

 Open access • Posted Content • DOI:10.1101/2020.09.29.318279

Reprogramming Epiblast Stem Cells into Pre-Implantation Blastocyst Cell-like Cells

— [Source link](#) 

Kiichiro Tomoda, Kiichiro Tomoda, Haiming Hu, Yoshiki Sahara ...+4 more authors

Institutions: Osaka Medical College, Gladstone Institutes, Otsuka Pharmaceutical, Kyoto University

Published on: 01 Oct 2020 - bioRxiv (Cold Spring Harbor Laboratory)

Topics: Epiblast, Cell potency, Reprogramming, Stem cell and Blastocyst

Related papers:

- [Reprogramming epiblast stem cells into pre-implantation blastocyst cell-like cells.](#)
- [Spatiotemporal Reconstruction of the Human Blastocyst by Single-Cell Gene-Expression Analysis Informs Induction of Naive Pluripotency](#)
- [Direct Induction of the Three Pre-implantation Blastocyst Cell Types from Fibroblasts.](#)
- [Defining the three cell lineages of the human blastocyst by single-cell RNA-seq](#)
- [Comparative Parallel Multi-Omics Analysis During the Induction of Pluripotent and Trophectoderm States](#)

Share this paper:    

View more about this paper here: <https://typeset.io/papers/reprogramming-epiblast-stem-cells-into-pre-implantation-2d8m1m8grx>

1 TITLE

2 **Reprogramming Epiblast Stem Cells into**
3 **Pre-Implantation Blastocyst Cell-like Cells**

4 **Kiichiro Tomoda^{1,2,3}, Haiming Hu⁴, Yoshiki Sahara^{4,5,6}, Hashimita Sanyal⁴, Minoru Takasato^{4,5}, and**
5 **Cody Kime^{4,*}**

6 1 The Gladstone Institutes, San Francisco, CA 94158, US.

7 2 Center for iPS Cell Research and Application, Kyoto 606-8507, Japan.

8 3 Osaka Medical College, Osaka 569-8686, Japan.

9 4 RIKEN Center for Biosystems Dynamics Research, Kobe 650-0047, Japan.

10 5 Laboratory of Molecular Cell Biology and Development, Department of Animal Development and Physiology, Graduate School of
11 Biostudies, Kyoto University, Kyoto 606-8501, Japan.

12 6 Department of Renal and Cardiovascular Research, New Drug Research Division, Otsuka Pharmaceutical Co. Ltd., Tokushima
13 771-0192, Japan.

14 * Lead Contact

15 **Corresponding Author:** Correspondence should be addressed to C.K. (cody.kime@riken.jp).

16

17 **ABSTRACT**

18 Recently, a new wave of synthetic embryo systems (SEs) have been established from cultured cells
19 toward efficient and ethical embryonic development research. We recently reported our epiblast stem cell
20 (EPISC) reprogramming SES that generates numerous blastocyst (BC)-like hemispheres (BCLH) with
21 pluripotent and extraembryonic cell features detected microscopically. Here, we further explored the
22 system over key time points with unprecedented single-cell RNA sequencing (scRNA-seq) analysis and
23 revealed broad induction of the 2C-like reporter *MERVL* and RNA velocity diverging three major
24 population regions with genetic expression resembling pluripotent epiblast (EPI), primitive endoderm (PE),
25 and trophoderm (TE). Enrichment of those three BC-like cell fates involved key regulons, zygotic
26 genome activation (ZGA) related genes, specific RNA splicing, and select cells meaningfully distinguished
27 critical regulons of model cells. This analysis confirms the induction of the extraembryonic cell populations
28 during the reprogramming and we anticipate that our unique BCLH SES and rich data may uncover new
29 facets of cell potency, improve developmental biology, and help biomedicine advance.

30

31 **KEYWORDS**

32 synthetic embryology; stem cells; reprogramming; early embryo; synthetic biology

33 INTRODUCTION

34 Researching early embryonic development was the basis for developmental biology and subsequent
35 stem cell biology. In recent decades embryology have shed light on the mammalian embryo with animal
36 models for broad significance and to address an ethically bound human embryology (Hyun et al., 2020;
37 Rossant and Tam, 2009, 2017). At first, early embryos were used to derive various pluripotent and
38 multipotent stem cell lines cultures with characteristics and chimeric potential analogous to the assumed
39 origin (Evans and Kaufman, 1981; Martin, 1981). However, the ability to form synthetic embryos from
40 cultured cells had been elusive until recent advances enabled *in vitro* Synthetic Embryo Systems (SEs).

41 SEs are part of a newly emerging field akin to organoids, but reflecting early embryology through
42 'embryoids' that are far more convincing (Harrison et al., 2017; Kime et al., 2018, 2019; Rivron et al.,
43 2018; Shahbazi and Zernicka-Goetz, 2018; Zheng et al., 2019). Several SEs exist and some focus on
44 modeling the blastocyst (BC) and its three layers of trophectoderm (TE), primitive endoderm (PE), and
45 pluripotent preimplantation epiblast (EPI). Some SEs utilize embryonic stem cell (ESC) and trophoblast
46 stem cell aggregations to model the pre/post-implantation embryo *in vitro*. Others SEs, including work
47 from our group, involve cell reprogramming or unique cell plasticity states that give rise to BC-like cysts
48 from single cultures. Various SE approaches building on BC-like cyst formation *in vitro* continue to be
49 developed and explored as each system pioneers to widen embryology at large.

50 In a related field, reprogramming cells with exogenous factors (e.g., transcription factors, small
51 molecules, cytokines, nutrients) pioneered new dimensions in cell biology by inducing donor cells to
52 desirable and generally unforeseen synthetic states (Davis et al., 1987; Kime et al., 2016, 2019;
53 Takahashi et al., 2007; Woogeng et al., 2020). Indeed, cell analogs of early embryonic BC-lineage cells
54 have been induced (Benchetrit et al., 2019; Kubaczka et al., 2015; Parenti et al., 2016; Takahashi and
55 Yamanaka, 2006). Our past epiblast stem cell (EPISC) reprogramming induced high-quality chimera-
56 forming naïve-like cells with X-chromosome reactivation (Kime et al., 2016). We recently showed that the
57 same reprogramming generated plates of BC-like hemispheres (BCLH) with KRT8+ (TROMA-1) TE-like
58 cells surrounding the Xa/Xa EPI-like naïve ESC region which also had a PE-like

59 GATA4+/GATA6+/PDGFRA+ population at its inner-face toward the putative blastocoel (Kime et al., 2018,
60 2019). However, detailed cellular gene expression and regulation of the converting BCLH cells to
61 resemble the three BC-lineage cells was previously unknown.

62 The BCLH SES can be easily set up and generates BCLH efficiently from EPISC cultures. We
63 established EPISC with the 2C-reporter *MERVL* and saw broad early expression prior to BCLH cyst-like
64 formation. We applied single cell RNA sequencing (scRNA-seq) and saw that on Day5, three distinct
65 regions of cells branched with gene expression resembling the blastocyst's TE, PE, and EPI lineages.
66 The three regions each had RNA velocity toward Day7 cells that extended those diverging regions and
67 further enriched convincing cells of the postulated BC-cell identities. Furthermore, RNA splicing regulation
68 and gene regulatory networks implicated significant cell reprogramming had occurred with germ and
69 zygotic genome activation (ZGA) signature genes. Herein we detail these observations and anticipate a
70 welcome interest in the relatively poorly explored aspect of EPISC SES reprogramming into much earlier
71 embryonic cells.

72 **RESULTS**

73 **Naïve ESC in 2iLIF may Stabilize MERVL+ Reporter Expression**

74 For this study, we found TE and PE scRNA-seq data from previous reports, shown later, and required
75 control naïve embryonic stem cells (ESCs). We therefore integrated BL6 ESCs with *MERVL::RFP*
76 reporters that were cultured in our modified media on laminin as previously reported (Kime et al., 2019).
77 Two distinct populations of ESCs stabilized; one with traditional naïve ESC dome-like morphology and
78 transient *MERVL::RFP* expression (Macfarlan et al., 2012), and the other with a unique larger cell
79 morphology and consistent range of *MERVL::RFP* expression (Figure 1A). Our scRNA-seq sample of the
80 culture confirmed our suspicion of a ‘duality’ because cells clustered into two distinct groups (Figure 1B)
81 that we bisected at the origin of UMAP_1: the left cluster termed ‘ESC’ and the right cluster with much
82 more *MERVL::RFP* ‘2C-like’ reporter expression (Figure 1B,C) termed ‘ESC2CL’. Although technically
83 cultured and sampled as one, the ESC2CL had higher scRNA-seq features and counts (Figure S1A).
84 When compared, both populations generally retained similar core pluripotency features (Figure 1D)
85 although differential gene markers could also be identified (Table S1, Table S2) with little outstanding
86 genes each group’s top 20 (Figure S1B). For technical similarity in scRNA-seq sampling of naïve ESC
87 controls and a common interest in ESC *MERVL* reporter activity, we used both ESC and ESC2CL
88 clusters through this study.

