcl9l
Fzd5
Lig4
Perp
Mycn
Sfrp1
Gdf9
Bmp4
Sall1
Kit
Tet1
Bmp7
Dll1
Nfya
Lef1
Kitl
Fgf10
Tdgf1
Gata6
Cdk2ap
Smc3
Sall3
Socs1
Aspm
Tbx3
Cdh2
Hells
Eomes
Piwil2
Epcam
Ldb1
Atrx
Apc
Cdh1
Otx2
Med21
Utf1
Rif1
Sox2
Mybl2
Mettl3
Dppa5a
Ascl2
Cd9
Med30
Med17
Setd6
Dvl1
Dppa4
Gdf3
Itga6
Zfp42
Kdm4c
Mettl14
Wdr62
Dppa2
Raf1
Med12
Rcor2
Med27
Spp1
Bcam
Med28
Leo1
Med6
Nodal
Nr0b1
Pelo
Ocln

Figure S5 Analyses related to the expression of pluripotency markers in the identified embryonic cells.

(A) Hierachical clustering by the connection specific index (CSI) of differentially-expressed pluripotency-associated genes, which were selected from the differentially-expressed gene list (One-sided Mann-Whitney U test, FDR<0.25). Genes expressed in fewer than 6 cells were further exclueded from the list. Three major module cliques (MC1-3) were identifed. The genes specific for DE (higher than both ME and Late EPI) and ME (higher than both DE and Late EPI) are highlighted in the purple and deep green, respectively. The genes enriched in both DE and ME (compared with EPI) are highlighted in the light green. Two submodules (sMC1-2) are also denoted.

(B) Heatmaps displaying the expression of pluripotency-associated genes. The upper panel displays the expression of each gene in each cell. The bars above the heatmap denote embryo membership and lineage. Genes are arranged in the same sequence as in (A). Cells are hierachically clustered. The lower panel is a heatmap showing the average expression values (after z-score normalization) of MC1-, MC2- and MC3- genes in each cell. Cells are arranged in the same order as in the upper panel.

(C) The co-expression network of pluripotency-associated genes based on the CSI values (CSI>0.8). MC1-, MC2- and MC3- genes are denoted by different colored nodes. Edge weights are proportional to the CSI values of two correlated nodes. Red lines indicate a positive correlation and green lines indicate a negative correlation.

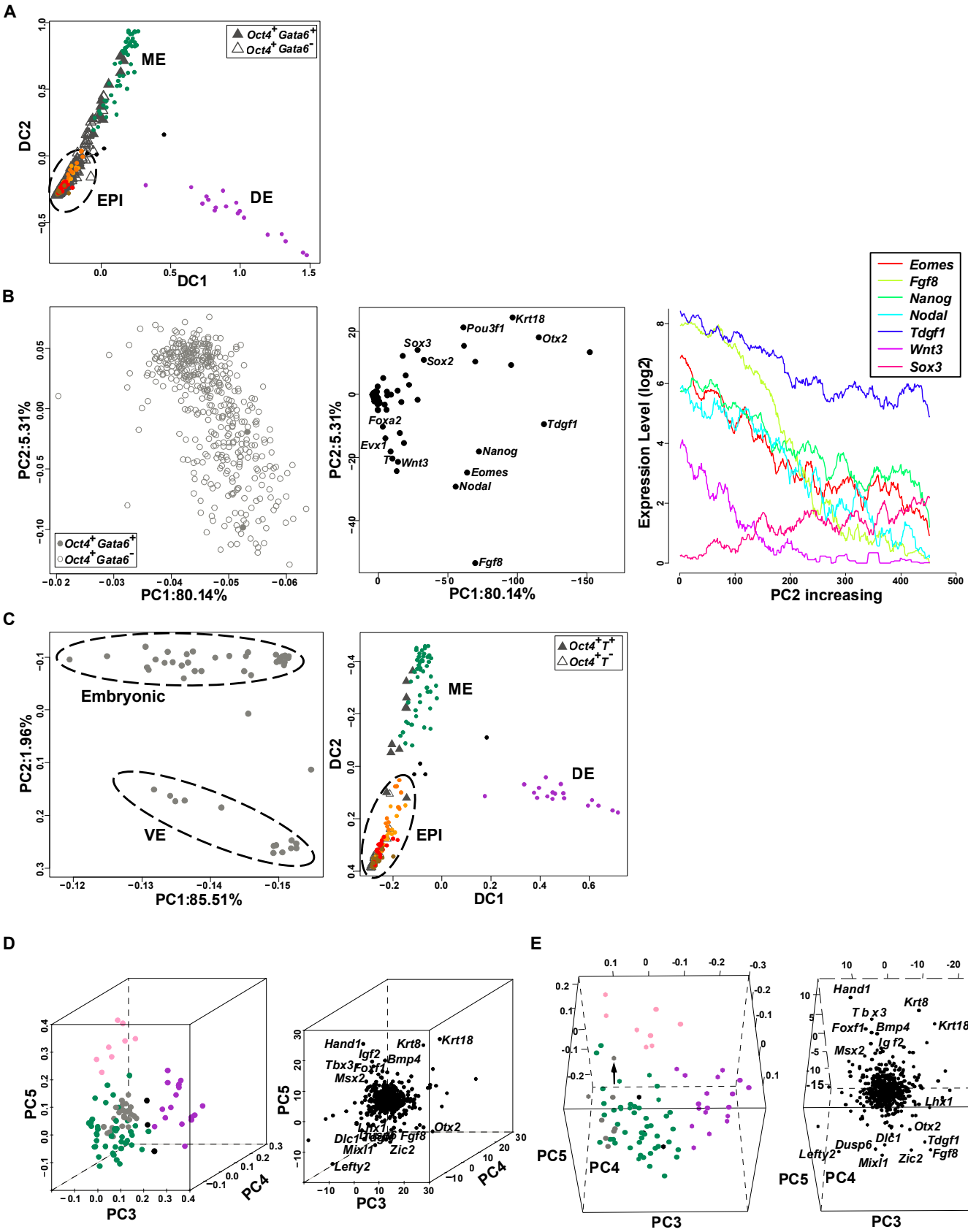
A
Oct4+Gata6+
Oct4+Gata6-
ME
EPI
DE
DC1
DC2
B
PC1:80.14%
PC2:5.31%
Oct4+Gata6+
Oct4+Gata6-
Krt18
Pou3f1
Otx2
Sox3
Sox2
Foxa2
Tdgf1
Evx1
Nanog
T
Wnt3
Eomes
Nodal
Fgf8
Eomes
Fgf8
Nanog
Nodal
Tdgf1
Wnt3
Sox3
Expression Level (log2)
PC2 increasing
C
Embryonic
VE
PC1:85.51%
PC2:1.96%
Oct4+T+
Oct4+T-
ME
DE
EPI
DC1
DC2
D
PC3
PC4
PC5
Hand1
Krt8
Krt18
Igf2
Bmp4
Tbx3
Foxf1
Msx2
Otx2
Lhx1
Dlc1
Tdgf1
Fgf8
Mixl1
Zic2
Lefty2
E
PC3
PC4
PC5
Hand1
Krt8
Tbx3
Foxf1
Bmp4
Krt18
Igf2
Msx2
Lhx1
Otx2
Dusp6
Dlc1
Tdgf1
Lefty2
Mixl1
Zic2
Fgf8

