

1 **Transgene-Free Ex Utero Derivation of A Human Post-Implantation** 2 **Embryo Model Solely from Genetically Unmodified Naïve PSCs**

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1 Abstract

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3 **Our ability to study early human post-implantation development remains highly limited due**

4 **to the ethical and technical challenges associated with intrauterine development of the human embryo**

5 **after implantation. Despite the great progress made on human gastruloids, axiolooids and in vitro**

6 **cultured blastoids, such elegant models do not constitute an integrated Stem cell-derived Embryo**

7 **Models (SEMs) that includes all the key extra-embryonic tissues of the early post-implantation**

8 **human conceptus (e.g., hypoblast, yolk-sac, trophoblasts, amnion, and extraembryonic mesoderm),**

9 **and thus, do not recapitulate post-implantation epiblast development within the context of these**

10 **extra-embryonic compartments. Mouse naïve pluripotent stem cells (PSCs) have recently been shown**

11 **to give rise to embryonic and extra-embryonic stem cells capable of self-assembling into post-**

12 **gastrulation mouse SEMs, while bypassing the blastocyst-like stage, and eventually initiating**

13 **organogenesis *ex utero*. Here, we implement critical adaptations to extend these finding to humans,**

14 **while using only genetically unmodified human naïve PSCs, thus circumventing the need for ectopic**

15 **expression of lineage promoting transgenes. Such integrated human SEMs recapitulate the**

16 **organization of all known compartments of early post-implantation stage human embryos, including**

17 **epiblast, hypoblast, extra-embryonic mesoderm, and trophoblast surrounding the latter layers. The**

18 **organized human SEMs recapitulate key hallmarks of post-implantation stage embryogenesis up to**

19 **13-14 days post-fertilization (dpf, Carnegie stage 6a), such as bilaminar disk formation, epiblast**

20 **lumenogenesis, amniogenesis, anterior-posterior symmetry breaking, PGC specification, primary**

21 **and secondary yolk sac formation, and extra-embryonic mesoderm expansion that defines a**

22 **chorionic cavity and a connective stalk. This new platform constitutes a tractable stem cell-based**

23 **model for experimentally interrogating previously inaccessible windows of human peri- and early**

24 **post-implantation development.**

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1 Introduction

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3 Our understanding of human early post-implantation development has been limited due to
4 difficulties in obtaining relevant embryo samples from these early stages of human gestation, and technical
5 and ethical challenges for *in vitro* development of donated blastocysts towards the post-implantation stages
6 while preserving the *in vivo* complexity of both embryonic and extra embryonic compartments^{1,2}. Most of
7 our knowledge of these stages has been gained solely from histological and anatomical descriptions in
8 embryological collections, and the experimental models for mechanistic research are lacking^{3,4}. Despite
9 the important recent advances in culturing the *in vitro* attached human embryos beyond implantation, these
10 culture systems are restricted in sample number and, overall, do not support normal development of
11 embryos beyond the initiation of early epiblast lumenogenesis⁵⁻⁷.

12

13 The ability to capture human pluripotent stem cells (PSCs) in different developmental states in
14 culture⁸⁻¹⁰ has opened the possibility of investigating early human embryogenesis using 3D stem-cell
15 derived embryo models. By controlling cellular composition and differentiation conditions, the recently
16 introduced elegant models such as gastruloids, blastoids, axioids, or amniotic sac embryoids¹¹⁻²³
17 recapitulate some aspects of early mammalian development in a modular manner. Among them, Blastocyst-
18 like structures (blastoids) are the only currently available human integrated (i.e., comprised of all embryonic
19 and extra-embryonic lineages) model of the human pre-implantation embryo, containing epiblast, primitive
20 endoderm (PrE), and trophectoderm (TE)²²⁻²⁵. However, so far, the attempts to advance the blastoids
21 towards post-implantation stages through *in vitro* culture did not progress beyond what has been achieved
22 with natural human blastocysts^{5-7,22-25}. Similarly, mouse blastoids do not develop into bona fide post-
23 implantation embryos even when transferred *in utero*²⁶.

24

25 Recently, mouse PSCs were shown to possess the ability to be coaxed *ex utero* into post-
26 gastrulation-stage SEMs, by co-aggregation of non-transduced naïve PSCs with naïve PSCs transiently
27 expressing the transcription factors Cdx2 and Gata4, to promote their priming towards TE and PrE lineages,
28 respectively^{27,28}. Such SEMs do not go through a blastocyst or an *in vitro* implantation-like stages but
29 develop directly into egg-cylinder shaped SEMs within complex extra-embryonic compartments and are
30 able to advance beyond gastrulation and reach early organogenesis stages of development as late as day
31 E8.5^{27,28}. These findings establish that naïve pluripotent cells²⁹⁻³¹ can serve as the sole source of embryonic
32 and extra-embryonic tissues in advanced bona fide “organ-filled” embryo models, and thus may enable the
33 generation of integrated SEMs from other mammalian species from which naïve PSCs have been stabilized,

1 including humans. Given that conditions for human naive pluripotency have been continually improved
2 and optimized^{32–38}, here we sought to test whether and how an equivalent approach could be implemented
3 with human naive PSCs to obtain human post-implantation SEMs that unequivocally acquire anatomical
4 compartments of pre-gastrulating human embryo with lineage marker expression and an adequate
5 emergence of the key developmental milestones.

6

7 **Optimizing human naive PSC priming towards extra-embryonic lineages competent** 8 **for post-implantation SEM formation**

9

10 In mouse, deriving SEMs that contain critical embryonic and extraembryonic compartments
11 requires optimal conditions and high-quality rapid induction of Primitive endoderm (PrE) and
12 Trophectoderm (TE) from naive pluripotent stem cells (PSC), which was successfully achieved by ectopic
13 expression of Gata4 and Cdx2, respectively^{27,28,39,40}. The latter feat²⁷ offered the crucial and exclusive
14 platform for us to demonstrate and unleash the self-organizing capacity of mouse stem cells to generate
15 post-gastrulation bona fide synthetic embryos with both organs and extra-embryonic compartments in a
16 unique ex utero set-up and only by starting from naive PSCs. This latter fact is of added importance, since
17 it showed that the establishment of pluripotent stem cells only, may be sufficient to make advanced embryo-
18 like structures in the petri dish from many mammals, including humans, without the need to derive TSC or
19 PRE/XEN lines from human embryo derived material, some of which are not yet established in humans or
20 did not prove competent to generate post-implantation mouse SEMs.

21

22 Hence, we first set out to establish a similar platform to obtain extraembryonic lineages through
23 transient expression of these transgenes in human naive PSCs (**Fig. 1a**), while bearing in mind that the early
24 post-implantation pre-gastrulation human, but not mouse, embryo already contains an extra embryonic
25 mesoderm compartment (ExEM)^{41–47}. To generate DOX (Doxycycline) inducible human PSCs for GATA4
26 or GATA6, regulators of PrE in mouse^{48,49} and of Pre and ExEM lineages in humans⁵⁰, we used a
27 PiggyBac system carrying M2Rtta and transcription factor of interest (**Extended Data Fig. 1a**).
28 Monoclonal and polyclonal human ESC and iPSCs lines were validated for GATA4 or GATA6 expression
29 upon DOX addition (**Extended Data Fig. 1b**). We aimed to use FACS staining for cell surface expression
30 of PDGFR α , which marks both PrE and ExEM lineages in humans⁵⁰, as an initial screening point for
31 identifying optimal conditions to rapidly and efficiently induced PrE and/or ExEM cells, which will be
32 followed by secondary characterization and validation. While Gata4 induction (iGata4) in mouse naive
33 2i/LIF conditions yielded dramatic upregulation of PDGFR α + cell fraction after 48 hours of DOX

1 supplementation (**Extended Data Fig. 1c**)⁵¹, induction of GATA4 and GATA6 expression in human naive
2 PSCs cultured in HENSM conditions, resulted <10% PDGFRa+ cells even after 6 days of follow-up (**Fig.**
3 **1b, Extended Data Fig. 1d**). Since WNT stimulation by GSK3B inhibition via CHIR99021 (CH) has been
4 shown to be the key stimulant for mouse and human PrE induction⁵²⁻⁵⁴, and given that CH is included in
5 mouse, but not in recent human naïve media conditions^{35,55,56}, we thought that HENSM conditions during
6 the induction phase might not be suitable for human cells. Hence, we screened for other conditions to
7 facilitate the induction of PDGFRa+ cells from naive human PSCs (**Fig. 1a**). The recently described mouse
8 PrE-derivation conditions (termed C10F4PDGF)⁵⁴, resulted in very low yield of PDGFRa induction
9 (Fig.1c). RACL induction media (RPMI based medium supplemented with ACTIVIN A, CHIR99021 and
10 LIF)⁵⁷ which has been used to prime human naïve PSCs towards PrE and ExEM state^{50,58}, or NACL media
11 (DMEM/F12/Neurobasal N2B27-based) that stabilizes naïve Endoderm (nEnd) cells generated in RACL
12 conditions⁵⁷, also led to low levels of PDGFRa+ cell fraction even after six days of induction in RACL
13 (**Fig. 1c, middle**) or NACL (**Extended Data Fig. 1e**).

14
15 Since ACTIVIN A is not required for priming cells towards PrE in mouse⁵⁴ and inhibits human
16 naïve PSC in vitro differentiation into ExEM cells⁵⁰, we omitted it from RACL condition (now termed
17 RCL). Remarkably, RCL media resulted in up to 60% PDGFRa+ cells in iGATA4 cells and up to 80%
18 PDGFRa+ from iGATA6 cells within 3-6 days (**Fig. 1c, right**). The slightly improved outcome when using
19 Gata6 overexpression is consistent with previous reports placing Gata6 as the most upstream regulator of
20 PrE identity in mouse and human cells^{48,59}. Remarkably however, high efficiencies of PDGFRa+ cell
21 formation was evident in RCL conditions from isogenic wild type (WT) cells without exogenous expression
22 of GATA6 (**Fig. 1d**), indicating that the transient transgene expression is not required for efficient
23 PDGFRa+ induction in human naïve PSC. Further optimization showed that 3 days of induction in RCL
24 condition followed by 3 days incubation in basal N2B27 conditions yielded comparable results (**Fig 1d –**
25 **right, Extended Data Fig. 1g**). Notably, incubating naïve PSCs in N2B27 media also yielded PDGFRa+
26 cells albeit at significantly lower levels (**Fig 1d, left**), and thus we focused on using RCL conditions for
27 further characterization analysis. The B27 supplement devoid of insulin in RCL was utilized herein as it
28 yielded slightly higher efficiency in PDGFRa+ cell induction compared to B27 with insulin (**Extended**
29 **Data Fig. 1f**), consistent with previous reports⁵⁷.

30
31 Our next step was to test for existence of PDGFRa+ PrE and/or ExEM cells in our RCL induction
32 protocol and to distinguish between them. Both immunostaining and RT-PCR analysis validated
33 endogenous expression of PrE marker genes in RCL conditions, including SOX17 which marks only the

1 PrE fraction, alongside GATA4, GATA6, and NID2 genes (**Extended Data Fig. 2 a, b**)⁵⁷. Markers of
2 definitive endoderm like GSC or HHEX were not induced from naïve PSCs in RCL conditions (**Extended**
3 **Data Fig. 3a**) thus excluding definitive endoderm cell formation⁵⁷. Applying RCL conditions on human
4 isogenic primed PSCs yield predominant definitive endoderm marker expression (GSC or HHEX)
5 (**Extended Data Fig. 1h**), consistent with previous findings⁵⁷. Immunostaining results for SOX17 and
6 BST2 markers that distinguish between PrE or ExEM lineages^{52,60}, respectively, confirmed the co-
7 emergence of PrE and ExEM from naïve PSCs under the same RCL induction protocol (with or without
8 GATA6 overexpression) (**Fig. 1e, Extended Data Fig. 3b**). GATA4 positively marked both SOX17+ PrE
9 and BST2+ ExEM populations as expected (**Extended Data Fig. 3c**)⁵⁰. BST2+ ExEM cell identity was
10 further validated by upregulation of FOXF1 protein which marks ExEM cells, but not PrE cells or residual
11 PSCs (**Extended Data Fig. 3d**)^{61,62}. Thus, we adopted RCL pre-treatment of naïve PSCs for co-
12 aggregation experiments with other lineages (**Fig. 2b**).

13
14 In parallel, we set out to find optimal conditions for deriving cells from the trophectoderm (TE)
15 lineage that can adequately aggregate with the other lineages and develop into post-implantation stage
16 human SEMs⁶³. Overexpression of Cdx2, a master regulator of TE lineage, can lead to formation of TSC
17 lines in mice^{39,64}. Moreover, its overexpression in mouse naïve PSCs has been shown to be efficient in
18 generating TSCs that remained viable and correctly integrated upon coaggregation with mouse naïve PSCs
19 and iGata4 cells and generated both chorionic and ectoplacental cone placental lineages^{27,28} (**Extended**
20 **Data Figure 4a-b**). Cdx2 has also been shown to be transiently expressed in the TE of the human
21 blastocysts and blastoids^{6,7,22,65}. Therefore, we generated DOX inducible CDX2 human ESC and iPSC
22 lines using a similar PiggyBac approach (**Extended Data Fig. 4c, d**), which were also subjected to lentiviral
23 labeling with a constitutively expressed tdTomato marker to better track cell viability and integration
24 patterns (**Extended Data Fig. 4e**). Remarkably, human iCDX2 cells pre-treated with DOX for 1 – 4 days
25 in either naïve HENSM or in different validated human TE/TSC induction conditions: PALY medium
26 (N2B27 supplemented with PD0325901, A83-01, and Y-27632)²², BAP(J) induction medium
27 (DMEM/F12 based medium with ALK4/5/7 inhibitor A83-01, FGF2 inhibitor PD173074, and BMP4
28 substituted with JAK inhibitor I after 24h)^{36,66}, or TSC maintenance medium (TSCm)⁶⁷, did not
29 meaningfully contribute and expand within the aggregates generated with induced PrE/ExEM and PSCs.
30 This can be judged by the loss of CK7+ or tdTomato-positive cells in early aggregates (**Extended Data**
31 **Fig. 4f, h**), likely due to a drastically reduced viability of iCDX2 cells upon treatment with DOX in different
32 conditions and clones used (**Extended Data Fig. 4i**), which was not encountered in mouse cells^{27,28,68}.

1 iCDX2 cells also expressed low levels of trophoblast markers when compared to WT non-transgenic
2 cells (**Extended Data Fig. 4g**)

3
4 In the mouse SEM model, established embryo derived TSC lines can substitute the iCdx2-induced
5 PSCs during aggregations without compromising the outcome^{27,69}. Therefore, we then proceeded to test
6 the established TSC lines derived from both naïve and primed hPSC^{70,71}. To be able to trace cells after
7 aggregation, TSC lines were either labelled with tdTomato via lentiviral transduction (**Extended Data Fig.**
8 **5a**) or stained for CK7 expression. In all employed aggregation conditions with either primed (pTSC)
9 (**Extended Data Fig. 5**) or naïve PSC derived TSC lines (nTSC) (**Extended Data Fig. 6**), the TSCs did
10 not generate an outer layer surrounding the aggregate, but rather formed focal clumps (**Extended Data Fig.**
11 **5b – e; Extended Data Fig. 6a – d**). The inability of human TSCs to integrate in putative SEMs, as opposed
12 to mouse TSCs, might stem from the fact that mouse TSC lines are Cdx2+ and correspond to earlier stages
13 of trophoblast development, than those isolated from human naïve or primed TSCs which are CDX2-
14 negative^{70–74}.

15
16 Recent studies suggest that GATA3 might have an important role for human TE induction⁷⁵,
17 thereby, we also established and tested induced GATA3 (iGATA3) human PSCs lines (**Extended Data**
18 **Fig. 7a–c**) and compared them to differentiation conditions using isogenic genetically unmodified cells.
19 RT-qPCR analysis showed higher expression levels of CDX2 and TACSTD2 in the non-transduced group
20 under BAP(J) conditions (**Extended Data Fig. 7d**). Immunofluorescence staining revealed that although
21 GATA3 overexpression induced endogenous expression of GATA2 TE marker, the cells did not uniformly
22 express TFAP2C, CDX2, and CK7, while in the absence of transgene overexpression we observed higher
23 and uniform expression of TFAP2C, CDX2, and higher occurrence of CK7 positive cells under BAP(J)
24 conditions (**Extended Data Fig. 7e, f**). Flow cytometry analysis for TACSTD2 (marker of early and late
25 TE) and ENPEP (expressed only in the late TE) showed the highest percentage of a double positive
26 population in non-transduced cells under BAP(J) conditions (**Fig. 1f; Extended Data Fig. 7g, right**)⁷³.
27 Addition of BMP4 on the first day of induction showed homogeneous double positive population while
28 exclusion of BMP4 showed that almost half of the population was ENPEP negative (**Extended Data Fig.**
29 **7g, right**), consistent with a previous report³⁶. Notably, following the induction and aggregation with naïve
30 PSCs and PrE/ExEM, iGATA3 cells remained viable but did not surround the aggregates (**Fig. 1g, left**). In
31 contrast, TE derived from genetically unmodified naïve PSC under the same BAP(J) protocol, uniformly
32 surrounded the aggregates (**Fig. 1g, right**), which is a critical criterion expected to be fulfilled in integrated
33 SEM⁷⁶. It is possible that the high ectopic expression produced by the PiggyBac system of GATA3 or
34 CDX2, in addition to the endogenous levels induced by the media used, leads to unfavorably high levels of

1 such factors which might derail the differentiation outcome, and is consistent with the findings in mouse
2 SEMs that cautioned from using highly expressing Cdx2 transgenic clones when generating mouse
3 TE/TSCs^{76,77}. The ability to derive relevant extra-embryonic lineages from genetically unmodified WT
4 human naïve PSCs are without the need for transgene overexpression are in line with the recent studies
5 demonstrating that human naïve pluripotent cells can be more easily coaxed to give rise to early progenitors
6 of PrE cells and TSCs when compared to mouse PSCs, which so far require ectopic transcription factor
7 overexpression^{35,36,58,74}. The latter is consistent with the observation that enhancers⁷⁸ of key TE and PrE
8 regulators (GATA3, GATA6, GATA4) are accessible in human, but not mouse naïve PSCs while being
9 transcriptionally inactive in both (**Extended Data Figure 8**).

11 **Human Post-Implantation SEM self-assembled exclusively from non-transgenic naïve PSCs**

13 Having established fast and efficient *in vitro* induction of the three extraembryonic lineages
14 comprising the pre-gastrulating natural human embryo, we proceeded to test their capacity to form embryo-
15 like structures solely from naïve pluripotent stem cells (nPSC) (**Fig. 2a**). To this end, we calibrated the
16 aggregation conditions, such as cell numbers needed and ratios within cell mixtures (**Extended Data Fig.**
17 **9a-c**), and the aggregation and growth media composition for different stages of SEM development
18 (**Extended Data Fig. 10**). Unlike for mouse where FBS presence in the first 3 days of SEM formation was
19 critical^{27,79}, serum free conditions were found favorable for human SEM (**Extended Data Fig. 10b, c**).
20 The protocol starting with 120 cells aggregated at the ratio 1:1:3 (nPSC: PrE/ExEM: TE) in basal N2B27
21 conditions supplemented with BSA, which was found critical to reduce human aggregate stickiness
22 (**Extended Data Fig 11a**)⁸⁰, for three days resulted in optimal aggregation, validated by the presence of
23 epiblast and extra-embryonic lineages in the aggregates via immunostaining (**Extended Data Fig. 12a**). To
24 further support unrestricted growth of the SEM and prevent TE attachment to the 2D surface, which disrupts
25 the morphology after day 3 of the protocol, we had to continue our culture using orbital shaking conditions
26 and optimal medium volume per well (**Extended Data Fig. 11b-d**)²⁷. The composition of the Ex-Utero
27 Culture Media 2 (EUCM2) was adapted from the mouse SEMs protocol²⁷, while using here incremental
28 FBS concentrations, ranging from 20% at day 3, 30% at day 4-6, and 50% at day 6-8, which were found
29 most optimal for human SEM growth (**Fig. 2b, Extended Data Fig. 12a-b**). We did not observe an
30 improved outcome when using roller-culture applied after day 6 with the same media composition,
31 compared to orbital shaking placed in static incubators (**Extended Data Fig. 11e**)²⁷, which is consistent
32 with observations in mice where roller culture platform was needed particularly for late gastrulation and
33 organogenesis stages⁸¹.

1
2 To study the development of our human SEMs in further detail and with an adequate reference, we
3 mostly relied on the data from the Virtual Human Embryo Project based on the Embryo Carnegie Stages as
4 the most detailed and relevant source available to date ^{82,83} (see Methods). During eight days of *ex utero*
5 culture, the aggregates grew extensively, forming a 3D spherical structure with evident tissue compartments
6 and inner cavities (**Fig. 2c**), which we then aimed to characterize in more detail by immunofluorescence
7 (**Video S1**). Strikingly, human SEM did not only express the respective lineage markers, but also
8 established the structures morphologically characteristic of *in utero* implanted embryos (**Fig. 2d, f**). From
9 the beginning of the *ex utero* culture, SEMs became surrounded by the Trophoblast (Tb) compartment,
10 marked by GATA3, CK7, and SDC1 that marks syncytiotrophoblast (STB) (Days 3, 4; **Fig. 2d, left;**
11 **Extended Data Fig. 12a – c**), which is reminiscent of human early *in utero* development by 8 dpf (day
12 post-fertilization) or Carnegie Stage 5 (CS5) (**Fig. 2a, left**). At this stage, the implanting embryo starts to
13 become surrounded by a layer of syncytiotrophoblast (STB), which directly invades maternal endometrium
14 and supports future histotrophic nutrition of the embryo. Notably, the co-aggregation protocol devised
15 herein does not result in a blastocoel cavity formation nor in a condensed ICM-like structure, as opposed
16 to human and mouse blastoid models (**Extended Data Fig. 13a-h**), indicating that human SEMs do not go
17 through a blastocyst-like stage, in agreement with observations in mouse SEM protocol (**Extended Data**
18 **Fig. 13i-j**) ^{27,79,84,85}. The efficiency of correctly organized human SEM was estimated at day 6 to be 2.9%
19 of the starting aggregates as judged by co-immunofluorescence for lineage markers and meeting
20 morphology criteria (see Methods) (**Fig. 2e, Extended Data fig 12c-d**). We note that the human SEMs
21 show a large degree of asynchrony, with up to 2 days difference in developmental staging for SEMs found
22 at day 6-8, leading to some more advanced and some earlier structures when evaluating SEMs at a specific
23 time point. Starting the human SEM protocol with human primed, rather than naïve, PSCs did not generate
24 equivalent SEMs (**Extended Data Fig. 9d**), as also seen in mouse models ^{27,86}.

25
26 In human and non-human primates (NHP), the inner cell mass segregates into two lineages, the
27 epiblast (Epi), formed by columnar cells expressing OCT4 ^{5–7,87}, and the hypoblast located underneath the
28 Epi (Hyp, comprised of cuboidal cells expressing SOX17, GATA6, GATA4, PDGFRa) ^{88–90} (CS4-5a, **Fig.**
29 **2a**). Importantly, in our human SEM, Epi and PrE cells segregated into two distinct compartments,
30 differentially expressing the respective lineage marker genes (OCT4 and SOX17) (**Fig. 2d**). Both Epi and
31 Hyp were surrounded by the Tb, marked by CK7 starting from day 3 (**Fig. 2d, Extended Data Fig. 12a**),
32 and SEMs drastically advanced morphologically by day 6 (**Extended Data Fig. 12b**). In particular, the Epi
33 initiated formation of the amniotic cavity (AC), whilst the hypoblast formed a yolk sac (YS) cavity,

1 establishing a bilaminar structure in between (**Fig. 2d, middle; Extended Data Fig. 12b**) reminiscent of
2 the 9-10 dpf human embryo (CS5b, **Fig. 2a**).

3
4 The early post-implantation pre-gastrulating human embryo already contains some extraembryonic
5 mesoderm (ExEM)^{41–47,91}. The ExEM contributes to all fetal membranes and participates in formation of
6 blood and placental vasculature^{91,92}. ExEM tissue becomes abundant between the primary yolk sac (PYS)
7 and the Tb by CS5a, forming a chorionic cavity (ChC) underneath the PYS by CS5c (11 dpf, **Fig. 2d –**
8 **middle scheme**). The latter was also observed upon close examination of day 6 SEMs, revealing the
9 presence of a cavity formed by an additional tissue layer between the YS and the Tb, that corresponds to
10 ChC, similar to what has been characterized in natural human embryos corresponding to these stages (**Fig.**
11 **2c, d**). Later on, the ExEM expands allowing the remodeling of the PYS into becoming what is termed the
12 secondary yolk sac (SYS) and formation of a connective stalk (St), the structure that crosses through the
13 chorionic cavity and holds the bilaminar disc to the chorion and later forms the umbilical cord (Florian,
14 1930). In human SEMs, we also observed 3D expansion of all the above mentioned lumina and growth of
15 the extraembryonic tissues (**Fig. 2c-d, f**), suggesting differentiation and remodeling of the PYS into SYS
16 alongside ExEM expansion, and formation of a connective stalk (**Fig. 2d, f; Video S1; Extended Data Fig.**
17 **14**).

18
19 By 11-12 dpf (CS5c) in human embryos, the embryonic disk segregates into a ventral
20 pseudostratified epiblast and a dorsal squamous amnion (Am) (**Fig. 2a, right panel**). The bilaminar epiblast
21 will give rise to the embryo proper, whereas the amnion will comprise the protective membrane surrounding
22 the fetus until birth⁹⁴. Strikingly, starting from day 6 and until day 8 of the *ex utero* culture (D6 – D8
23 SEMs), the dorsal segment of the epiblast acquired squamous morphology resembling the amnion-like
24 layer, and the bilaminar structure adapted a disk shape (**Fig. 2d, f; Extended Data Fig. 14; Video S1**),
25 resembling the key hallmark of the *in utero* human post-implantation development in preparation for
26 gastrulation. Altogether, our approach exploits the developmental plasticity of genetically unmodified
27 transgene-free human naïve PSCs, demonstrating their capacity to self-assemble into early post-
28 implantation human embryo models that comprise both embryonic and extraembryonic compartments, as
29 we characterize and validate further below.

30
31 **Human SEMs undergo epiblast morphogenesis and form a bilaminar disk, amnion and**
32 **PGCs**

33

1 We then aimed to characterize development of key lineages in SEMs in more detail. As the epiblast
2 is derived from initially naïve PSCs, we checked whether they undergo priming after co-aggregation with
3 other lineages and being placed in N2B27 basal conditions. Indeed, shortly after co-aggregation, we noted
4 loss of expression of naïve marker genes (DNMT3 and STELLA) that were originally expressed in the
5 starting naïve PSCs in HENSM conditions (**Fig. 3a**), and upregulation of the primed pluripotency marker
6 OTX2 by day 3, alongside maintaining the expression of OCT4 and SOX2 pluripotency factors that mark
7 both naïve and primed pluripotency states (**Fig. 3a, b**)^{95,96}. Consistent with developmental progression, by
8 day 4, PSCs formed an evident epiblast tissue inside the SEM which considerably grew during subsequent
9 development (**Fig. 3c, Video S2**). Electron micrographs of the *in utero* rhesus monkey embryos indicate
10 Epi polarization approximately at the time of implantation⁹⁷. *In vitro* attached and cultured (IVC) human
11 blastocysts showed Epi polarization and lumenogenesis (evident by apical Podocalaxyn localization)
12 starting from 10 dpf⁵, while in our SEM, the Epi established apicobasal polarity from day 6, as judged by
13 the apical localization of phosphorylated Erzin, Podocalaxyn (**Fig. 3e**), and aPKC (**Extended Data Fig.**
14 **15a**), as well as alignment of the Epi cells towards the emerging cavity (**Extended Data Fig. 15b**). The
15 timing of lumenogenesis at day 6 also corresponded to a significant increase in the Epi cell number (**Fig.**
16 **3d**), in agreement with the histological descriptions of the *in utero* human embryo⁸².

17
18 Early emergence of the anterior-posterior (AP) axis is prevalent in mammals when a proportion of
19 Epi cells initiates expression of BRACHYURY (T) at the prospective posterior side of the embryonic disk
20⁹⁸. We also checked for the expression of BRACHYURY (T) in human SEMs and identified a
21 T/BRACHYURY-positive population of Epi cells in the posterior part of the SEM epiblast (**Fig. 3f,**
22 **Extended Data Fig 15d**). The emergence of the anterior visceral endoderm (AVE), which constitutes the
23 anterior signaling center for epiblast patterning, was seen by the expression of CER1 in the Epi-adjacent
24 part of the VE from day 6 (**Extended Data Fig. 15c, d**). The previously known vesicular staining pattern
25 of CER1^{99,100} was evident in human SEMs (**Extended Data Fig. 15c**). Co-immunostaining for these
26 markers further supported establishment of the AP axis and symmetry breaking by day 8 in human SEMs,
27 where T/BRA-positive Epi cells were found in the region opposite to Cer1-positive AVE (**Fig. 3f**).

28
29 From day 6 on, the Epi exhibited patterning of early amniotic sac formation with a dorsal squamous
30 cell population corresponding to the putative amnion, and ventral columnar pseudostratified epiblast cells
31 (**Extended Data Fig. 15e, f**). Co-immunostaining for TFAP2A and ISL1 in day 8 SEMs, revealed their co-
32 localization in multiple squamous dorsal cells, also depleted of SOX2 expression, confirming their amnion
33 identity by localization, morphology, and gene expression (**Fig. 3g; Video S3**)^{101–104}. Based on the cell

1 morphology and localization, amnion can be distinguished from the rest of the Epi between 10 and 12 dpf
2 of human *in utero* development⁸². Moreover, by day 8 in SEM, the Epi acquired an apparent disk shape
3 with an enlarged amniotic cavity, while the amnion formed a thinner squamous shaped epithelium highly
4 resembling *in utero* embryo morphology as documented in Carnegie collection at CS6a (**Fig. 3h – k, Video**
5 **S3**). Lastly, we asked whether advanced *in utero*-like development of the embryonic disk would also lead
6 to induction of early primordial germ cells (PGCs)^{105,106}, as was seen in mouse SEMs derived from naïve
7 PSCs²⁷. Co-immunostaining for several PGC markers identified a rare population of cells positive for
8 OCT4, SOX17, and BLIMP1 in the dorsal Epi region of the SEM at day 8 (**Fig. 3l; Extended Data Fig.**
9 **15g**), which unequivocally constitutes a specific signature of human PGCs^{107–109}.

10

11 **Human SEMs recapitulate development of yolk sac and scaffolding of the chorion cavity by** 12 **the extra-embryonic mesoderm.**

13

14 The yolk sac starts forming from the hypoblast between CS4 and CS5 in human. The part of
15 hypoblast located underneath the epiblast (forming a characteristic bilaminar disc), belongs to the visceral
16 endoderm (VE) and is a dynamic signaling center for epiblast patterning. It is connected to the parietal
17 endoderm (PE), forming the inner cavity, primary yolk sac (PYS), which becomes reorganized during
18 development^{42,46}. Eventually, yolk sac serves multiple functions for the growing embryo, supplying
19 nutrients, and the blood cell progenitors during the embryonic period until the placenta takes over⁴².
20 Formation of the yolk sac cavity was frequently seen in structures with all segregated lineages and became
21 more prominent at day 6 of the *ex utero* development (**Fig. 4**). Once formed, SOX17-positive yolk sac was
22 comprised of the columnal VE in the Epi proximity, and the squamous PE lining the opposing side of the
23 cavity (**Fig. 4a – c**), recapitulating VE and PE cell morphology in PYS of CS5c natural embryos (**Fig. 4c**).
24 Both VE and PE acquired apicobasal polarity, as judged by the apical localization of aPKC (**Extended**
25 **Data Fig. 16a; Video S4**), which agrees with the electron micrographs of the hypoblast cell morphology
26 in implantation stage rhesus monkey embryos⁹⁷. Consistent with polarity, cell shape, and localization in
27 natural embryos, SOX17-positive hypoblast also co-expressed primitive endoderm markers GATA6 and
28 GATA4 (**Fig. 4d, e; Extended Data Fig. 16d**).

29

30 Next, we aimed to characterize the above mentioned OCT4-negative and SOX17-negative cell
31 population beneath the yolk sac (**Fig. 2d**). Interestingly, these cells had mesenchymal rather than epithelial
32 morphology, extending cell protrusions towards the surrounding tissues and forming an intermediate mesh-
33 like 3D structure (**Extended Data Fig. 16b**). We then checked the expression of multiple lineage-specific

1 transcription factors, differentially marking ExEM vs. YS during the relevant stages in marmoset embryos
2 ⁹⁰. The ExEM cells expressed GATA6 and GATA4, but not SOX17 (**Fig. 4e; Extended Data Fig. 16d, e**),
3 consistent with the ExEM expression profile from marmoset embryos ⁹⁰ (**Extended Data Fig. 16c**) and
4 human PSC in vitro derived ExEM cells ⁵⁰. The observed lower GATA6 level in ExEM cells compared to
5 PrE cells (**Fig. 4e, Extended Data Fig. 16d**), is also in agreement with the expression for those lineages in
6 the *in utero* marmoset embryos ⁹⁰ (**Extended Data Fig. 16c**). Immunostaining for additional mesenchymal
7 markers, such as BST2 ⁵⁰, VIM ^{87,110} and FOXF1 ⁶², which allow distinguishing ExEM from PrE, further
8 validated the ExEM Identity of these cells in day 6 – 8 SEMs (**Fig. 4f-g, Extended Data Fig. 16e-f; Video**
9 **S5**). Hence, we concluded that the inner cavity of the ExEM represents the chorionic cavity, which is formed
10 by remodeling of a mesh like population of mesenchymal cells predominantly visible starting from day 6
11 SEMs that corresponds to the human extraembryonic mesoderm.

