In vitro generation of mouse polarised embryo-like structures from embryonic and trophoblast stem cells

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Key references:

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2) Bedzhov, I. and Zernicka-Goetz, M. (2014) 'Self-Organizing Properties of Mouse Pluripotent Cells Initiate Morphogenesis upon Implantation.', *Cell*. Elsevier Inc., 156(5), pp. 1032–44. doi: 10.1016/j.cell.2014.01.023.

EDITORIAL SUMMARY:

Directing assembly of extra-embryonic trophoblast stem cells with embryonic stem cells to recapitulate post-implantation mouse embryogenesis.

SUMMARY

Mammalian embryogenesis requires the coordination of embryonic and extra-embryonic tissues to enable implantation into the uterus and post-implantation development to establish the body plan. Mouse embryonic stem cells (ESCs) are a useful tool for studying pluripotent embryonic tissue in vitro. However, they cannot undertake correct embryogenesis alone. Many attempts to model the early embryo in vitro involve the aggregation of ESCs into spheroids of variable size and cell number that undertake germ-layer specification but fail to recapitulate the characteristic architecture and arrangement of tissues of the early embryo. Here, we describe a protocol to generate the first embryo-

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like structures by directing the assembly of mouse ESCs and extra-embryonic trophoblast stem cells (TSCs) in a 3D extracellular matrix (ECM) into structures we call 'polarized embryo-like structures'. By establishing the medium and culture conditions needed to support the growth of both stem cell types simultaneously, embryonic architecture is generated within 4 d of co-culture. This protocol can be performed by those proficient in standard ESC culture techniques and can be used in developmental studies to investigate the interactions between embryonic and extra-embryonic tissues during mammalian development.

INTRODUCTION

At implantation, the mouse embryo, known as a blastocyst, is made up of three distinct tissues, which are arranged in a specific configuration to develop into a structure known as the egg cylinder. One of these tissues is the pluripotent embryonic epiblast, which gives rise to the fetus and forms a cupshaped epithelium at the distal side of the conceptus. Abutting the epiblast is one of two extraembryonic tissues, the extra-embryonic ectoderm (ExE), which is derived from the trophectoderm and positioned at the proximal side of the embryo, giving rise to the fetal portion of the placenta. The third tissue, the visceral endoderm (VE), gives rise to the yolk sac of the embryo. This forms a monolayer of cells that envelops the other two tissue compartments. These extra-embryonic tissues not only nourish the embryo as it grows, they also provide essential signals to pattern the epiblast, ensuring that the body plan is established correctly. In this protocol, we describe a system to model the early development of the mouse embryo ex vivo that utilizes cells derived from both embryonic and extra-embryonic tissues to mimic in vivo embryogenesis.

Modelling embryogenesis with ESCs alone

Mouse embryonic stem cells (ESCs) can be derived from the pre-implantation blastocyst and are transcriptionally equivalent to the epiblast of the mouse embryo just before implantation, at embryonic day 4.5 (E4.5)[1]. These cells are a useful tool to investigate the early development of the pluripotent embryonic tissue in the first few days of embryogenesis *in vitro*. However, when allowed to differentiate in 2D adherent culture, these cells cannot undertake embryogenesis without the support of the *in vivo* environment. Instead, they differentiate into neural tissue[2] or can be coaxed into generating other lineages by the administration of growth factors to bias cell fate[3,4]. When cultured in 3D with extracellular matrix (ECM), mammalian ESCs and their reprogrammed counterparts, induced pluripotent stem cells have been shown to develop with time into organ-like structures including fully organised retina and brain-like tissue[5–9]. The generation of these structures, termed 'organoids', occurs through self-assembly/self-organisation of cells from an initially

homogenous pluripotent population. Whilst some organoid structures exhibit endogenous axial patterning[6,10], ESCs in culture fail to reproducibly establish body axes without exogenous signals and do not generate a structure resembling whole-embryonic architecture. Initial attempts to model early post-implantation mouse embryogenesis focussed on the use of ESCs alone to generate 3D aggregates of hundreds of cells. This technique leads to the formation of 'embryoid bodies' that can spontaneously induce domains of polarised gene expression[11]. Some of these structures have been termed 'gastruloids' as they sometimes initiate the regionalised expression of germ layer markers associated with the onset of gastrulation during embryogenesis[12,13]. However, these structures do not develop as embryos and, consequently, do not recapitulate the specific architecture of the embryo at post-implantation stages, nor do they represent the extra-embryonic lineages present in the mammalian embryo.