89 **The BCLH SES Induces 2C-reporter, XGFP, and Three Regions of Blastocyst-like Lineage Cells**

90 We previously generated (Kime et al., 2018, 2019) EPISC with XGFP and *MERVL::RFP* reporters that are
91 completely off when viewed in fluorescence microscopy (Figure 2A). We induced BCLH reprogramming
92 with and sampled the Day5 and Day7 reprogramming cells along with the starting EPISC and the duality
93 ESC/ESC2CL for scRNA-seq with as standard workflow including SkewC (Abugessaisa et al., 2020) to
94 select high quality cells for analysis (Figure S2A). On Day4 we rarely spotted XGFP while many cells
95 showed *MERVL::RFP* activation that continued through Day5 and Day7 as XGFP activated (Figure 2B).
96 The trend of full colonies expressing the *MERVL* reporter was also visible with a rapidly degrading
97 D2nRFP (Kime et al., 2018; Li et al., 1998) (Figure S2B).

98 We prepared our scRNA-seq samples in Seurat (Butler et al., 2018) with SCTransform and found a
99 clear trend of three regions of cells on Day5 that clustered closely to similar expanding and surrounding
100 regions on Day7 (Figure 2C). In general, cells in the three regions often clustered together while Day7
101 cells had more distinguished separation (Figure 2C). The EPISC, the ESC, and the ESC2CL cells
102 clustered separately (Figure 2C). Surprisingly, many reprogramming cells in all regions had enriched the
103 *MERVL::RFP* expression (Figure 2D,E), while mostly one specific region of Day7 cells expressed the
104 XGFP reporter (Figure 2F) grossly related to naïve pluripotency and the ICM.

105 Checking gene expression revealed that common late BC-lineage cell markers were enriched and
106 relatively focused in the cells of three separated regions with Pluripotent/Epiblast-like, TE-like, and PE-
107 like genes (Figure 2H,I,J). The Epiblast-like region colocalized with the XGFP expression (Figure 2F) and
108 enriched for pluripotent genes (*Pou5f1*, *Zfp42*, *TdGF1*, *Nr0b1*, *Klf2*) (Figure 2H). Importantly, XGFP was
109 detected in the cells where *Klf2*, *Klf4* and *Prdm14* were expressed (Figure 2F,H) relating the importance
110 of those genes to reactivate the inactive X chromosome as previously reported (Gillich et al., 2012; Kime
111 et al., 2016). In the TE-like region, we observed numerous important TE establishing genes (*Ets2*, *Tfap2c*,
112 *Gata2*, *Gata3*, *Elf4*) (Figure 2I) with remarkable expression of *Krt8* and *Krt18* recently reported to
113 organize extraembryonic fate determination in the compacting and polarizing embryo (Lim et al., 2020).
114 Also, the smaller PE-like region there had milder yet focused enrichment for important PE genes (*Pdgfra*,
115 *Gata4*, *Gata6*, *Fgfr1*, *Lifr*, *Lama1*) (Figure 2J).

116 The 2C-like *MERVL* reporter in ESCs has been used in several studies yet its use in naïve ESCs has
117 been limited due to an unclear relationship to ZGA early embryonic-like plasticity. In our SESs we found
118 utility with this reporter (Kime et al., 2018, 2019) related to heightened cell plasticity and therefore
119 checked numerous recently reported ZGA-like regulators and ZGA signature genes derived from powerful
120 screens (Alda-Catalinas et al., 2020). Many ZGA-like regulators were induced in reprogramming cells
121 (Figure 2K), and ZGA signature genes were often highly expressed broadly or regionally in Day5 and
122 Day7 reprogramming cells (Figure S2C), while many were not expressed in the stem cell controls
123 including the ESC2CL cells (Figure S2C). As such, *MERVL* reporter ESCs may have a narrower

124 threshold to activate and reflect smaller differences while de novo MERVL reporter activation in
125 reprogramming cells might indicate a greater composition of ZGA-like genomic remodeling.

126 **Three Regions of Blastocyst-likeness Enrich Over Time**

127 To investigate the state of cells on Day5 and Day7, we employed RNA Velocity with Velocyto (La Manno
128 et al., 2018) to determine the 'direction' of cell change and view RNA splice variation. The EPISC and the
129 ESC/ESC2CL had RNA velocities pointing inward, demonstrating stable states (Figure 3A). As we
130 anticipated there was an obvious trend of RNA Velocity from Day5 toward Day7 among the three regions,
131 all three of which pointed away from each other (Figure 3A). Perhaps reflecting the PE formation of a BC,
132 the ratio of cells driving into the PE-like region was fewer and distributed between the Epiblast-like and
133 extraembryonic-like regions (Figure 2H,J, Figure 3A).

134 Discriminating unspliced (u) pre-mRNA vs spliced (s) mRNAs ratios with scRNA-seq and Velocyto
135 allows for a comprehensive understanding of important state-specific splice mechanisms beyond simple
136 RNA detection. *Tdgf1* is an early BC ICM gene (Pfister et al., 2007) often detected in ESCs, however
137 *Tdgf1* was mostly unspliced in the ESC/ESC2CL while strongly spliced and enriched in the Epiblast-like
138 region of reprogramming cells (Figure 3A,B) where other expression seems to implicate more ICM-like
139 expression. The germ programming genes *Prdm1 (Blimp1)* and *Smc1b* were also splice-enriched in
140 reprogramming cells (Figure S3A) supporting previous consideration of the importance of germ genes
141 toward higher plasticity in our SES reprogramming (Kime et al., 2018, 2019). As seen in base expression
142 (Figure 2K), the ZGA-like regulators *Dppa2*, *Dppa4*, *Tsc22d4*, *Smarca5*, and *Smad1*, were induced
143 across the reprogramming cells with splicing preference (Figure S3B) and the ESC2CL cells had spliced
144 *Dppa2* more efficiently than the ESC. Similar region-specific splice mechanism preference was found with
145 TE and PE genes *Fgfr2* and *Lama1* (Figure 3B) for translation in putative cells. *Pard6b* and *Lifr* followed a
146 similar trend while enriched in reprogramming cells' regions (Figure S3C). Taken together, the regulation
147 of splice mechanisms and time-based BC-like cell region specification is apparent and related to pre-BC
148 stage regulators.

149 To investigate reprogramming cells downstream gene regulation, we randomly downsampled the three

150 regions to similar cell numbers after Seurat clusters 2:TE-like, 5:PE-like, and 4:Epiblast-like with Day-
151 specific labeling (Figure 3C). A heatmap of the top 100 global variable genes confirmed that each cluster
152 had a unique pattern of expression (Figure 3D). We then performed SCENIC (Aibar et al., 2017) analysis
153 to determine cell regulons. Expectedly, each region had distinct patterning that generally enriched from
154 Day5 to Day7 (Figure S3D). The regulons were then binarized to clarify interpretation. The pluripotent
155 Epiblast-like population had largely lost primed pluripotency EPISC-specific regulons (e.g., Pou3f1)
156 (Buecker et al., 2014) and reprogrammed with remarkably similar regulation to the ESC/ESC2CL with
157 well-known naïve pluripotency regulons (e.g. Mybl2, Esrrb, Klf2, Klf4) and shared broader pluripotency
158 continuum regulons (e.g., Sox2, Nanog, Pou5f1) with EPISC (Figure 3E) (Nichols and Smith, 2009;
159 Weinberger et al., 2016). Not surprisingly, the ESC and ESC2CL populations had few differences at the
160 regulatory level.

161 As anticipated, the PE-like and TE-like cells shared important extraembryonic regulons (e.g., Klf6,
162 Kdm5a, Creb3, Elf1, Elf4) (Figure 3E) (Burton et al., 2013; Krendl et al., 2017; Rivron et al., 2018; Yang et
163 al., 2013). Furthermore, the TE-like region had enriched significant TE-specific regulon activity (e.g.,
164 Gata2, Gata3, Ascl2, Pparg) (Figure 3E) including Cdx2 regulon (Figure S3D) (Home et al., 2017; Krendl
165 et al., 2017; Ralston et al., 2010). Also, the PE-like region had some enriched PE-specific regulon activity
166 including Gata6 and Hnf1b (Figure S3D) (Lo Nigro et al., 2017). Average gene expression for the same
167 transcription factors of regulon analysis reflected a similar pattern (Figure 3F), although far less specific,
168 highlighting the value of specific downstream regulation analyses when comparing cells (Aibar et al.,
169 2017; Woogeng et al., 2020).

170 The gene regulation and RNA splice differences raised uncanny distinction of the regions. We
171 investigated numerous mouse RNA Spliceosome genes (Kanehisa and Goto, 2000) and found some with
172 discrete differences among the day-specific regions (Figure S4A). The Mbnl splice factors that repress
173 naïve pluripotency-specific splicing (Han et al., 2013) were only active in the TE-like and PE-like region
174 cells (Figure S4A). Also, Mbnl3 is a core trophoblast gene induced by Gata3 and Cdx2 (Ralston et al.,
175 2010), and was neatly expressed in the TE-like region. Interestingly, *Mbnl2* was one of the top20 markers

176 for the TE-like region among other TE genes (Figure S4B).

177 Taken together, the three diverging regions of BCLH reprogramming cells formed over time with specific
178 epigenetic splicing, expression, and downstream regulation that grossly reflected the three BC-cell
179 lineages; such may explain the controlled order and development of BCLH observed in the cell culture
180 plate (Kime et al., 2019).

181 **Some BCLH Cells Adopt the Regulatory Networks of Established Models**

182 BCLH SES reprogramming induces many cells on the plate regulated spatially to resemble BCLHs (Kime
183 et al., 2019). To better isolate BC-like cells *in silico*, we selected the induced cells that were most
184 ICM/Epiblast-like (iEPI), TE-like (iTE), and PE-like (iPE) cells based on critical gene expression criteria
185 (see methods). We also included comparable numbers of the ESCs/ESC2CL cells and starting EPISC.
186 For established TE and PE model cell data, we sourced a loom file (Posfai et al., 2020) that was built
187 from established reports' scRNA-seq data. We then merged samples and normalized features and counts
188 to integrate the data fairly (see methods).