Figure S6 Validation of our results using published datasets.

(A) A diffusion map of Scialdone et al.'s 481 E6.5 embryonic cells together with our 214 identified embryonic cells using 90 germ-layer markers. Our cells are colored to denote lineages. Scialdone et al.'s embryonic cells were identified in Figure S1B and colored in the grey. Their *Oct4*$^{+}$*Gata6*$^{+}$ cells are denoted by solid triangles, and their *Oct4*$^{+}$*Gata6*$^{-}$ cells are denoted by hollow triangles. 452 of their cells clustered with our EPI cells and 29 of their cells clustered with our ME cells, the former are circled by a dashed ellipse. None of their cells clustered with our DE cells. 10 out of 13 of Scialdone et al.'s *Oct4*$^{+}$*Gata6*$^{+}$ cells were in the ME cluster, consistent with the notion that the majority of *Oct4*$^{+}$*Gata6*$^{+}$ cells are cells from the PS region.

(B) PCA of Scialdone et al.'s 452 cells in the EPI cluster using $\log_2$(RPKM+1) values of 90 germ-layer markers. The left panel is PC projections of cells and the middle panel is PC loadings of genes. The right panel is moving average analyses performed for the genes displayed in Figure 2D. Cells are ordered according to their PC2 projections. The distribution and changing trends of mesendodermal genes *Fgf8*, *Nodal*, *Eomes*, *Nanog*, *Tdgf1*, *Wnt3* and neuroectodermal gene *Sox3* along the PC2 axis are similar to those in Figure 2D.

(C) Identification and characterization of Nakamura et al.(2)'s E5.5 and E6.5 EPI cells. Nakamura et al.'s EPI cells could be identified by PCA using either transcriptome data (the left panel) or specific markers we identified in Table S2 (data not shown). 34 out of 49 cells should be embryonic cells (denoted by "Embryonic"), and were subject to diffusion map analyses together with our identified 214 embryonic cells using $\log_2$(RPKM+1) values of 90 germ-layer markers (the right panel). Nakamura et al.'s cells in the ME cluster are mainly *Oct4*$^{+}$*T*$^{+}$ cells, suggesting that these cells are ME cells from the PS region. These cells were all from E6.5 embryos. None of their cells clustered with our DE cells.

(D) Characterization of Scialdone et al. 's E6.5 ME cells by PCA with our MEN cells using 822 germ-layer markers. Scialdone et al. 's E6.5 ME cells are identified in (A) as cells in the ME cluster, and colored in the grey. Our cells are the same MEN cells analyzed in Figure 6A, colored by the purple, deep green, light rose and black to denote DE, ME, EXEM and Other, respectively. Scialdone's E6.5 ME cells clustered with our ME cells. No cells clustered with our EXEM cells.

(E) Characterization of Nakamura's E6.5 ME cells by PCA with our MEN cells using 822 germ-layer markers. Nakamura's E6.5 ME cells are identified in (C) as cells in the ME cluster, and colored in the grey. Our cells are the same MEN cells analyzed in Figure 6A, colored by the purple, deep green, light green and black to denote DE, ME, EXEM and Other, respectively. Nakamura's E6.5 ME cells clustered with our ME cells. One of the cells were near the cluster of our EXEM cells, and was indicated by an arrow.