12
13 Histological descriptions of human and NHP embryos suggest the remodeling of the PYS cavity
14 by the ExEM expansion with a subsequent pinching off and vesiculation of the PYS remnants, resulting in
15 the formation of the secondary yolk sac (SYS) ⁴⁶. In some of the day 8 SEMs, BST2 and FOXF1 positive
16 cells formed a complex filamentous meshwork with multiple cavities, contributing to the SEM complex
17 architecture (**Fig. 4h, i; Extended Data Fig. 17**). Closer examination of these structures revealed clusters
18 of SOX17 positive cells entrapped between ExEM cells, suggesting residual PYS cells after the tissue
19 remodeling (**Fig. 4j-k; Extended Data Fig. 17**). Moreover, an ExEM cell population connecting the
20 bilaminar disk to the trophectoderm could be seen in day 8 SEMs, resembling a connective stalk structure
21 (**Fig. 4h-I, Fig. 2d, Extended Data Fig. 14**), which contributes to the future umbilical cord in natural
22 human embryos ⁹³.

23

24 **Trophoblast integration and maturation in human SEMs**

25

26 *In utero*, the human embryo develops surrounded by the trophoblast, which is essential for truly
27 integrated experimental models of early implantation-stage development. The early days of trophoblast
28 development during implantation are partially characterized in human *in vivo*. The fusion of trophoblast
29 leads to formation of an outer syncytium, morphologically seen shortly after implantation on the earliest
30 Carnegie data. Immunofluorescence analysis shows that the majority of human SEMs generated with our
31 protocol are surrounded by the trophoblast with very high efficiency (79-84%), expressing multiple
32 trophoblast marker genes, such as GATA3, CK7, and SDC1, the latter being characteristic for syncytium
33 (**Fig. 5a; Extended Data Fig. 18a-d**) ³⁶. Marker gene expression and cell morphology further indicated

1 that the outer trophoblast layer is formed by syncytiotrophoblast, confirming the development of post-
2 implantation trophoblast in human SEMs (**Fig. 5a; Extended Data Fig. 18a-d**). Notably, SDC1 is not
3 expressed on the starting TE cells upon BAP(J) induction prior to the aggregation, indicating that
4 maturation of the TE cells occurs in the aggregates (**Extended Data Fig. 7e**).

5
6 The lacunar phase of trophoblast development begins around 14 dpf, when the fluid-filled spaces
7 form within the trophoblast syncytium, merging and partitioning the trophoblast into trabeculae¹¹¹, later
8 contributing to the placental villi (**Fig. 5b**). Remarkably, we observed multiple cavities with variable sizes
9 forming inside the syncytiotrophoblast and were predominantly located at the SEM periphery (**Fig 5b-d, e**
10 **top panel; Video S6**), which resembled the trophoblast lacunae on the embryo periphery in utero at CS5c
11 (**Fig. 5d**).

12
13 Another important function of the trophoblast syncytium is production of the hormones, such as
14 human chorionic gonadotropin beta (HCGB), which maintains multiple physiological aspects of pregnancy.
15 The immunostaining for HCGB demonstrated abundance of the hormone protein in surrounding trophoblast
16 cells of the SEM, enriched inside the intracellular vesicles³⁶ (**Fig. 5c; Extended Data Fig. 18e**). We
17 additionally confirmed its secretion by detection of soluble HCG in media in which day 7 SEMs were
18 placed for 24h until day 8 (**Extended Data Fig. 18g**). Lastly, we checked whether the syncytium in our
19 SEMs is multinuclear, as it is typically seen during early placental development¹¹². Phase-images alongside
20 co-immunostaining of the trophoblast marker SDC1 enabled us to see clearer the trophoblast cell shape
21 forming a layer of thick outer syncytium (**Fig. 5e, bottom**). Co-immunostaining with F-ACTIN, that helps
22 define individual cell membrane boundaries, and DAPI, showed that the trophoblast syncytium indeed has
23 multiple nuclei (**Fig. 5f; Extended Data Fig. 18f**). Collectively, these findings confirm proper integration,
24 maturation, and localization of Tb cells in our SEMs.

25 26 **scRNA-seq analysis validates human SEM cellular composition and identity**

27
28 To further validate and examine the milieu of cell types present in the human SEMs generated
29 herein in a more unbiased manner, we performed a single cell transcriptomic analysis by Chromium 10X
30 scRNA-seq on selected day 4, 6 and 8 SEMs. We analyzed ~4,000-8000 cells from pooled high-quality
31 SEMs manually selected at these experimental timepoints. After quality control and strict filtering, a total
32 of 12,190 single cells were used for subsequent analyses (**Extended Data Fig. 19a-c**). Uniform Manifold
33 Approximation and Projection (UMAP) analysis using Seurat package identified a total of 13 separate cell
34 clusters (**Fig. 6a**). Cluster annotation was performed based on expression (+) or lack-of expression (-) of

1 previously defined lineage-specific markers that allowed us to annotate all 13 identified cell clusters (**Fig.**
2 **6 b,c**): Epiblast (Epi – in total four clusters: OCT4+, SOX2+), Yolk-Sac/Hypoblast (YS/Hb – in total three
3 clusters: SOX17+, APOA1+, LINC00261 in addition to GATA6+, GATA4+, PDGFRA+), Extra embryonic
4 mesoderm (ExEM – in total four clusters: FOXF1+, VIM+, BST2+, in addition to GATA6+, GATA4+,
5 PDGFRA+), amnion (Am – one cluster: ISL1+, GABRP+, VTCN1+), and trophoblast (Tb - one cluster:
6 GATA3+, SDC1+, CPM+) (**Fig. 6a,b**).

7
8 From the four annotated Epi clusters (all expressing CD24 primed pluripotency marker alongside
9 OCT4 (POU5F1) and SOX2 (**Extended Data Figure 20a- top row**), we subclassified two of them. The
10 first one, which we termed Posterior epiblast cluster (cluster 4), was marked by upregulation of TBXT
11 (BRACHYURY/T), co-expression of MIXL1, EOMES, MESP1 and WNT8A (**Extended Data Fig. 20a,**
12 **second row**), which are all markers of epithelial to mesenchymal transition (EMT) process, known to
13 accompany upregulation of brachyury during peri-gastrulation in mammalian species¹¹³. The second Epi
14 subcluster (cluster 7) we termed as “committed epiblast” as it was marked by upregulation of ZIC2, ZEB2
15 and VIM lineage commitment marker expression, and absence of NANOG while maintaining OCT4 and
16 SOX2 pluripotency markers (**Extended Data Fig. 20a, bottom row**). Rare PGCs
17 (POU5F1+PRDM1+SOX17+ cells) (n=27) could be also identified within epiblast clusters, most of them
18 (n=19) in cluster 4 (**Fig. 6d**). The fact that PGCs did not create their own subcluster likely results from their
19 relative scarcity within SEMs, as was observed in mouse SEMs^{76,114}. However, these markers are definitive
20 for human PGC identity¹¹⁵ and confirm our immunostaining based detection results for human PGCs in
21 SEMs (**Fig. 3I**). Although we had technical problems in maintaining high trophoblast viability and recovery
22 following SEM enzymatic dissociation, we still observed a well separated cluster expressing lineage
23 markers of trophoblast identity (Tb- cluster 12) (**Extended Data Fig 20b**). While amnion cells can share
24 certain markers with Tb (like GATA3 and TFAP2A)^{87,101}, they formed a separate cluster which also
25 expressed amnion markers like ISL1, GABRP and VTCN1, that are specific to amnion but not to Tb
26 (**Extended Data Fig. 20c**)²⁷. The latter is consistent with amnion immunostaining results in human SEMs
27 shown herein (**Fig. 3g**). It has been hypothesized that human amnion might be a significant source of BMP4,
28 analogous to the extra embryonic ectoderm in mice that serves as a signaling secreting source of Bmp4 and
29 Furin secretion^{87,103,116}. We note that in our scRNA-seq data BMP4 and FURIN are expressed in a fraction
30 of human SEM derived amnion cells (**Extended Data Fig. 20d**).

31
32 Among the three different yolk-sac (YS) clusters that commonly expressed GATA4, GATA6,
33 PDGFRA and APOA1 (clusters 3,5 and 9), we found that recently identified markers of secondary yolk-
34 sac (SYS) in marmoset⁹⁰, TTR, APOB and GSTA1, are expressed more specifically only in cluster 9,

1 supporting its subclassification as SYS (**Extended Data Fig. 21a, Fig. 6a**). STC1, LHX1 are absent in SYS
2 (cluster 9), but not in primary yolk-sac (clusters 3 and 5) also consistent with findings in marmoset.⁹⁰ Lack
3 of uniform HHEX and GSC among most cells in all YS clusters, is consistent with primitive, rather than
4 definitive, endoderm identity for these three YS related clusters (**Extended Data Fig. 21a**)⁵². The co-
5 expression of DKK1 and LHX1 detected via scRNA-seq alongside CER1 expression among some of the
6 SOX17+ cluster 3 YS/Hb cells, supports the validity of AVE cell identity in SEMs (**Extended Data Fig.**
7 **21a – black arrows in second row**). Among the four identified ExEM clusters, based on FOXF1 and BST2
8 expression (**Fig. 6b**), HAND1, LEF1 and BMP4 were also expressed consistent with
9 mesenchymal/mesodermal like identity (**Extended Data Fig. 21b**). However, we noted that ExEM clusters
10 0 and 6, but not ExEM cluster 2 and 8, expressed significantly higher levels of extra cellular matrix related
11 genes (COL3A, COL1A1, COL1A2, COL6A2 and FIBRONECTIN1 (FN1)) (**Extended Data Fig. 21b-c**)
12 suggesting the possibility that that ExEM clusters 2 and 8 might represent a relatively earlier and less mature
13 ExEM lineage identity. While ExEM cluster 8 cells express CER1, DKK1 and LHX1 AVE markers, they
14 lack other key VE/AVE markers like SOX17 and APOA1 and thus were not annotated as AVE, but rather
15 as EXEM cells as they predominantly express mesenchymal signature (**Extended Data Fig. 21a – second**
16 **row; b-first row**). The latter is consistent with CER1, DKK1 and LHX1 being co-expressed also in extra-
17 embryonic mesodermal cells as detected in primate⁹⁰ and gastrulating human embryo datasets¹¹⁷.

18
19 We next conducted a comparison analysis of the transcriptional profile of cells in the human SEMs
20 dataset to an integrated human embryonic reference¹⁰¹ consisting of 5 human embryonic data sets spanning
21 *in vitro* cultured human blastocysts^{24,118,119}, 3D- *in vitro* cultured human blastocysts until pre-gastrulation
22 stages¹²⁰, and a Carnegie Stage 7 (CS7) 16-19dpf human gastrula¹¹⁷ (**Fig. 6e**). UMAP projection confirmed
23 the annotated identity of the SEM clusters and validated their resemblance to the transcriptome and cell
24 type composition of early post-implanted human embryos, including those derived following *in vitro*
25 attached growth (**Fig. 6e; Extended Data Fig. 19d, e**). Projection of SEMs cells onto the human embryonic
26 reference further highlighted that the transcriptional profile of cells differed from human pre-implantation
27 embryos as expected, and all cells corresponded to a post-implanted lineage (Fig. 6e). This analysis
28 validated the detection of post-implantation Epi, YS, ExEM and Tb, as well as emergence of other
29 transcriptomic states, such as amnion, Hb/YS and posterior Epi (**Fig. 6e; Extended Data Fig. 19d, e**).
30 Overall, despite of the known limitations of cross-dataset, cross-platform comparisons, the single cell
31 transcriptomic analysis presented above supports the conclusion that human SEMs recapitulate lineage
32 differentiation of the early human post-implantation embryo.

33

34 Discussion

1
2 Studying early human post-implantation development is crucial for understanding the basis of
3 normal human embryogenesis as well as developmental birth defects, since around a third of human
4 pregnancies fail between implantation and the fourth week of gestation ¹²¹. Moreover, it is becoming
5 increasingly clear that devising and optimizing protocols for 2D *in vitro* human ESC and iPSC
6 differentiation into mature cell types, would greatly benefit from understanding the key mechanical,
7 transcriptional, and signaling pathways active during early embryogenesis, to further improve the quality
8 and efficiency of differentiation protocols ¹²². Such research endeavors would require large numbers of
9 donated human embryo derived materials from early post-implantation stages but justified ethical barriers
10 and the scarcity of such samples, make conducting the mechanistic human embryo-based research ethically
11 and technically challenging. Given the capacity to generate stem cells from human embryonic and
12 extraembryonic components, generating a human stem-cell derived model capable of mimicking the key
13 developmental milestones occurring during the early post-implantation stages is becoming a necessary key
14 element of a growing toolbox of SEMs^{1,2}.

15
16 Here, we readapted an equivalent approach to that recently described in mice ^{27,28} and generated
17 self-organizing human post-implantation SEMs exclusively from naïve PSCs, but without the need to
18 genetically modify or overexpress exogenous lineage factors for priming the naïve PSCs towards the three
19 different extra-embryonic lineages prevalent at these developmental stages, contrary to what is currently
20 still inevitable in mouse SEM derivation protocols ^{27,28}. The latter finding further underscores the self-
21 organization capability of naïve pluripotent stem cells to generate both embryonic and all extra-embryonic
22 compartments ^{36,50,57}, including the ExEM found in human, but not mouse, early pre-gastrulation stages.
23 The human SEMs generated herein mimic the 3D architecture and key developmental landmarks of *in utero*
24 developed natural human embryos from day 7-8 to day 13-14 post-fertilization (Carnegie stage 5a-6a). We
25 observed proper spatial allocation of cell lineages into defined embryonic and extra-embryonic
26 compartments in the complete absence of fertilization or interaction with maternal tissues and without the
27 need of providing external targeted signaling pathway induction during the self-organization of the
28 aggregated cells.

29
30 The fact that human (and mouse) SEM formation bypasses the blastocyst-like stage and that the *ex*
31 *vivo* protocols for growing natural human blastocysts into the authentic 14 dpf stage are still lacking, makes
32 donated fetal materials from 7-14 dpf as the only relevant control for benchmarking the SEM describe
33 herein. Obviously, obtaining new 7-14 dpf human embryo samples is not a viable option from both technical
34 and ethical reasons. However, this limitation is partially mitigated by the available Carnegie and other

1 embryo atlas collections^{82,83}, as well as the ability to compare human development to NHP embryo models
2 and datasets. Moreover, we emphasize that we are only trying to make a reproducible embryo model with
3 recognizable recapitulation of the key milestones and the critical embryonic structures, which will be
4 helpful for research purposes even if it does not entirely resemble the natural human embryo. At the level
5 of structure, our human SEM resembles the situation in utero.

6

7 Naïve pluripotent stem cell growth conditions typically utilize FGF/MEK signaling inhibition,
8 which leads to the loss of imprinting after extended passaging^{123,124}, perturbing the developmental potential
9 of such cells and thus might partially underlie the low efficiency yield and SEM quality. This risk may
10 possibly be mitigated in the future by using naïve conditions with titrating down the concentration of
11 FGF/MEK pathway inhibitors or using alternative naïve conditions that do not target this pathway^{123,125}. At
12 this moment, the reduced efficiency and heterogeneity observed during the formation of human SEMs is a
13 complicating factor that needs to be overcome to facilitate the use of such platforms as viable experimental
14 set-ups. Nevertheless, the emergence of well-defined complete structures suggests that this will likely be
15 possible. It is plausible that upon further experimentation and mechanistic understanding of self-
16 organization, the efficiency and variability in SEM formation can be improved in the future. It is also likely
17 that alternative *ex utero* culture platforms, aggregation strategies, or growth conditions will yield similar or
18 enhanced results relative to the ones reported herein with human SEMs.

19

20 Testing of whether human SEMs described here can develop further towards completing
21 gastrulation and initiating organogenesis as achieved with mouse SEMs, may be of critical experimental
22 importance and may offer insights into previously inaccessible windows of early human development^{1,2}.

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1 **Acknowledgments:** J.H.H lab was funded by Pascal and Ilana Mantoux; Flight Attendant Medical
2 Research Institute (FAMRI); MBZUAI-WIS Program, ISF; Minerva, Israel Cancer Research Fund (ICRF),
3 Kimmel Stem Cell Research Center, and a sponsored research program by Renewal Bio Ltd. F.L. lab is
4 supported by the Ming Wai Lau Center for Reparative Medicine, Ragnar Söderbergs Stiftelse, Wallenberg
5 Academy Fellow, Center for Innovative Medicine and Karolinska Institutet SFO Stem Cells and
6 Regenerative Medicine. S.P. lab is supported by the Swedish Research Council, Swedish Society for
7 Medical Research. S.P. holds the Canada Research Chair in Functional Genomics of Reproduction and
8 Development (950-233204),

9
10 **Author contributions:** B.O and E.W established the SEM aggregation conditions and protocols for ex
11 utero culture, designed and conducted most of the wet lab work and contributed on manuscript elaboration.
12 B.O established the human stem cell conditions, and inductions. E.W and V.B conducted most embryo
13 immunostaining and confocal imaging. V.B made light sheet microscopy analysis and helped writing the
14 manuscript. B.O. generated cell lines with assistance from S.V and optimized the final SEM protocol.
15 A.A.C conducted some aggregation experiments and roller culture adaptation for the system,
16 immunostaining and microscopy and sample preparation for 10X scRNA-seq experiments. M.Y.C helped
17 on human stem cell culture expansion and optimizations. C.Z conducted the scRNAseq comparative
18 analysis to previous human and NHP datasets, under the supervision of F.L and S.P. S.T contributed on
19 optimization of lineage inductions. R.C generated MEF and other critical reagents for stem cell
20 maintenance. S.A and D.L conducted immunostainings and RT-PCR. F.R, C.J, M.R assisted in
21 immunostainings. N.L assisted on lentivirus production and flow cytometry experiments. E.A supervised
22 flow cytometry and sorting experiments. T.S helped in bioinformatic analysis. S.V generated plasmids.
23 A.A.C, M.K. and H.K.S performed RNA library preparation and sequencing. S.A provided input on
24 optimizing light sheet microscopy experimentation and analysis. N.N. – conducted and supervised
25 bioinformatics analyses. J.H.H. conceived the idea for the project, supervised data analysis and manuscript
26 writing.

27
28 **Declaration of interests:** J.H.H has submitted patent applications relevant to the findings reported herein
29 and is a chief scientific adviser of Renewal Bio Ltd. that has licensed technologies described herein.

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31
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34

1 **Figure Legends**

2

3 **Figure 1. Optimizing human naïve PSC differentiation towards extra-embryonic lineages competent**
4 **for early post-implantation SEM generation. a**, left, scheme of the tested induction (iGATA4, iGATA6,
5 iCDX2, iGATA3) and media conditions for generating the three different needed extraembryonic lineages
6 from naïve pluripotent stem cells (nPSC). Right, scheme of the early human post-implantation embryo with
7 the epiblast (cyan), hypoblast (yellow), ExEM (grey), and trophoblast (magenta) compartments. **b**, Flow
8 Cytometry (FACS) plots of PDGFRa-APC versus SSC for PrE/ExEM induction using iGATA4 with DOX
9 in HENSM after 6 days (right) and the control condition of naïve cells (WT cells without iGATA4, left). **c**,
10 from left to right, FACS plots of PDGFRa-APC for PrE/ExEM induction using iGATA4 and iGATA6 with
11 DOX for 6 days in different media conditions as indicated (C10F4PDGF, RACL, RCL). **d**, from left to
12 right, FACS plots of PDGFRa-APC for PrE/ExEM using WT naïve PSCs induced 6 days in different media
13 conditions (N2B27, RCL, and RCL for 3 days followed by 3 days of N2B27). **e**, immunofluorescence
14 images of WT nPSCs induced in RCL media for SOX17 (yellow) and BST2 (red). Two distinct cell
15 populations emerge with mutually exclusive expression pattern (outlined); scale bar, 100 μ m. **f**, FACS plots
16 of ENPEP against TACSTD2 for trophoctoderm (TE) lineage induction of HENSM naïve PSCs in BAP(J)
17 regimen for 3 days. **g**, immunofluorescence images of day 6 SEM aggregates stained for OCT4 (cyan),
18 SOX17 (yellow) and SDC1 (magenta). Aggregates made with iGATA3 in DOX for 72h in BAP(J), showed
19 no outer surrounding trophoblasts in the obtained aggregates (left). In contrast, WT nPSCs in BAP(J)
20 showed very high efficiency in yielding aggregates with outer layer of surrounding trophoblast (marked by
21 SDC1 – magenta). Scale bars 100 μ m. RACL **R**PMI based medium with **C**HIR99021, **A**CTIVIN, and **L**IF;
22 RCL, **R**PMI based medium with **C**HIR99021 and **L**IF without **A**CTIVIN; HENSM, **H**uman **E**nhanced
23 **N**àive **S**tem cell **M**edia; BAP(J), DMEM/F12 based medium with ALK4/5/7 inhibitor **A**83-01, ERKi/MEKi
24 **P**D0325901, and **B**MP4 substituted with **J**AK inhibitor I after 24h.

25

26 **Figure 2. Self-assembly of human Post-Implantation SEM exclusively from non-transgenic naïve**
27 **PSCs. a**, anatomical sections taken from the Carnegie collection and schemes of the human embryo
28 development between 7 and 14 days post-fertilization (dpf); left to right, Carnegie Stages (CS) CS5a (7-8
29 dpf), CS5b (9-10 dpf), CS5c (11-12 dpf), and CS6a (13-14 dpf). **b**, scheme of the protocol for generating
30 human early post-implantation SEMs. Hypoblast/ExEM (yellow/grey), epiblast (cyan), and trophoblast
31 (magenta) lineage priming for 3 days from naïve hPSCs in HENSM media is followed by aggregation
32 (counted as day 0) in Aggrewell 400 at the 1:1:3 nPSC: PrE/ExEM: TE ratio in N2B27. Starting from day
33 3, SEMs are transferred to non-adherent 6-well plates on a shaker and cultured in *Ex-Utero* Culture Medium
34 2 (EUCM2) with increasing concentrations of FBS until day 8 (see Methods). **c**, representative brightfield

1 images of SEMs growing between days 0 and 8 showing formation of the inner embryonic structures, which
2 were defined based on the lineage-specific immunofluorescence (see **Extended Data Figure 12b**). Scale
3 was maintained to show the growth. **d**, from left to right, immunofluorescence images of SEMs from days
4 4, 6, and 8 of the protocol, showing OCT4 (cyan), SOX17 (yellow), CK7 (magenta), and nuclei (DAPI,
5 white), labeling different embryonic structures (marked with arrows). **e**, quantification of the SEM
6 derivation efficiency across three independent experimental replicates. **f**, 3D reconstruction of the day 8
7 SEM shown in **d** (right) with segmented epiblast, (OCT4, cyan), hypoblast (SOX17, yellow), CK7
8 (magenta) and nuclei (DAPI). Bottom, segmentation of the epiblast and hypoblast shown in 0 and 90°
9 degrees of rotation. See also **Video S1**. Epi, epiblast; AC, amniotic cavity; Am, amnion; TE, trophoctoderm;
10 Tb, trophoblast; PrE, primitive endoderm; Hb, hypoblast; PYS, primary yolk sac; VE, visceral endoderm;
11 PE, parietal endoderm; SYS, secondary yolk sac; ExEM, extraembryonic mesoderm; ChC, chorionic
12 cavity; Sk, stalk; L, lacunae. Scale bars, 50 μm.

13

14 **Figure 3. Human SEMs undergo epiblast morphogenesis and form a bilaminar disk with amnion and**
15 **PGCs. a**, immunofluorescence images of naïve PSCs in HENSM medium, showing expression of OCT4
16 (cyan), DNMT3L (green, top), STELLA (green, middle), but not OTX2 (red). **b**, immunofluorescence
17 images of Day 3 SEMs in N2B27 showing expression of OCT4 (cyan) and OTX2 (red), but not DNMT3L
18 (green, top) or STELLA (green, middle). **c**, immunofluorescence images of SEMs developing from day 4
19 to day 8 showing epiblast (OCT4, cyan), hypoblast (SOX17 or GATA6, yellow), and trophoblast (CK7,
20 SDC1 or GATA3, magenta). Right, zoom into the epiblast. Single- and double- asterisks mark proamniotic
21 and amniotic cavities, respectively. **d**, quantification of epiblast growth by counting cell numbers in day 4,
22 6, and 8 SEMs. p-values, two-sided Mann-Whitney U-test. **e**, immunofluorescence images of day 6 SEMs
23 showing apical cell markers: phospho-Ezrin/Radixin/Moesin (ph-ERM, red; top), Podocalaxyn (PODXL,
24 red; bottom), and F-actin (green). **f**, immunofluorescence image, showing Anterior-Posterior (A-P) axis,
25 marked by expression of T/BRA (red) and CER1 (green) on the opposite sides of the SEM in day 8. Right,
26 zoom into the epiblast (OCT4, cyan). **g**, immunofluorescence image, showing amnion, marked by co-
27 expression of TFAP2A (magenta) and ISL1 (green) in SOX2-negative cells (cyan) of the day 8 SEM. Right,
28 zoom into the epiblast region; arrows point at the amnion cells. **h**, immunofluorescence image, showing
29 putative amnion in day 7 SEM, characterized by squamous OCT4-positive cells; OCT4 (cyan), DAPI
30 (white). **i**, histological sections from the Carnegie collection of the human embryo at Carnegie Stage (CS)
31 CS5c highlighting the start of amnion (Am) formation. **j**, immunofluorescence image, showing amnion in
32 day 8 SEMs, characterized by squamous cells expressing TFAP2A (magenta), but not SOX2 (cyan); nuclei
33 (DAPI, white). See also **Video S2**. **k**, histological sections taken from the Carnegie collection of the human
34 embryo at Carnegie Stage (CS) CS6 highlighting amnion (Am) located dorsally to the bilaminar disk. **l**,

1 immunofluorescence images (Z slices 22 and 26, top and bottom, respectively) of day 8 SEM, showing
2 progenitor germ cells (PGCs) with co-expression of OCT4 (cyan), SOX17 (yellow), and BLIMP1 (red).
3 Right, zoom into the individual PGCs, marked with arrows. Inset, brightfield image, embryo perimeter is
4 outlined. Single- and double- asterisks mark proamniotic and amniotic cavities, respectively. Scale bars, 50
5 μm , 25 μm (e, right), 10 μm (l, right).

6
7 **Figure 4. Human SEMs recapitulate development of yolk sac lumenogenesis and extra-embryonic**
8 **mesoderm scaffolding. a**, immunofluorescence image, showing yolk sac (YS) parietal (PE) and visceral
9 (VE) endoderm (SOX17, yellow) in day 6 SEM. Nuclei (DAPI, blue), F-actin (white). Right, zoom into
10 cuboidal VE and squamous PE cells. See also **Video S4. b**, quantification of cell aspect ratio (height/width)
11 in VE (n = 14) and PE (n = 12) cells. **c**, Histological section taken from the Carnegie collection of the
12 human embryo at Carnegie Stage 5c with a primitive yolk sac (PYS), cuboidal visceral endoderm (VE, top
13 right), and squamous parietal endoderm (PE, bottom right). **d**, immunofluorescence images of day 6 SEMs
14 showing epiblast (OCT4, cyan), hypoblast (SOX17, GATA6, yellow), and trophoblast (CK7, GATA3,
15 magenta); nuclei (DAPI, white). Right, 2x zoom into the yolk sac (YS) region with extraembryonic
16 mesoderm (ExEM). White arrows point at the VE and PE. **e**, immunofluorescence image of day 6 SEM
17 showing formation of chorionic cavity (ChC) surrounded by ExEM (outlined) expressing GATA6 (red) and
18 not SOX17 (yellow); nuclei (DAPI, white). Note lower expression levels of GATA6 in ExEM relative to
19 YS cells. **f**, immunofluorescence image of day 6 SEM showing expression of BST2 in the cell membrane
20 (red) in ExEM underneath the YS (SOX17, yellow); OCT4 (cyan), nuclei (DAPI, white). Right, zoom into
21 the ExEM region. **g**, immunofluorescence image of day 8 SEM showing expression of VIM (red) in ExEM
22 underneath the YS (SOX17, yellow); OCT4 (cyan), nuclei (DAPI, white). Right, zoom into the ExEM
23 region. The boundaries between the YS, ExEM, and trophoblast (Tb) are outlined. See also **Video S5. h**,
24 schematic of the 14 dpf human embryo showing ExEM in grey; Am, amnion; Sk, stalk; SYS, secondary
25 yolk sac. **i**, brightfield and immunofluorescence images of day 8 SEM in different Z slices (Z 42 and 67)
26 showing FOXF1 (green), GATA6 (red), and OCT4 (cyan). Arrows point to epiblast (Epi), ChC, ExEM,
27 YS, Sk, and a secondary yolk sac (SYS); scale bar, 100 μm . **j**, immunofluorescence image of day 8 SEM
28 with a cavitated ExEM marked by BTS2 (white), and SYS; OCT4 (cyan), SOX17 (yellow). Red arrows
29 point at the SOX17+ PYS cell remnants. See also sequential sections from the same SEM in **Extended**
30 **Data Fig. 17. k**, histological section taken from the Carnegie collection of the human embryo at Carnegie
31 Stage 6 showing filamentous ExEM and the secondary yolk sac (SYS). Scale bars, 50 μm , 10 μm (zoom:
32 a, c, f).

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1 **Figure 5. Trophoblast compartment integration and maturation in human SEMs.** **a**, top,
2 immunofluorescence images of day 6 SEMs showing epiblast (OCT4, cyan), hypoblast (SOX17 or GATA6,
3 yellow), and trophoblast (GATA3, CK7 or SDC1, magenta). Bottom, single-channel images of the
4 trophoblast, surrounding the SEMs. **b**, scheme of the 11-12 dpf human embryo showing multinucleated
5 syncytiotrophoblast (magenta) with lacunae. **c**, left, maximum intensity projection image of day 6 SEMs
6 showing HCGB expression in the outer cells; F-ACTIN (red). Right, immunofluorescence image of the
7 same SEM showing intracellular lacunae (marked with asterisks) inside the outer syncytiotrophoblast cell
8 layer; nuclei (DAPI, white). **d**, histological section and 3D reconstruction (top right) from the Carnegie
9 collection of a human embryo at Carnegie Stage (CS) 5c, showing lacunae in the syncytiotrophoblast
10 (asterisks). **e**, brightfield and immunofluorescence images of two different Z planes (number 3 and 11, top
11 and bottom panels, respectively) of day 6 SEMs showing epiblast (OCT4, cyan), hypoblast (SOX17, or
12 GATA6, yellow), and trophoblast (SDC1, magenta). Top row - Lacunae are outlined and marked with
13 asterisks. Right, zoom into the lacunae (top). Bottom row - the outer syncytiotrophoblast layer (bottom), its
14 thickness is marked with brackets. **f**, immunofluorescence image of day 6 SEM showing CK7 (magenta),
15 F-ACTIN (red), and nuclei (DAPI, white). Bottom, zoom into the multinucleated syncytiotrophoblast cell;
16 arrows point at multiple nuclei inside the single cell. Scale bars, 50 μ m, 10 μ m (e, zoom).

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18 **Figure 6. scRNA-seq analysis validates key cell type identity comprising the human SEM.**

19 **a**, UMAP plot displaying individual human SEM derived cells. Points are colored according to their
20 assigned cell cluster. All 13 clusters representing multiple cell types were identified based on known marker
21 genes and overlap with gene signatures, as indicated. ExEM: Extra-embryonic Mesoderm; YS: Yolk-Sac;
22 Hb: Hypoblast; SYS: Secondary Yolk Sac; Am: Amnion; Tb: Trophoblast. **b**, Normalized expression of
23 key marker genes projected on SEM UMAP. Cell type cluster highlighted is indicated in red, along with
24 the corresponding cluster number. **c**, Dot-plot illustrating the expression of key marker genes across the 13
25 clusters. Cell types as in (A) and (B). Color shade indicates average expression. Dot size indicates the
26 percentage of cells in the cluster which express the marker highlighted. **d**, left: POU5F1+PRDM1+SOX17+
27 cells (n=27) can be identified in Epi clusters, with majority located in cluster 4 (n=19). Right: Normalized
28 expression heatmap of PGC markers (SOX17, PRDM1, NANOS3, NANOG, POU5F1). 9 cells were
29 positive for POU5F1, PRDM1, SOX17 and NANOS3, again confirming their identity as human PGCs. **e**,
30 Left: UMAP projection of integrated human embryonic reference ¹⁰¹ consisting of 5 human embryonic data
31 sets spanning in vitro cultured human blastocysts ^{24,118,119}, 3D- in vitro cultured human blastocysts until pre-
32 gastrulation stages ¹²⁰, and a Carnegie Stage 7 (CS7) 16-19dpf human gastrula ¹¹⁷. The color of each data
33 point corresponds to the cell annotations retrieved from the respective publication. Right: The grey data
34 points represent embryonic reference cells, as in the left panel. Each colored triangle represents the

1 projection position of representing neighborhood nodes from SEM cells onto the human embryonic
2 reference UMAP space.