189 Seurat UMAP clustering from gene expression generally showed distinct populations based on type
190 although some batch effects were obviated separating TE samples that had different origins (Figure 4A).
191 Some TE and PE cells clustered together, as was seen with some iTE and iPE cells, likely based on
192 common extraembryonic expression. Excitingly, analysis with SCENIC showed distinct trends among
193 putative similar cells (Figure S4C) and SCENIC binarized regulon analysis revealed, again, the distinction
194 of three regions of BC-like lineage cells that resembled putative models but with more established BC-like
195 cell factors (Figure 4B). The iEPI cells were regulated alike the ESC/ESC2CL, having lost nearly all
196 EPISC-specific regulons and acquiring those of naïve pluripotency. The iPE were regulated to have PE
197 regulons at similar or low levels and the iTE were regulated to have many of the TE regulons. Expectedly,
198 the induced extraembryonic-like cells (iPE/iTE) had many regulons shared with the extraembryonic PE/TE
199 cell models (Figure 4B) and some differences that appeared to come from batch effect. It was clear that
200 the iPE and iTE samples had lost many EPISC regulons that were also not found in the PE and TE model
201 data, although some remained (Figure 4B). Checking average gene expression for the transcription

202 factors of the regulons expectedly showed a similar pattern with less distinction (Figure 4C), highlighting
203 the importance and power of gene regulatory networks to determine a cell identity (Aibar et al., 2017). We
204 imported the binarized regulon activity tables to the Seurat object and used FindMarkers to identify
205 pluripotent, PE, and TE markers, select the top 10 of each, and plot the associated activity (Figure 4D).
206 Indeed, nearly all top markers discovered were highly reported genes for their correlating cell states (e.g.,
207 Klf2, Mybl2, Nanog, Prdm14 :: Cdx2, Ets2, Gata2, Gata3 :: Sox17, Sox7, Gata4, Gata6), and were
208 regulated relatively neatly among induced and model populations (Figure 4D). Notably, the iPE population
209 had more iTE/TE extraembryonic regulons than the PE population (Figure 4B,D). In general, each of the
210 three BC-like regions in BCLH appeared to be regulated by the critical transcription factors of their
211 putative embryonic equivalents.

212 As shown previously, re-clustering cells based on SCENIC regulon activity can provide clearer identity-
213 based clustering (Aibar et al., 2017; Posfai et al., 2020). Upon doing so in tSNE map, a pattern of cells
214 reflecting the reprogramming and cell states had clarified (Figure 4E) and most of the batch effects
215 between the TE cells was reduced, although some model PE and TE cells still mixed. As seen with the
216 regulon heatmaps, the ESC and ESC2CL cells nearly shared the same tSNE space than when based on
217 gene expression, strengthening the notion that these cells were more similar at the gene regulatory level
218 when cultured in 2iLIF (Figure 4E); expectedly, the iEPI cells now clustered very close to the
219 ESC/ESC2CL cells (Figure 4E). The iTE and iPE cells had dimensionally moved toward the TE and PE
220 models with some iPE cells remarkably close to the PE model cluster (Figure 4E). To view individual BC-
221 lineage critical regulon activity (Figure 4D), we prepared three tSNE plots with lineage-specific regulons
222 on each plot that explained the regulatory activity responsible for the diverse cell types (Figure 4F).

223 At last, select cells of the three major regions of cells on the plate had iEPI, iPE, and iTE, cells present
224 that had undergone remarkable cell reprogramming from primed pluripotent EPISC toward model cells of
225 earlier embryos with sophisticated gene regulation at the regulon and RNA splicing levels.

226 **DISCUSSION**

227 Until now we had seen self-assembly and order to resemble BCs in the BCLH SES (Kime et al., 2018,
228 2019). In addition to confirming those observations, this study provided numerous aspects of gene
229 expression, RNA splicing regulation, and gene regulator network level that greatly strengthened our
230 understanding that EPISC can reprogram to represent BCs. The numerous ZGA signature and germ
231 program related genes provide further inspiration to wonder how the cell reprogramming is engaged
232 although anticipated Dux and Zscan4 expression was not seen at these time points.

233 The BCLH SES is unique from its cell origin, defined conditions, and output. Given that the MERVL
234 reporter activated early on, and Day5 cells neatly branched toward Day7, we anticipate that an earlier
235 time-point of a unified reprogramming precursor cell may exist for exploit. Discovery and optimization of
236 that cell, which reprograms from EPISC, may further advance the clarity and distinction of this SES. We
237 anticipate that lineage tracing based on the Day5 data may help discern key precursors and emerging
238 populations for study and optimization. We would also like to include early BC ICM cells and BC Epiblast-
239 specific cells for scRNA-seq analysis that we could not presently access.

240 The MERVL reporter has had significant utility in our SESs and in the BCLH system it is more broadly
241 induced than in the induced BC-like cysts (iBLC) (Kime et al., 2019) where the defined conditions are
242 modified to different in phases. Conversely, BCLH SES cells proceed more rapidly to less organized BC-
243 like hemispheres instead of puckering from the plate as floating self-organizing cysts. Since the MERVL
244 reporter was highly active in all three regions of this study across Day5 and Day7, it provided some clues
245 about unique early embryonic programs that may be engaging the genome. Surprisingly, the ESC2CL
246 cells in our base condition with apparent *MERVL::RFP* expression had some interesting differences from
247 ESCs in gene expression, but at the regulatory level they near-completely collapsed to the same cluster.
248 We speculate that the MERVL reporter may have significant utility in cell reprogramming that had
249 numerous ZGA related genes and we wonder if our ESC data challenges the reporter's value in 2iLIF.

250 *Cell Reprogramming*

251 In general, the reprogramming cells grossly lost their donor cell state and took on the programs of early

252 BC-like cell lineages which reflected prior observations with high detail. The XGFP+ iEPI population was
253 previously shown as readily potentiated for high contribution in chimeric embryos (Kime et al., 2016).
254 Although the TE-like and PE-like regions could be identified and had convincing cells therein, we wonder
255 if such cells could seed trophoblast stem cell or XEN cell cultures if transferred to appropriate conditions.
256 In the BCLH SES, the TE-like region generally had low *Cdx2* expression despite the specific enrichment
257 of the *Cdx2* regulon, perhaps related to abundant keratin expression and TE-related transcription factor
258 involvement. Interestingly, the TE can be specified independent of *Cdx2* (Wu et al., 2010). Although *Cdx2*
259 is not required for blastocyst formation (Meissner and Jaenisch, 2006), its role in implantation is important
260 and distinct CDX2+ TE is roundly regarded (Strumpf et al., 2005). The PE-like region was less
261 distinguished and had significant overlap between the Epiblast-like and TE-like regions; perhaps the
262 molecular distinction of the emerging iPE is as complicated as its natural ICM-to-extraembryonic transition.
263 Traces of gene expression throughout this study suggested that iPE and PE-like region cells shared a
264 pluripotent-like origin with the iEPI population reminiscent to the GATA4+, GATA6+, PDGFRA+ positive
265 cells arising at the inner-face of BCLH pluripotent cells (Kime et al., 2019). Consideration for PE cells has
266 weighed heavily on various SESs and we suspect that correct hypoblast formation will remain a hinge
267 point for healthy embryoid development.

268 **AUTHOR CONTRIBUTIONS**

269 Conceptualization, C.K.; Methodology, C.K., K.T.; Experimentation, C.K., H.H., H.S., Y.S.; Formal
270 Analysis, C.K., K.T.; Investigation, C.K.; Resources, C.K., M.T., Y.S., K.T.; Writing – Original Draft, C.K.;
271 Writing – Revision & Editing, C.K., K.T.; Visualization, C.K.; Project Supervision, C.K.; Bioinformatics:
272 C.K.; Project Administration and Funding: C.K.

273 **ACKNOWLEDGEMENTS**

274 We are grateful for the support from RIKEN Center for Integrative Medical Sciences for specific training
275 and research environment with support from Erik Arner, Piero Carninci, Imad Abugessaisa, Akira
276 Hasegawa, and Teruaki Kitakura. We also thank Osamu Nishimura and the Shigehiro Kuraku Lab at
277 RIKEN for providing a HPC for Cell Ranger processing. We thank Eszter Posfai of Princeton University,
278 and both Vincent Pasque and Adrian Janiszewski of Katholieke Universiteit (KU) Leuven for providing
279 important control data and thoughtful discussions.

280 *Funding Sources*

281 This study was primarily funded by the RIKEN Center for Biosystems Dynamics Research Organoid
282 Project.

283 **LEGENDS**

284 **Figure 1: Mouse ESCs and MERVL Reporter Expression**

285 **A)** A duality of ESC culture in 2iLIF viewed with brightfield imaging (top) and *MERVL::RFP* expression
286 (bottom). *Scale bars = 200 μ m.*

287 **B)** UMAP based gene expression feature plot for transgenic *MERVL::RFP*.

288 **C)** UMAP plot with newly labeled ESC and ESC2CL populations.

289 **D)** UMAP based gene expression feature plots for pluripotency genes.