Supplemental Tables and Descritpions of Supplemental Tables

Table S1 Single-cell RNA-Seq transcript counts of 236 cells. The values of log_2(RPKM+1) are represented. The embryo membership, the stage and lineages of the cells are also shown.

Table S2 Genes specifically expressed in the EPI, VE and EXE.
The expression level of each gene was compared among different groups and cell-type specific genes (Mann-Whitney U test, FDR<0.05) are listed. Sheet 1 lists 6 categories of specific genes in our data. Sheet 2 lists the same 6 categories of specific genes in Scialdone et al.'s data. Sheet 3 lists common specific genes of our data and Scialdone et al.'s data. The differentially-expressed Fgf ligands and receptors, as well as the Gene Ontology analyses of each category of specific genes are also shown in Sheet 3.

Table S3 Expression of germ-layer markers in EPI cells and analyses related to preMEN cells. Sheet 1 contains Log_2(RPKM+1) values of 90 germ-layer specific markers in 108 EPI cells from E5.5 and E6.5_Early embryos (I, II and III). Sheet 2 lists genes that were significantly downregulated or upregulated across PC2 in Figure 2B (Mann-Kendall test, FDR<0.1). Sheet 3 contains 30-ΔCt [30-(Ct-Ct_{Gapdh})] values of *Pou5f1* and 45 germ-layer specific markers in 98 *Oct4*$^+$ cells from E5.5 and E6.5_Early embryos (IV, V and VI). The 90 germ-layer markers in Figure 2A were all examined, but only 45 of the 90 markers were detected in at least one cell here (Ct<40 was considered detected, and Ct>40 was replaced by "0"). Sheet 4 lists genes that were significantly downregulated or upregulted across PC2 in Figure S6B (Mann-Kendall test, FDR<0.1). Sheet 5 lists common genes of respective columns of Sheet 2 and Sheet 4 (common genes enriched in our preMEN cells and Scialdone et al.'s preMEN cells, and common genes downregulated in our preMEN cells and Scialdone et al.'s preMEN cells).

Table S4 Information about the embryonic membership, stage and the number of cells analyzed. The table lists the designations of each embryo, their stage and related figures, as well as the numbers of cells analyzed in them.

	E5.5				**E6.5_Early**		**E6.5_Late**				**Total**
Designation in the manuscript	**I**	**II**	**IV**	**V**	**III**	**VI**	**VII**	**VIII**	**IX**	**X**	
Figures involved	**1,2,4,S1,S4-S6**	**1,2,3A,4,S1,S4-S6**	**S2,3A,3C**	**S2,3A,3C**	**1,2,3A,4,S1,S4-S6**	**S2,3A,3C**	**3A,4,5,3C,3D,6,S3,S4-S6**	**3A,4,5,3C,6,S3,S4-S6**	**3A,3C,S3**	**3A,4,5,3C,6,S3,S4-S6**	
***Oct4*/*Gata6*/*Hand1* examined by qRT-PCR**	**partial**[a]	+	+	+	+	+	+	+	+	+	
Germ layer markers examined by qRT-PCR			+	+		+	+	+	+	+	
The number of cells with germ layer markers examined			**26**	**30**		**42**	**112**	**112**	**29**	**90**	**441**
transcriptome examined by RNA-Seq	+	+			+		+	+		+	
the number of cells examined by RNA-Seq	**21**	**29**			**74**		**14**	**54**		**44**	**236**

[a] : The expression of *Oct4*, *Gata6* and *Hand1* is only partially analyzed in embryo I, causing cells from it not used in the statistical analysis in Figure 3A.

Table S5 qPCR data of examined cells

Shown are 30-ΔCt [30-(Ct-Ct_{Gapdh})] values of 65 germ-layer markers and 5 pluripotency markers (marked by grey) in 441 cells analyzed by single-cell high-throughput qRT-PCR. Cells were from 7 embryos spanning E5.5, E6.5_Early to E6.5_Late stages. *Oct4*$^{+}$*Gata6*$^{-}$*Hand1*$^{-}$ cells are marked in the red, *Oct4*$^{+}$*Gata6*$^{+}$*Hand1*$^{-}$ cells are marked in the deep green, *Oct4*$^{+}$*Gata6*$^{+}$*Hand1*$^{+}$ cells are marked in the light green, and *Oct4*$^{+}$*Gata6*$^{-}$*Hand1*$^{+}$ cells are marked in the rose. The 90 germ-cell markers in Figure 2A were all examined but only 65 of them were detected in at least 1 of these cells (Ct<40 was considered detected, and Ct>40 was replaced by "0"). Data in this table include data in Sheet 3 of Table S3, which is related to Figure S2. The expression of pluripotency markers is related to Figure 3C. The expression of germ-layer markers in E6.5_Late embryos (VII, VIII, IX and X) is related to Figure S3, which is for identification of MEN cells.

Table S6 Analysis of DE and ME cells using single-cell RNA-Seq data.

