

1 **Bovine blastocyst like structures derived from stem cell cultures**

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33 Understanding blastocyst formation and implantation is critical for improving farm animal
34 reproduction but is hampered by a limited supply of embryos. We developed an efficient method
35 to generate bovine blastocyst-like structures (termed blastoids) via the assembly of trophoblast
36 stem cells and expanded potential stem cells. Bovine blastoids resemble blastocysts in
37 morphology, cell composition, single-cell transcriptomes, and represent an accessible in vitro
38 model for studying bovine embryogenesis.

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40 Blastoids, were initially developed in mice by assembling embryonic stem cells (ESCs)¹
41 or extended pluripotent stem cells (EPSCs)² with trophoblast stem cells (TSCs), or through
42 EPSC differentiation and self-organization³, have also been successfully generated in humans⁴⁻⁸.
43 To date, however, blastoids from other species have not been reported. Recently, several types of
44 pluripotent stem cells (PSCs), including EPSCs, have been derived from *Bos taurus* blastocysts⁹⁻
45 ¹⁵, which hold great potential to advance animal agriculture¹⁶. Surprisingly, we found a bovine
46 EPSC condition^{13, 17} could support de novo derivation and long-term culture of bovine
47 trophoblast stem cells (TSCs) (Wang et al., manuscript co-submitted). The availability of bovine
48 EPSCs and TSCs prompted us to test whether bovine blastoids could be generated through 3D
49 assembly (**Extended Data Fig. 1a**).

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51 To develop a condition that supports bovine blastoid formation, we adapted the FAC
52 (FGF2, Activin-A and CHIR99021) medium¹⁸, as this medium supports the differentiation of
53 hypoblast-like cells (HLCs) from naïve human PSCs⁴, and added the leukemia inhibitory factor
54 (LIF) that is known to improve preimplantation bovine embryo development¹⁹ (FACL). FGF
55 signaling level can bias the fate of inner cell mass (ICM) likely acting through the MEK-ERK
56 pathway^{20, 21}, where high level of FGF directs ICM cells towards the hypoblast (HYPO, or
57 primitive endoderm [PE]) lineage²². To support both HYPO and epiblast (EPI) lineages, we
58 optimized FGF signaling by lowering FGF2 concentration and including a low dose of a MEK
59 inhibitor (PD0325901, 0.3 μ M), as MEK inhibition has been shown to suppress HYPO fate in
60 bovine embryos in a dose dependent manner²³. This optimized condition, termed titrated
61 FACL+PD03 (tFACL+PD) (see Methods), supported the formation of bovine blastoids with high
62 efficiency (64.2 \pm 7.6%) within 4 days (**Fig. 1a, b, and Extended Data Fig. 1b-h**).
63 Morphologically each bovine blastoid contains a cavity, an outer trophectoderm (TE)-like layer
64 and an ICM-like compartment, which resembles bovine blastocysts produced by *in vitro*
65 fertilization (IVF) (**Fig. 1b, and Supplementary Video 1**). Blastocoele and ICM sizes of day-4
66 bovine blastoids reached diameters equivalent to day-8 IVF blastocysts (**Fig. 1c, d**). We
67 performed immunofluorescence (IF) analysis and found bovine blastoids expressed markers
68 characteristic of EPI (SOX2), HYPO (SOX17) and trophectoderm (TE) (GATA3, KRT18, and
69 CDX2) lineages, and stained positive for a tight junction marker ZO1(TJP1) and an apical
70 marker F-actin (Phalloidin) (**Fig. 1e, Extended Data Fig. 2**). Despite the similarities, we found
71 that the expression levels of some lineage markers were different between blastocysts and
72 blastoids when quantified via IF, with blastoid trophoblast-like cells (TLCs) expressing higher

73 levels of CDX2 and HLCs, and epiblast-like cells (ELCs) expressing lower levels of SOX17 and
74 SOX2 when compared to their corresponding cell types in blastocysts produced by *in vitro*
75 fertilization (IVF) (**Extended Data Fig. 2e**). Analysis of lineage composition in bovine blastoids
76 by flow cytometry further revealed that on average $49.67 \pm 3.29\%$, $31.47 \pm 2.54\%$, $6.58 \pm 1.85\%$
77 cells stained positive for SOX2 (EPI), CDX2 (TE), and SOX17 (HYPO), respectively (**Fig. 1f**,
78 **Extended Data Fig. 3**).

79
80 Next, we evaluated the *in vitro* growth of blastoids and blastocysts under a 3D rotating
81 culture (see Methods). We found trophoblast cells and cavities in both IVF blastocysts and
82 blastoids continued to proliferate and expand over a period of more than 2 weeks, which were
83 also accompanied by an increase in the ICM size (**Fig. 1g-j**, **Extended Data Fig. 4**, and
84 **Supplementary Video 2**). To evaluate whether blastoids can establish pregnancy, we performed
85 embryo transfer to synchronized surrogates (see Methods). Interestingly, we were able to detect
86 the anti-luteolytic hormone interferon-tau ($\text{INF}\tau$) in the surrogate blood. $\text{INF}\tau$ is the signal for
87 maternal recognition of pregnancy in ruminants, which acts by blocking prostaglandin release
88 from the uterus and allowing the corpus luteum to persist and the pregnancy to be maintained²⁴⁻²⁶
89 (**Fig. 1k**). $\text{INF}\tau$ was measured at concentrations of $56.53 \pm 25.13 \text{ pm/ml}$ in 2 out of 4 surrogates 7
90 days following blastoid transfer, which were comparable to those from IVF blastocyst transfers
91 ($78.36 \pm 21.54 \text{ pm/ml}$) in 2 out of 5 surrogates (**Fig. 1l**).

92
93 To determine the transcriptional states of bovine blastoid cells, we performed single-cell
94 RNA-sequencing (scRNA-seq) using the 10x Genomics Chromium platform and carried out
95 integrated analysis with Smart-seq2 single-cell transcriptomes derived from zygote²⁷, 2-cell²⁷, 8-
96 cell²⁸, 16-cell²⁸, morula²⁷ and day 7.5 blastocyst stage IVF bovine embryos²⁷ as well as *in vivo*
97 bovine blastocysts (see data availability). Joint uniform manifold approximation and projection
98 (UMAP) embedding revealed blastoid-derived cells clustered with blastocyst-derived cells (**Fig.**
99 **2a, b**). To further evaluate the temporal identity of blastoid cells, we performed pseudo bulk
100 analysis on the 10x blastoid data to compensate for the differences in sequencing depth to Smart-
101 seq2 data. For this analysis we also included datasets from bovine early gastrulation-stage
102 embryos²⁹. We found that different embryo datasets were orderly arranged on the PCA plot
103 according to their developmental time and blastoid cells were mapped closer to blastocyst cells
104 (**Fig. 2c, d**, and **Extended Data Fig. 5**).

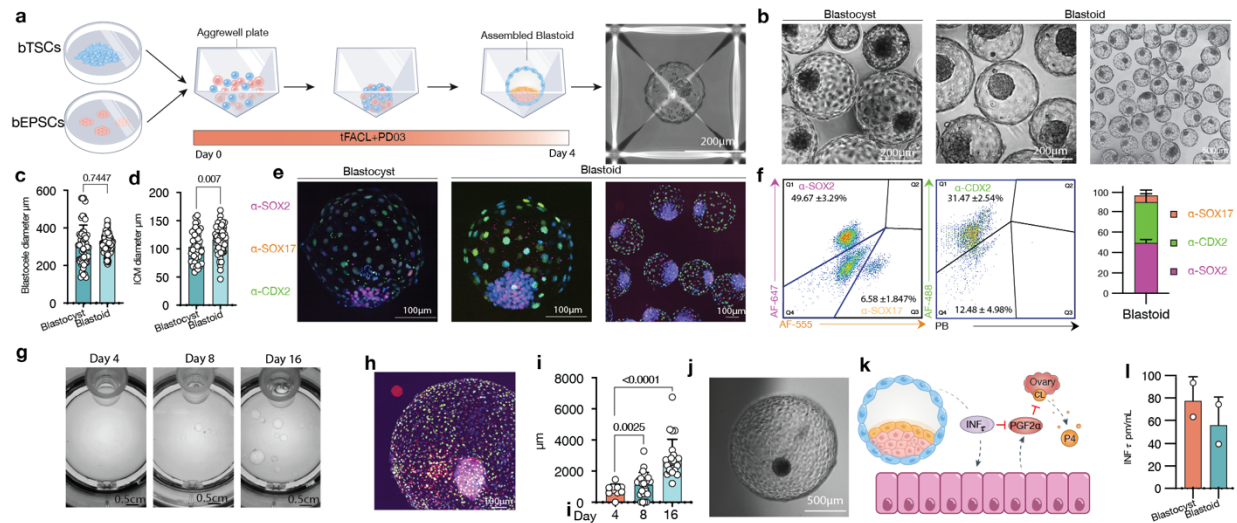
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106 We annotated the six identified cell clusters based on marker gene expression and overlap
107 with cells from bovine embryos (**Fig. 2e-g**). Cluster 3 expresses TE markers, e.g., GATA2 and
108 GATA3, and is annotated as TLCs; Cluster 4 expresses HYPO markers, e.g., GATA4 and
109 SOX17, and thus represents HLCs; Three clusters (0, 1, 2) express EPI markers, e.g., SOX2 and
110 LIN28a, and are designated as ELCs; Cluster 5 is mostly composed of cells from pre-blastocyst
111 stage embryos (named pre-lineage), and each blastoid cluster expressed lineage specific cadherin
112 and tight junction markers (**Fig. 2e-h**, **Extended Data Fig. 6**). To evaluate the relationship

113 between clusters, we performed pseudo time analysis, which predicted the differentiation
114 trajectories from pre-lineage cluster to blastocyst and blastoid lineages (**Fig. 2i, Extended Data**
115 **Fig. 7**). Finally, cross-species comparison revealed similarities and differences of bovine
116 blastoids with human blastoids and blastocysts (**Extended Data Fig. 8**).