290 **Figure 2: BCLH Cell Induction and Regional Gene Expression**

291 **A)** EPISC culture with *MERVL::RFP* and XGFP reporters viewed with brightfield imaging (top)
292 *MERVL::RFP* expression (mid), and XGFP expression (bot). *Scale bars = 200 μ m.*

293 **B)** BCLH reprogramming from EPISC with *MERVL::RFP* and XGFP reporters imaged on Day4, Day5,
294 and Day7, for brightfield (top), *MERVL::RFP* expression (mid), and XGFP expression (bot). *Scale bars =*
295 *100 μ m/Day4, and 200 μ m/Day5:Day7.*

296 **C)** UMAP plot clustering of EPISC, ESC, ESC2CL, Day5, and Day7 samples.

297 **D)** Violin plot of transgenic *MERVL::RFP* reporter expression in EPISC, ESC, ESC2CL, Day5, and Day7
298 samples.

299 **E)** UMAP based gene expression feature plot for transgenic *MERVL::RFP*.

300 **F)** UMAP based gene expression feature plot for transgenic XGFP.

301 **H,I,J)** Multiple colored UMAP based gene expression feature plots for Pluripotent/Epiblast genes (H,
302 green), TE genes (I, purple), and PE genes (J, burgundy) associated for BC-like regional likeness.

303 **K)** UMAP based gene expression feature plots for ZGA-like regulators.

304 **Figure 3: BCLH Three Region RNA Velocity and Gene Regulation**

305 **A)** UMAP based RNA Velocity plot for EPISC, ESC, ESC2CL, Day5, and Day7 samples with BC-like
306 regions labeled based on regional gene expression (Figure 2).

307 **B)** UMAP based RNA splicing plots for spliced (s), unspliced (u) reads, with Cell Type coloring (Figure
308 3A) and residual (resid) unspliced expression shown. RNAs detected are regionally labeled by color for

309 pluripotency (green), TE (purple), and PE (burgundy).

310 **C)** UMAP plot of downsampled day-specific cells and control cells for use in gene heatmap (Figure 3D)

311 and SCENIC regulon analysis (Figure 3E,F, Figure S3D).

312 **D)** Heatmap(DoHeatmap) plot of the top 100 variable features of all cells, ordered by day-specific regions

313 of cells and control cells.

314 **E)** SCENIC Binarized regulons with heatmap(pheatmap) clustering and regulon activity (red scale).

315 **F)** Heatmap(pheatmap) of the row-matched transcription factor average expression (log-transformed) for

316 the regulons of Figure 3E.

317 **Figure 4: Select BCLH SES Cells Reprogram Meaningfully Close to Model Cells**

318 **A)** UMAP based plot for EPISC, ESC, ESC2CL, iEPI, iPE, iTE, PE, and TE samples.

319 **B)** SCENIC Binarized regulons with heatmap(pheatmap) regulon activity (red scale).

320 **C)** Heatmap(pheatmap) of the row-matched transcription factor average expression (log-transformed) for

321 the regulons of Figure 4B.

322 **D)** Pluripotent, PE, and TE, combined Top 10 markers based on Binarized Regulons.

323 **E)** SCENIC tSNE plot based on regulon activity for each sample.

324 **F)** Average Regulon activity in RGB color for pluripotency regulons (red), TE regulons (green), and PE

325 regulons (blue) across SCENIC tSNE plot (Figure 4E).

326 **Figure S1: Related to Figure 1**

327 **A)** Seurat violin plots for features and counts of ESC and ESC2CL samples.

328 **B)** Heatmap(pheatmap) of the average gene expression of the ESC top 20 markers combined with

329 ESC2CL top 20 markers.

330 **Figure S2: Related to Figure 2**

331 **A)** An overview of the Primary and Secondary Analysis of scRNA samples in this study.

332 **B)** BCLH reprogramming from EPISC with *MERVL::D2nRFP* and XGFP reporters imaged on Day4 for

333 brightfield (top), *MERVL::D2nRFP* expression (mid), and XGFP expression (bot). *Scale bar = 100μm*.

334 **K)** UMAP based gene expression feature plots for ZGA signature genes.

335 **Figure S3: Related to Figure 3**

336 **A)** UMAP based RNA splicing plots for spliced (s), unspliced (u) reads, with Cell Type coloring (Figure
337 3A) and residual (resid) unspliced expression shown for germ program factors.

338 **B)** UMAP based RNA splicing plots for spliced (s), unspliced (u) reads, with Cell Type coloring (Figure
339 3A) and residual (resid) unspliced expression shown for germ ZGA-like regulators.

340 **C)** UMAP based RNA splicing plots for spliced (s), unspliced (u) reads, with Cell Type coloring (Figure
341 3A) and residual (resid) unspliced expression for TE (purple) and PE (burgundy) genes.

342 **D)** SCENIC total AUC regulon activity by day-specific cells and control cells.

343 **Figure S4: Related to Figure 3, Figure 4**

344 **A)** Heatmap(DoHeatmap) plot of notable RNA splicing (spliceosome) factors of all cells, ordered by the
345 day-specific cells and control cells.

346 **B)** Heatmap(DoHeatmap) plot of the combined top20 markers per region (2: TE-like, 5: PE-like, and 4:
347 EPI-like) ordered by day-specific cells and control cells.

348 **C)** SCENIC total AUC regulon activity for select iTE, iPE, and iEPI, and control TE, PE, ESC/ESC2CL,
349 and EPISC.

350 **Table S1: ESC Markers**

351 ESC markers discovered from FindMarkers in Seurat.

352 **Table S2: ESC2CL Markers**

353 ESC2CL markers discovered from FindMarkers in Seurat.

354 **EXPERIMENTAL PROCEDURES**

355 **EPISC Culture and BCLH Reprogramming**

356 EPISCs culture and reprogramming was performed as described previously (Kime et al., 2016, 2019),
357 and reprogramming included Sodium Pyruvate.

358 **ESC Culture and MERVL Reporter Integration**

359 BL6 ESCs were converted from 3iLIF conditions and cultured in 2iLIF conditions with CTSFES basal
360 media as described previously (Kime et al., 2019) on iMatrix511 coated 6-well plates. Cells were
361 integrated and selected for *MERVL::RFP* reporters (mCherry and D2nmCherry) in piggyback vectors the
362 same as with EPISC in our previous study (Kime et al., 2019). After several passages, the two different
363 populations of cells became obvious and stabilized.

364 **scRNA-seq Sampling and Processing**

365 Cells were dissociated and passed through cell screen cuvettes to isolate mostly healthy single-cells that
366 were prepared with 10x Chromium Single Cell 3' Library & Gel Bead Kit V3.0. Sample libraries were
367 finalized and sequenced on one HiSeq X lane (150bp PE; Macrogen) for each. Standard Cell Ranger
368 protocol detected sample chemistry and produced 'possorted' BAM files from which the subsequent
369 Primary Analysis workflow in Figure S2A was performed.

370 To match loom data counts for TE and PE control cells (Posfai et al., 2020), we prepared our data
371 tables similarly with DESeq2 size factor normalization prior to merging samples. The iEPI, iTE, and iPE
372 cells were selected with the following criteria:

373 iEPI: $Zfp42 > 0.1$ & $Klf2 > 0.1$ & $EGFP > 0$ & $Prdm14 > 0.001$

374 iTE: $Cdx2 > 0$ & $Gata2 > 0$

375 iPE: $Gata6 > 0$ & ($Sox7 > 0$ | $Gata4 > 0$ | $Sox17 > 0$)