Sheet 1 lists the expression of 90 germ-layer markers in 214 identified embryonic cells, which contained 108 EPI cells from E5.5 and E6.5_Early embryos and 106 cells from E6.5_Late embryos. Sheet 2 lists names of 822 germ-layer markers. Sheet 3 lists differentially-expressed genes between Early EPI (including Early preMEN and the rest of Early EPI) and Late EPI (including Late preMEN and the rest of Late EPI) groups, and among Late EPI, DE and ME groups. Cells from embryos VII/VIII were considered as one batch and cells from the embryo X were considered as the other batch, and the two batches of EPI, DE and ME cells were compared separately. The common differentially-expressed genes were obtained (Mann-Whitney U test, FDR<0.25). Sheet 4 lists GO analyses of differentially-expressed genes in Sheet 3. The terms mentioned in the text are marked in the red. Sheets 5, 6 and 7 list differentially-expressed Wnt signaling molecules, transcription factors, and pluripotency-associated genes, respectively, which were all selected from Sheet 3.

Table S7 Analysis of differentially-expressed transcription factors and secreted factors between EXEM and the rest of MEN cells.

Transcription factors or secreted factors were compared between 8 EXEM cells and the rest of MEN cells (identified in Figure 6A). Differentially-expressed transcription factors and secreted factors (One-sided Mann-Whitney U test, FDR<0.25) are shown. The lists of transcription factors (TFs, GO: 0003700) and secreted factors (http://www.uniprot.org, keyword:"Secreted [KW-0964]") are also shown.

Supplemental Experimental Procedures

Selection of germ-layer markers

The germ layer-markers were divided into 5 groups as shown in Figure 2A. The first group included markers of the anterior mesendoderm, definitive endoderm, anterior primitive streak and node (AME/DE/APS/Node) (*Sfrp1*, *Cited2*, *Lbh*, *Fzd8*, *Lefty1*, *Hhex*, *Foxa2*, *Gsc*, *Sfrp5*, *Hesx1*, *Cer1*, *Lhx1*, *Dkk1*, *Sox17*, *Bmp7*, *Chrd* and *Nog*) (3-5). Some markers for VE (*Afp*, *Pga5*, *Tbx3*, *Prdm1*, *Pdgfra*, *Gata4*, *Gata6* and *Sox7*) (6-11) were also included because the VE shares many characteristics with the DE (12) and therefore markers for the VE might also be expressed in the DE. The second group was markers reported to express in the posterior PS (*Evx1*, *Cdx4* and *Cdx2*) (3). The third group contained other markers for the PS or the posterior EPI (*Tdgf1*, *Ifitm3*, *Ifitm1*, *Eomes*, *Nanog*, *Lefty2*, *Fgf8*, *Nodal*, *Bmp4*, *Wnt3*, *Wnt5b*, *T*, *Wnt8a*, *Myh6*, *Mixl1*, *Fzd10*, *Mesp1*, *Myl2*, *Mesp2*, *Myod1*, *Meox1*, *Myf5*, *Bmp8b*, *Tgfb1i1*, *Hand1*, *Tbx20*, *Aldh1a2*, *Tbx4*, *Hoxb1*, *Tbx6* and *Nkx2-5*) (3,13-22). Some third-group markers were reported to be NE markers (*Meis1*, *Meis2*, *Irx5* and *Cdh2*) (23,24). However, they were also demonstrated to express in the mesoderm or mesenchyme (25-29). The fourth group contained markers for the NE (*Otx2*, *Pou3f1*, *Churc1*, *Sox2*, *Sox3*, *Zic2*, *Nes*, *Fabp7*, *Otx1*, *Neurod1*, *Foxg1*, *Lhx2*, *Pax6*, *Six1*, *Sox1*, *Pax2*, *Neurog2*, *Six3*, *Gbx2*, *Hoxb4*, *Dach1*, *Olig3*, *Hoxa1*, *Zic1* and *Hoxc8*) (13-15,23,30-32). And the fifth group contained epidermis markers (*Krt8* and *Krt18*) (14).

qRT-PCR

The sequence of each marker was copied from the IGV software, taking sequencing reads coverage into consideration. Taqman probes and primers were designed and produced by commercial suppliers (Shinegene or Genepharma), diluted into 20 μM for each mixture and stored. 0.1μL of the mixture was used for a 10 μL reaction system for single-cell qRT-PCR, and 1.25μL of the mixture was used for a 5μL reaction system for single-cell high-throughput qRT-PCR.

Embryo Immunostaining

Embryos were dehydrated in a 50%, 80% and 100% methanol/PBS series for 10 min each step. After further wash with 100% methanol for 10 min, embryos were chilled and bleached in 5% H_2O_2/20% DMSO/methanol for 2 h at $4^{O}C$. Then embryos were washed in 100% methanol for 10 min three times, incubated in 20% DMSO/methanol for 30 min, rehydrated in a 80%, 50% and 0% methanol/PBS series for 10 min each step, and permeabilized in PBS/0.2% Triton X-100 for 30 min twice. Pretreated samples were incubated in PBS/0.2% Triton X-100/20% DMSO/0.3M glycine at room temperature (RT) overnight, then blocked in PBS/0.2% Triton X-100/ 10% DMSO/ 6% Donkey Serum at RT for 2 h. Samples were washed in PBS/0.2% Tween-20 with 10 μg/ml heparin (PTwH) for 1 h twice, then incubated in primary antibody dilutions in PTwH/5% DMSO/3% Donkey Serum at RT overnight. Samples were then washed in PTwH for 15 min 8 times, and incubated in secondary antibody dilutions in PTwH/3% Donkey Serum at RT for 2 h. Samples were finally washed in PTwH for 15 min 4 times before mounting on the glass slide and imaging.