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118 In sum, here we report an efficient and robust protocol to generate bovine blastoids by
119 assembling EPSCs and TSCs that can self-organize and faithfully recreate all blastocyst lineages.
120 The bovine blastoids show resemblance to bovine blastocysts in morphology, size, cell number,
121 lineage composition and allocation, and could produce maternal recognition signal upon transfer
122 to recipient cows. The bovine blastoids represent a valuable model to study early embryo
123 development and understand the causes of early embryonic loss. Upon further optimization,
124 bovine blastoid technology could lead to the development of new artificial reproductive
125 technologies for cattle breeding, which may enable a paradigm shift in livestock reproduction.

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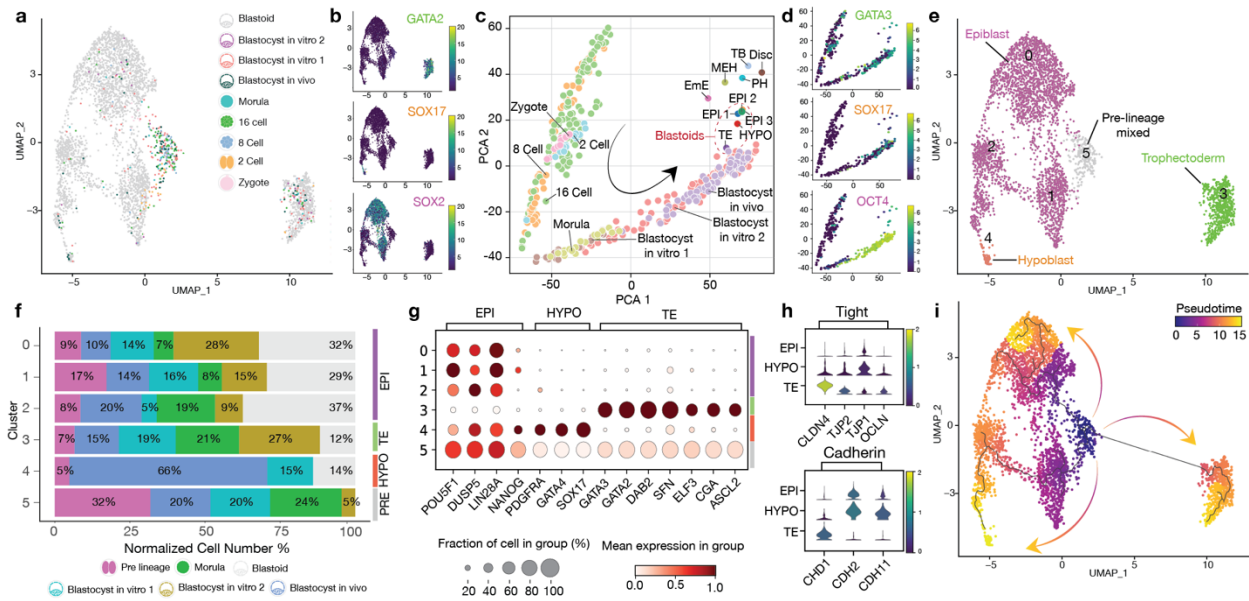
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Figure 1. Assembly of bovine blastoids from EPSCs and TSCs cultures. **a.** Illustration of the assembly process via bovine EPSCs and TSCs aggregation. **b.** Phase-contrast image comparing blastoids vs blastocysts. **c.** Blastocoele diameter measurement. **d.** Inner cell mass (ICM) diameter measurement. **e.** Immunostaining for epiblast marker SOX2 (magenta, EPI), hypoblast marker SOX17 (red, HYPO) and trophoblast marker CDX2 (green, TS), individual markers in Extended Data 1 and 2. **f.** Flow cytometry quantification of single cell dissociated blastoids showing the relative quantities for each lineage, left panel cells are gated from SOX2 and SOX17 negative (Q4) cells in right panel, n=3. **g.** Snapshots of in vitro growth of blastoids in rotating culture system (Clinostar Incubator, Celvivo). **h.** Representative image via immunostaining of all three lineages as in e, individual markers in Extended Data figure 4. **i.** Blastoid diameter quantification. **j.** representative micrographs of in vitro grown blastoid. **k.** A schematic of the maternal recognition of the action of pregnancy signal interferon TAU (INF τ). **l.** Enzyme-linked immunosorbent assay (ELISA) measurement of (INF τ) in surrogate recipients following embryo transfers. PGF2 α : Prostaglandin F2 α . P4: Progesterone.

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Figure 2. Single cell characterization of bovine assembled blastoids. **a.** Joint uniform manifold approximation and projection (UMAP) embedding of 10x Genomics single-cell transcriptomes of bovine blastoids (grey) and bovine zygote (pink), 2 cell (orange), 8 cell (blue), 16 cell (green), Morula (cyan) and *in vivo* and *in vitro* Blastocyst stage embryos (purple, dark green, light red). **b.** UMAP Heatmap showing expression of Trophectoderm (TE), Hypoblast (HYPO), and epiblast (EPI) markers, GATA2, SOX17 and SOX2, respectively **c.** Principal component analysis (PCA) of pseudo bulk conversion of blastoid data. Gastrulation markers²⁹: Disc: Embryonic disc (Day 14 Stage 4). EmE: Embryonic ectoderm (Day 14 Stage 5). MEH: Mesoderm, endoderm and visceral hypoblast. (Day 14 Stage 5). PH: Parietal hypoblast. (Day 14 Stage 5). TB: Trophoblast. (Day 14 Stage 5). **d.** PCA heatmaps showing expression of Trophectoderm (TE), Hypoblast (HYPO), and epiblast (EPI) markers, GATA3, SOX17 and OCT4 (also known as POU5F1), respectively. **e.** Major cluster classification based on marker expression. **f.** Normalized percentage of cells in each cluster. **g.** Dot plot indicating the expression of markers of epiblast (EPI), trophectoderm (TE) and hypoblast (HYPO). **h.** Violin plot of lineage specific cell junction markers. **i.** RNA velocity pseudotime analysis depicting the cell trajectories.

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260 **Materials and Methods**

261

262 **Bovine EPSCs stem cell culture**

263 Bovine female ESCs cultured in NBFR¹⁰ were adapted to the bEPSC³⁰ condition via culture
264 adaptation for a minimum of 5 passages, until a doom morphology was visible. Cell cultures
265 were performed in 0.1% gelatin-coated 6 well plates with 5×10^5 irradiated MEF / STO per well.
266 Upon passaging cells were washed with 1xPBS and dissociated with TrypLE (Thermo Fisher)
267 for 3 minutes at 37°C, cells were then collected with 0.05% BSA in DMEM-F12 (Thermo Fisher)
268 and centrifuged at 1000xg for 3 minutes and resuspended in 1ml of media per 9.6cm². Each
269 passage cells were count using Countess II (Thermo Fisher) and plated at a density of
270 30,000cells/cm², at this plating ratio cells were passaged every 4 days. Upon plating cells were
271 treated with the CETP cocktail³¹, 50 nM chroman-1 (C, Tochriss), 5 μM emricasan (E,
272 Selleckchem), 0.7 μM trans-ISRIB (T, Tochriss), and polyamine supplement (P, Thermo) diluted
273 1x (CETP) was routinely used during the first 12h after passaging. Fresh culture media was
274 added every day. Cells were cultured at 37°C in a 5% CO₂ humidified incubator. Cells were
275 cryopreserved in bEPSC media with 10% DMSO at 0.5×10^6 cells per ml. Detailed description
276 of medias are in Supplementary Table 1.

277

278 **Bovine TSCs stem cell culture**

279 TSCs were cultured in LCDM media (Wang et al., manuscript co-submitted) as stated above
280 with slight modifications (Supplementary Table 1). Upon passaging cells media was removed
281 and treated with Accumax (Thermo Fisher) for 5 minutes at 37°C (No 1xPBS wash), cells were
282 collected with the same volume of bTSC medium and gently lifted of the plate using a wide
283 opening p100 pipette tip and gentle force. Cells were split in a 1:3 ratio and plated in mouse
284 feeder cells with CETP. Only one ml of media was plated in a 6 well for the first 24h to facilitate
285 TSCs attachment. TSCs do not survive well single cell dissociation and tend to form
286 trophospheres if not plated correctly. These steps are critical for the continuous culture and
287 expansion of these cells. Cells were incubated at 37°C in a 5% CO₂ humidified incubator. Cells
288 were cryopreserved in CoolCell 1x cell freezing vial containers (Corning) in 45 % LCDM 45%
289 FBS and 10% DMSO orProFreeze Freezing medium (Lonza, 12-769E) at 2×10^6 cells per ml.