376 **Microscopy**

377 Brightfield and live cell RFP and GFP fluorescence was imaged with a Olympus IX71 Microscope.

378 17

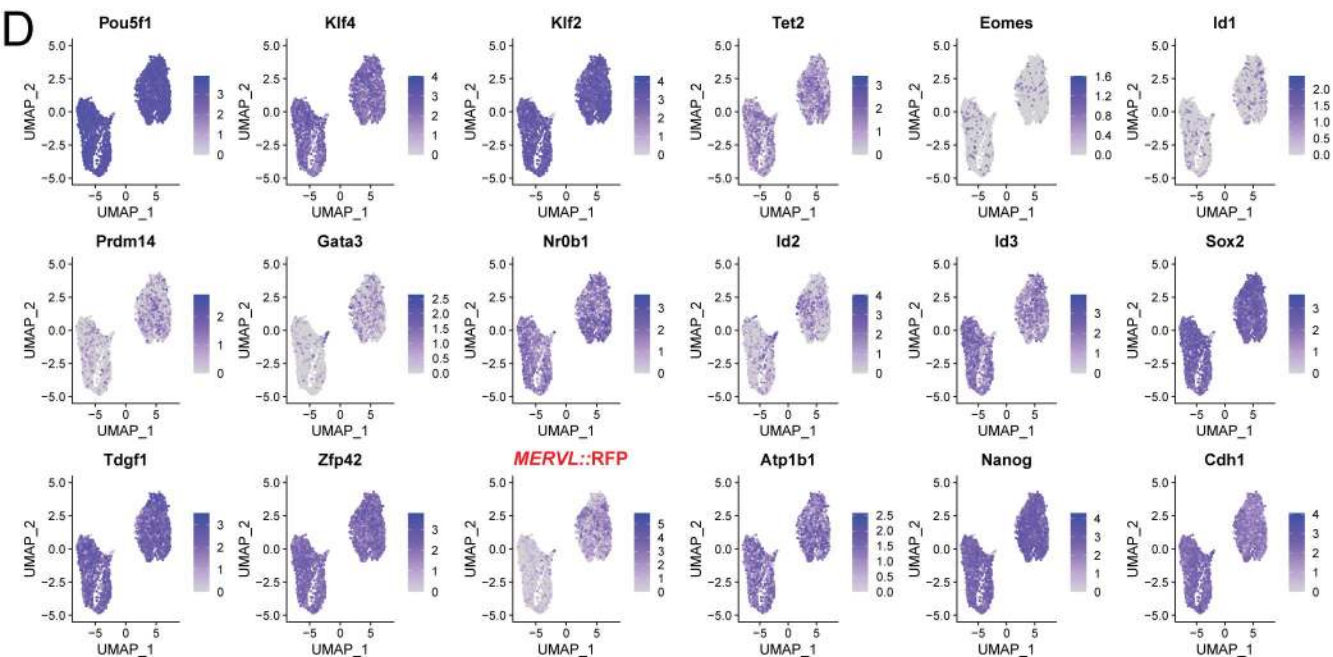
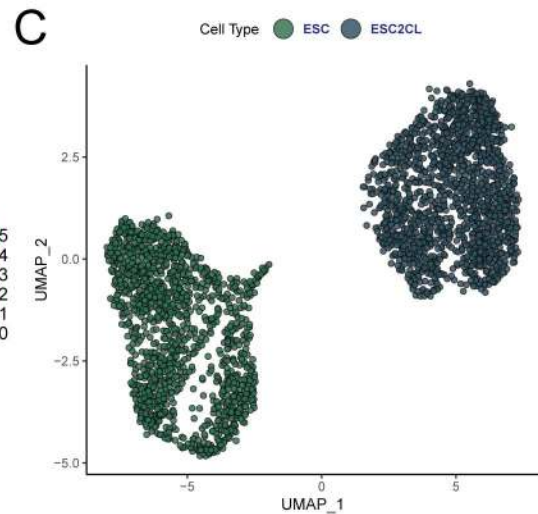
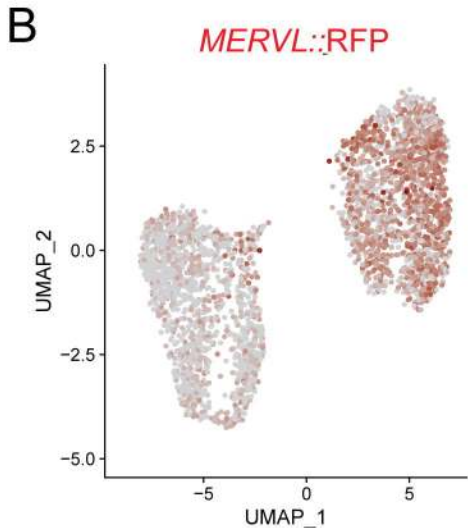
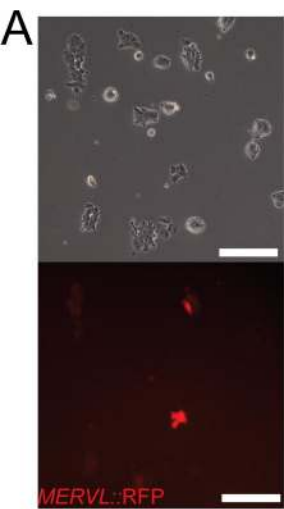
379 **REFERENCES**

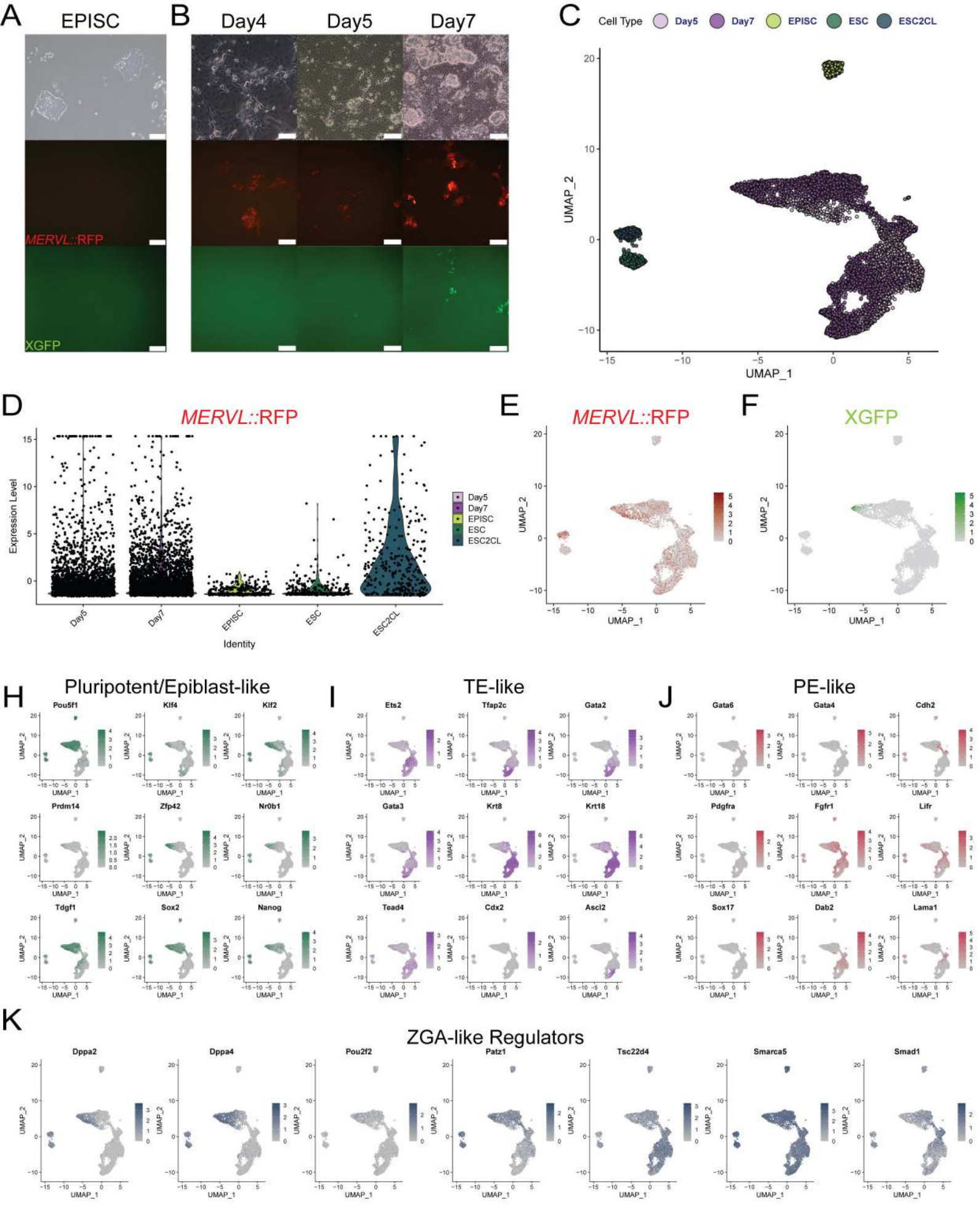
- 380 Abugessaisa, I., Noguchi, S., Cardon, M., Hasegawa, A., Watanabe, K., Takahashi, M., Suzuki, H.,
381 Katayama, S., Kere, J., and Kasukawa, T. (2020). Quality assessment of single-cell RNA sequencing
382 data by coverage skewness analysis. *BioRxiv* 2019.12.31.890269.
- 383 Aibar, S., González-Blas, C.B., Moerman, T., Huynh-Thu, V.A., Imrichova, H., Hulselmans, G., Rambow,
384 F., Marine, J.-C., Geurts, P., Aerts, J., et al. (2017). SCENIC: single-cell regulatory network inference and
385 clustering. *Nat. Methods* *14*, 1083–1086.
- 386 Alda-Catalinas, C., Bredikhin, D., Hernando-Herraez, I., Santos, F., Kubinyecz, O., Eckersley-Maslin,
387 M.A., Stegle, O., and Reik, W. (2020). A Single-Cell Transcriptomics CRISPR-Activation Screen Identifies
388 Epigenetic Regulators of the Zygotic Genome Activation Program. *Cell Syst.* *11*, 25-41.e9.
- 389 Benchetrit, H., Jaber, M., Zayat, V., Sebban, S., Pushett, A., Makedonski, K., Zakheim, Z., Radwan, A.,
390 Maoz, N., Lasry, R., et al. (2019). Direct Induction of the Three Pre-implantation Blastocyst Cell Types
391 from Fibroblasts. *Cell Stem Cell*.
- 392 Buecker, C., Srinivasan, R., Wu, Z., Calo, E., Acampora, D., Faial, T., Simeone, A., Tan, M., Swigut, T.,
393 and Wysocka, J. (2014). Reorganization of Enhancer Patterns in Transition from Naive to Primed
394 Pluripotency. *Cell Stem Cell* *14*, 838–853.
- 395 Burton, A., Muller, J., Tu, S., Padilla-Longoria, P., Guccione, E., and Torres-Padilla, M.-E. (2013). Single-
396 Cell Profiling of Epigenetic Modifiers Identifies PRDM14 as an Inducer of Cell Fate in the Mammalian
397 Embryo. *Cell Rep.* *5*, 687–701.
- 398 Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018). Integrating single-cell
399 transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* *36*, 411–420.
- 400 Davis, R.L., Weintraub, H., and Lassar, A.B. (1987). Expression of a single transfected cDNA converts
401 fibroblasts to myoblasts. *Cell* *51*, 987–1000.
- 402 Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse
403 embryos. *Nature* *292*, 154–156.
- 404 Gillich, A., Bao, S., Grabole, N., Hayashi, K., Trotter, M.W.B., Pasque, V., Magnúsdóttir, E., and Surani,
405 M.A. (2012). Epiblast Stem Cell-Based System Reveals Reprogramming Synergy of Germline Factors.
406 *Cell Stem Cell* *10*, 425–439.
- 407 Han, H., Irimia, M., Ross, P.J., Sung, H.-K., Alipanahi, B., David, L., Golipour, A., Gabut, M., Michael, I.P.,
408 Nachman, E.N., et al. (2013). MBNL proteins repress ES-cell-specific alternative splicing and
409 reprogramming. *Nature* *498*, 241–245.
- 410 Harrison, S.E., Sozen, B., Christodoulou, N., Kyprianou, C., and Zernicka-Goetz, M. (2017). Assembly of
411 embryonic and extraembryonic stem cells to mimic embryogenesis in vitro. *Science* *356*, eaal1810.
- 412 Home, P., Kumar, R.P., Ganguly, A., Saha, B., Milano-Foster, J., Bhattacharya, B., Ray, S.,
413 Gunewardena, S., Paul, A., Camper, S.A., et al. (2017). Genetic redundancy of GATA factors in the
414 extraembryonic trophoblast lineage ensures the progression of preimplantation and postimplantation
415 mammalian development. *Dev. Camb. Engl.* *144*, 876–888.