Taqman probes and primers for qRT-PCR

Gene Name	Forward Primer	Reverse Primer	Probe

AFP	CTCAGCAGAGCTGATCGACCT	ACCATTTCTCCTCGCTGAGC	AAGATGGTGAGCATTGCCTCCACG
Bmp4	CCATACCTTGACCCGCAGA	ATAGTGAATGGCGACGGCA	CAAACGTAGTCCCAAGCATCACCCA
Bmp7	GTTTCTGGAACCCTTACATGCTT	GAGCCAACAGACCAACCTCTC	TTGGGGAGGTGAGGGGAAGGCT
Bmp8b	GCATCTACCCACTGAACTCCTG	TATCTGGCTTCATCAGATGTACCA	ACTCCACCAACCACGCCACTATGC
Cdh2	ATATTCTGTTGCATATATCGATCGG	ATCCTAAAATCATTCGCCAAGAG	AGCAGTGAGCGTGGTCAGCATGG
Cdx2	ACCGCAGAGCCAAGGAGA	GAGGCTGGGAAGGTTGTGG	CGGCTGTGGAGGCTGTTGTTGC
Cdx4	GGAGGTTCCGTGCAAAGTGA	TATCTCGATTGGCTGAAATCCA	CTCCATCAGCCCCGGAGAACTGC
Cer1	CTGGTGCAGTTCAGCATCAAG	ATGCAGTTCCATTCGTTTTCC	TTGGTGGGCGAGCAGTGGGA
Chrd	ATGAGCTGTATTACCTGCAGATGTG	GACTCTCCTTCCCTGAGCCAC	ACCCCACTGTGAGCGGGACGATT
Churc1	TGAGATGGCTCAGTGGGTAAGA	TGTGGTTGCTGGGATTTGAAC	ACCCGACTGCTCTTCCGAAGGTCC
Dach1	GCATAGAGCGCAAATATGGTTATA	ATTTGTGTTTTGGTGTAAGCAAGTA	AATTGTGCCCATTTTATGGTCCCCA
Dkk1	GGGAGTTCTCTATGAGGGCG	GAGCCGCACTCCTCATCTTC	CAACTACCAGCCCTACCCTTGCGC
Dppa2	AAACAAACAAAAACCTGGCTGTC	AGAGATGGATGCTGATTTGTCAAC	TCATCAGAAGCCCCTCCTCCTGTCTC
Dppa4	ACTCTTGTGGTGTGATCAGCAGA	GCACCTTCAAAGGCAGAGATG	TGCGGCTGACGCTGAGACTGATAGA
Eomes	TATCTTCTGCCAGGGCCTATAGAG	GCAAGGAATTGAAGGCAGTGAG	CTGAGCCTGAGAGTCAAGCGGGGA
Evx1	GCTGTGTATGCAGAGCAGGTAGA	CCCCTAAATGGCTGGGATGT	TTAATCTTCACCAGCTTTTCCAACGCATG
Fabp7	TTTTGTGAATTACGGTGGTGG	AAAGCATCTACCATCCTTAACCG	CCCGAGTTCCTCCAGTTCCTGCC
Fgf4	CCCTGTTCTGATGGGATTCTCT	TCCAAAGATACAGTCTTGTCCCTG	TTGCTTCAGGCAGGCTGTGGTCC
Fgf8	TTGTGGAGACCGATACTTTTGG	AATACGCAGTCCTTGCCTTTG	CAATTAGCTTCCCCTTCTTGTTCATGCA
Foxa2	CCAACAAGATGCTGACGCTG	GAAGGAGAGAGAGTGGCGGA	ATCTATCAGTGGATCATGGACCTCTTCCC
Foxg1	ACAAACGAAAAAGGAAGGTTGTTTA	TGAAGGCAATCCTTAATTTTGTCTC	TTGGCAACACTGCCCATTCAATTGA
Gapdh	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA	CCGCCTGGAGAAACCTGCCAAGTATG
Gata4	CCTGGAAGACACCCCAATCT	GGTAGTGTCCCGTCCCATCT	CAGAGTGGGGTGGACATGGCCC
Gata6	CCAACCCCGAGAACAGTGAC	CACCAAGAATCCTGTCGCAC	CAGGTCAAGACGGCCTCTACATAGGTGTC
Gbx2	CTGGAACCAGATTTTTACTTGCAC	AGTACCTCTCCCTGACCGAGC	TCGCTGAGTTTGAGGGCGTGGG
Gdf3	GTTGAACTGGAGCTGACCTTGA	GTGGTTGCTCTGTCTGTGGTTC	CCCAGGGACAGACCGCAGAAAAAG
Hand1	CACCACCTACCACCGCAGT	CTGAGCCTTTTCGTTTGGG	CCCGATGCCAGGCCGAGTCA
Hhex	TACACGCACGCCCTACTCC	ACCTCACTTGACCGCCTTTC	CCCTTGCTCTGGAGCCCCTTCC
Hoxb1	TGTGGGTCAGTCGGAAGGA	AGGTTGCGGTCTGCTCAGTT	CGGCTCAGGGCCATATCCTCCG
Ifitm1	GGAAGATGGTGGGTGATACGAC	CTAATGGCACAGACAACGATGAC	CGCCTCCACCGCCAAGTGC
Ifitm3	CGTGAAGTCTAGGGATCGGAA	GCTGAGGACCAAGGTGCTG	ATGGTGGGTGATGTGACTGGAGCC
Irx5	CATTTCAATGGATTAAACCAGACG	TTGCACAGGTCTAGCTGAGACTG	TTGAATCGTGCGGACGTTTTGGC
Krt18	CAGGAATATGAAGCCCTCTTGAA	GGCATCGTTGAGACTGAAATCTT	ATTGCCACCTACCGCCGCTTGC
Krt8	GAGTCTGGGATGCAGAACATGA	TCCCCCATAGGATGAACTCAGT	CATTCATACGAAGACCACCAGCGGC
Lefty1	CACAAGTTGGTTCGTTTCGC	CATCGGGTGCCTTCAGTCA	CCTTGAGCTCCATAGTCCTTGAGGTCC
Lhx1	ACACGGTCTGGCTCTTCACTG	AAAAGTCGAGCGACTGTGGG	CTGGACGAGCATCCTGGCTTCAGTC
Lhx2	TTTGAGCAACTAACTAACCACGTTT	CAAGTAATTCCTCAGCACACACC	AGGATCTCGCCTGGAAACAGAGGGA
Meis1	GTATAAGGTGATGGTTGTTGTTGC	CGTGTGTGTTTAGAAGCCTAACTG	TGGGGACGATGATGCTTGATGTGA
Meis2	CTAGCCGCTGGAGTTAGTGATG	CTGTTGGGCCAATGAGAAAAG	TCAGACAAGTGTCCATCTCGCTCCG
Meox1	CCAAAACCAAGAAAGACTGAAGGA	GGTGGGTCCAGAGATGTGAGA	AGTTCCCACCACCCGGTCCCAC
Mesp1	ATCCCAGGAAAGGCAGGAA	GAAGGTGCCAAGACCAAAGG	CAGTCCCTCATCTCCGCTCTTCAGCA