290

291 **Blastoid formation**

292 For blastoid formation EPSCs cells were collected as stated above. Bovine TSCs were washed
293 with 1x PBS, dissociated with Trypsin for 10 minutes at 37 °C, and inactivated with DMEM-F12
294 containing 10% fetal bovine serum (FBS). Cells were washed twice and on final resuspension in
295 their normal culture media with 1x CETP and 10 UI per ml of DNase I (Thermo Fisher). To
296 deplete MEF, cells were placed in precoated 12 well plates (Corning) with 0.1% gelatin and
297 incubated for 15 minutes at 37°C. Single cell dissociation was made by gentle but constant
298 pipetting and by passing the cells through a glass capillary attached to a p200 pipette tip, pulled
299 to an inner diameter of 40-60μm (micropipette puller, Sutter Instruments). After this, cells were
300 collected and strained using a 70μm and then a 37μm cell strainers (Corning). This same single
301 cell dissociation procedure was used for blastoids processing. Cells were stained with 1x trypan
302 blue and manually counted in a Neubauer chamber. Current protocol is optimized for 16 bEPSCs
303 and 16 bTSCs per well in a ~1200 well Aggrewell 400 microwell culture plate (Stemcell
304 technologies) for a total of 19,200 of each cell types per well. Each well was precoated with
305 500μl of Anti-Adherence Rinsing Solution (Stemcell technologies) and spun for 5 minutes at

306 1500 ref. Wells were rinsed with 1ml of PBS just before aggregation. An appropriate number of
307 cells for the wells to be aggregated were centrifuged at 1000xg for 3 minutes and resuspended in
308 1ml of tFACL + 0.3 μ M PD03 media per well, supplemented with 1x CETP. To ensure even
309 distribution, each microwell was gently mixed by pipetting with a P200 pipette to ensure equal
310 distribution of the cells along the microwell, then the plate was centrifuged at 1300xg for 2
311 minutes and put in a humidified incubator at 37°C with 5% CO₂ and 5% Oxygen. As MEK
312 inhibition inhibits hypoblast differentiation a gradual decrease can be done if higher numbers of
313 hypoblast cells are desired from 0.3 to 0.125 μ M.

314

315 **In vitro fertilization**

316 Bovine IVF was performed as previously described³² with modifications. Briefly oocytes were
317 collected at a commercial abattoir (DeSoto Biosciences) and shipped in an MOFA metal bead
318 incubator (MOFA Global) at 38.5°C overnight in sealed sterile vials containing 5% CO₂ in air-
319 equilibrated Medium 199 with Earle's salts (Thermo Fisher), supplemented with 10% fetal
320 bovine serum (Hyclone), 1% penicillin–streptomycin (Invitrogen), 0.2-mM sodium pyruvate, 2-
321 mM L-glutamine (Sigma), and 5.0 mg/mL of Folltropin (Vetoquinol). The oocytes were matured
322 in this medium for 22 to 24 hours. Matured oocytes were washed twice in warm Tyrode lactate
323 (TL) HEPES supplemented with 50 mg/mL of gentamicin (Invitrogen) while being handled on a
324 stereomicroscope (Nikon) equipped with a 38.5°C stage warmer. In vitro fertilization was
325 conducted using a 2-hour pre-equilibrated IVF medium modified TL medium supplemented with
326 250-mM sodium pyruvate, 1% penicillin–streptomycin, 6 mg/mL of fatty acid–free BSA
327 (Sigma), 20-mM penicillamine, 10-mM hypotaurine, and 10 mg/mL of heparin (Sigma) at 38.5
328 C, 5% CO₂ in a humidified air incubator. Frozen semen (Bovine-elite) was thawed at 35°C for 1
329 minute, then separated by centrifugation at 200xg for 20 minutes in a density gradient medium
330 (Isolate, Irvine Scientific) 50% upper and 90% lower. Supernatant was removed; sperm pellet
331 was resuspended in 2-mL modified Tyrode's medium and centrifuged at 200 g for 10 minutes to
332 wash. The sperm pellet was removed and placed into a warm 0.65-mL microtube before bulk
333 fertilizing in Nunc four-well multidishes (VWR) containing up to 50 matured oocytes per well at
334 a concentration of 1.0x10⁶ sperm/mL. 18 hours after insemination, oocytes were cleaned of
335 cumulus cells by constant pipetting for 3-minutes in vortex in 100 μ l drop of TL HEPES with
336 0.05% Hyaluronidase (Sigma), washed in TL HEPES, and then cultured in 500 μ l of IVC media
337 (IVF-Biosciences) supplemented with 0.5xN2B27 (Thermo Fisher) and FLI¹⁹ under mineral oil
338 (Irvine Scientific) cultured until the blastocyst stage. Cleavage rates were recorded on Day 2, and
339 viable embryos were separated from nonviable embryos. Blastocyst rates were recorded on Day
340 8 after IVF.

341

342 **Immunofluorescent staining.**

343 Samples (Cells, single cells, blastoids and blastocysts) were fixed with 4% paraformaldehyde
344 (PFA) in 1xDPBS for 20 min at room temperature, washed in wash buffer (0.1% Triton X-100,
345 5% BSA in 1xDPBS) for 15 minutes and permeabilized with 1% Triton X-100 in PBS for 1 h.
346 For phosphor antibodies samples were treated with 0.5% SDS for 1h. Samples were then blocked
347 with blocking buffer (PBS containing 5% Donkey serum, 5% BSA, and 0.1% Triton X-100) at
348 room temperature for 1 h, or overnight at 4 °C. Because of the large number of blastoids, to
349 facilitate processing blastoids were gently washed out of the aggrewell plate and separated from
350 cell debris using a 100 μ M reversible strainer (Stem cells), blastoids were then placed in a 70 μ m
351 strainer (Corning) in a 6 well plate containing wash buffer and the strainer was moved from one

352 well to another between steps. Primary antibodies were diluted in blocking buffer according to
353 supplementary table 1. Blastoids were incubated in primary antibodies in 96 wells for 2 h at
354 room temperature or overnight at 4 °C. Samples were washed three times for 15 minutes with
355 wash buffer, and incubated with fluorescent-dye conjugated secondary antibodies (AF-488, AF-
356 555 or AF-647, Invitrogen) diluted in blocking buffer (1:300 dilution) for 2 h at room
357 temperature or overnight at 4 °C. Samples were washed three times with PBS-T. Finally, cells
358 were counterstained with 300 nM 4',6-diamidino-2-phenylindole (DAPI) solution at room
359 temperature for 20 min. Phalloidin was directly stained along with other secondary antibodies in
360 blocking buffer.

361 **Imaging**

362 Phase contrast images were taken using a hybrid microscope (Echo Laboratories, CA) equipped
363 with objective x2/0.06 numerical aperture (NA) air, x4/0.13 NA air, x10/0.7 NA air and 20x/0.05
364 NA air. Fluorescence imaging was performed on 8 well μ -siles (Ibidi) on a Nikon CSU-W1
365 spinning-disk super resolution by optical pixel reassignment (SoRa) confocal microscope with
366 objectives x4/0.13 NA, a working distance (WD) of 17.1nm, air; \times 20/0.45 NA, WD 8.9–6.9 nm,
367 air; \times 40/0.6 NA, WD 3.6–2.85 nm, air.

368

369 **Imaging analysis**

370 Imaging experiments were repeated at least twice, with consistent results. In the figure captions n
371 denotes the number of biological repeats. Raw images were first processed in Fiji³³ to create
372 maximal intensity projection (MIP) and an export of representative images. Nuclear
373 segmentation was performed in Ilastik. MIP images and segmentation masks were processed in
374 MATLAB (R2022a) using custom code, which is available in a public repository. Nuclear
375 localized fluorescence intensity was computed for each cell in each field, and the value was then
376 normalized to the DAPI intensity of the same cell. Intensity values of all cells were plotted as
377 mean \pm s.d. Total cells and CDX2, SOX2 and SOX17 positive cell numbers were calculated with
378 Imaris (v.9.9, Oxford).

379

380 **Flow Cytometry**

381 Blastoids were collected under a stereo microscope and single cell dissociated as stated above for
382 the TSCs. Strained single cells were processed as stated above for immunofluorescent staining
383 performing wash steps in 1.5ml Eppendorf tubes on a 90° centrifuge. Flow cytometry was
384 performed using the appropriate unstained and single stain controls in a DBiosciences LSR II
385 flow cytometer and analyzed using Flow Jo. Gating Strategy is shown in Extended Data figure 3.