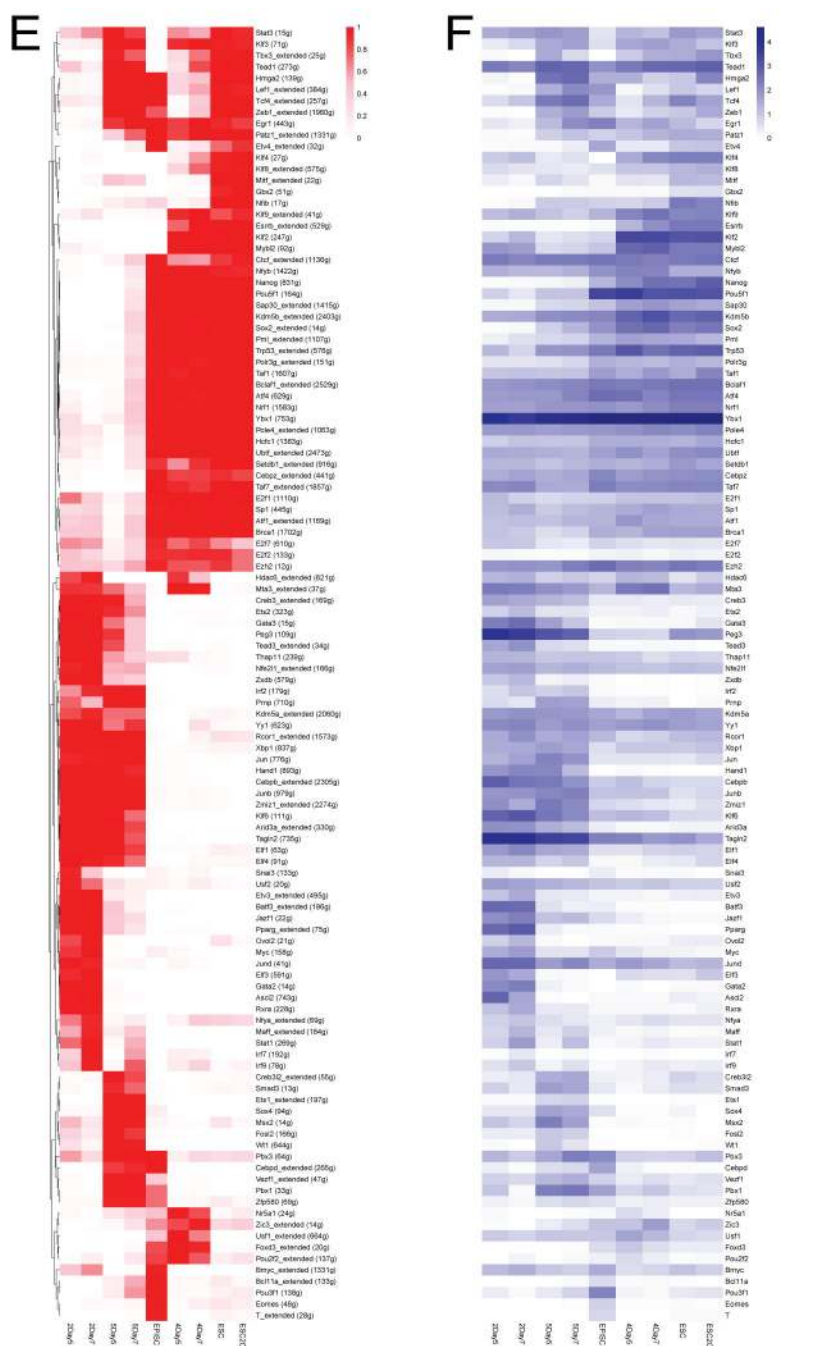
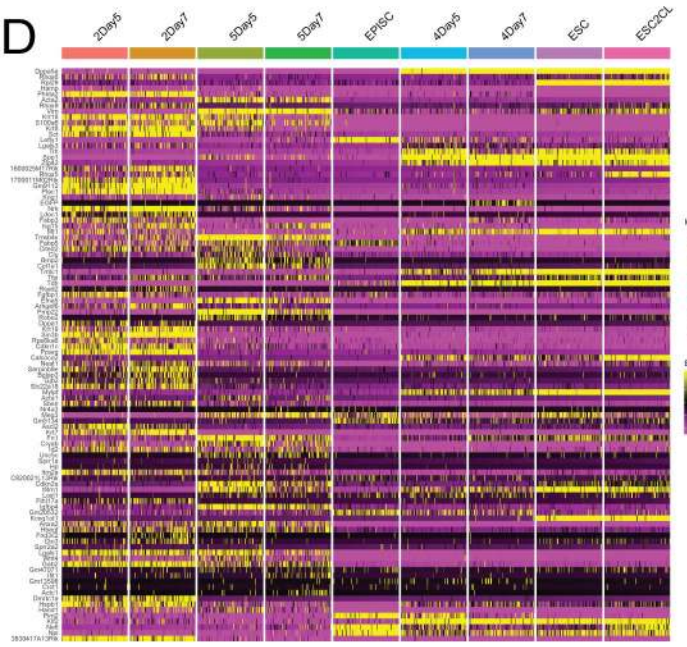
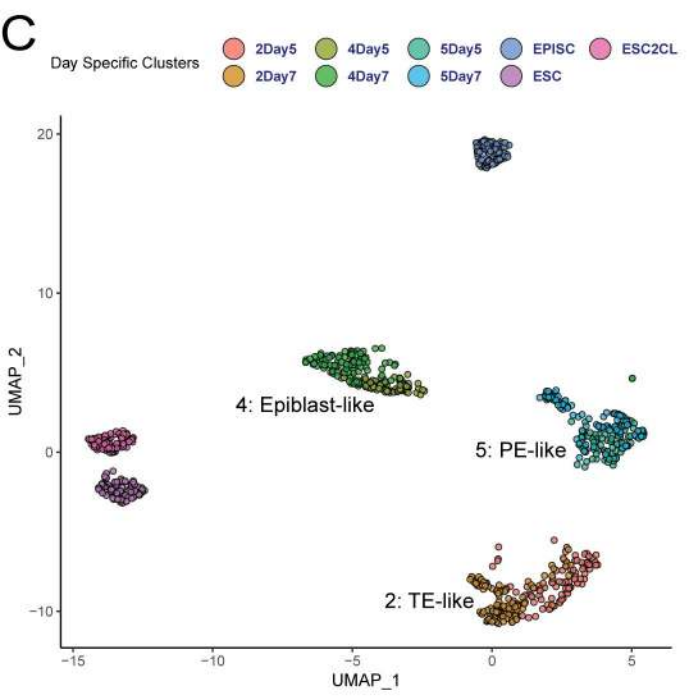
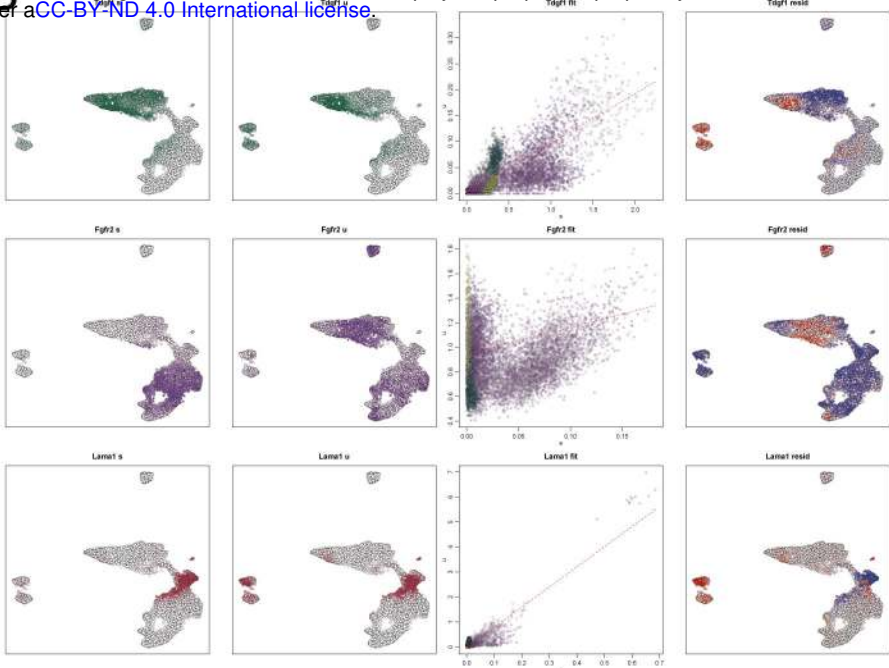
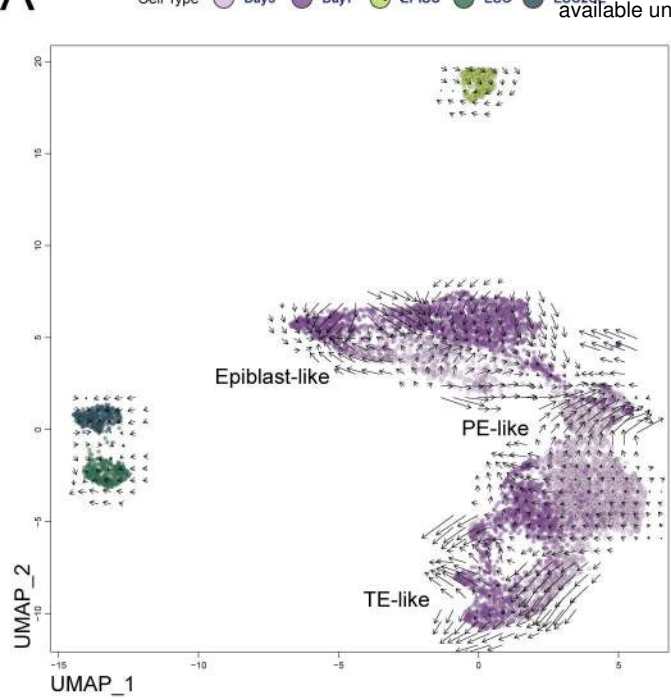
- 416 Hyun, I., Munsie, M., Pera, M.F., Rivron, N.C., and Rossant, J. (2020). Toward Guidelines for Research
417 on Human Embryo Models Formed from Stem Cells. *Stem Cell Rep.* *14*, 169–174.
- 418 Kanehisa, M., and Goto, S. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids*
419 *Res.* *28*, 27–30.
- 420 Kime, C., Sakaki-Yumoto, M., Goodrich, L., Hayashi, Y., Sami, S., Derynck, R., Asahi, M., Panning, B.,
421 Yamanaka, S., and Tomoda, K. (2016). Autotaxin-mediated lipid signaling intersects with LIF and BMP
422 signaling to promote the naive pluripotency transcription factor program. *Proc. Natl. Acad. Sci.* *113*,
423 12478–12483.
- 424 Kime, C., Kiyonari, H., Ohtsuka, S., Kohbayashi, E., Asahi, M., Yamanaka, S., Takahashi, M., and
425 Tomoda, K. (2018). Implantation-Competent Blastocyst-Like Structures from Mouse Pluripotent Stem
426 Cells. *BioRxiv* 309542.
- 427 Kime, C., Kiyonari, H., Ohtsuka, S., Kohbayashi, E., Asahi, M., Yamanaka, S., Takahashi, M., and
428 Tomoda, K. (2019). Induced 2C Expression and Implantation-Competent Blastocyst-like Cysts from
429 Primed Pluripotent Stem Cells. *Stem Cell Rep.* *0*.
- 430 Krendl, C., Shaposhnikov, D., Rishko, V., Ori, C., Ziegenhain, C., Sass, S., Simon, L., Müller, N.S., Straub,
431 T., Brooks, K.E., et al. (2017). GATA2/3-TFAP2A/C transcription factor network couples human
432 pluripotent stem cell differentiation to trophectoderm with repression of pluripotency. *Proc. Natl. Acad. Sci.*
433 *114*, E9579–E9588.
- 434 Kubaczka, C., Senner, C.E., Cierlitz, M., Araúzo-Bravo, M.J., Kuckenberger, P., Peitz, M., Hemberger, M.,
435 and Schorle, H. (2015). Direct Induction of Trophoblast Stem Cells from Murine Fibroblasts. *Cell Stem*
436 *Cell* *17*, 557–568.
- 437 La Manno, G., Soldatov, R., Zeisel, A., Braun, E., Hochgerner, H., Petukhov, V., Lidschreiber, K., Kastrioti,
438 M.E., Lönnerberg, P., Furlan, A., et al. (2018). RNA velocity of single cells. *Nature* *560*, 494–498.
- 439 Li, X., Zhao, X., Fang, Y., Jiang, X., Duong, T., Fan, C., Huang, C.-C., and Kain, S.R. (1998). Generation
440 of Destabilized Green Fluorescent Protein as a Transcription Reporter. *J. Biol. Chem.* *273*, 34970–34975.
- 441 Lim, H.Y.G., Alvarez, Y.D., Gasnier, M., Wang, Y., Tetlak, P., Bissiere, S., Wang, H., Biro, M., and
442 Plachta, N. (2020). Keratins are asymmetrically inherited fate determinants in the mammalian embryo.
443 *Nature* 1–6.
- 444 Lo Nigro, A., de Jaime-Soguero, A., Khoueiry, R., Cho, D.S., Ferlazzo, G.M., Perini, I., Abon Escalona, V.,
445 Aranguren, X.L., Chuva de Sousa Lopes, S.M., Koh, K.P., et al. (2017). PDGFR α + Cells in Embryonic
446 Stem Cell Cultures Represent the In Vitro Equivalent of the Pre-implantation Primitive Endoderm
447 Precursors. *Stem Cell Rep.* *8*, 318–333.
- 448 Macfarlan, T.S., Gifford, W.D., Driscoll, S., Lettieri, K., Rowe, H.M., Bonanomi, D., Firth, A., Singer, O.,
449 Trono, D., and Pfaff, S.L. (2012). Embryonic stem cell potency fluctuates with endogenous retrovirus
450 activity. *Nature* *487*, 57–63.
- 451 Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium
452 conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U A* *78*, 7634–7638.
- 453 Meissner, A., and Jaenisch, R. (2006). Generation of nuclear transfer-derived pluripotent ES cells from
454 cloned Cdx2-deficient blastocysts. *Nature* *439*, 212–215.