Mesp2	TATGGCCTTACCTGCACCCT	TGTCACAGAAGAGGCAGATAAAGG	TGTGGATTTGGGCTGCCTTGGA
Nanog	GCTCCGCTCCATAACTTCG	CTTGTGGGGTGCTAAAATGC	CTTTCTGCAGCCTTACGTACAGTTGCA
Nes	CTTGCTAGCCCTGCCTGTCTAC	ACTCATCATTGCTGCTCCTCTG	TAGCACCACCTGCACAGGGTCTGG
Nodal	TCATTTGCCAGACAGAAGCC	TAGGACACTCGCCCTCACAG	CATTGTACTGCTTGGGGTAGATGATCCA
Nog	AGGGTCAAAGATAGGGTCTGGAT	CGGCCAGCACTATCTACACATC	TCGATGAGGTCCACCAGGGGCA
Otx1	TGCCGATTGCTTGGATTACA	CAGTCGGGAGAGTTGAAATTGA	CCGCTGCTTCCTCTGCCTGGAA
Otx2	CGTCCATCTCCCCACTGTCT	TGAGCCAGCATAGCCTTGAC	CCCTTGTCCACTTCCTCCTCCTGC
Pax2	CCTGTAGTTGCTCTCTCGGTAGC	TTTCTGTTATTTCGGGTCGTCAC	CTCCCTGCATGTCTCCTCAACCGTG
Pax6	TCACTTTGTAACTGTCCTGAACTGG	TCCAACTGATACCGTGCCTTC	CCGGGAATGGACTAGAACCAAGGACC
Pga5	CTGAAGGAGGCTCAACACTCAT	GGCTGTATTTCACCGTGTTTG	CCAGACCAATCCTGTTATTTGCCCG
Pou3f1	GGTTTTTATTTATTCGTGGAGCC	CCGATAAATACAGCATACAGCG	TCGGCTCCTGGGGTCCTTCTAACTC
Pou5f1	GAGGAAGCCGACAACAATGA	TCCAGACTCCACCTCACACG	TCAGGAGATATGCAAATCGGAGACCC
Prdm1	ATCCAGAAATGTCAGTCTTGTTGG	AACCAGAGATTCCACACATGCTAG	CAGCCCAGTGACAGCTTGCTTGCTT
Sfrp1	ACAACCTCAGCCACAACTTTCTC	GGACACTCGTGGTTTTTCATTCT	TGAAGAGCCAGTACCTGCTGACAGCCA
Sfrp5	GGGACCGAAAGTTGATTGGA	TGCCCGTCAGGTTGTCTAAC	CCCCTTAAAGCGCAAGGACACCAA
Six1	AGAATTGTCCCTGGCTTCTATGTAC	GTGGACTTGGGTTCCTAAGTGG	TGTCCCCTAGATTGCTGCGGTTGG
Six3	ACCCCACCACCACCTACTACC	CAACATCAGCGTTTGTGACTGTC	CAAAGGACCGCGACGCCAACA
Sox1	ATGGGTTGTGCTCAGTGGTG	AATCAAAGGCACGCTGTCTTG	CCAAGGATAGGGACAACAGCCACCG
Sox17	GGAAGTGTGTAACACTGCTTCTGG	GCTCAGCGGTCTACTATTGCAAC	TCGGGTCGGCAACCGTCAAATG
Sox2	CATGACCAGCTCGCAGACC	GCCTCGGACTTGACCACAG	CATGTCCTACTCGCAGCAGGGCA
Sox3	TCAGAAGCCAGCGGCTCTAG	CGAAAGTTTGGCCGTAACTGTC	AAGTCCCATTTCCGCTGCTCGGG
T	ACAGTCACAGACATTTCTGACAGC	TCACATAGATGGGGGTGACAC	TATGACACGGCCCAAAGCCTCCTC
Tbx20	CTGACGCTGGAGTGAAAAATG	CAAAGTCTGGTGTCTCTCTTCTGAG	AGGACTTTGGTTCCCAGGGCAGAGA
Tbx3	AAGTCCCATTATCCTCAACCTTG	ACAAGCGCTCAGATAAAAATCAAC	CAAGGCGGCAACCAGGAGGAAG
Tbx6	CCGCTACCCTGATTTGGATACT	CCTTCCACAGTTCCTGGTTCTC	TTTGCCCCTTCTCCCATCTGCTCTG
Tdgf1	CATTTGGGACCAGAAAGAACC	AGGGCAGGCACAGAAGGAC	TCGGTCTTTCCAGTTTGTGCCTTCC
Tgfb1i1	TACTGCCAGCCTTGCTTCCT	AGTCTTTTTCACGAATGTGTTTTTG	CTCTTCGGCTGACCTCCTCTTGGGA
Wnt3	CCTTCGCAGTCACACGCTC	CCCCACTTCCAGCCTTCTC	CTCCACCATCTGCGGCTGTGACTC
Wnt5b	GCCAAGACGGGCATCAGA	GCGCTCACTGCATACGTGAA	CAGCACCGTGGACAACACATCTGTCTTT
Zic2	CATTTGTAAACTCCCGGATTGC	GGAAAAAGAAAAGGCCCATCAC	CCTCCCGCCTTTCTCCCATTTCC
Tnfrsf19	TTGACCTGACTGATTCAAAAGTCCT	ACTGGTATAGAGAGGCATCTAGGCA	TCCTCCATCTTGTCCTGGCTTCTGC
Ppp4r4	CAAGTGTGCTAGTAAAAGTTCCACC	CGAGTTCGGAATGAGTCATCA	ACACGTCTTCCGTCTCAGGGCTGGT
Rreb1	GGGAACGGCCTTACATCTG	TCTTCTCAATGTCCTTCCTGGT	CACTACCCATTCACGGTCAAAGCCA
Fat3	AAGTTATCTCAGCATTTGTACCAAT	GGTAACAGTGCCTGAGGACA	TTGCTGGTGGCAAAAACAGACA
Olig1	CCCCACTCCCTGGCAATTAA	TTCTGGCTCTAAACAGGTGGGAT	AGCGGGTGTTCCAAGGAGCGATGTA