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1 **Extended Data Figure Legends**

2 **Extended Data Figure 1. Optimization of extra embryonic lineage induction using transient**
3 **overexpression of GATA4 and GATA6. a**, scheme of the donor plasmid vector used for genomic
4 integration of the DOX-inducible iGATA4 or iGATA6 overexpression in human PSCs. **b**, Representative
5 immunofluorescence images of mono- and polyclonal iGATA4 (left) and iGATA6 (right) WIBR3 hESC
6 (W3) clones, showing uniform expression of GATA4 (green) and GATA6 (green) only in response to DOX;
7 nuclei (DAPI, blue). Scale bars, 50 μ m. **c**, Flow Cytometry (FACS) plots of PDGFRa-PE/Cy7 marking
8 primitive endoderm (PrE) induction from mouse embryonic stem cells (ESC) using iGata4 with DOX for
9 48 hours in 2i/Lif medium (right) versus the control condition (without DOX). **d**, FACS plots of PDGFRa-
10 APC for putative PrE/ExEM induction from human naïve pluripotent stem cells (nPSCs) using iGATA6
11 with DOX in different media (N2B27 and HENSM) as indicated. **e**, Representative FACS plot of PDGFRa-
12 APC for putative PrE/ExEM induction from iGATA4 nPSCs in NACL media. **f**, Representative FACS
13 plots of PDGFRa-APC for putative PrE/ExEM PrE induction of wild type (WT, non-genetically modified)
14 nPSCs in RCL media with B27 with insulin (left) or B27 without insulin (right). **g**, Representative FACS
15 plots of PDGFRa-APC for putative PrE/ExEM induction from human nPSCs after three days in RCL
16 medium (conventional 2D conditions) followed by three days of RCL in aggregation setting (3D -left) or
17 N2B27 (right). **h**, qRT-PCR gene expression (normalized by GAPDH and ACTIN) of the endodermal
18 markers for PrE/ExEM induction in RCL medium starting from naïve (blue) vs primed (red) human PSC.
19 Naïve human PSCs maintained in HENSM (white) were used as a reference control (set as 1). (DE) - definitive
20 endoderm.

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22 **Extended Data Figure 2. Immunostaining for PrE signature in naïve PSC derived cells in RCL**
23 **conditions. a, b**, immunofluorescence images of WT and iGATA6 naïve PCS cells with and without DOX
24 induced in RCL media for 3 or 6 days as indicated (**a** and **b**, respectively), showing expression of the
25 indicated PrE markers (red), including SOX17, and nuclei (DAPI, blue). Scale bars, 100 μ m.

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27 **Extended Data Figure 3. Optimization of PrE/ExEM induction protocol from human naïve PSCs. a**,
28 RT-qPCR gene expression (normalized by GAPDH and ACTIN) of the endodermal marker genes, in RCL
29 (yellow), RACL (blue), NACL (dark blue), and N2B27 (grey) media conditions versus naïve PCSs used as
30 reference control (white). PrE-/ExEM and definitive endoderm (DE)-specific genes are separately
31 underlined. **b**, immunofluorescence images iGATA6 nPSCs induced in RCL media for 6 days (with or
32 without DOX), showing expression of SOX17 (yellow), BST2 (red), and GATA4 (green) (see also **Fig.**
33 **1e**). In both set-ups, BST2-positive (ExEM) and SOX17 – positive (PrE) cell populations are mutually
34 exclusive. Scale bars, 200 μ m. **c**, immunofluorescence images of WT WIBR3 (W3) nPSCs induced in RCL

1 media for 6 days, showing expression of GATA4 (green), FOXF1 (blue), and BST2 (white); nuclei (DAPI,
2 white; left panel). As expected, GATA4 marks both SOX17+ PrE and BST2+ ExEM populations. Scale
3 bars, 500 μ m. **d**, left, immunofluorescence images of iGATA6 nPSCs induced with DOX in RCL media
4 for 3 days (top) and 6 days (bottom). **d**, Immunofluorescence images of WT nPSCs in RCL media for 6
5 days (bottom). SOX17 (yellow), BST2 (red), nuclei (DAPI, white). Scale bars, 100 μ m. Please note that
6 the BST2+ cells are GATA4+/FOXF1+, excluding the possibility that they represent residual pluripotent
7 cells in the RCL induced cultures.

8

9 **Extended Data Figure 4. Testing Tb induction using transient overexpression of CDX2 in human**
10 **naïve PSCs. a**, relative proportion of the indicated cell types among extra-embryonic cells of mouse natural
11 embryos grown in utero or ex utero and in day 8 mouse SEMs generated from iCdx2 mouse naïve ESCs
12 (and not embryo derived mouse TSC lines)⁷⁶. Three pooled samples are presented: *in utero* natural embryos
13 (n = 2401 cells), *ex utero* natural embryos (n = 1382), and mouse iCdx2 day 8 SEMs (n = 6249). The cell
14 types: Chorion, Intermediate-Chorion, Chorion Progenitors, Uncommitted Ectoplacental-Cone Cells
15 (EPC), Trophoblast Giant Cells (TGC) progenitors, parietal trophoblast giant cells (pTGC), spiral artery
16 associated trophoblast giant cells (SpA-TGC), and junctional zone spongiotrophoblast cells (SpT-Gly)
17 based on previously published similar analysis and annotations⁷⁷. **b**, frequencies of the cell types presented
18 in **(a)**, showing a significant reduction of TGC-progenitors and pTGCs in *ex utero* embryos (natural and
19 SEM), compared to *in utero* embryos. Shaded area represents 95% confidence interval. This analysis
20 confirmed that naïve PSCs derived TSC lineage following Cdx2 overexpression under optimized conditions
21 developed in⁷⁶, can contribute to both the chorionic and ectoplacental cone lineages in mouse SEMs
22 generated exclusively from mouse naïve PSCs. **c**, scheme of the donor plasmid vector used for genomic
23 integration of the DOX-inducible CDX2 overexpression in human PSCs. **d**, immunofluorescence images
24 of iCDX2 cells, showing CDX2 (green) expression only in response to DOX; nuclei (DAPI, blue). Scale
25 bars, 50 μ m. **e**, images of iCDX2 (left) and WT (right) human W3 ESCs in HENSM conditions, showing
26 live fluorescence of tdTomato (red) after transfection with lentiviral particles carrying the fluorophore.
27 Scale bars, 50 μ m. **f**, top, the scheme of the experiment, where naïve PSCs and Hypoblast/ExEM induced
28 cells were aggregated with iCDX2 cells, induced by DOX for 48h in three different media, PALY (N2B27
29 supplemented with PD0325901, A83-01, hLIF, and Y-27632)²², TSCm⁷⁰, and BAP(J) (DMEM/F12 based
30 medium with ALK4/5/7 inhibitor A83-01, FGF2 inhibitor PD0325901, and BMP4 substituted with JAK
31 inhibitor I after 24h). **f**, bottom, immunofluorescence images showing no surrounding trophoblast in
32 aggregates with iCDX2 cells, regardless of the media conditions; epiblast (OCT4, cyan), hypoblast
33 (GATA4, SOX17, yellow), trophoblast (CK7, SDC1, magenta), nuclei (DAPI, blue). Scale bars (from left
34 to right), 100 μ m, 100 μ m, 50 μ m. **g**, RT-qPCR gene expression (normalized by GAPDH and ACTIN) of

1 iCDX2 (blue) and WT (red) cells in TSCm conditions for one, three, and six days versus naïve PSCs
2 maintained in HENSM conditions and used as a reference control (white). **h**, top, the scheme of the
3 experiment, in which naïve PSCs and Hypoblast/ExEM induced cells were aggregated with WT or iCDX2
4 tdTomato-labelled nPSC induced towards trophoblast (TE) in PALY media with or without DOX as
5 indicated. **h**, bottom, brightfield and live fluorescence images of day 4 – 7 aggregates showing localization
6 of the trophoblast. Scale bar, 200 μm . **i**, Phase contrast microscopy images showing iCDX2 cells induced
7 for 72h in different media (HENSM, BAP(J), TSCm) with or without DOX, showing reduced viability upon
8 iCDX2 transgene overexpression. In all conditions, 750,000 iCDX2 cells were seeded in 10 cm Matrigel
9 coated plates, and DOX induction was started 24h after seeding. Scale bar, 100 μm .

10

11 **Extended Data Figure 5. Testing SEM aggregations with human conventional trophoblast stem cell**

12 **lines. a**, scheme depicting aggregation protocol of epiblast (naïve hPSCs in HENSM media) and PrE/ExEM
13 cells with tdTomato-expressing validated human TSC line derived from human primed ESCs (termed
14 pTSC). **b**, brightfield images and live fluorescence of tdTomato (red) in aggregates with labeled TSCs;
15 scale bar, 200 μm . Right, zoom into the several SEMs with tdTomato signal. Scale bar, 50 μm . The
16 aggregates with TSCs do not form a uniformly surrounding TB layer, but rather remain as isolated clumps.
17 **c**, immunofluorescence images of day 8 aggregates, showing OCT4 (cyan), SDC1 (magenta), and TSCs,
18 labelled by tdTomato; nuclei (DAPI, white). Scale bar, 50 μm . White arrows highlight TSC isolated clumps.
19 **d**, sequential aggregation of nPSCs (in HENSM) with tdTomato-expressing primed PSC derived TSC line
20 on the first or second days of the aggregation protocol. **e**, brightfield images and live fluorescence of
21 tdTomato (red) in day 5 and 8 aggregates with TSCs, added on the first (top) or second (bottom) days of
22 aggregation; scale bar, 200 μm . Right, zoom into the several SEMs with tdTomato signal. Scale bar, 50
23 μm . The aggregates with TSCs do not form a uniformly surrounding TB layer, but rather remain as isolated
24 clumps.

25

26 **Extended Data Figure 6. Testing SEM aggregations with naïve PSC derived human trophoblast stem**

27 **cell lines. a**, immunofluorescence images validating correct expression of TSC marker genes in colonies of
28 a WIBR3 (W3) naïve hESC derived TSC line (termed nTSC). CK7, TFAP2C, GATA3, CDX2 (all in
29 green); DAPI (blue). Scale bar, 100 μm . **b**, scheme for aggregation protocol of naïve pluripotent stem cells
30 (nPSCs) in HENSM media, naïve-derived trophoblast stem cells (nTSCs), and nPSCs induced in RCL
31 towards PrE/ExEM for 3 days. **c**, immunofluorescence images showing rare expression of CK7, GATA3,
32 and SDC1 (red) trophoblast markers in the aggregates. nuclei (DAPI, white); Left column scale bar, 500
33 μm ; Right, zoom into several aggregates with CK7 expression; scale bar, 100 μm . **d**, immunofluorescence
34 image from (**upper left panel in c**), showing Epi (OCT4, cyan) and PrE (SOX17, yellow) with CK7 (red);

1 nuclei (DAPI, white). Zoom ins from the indicated regions are shown. Although some aggregates express
2 lineage markers, they do not organize into embryoid-like structures and are not uniformly surrounded by
3 the trophoblast compartment. Scale bar, 500 μm ; bottom, 100 μm .

4

5 **Extended Data Figure 7. Evaluating trophoblast induction using transient overexpression of GATA3**

6 **in human naïve PSCs. a**, scheme of the donor plasmid vector used for genomic integration of the DOX-

7 inducible iGATA3 overexpression transgene. **b**, immunofluorescence images of iGATA3 cells, showing

8 uniform GATA3 expression (green) only in response to DOX; nuclei (DAPI, blue). Scale bars, 100 μm . **c**,

9 brightfield images of WT (top) and iGATA3 (bottom) cells after incubation in BAP(J) media for three days.

10 Scale bar, 200 μm . **d**, qRT-PCR gene expression (normalized by GAPDH and ACTIN) of the trophoblast

11 markers for WT naïve pluripotent stem cells (nPSCs) in BAP(J) media (purple) and iGATA3 nPSC cells

12 induced by DOX in BAP(J) media (yellow), versus nPSCs maintained in HENSM media and used as a

13 reference control (set as 1) (white). **e**, immunofluorescence images showing different patterns of expression

14 of trophoblast marker genes in the wild type (WT) nPSCs incubated in BAP(J) media for three days (top)

15 versus iGATA3 cells, induced by DOX in BAP(J) media (bottom). GATA3 (magenta), TFAP2C (magenta),

16 GATA2, CDX2, SDC1, and HCGB (all in green), nuclei (DAPI, blue). Scale bar, 100 μm . **f**,

17 immunofluorescence images showing uniform expression of CK7 in colonies of WT nPSCs incubated in

18 BAP(J) media for three days (top), whereas colonies of iGATA3 cells induced by DOX in BAP(J) media

19 express CK7 heterogeneously, with most of them being negative for CK7. Scale bar, 100 μm . **g**, from left

20 to right, FACS plots of ENPEP versus TACSTD2 for trophoblast (Tb) priming using iGATA3 induction in

21 different media (AP(J) with and without DOX, BAP(J) with and without DOX), and using WT nPSC

22 priming to trophectoderm in AP(J) and BAP(J) regimens. Percentage of double positive population is

23 indicated. This result shows that transient expression of the GATA3 transgene is not required for Tb

24 differentiation and that it can be achieved with BAP(J) media using WT nPSCs as starting cells.

25

26 **Extended Data Figure 8. Enhancer accessibility of extra-embryonic lineage master regulators**

27 **GATA3, GATA4 and GATA6 in human but not mouse naïve PSCs.** ATAC-seq and RNA-seq of

28 GATA3, GATA4 and GATA6 genes in human vs. mouse, as measured in naïve PSCs and in multiple

29 differentiated cell types (as indicated). HENSM conditions were used for human naïve PSCs. Known

30 enhancers are marked in grey bars at the bottom. Putative enhancers are marked in red or green: (1) green

31 indicates potential enhancers that are already open in naïve stem cells, (2) red indicates enhancers that are

32 closed in naïve pluripotent stem cells, but open in at least one differentiated cell state. Putative enhancers

33 in the approximate regions of the genes were manually selected. Open regions that overlap with promoter

34 or exon were excluded from the analysis. RNA-seq of the indicated genes in naïve PSCs are shown. The

1 latter shows that while Gata6, Gat3 and Gata4 master regulators of extra-embryonic lineage
2 differentiation^{63,127} are not transcribed in mouse and human naïve PSCs, their enhancers are predominantly
3 more accessible in human naïve PSCs, but not in mouse naïve PSCs.

4

5 **Extended Data Figure 9. Optimization of the cell numbers co-aggregated for conducive human SEM**

6 **formation exclusively from human naïve PSCs. a**, scheme of the protocol: induced PrE/ExEM cells

7 (yellow), WT naïve pluripotent stem cells (nPSCs) maintained in HENSM medium (cyan), and WT nPSC

8 induced towards trophoblast with BAP(J) medium (magenta) were aggregated on Aggrewell 400 platform

9 at different ratios in N2B27 and grown as indicated in the scheme until day 6. **b**, left, brightfield images of

10 day 6 SEMs, aggregated from total 60 cells at 1:1:3 (nPSC: PrE/ExEM: TE) cell ratio, showing mostly

11 small and fragmented aggregates. Right, brightfield images of day 6 SEMs, aggregated from total 120 cells

12 at 1:1:3 (nPSC: PrE/ExEM: TE) cell ratio, showing lower fragmentation tendency and more frequent

13 formation of bigger aggregates (this condition was chosen for SEM generation). Scale bar, 1000 μ m; zoom,

14 125 μ m. **c**, immunofluorescence images of day 6 SEMs showing epiblast (OCT4, cyan), hypoblast (SOX17,

15 yellow), and trophoblast (SDC1, magenta); nuclei (DAPI, white). Left, Day 6 SEMs aggregated from total

16 132 cells at 4:2:7 (nPSC: PrE/ExEM :TE) cell ratio, or , right, from total 160 cells at 2:1:5 (nPSC:

17 PrE/ExEM :TE) cell ratio showing inadequate organization of SEMs, when compared to optimized

18 conditions (120 cells at 1:1:3 nPSC: PrE/ExEM: TE) as shown in **Extended Data Figure 12c**. Scale bars,

19 100 μ m. **d**, left, scheme of the experiment testing the capacity of cells, differentiated from human WIBR3

20 primed PSCs, to form equivalent SEMs to those obtained from isogenic naïve PSCs expanded in HENSM

21 conditions. Right, immunofluorescence images of day 6 SEMs showing OCT4 (cyan), SOX17 (yellow),

22 CK7 (magenta), and nuclei (DAPI, white). Scale bar, 200 μ m. The resulting aggregates when starting with

23 isogenic primed PSCs, did not present organization and maturation of the key embryonic and extra-

24 embryonic compartments as seen when starting with naïve PSCs (please compare to **Fig. 2**).

25

26 **Extended Data Figure 10. Optimization of the aggregation conditions for human SEM generation**

27 **from naïve PSCs. a**, scheme of the protocol: PrE/ExEM induced cells in RCL medium (yellow), WT naïve

28 pluripotent stem cells (nPSCs) maintained in HENSM medium (cyan), and iGATA3 trophoblast cells

29 induced with DOX in BAP(J) medium (magenta) were aggregated in different media in Aggrewell 400

30 plate at 1:1:3 (nPSC: PrE/ExEM: TE) ratio and cultured until day 6. **b**, from bottom to top, representative

31 brightfield images of day 2 and 4 SEM, aggregated in DMEM-F12:Neurobasal (1:1) supplemented with

32 20% FBS or N2B27, CMRL 1066 base medium supplemented with 20% FBS or N2B27, CMRL

33 supplemented with N2B27 and extra-added 3 mg/ml D-Glucose, and DMEM F12: Neurobasal (3:1).

34 Although addition of FBS is beneficial for SEM growth, FBS impairs aggregation by day 4, and the best

1 aggregation efficiency was consistently observed in DMEM-F12:neurobasal supplemented with N2B27
2 without FBS. **c**, representative brightfield images showing of SEM morphology at day 6 after aggregation
3 in DMEM-F12:Neurobasal and CMRL supplemented with 20% FBS. Addition of FBS impaired formation
4 of the aggregates while N2B27 conditions allowed better human PSC derived aggregate growth. Scale bars,
5 200 μm .

6
7 **Extended Data Figure 11. Optimization of human SEM culture conditions.** **a**, brightfield images of day
8 2 SEM aggregates inside the Aggrewell 400 plate; addition of BSA to the aggregation medium prevents
9 attachment of the aggregates to the plate edges (white arrows). Scale bar, 400 μm . Optimal BSA
10 concentration chosen for further experimentation highlighted by a green box. **b**, growth of SEMs in 6-well
11 non-adherent plates without shaking leads to their attachment to the plate and disruption of morphology.
12 Scale bar, 200 μm . **c**, scheme of the experiment in which volume of the culture media on the non-adherent
13 6-well dish, using orbital shaking (day 4-8), was optimized. **d**, brightfield images of multiple SEMs showing
14 that the rate of their clumping with each other is dependent on the media volume (1 – 6 ml). The optimal
15 suspension condition is outlined in green (3ml). Scale bar, 200 μm . **e**, top, the scheme of roller culture test,
16 where aggregates were generated as described (see Methods), cultured until day 6 on a shaker, followed by
17 roller culture in EUCM2 50% FBS 20% O₂ 5% CO₂. Bottom, immunofluorescence images of day 7 SEMs
18 in this regimen, showing Epi (OCT4, cyan), hypoblast (SOX17, yellow), and trophoblast (CK7, magenta);
19 nuclei (DAPI, white), brightfield (BF); scale bar, 200 μm . Right, example of the SEM in which epiblast,
20 hypoblast, and trophoblast are adequately compartmentalized; scale bar, 50 μm .

21
22 **Extended Data Figure 12. Characterization of epiblast, hypoblast, and trophoblast lineages in SEMs.**
23 **a**, from left to right, representative merged brightfield (BF) and immunofluorescence images of day 3 SEMs
24 showing three lineages, epiblast (OCT4, cyan), hypoblast (SOX17, yellow), and trophoblast (SCD1,
25 magenta) merged with a nuclei channel (DAPI, white). **b**, representative merged brightfield and
26 immunofluorescence images of multiple day 6 SEMs, showing epiblast (OCT4, cyan), hypoblast (SOX17,
27 yellow), and trophoblast (CK7, magenta) merged with a nuclei channel (DAPI, white). **c**,
28 immunofluorescence images of day 6 SEMs with segregated Epi (OCT4, cyan) and hypoblast (SOX17,
29 yellow), and surrounded by trophoblast (CK7, magenta, outlined). Left panel shows a wide field image to
30 identify adequate structures (dotted square). Right panel zooms into the structure of interest **d**,
31 immunofluorescence images of common examples of mis-developed day 6 SEMs with no trophoblast (1),
32 or no epiblast and hypoblast (2), or no epiblast (3). Epiblast (OCT4, cyan), hypoblast (SOX17, yellow), and
33 trophoblast (SDC1, magenta); nuclei (DAPI, white). Scale bars, 50 μm , 200 μm (c right, d right).

34

1 **Extended Data Figure 13. Human and mouse SEMs bypass blastocyst-like stage.** a, scheme showing
2 the distinction between human SEM (top) and human blastoid (bottom) aggregation protocols: as opposed
3 to blastoids, SEMs do not form a blastocoel cavity. Epiblast (cyan), hypoblast (yellow), trophoblast
4 (magenta). **b**, representative brightfield image of a blastoid with blastocoel cavity (BC). Scale bar, 100 μm .
5 **c**, representative examples of brightfield images of day 3 and day 4 SEMs showing no blastocoel cavity.
6 Scale bar, 200 μm . **d**, brightfield images of day 4 human blastoids with a BC cavity. Scale bar, 200 μm . **e**-
7 **f**, enlarged brightfield (BF) images of the SEMs showing no blastocoel cavity at day 3 or day 4 human
8 SEMs (**f**). Scale bar, 100 μm ; zoom in, 50 μm . **g-h**, brightfield and immunofluorescence images of day 3
9 (**g**) and day 4 (**h**) human SEMs showing developmental progression of epiblast (OCT4, cyan), hypoblast
10 (SOX17, yellow), and trophoblast (CK7, magenta); nuclei (DAPI, white). Scale bars, 50 μm **i**, scheme
11 showing mouse SEM (also known as sEmbryo) aggregation with murine stem cells as described in ²⁷. **j**,
12 immunofluorescence images showing epiblast (OCT4, magenta), hypoblast (SOX17, red), and
13 trophectoderm (CDX2, green); Like human SEMs, mouse SEMs (sEmbryo models) do not form a BC.
14 Notably mouse SEMs are predominantly surrounded by the PrE, rather than TE by Day 2 of the protocol.
15 Scale bars, 50 μm ; zoom in, 20 μm .

16
17 **Extended Data Figure 14. 3D structure of a human SEM at day 8.** **a**, individual Z-planes of the 3D
18 immunofluorescence image of day 8 human SEM. Epiblast (OCT4, cyan), hypoblast (SOX17, yellow), and
19 trophoblast (CK7, magenta); nuclei (DAPI, white). AC, amniotic cavity; Am, amnion; ExEM,
20 extraembryonic mesoderm; SYS, secondary yolk sac; ChC, chorionic cavity; STB, syncytiotrophoblast. Z-
21 step, 20 μm ; scale bar, 50 μm . See also **Video S1**.

22
23 **Extended Data Figure 15. Characterization of the epiblast in human SEM.** **a**, immunofluorescence
24 image of day 6 SEM showing aPKC (green), OCT4 (cyan), F-ACTIN (red), nuclei (DAPI, white). Scale
25 bars, 50 μm , 12.5 μm (zoom in, right). **b**, left, immunofluorescence image of day 6 SEM showing OCT4
26 (cyan), F-ACTIN (red), and pHERM (green); alignment of epi cells in a single 2D plane is marked with
27 dashed lines; asterisk, pro-amniotic cavity. Scale bar, 25 μm . **b**, right, the angle between the epiblast cell
28 axis and the pro-amniotic cavity was quantified; the plot shows the radial histogram of the angle values.
29 The latter shows predominant alignment of epi cells towards the center of the emerging cavity. **c**,
30 immunofluorescence image of day 6 SEM showing CER1 (green) localization inside the intracellular
31 vesicles; OCT4 (cyan), nuclei (DAPI, white). Right, zoom into hypoblast, arrows mark apical side of the
32 visceral endoderm cells with CER1 vesicles. Scale bar, 50 μm , 12.5 μm (4x zoom in, right). **d**,
33 immunofluorescence image of day 6 SEM showing T/Bra expression (red); OCT4 (cyan), nuclei (DAPI,
34 white). Right, zoom into the posterior epiblast, arrows mark individual T/Bra-positive cells. Scale bar, 50

1 μm , 12.5 μm (4x zoom in, right). **e**, immunofluorescence image of day 6 SEM showing F-ACTIN (white)
2 and nuclei (DAPI, blue). Right, zoom into epiblast with squamous cells (sEpi) in the top and
3 cylindrical/columnar cells (cEpi) in the bottom parts of the epiblast. Scale bar, 50 μm ; zoom in, 25 μm . **f**,
4 immunofluorescence image of day 6 SEM showing F-ACTIN (white) and OCT4 (cyan). Right, zoom into
5 epiblast with squamous cells in the top and cylindrical cells in the bottom parts of the epiblast. Scale bar,
6 50 μm ; zoom in, 25 μm . **g**, brightfield and immunofluorescence images of day 8 SEM showing NANOG
7 (cyan), SOX17 (yellow), and nuclei (DAPI, white). Right, zoom onto PGCs in day 8 human SEMs, co-
8 expressing NANOG and SOX17 (cells marked with arrows). Scale bars, 50 μm .

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10 **Extended Data Figure 16. Characterization of hypoblast and extraembryonic mesoderm in human**
11 **SEMs. a**, immunofluorescence image of day 6 SEM showing apical polarity of the visceral and parietal
12 hypoblast (SOX17, yellow); aPKC (heat gradient), F-ACTIN (white). Scale bar, 50 μm ; zoom in, 10 μm .
13 See also **Video S3. b**, immunofluorescence image of day 6 SEM showing mesenchymal-like cells
14 underneath the yolk sac; OCT4 (cyan), F-ACTIN (red), and nuclei (DAPI, white). Right, zoom into the
15 region underneath the yolk sac, shown in different Z-planes (number 50, 60, and 70). Arrows point at the
16 cells between yolk sac and the trophoblast compartments. Scale bar, 50 μm ; zoom in, 25 μm . See also
17 **Video S2. c**, heatmap of GATA6 and SOX17 gene expression across extraembryonic tissues in marmoset,
18 corresponding to the Carnegie stages (CS) 5-7), extracted from previously published study related gene
19 expression dataset ⁹⁰. Am, amnion; Tb, trophoblast; VE, visceral endoderm; SYS, secondary yolk sac;
20 ExEM, extraembryonic mesoderm. **d**, immunofluorescence image of day 6 SEM showing OCT4 (cyan),
21 GATA4 (red), SOX17 (yellow), and nuclei (DAPI). Right, zoom on ExEM cells expressing GATA4, but
22 not SOX17. YS, yolk sac. Scale bar, 50 μm ; zoom in, 10 μm . **e**, immunofluorescence images of day 6 SEMs
23 showing chorionic cavity surrounded by ExEM (outlined), negative for SOX17 (yellow), but expressing
24 BST2 (top, red) and GATA6 (bottom, red); OCT4 (cyan), nuclei (DAPI, white). Scale bar, 50 μm . **f**, left,
25 immunofluorescence image of Z slice from day 8 SEM and the zoom into the ExEM region showing VIM
26 (red) expression and nuclei (DAPI, white). Right, merged brightfield and maximum intensity projection
27 showing VIM (red) expression. Scale bar, 50 μm ; zoom in, 10 μm .

28

29 **Extended Data Figure 17. 3D structure highlighting the ExEM in human SEM at day 8. a**, individual
30 Z-planes of the 3D immunofluorescence image of day 8 human SEM showing OCT4 (cyan), SOX17
31 (yellow), and BST2 (white). Epi, epiblast; YS, yolk sac; ExEM, extraembryonic mesoderm; SYS,
32 secondary yolk sac; Z-step, 20 μm ; scale bar, 50 μm .

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34 **Extended Data Figure 18. Characterization of the trophoblast compartment in human SEM. a**,

1 brightfield image overlaid with the immunofluorescence of day 8 SEM showing the outer trophoblast
2 (CK7, magenta) surrounding the entire SEM. Right, zoom into the multinucleated trophoblast layer. Scale
3 bars, 50 μ m. **b**, the maximum intensity projection (Maxi. Proj.) of the immunofluorescence image of day 8
4 SEM showing trophoblast (GATA3, magenta) and nuclei, (DAPI, white). Scale bar, 50 μ m. **c**,
5 quantification of the percentage SEMs surrounded by trophoblast lineage (generated from WT naïve PSCs
6 following 3 day BAP(J) protocol), as judged by the expression of SDC1, CK7, and GATA3 of day 6, across
7 two independent experimental replicates. The total number of SEMs used for quantification are: SD1
8 (n1=525 and n2=769); CK7 (n1=299 and n2=466) and GATA3 (n1=150 and n2=337). **d**, representative
9 immunofluorescence images of multiple day 6 SEMs showing epiblast (OCT4, cyan), hypoblast (SOX17
10 and GATA6, yellow), and trophoblast (SDC1 and GATA3, magenta) surrounding the SEMs; nuclei (DAPI,
11 white), that were used for calculation presented in (c). Scale bar, 200 μ m. **e**, immunofluorescence image of
12 day 6 SEM showing HCGB (green) and CK7 (magenta) staining. HCGB positive syncytiotrophoblast cells
13 (right, zoom) are highlighted. nuclei (DAPI, white), F-actin (white, right zoom). Arrows point at the outer
14 syncytiotrophoblast cell surface. Scale bars, 50 μ m. **f**, immunofluorescence image of a human SEM
15 showing multinucleated HCGB-positive syncytiotrophoblast; HCGB (green), F-ACTIN (red), nuclei
16 (DAPI, white). Arrows point at multiple nuclei inside the same single cell as validated following F-actin
17 staining. Scale bars, 50 μ m. **g**, pregnancy test run on spent medium of the day 8 SEMs (Day 8- right)
18 compared to unspent medium as a negative control (CTR-left) which shows the secretion of HCGB from
19 the syncytiotrophoblast of day7-8 human SEM to the culture medium.

20

21 **Extended Data Figure 19. Human SEM UMAP quality assessment of scRNA-seq experiment.**

22 **a**, Violin plots indicating the number of genes, the unique molecular identifiers (UMIs), and the percentage
23 of mitochondrial reads obtained per sample prior to filtering. **b**, Violin plots indicating the number of genes
24 and the unique molecular identifiers (UMIs) obtained per cluster, after filtering cells with less than 1000
25 identified genes. **c**, UMAP plot displaying individual cells of the different SEM samples as indicated. Left:
26 Colors indicate different samples. Right: Colors indicate the 13 identified clusters as indicated in **Fig. 6a**.
27 The number of single cells in each sample is indicated. **d**, UMAP projection of the assembled human
28 embryonic reference dataset with color indicating data source (related to **Fig. 6e**). **e**, Highlights the projected
29 SEMs cells on the embryonic reference UMAP space stratified by the corresponding raw Seurat clusters.
30 The grey data points represent embryonic reference cells or unselected neighborhood nodes, while each red
31 triangle corresponds to the projection of representative neighborhood nodes from each SEM raw cluster
32 onto the UMAP space (related to **Fig. 6e**).

33

34 **Extended Data Figure 20. Identification and validation of specific cell sub-types in human SEMs.**

1 **a**, UMAP of the four annotated epiblast clusters (1,4,7 & 11) alongside normalized expression of key
2 marker genes. From the four Epi clusters we subclassified 2 of them. The first we termed Posterior epiblast
3 cluster (#4) was marked by upregulation of TBXT (Brachyury), MIXL1, EOMES, MESP1 and WNT8a
4 which are markers of EMT. The second we termed as “committed epiblast” cluster (#7) and was marked
5 by ZIC2, ZEB2, VIM lineage commitment marker expression and absence of NANOG while maintaining
6 OCT4 and SOX2 expression. **b**, Normalized expression of key trophoblast marker genes projected on SEM
7 UMAP. Trophoblast (Tb) cluster (number 12) is highlighted in red. **c**, Normalized expression of key amnion
8 marker genes projected on human SEM related UMAP. Amnion cluster (number 10) is highlighted in red.
9 **d**, Expression of BMP4 and/or FURIN in Amnion (Am) and Trophoblast (Tb) clusters.

10

11 **Extended Data Figure 21. Identification of Primary and Secondary Yolk-sac, and Extra-Embryonic**
12 **Mesoderm lineages.**

13 **a**, Normalized expression of key Yolk-Sac (YS) marker genes projected on SEM UMAP. GATA4, GATA6,
14 PDGFRA & APOA1 are expressed both in primary and secondary yolk-sac. TTR, APOB & GSTA1 are
15 expressed more specifically in the secondary Yolk-sac consistent with findings in marmoset⁹⁰. STC1,
16 LHX1 are absent in secondary yolk-sac cluster, but not in primary yolk-sac consistent with findings in
17 marmoset⁹⁰. The co-expression of DKK1 and LHX1 detected via scRNA-seq alongside CER1 expression
18 among some of the cluster 9 SOX17+ YS cells (arrows) marks AVE cells. **b**, Normalized expression of
19 key extra-embryonic mesodermal genes projected on SEM UMAP. Cells in all four ExEM clusters (0, 2, 6
20 and 8) express general mesodermal genes such as HAND1, LEF1 and BMP4, while only cells in clusters 0
21 and 6 express typical extra cellular matrix related genes such as collagen genes and POSTN. **c**, differentially
22 expressed genes between extra-embryonic mesoderm (ExEM) clusters 2 and 8 vs. (ExEM clusters 0 and 6.
23 Most significant differentially expressed genes (adjusted p-value < 0.05 & $\text{asb}(\log_2\text{Fold-change}) > 1$) are
24 marked in red. Clusters 2 and 8, termed Early ExEM cells, expressed much lower level of extra cellular
25 matrix related genes and remodeling enzymes, indicative of their putative relatively less mature cellular
26 state.