386

387 **In vitro growth**

388 Prior to use for bovine blastoid culture, the water beads, inside the humidity chamber of the
389 ClinoReactor (CelVivo), were hydrated with sterile water (Corning) overnight at 4°C. Once
390 hydrated and the growth chamber was filled with N2B27 basal media, and the reactor chamber
391 was equilibrated for 1h at 37°C before exchanging for culture media. For rotating-culture
392 blastoids were collected at day 4 post aggregation and placed in pre-equilibrated ClinoReactors
393 in 10ml of tFACL+PD03 media and 1x CETP (Supplementary table 1). ClinoReactors were
394 placed in the ClinoStar incubator at 37 °C with a gas mix of 5%CO₂, 5% O₂ and air. The rotation
395 speed was set between 10 and 12 rpm and was lowered progressively as the blastoids expanded.
396 Optimal growth conditions were achieved by exchanging media every four days. Blastoid and

397 blastocysts growth was also tested on N2B27 with rock inhibitor (Y27632) and activin A as
398 reported in ³⁴. (Extended Data Figure 1 h, I)

399

400 **Embryo Transfer**

401 Surrogate cows were synchronized with an intramuscular (IM) an injection of ovulation-inducing
402 gonadotropin-release hormone (GnRH, Fertagyl), followed by a standard 7-day vaginal
403 controlled drug internal release (CIDR) of progesterone. Upon CIDR removal, one dose of
404 prostaglandin (Lutaluse) was administered. 48 hours after CIDR removal another dose of GnRH
405 was administered via IM injection. A cohort of 15-20 bovine blastoids or 12-15 control IVF
406 blastocysts were loaded into 0.5 mL straws in prewarmed Holding medium (ViGro) and
407 transferred non-surgically to the uterine horn ipsilateral to the ovary with the corpus luteum (CL)
408 as detected by transrectal ultrasound. 7 days after transfer, blastoids where be recovered by
409 standard non-surgical flush with lactated ringers' solution supplemented with 1% fetal bovine
410 serum. All recipients were treated with prostaglandin (Lutaluse) after flushing.

411

412 **Quantitative measurement of Bovine IFN- τ in blood**

413 Blood samples from surrogate and controls were drawn from the coccygeal vein using serum
414 separator tubes. The samples were immediately placed in refrigerator overnight before
415 centrifugation for 15 minutes at 1000 \times g. IFN τ in the serum was determined by Bovine
416 Interferon-Tau ELISA Kit (CSB-E 16948B) according to manufacturer's protocol. Briefly, each
417 well was added 100 μ L standard or sample and incubated for 2 hours at 37 $^{\circ}$. Then, liquid was
418 removed and 100 μ L Biotin-antibody (1X) was added to each well, incubating 1 hour at 37 $^{\circ}$.
419 After aspirating the wells, 200 μ L Wash Buffer was used to wash the wells for three times. After
420 last wash, the plate was inverted and blotted against clean paper towels to remove any remaining
421 Wash Buffer. 100 μ L HRP-avidin (1X) was added to each well and incubated for 1 hour at 37 $^{\circ}$.
422 200 μ L Wash Buffer was used to wash the wells for five times. 90 μ L TMB Substrate was added
423 and incubated for 20 minutes at 37 $^{\circ}$. Protect from light. 50 μ L Stop Solution was added to each
424 well, gently tapping plate to ensure thorough mixing. The plate was measured using microplate
425 reader set to 450 nm.

426

427 **Single-cell RNA-Seq library generation.**

428 Bovine blastoids were single cell dissociated and strained cells were prepared as stated adobe.
429 Cells were washed in PBS containing 0.04% BSA and centrifuged at 90 $^{\circ}$ x500g for 5 min. Cell
430 were resuspended in PBS containing 0.04% BSA at a single cell suspension of 1,000 cells/ μ L.
431 Cells were loaded into a 10x Genomics Chromium Chip following manufacturer instruction (10x
432 Genomics, Pleasanton, CA, Chromium Next GEM Single Cell 3 \times GEM, Library & Gel Bead
433 Kit v3.1) and sequenced by Illumina NextSeq 500/550 sequencing systems (Illumina).

434

435 **Published single-cell data collection**

436 We collected single-cell sequencing data from published literature for comparative analysis. Two
437 Bovine IVF single-cell sequencing raw FASTQ data were downloaded from the GEO database,
438 including 179 IVF cells³⁵ sequenced using Smart-seq2 and 98 IVF cells³⁶ sequenced using
439 STRT-seq.

440

441 **Pre-processing single-cell data**

442 For 10X Genomics single-cell data, we used the Cell Ranger pipeline (v.3.1.0) with default
443 parameters to generate the expression count matrix. The bovine reference genome and gene
444 annotation file were downloaded from Ensembl database (UMD3.1) and generated by Cell
445 Ranger mkfastq with default parameters. Seurat³⁷ (3.1.4) was used to single-cell quality control.
446 To reduce multiplets and dead cells, we screened cells with expressed gene numbers between
447 200 and 6000, unique molecular identifiers (UMIs) between 5000 and 30,000, and mitochondrial
448 RNA genes counts below 15 percent.

449
450 For public Smart-seq2 and STRT-seq data, raw FASTQ reads were trimmed using Trim Galore
451 (0.6.4, https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with default
452 parameters. In order to minimize processing differences, trimmed reads were aligned to the same
453 genome reference (UMD3.1) by using HISAT2³⁸ (2.1.0) with default parameters. Read counts
454 per gene were annotated by HTSeq-count³⁹ software (2.0.2) using the same gene annotation files
455 (UMD3.1). Then, transcripts per million (TPM) were calculated to reduce gene length
456 differences. Also, dead cells were removed by filter mitochondrial gene counts content below
457 15%.

458
459 **Normalization and dimensionality reduction**
460 We used log-percentage value to normalize each single-cell expression matrix, which can reduce
461 the bias of gene expression values caused by different sequencing depths and sequencing
462 methods. In order to reducing the dimension of feature genes and improving the efficiency and
463 accuracy of integration, the variance and mean of genes in each single-cell cohort were used to
464 fit local polynomial regression and filter the top 2000 variable feature genes⁴⁰.

465
466 **Data integration and clustering**
467 The Find Integration Anchors model in the Seurat package was used to find the similarity anchor
468 structure between different single-cell data. Then, we completed the data integration according to
469 the anchors information with 80 dimensions, 20 anchors, 40 candidate cells, and reciprocal PCA
470 for dimensionality reduction ('dims = 1:80, k.anchor = 20, k.filter = 40, reduction = "rpca").
471 Single cells were clustered using the shared nearest neighbor (SNN) modularity optimization-
472 based clustering algorithm in Seurat package, with 90 Principal Component (PC) and 0.6
473 resolution. Then, Uniform manifold approximation and projection (UMAP) was used to reduce
474 the dimensions and show the visualize figure with non-default parameters: 'dims = 1:90'.

475
476 **Gene function annotation**
477 Gene ontology (GO)⁴¹ terms and Kyoto encyclopedia of genes and genomes (KEGG)⁴² pathways
478 enrichment were performed using clusterProfiler⁴³ (3.14.3; org.Bt.eg.db v 3.10.0) with
479 parameter: 'pvalueCutoff = 0.05'.

480
481 **Pseudotime construction**
482 Monocle3⁴⁴ (0.2.3.0) was used for pseudotime analysis, with the UMI matrix and UMAP
483 embedding matrix generated by Seurat as input. Cell pseudotime trend was learnt by using cells
484 in all clusters to generate a single and acyclic structure graph ('use_partition = F, close_loop =
485 F').

486
487 **Data availability**

488 8 cell and 16 cell from GSE99210 (Single-cell RNA sequencing reveals developmental
489 heterogeneity of blastomeres during major genome activation in bovine embryos)²⁸. Zygote, 2
490 cell, 8 cell, morula and blastocyst from PRJNA727165 (Reprogramming barriers in bovine cells
491 nuclear transfer revealed by single-cell RNA-seq analysis)²⁷. Raw unprocessed data of
492 gastrulation embryos was obtained from Dr. Peter L. Pfeffer²⁹. *In vivo* blastocyst and *in vitro*
493 blastocyst1 datasets were obtained from Dr. Zongliang Jiang (GSE215409). Bovine blastoid
494 single cell raw and processed data have been deposited in the Gene Expression Omnibus under
495 accession code (GSE221248).

496

497 **Author contributions**

498 C.A.P-A., Y.Wang., Z.J. and J.W. conceptualized, designed, analyzed, and interpreted the
499 experimental results. C.A.P-A., Y.Wang. and Y.Wei. performed blastoid generation experiments.
500 M.S. helped with in vitro fertilization of bovine embryos. Y.W. and L.Y. helped with
501 immunostaining. C.A.P-A. and Y.Wang. performed extended in vitro culture of bovine
502 blastocysts and blastoids. C.A.P-A., G.S., Y.Wang. and Z.J. performed embryo transfer
503 experiments. J.Y. and B.L. prepared scRNA-seq library. C.A.P-A., L.I. and A.E.R.O. performed
504 scRNA-seq analysis. Z.J. and J.W. supervised the study. C.A.P-A., Z.J. and J.W. wrote the
505 manuscript with inputs from all authors.

506

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517

518 **Conflict of interests**

519 C.A.P-A., Y.Wang., Y.Wei., Z.J. and J.W. are co-inventors on US provisional patent application
520 63/370,068 relating to the Bovine blastocysts like structures and uses thereof.