Supplemental References

1. Scialdone A, T. Y., Jawaid W, Moignard V, Wilson NK, Macaulay IC, Marioni JC, Göttgens B. (2016) Resolving early mesoderm diversification through single-cell expression profiling. *Nature* **535**, 289-293
2. Nakamura, T., Okamoto, I., Sasaki, K., Yabuta, Y., Iwatani, C., Tsuchiya, H., Seita, Y., Nakamura, S., Yamamoto, T., and Saitou, M. (2016) A developmental coordinate of pluripotency among mice, monkeys and humans. *Nature* **537**, 57-62

3. Kojima, Y., Kaufman-Francis, K., Studdert, J. B., Steiner, K. A., Power, M. D., Loebel, D. A., Jones, V., Hor, A., de Alencastro, G., Logan, G. J., Teber, E. T., Tam, O. H., Stutz, M. D., Alexander, I. E., Pickett, H. A., and Tam, P. P. (2014) The transcriptional and functional properties of mouse epiblast stem cells resemble the anterior primitive streak. *Cell stem cell* **14**, 107-120
4. Davidson, B. P., and Tam, P. P. (2000) The node of the mouse embryo. *Current biology : CB* **10**, R617-619
5. Hermesz, E., Mackem, S., and Mahon, K. A. (1996) Rpx: a novel anterior-restricted homeobox gene progressively activated in the prechordal plate, anterior neural plate and Rathke's pouch of the mouse embryo. *Development* **122**, 41-52
6. Kwon, G. S., Fraser, S. T., Eakin, G. S., Mangano, M., Isern, J., Sahr, K. E., Hadjantonakis, A. K., and Baron, M. H. (2006) Tg(Afp-GFP) expression marks primitive and definitive endoderm lineages during mouse development. *Developmental dynamics : an official publication of the American Association of Anatomists* **235**, 2549-2558
7. Ogaki, S., Harada, S., Shiraki, N., Kume, K., and Kume, S. (2011) An expression profile analysis of ES cell-derived definitive endodermal cells and Pdx1-expressing cells. *BMC developmental biology* **11**, 13
8. Lu, R., Yang, A., and Jin, Y. (2011) Dual functions of T-box 3 (Tbx3) in the control of self-renewal and extraembryonic endoderm differentiation in mouse embryonic stem cells. *The Journal of biological chemistry* **286**, 8425-8436
9. Orr-Urtreger, A., Bedford, M. T., Do, M. S., Eisenbach, L., and Lonai, P. (1992) Developmental expression of the alpha receptor for platelet-derived growth factor, which is deleted in the embryonic lethal Patch mutation. *Development* **115**, 289-303
10. Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S. C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A., Saitou, M., and Surani, M. A. (2005) Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* **436**, 207-213
11. Kanai-Azuma, M., Kanai, Y., Gad, J. M., Tajima, Y., Taya, C., Kurohmaru, M., Sanai, Y., Yonekawa, H., Yazaki, K., Tam, P. P. L., and Hayashi, Y. (2002) Depletion of definitive gut endoderm in Sox17-null mutant mice. *Development* **129**, 2367-2379
12. Sherwood, R. I., Jitianu, C., Cleaver, O., Shaywitz, D. A., Lamenzo, J. O., Chen, A. E., Golub, T. R., and Melton, D. A. (2007) Prospective isolation and global gene expression analysis of definitive and visceral endoderm. *Dev Biol* **304**, 541-555
13. Chng, Z. Z., Teo, A., Pedersen, R. A., and Vallier, L. (2010) SIP1 Mediates Cell-Fate Decisions between Neuroectoderm and Mesendoderm in Human Pluripotent Stem Cells. *Cell Stem Cell* **6**, 59-70
14. Li, L. Y., Liu, C., Biechele, S., Zhu, Q. Q., Song, L., Lanner, F., Jing, N. H., and Rossant, J. (2013) Location of transient ectodermal progenitor potential in mouse development. *Development* **140**, 4533-4543
15. Vallier, L., Reynolds, D., and Pederson, R. A. (2004) Nodal inhibits differentiation