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1 **Supplementary Spreadsheet Legends**

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3 **Supplementary Spreadsheet 1. Summary of the microscopy parameters used for imaging in**

4 **this study.** The spreadsheet provides information of the type of the microscope, used detection

5 objectives, laser lines, and the voxel size for acquisition of the images published herein.

6

7 **Supplementary Spreadsheet 2 - PCR primers used in this study.**

8 Primer names, DNA sequences from 5' to 3' end, and the cited reference (when relevant).

9

10 **Supplementary Spreadsheet 3. Human SEM scRNA-seq analysis related gene expression**

11 **list.**

12 Top markers (average $\log_2(\text{Fold-change}) > 0.25$) of each of the 13 cell clusters in human SEMs,

13 as identified by Seurat package.

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1 **Supplementary Video Legends**

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3 **Video S1. 3D reconstruction of the human SEM at day 8.**

4 The video shows 3D rendering of the embryonic and extraembryonic tissue structures comprising day 8
5 human SEM. Immunofluorescence for epiblast (OCT4, cyan), yolk sac (SOX17, yellow), trophoblast (CK7,
6 magenta), and nuclei (DAPI, white). 0 – 8 sec, 3D view of the SEM shape and the outer trophoblast with
7 enlarged multinuclear cells (also seen on the slices from 22 sec). 8 – 22 sec and 39 – 42 sec, 3D segmentation
8 of the epiblast (cyan) and hypoblast (yellow) is shown with the DAPI immunofluorescence signal on the
9 background. 22 sec – 38 sec, slicing through the 3D volume showing the inner structure of the SEM with
10 bilaminar disk with amnion, connected to the outer trophoblast, the yolk sac with the cavity, and the
11 surrounding connective tissues with extensive intercellular space. Immunofluorescence signal and tissue
12 segmentation are outlined. The image was acquired with Zeiss Z7 light-sheet microscope (see Methods)
13 and processed with Imaris v10.0.0.

14

15 **Video S2. 3D reconstruction of the human SEM at day 6 demonstrating pro-amniotic cavity** 16 **formation within the epiblast.**

17 3D reconstruction of the human SEM at day 6 shows 3D shape of the day 6 SEM (0 – 12 sec) and early
18 formation of the proamniotic cavity in the center of the epiblast (12 – 18 sec). Immunofluorescence for
19 OCT4 (epiblast, cyan), F-actin (red), and nuclei (DAPI, white). The image was acquired with Zeiss LSM
20 800 microscope (see Methods) and processed with Imaris v10.0.0.

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22 **Video S3. 3D reconstruction of the human SEM at day 8 shows embryonic disk and amnion** 23 **formation.**

24 3D reconstruction of the human SEM at the day 8, showing embryonic disk (SOX2, cyan) and amnion
25 (TFAP2C, magenta). The segmentation of epiblast (cyan) and amnion (pink) is denoted as the semi-
26 transparent outline together with the immunofluorescence signal. 17 – 21 sec, human SEM epiblast has a
27 disk shape. The image was acquired with Zeiss Z7 light-sheet microscope (see Methods) and processed
28 with Imaris v10.0.0.

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30 **Video S4. 3D reconstruction of the human SEM at day 6 shows yolk sac morphology.**

31 3D reconstruction of the human day 6 SEM showing yolk sac development (marked by SOX17, yellow). 5
32 – 18 sec, zoom into the visceral and parietal yolk sac cells having columnar and squamous cell shape,
33 respectively, and apical cell polarity (aPKC, green). F-actin (red), nuclei (DAPI, white). The image was
34 acquired with Zeiss LSM 700 microscope (see Methods) and processed with Imaris v10.0.0.

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Video S5. 3D reconstruction of the human SEM at day 8 shows extraembryonic mesoderm cells integration underneath the yolk sac.

3D reconstruction of the human SEM at the day 8 showing extraembryonic mesoderm cells located underneath the yolk sac (yellow) and marked by expression of VIM (red). OCT4 (epiblast, cyan), nuclei (DAPI, grey). The image was acquired with Zeiss LSM 700 microscope (see Methods) and processed with Imaris v10.0.0.

Video S6. 3D reconstruction of human SEM at day 8 shows development of the syncytial trophoblast and lacunae formation.

3D reconstruction of the human SEM at the day 8 showing development of the syncytial trophoblast, expressing both SDC1 (magenta) and HCGB (green) with multiple lacunae. F-actin (red), nuclei (DAPI, grey). 11 – 19 sec, slicing through the 3D volume showing inner structure of the multiple trophoblast lacunae. The image was acquired with Zeiss LSM 700 microscope (see Methods) and processed with Imaris v10.0.0.

1 **Methods**

3 **Ethics**

4 All experiments reported herein involving human PSCs were conducted following obtaining Weizmann
5 Institute IRB approval (1868-2) to generate human Stem cell-derived Embryo Models (termed “SEMs” or
6 “Stembroids”) from WIBR1, WIBR2 and WIBR3 human ESC lines ¹²⁸ and from iPSC lines following
7 obtaining donor informed consent to make genetically unmodified iPS lines from donated blood (Weizmann
8 Institute IRB approval – 1871-2). All the experiments reported herein follow the latest ISSCR guidelines
9 released in 2021¹²⁶. This study does not involve derivation of new human ESC lines, does not use any
10 newly obtained samples from fetal abortions, and does not use any newly donated human blastocysts.
11 Further, this study does not involve in utero transfer of any human SEMs into any other species, consistent
12 with ISSCR guidelines and Israeli legislation. Finally, all the human SEMs described herein do not
13 correspond to developmental stages beyond 14 dpf.

15 **Data reporting**

16 No statistical methods were used to predetermine sample size. Samples were randomly allocated when
17 placed in the different growth conditions. Other experiments were not randomized. The investigators were
18 not blinded to allocation during experiments and outcome assessment since there was no relevant scientific
19 reason to do so.

21 **Stem cell lines**

22 The following already established human ESC lines were used: WIBR3 (WT female), WIBR2 (WT
23 female) and WIBR1 (WT male) human ES lines ¹²⁸. Genetically unmodified JH33 and JH22 human
24 induced pluripotent stem cell (iPSC) lines made from a healthy adult Caucasian/Middle Eastern male were
25 also used to generate human SEMs. Previously established mouse Tet-ON iGata4 KH2-WT ESC line was
26 used for comparing induction efficiency of PDGFRa⁺ cells ²⁷. All cell lines were routinely checked for
27 Mycoplasma contaminations every month (Lonza–MycoAlert), and all samples analyzed in this study were
28 not contaminated.

30 **Human naïve ESC in vitro culture conditions**

31 Golden stocks of human ESCs were cultured on feeder layer of irradiated mouse embryonic
32 fibroblast (MEFs) and maintained in conventional human FGF/KSR primed conditions. FGF/KSR
33 conditions on MEF substrate: DMEM-F12 (Invitrogen 10829) supplemented with 20% Knockout Serum
34 Replacement (Invitrogen 10828-028), 1mM GlutaMAX (Gibco 35050061), 1% nonessential amino acids

1 (BI 01-340-1B), 1% Sodium-pyruvate (BI 03-042-1B), 1% Penicillin-Streptomycin (BI 03-031-1B) and 8
2 ng/mL bFGF (Peprotech 100-18B-1MG).

3
4 To reprogram primed PSCs to a naïve state, human PSCs were maintained and expanded as in
5 Bayerl et al³⁵, in serum-free HENSM on plates coated with 1% Matrigel (corning 356231) or MEF/gelatin-
6 coated plates. At least three passages in HENSM conditions were applied before cells were used for
7 experiments. Human naïve ESC lines were used for up to 10 passages in HENSM conditions. For
8 maintenance of ESCs in naïve HENSM conditions, cells were passaged every 3-5 days using TrypLE (Gibco
9 12604054). Naïve and primed hPSCs were expanded and induced into different lineages in a 5% CO₂
10 incubator at 5% O₂ at 37C.

11
12 **Generation of iGATA4, iGATA6, iCDX2 and iGATA3 human ESCs clones**

13 To generate Tet-ON inducible lines, we employed a PiggyBac plasmid expressing cDNA insert adba
14 transposase vector of choice under the control of a doxycycline-inducible promotor (a kind gift from Volker
15 Busskamp, Addgene plasmid #104454). The donor vector carries M2Rtta and a site for cDNA insert of
16 transcription factor of interest. We used this vector to generate 4 different DOX inducible lines in WIBR3
17 WT human female ESCs: human iCDX2 or human iGATA3 (to promote human PSC differentiation
18 towards trophoctoderm) and human iGATA4 or human iGATA6 (to promote human primitive endoderm/
19 extra embryonic mesoderm priming from human PSCs). Puromycin selection was applied for
20 approximately 6-8 days. Resistant clones were picked and cultured for downstream characterization.
21 Insertion was validated by immunostaining after DOX (2µg/ml) induction of the gene of interest. Transgene
22 expression was verified to be specifically detected only after DOX addition in the corresponding lines.
23 Detailed generation, characterization and validation of these lines can be found on (Extended Data Figure
24 1, 3, 7). Generation of fluorescent labeled WIBR3 iCDX2 line was made after transduction with lentivirus
25 constitutively expressing tdTomato protein. For lentivirus generation, HEK293T cells were plated on 10
26 cm dishes filled with 10 ml DMEM 10% FBS and Pen/Strep, at a density of 5.5 million cells per plate. On
27 the next day, cells were transfected with Second-generation lentiviral vectors (Addgene 8455 and 8455),
28 using X-tremeGENE 9 transfection reagent, along with 16 µg of the target plasmid for the transduced
29 fluorescent protein (tdTomato). The supernatant containing the virus was collected 48hr following
30 transfection, filtered using 0.45µm filter and concentrated by ultracentrifugation. Human PSCs were plated
31 in mTESR medium on Matrigel coated 6-well plates at low density, next day they were transduced with
32 lentivirus in the presence of protamine sulfate (10 µg/ml) for 6 hours, afterwards medium was exchanged.
33 After 2 days, the infected human ESCs were expanded for 1 passage and the positive population was sorted
34 using FACS and further expanded for experimentation.

1

2 **Derivation of a stable human TSC line from human ESCs**

3 TSC lines were produced from Naïve (HENSM) (nTSC) and primed conditions (pTSC) according
4 to Okae et al and Viukov et al, respectively ^{70,71}. Briefly, human naïve WIBR3 ESCs were expanded at
5 least 3 passages in naïve or primed conditions and then transferred into TSC media (TSCm) on 1% Matrigel
6 (corning 356231) -coated plates. After 3 passages, stable TSC lines could be established and could be
7 passaged 70 times ^{129,130}. Cells were expanded in a 5% CO₂ incubator at 5% O₂. For maintenance, human
8 TSC were passaged with TrypLE (Gibco 12604054) when reached 70%-80% confluency. Immunostaining
9 was performed to confirm human TSC identity: human TSC cells were negative for CDX2, and positive
10 for Cytokeratin7 (CK7) GATA3 and TFAP2C, consistent with previous reports. Only confirmed lines were
11 used for SEM experiments. Human TSC media (TSCm) used herein was previously described in ⁷⁰ with
12 slight modifications: 470 ml DMEM/F12 (Invitrogen 21331), 5 ml Commercial N2 supplement (Invitrogen
13 17502048), 10 ml B27 supplement (Invitrogen 17504-044), 5 mL Sodium Pyruvate (Biological Industries
14 03-042-1B), 5 mL Penicillin/Streptomycin (Biological Industries 03-033-1B) 5ml (Biological Industries
15 03-033-1B), 5 mL GlutaMAX (Invitrogen 35050061), 5 mL NEAA (Biological Industries 01-340-1B), 50
16 µg/ml L-ascorbic acid 2-phosphate (Sigma A8960), 50ng/ml Human EGF (Peprotech AF-100-15), 0.75- 1
17 µM TGFRI A83-01 (Axon 1421), 2 µM GSK3i CHIR99021 (Axon Medchem 1386), and 5 µM ROCKi
18 Y27632 (Axon 1683).

19

20 **Derivation of human trophectoderm (TE) from human naïve PSCs for SEM generation**

21 Human TE cells were obtained from human naïve PSCs expanded in human HENSM conditions
22 for at least 3 passages, 24 hours before the induced cells were plated in HENSM on 1% Matrigel (corning
23 356231) -coated plates supplemented with 10 µM ROCKi Y27632 (Axon 1683), next day the protocol was
24 started. The 3-day protocol for human TE induction was previously described and is adapted from Io et al³⁶
25 as follows: BAP(J) media was used for 72 hours. This medium consisted on 2 µM TGFRI A83-01 and 2
26 µM MEKi/ERKi PD0325901 base, which was complemented the first 24h with 10ng/ml Human
27 recombinant BMP4 (Peprotech) and then substituted with for 1µM JAK inhibitor I (Calbiochem 420099)
28 on day 2 and 3. The base medium consisted on: 470 ml of 1:1 mix of Neurobasal (Invitrogen 21103-049)
29 and DMEM/F12 (Invitrogen 21331), 5 ml penicillin-streptomycin (Biological Industries 03-033-1B), 5 ml
30 GlutaMAX (Invitrogen 35050061), 5 ml NEAA (Biological Industries 01-340-1B), 5 ml Sodium Pyruvate
31 (Biological Industries 03-042-1B), 10 ml B27 supplement (Gibco 17504-044), 5 ml N2 supplement
32 (Invitrogen 17502048), 2 µM TGFRI A83-01 (Axon Medchem A83-01), 2 µM MEKi/ERKi PD0325901
33 (Axon Medchem 1408). WT human naïve PSCs or iCDX2/iGATA3 cell lines were tested for human TE

1 induction in the presence or absence of DOX as indicated. All the process was incubated in a 37C incubator
2 with 5% O₂ and 5% CO₂.

3 4 **Primitive Endoderm (PrE) and Extra-Embryonic Mesoderm (ExEM) induction from human naïve** 5 **PSCs**

6 Pre/ExEM cells were induced from human naïve PSCs expanded in HENSM conditions for at least 3
7 passages as described above. For induction HENSM cells were plated on gelatin-MEF coated plates the
8 day before induction with 10 µM ROCKi Y27632, next day medium was changed to RCL for 72h¹³¹. RCL
9 is composed of: 480 ml RPMI media (GIBCO 21875-03), 10 ml B27 minus insulin supplement (Invitrogen
10 A18956-01), 1 mM GlutaMAX (Invitrogen), 1 % penicillin-streptomycin (Invitrogen), 3 µM CHIR (Axon
11 Medchem 1386) and 10 ng/ml LIF (Peprotech 300-05). RCL medium contains the same composition as
12 RACL but without adding recombinant Activin. WT human naïve PSCs or iGATA4/iGATA6 cell lines
13 were employed for induction in the presence or absence of DOX as indicated. All the process was incubated
14 in a 37C incubator with 5% O₂ and 5% CO₂.

15 16 **Generation of human stem cell embryo models (SEMs)**

17 A step-by-step detailed protocol will be accompanying this work upon final publication. Briefly, To generate
18 SEMs from human naïve PSCs, three starting cell mixtures were co-aggregated using AggreWell 24-well
19 plate 400 (STEMCELL Technologies 34415):

- 20 1) Naïve PSC (nPSC) WT cells cultured in HENSM medium in a 5% CO₂ incubator at 5% O₂ at
21 37C.
- 22 2) For the primitive endoderm and extra-embryonic mesoderm compartments (PrE/ExEM), naïve
23 WT cells were plated on irradiated MEF (mouse embryonic fibroblast conditions)/Gelatin
24 coated plates in HENSM supplemented with ROCKi 10 µM (Axon Medchem 1683). The next
25 day, cells were washed with PBS twice (without harvesting), and HENSM was replaced by
26 RCL medium. RCL was kept for 72h with 24h medium exchanges in a CO₂ incubator at 5%
27 O₂ at 37C.
- 28 3) For the trophectoderm lineage, naïve WT cells were plated on feeder free conditions (Matrigel)
29 in HENSM supplemented with ROCKi 10 µM. The next day, cells were washed with PBS
30 twice (without harvesting), and HENSM was replaced with BAP medium for 24 hours,
31 following by replacement with APJ for another 48 hours in a 5% CO₂ incubator at 5% O₂ at
32 37C (termed BAP(J) protocol).

33

1 Co-aggregation was defined as time point 0 of the protocol. 12-24h before aggregation all donor
2 cells were supplemented with ROCKi 10 μ M (Axon Medchem 1683). At the day of aggregation (day 0),
3 AggreWell 400 24-well plate preparation was done according to manufacturer instructions. Briefly, 500 μ l
4 of anti-adherence rinsing solution (STEMCELL Technologies 07010) was added to each well, the plate
5 was centrifuged at 2,000g for 5 minutes and incubated 30 min at room temperature. Subsequently, rinsing
6 solution was removed and the plate was washed with PBS. Each well was filled with 500 μ L of aggregation
7 medium and kept at 37° C for medium equilibration. Aggregation medium (BSA supplemented N2B@7
8 media) consisted in 500ml 1:1 mix of Neurobasal (Invitrogen 21103-049) and DMEM/F12 (Invitrogen
9 21331), 5 ml penicillin-streptomycin (Biological Industries 03-033-1B), 5 ml GlutaMAX (Invitrogen
10 35050061), 5 ml NEAA (Biological Industries 01-340-1B), 5 ml Sodium Pyruvate (Biological Industries
11 03-042-1B), 10 ml B27 supplement (Invitrogen 17504-044), 5 ml N2 supplement (Invitrogen 17502048),
12 1 ml β -mercaptoethanol 50mM (Gibco 31350-010), 2.25ml of BSA solution 35% (Merck 9048-46-8).

13
14 The three cell populations were collected with TrypLE (Thermo Fisher 12604054) (3 minutes for
15 the HENSM and RCL-induced cell populations, and 5 minutes for the BAP(J)-induced cells) at 37° C,
16 Afterwards TrypLE was removed with vacuum and the cells were incubated for two minutes at room
17 temperature and cells were subsequently collected with PBS. Cells were centrifuged at 1300 rpm for 3-5
18 minutes and resuspended in aggregation medium. Next, RCL-induced cells were plated on gelatinized
19 tissue culture plates on MEF medium consisting on 500ml DMEM (Gibco 41965-039) 20% FBS (Sigma,
20 F7524-500ml), 5 ml penicillin-streptomycin (Biological Industries 03-033-1B), 5 ml GlutaMAX
21 (Invitrogen 35050061), 5 ml NEAA (Biological Industries 01-340-1B), 5 ml Sodium Pyruvate (Biological
22 Industries 03-042-1B), for MEF depletion for 30 minutes at 37° C. At the end of MEF depletion, the
23 supernatant was collected and passed through a 70 μ M cell strainer, and all three cell types were centrifuged
24 separately and resuspended and passed through a 70 μ M cell strainer in N2B27 medium. The three cell
25 fractions were counted and combined as follows in an Aggrewell 400 plate: Ratio of 1:1:3 (HENSM: RCL:
26 BAPJ) or (Epi: PrE/ExEM: TE) = 28800 Epi (HENSM) cells, 28800 Pre/ExEM (RCL) cells, and 86400 TE
27 (BAPJ) cells per 24-well . Cell number per single microwell = 120 cells. Cells were prepared on a 2x
28 concentration with 20 μ M ROCKi, 500 μ l of cell-mix suspension was gently added drop wise to each well
29 of the AggreWell plate (1ml final volume 10 μ M final ROCKi concentration). The plate was centrifuged at
30 100g for 3 minutes and incubated at 37 °C in hypoxic conditions (5% O₂ and 5% CO₂)

31
32 Next day (day 1), 900 μ L of medium were gently removed from each well and replaced with 1 ml
33 of pre-equilibrated aggregation (N2B27-BSA) medium. The same volume of medium was replaced at day
34 2. At aggregation day 3, aggregates were gently transferred to 6-well cell suspension culture plates (Greiner,

1 657185) filled with 3 ml of pre-equilibrated EUCM2 (20% FBS) per well and placed on an orbital shaker
2 rotating at 60 rpm (Thermo Scientific 88881102 + 88881123) located inside a 5% CO₂ incubator in 20%
3 O₂. On day 4, 2 ml of medium were gently removed per well and were replaced with 2 ml of pre-heated
4 EUCM 2 (with 30% FBS). Same procedure was repeated on day 5, refreshing with 2 ml of EUCM2. After
5 6 days, 2 ml of medium were gently removed per six-well and were replaced with 2 ml of pre-heated
6 EUCM2 with 50% FBS. Same procedure was repeated at day 7 and cultures were finished at day 8 post-
7 aggregation. Alternatively, human SEMs can be cultured from day 6 to 8 using the roller culture platform
8 adapted to an electronic gas regulator module using EUCM2 50% FBS with similar outcome. EUCM2 is
9 formulated as follows: Advanced DMEM/F12 (GIBCO 21331-020), extra added 1 mM Sodium pyruvate
10 (Sigma-Aldrich, S8636), 0.5% CMRL media (GIBCO 11530037), extra added 1 mg/mL D(+)-Glucose
11 Monohydrate (J.T. Baker - 0113) (e.g. add 500mg per 500mL media), 1 mM GlutaMAX (GIBCO,
12 35050061), 1% penicillin streptomycin (Biological Industries – Sartorius 03-031-1B), 1x of ITS-X
13 supplement (Thermo Fisher Scientific 51500-056), 8 nM B-estradiol (Sigma-Aldrich, E8875), 200 ng/ml
14 progesterone (Sigma-Aldrich, P0130), 25 μM N-acetyl-L-cysteine (Sigma-Aldrich, A7250), 20-50% FBS
15 (Sigma Aldrich F7524 – heat inactivated and filtered) as indicated in **Fig. 2b**. Culture media was pre-heated
16 for at least an hour by placing it inside a CO₂ incubator at 37°C.

17

18 **Human blastoid generation and PALLY/PALY conditions**

19 Human blastoids were generated according to Kagawa et al²² with few modifications. WIBR3 hESCs were
20 grown in HENSM for at least 3 passages on feeder free conditions (1% Matrigel coated plates) and were
21 used for generating human blastoids. After 3 days of growth of naïve PSC were harvested and counted and
22 55 cells were seeded per microwell (total of 66,000 cells were seeded per 24-well in 1 ml of medium)
23 AggreWell 400 24-well (Stemcell Technologies cat 34415) in N2B27-BSA supplemented with 10 μM
24 ROCKi Y27632. The next day medium was changed to PALLY consisting of N2B27 base with 1 μM
25 MEKi/ERKi PD0325901 (Axon Medchem 1408), 1 μM TGFRi A83-01 (Axon Medchem A83-01), 1 μM
26 LPA (Tocris, 3854), LIF 10ng/ml and 10 μM ROCKi Y27632. This medium was repeated on day 2, but on
27 day 3 medium was changed for LY (1 μM LPA (Tocris, 3854) and 10 μM ROCKi Y27632) for another
28 48h. Afterwards human blastoids were manually selected and collected for further analysis. The entire
29 was conducted in 5% O₂ and 5% CO₂ conditions.

30

31 **Mouse SEM generation**

32 Mouse SEM aggregations were made according to Tarazi et al²⁷. Mouse animal experiments
33 pertained only to mouse SEM and were performed according to the Animal Protection Guidelines of
34 Weizmann Institute of Science and approved by the following Weizmann Institute IACUC (#01390120-1,

1 01330120-2, 33520117-2). Mouse aggregation medium was also tested for human SEMs but was found
2 inappropriate. Mouse aggregation medium (mouse AM) consisted of 1x DMEM (GIBCO-41965)
3 supplemented with 20% FBS (Sigma), 1 mM GlutaMAX (GIBCO, 35050061), 1% penicillin streptomycin
4 (Biological Industries – Sartorius 03-031-1B), 1% Sodium Pyruvate (Biological Industries – Sartorius 03-
5 042-1B), 1% non-essential amino acids (Biological Industries – Sartorius 01-340-1B) and 0.1 mM β -
6 mercaptoethanol (Thermo 31350010).

7

8 **Morphological evaluation of human early development and efficiency calculations**

9 Assessment of appropriate human development was performed by careful analysis of available *in-*
10 *utero* histological embryo collections (predominantly Carnegie collection), taking in account different
11 epithelium morphology and structure organization through different stages of development. Furthermore,
12 available work on primate development was employed as a reference for anatomical structure^{132–134},
13 specific markers of each of the compartments was inferred from previous ex-utero human development
14 works^{135,136}, in-vitro differentiation protocols and primate existing databases¹³².

15 Most of human histological descriptions and figures used for this paper are mentioned in the virtual
16 human embryo website (<https://www.ehd.org/virtual-human-embryo/>) and available human embryology
17 textbooks (Langman, Larsen, Carlson).

18 All human in utero data and figures included in this study were made only from Carnegie
19 collections after obtaining the appropriate copyright approvals (in process). Only SEMs presenting all the
20 previously defined features were considered as properly developed. Percentage of human SEMs generation
21 is calculated based on the number of properly developed structures observed per random fields of view at
22 a specific time point on independent experiments, while always relying on immunofluorescence to
23 corroborate the 3-lineage contribution.

24

25 **Quantification and Statistical Analysis**

26 Statistical analyses of real time PCRs were performed in QuantStudio software v1.3 and visualized
27 in GraphPad Prism 7. Visualization and statistical analyses of the cell numbers and SEM efficiencies were
28 performed with Python 3.8.5 software using scipy and seaborn libraries. Boxplot graphs indicate medians
29 with interquartile ranges, the whiskers mark distribution range. The barplots show average values plus s.d.
30 The dots mark individual numerical values used for visualization of the data distributions and analyses.
31 Significant difference between two samples was evaluated by the two-sided Mann-Whitney test for non-
32 normally distributed data. $p < 0.05$ was considered as statistically significant.

33

34 **Immunofluorescence**

1 Cells were fixed in 4% paraformaldehyde in PBS at RT for 10 minutes. Samples were then
2 washed 3 times in PBS, permeabilized in PBST (PBS with 0.1% Triton X-100) for 10 min, blocked in
3 PBS/0.05% Tween/5% fetal bovine serum/1% bovine serum albumin for 1h and incubated with primary
4 antibodies diluted in blocking solution at 4°C overnight. Subsequently, cells were washed in PBS/0.05%
5 (three times, 5 min each) and incubated with Alexa Fluor (488, 568 and/or 647)-conjugated secondary
6 antibodies (Jackson ImmunoResearch) diluted in blocking solution (1:200). Samples were counterstained
7 with 1 µg/ml DAPI for 10 min at RT, washed with PBS three times (5 min each) and mounted with Shandon
8 Immuno-Mount (Thermo Scientific). The antibodies and dilutions employed for cell immunofluorescence
9 were the following: Rabbit polyclonal anti-Cdx2 (Cell Signaling Cat# 3977) 1:200; Mouse monoclonal
10 anti-Cdx2 (Biogenex Cat# MU392A-UC) 1:200; Rabbit polyclonal anti Gata4 (Abcam Cat# Ab84593)
11 1:120; Rabbit monoclonal anti-Foxa2 (Abcam Cat# Ab108422) 1:100; Goat polyclonal anti-Sox17 (R&D
12 Cat# AF1924) 1:200; Mouse monoclonal anti-Oct4 (clone C-10) (Santa Cruz Cat# SC-5279) 1:200; Rabbit
13 monoclonal Cdx2 (Abcam Cat# ab76541) 1:200; Goat Monoclonal Tfp2c (R&D Cat# AF5059) 1:200;
14 Rabbit monoclonal Cytokeratin 7 (Abcam Cat# ab181598) 1:200; Rabbit monoclonal Cytokeratin 7
15 (Abcam Cat# ab68459) 1:200; Rabbit monoclonal Nanog (Abcam Cat# ab109250) 1:200; Mouse
16 monoclonal Gata3 (Invitrogen Cat# MA1-028) 1:200; Goat polyclonal Gata3 (R&D Cat# AF2605) 1:200;
17 Mouse monoclonal HCG-Beta (Abcam Cat# ab9582) 1:200; Rabbit monoclonal Gata6 (Cell signaling Cat#
18 5951) 1:200; Goat polyclonal Oct3/4 (R&D Cat# AF1759) 1:200; Goat polyclonal Gata6 (R&D Cat#
19 AF1700) 1:200; Rabbit monoclonal Syndecan1 (Abcam Cat# ab128936) 1:500; Rabbit monoclonal
20 PDGFR-A (Abcam Cat# ab134123) 1:100; Goat polyclonal Nidogen2 (R&D Cat# AF3385) 1:100.

21

22 **Flow cytometry**

23 Flow cytometry analysis were done on a BD FACS-Aria III. Cells were harvested with TrypLE
24 and washed once with PBS afterwards they incubated for half an hour with conjugated primary antibodies
25 (5ul) on 100ul PBS/0.5% BSA. The primary antibodies used are as follows: Mouse monoclonal TROP2-
26 488 labeled (R&D Cat# FAB650G); Mouse monoclonal TROP2-PE labeled (R&D Cat# FAB60P); Mouse
27 monoclonal CD249 (ENPEP)-BV421 labeled (BD Cat# 744872); Rat monoclonal anti mouse CD140a
28 (PDFGR-a)-PE/Cy7 labeled (BioLegend Cat# 135912); Mouse monoclonal anti human CD140a (PDFGR-
29 a)-PE/Cy7 labeled (BioLegend Cat# 323508); Mouse monoclonal anti human CD140a (PDFGR-a)-APC
30 labeled (BioLegend Cat# 323512). FSC and SSC singlets were gated, and only single cells were considering
31 for all analyses. An unstained control was employed to determine the negative/positive populations for all
32 antibodies, ensuring that 100% of the unstained population was allocated on the negative area of the
33 histogram/dot plot.

34

1 **Confocal microscopy**

2 The immunofluorescence images were acquired using a Zeiss LSM 700, as well as a LSM 800
3 inverted confocal microscopes (Zeiss), both equipped with 405 nm, 488 nm, 555 nm and 635 nm solid state
4 lasers, using a Plan-Apochromat 20× air objective (numerical aperture 0.8) or an EC Plan Neofluar 10× air
5 objective (numerical aperture 0.3). For a detailed description of the imaging parameters, see **Table S1**.
6 Images and maximum intensity projections were processed using Fiji ¹³⁷, Zen 2 blue edition software 2011
7 (Zeiss), and Adobe Illustrator CC.

8 **Light-sheet microscopy**

9 The immunofluorescence images were acquired using Zeiss Z7 light-sheet microscope, equipped
10 with 405 nm, 488 nm, 561 nm, and 638 nm lasers, using a single water 20x Plan-Apochromat (numerical
11 aperture 1.0) detection objective (Zeiss) and two air 10x Plan-Apochromat (numerical aperture 0.2)
12 illumination objectives (Zeiss). The single sample was imaged at a time, mounted in 1% low-melting
13 temperature agarose inside the imaging chamber filled with PBS. Light-sheet volumes along the Z-axis
14 were acquired in a dual scanning mode, using a pivot scan. Light-sheet thickness was set to 3.77 μm, and
15 laser power in the 1 – 50% range was applied. Frame size, 1920x1920 px, exposure time, 50 msec. The
16 light-sheets for left- and right-side illuminations were adjusted independently inside the sample volume for
17 each channel based on the signal intensity in the focal plane of the detection lens. See also **Table S1**.

18 **Electronically controlled ex utero roller culture platform**

19 Human SEMs can be kept in the ex utero electronically controlled roller culture platform after day
20 6 ¹³⁸ which provides continuous flow of oxygenating gas. The system consists of an electronic gas
21 modulating unit (Arad Technologies) adapted to the roller culture unit from B.T.C. Engineering, – Cullum
22 Starr Precision Engineering Ltd - UK)¹³⁹, as previously described ¹³⁸. On day 7, all human SEMs from one
23 well of the 6-well plate were picked and transferred to glass culture bottles (50-100 structures per bottle)
24 containing 4 mL of fresh EUCM2 50% FBS. The bottles were placed on the rolling culture system, rotating
25 at 30 revolutions per minute at 37°C, and continuously gassed with an atmosphere of 21% O₂, 5% CO₂ at
26 6.5-8 pounds per square inch (psi). Bottles were kept inside glass culture bottles rotating on a spinning
27 wheel allocated inside a “precision” incubator system (BTC01 model with gas bubbler kit - by B.T.C.
28 Engineering, – Cullum Starr Precision Engineering Ltd - UK). (BTC 04). Gas flows from the gas mix box
29 through the inlet into the humidifier water bottle, and then to the inside of the bottles in the rotating drum.
30 The rate of bubbles created inside an outlet-test tube filled with water is used to monitor speed of the gas
31 flow. The bubble rate was adjusted using the valve on the lid of the water bottle to the first point where
32 continuous bubbling is observed, which generally corresponds to 0.06-0.1 psi and 50-75ml/min gas flow.
33
34

1 A black cloth was used cover the incubator to provide protection against phototoxicity. 2 ml of EUCM2
2 with 50% FBS was used in the roller culture for human SEMs at these stages.