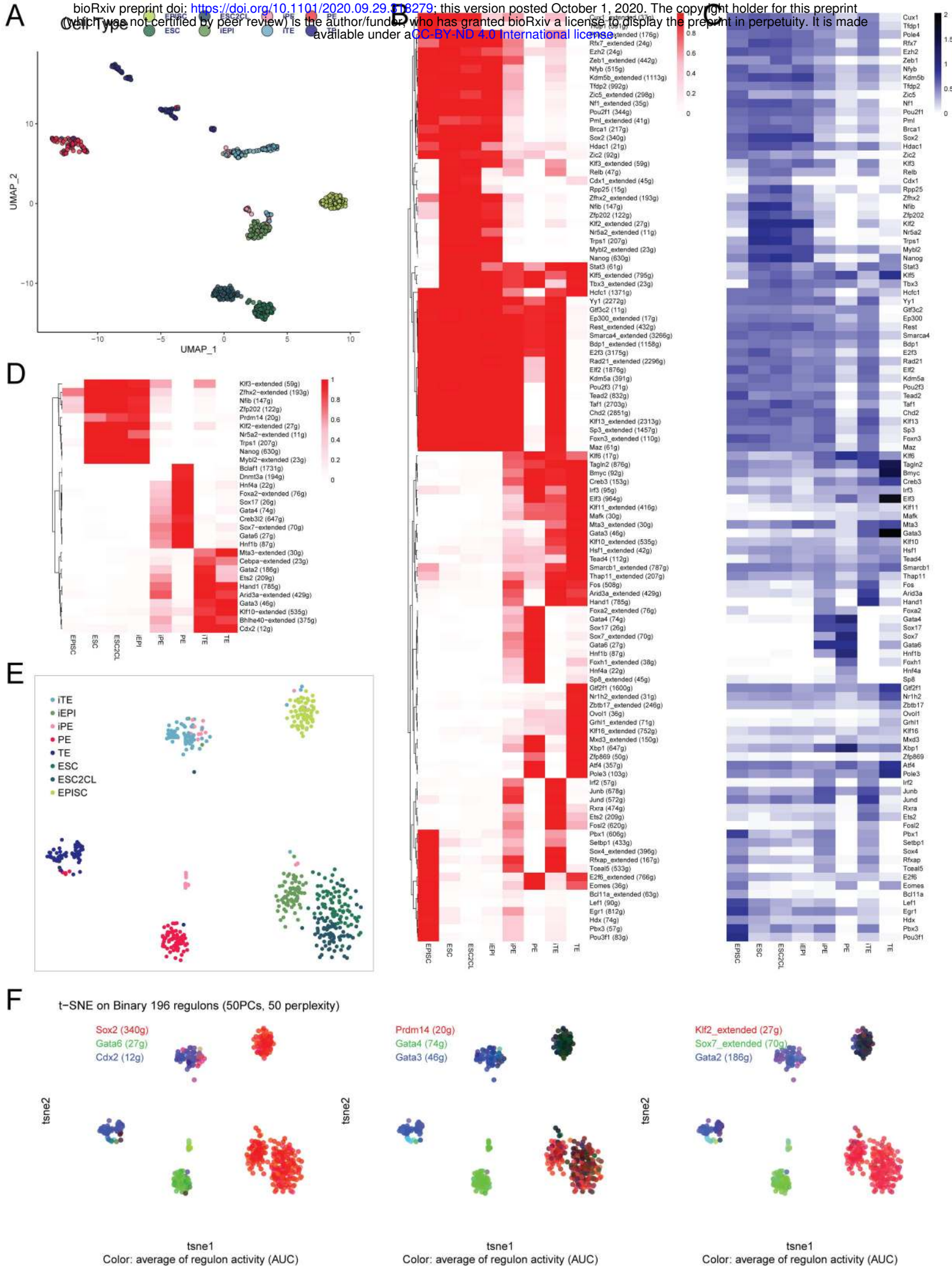
- 455 Nichols, J., and Smith, A. (2009). Naive and primed pluripotent states. *Cell Stem Cell* 4, 487–492.
- 456 Parenti, A., Halbisen, M.A., Wang, K., Latham, K., and Ralston, A. (2016). OSKM Induce Extraembryonic
457 Endoderm Stem Cells in Parallel to Induced Pluripotent Stem Cells. *Stem Cell Rep.* 6, 447–455.
- 458 Pfister, S., Steiner, K.A., and Tam, P.P.L. (2007). Gene expression pattern and progression of
459 embryogenesis in the immediate post-implantation period of mouse development. *Gene Expr. Patterns* 7,
460 558–573.
- 461 Posfai, E., Schell, J.P., Janiszewski, A., Rovic, I., Murray, A., Bradshaw, B., Pardon, T., Bakkali, M.E.,
462 Talon, I., Geest, N.D., et al. (2020). Defining totipotency using criteria of increasing stringency. *BioRxiv*
463 2020.03.02.972893.
- 464 Ralston, A., Cox, B.J., Nishioka, N., Sasaki, H., Chea, E., Rugg-Gunn, P., Guo, G., Robson, P., Draper,
465 J.S., and Rossant, J. (2010). *Gata3* regulates trophoblast development downstream of *Tead4* and in
466 parallel to *Cdx2*. *Development* 137, 395–403.
- 467 Rivron, N.C., Frias-Aldeguer, J., Vrij, E.J., Boisset, J.-C., Korving, J., Vivié, J., Truckenmüller, R.K.,
468 Oudenaarden, A. van, Blitterswijk, C.A. van, and Geijsen, N. (2018). Blastocyst-like structures generated
469 solely from stem cells. *Nature* 557, 106–111.
- 470 Rossant, J., and Tam, P.P. (2009). Blastocyst lineage formation, early embryonic asymmetries and axis
471 patterning in the mouse. *Development* 136, 701–713.
- 472 Rossant, J., and Tam, P.P.L. (2017). New Insights into Early Human Development: Lessons for Stem Cell
473 Derivation and Differentiation. *Cell Stem Cell* 20, 18–28.
- 474 Shahbazi, M.N., and Zernicka-Goetz, M. (2018). Deconstructing and reconstructing the mouse and
475 human early embryo. *Nat. Cell Biol.* 20, 878.
- 476 Strumpf, D., Mao, C.-A., Yamanaka, Y., Ralston, A., Chawengsaksophak, K., Beck, F., and Rossant, J.
477 (2005). *Cdx2* is required for correct cell fate specification and differentiation of trophectoderm in the
478 mouse blastocyst. *Development* 132, 2093–2102.
- 479 Takahashi, K., and Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and
480 Adult Fibroblast Cultures by Defined Factors. *Cell* 126, 663–676.
- 481 Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007).
482 Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* 131, 861–872.
- 483 Weinberger, L., Ayyash, M., Novershtern, N., and Hanna, J.H. (2016). Dynamic stem cell states: naive to
484 primed pluripotency in rodents and humans. *Nat. Rev. Mol. Cell Biol.* 17, 155–169.
- 485 Woogeng, I.N., Abugessaisa, I., Tachibana, A., Sahara, Y., Hon, C.-C., Hasegawa, A., Kaczkowski, B.,
486 Sakai, N., Nishida, M., Hu, H., et al. (2020). Inducing Human Retinal Pigment Epithelium-like Cells from
487 Somatic Tissue. *BioRxiv* 2020.07.27.215103.
- 488 Wu, G., Gentile, L., Fuchikami, T., Sutter, J., Psathaki, K., Esteves, T.C., Araúzo-Bravo, M.J., Ortmeier,
489 C., Verberk, G., Abe, K., et al. (2010). Initiation of trophoblast lineage specification in mouse embryos
490 is independent of *Cdx2*. *Development* 137, 4159–4169.