521

522 **Supplementary Table 1.**

- 523 1. Culture media
- 524 2. Material lists
- 525 3. Antibodies

526

527 **Supplementary Video 1. Bovine blastoid Z plane overview.**

528

529 **Supplementary Video 2. Bovine blastoid 3D rotating culture on day 16.**

530

531 **Extended Data figure 1. Stem cell cultures and Blastoid media optimization. a.**

532 Immunostaining of bovine EPSCs and TSCs for epiblast marker SOX2 (cyan), hypoblast marker
533 SOX17(red) and trophectoderm marker CDX2 (green). **b.** Quantification of blastoid formation

534 efficiency. Immunostaining for epiblast marker SOX2 (magenta), hypoblast marker SOX17(red)
535 and trophectoderm marker CDX2(green) and marker quantification **c-d**. FACL. **e-f**. tFACL. **g-h**.
536 FACL+ PD.

537

538 **Extended Data figure 2. Bovine blastoids immunostaining characterization and comparison**
539 **to IVF blastocysts.** Immunostaining for epiblast marker SOX2 (magenta), hypoblast marker
540 SOX17(red) and trophectoderm marker CDX2(green) **a-b**. IVF Blastocysts and **c-d**. Blastoids. **e**.
541 DAPI normalized relative intensity quantification of side-by-side staining and imaging of
542 blastocysts and blastoids n=5, mean \pm s.d. Immunostaining for epiblast marker SOX2 (red), and
543 trophectoderm marker gata3(magenta). **f**. Blastocyst and **g**. Blastoid. Immunostaining for
544 trophectoderm markers CDX2(green) and Keratin 18 (red). **f**. Blastocyst and **g**. Blastoid. J.
545 Immunostaining for phospho-STAT3(red). **k**. Immunostaining for tight junction marker
546 ZO1(TJP1, green) and apical marker F-actin (Phalloidin, Red).

547

548 **Extended Data figure 3. Blastoids lineage quantification by flow cytometry.** **a**. Unstained
549 control. **b**. Imaging examples of stained cells quantified by flow cytometry. **c-d**. Lineage
550 quantification for epiblast marker SOX2 (AF-647), hypoblast marker SOX17(AF-555, DsRed
551 channel) and trophectoderm marker CDX2(AF-488, GFP channel), autofluorescence control
552 (Pacific blue) and tSNE plots of each of the quantified markers for days 3 and 4 of protocol.

553

554 **Extended Data figure 4. 3D in vitro growth culture immunofluorescence staining.**
555 Immunostaining of bovine blastoids grown in the ClinoStar incubator at day 16 for epiblast
556 marker SOX2 (magenta), hypoblast marker SOX17(red) and trophectoderm marker
557 CDX2(green) in **a**. tFACL+PD media. **b**. A 1 to 1 mix of FACL and tFACL+PD. **c-d**. Phase-
558 contrast image of bovine blastoids grown in the ClinoStar incubator. **e**. Bovine IVF blastocyst
559 grown in in the ClinoStar incubator at day 16 for stained as in a-b. **f-g**. Phase-contrast image of
560 in vitro grown bovine blastocyst. **h-i**. Quantification of invitro grown blastoids and blastocysts
561 on N2NB27 with rock inhibitor (Y27632) and activin A as reported in ³⁴.

562 **Extended Data figure 5.** Principal component analysis (PCA) heatmaps of pseudo bulk
563 conversion of blastoid data. **a**. Color by dataset. Epiblast markers: **b**. NANOG. **c**.
564 POU5F1(OCT4). **d**. PRDM14. Hypoblast markers: **e**. SOX17. **f**. GATA4. **g**. PDGFRA. **h**. FN1. **i**.
565 HNF4A. **j**. HNF1B. **k**. FOXA2. **i**. COL4A1. **m**. MSX2. Trophectoderm markers: **n**. GATA3. **m**.
566 GATA2. **p**. DAB2. **q**. KRT19. **r**. OVOL1 **s**. GRHL1.

567

568 **Extended Data figure 6. Blastoid TS and ES sub clustering analysis.** **a**. UMAP of blastoid
569 data and clustering analysis. **b**. Cluster allocation. **c**. Heatmap of epiblast marker
570 POU5F1(OCT4). **d**.
571 Heatmap of trophectoderm marker (GATA3). **e**. Heatmap of hypoblast marker SOX17. **f**.
572 UMAP of epiblast subclusters **g**. Violin plot comparison of different signaling markers. **h**. Violin
573 plot comparison of different pluripotency markers. **i**. UMAP of trophectoderm subclusters **g**.
574 Violin plot comparison of different signaling markers. **h**. Violin plot comparison of different
575 pluripotency markers. **i**. Heatmap of epiblast marker POU5F1(OCT4) within the TSC subcluster
576 indicating an early blastocyst like subpopulation. **m**. Heatmap of INFT transcript INFT2
577 expression within TSC subcluster.

578

579 **Extended Data figure 7. RNA velocity and pathway analysis a-o.** Expression heatmap and
580 pseudotime analysis of different markers. **p-r.** Alluvial diagram of Go pathways of differentially
581 expressed genes (DEG) in each cluster **s-u.** Alluvial diagram of KEGG pathway of DEGs.

582
583 **Extended Data figure 8. Human blastoid, blastocyst and Bovine blastoid scRNA-seq**
584 **comparison. a-c.** UMAP of data integration. **d.** Heatmap of trophectoderm marker (GATA3). **e.**
585 Heatmap of epiblast marker POU5F1(OCT4). **f.** Heatmap of hypoblast marker SOX17. **g.** Violin
586 plot comparison of different signaling markers. **h.** Violin plot comparison of different
587 pluripotency markers.
588