4 **RNA extraction & RT-PCR analysis**

5 Total RNA was isolated using RNeasy mini kit (Qiagen) following manufacturer instructions.
6 After, 1 µg of total RNA was reverse transcribed using a High-Capacity Reverse Transcription Kit (Applied
7 Biosystems). RT-PCR was performed in triplicate using SYBR Green PCR Master Mix (Qiagen) and run
8 on Viiia7 platform (Applied Biosystems). Values were normalization to Actin and/or Gapdh across all
9 experiments. Data are presented as fold difference compared to reference sample, which is set as 1. RT-
10 PCR primer list used listed in **Table S2**.

12 **Chromium 10X single cell RNA sequencing**

13 Human SEMs grown ex utero were manually selected and harvested for single cell RNA
14 sequencing (**Table S3**) using the Chromium Next GEM Single Cell 3' platform (V3.1). All human SEMs
15 analyzed by scRNA-seq were generated by co-aggregating WT WIBR3 naïve ESC lines grown in HENSM
16 with RCL-induced or BAPJ-induced WT cells. All SEMs samples were processed including
17 extraembryonic compartments without any dissection. SEMs were dissociated with Trypsin-EDTA solution
18 C (0.05%) for 10 minutes (Biological Industries; 030501B). Trypsin was neutralized using media with 10%
19 FBS, and cells were washed and resuspended in 1x PBS with 400 µg/ml BSA. Cell suspension was filtered
20 with a 100 µm cell strainer to remove cell clumps. Cell viability of at least 90% was determined by trypan
21 blue staining for all samples. Cells were diluted at a final concentration of 1000 cells/µL in 1x PBS with
22 400 µg/ml BSA. scRNA-seq libraries were generated using the 10x Genomics Chromium v3.1 Dual Index
23 system (5000 cell target cell recovery) and sequenced using Illumina NovaSeq 6000 platform according to
24 the manufacturer's instructions.

26 **10X Single cell RNA-seq analysis**

27 10x Genomics data analysis was performed using Cell Ranger 7.1.0 software (10x Genomics) for
28 pre-processing of raw sequencing data, and Seurat 4.3.0 for downstream analysis. To filter out low-
29 expressing single cells, possible doublets produced during the 10x sample processing, or single cells with
30 extensive mitochondrial expression, we filtered out cells with under 1000 expressing genes, over 8,000
31 expressing genes and over 15% mitochondrial gene expression. Seurat integrated analysis and anchoring
32 of all individual samples was performed and then normalized by log-normalization using a scale-factor of
33 10,000. The top 2,000 variable genes were identified by the variance stabilizing transformation method,
34 and subsequently scaled and centered. Principal components analysis was performed for dimensional

1 examination using the ‘elbow’ method. The first 10 dimensions showed the majority of data variability.
2 Therefore, UMAP dimensional reduction was performed on the first 10 dimensions in all samples. Clusters
3 were detected using Seurat Find Clusters function, with resolution parameter =0.5. Dot-plot describing
4 expression and prevalence of specific genes was generated using Seurat DotPlot() function. Projection of
5 selected genes on SEM UMAP was generated with Seurat FeaturePlot() function.

6 7 **Projection on Human Embryo Reference**

8 The human embryo reference was built by integrating previously published datasets consisting of 5 human
9 embryonic data sets spanning in vitro cultured human blastocysts^{24,118,119}, 3D- in vitro cultured human
10 blastocysts until pre-gastrulation stages¹²⁰, and a Carnegie Stage 7 (CS7) 16-19dpf human gastrula¹¹⁷, as
11 previously described¹⁰¹ (manuscript in final preparation). The raw counts for cells of human SEMs were
12 aggregated within neighborhood nodes as calculated by Milo (Dann et al 2022) resulting in 945 nodes,
13 followed by projecting the summed counts matrix onto the assembled Human embryo reference (**Fig. 6e**).

14 15 **Mouse Extra-embryonic annotation analysis**

16 Rhox5 positive cells (>1 counts) were chosen for the analysis of extra-embryonic tissue. The cells were
17 annotated based on marker genes as previously conducted in⁷⁷, such that if at least 4 markers (3 in the case
18 of SpA-TGC and SpT-Gly) were expressed (>0 counts), the cell was annotated in that category. 26% of the
19 annotated cells, were annotated by multiple categories. The markers are as following: Chorion (Irx4, Esx1,
20 Id1, Id3, Phlda2, Klfh13), Chorion progenitors (Sox3, Dusp6, Nat8l, Bmp4, Sox2, Esrrb, Eomes),
21 Intermediate Chorion (Ascl2, Fgfr2, Cited1, Gjb3, Ndr1, Irx2, Irx3), uncommitted EPC (Chsy1, Gjb3,
22 Krt19, Lgals1, Cald1, Ctsl), SpA-TGC (Ctla2a, Pecam1, Ramp3, Igfbp7, Nos3), SpT-Gly (Dlx3, Car2,
23 Ncam1, Pcdh12, Tpbpa), TGC-progenitors (Adm, Fos11, Hand1, Trpm5, Maged2, Prl5a1), p-TGC (Star,
24 Serpinb9d, Hsd3b6, Rhox6, Cts7) as in⁷⁷. R ggplot was used to generated scatter plot, along with
25 geom_smooth(method="lm").

26 27 **Mouse and Human IGV Analysis**

28 Bulk ATAC-seq and RNA-seq profiles in the proximity of selected genes (GATA3, GATA4, GATA6)
29 are presented using IGV genome browser. Mouse datasets were taken from published datasets¹⁴⁰⁻¹⁴² and
30 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181053>, and so were human datasets^{125,143,144}.
31 Mouse enhancers were taken from¹⁴¹, human enhancers were taken from GeneHancer¹⁴⁵. Open regions
32 that overlap with promoter or exon were excluded from the analysis. Potential enhancers in these regions
33 were manually curated.

34

1 **Data and code availability**

2 All scRNA-seq data are deposited under GEO: **XXXXXXXXXX**

3 Any other information required to reanalyze the data reported in this work is available upon request from
4 the corresponding authors upon request authors.

5

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7

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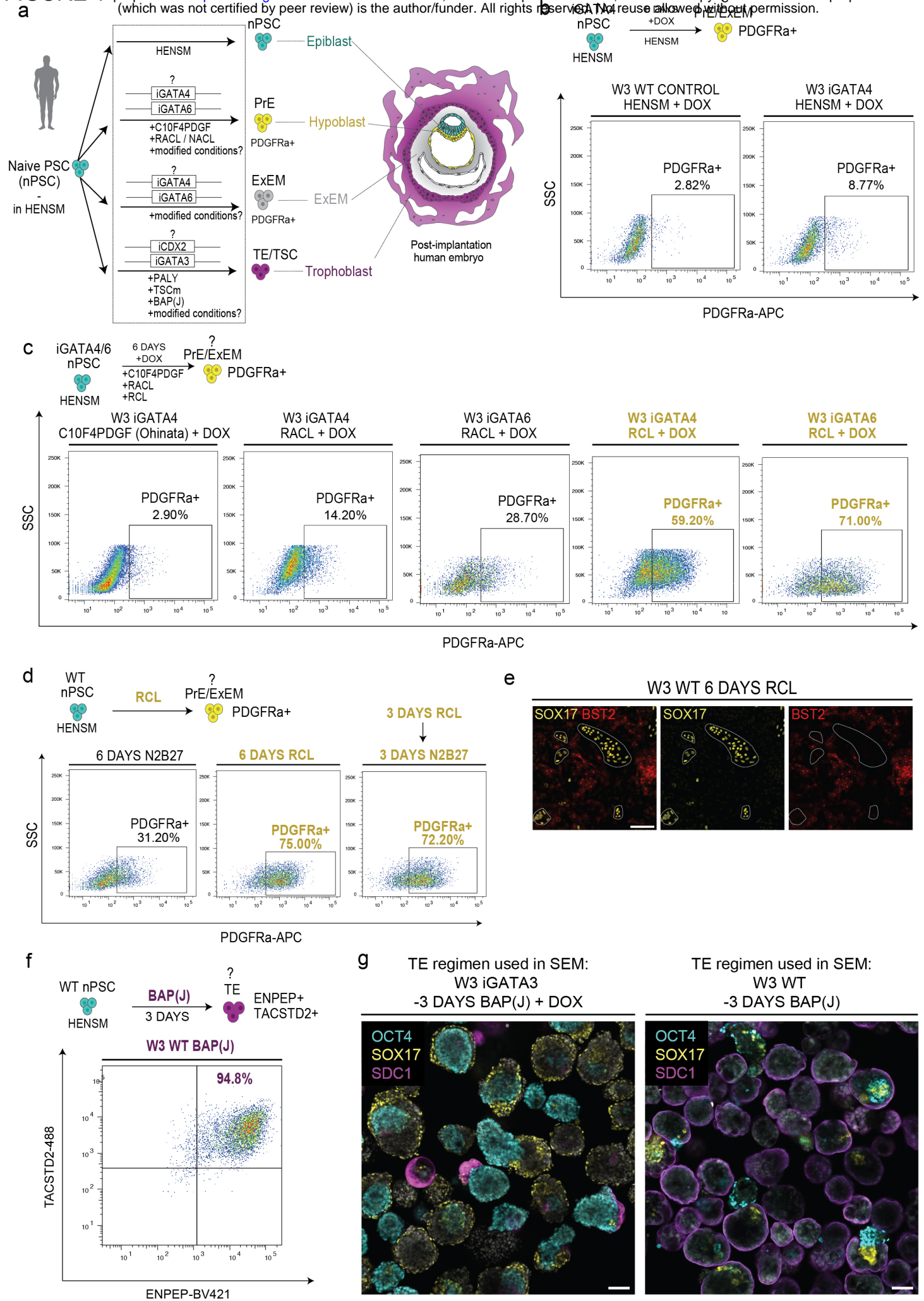
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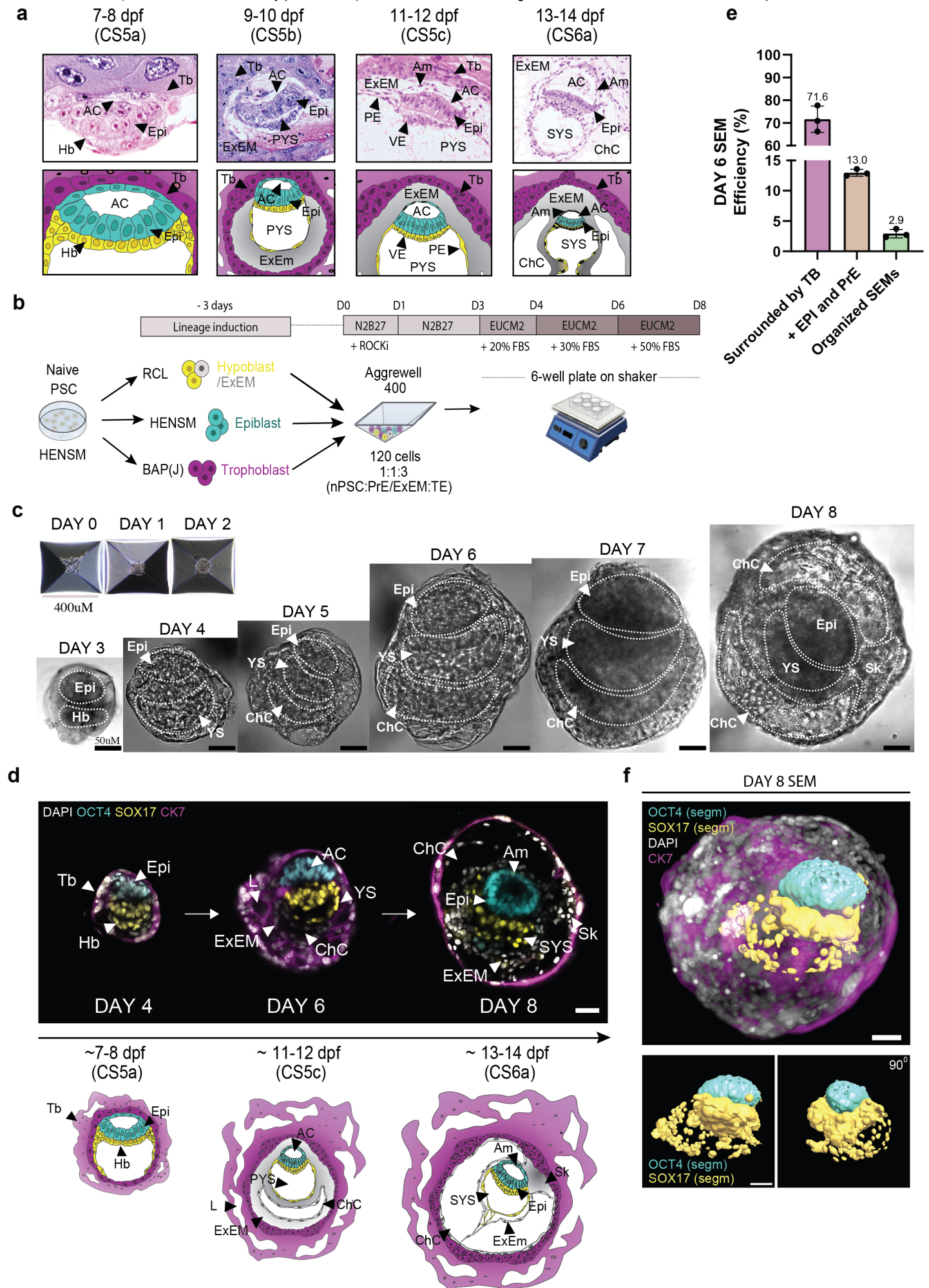
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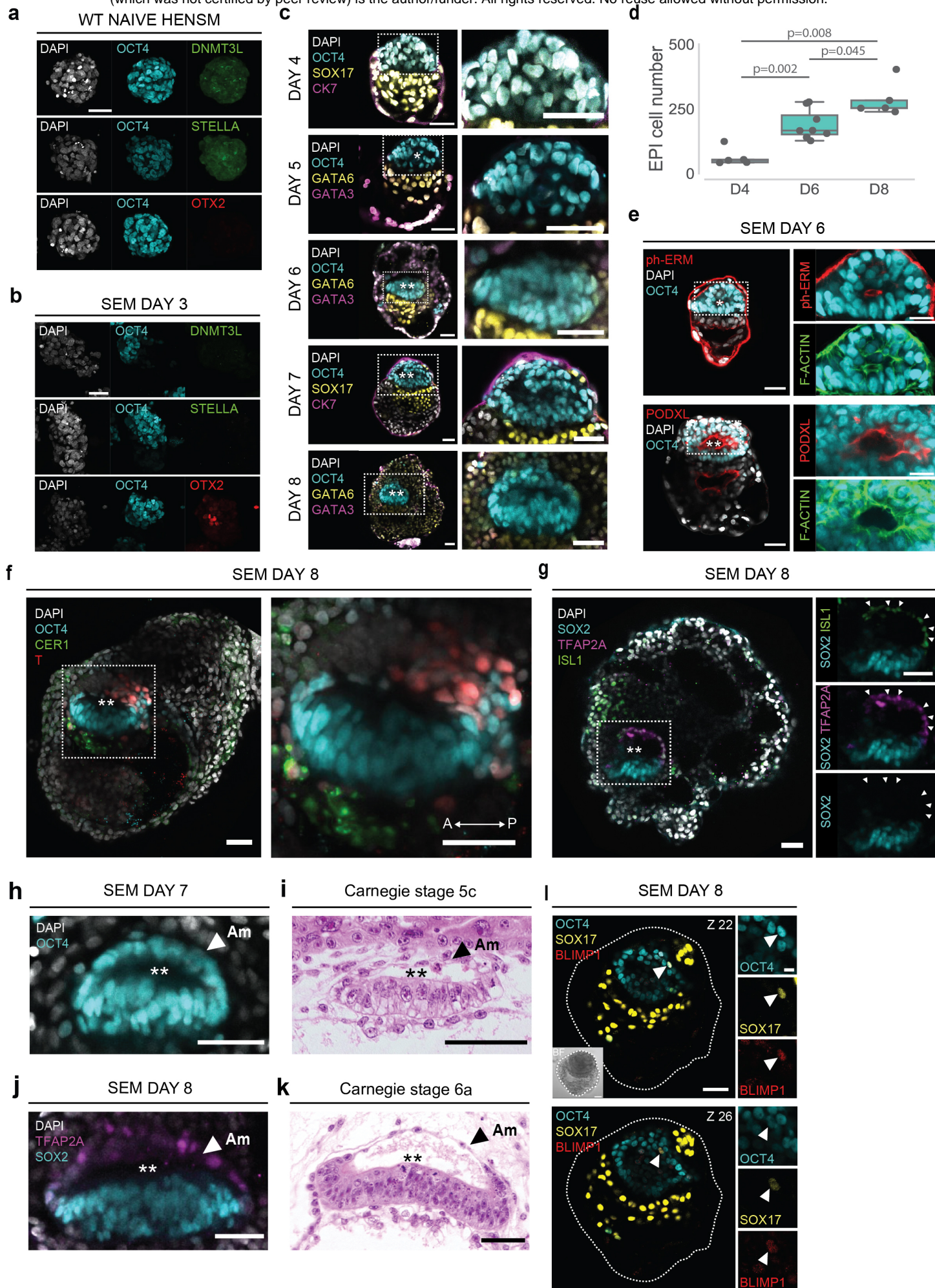
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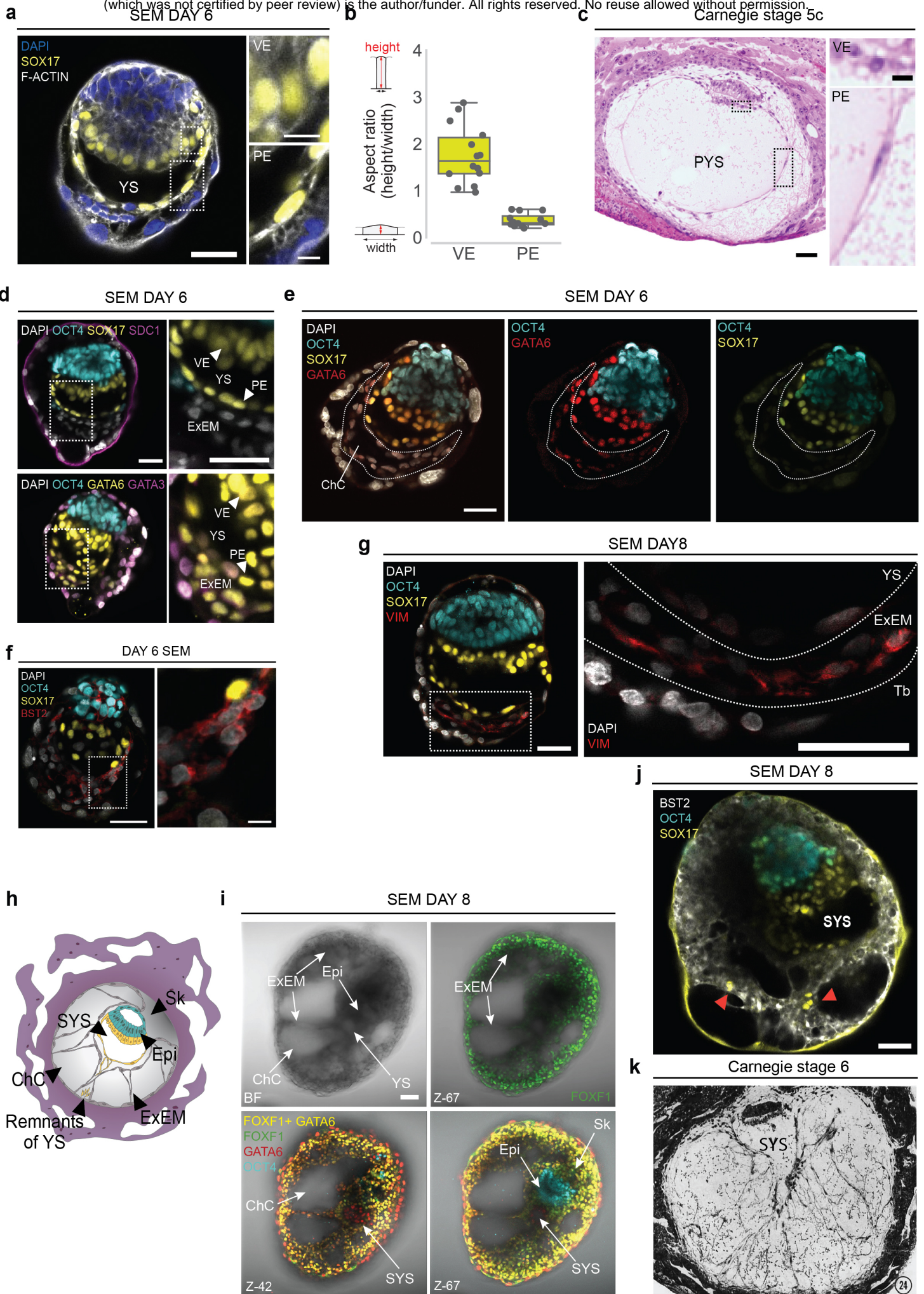
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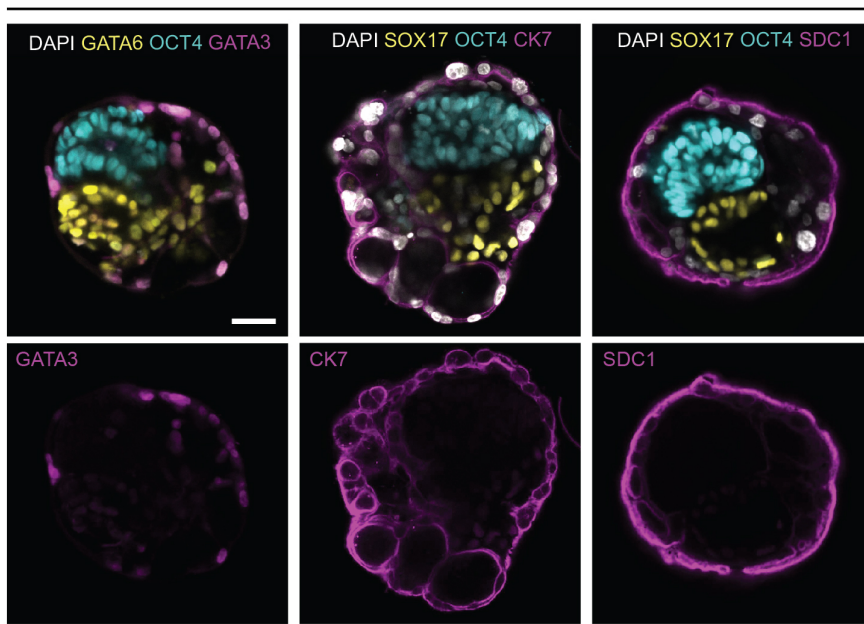




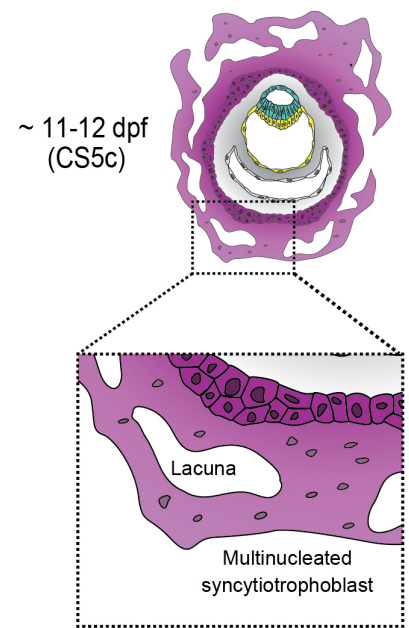




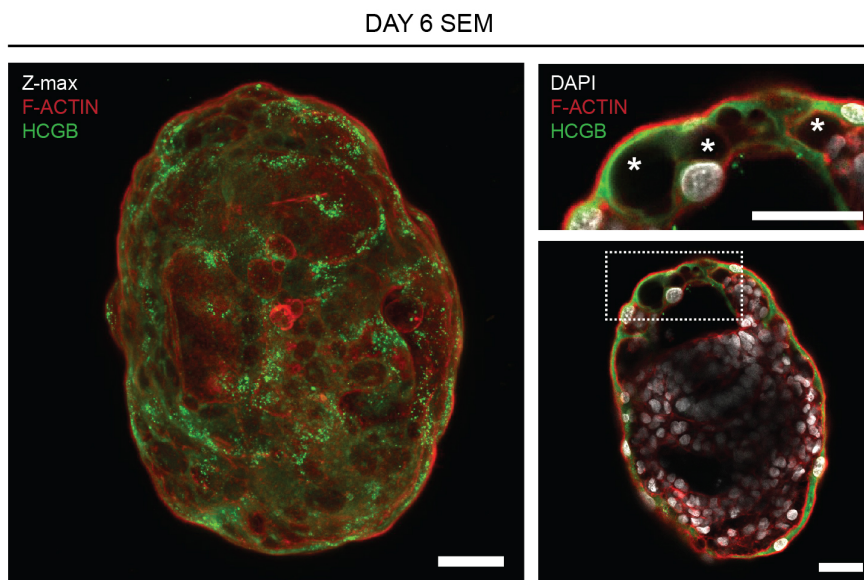
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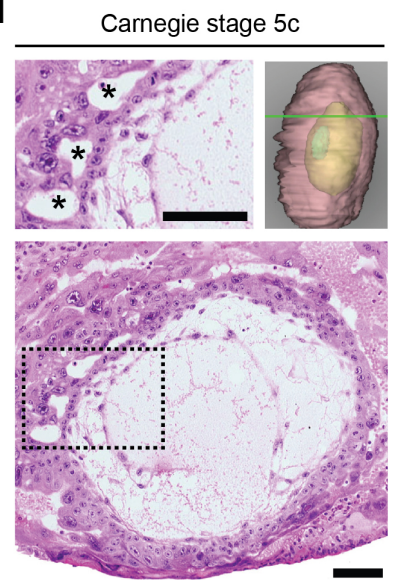
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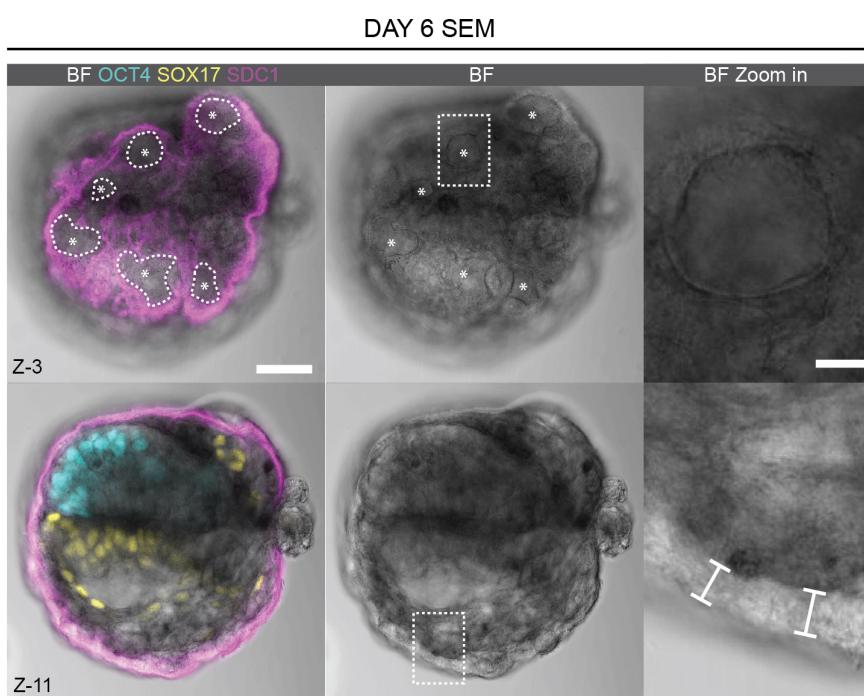
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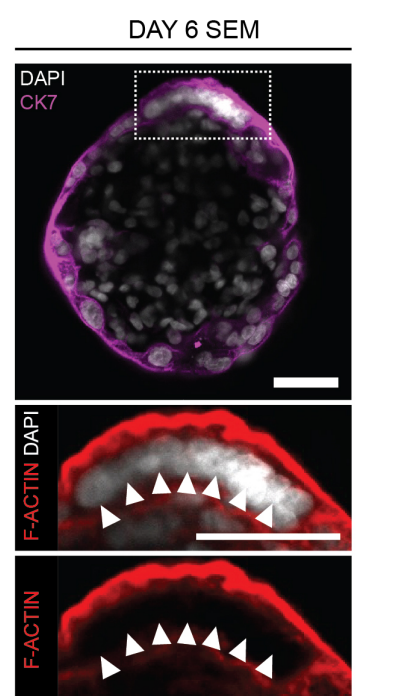
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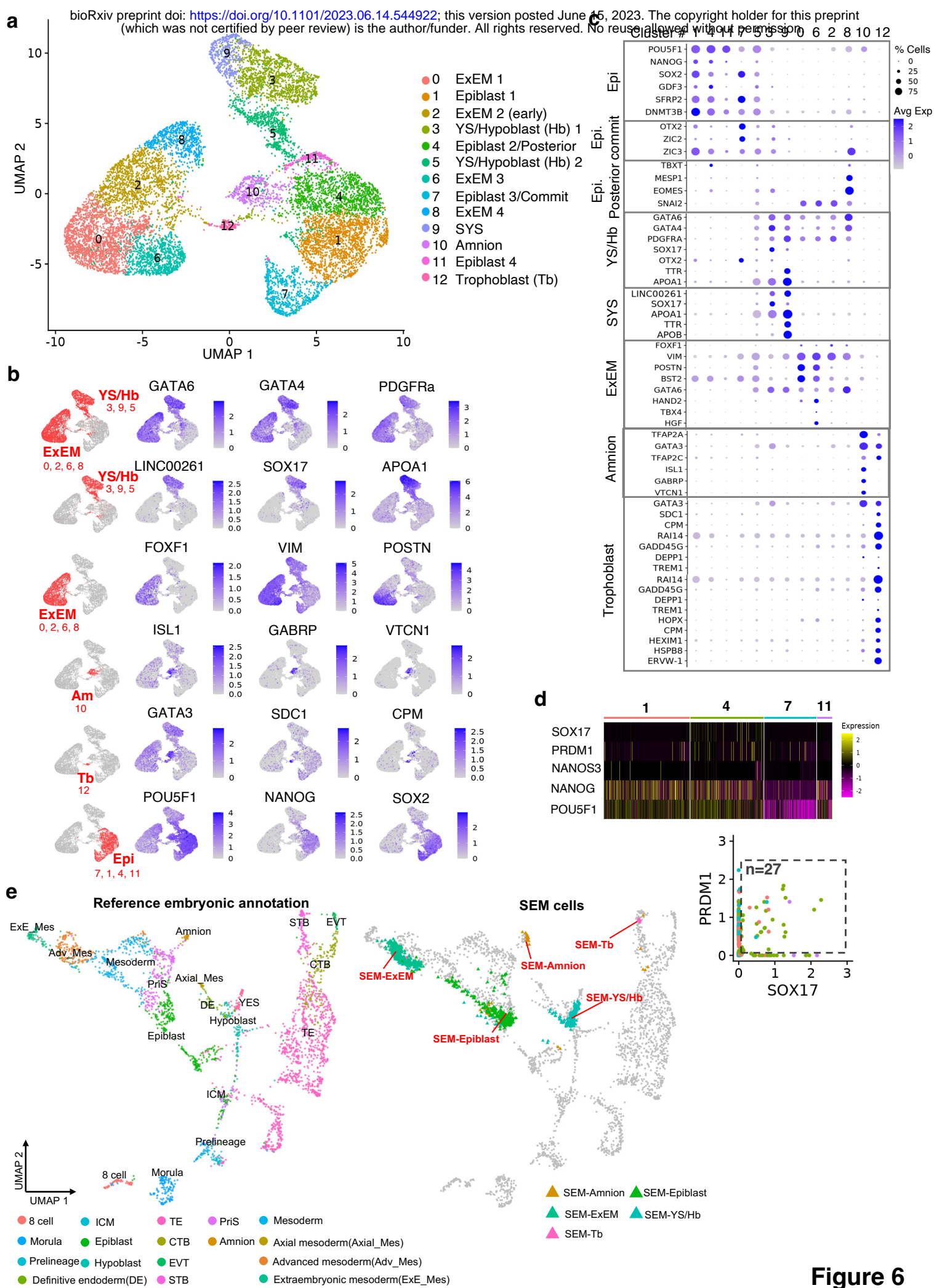
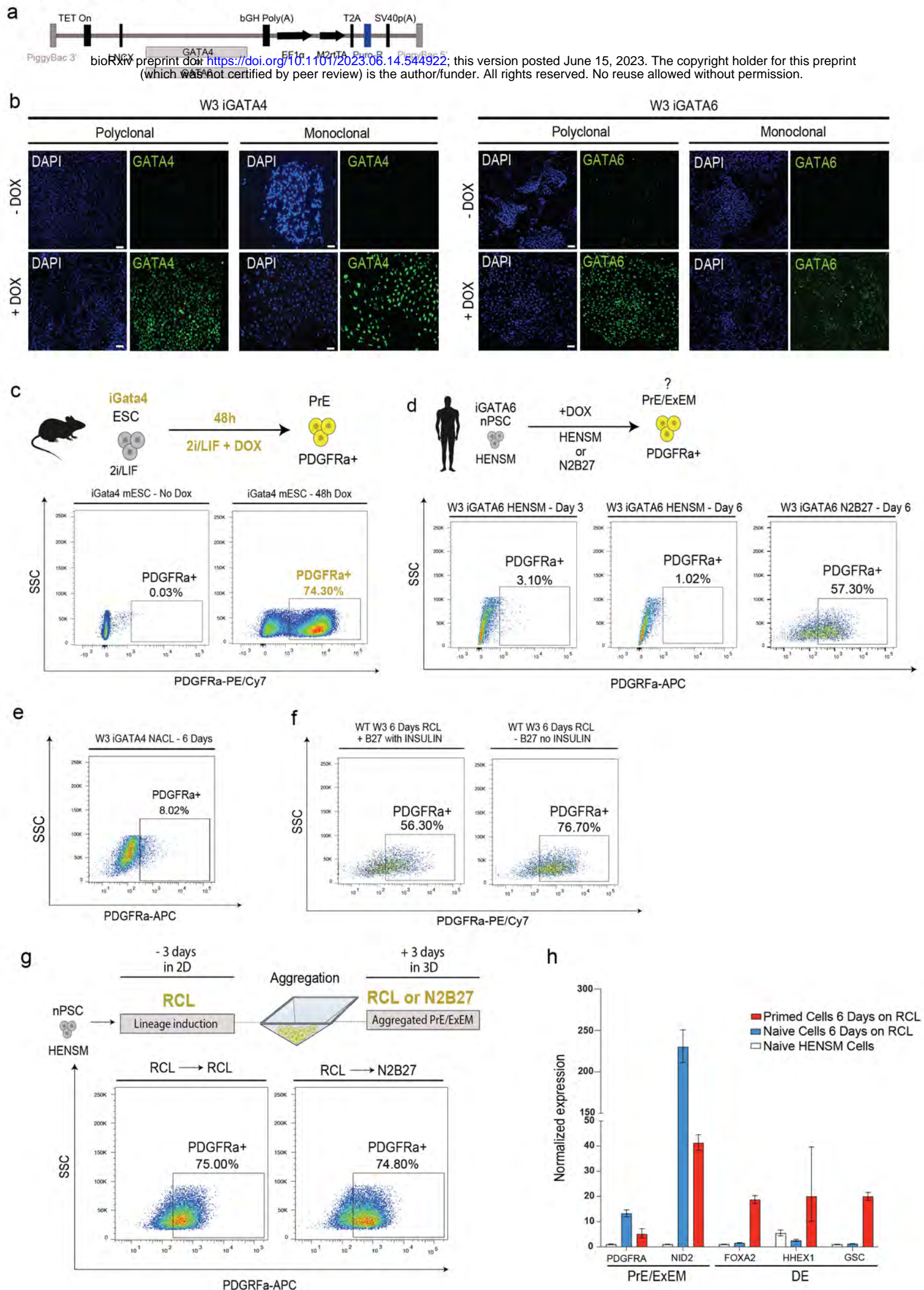