When embedded in ECM and when starting from individual cells, ESCs more faithfully mimic embryonic architecture. They develop to make a 'rosette-like' structure similar to the embryonic epiblast at the time of implantation[14]. Just as in the embryo, β1-integrin signalling mediated by laminin in the ECM leads to cell polarisation, which in turn underpins the formation of the ESC 'rosette'. At the centre of the rosette, a lumen opens analogous to the opening of the pro-amniotic cavity during embryogenesis[14].

Development of the protocol

Given that the embryo at early stages comprises extra-embryonic tissues in addition to the embryonic epiblast, the extra-embryonic ectoderm (ExE) and the visceral endoderm (VE) and these have been demonstrated to be important for establishing the body plan[15], we introduced trophoblast stem cells (TSCs) derived from the ExE of the embryo[16] into co-culture with ESCs embedded in ECM[17]. We also developed a medium to support the culture of ESCs and TSCs simultaneously, and used ECM as a substitute for the basement membrane secreted by the VE, which we showed previously to be critical for epiblast polarisation and lumenogenesis *in vivo*[14].

We demonstrated that ESCs and TSCs in this system could self-assemble into structures that mimicked embryo architecture within 4 days of culture[17]. These structures underwent similar morphogenesis to give rise to a cavity akin to the pro-amniotic cavity in the mouse egg cylinder, which is shared between the embryonic and extra-embryonic tissues. Despite the fact that only 22% of all structures formed comprised both ESCs and TSCs; when these 2 types of stem cell met, more than 90% of the structures developed a characteristic embryo-like morphology demonstrating the robustness of this model system[17]. Here we provide a step-by-step protocol for generating these

'polarised embryo-like structures', also called 'ETS-embryos' (ESCs and TSCs in culture), using custom developed media, ECM and optical plastic for high-resolution imaging of structures. This method demonstrates a new tool that can be used to investigate early mouse embryogenesis using a tractable stem cell model.

Potential applications of polarised embryo-like structures and comparison to other methods

We showed that our polarised embryo-like structures (ETS-embryos) recapitulate pro-amniotic cavity formation in the mouse embryo and therefore can be used to study how this cavity develops (Fig. 1g-h). They also recapitulate cell fate decisions and axial patterning events associated with early post-implantation development[17]. We showed that they can develop an area of regionalised mesoderm at the boundary between ESC and TSC compartments, which is analogous to the domain which marks the onset of gastrulation at the proximo-posterior side of the epiblast[18]. Opposite this region of mesoderm, cells acquire a more anterior-like fate, upregulating genes normally expressed in the anterior epiblast of the E6.5 embryo, such as *Pou3f1, Oct4*, *Slc7a3* and *Utf1* [17,19,20]. This indicates changes in the transcriptional program that reflects a gradient of cell fates across the embryonic compartment. Further development of these polarised embryo-like structures leads to specification of a small number of Primordial Germ Cell (PGC)-like cells. Amazingly, these PGC-like cells become specified in the correct spatial location, at the boundary between ESC and TSC compartments, within the T/Brachyury-positive domain [17]. We showed that this PGC-like cell specification occurs via BMP signalling, as it does in the embryo, and both induction of PGC and mesoderm markers in are preceded by the localised induction of Wnt[21,22]. Thus, polarised embryo-like structures mimic the spatial and temporal events of early mouse embryogenesis[17].

In addition to being a useful tool to study cavitation in the embryo, because polarised embryo-like structures can break symmetry and specify mesoderm in a regionalised manner, these structures can be used to study how mesoderm becomes specified. In comparison to embryoid bodies and the gastruloids derived from them, ETS-embryos stand out, in that both whole embryo architecture and germ layer specification occur as a result of the activity of endogenous signals provided by the TSC-derived extra-embryonic compartment. This is in contrast to coaxing ESCs in 3D aggregates to differentiate by supplementing the culture medium with growth factors such as recombinant Wnt or BMP[11–13]. In this respect, ETS embryos are useful for the study of how embryonic-and extra-embryonic tissues interact in the embryo to specify embryo architecture and the future body axes. Furthermore, the identity and role of endogenous signals in the these embryo-like structures may be deduced by adding inhibitors of known morphogens to the culture system, and monitoring the effects on mesoderm specification.