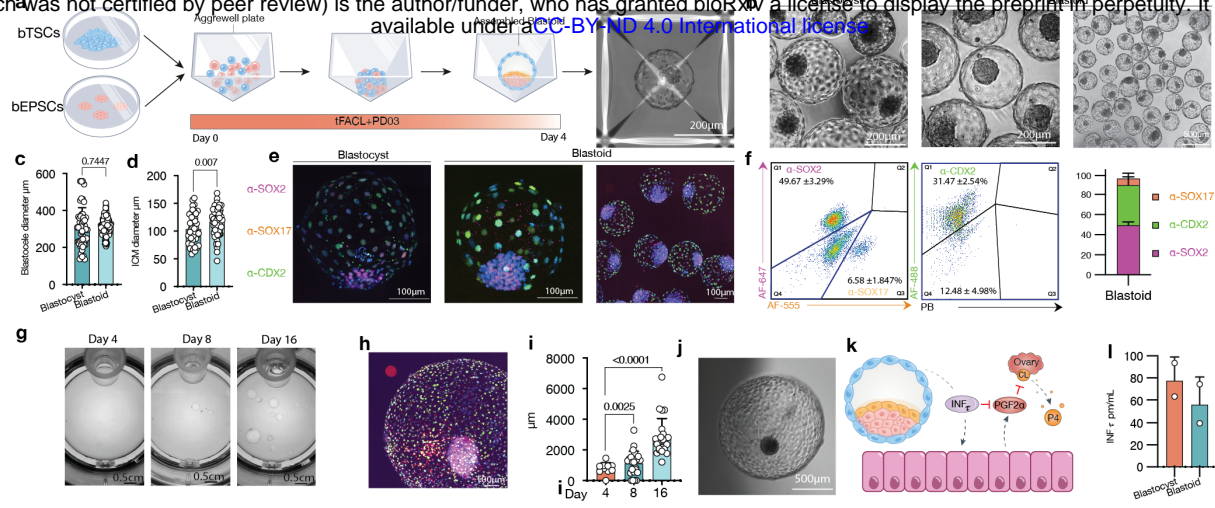


Figure 1

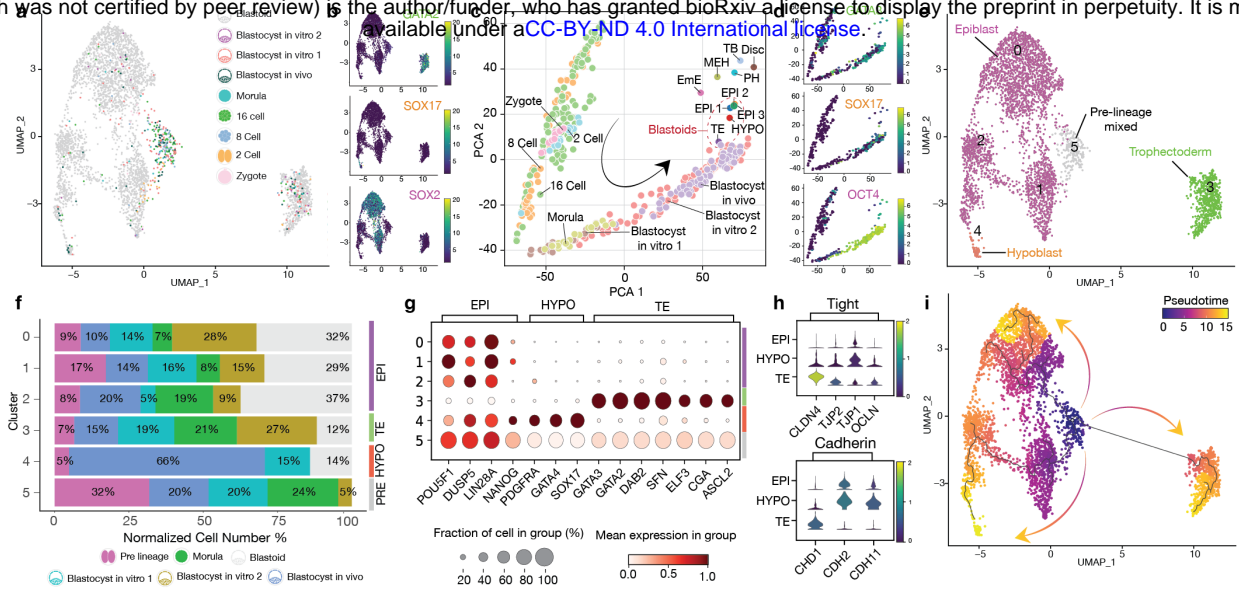
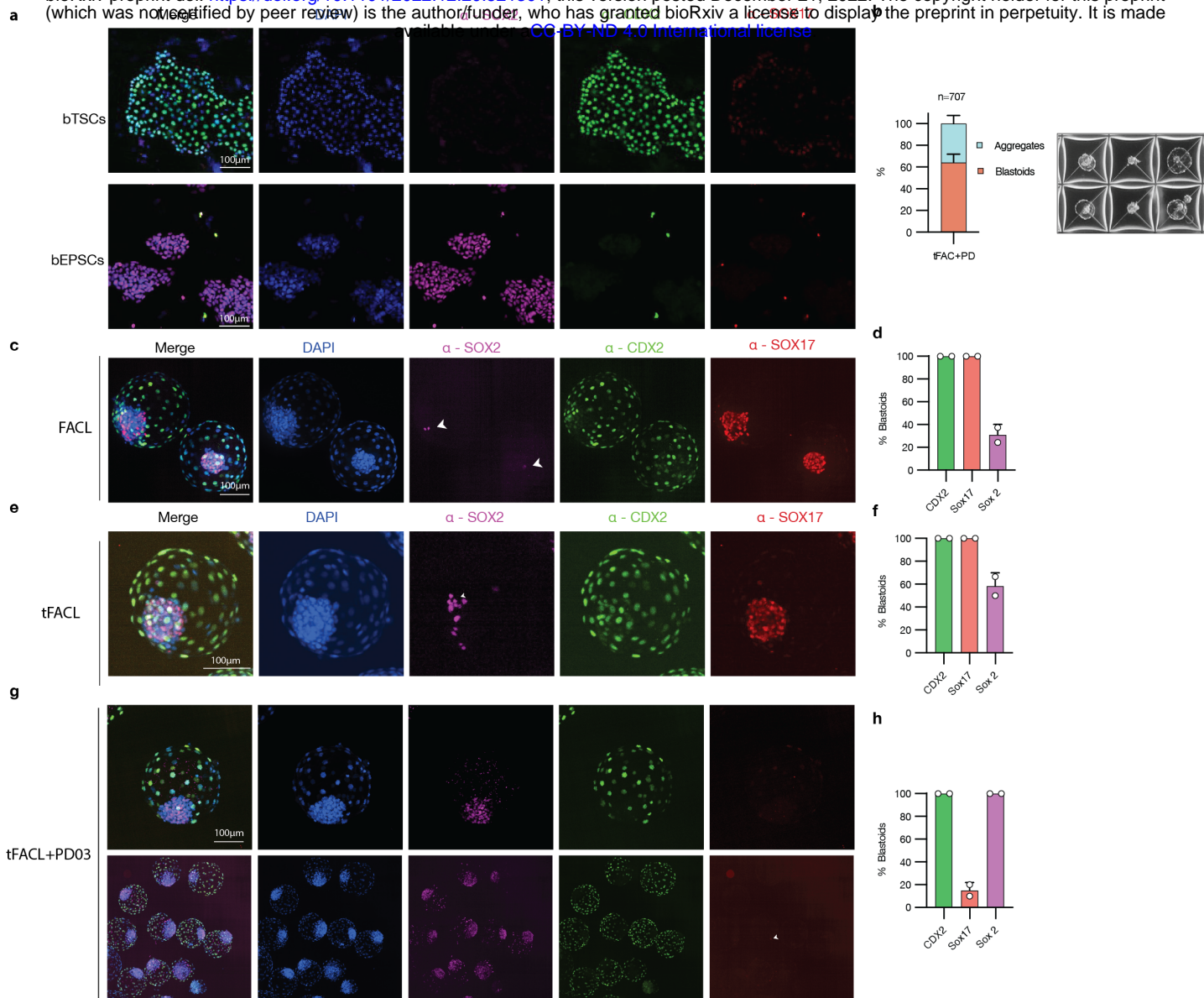