Figure 6

Extended Data Figure 1



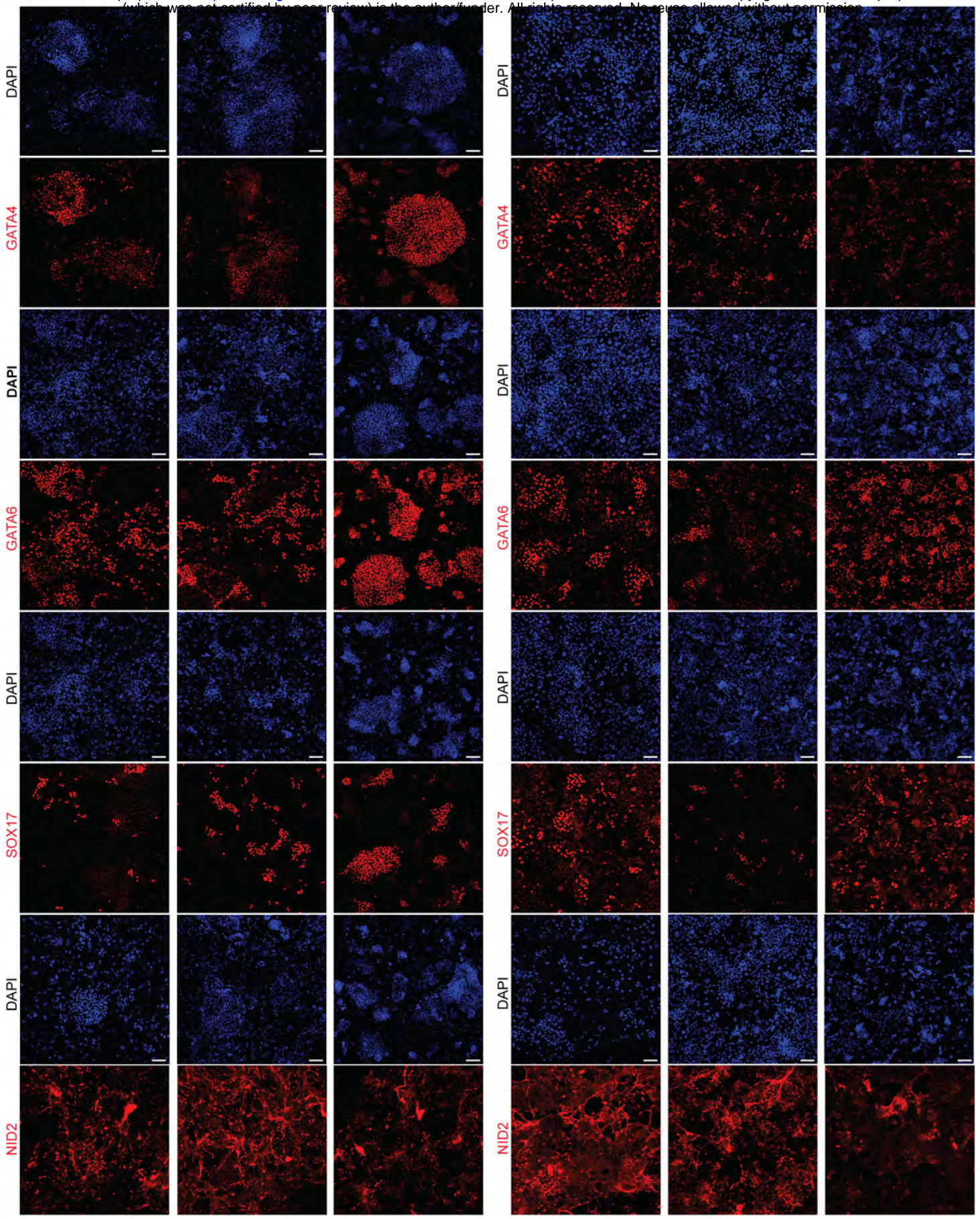
Extended Data Figure 2

a 3 DAYS RCL

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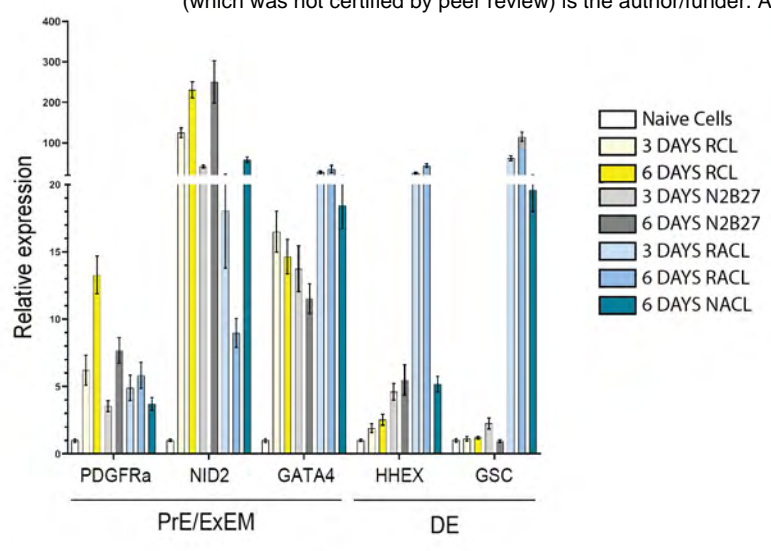
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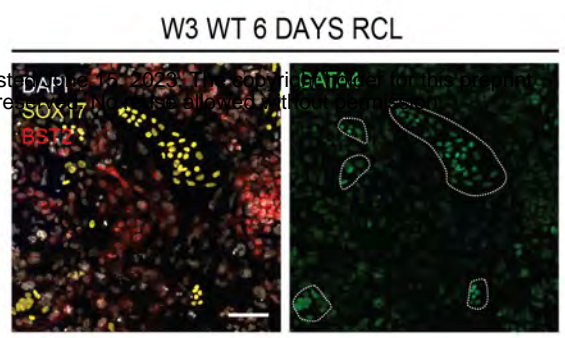
Extended Data Figure 3

a

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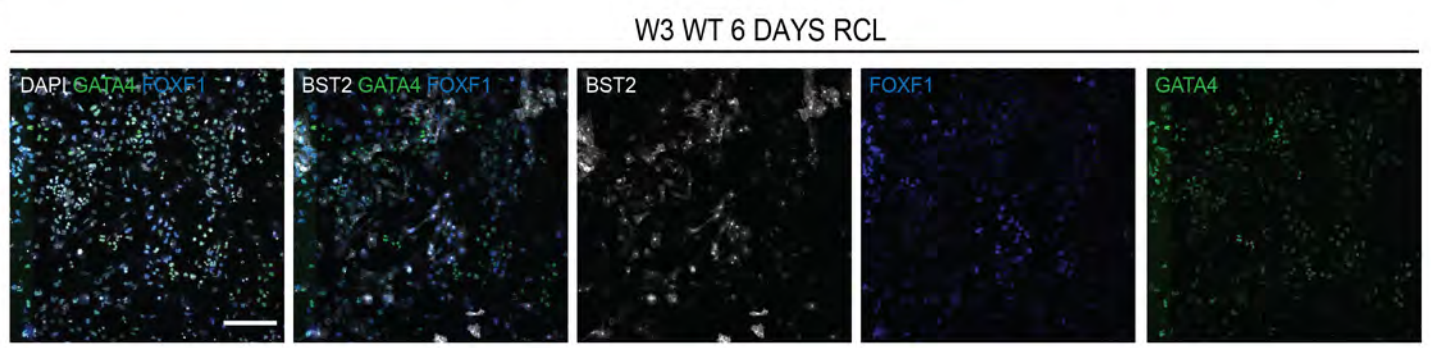
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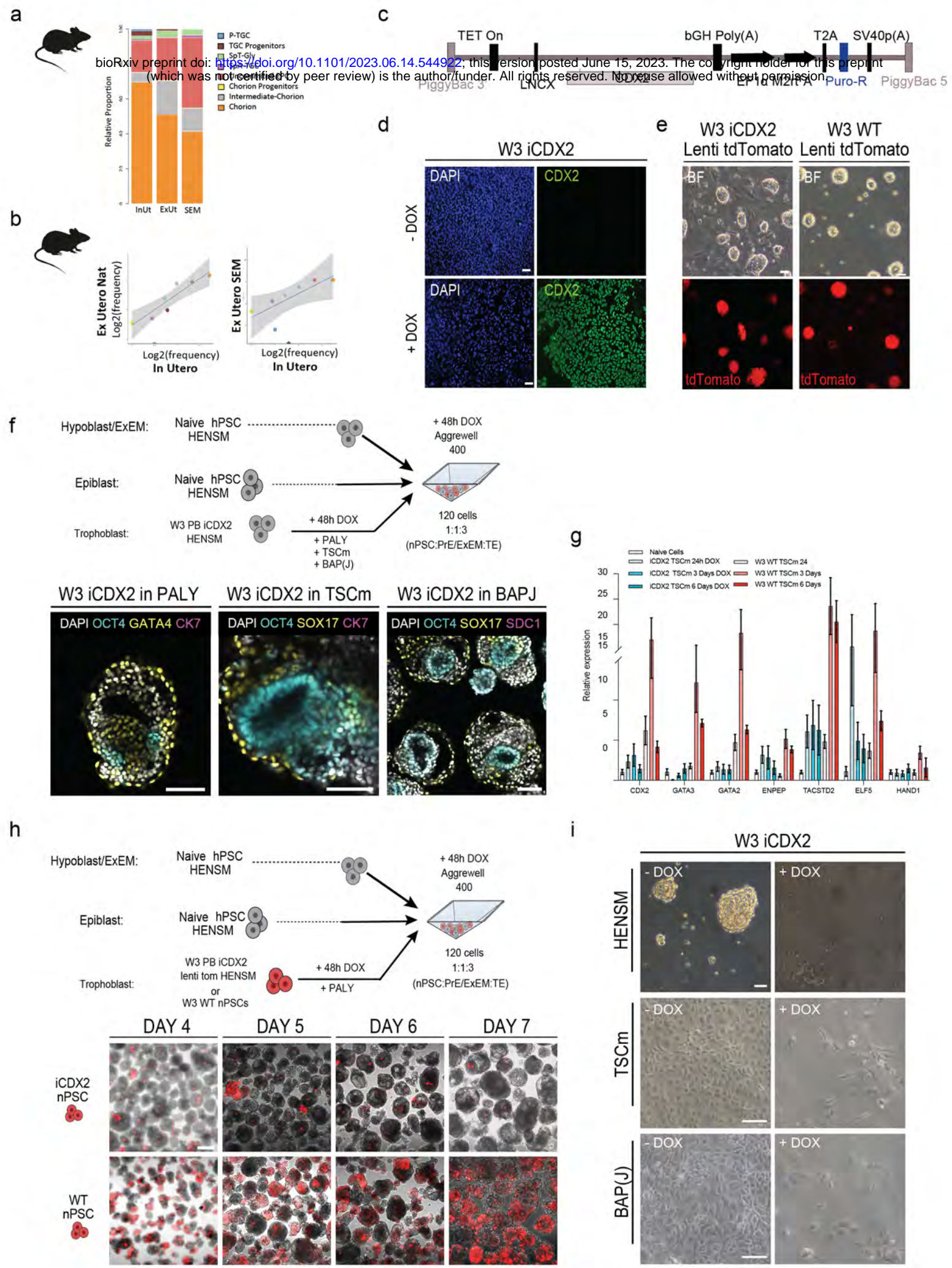
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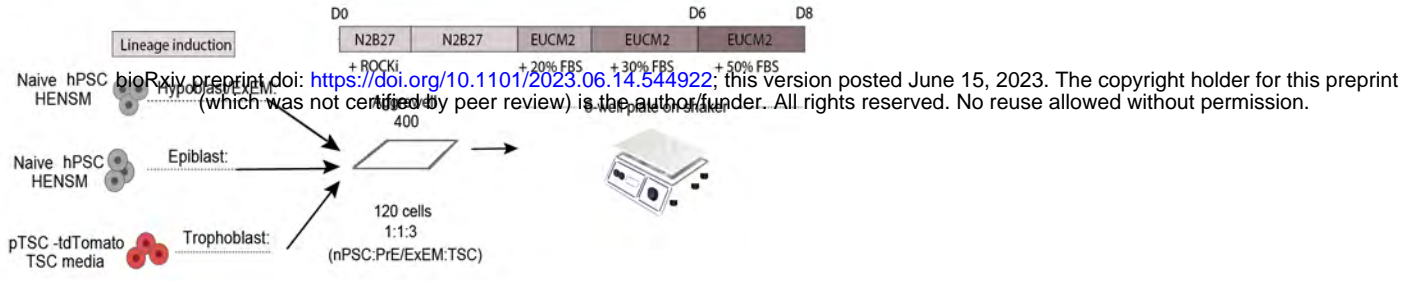


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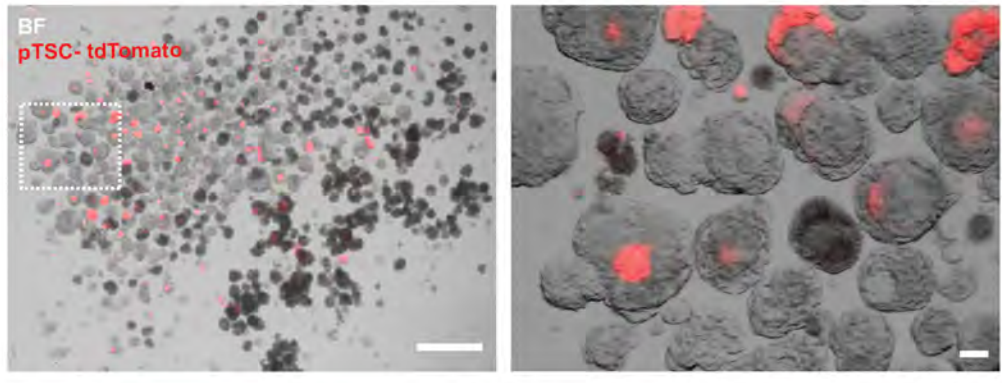
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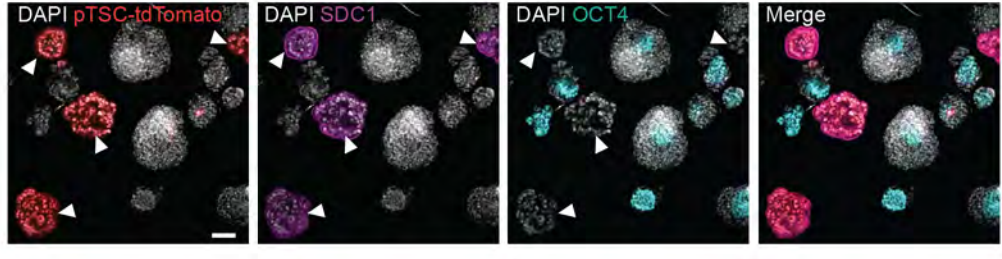
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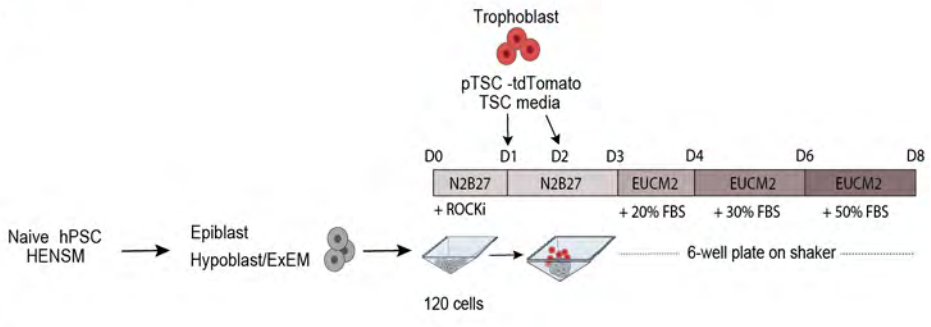


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DAY 8



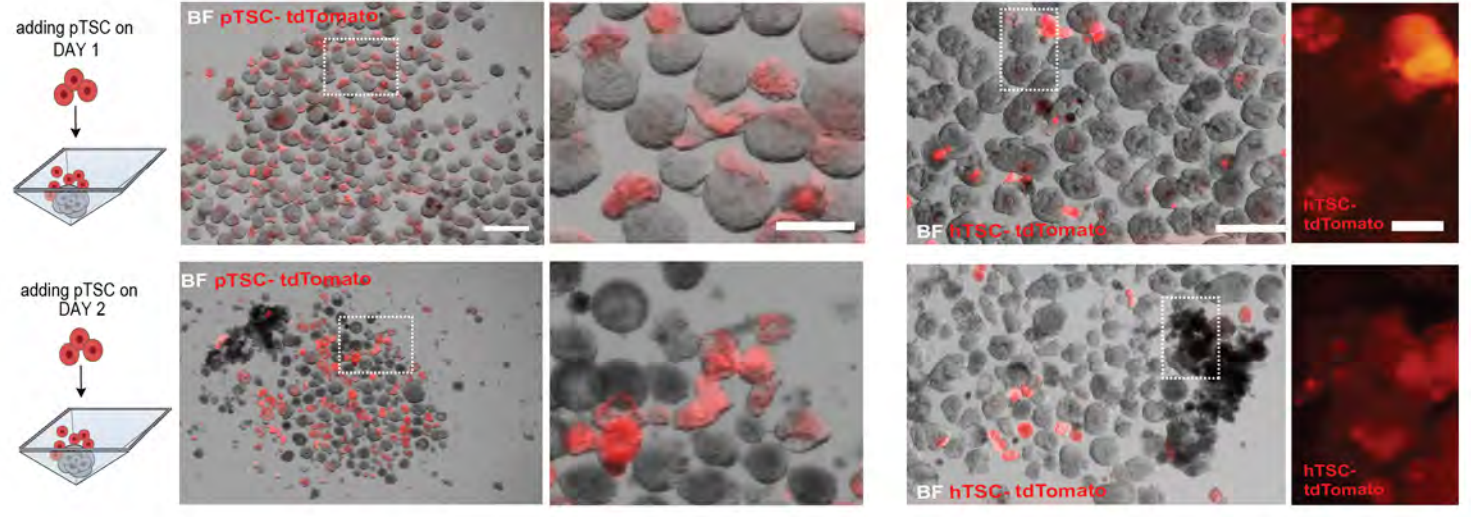
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DAY 5

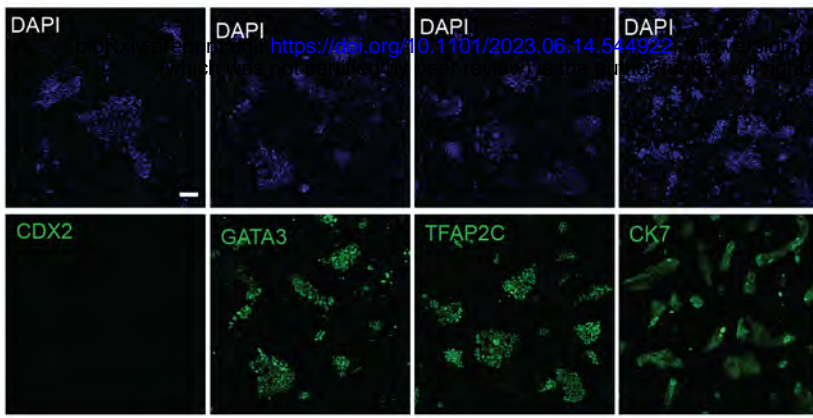
DAY 8



Extended Data Figure 6

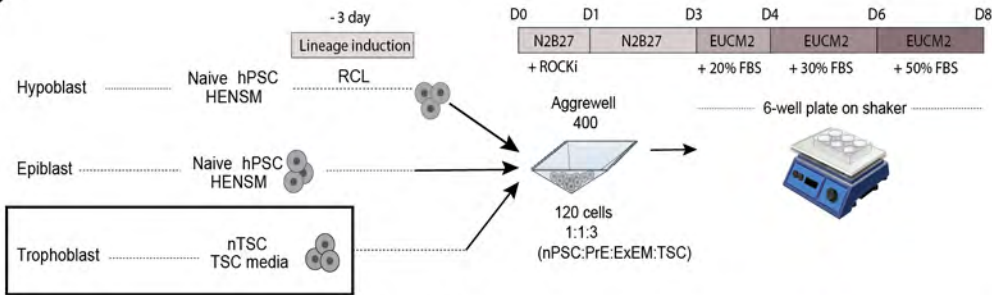
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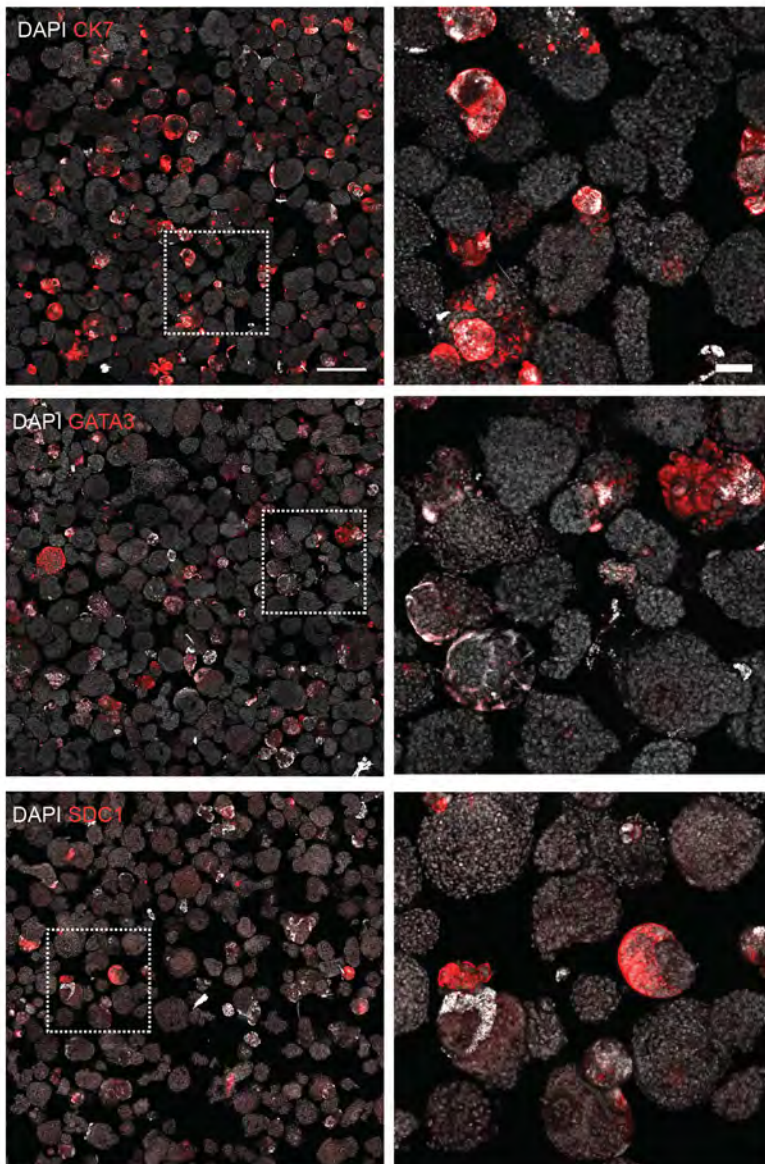
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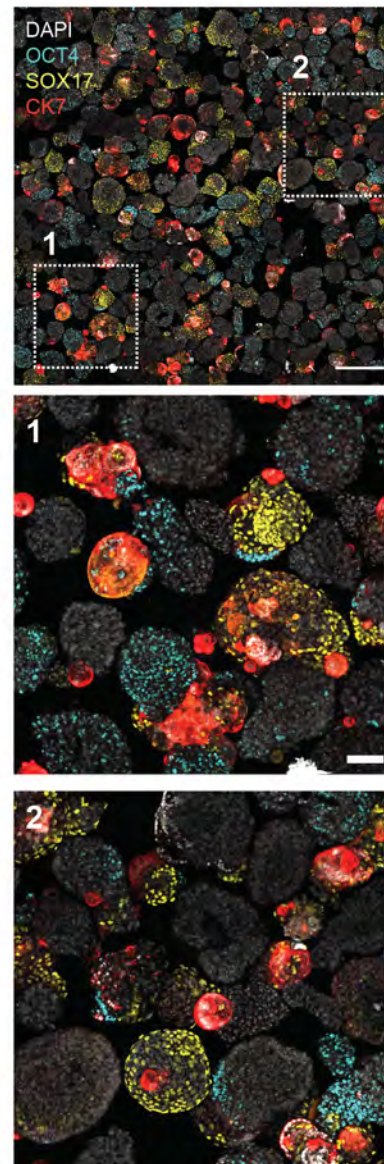
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SEM DAY 6



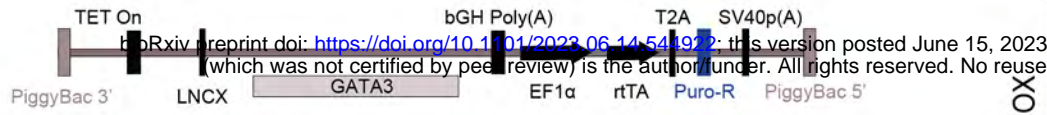
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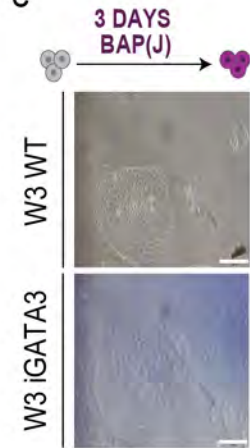


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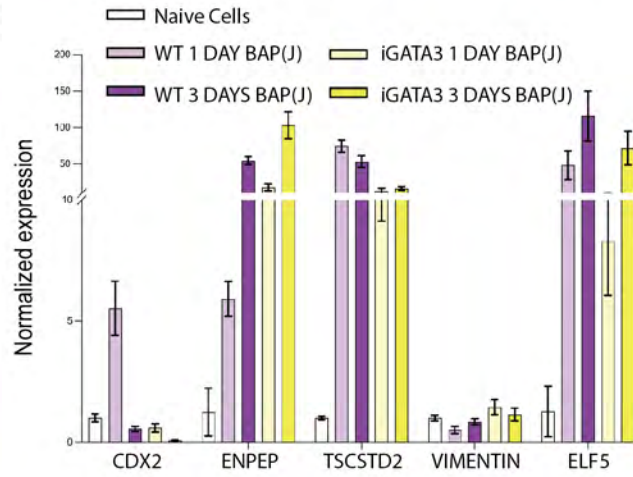
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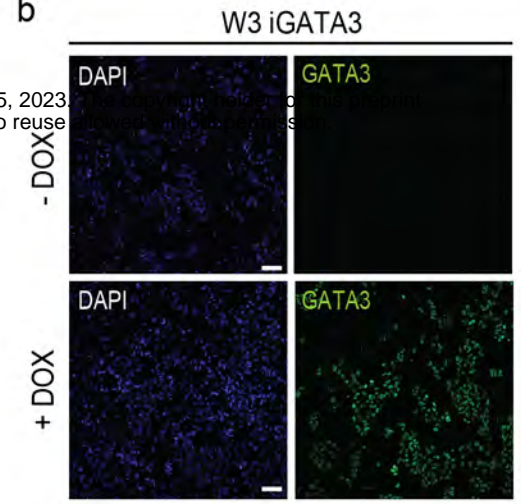
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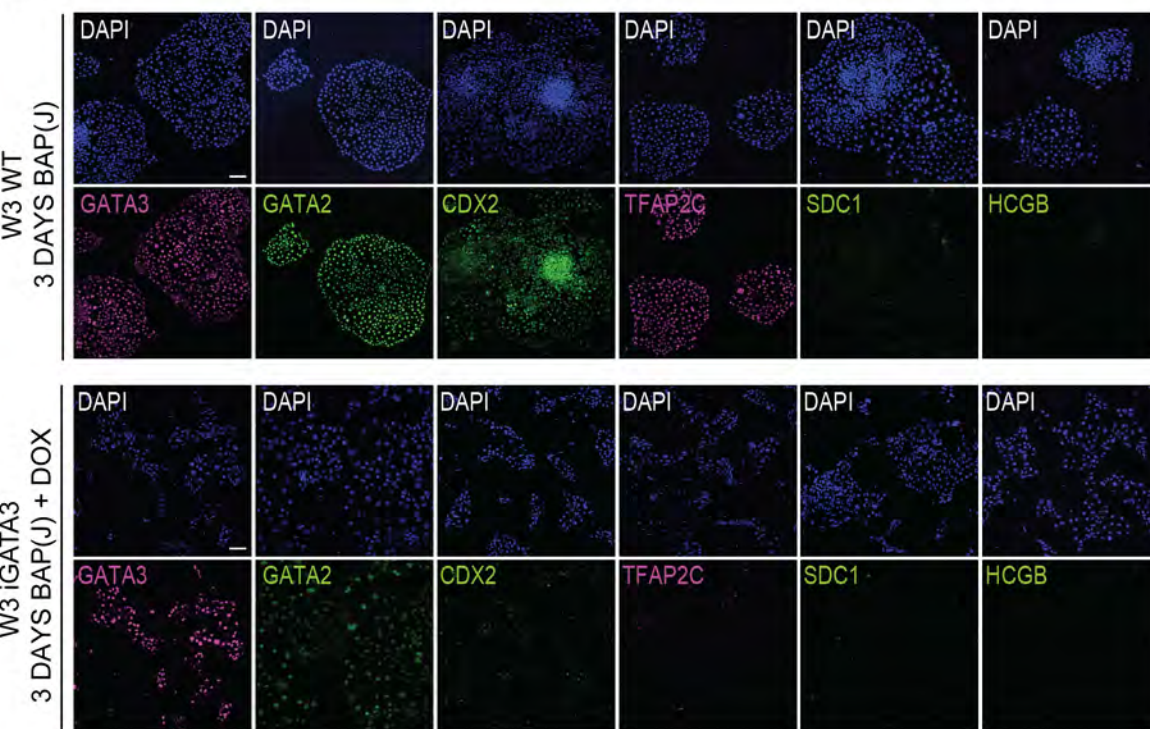
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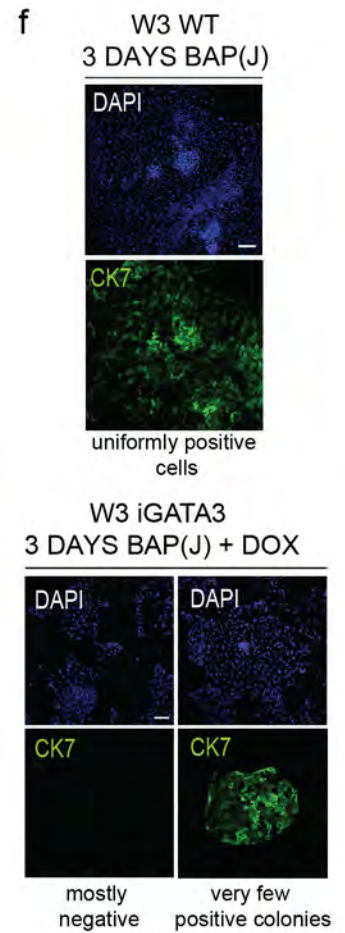
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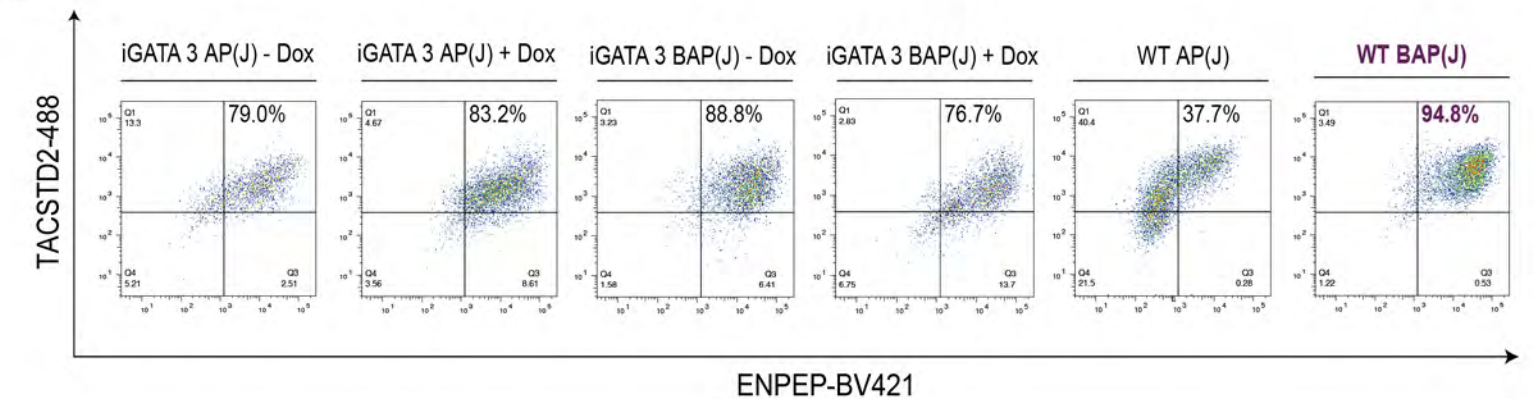
e