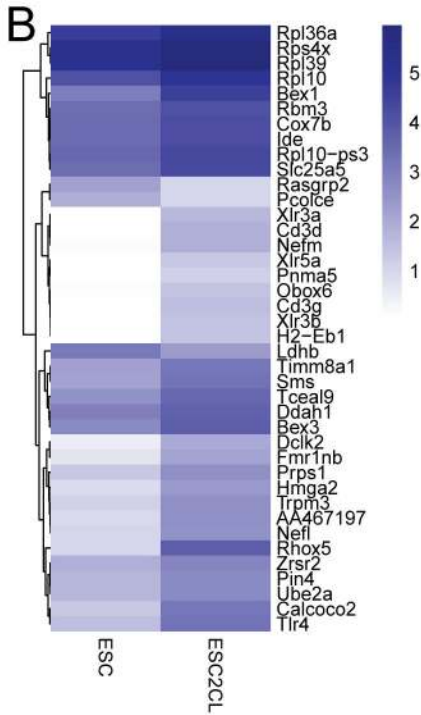
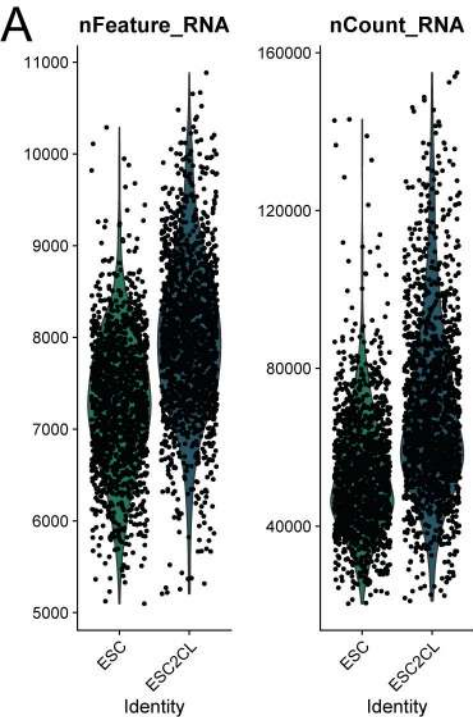
- 491 Yang, Y., JIN, Y., MARTYN, A.C., LIN, P., SONG, Y., CHEN, F., HU, L., CUI, C., LI, X., LI, Q., et al.
492 (2013). Expression Pattern Implicates a Potential Role for Luman Recruitment Factor in the Process of
493 Implantation in Uteri and Development of Preimplantation Embryos in Mice. *J. Reprod. Dev.* *59*, 245–251.
- 494 Zheng, Y., Xue, X., Shao, Y., Wang, S., Esfahani, S.N., Li, Z., Muncie, J.M., Lakins, J.N., Weaver, V.M.,
495 Gumucio, D.L., et al. (2019). Controlled modelling of human epiblast and amnion development using stem
496 cells. *Nature* *573*, 421–425.
- 497

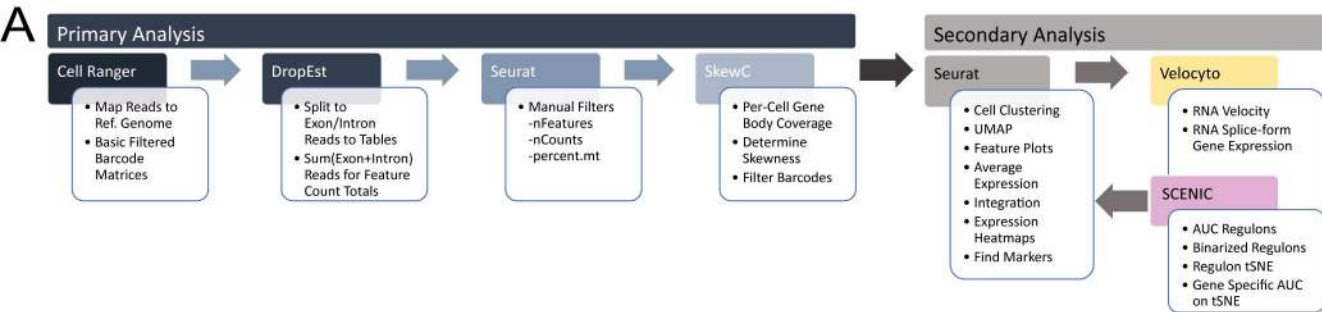




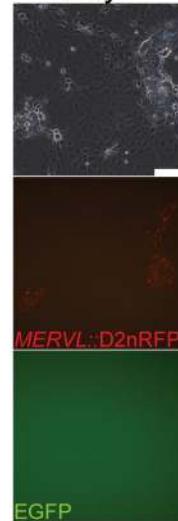








B Day4



C

ZGA Signature Genes