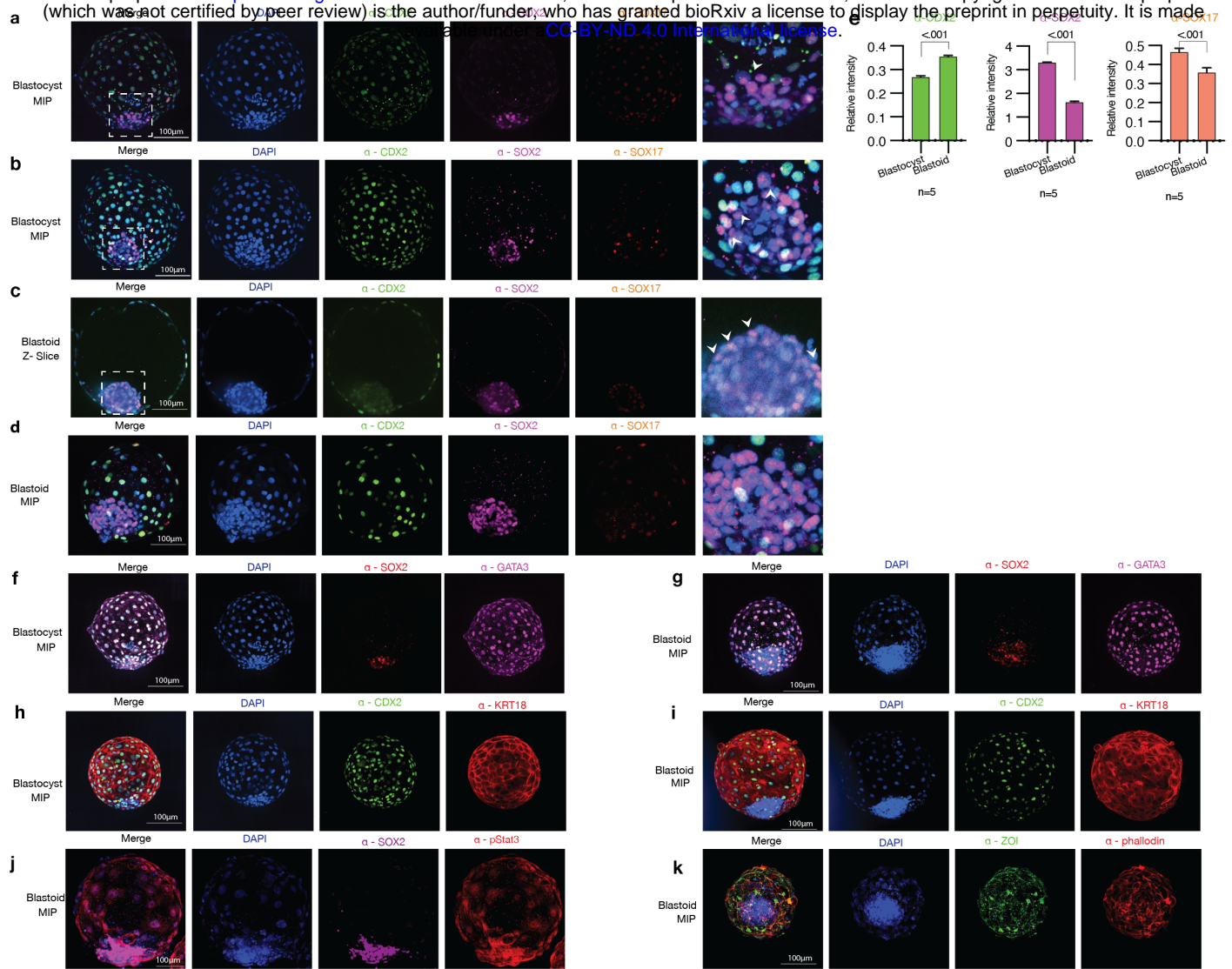
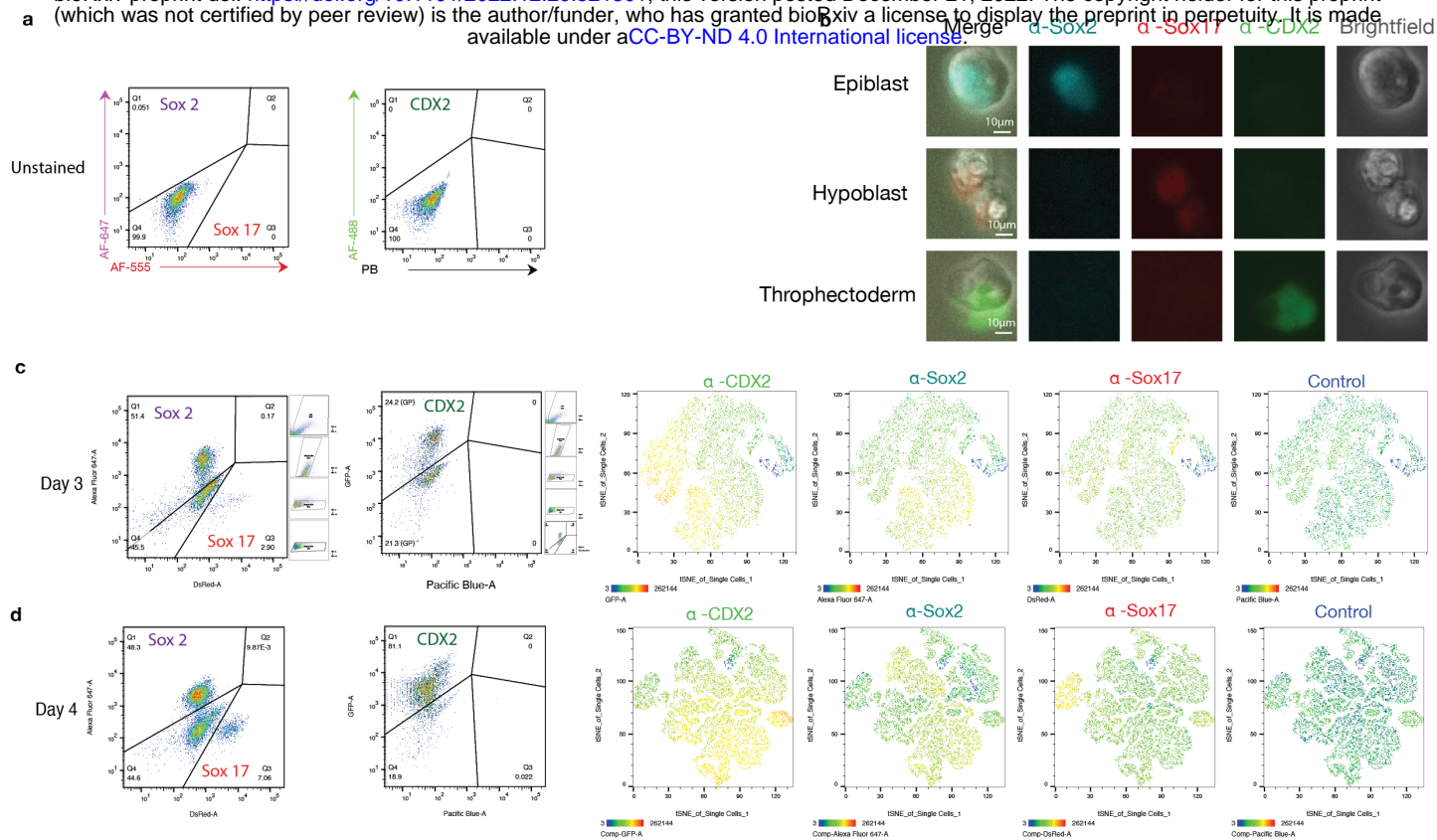
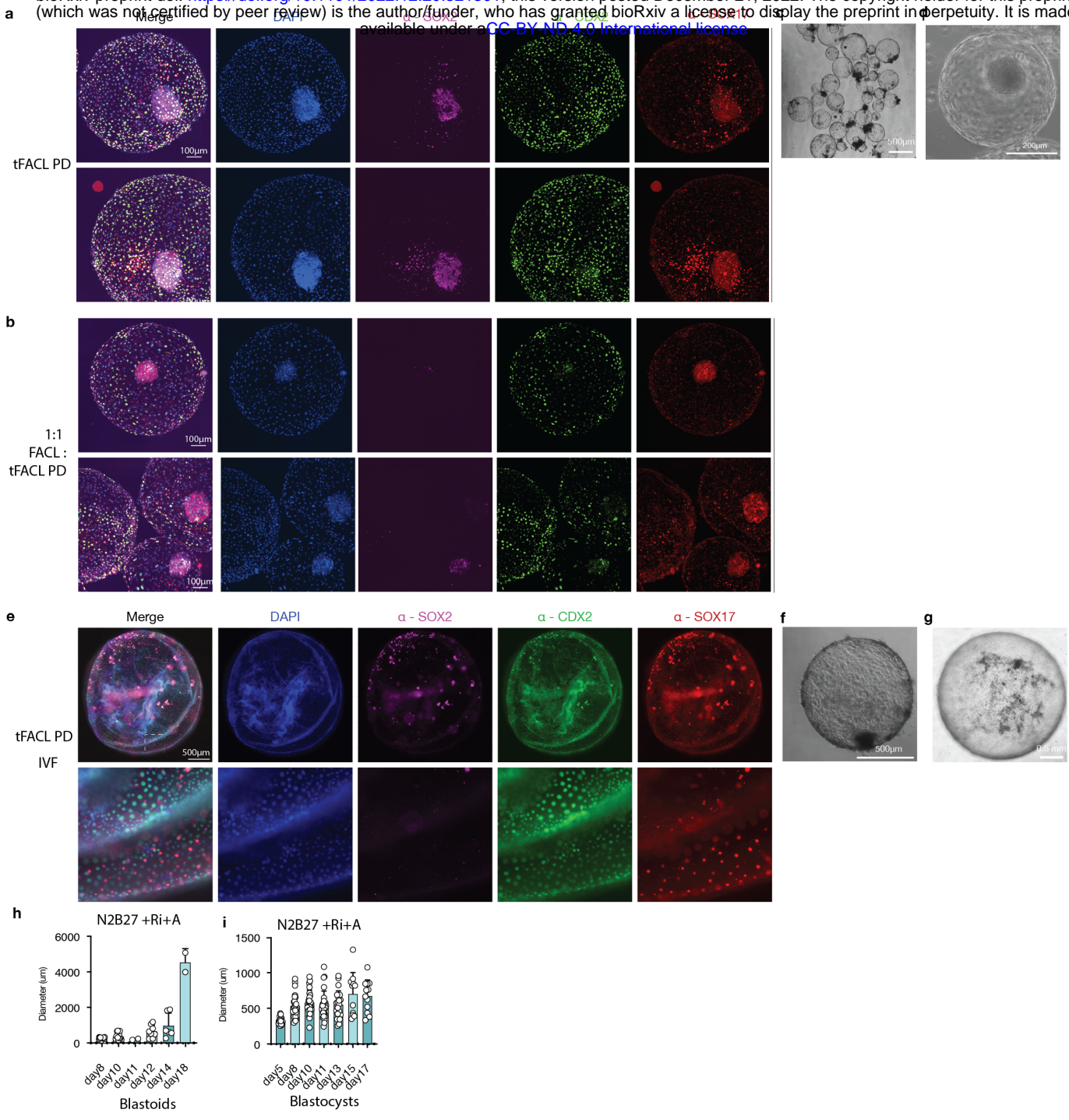