of human embryonic stem cells along the neuroectodermal default pathway. *Dev Biol* **275**, 403-421

16. Arnold, S. J., and Robertson, E. J. (2009) Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo. *Nat Rev Mol Cell Bio* **10**, 91-103
17. Hart, A. H., Hartley, L., Ibrahim, M., and Robb, L. (2004) Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. *Dev Dynam* **230**, 187-198
18. Liberatore, C. M., Searcy-Schrick, R. D., and Yutzey, K. E. (2000) Ventricular expression of tbx5 inhibits normal heart chamber development. *Dev Biol* **223**, 169-180
19. Hochgreb, T., Linhares, V. L., Menezes, D. C., Sampaio, A. C., Yan, C. Y. I., Cardoso, W. V., Rosenthal, N., and Xavier-Neto, J. (2003) A caudorostral wave of RALDH2 conveys anteroposterior information to the cardiac field. *Development* **130**, 5363-5374
20. Brunskill, E. W., Witte, D. P., Yutzey, K. E., and Potter, S. S. (2001) Novel cell lines promote the discovery of genes involved in early heart development. *Dev Biol* **235**, 507-520
21. Takada, S., Stark, K. L., Shea, M. J., Vassileva, G., Mcmahon, J. A., and Mcmahon, A. P. (1994) Wnt-3a Regulates Somite And Tailbud Formation In the Mouse Embryo. *Gene Dev* **8**, 174-189
22. Kraus, F., Haenig, B., and Kispert, A. (2001) Cloning and expression analysis of the mouse T-box gene Tbx20. *Mech Develop* **100**, 87-91
23. Pankratz, M. T., Li, X. J., LaVaute, T. M., Lyons, E. A., Chen, X., and Zhang, S. C. (2007) Directed neural differentiation of human embryonic stem cells via an obligated primitive anterior stage. *Stem Cells* **25**, 1511-1520
24. Cohen, D. R., Cheng, C. W., Cheng, S. H., and Hui, C. C. (2000) Expression of two novel mouse Iroquois homeobox genes during neurogenesis. *Mech Develop* **91**, 317-321
25. Tamplin, O. J., Kinzel, D., Cox, B. J., Bell, C. E., Rossant, J., and Lickert, H. (2008) Microarray analysis of Foxa2 mutant mouse embryos reveals novel gene expression and inductive roles for the gastrula organizer and its derivatives. *Bmc Genomics* **9**, 511
26. Cecconi, F., Proetzel, G., AlvarezBolado, G., Jay, D., and Gruss, P. (1997) Expression of Meis2, a Knotted-related murine homeobox gene, indicates a role in the differentiation of the forebrain and the somitic mesoderm. *Dev Dynam* **210**, 184-190
27. Wang, X. K., Emelyanov, A., Sleptsova-Friedrich, I., Korzh, V., and Gong, Z. Y. (2001) Expression of two novel zebrafish iroquois homologues (ziro1 and ziro5) during early development of axial structures and central nervous system. *Mech Develop* **105**, 191-195
28. Christoffels, V. M., Keijser, A. G. M., Houweling, A. C., Clout, D. E. W., and Moorman, A. F. M. (2000) Patterning the embryonic heart: Identification of five mouse Iroquois homeobox genes in the developing heart. *Dev Biol* **224**, 263-274

29. Sumi, T., Tsuneyoshi, N., Nakatsuji, N., and Suemori, H. (2008) Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/beta-catenin, Activin/Nodal and BMP signaling. *Development* **135**, 2969-2979
30. Smith, J. R., Vallier, L., Lupo, G., Alexander, M., Harris, W. A., and Pedersen, R. A. (2008) Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm. *Dev Biol* **313**, 107-117
31. Brown, L. Y., Kottmann, A. H., and Brown, S. (2003) Immunolocalization of Zic2 expression in the developing mouse forebrain. *Gene Expr Patterns* **3**, 361-367
32. Puschel, A. W., Westerfield, M., and Dressler, G. R. (1992) Comparative-Analysis Of Pax-2 Protein Distributions during Neurulation In Mice And Zebrafish. *Mech Develop* **38**, 197-208