f



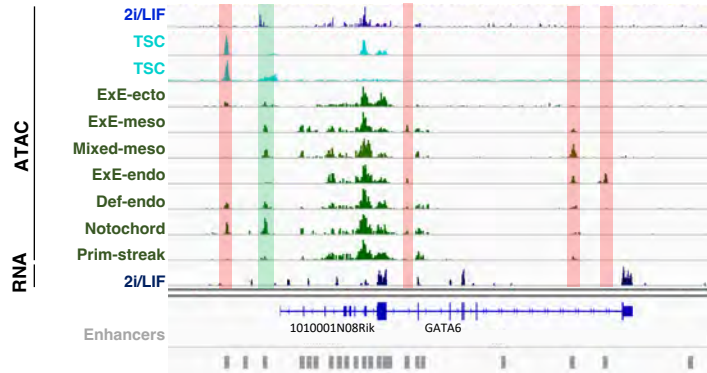
g



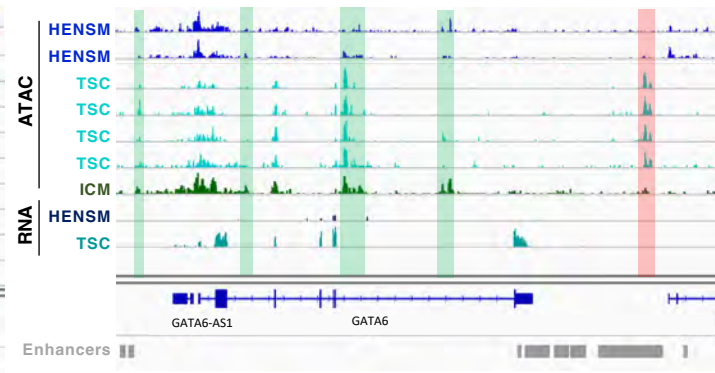
Extended Data Figure 8



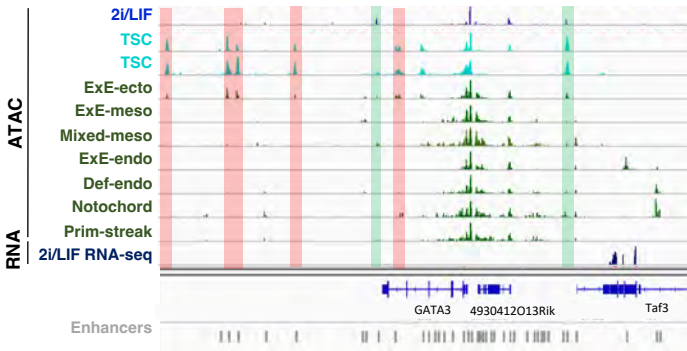
Gata6



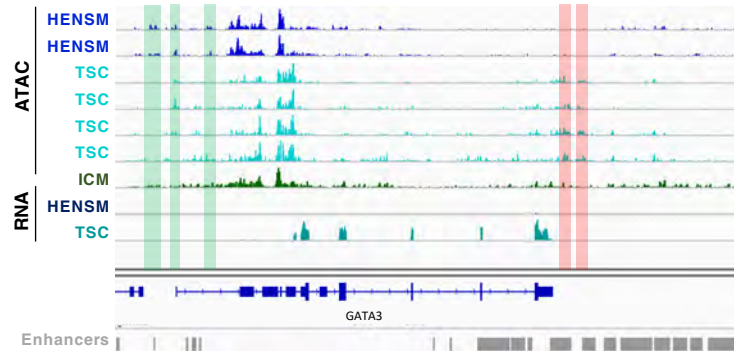
GATA6



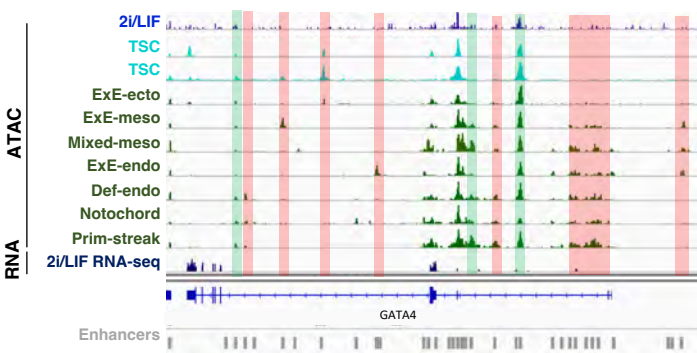
Gata3



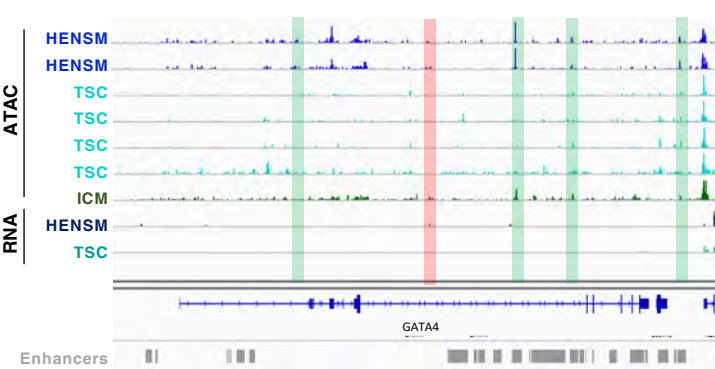
GATA3



Gata4

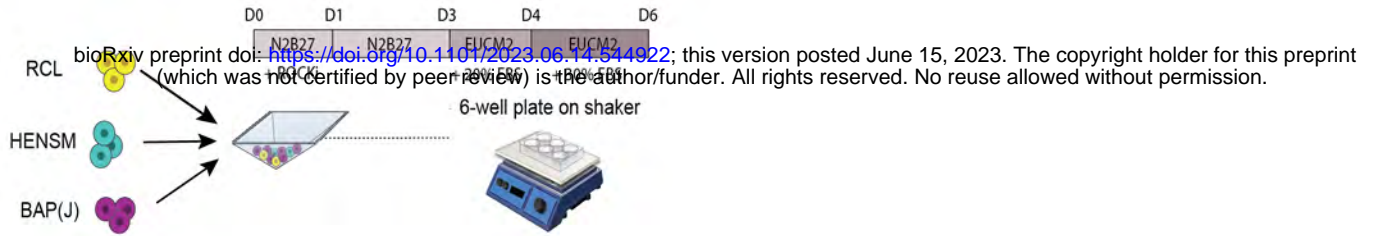


GATA4



Extended Data Figure 9

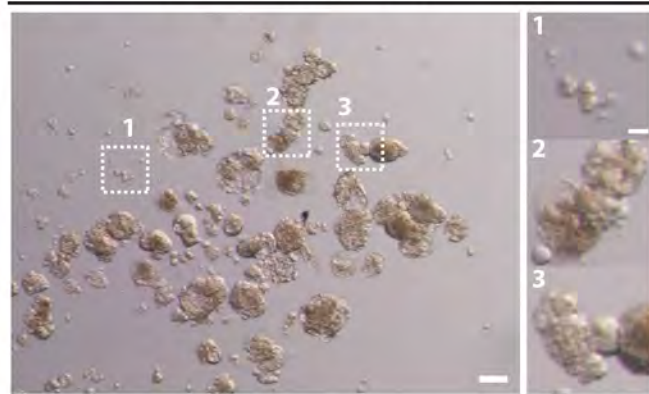
a



b

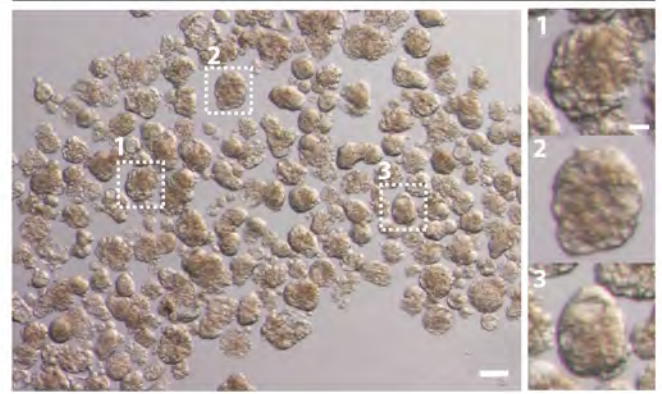
60 cells

1:1:3 (nPSC:PrE/ExEM:TE) SEM DAY6



120 cells

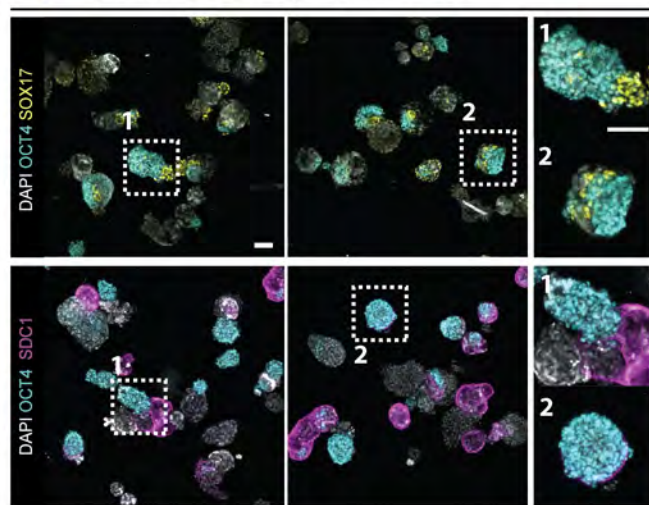
1:1:3 (nPSC:PrE/ExEM:TE) SEM DAY6



c

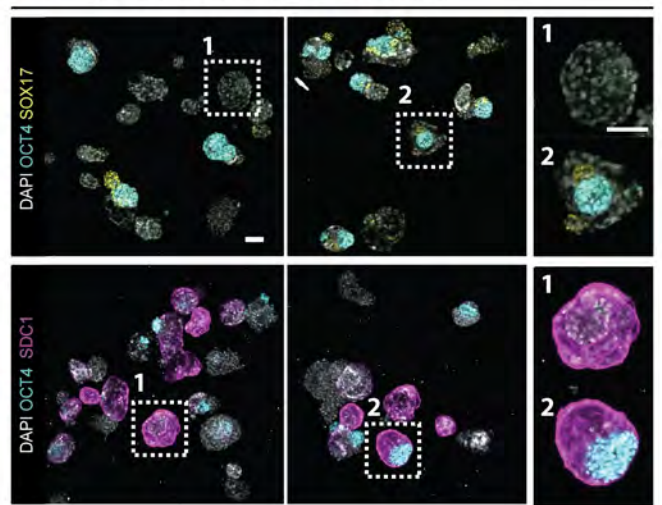
132 cells

4:2:7 (nPSC:PrE/ExEM:TE) SEM DAY6



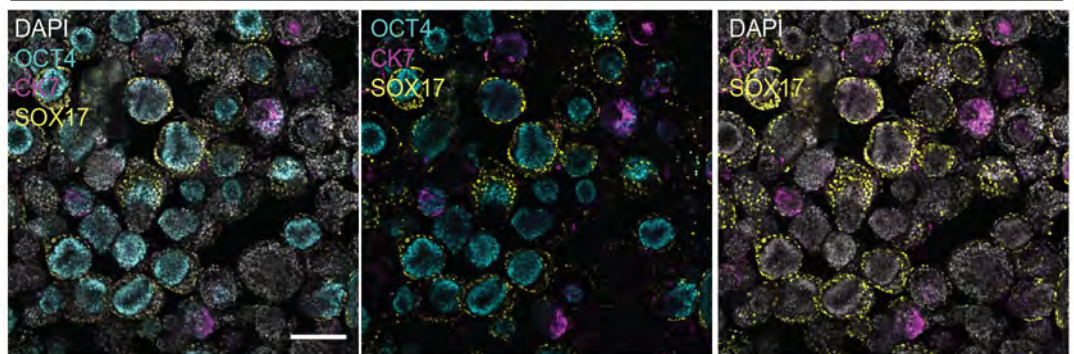
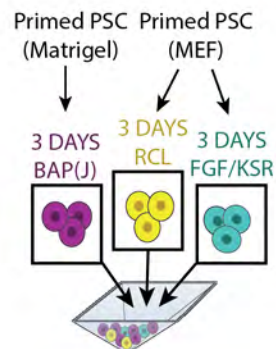
160 cells

2:1:5 (nPSC:PrE/ExEM:TE) SEM DAY6



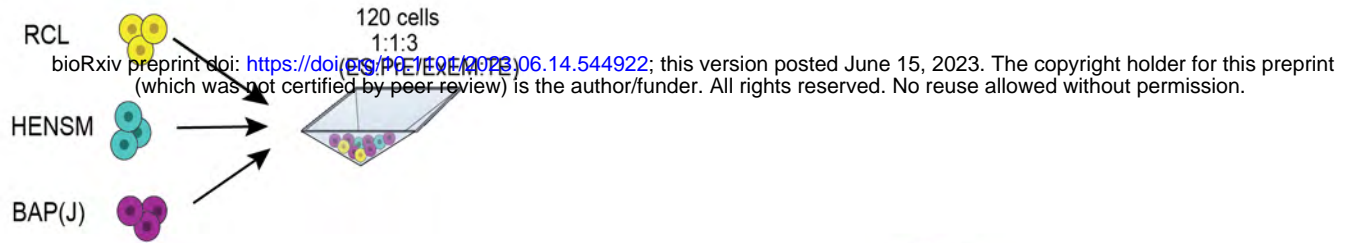
d

DAY 6 SEM

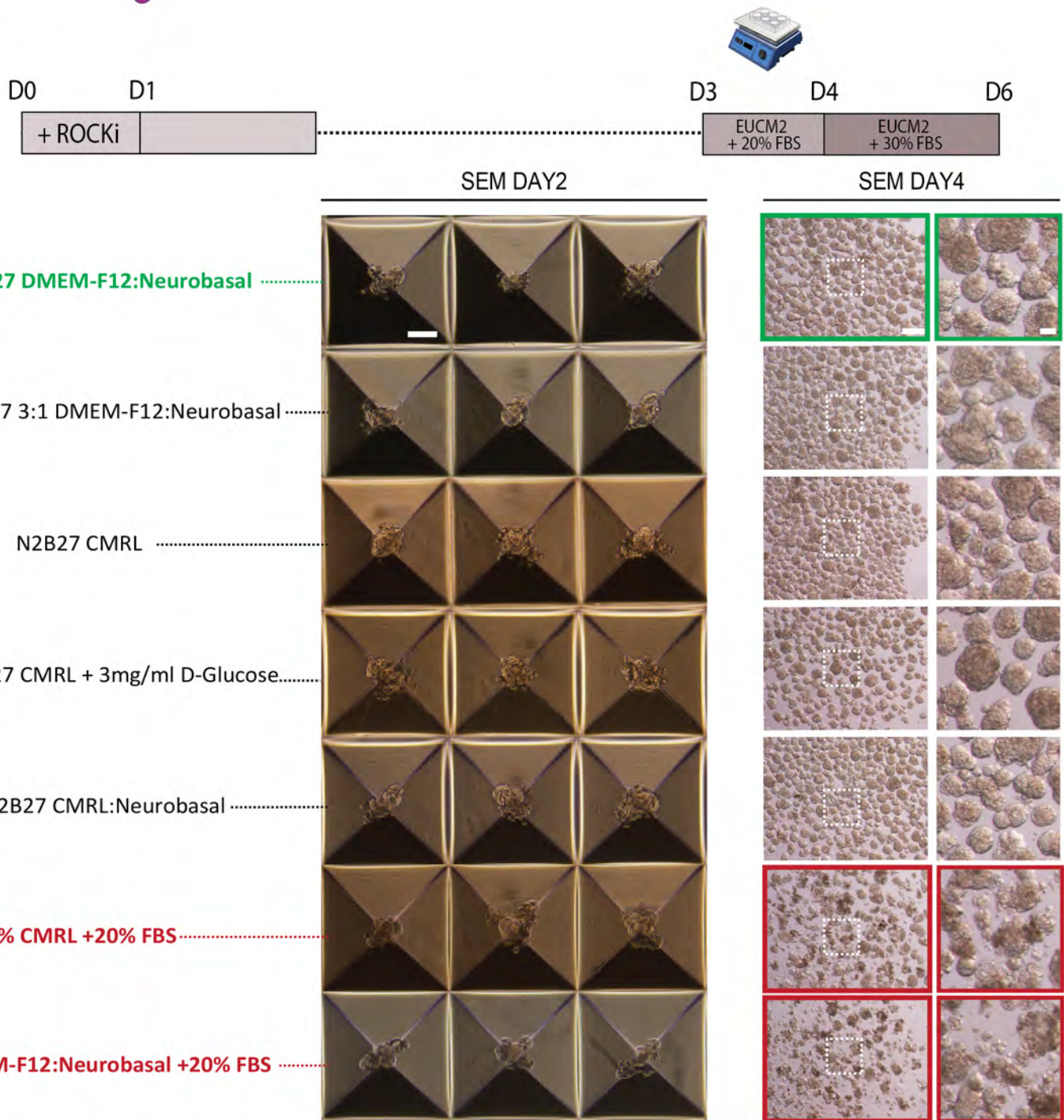


Extended Data Figure 10

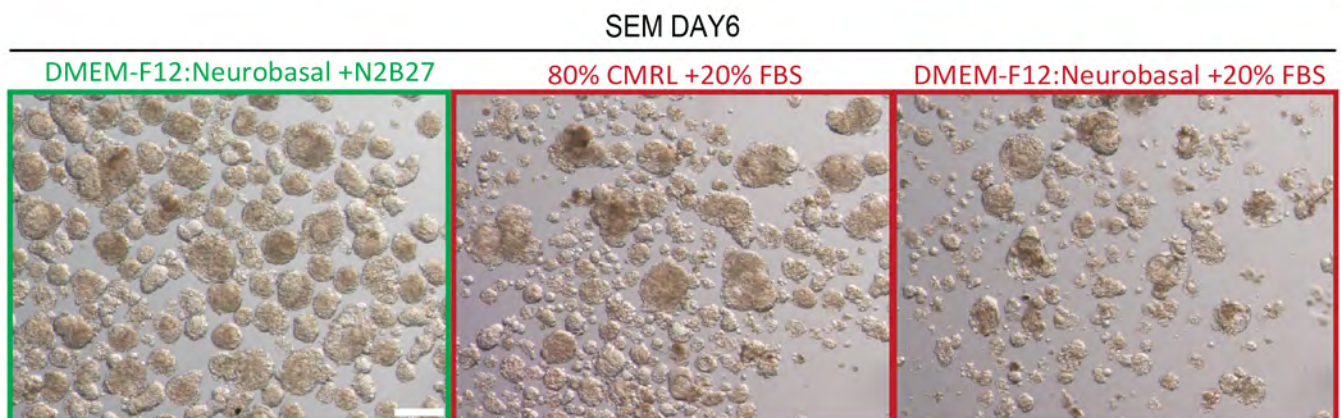
a



b

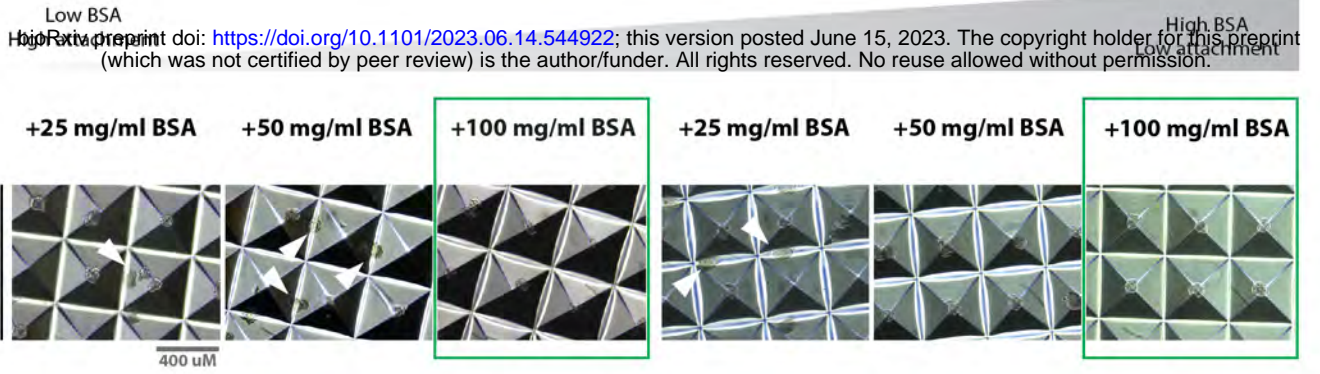


c

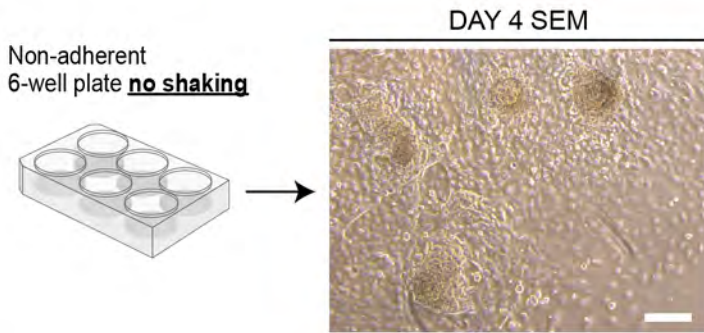


Extended Data Figure 11

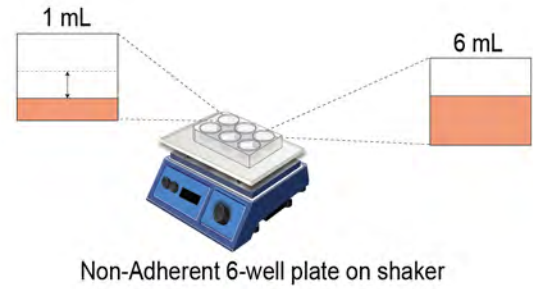
a



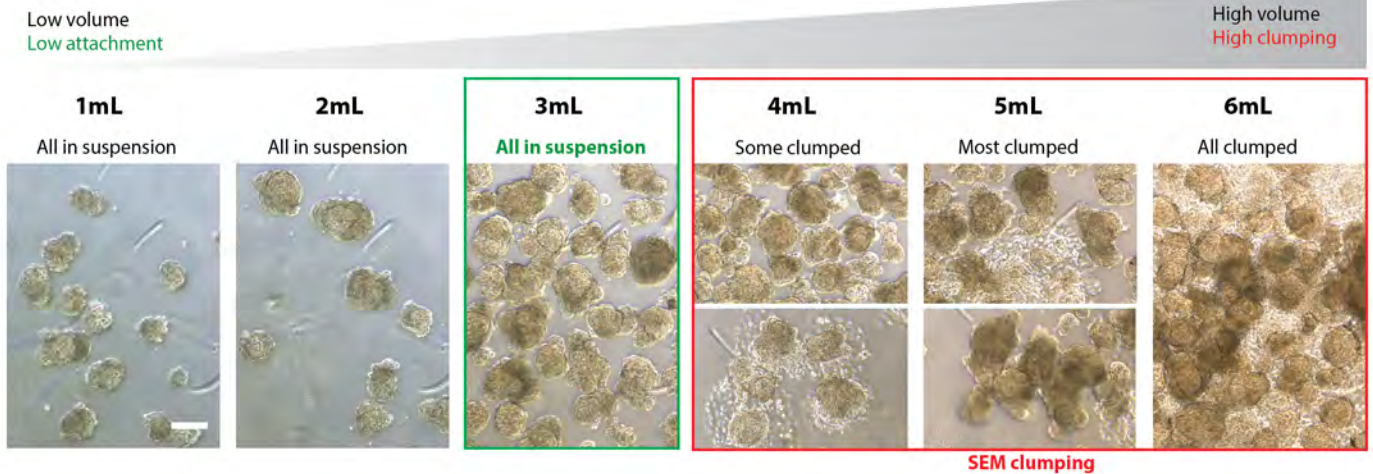
b



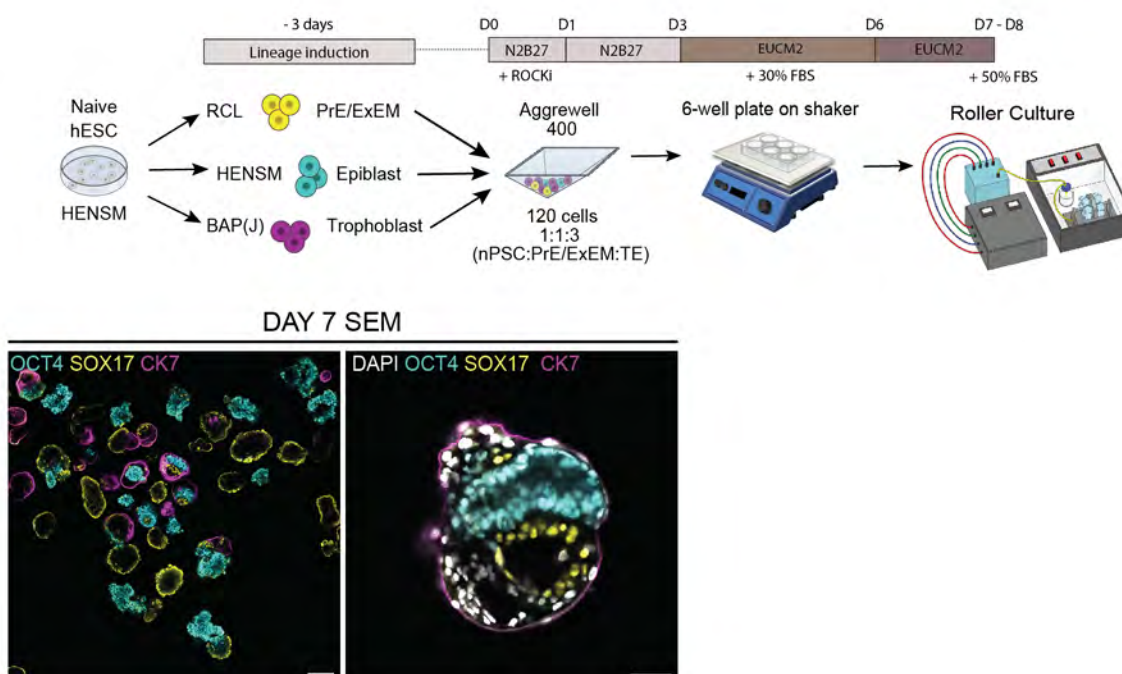
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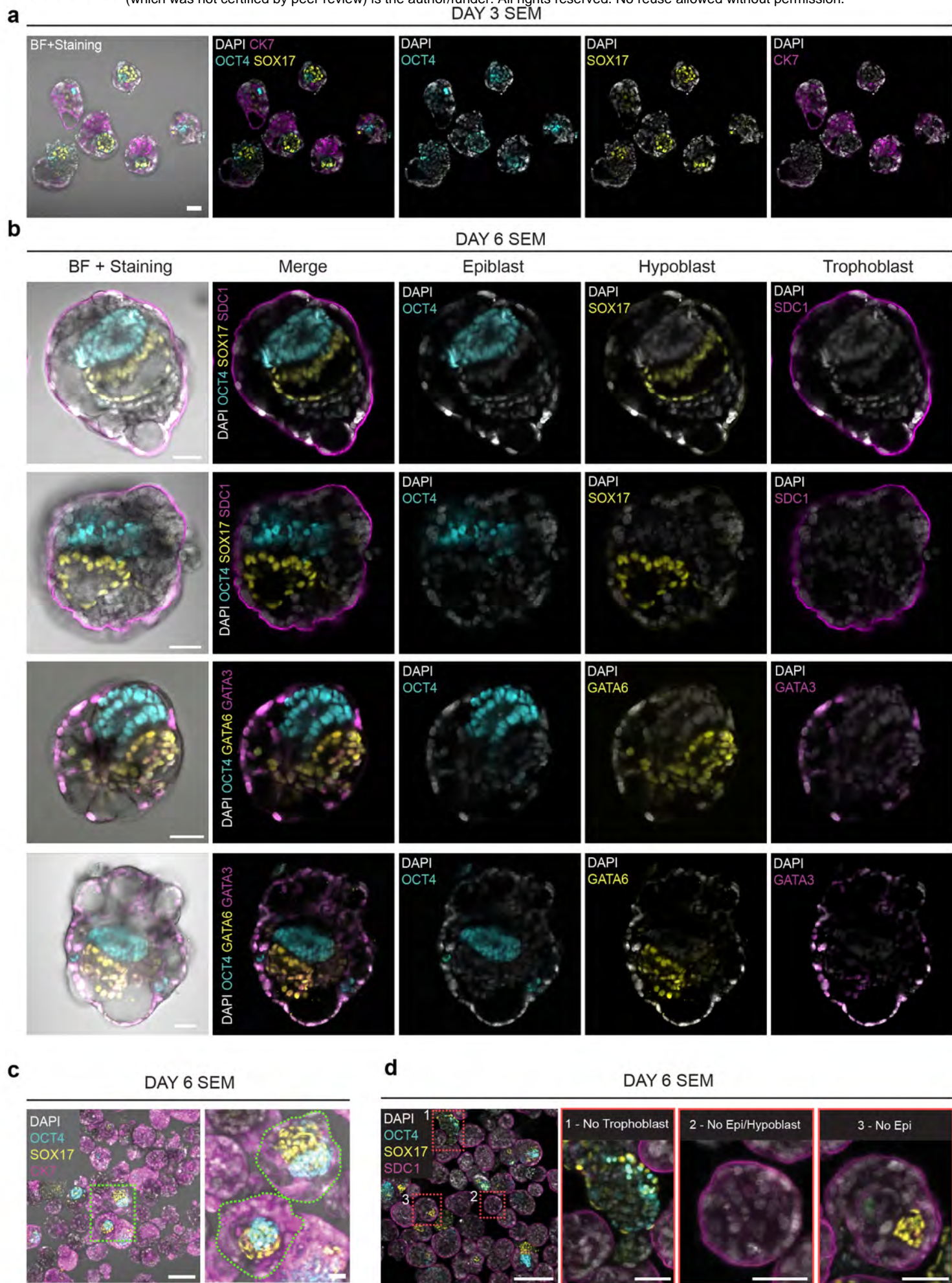