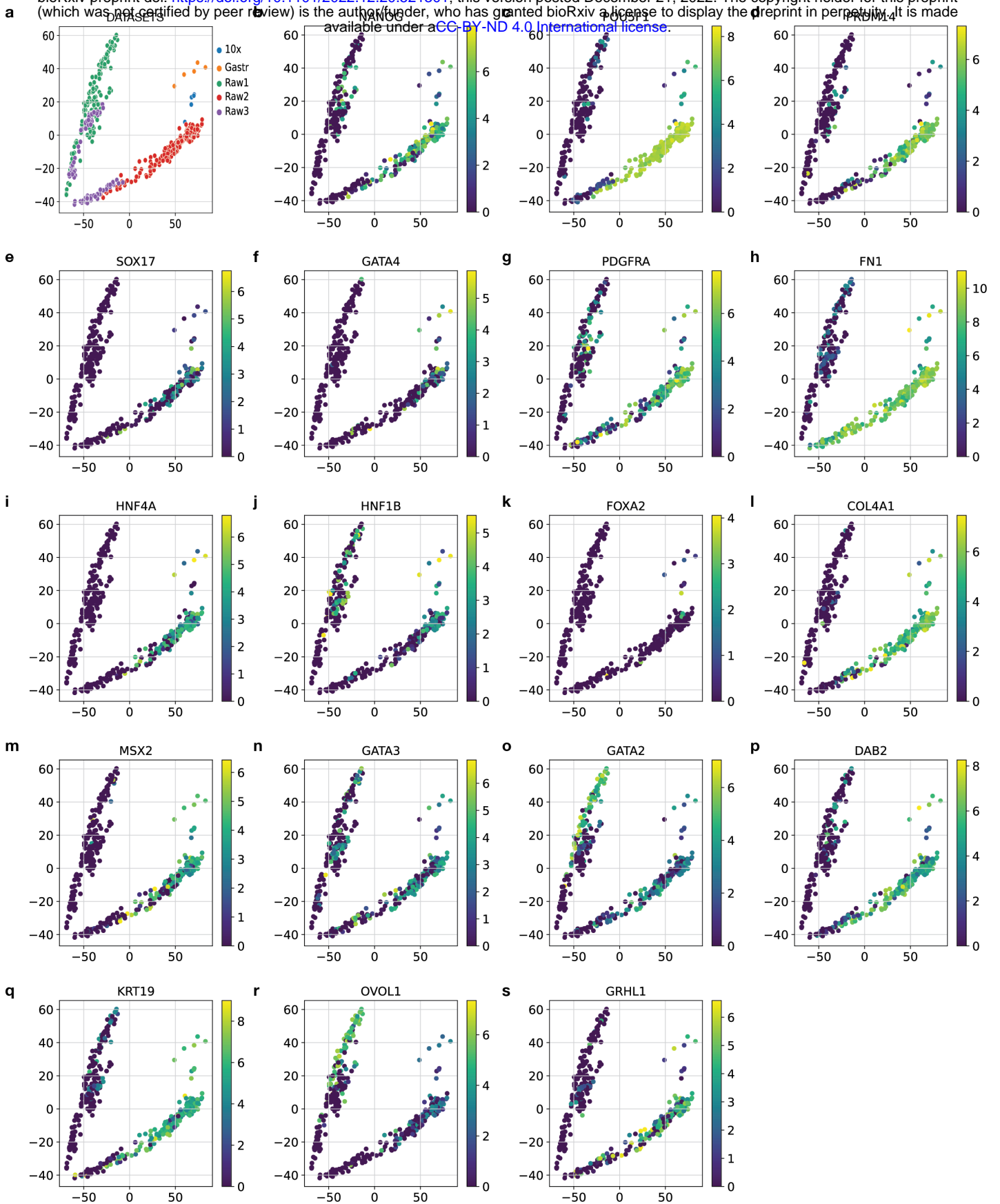
Figure 2



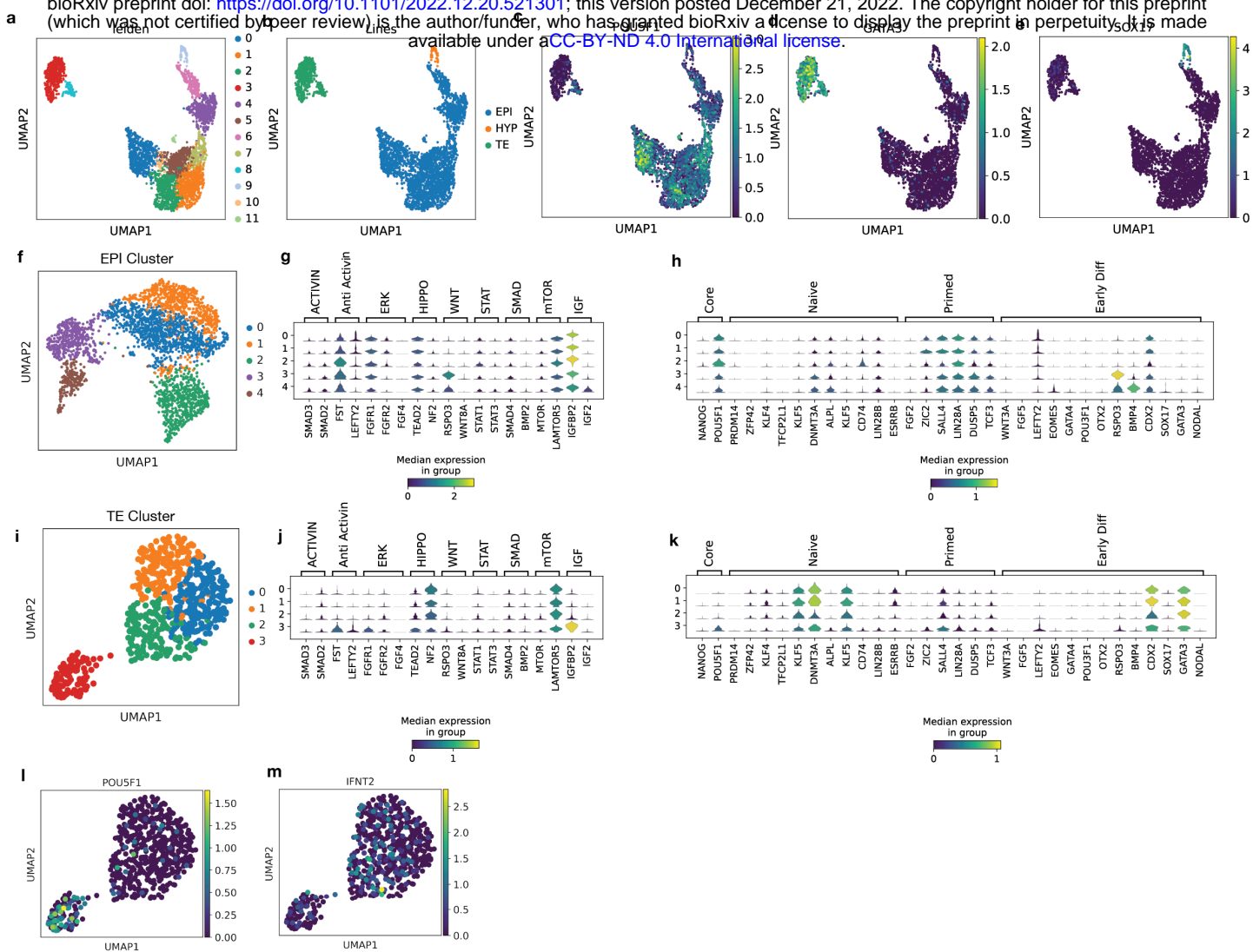




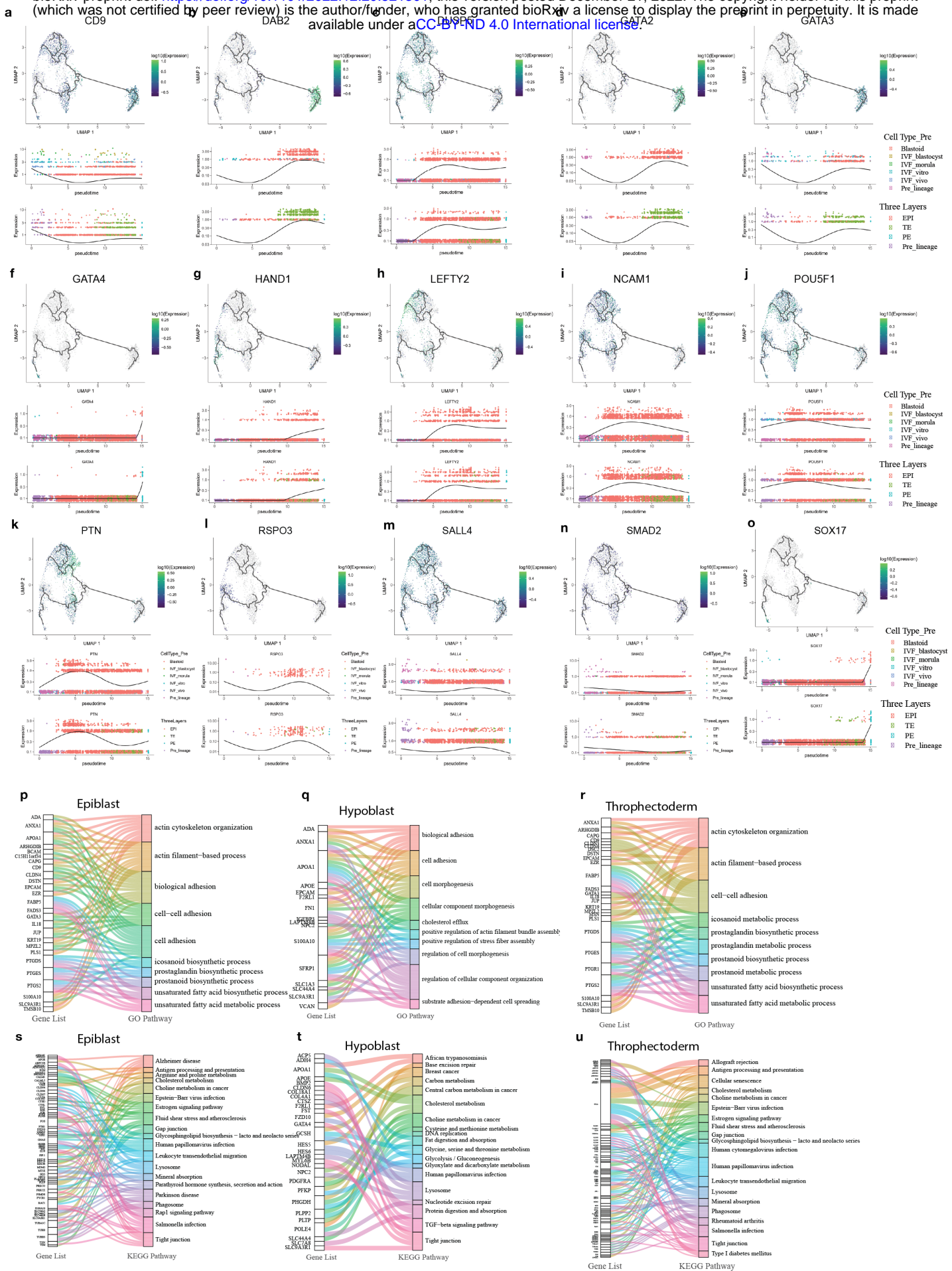




Extended Data Figure 5.



Extended Data Figure 6.



Extended Data Figure 7.