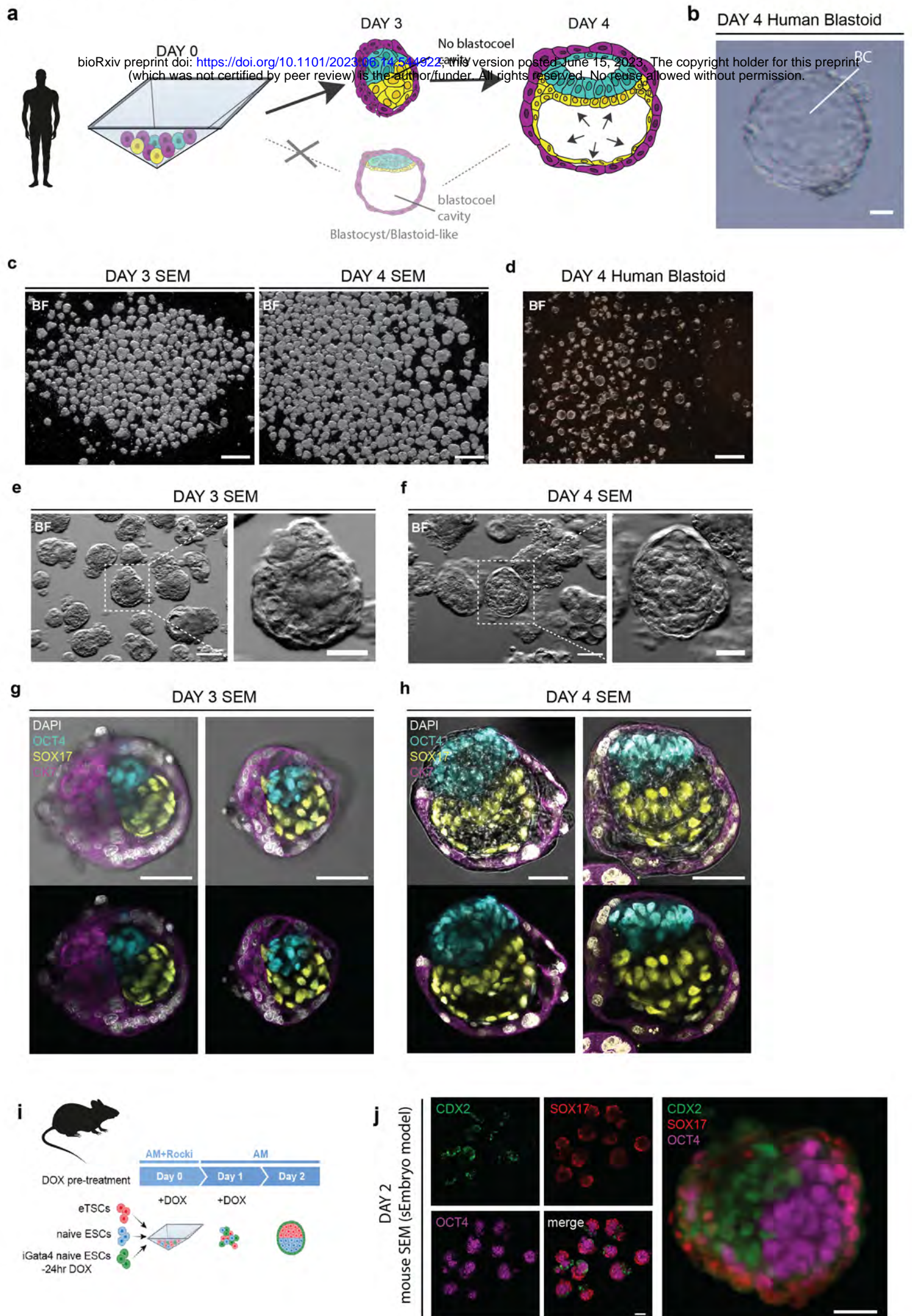
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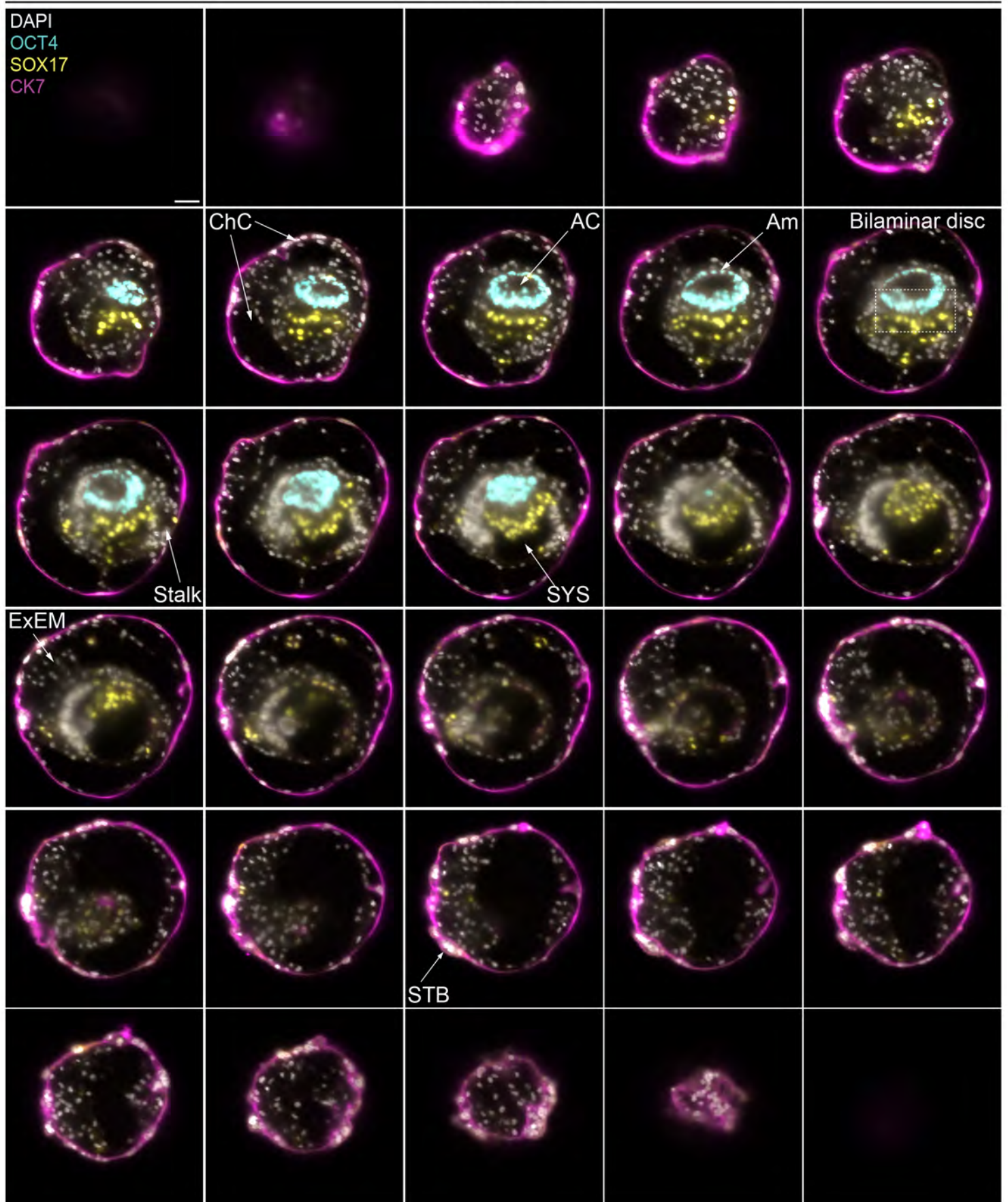
e



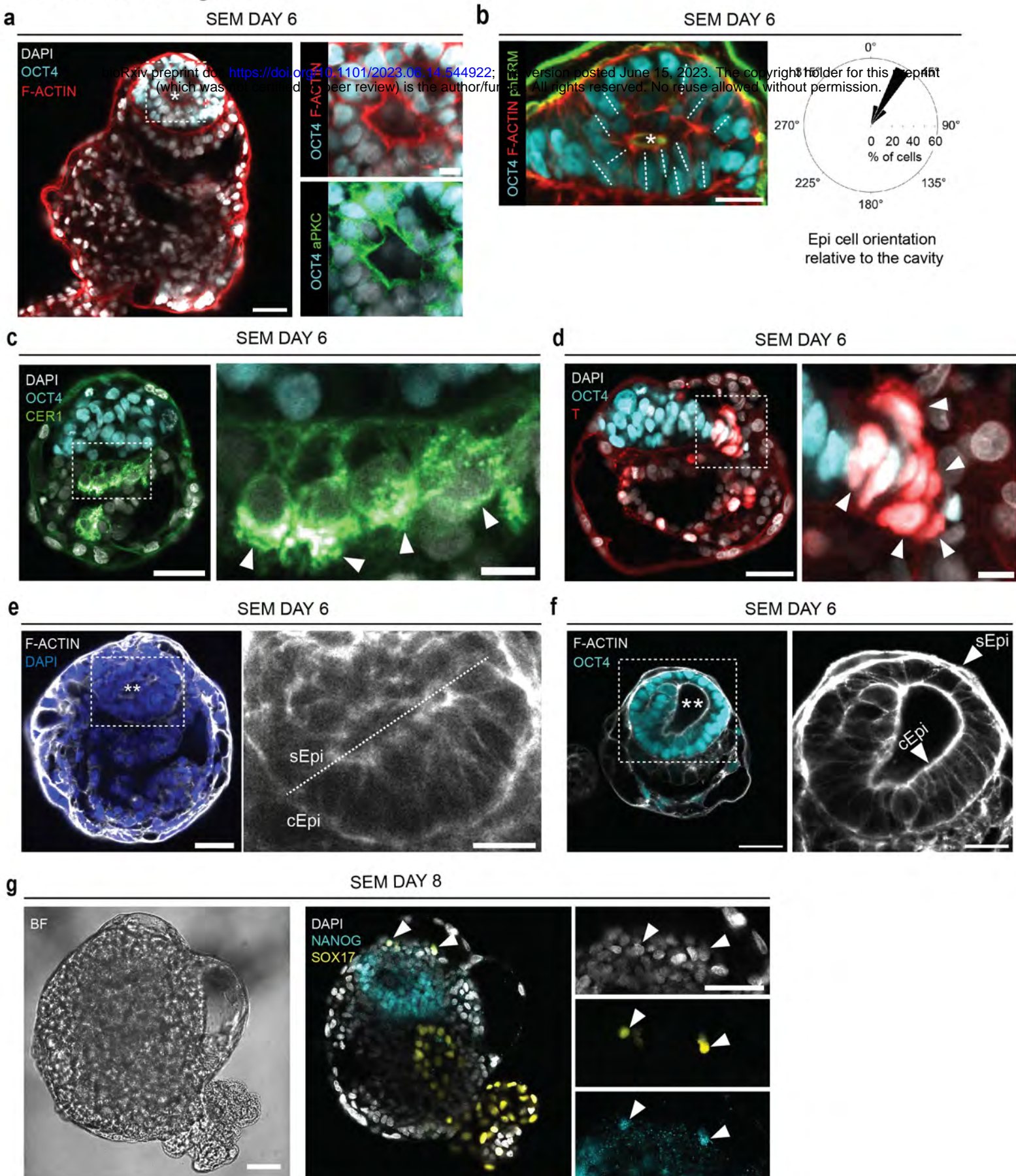




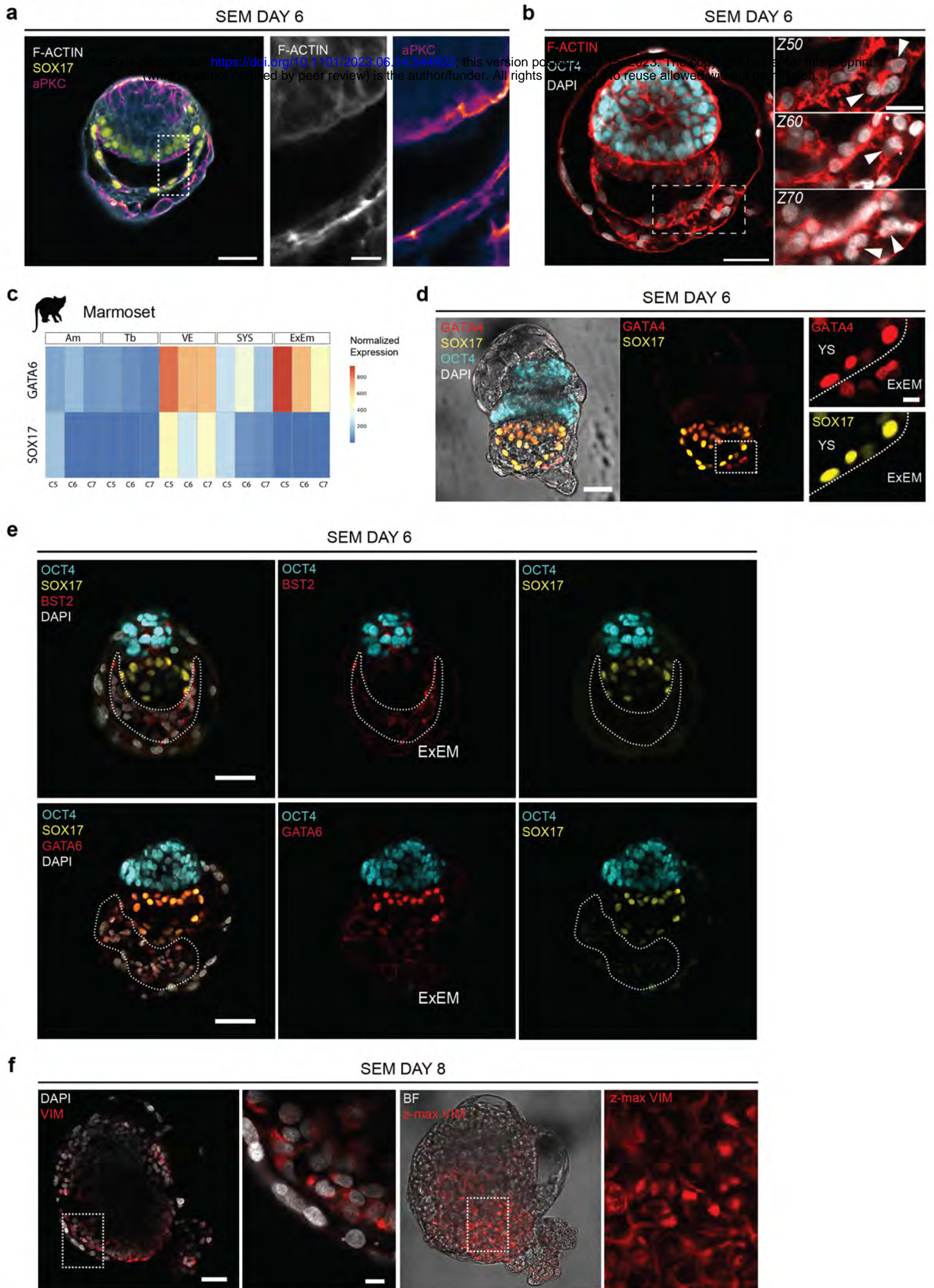
SEM DAY 8



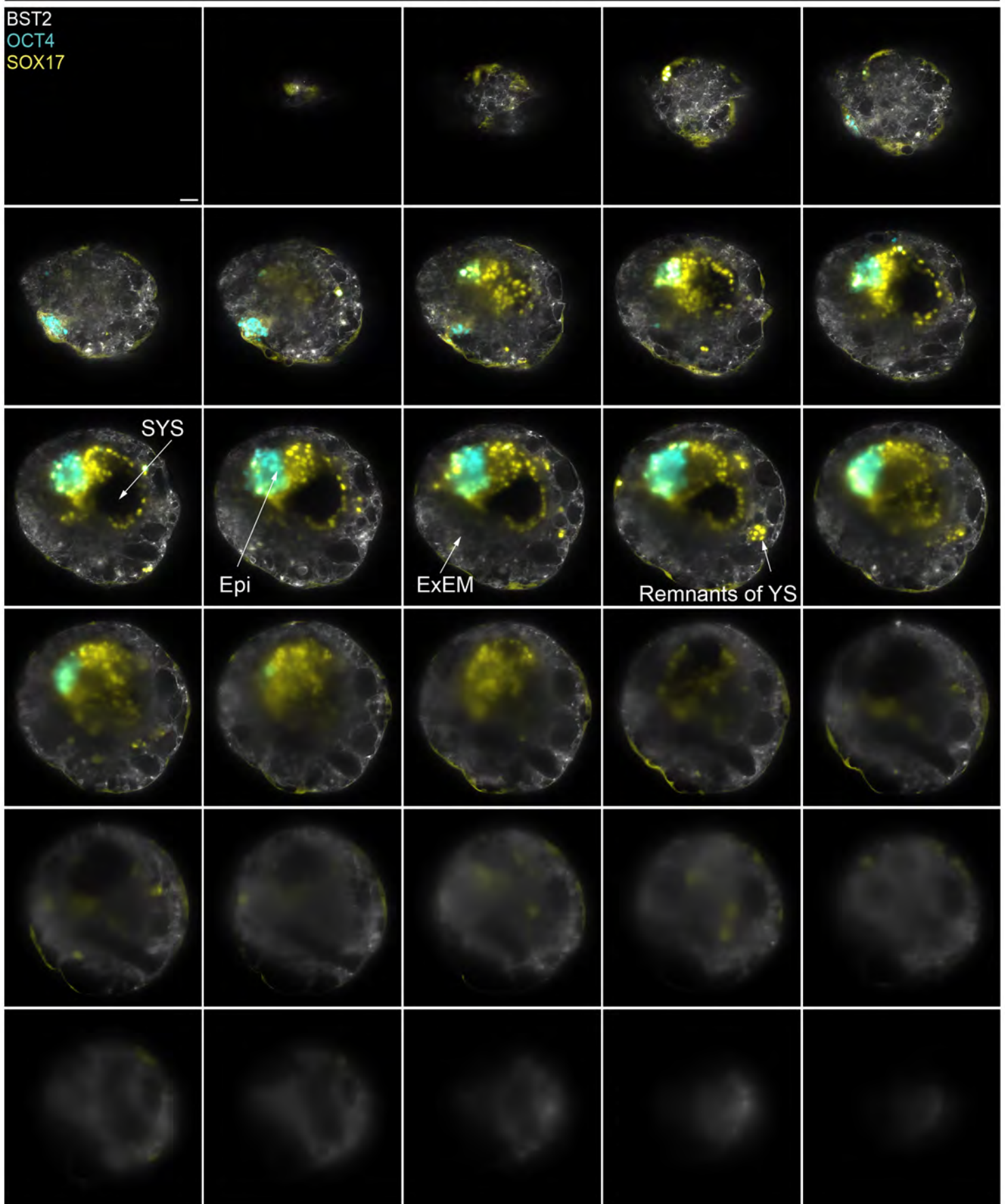
Extended Data Figure 15



Extended Data Figure 16

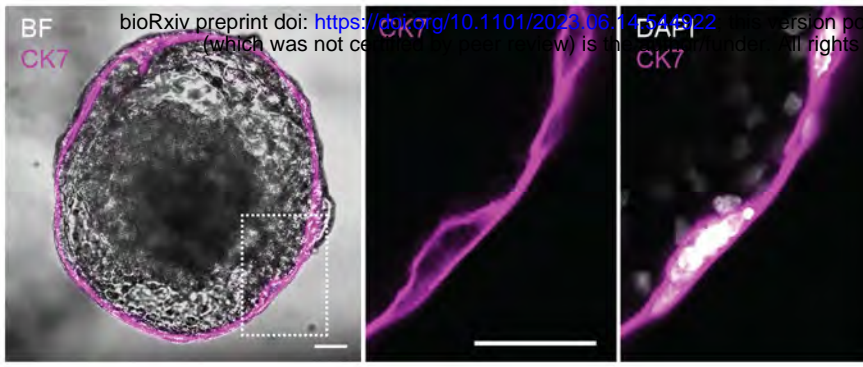


DAY 8 SEM

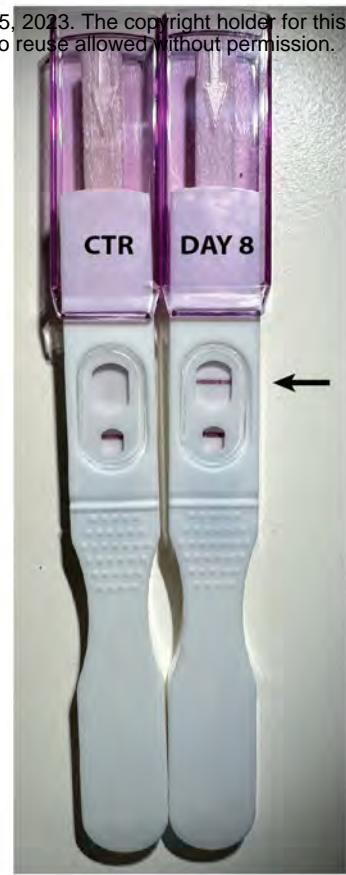


Extended Data Figure 18

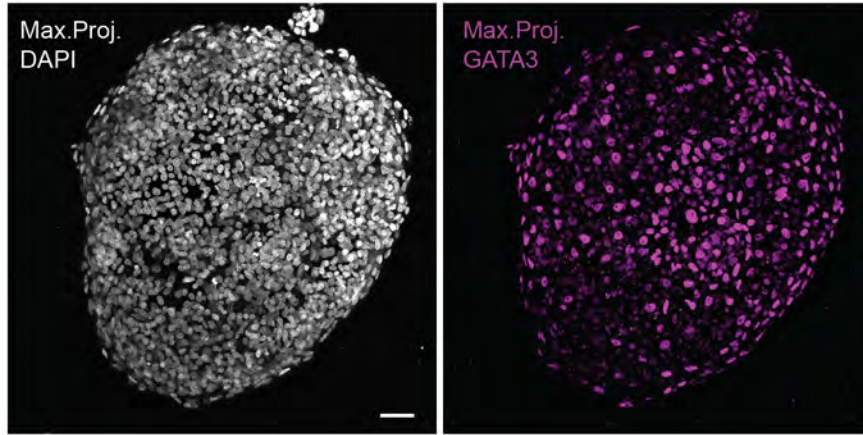
a SEM DAY 8



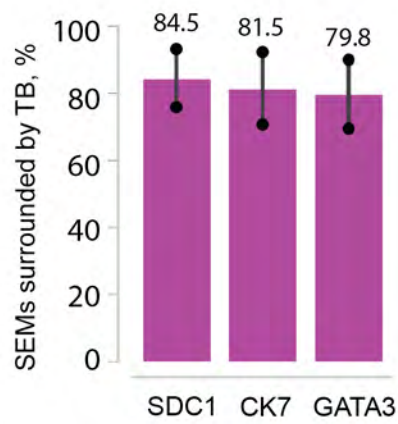
g



b SEM DAY 8

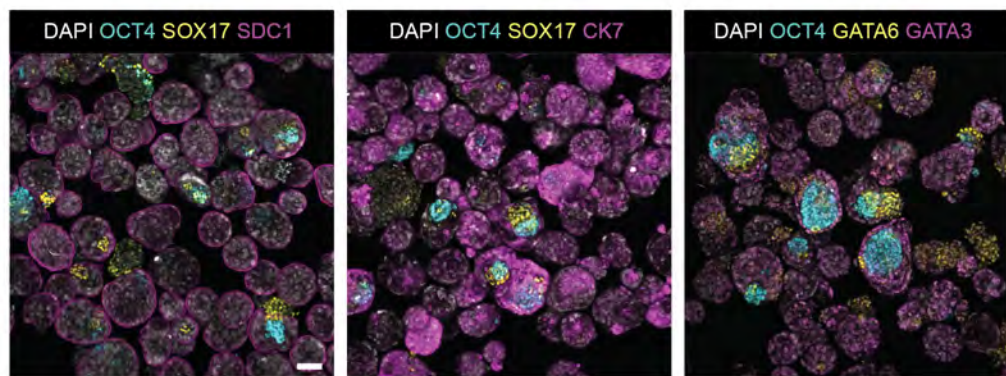


c



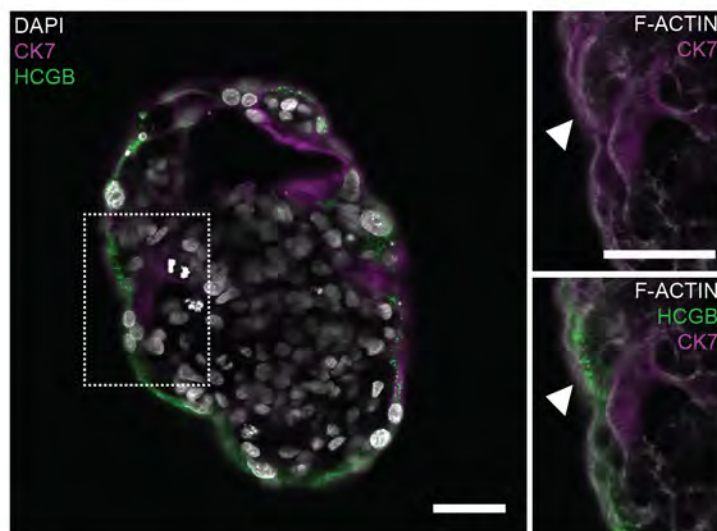
d

SEM DAY 6



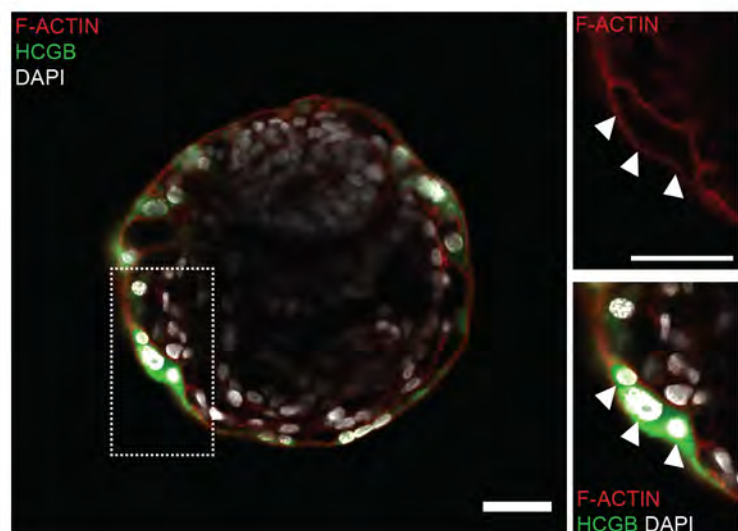
e

SEM DAY 6

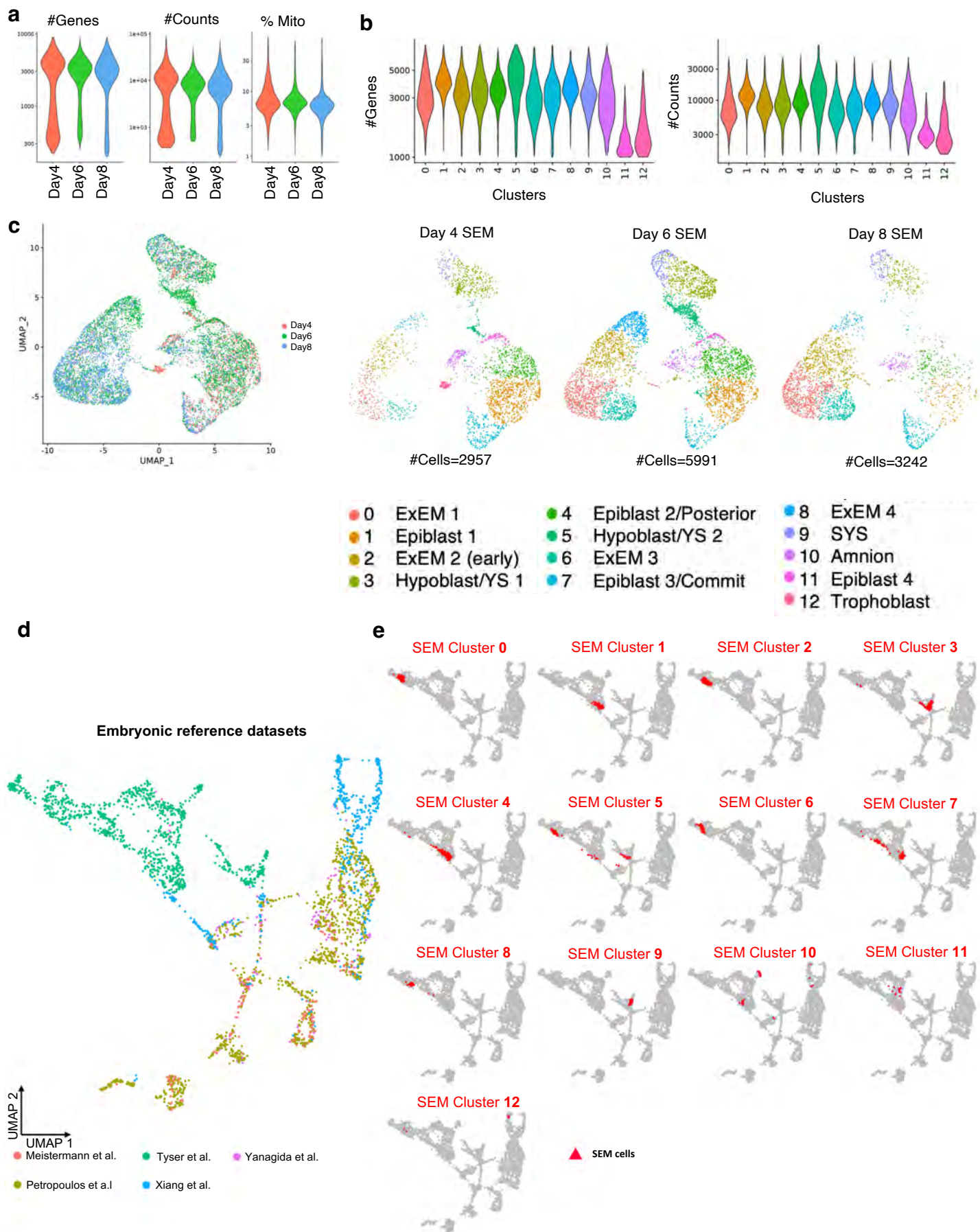


f

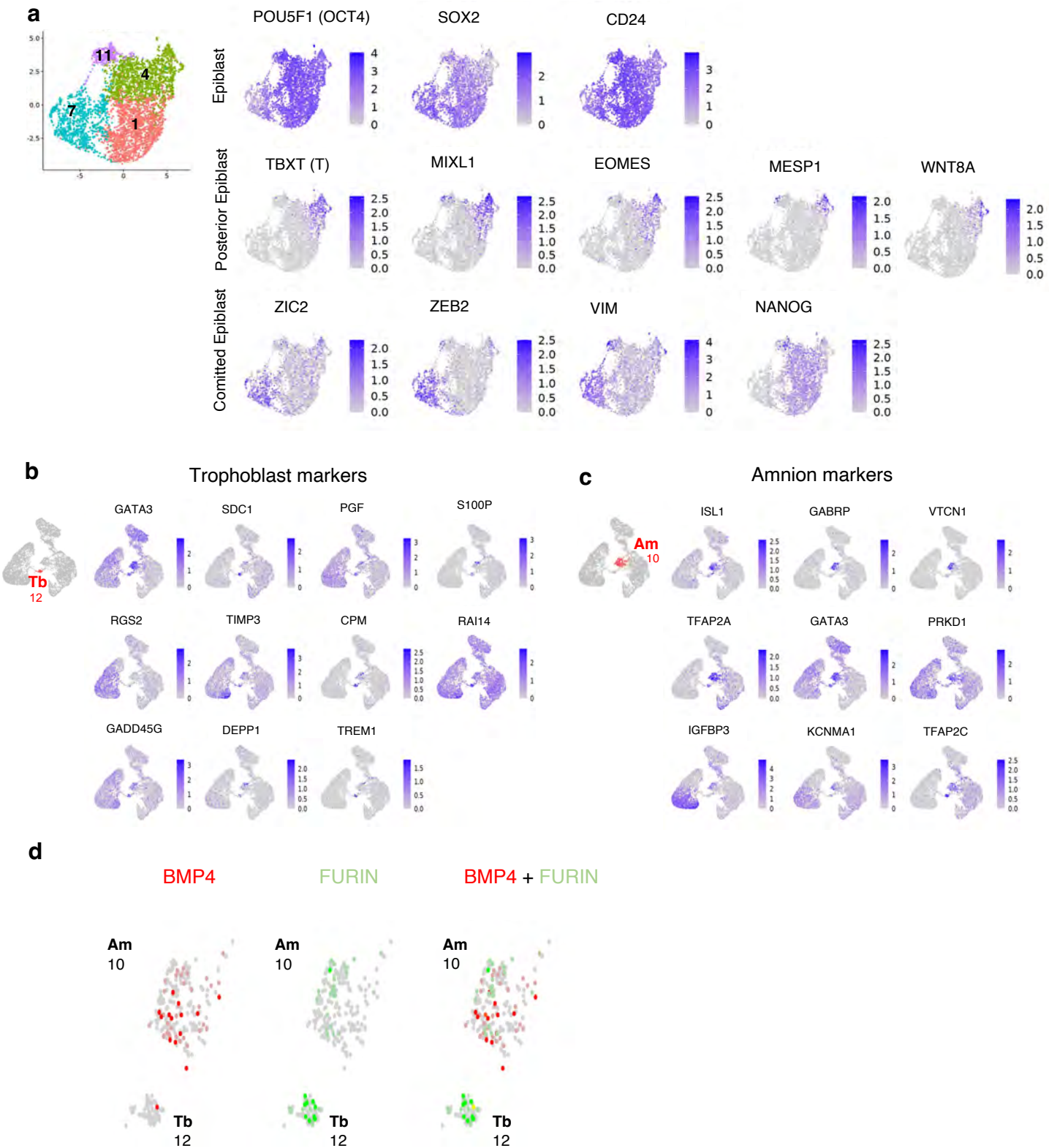
SEM DAY 6



Extended Data Figure 19



Extended Data Figure 20



Extended Data Figure 21