Embryoid bodies and gastruloids which are derived from them comprise only one cell type. As such, they may be useful in the study of how ESCs differentiate in response to the delivery of an exogenous signal. Moreover, although polarised embryo-like structures lack a VE layer, which is critical for natural embryo development, the fact that they are more simple than the real embryo but still comprise embryonic and extra-embryonic tissues aids investigation of the interaction between the epiblast and the ExE. This is because the absence of the VE means it cannot confound results by producing its own signals which affect embryogenesis.

In addition to being a valuable tool to investigate the patterning of the body axes, ETS embryos also have several other potential uses in developmental studies. This *in vitro* model of the mouse embryo can complement work *in vivo* as the amenability of ESCs and TSCs to genetic manipulation means that they can be used to generate embryo-like structures that are knockout for a particular gene of interest. This may be an efficient way to reveal novel functions of genes in early embryogenesis, and to perform molecular studies, without producing knockout animals which is both expensive and laborious. This approach may also aid in the identification and study of 'hidden' developmental phenotypes that might be masked in the mouse as maternally produced protein can compensate when the zygotic genome is compromised. Furthermore, the tissue-specific role of a gene can be easily probed in this system by knocking it out in either the embryonic or extra-embryonic cell line only. Whilst tissue-specific knockouts of mice may require the generation of a construct including a tissue specific promoter, this can be avoided using this embryo-like model as constitutive promoter systems can be introduced into each cell line independently.

Current limitations of the protocol and potential future directions

By mixing ESCs and TSCs in suspension and allowing 3D structures to grow, the ETS embryo culture is heterogeneous. On average, 22% of all structures in a single well are comprised of both stem cell types, whereas the majority of other structures comprise only ESCs and TSCs[17]. Despite this, by plating the recommended cell numbers, a yield of >100 polarised embryo-like structures can be achieved from a single experiment in a single 8-well μ-plate (Ibidi). This is more than one can analyse per day. We have not tried to optimise this method to generate embryo-like structures that would develop beyond early post-implantation stages. This may require culturing conditions allowing more efficient exchange of gas and nutrients to permit the growth of larger, more complex structures akin to embryos at later developmental stages. As polarised embryo-like structures are structurally robust enough to survive manual removal from the Matrigel after 4 days in culture, they could be maintained

in suspension using a rotary orbital shaker, or rotation culture using a spinning bioreactor[23]. Alternatively, a perfusion bioreactor could support structures remaining embedded in ECM.

In addition, the protocol in its current form yields embryo-like structures without a layer to represent the VE, which is the precursor to the yolk-sac. One improvement would be to establish a protocol to include extra-embryonic stem cells derived from the primitive endoderm ('XEN' cells) into co-culture. To do this, the medium composition described here may need to be altered to support the growth and proliferation of all three cell types. Furthermore, it has been shown that XEN cells in conventional culture are more equivalent to the primitive endoderm of the pre-implantation mouse embryo [24,25] but cells which are equivalent to the post-implantation VE lineage might be required. Several studies have shown that XEN cells can be coaxed to become more VE-like when treated with BMP [26,27], and so it may be that XEN cells would require 'priming' towards a VE-like state in this way before they can be used to generate VE tissue in our embryo-like structures. The use of Matrigel in the current protocol partially compensates for the VE, as this tissue is known to secrete the basement membrane essential for epiblast polarisation and lumenogenesis[14]. If VE-like cells were to be incorporated into our embryo-like structures, it is likely that these cells will secrete ECM components, thus providing the culture with exogenous sources of ECM.

Experimental design

Medium optimisation. Our approach is based on that developed by Bedzhov & Zernicka-Goetz[14] in which epiblast architecture is mimicked using ESCs in ECM by introducing TSCs into the system. To develop a medium that would support both types of stem cells in these conditions, we began by mixing 25% Neurobasal A, 25% DMEM/F-12 (Gibco Life-Technologies) and 50% MegaCell RPMI medium (Sigma) (vol/vol). We found that medium based on one of these components alone was not sufficient to support the growth of both cell types simultaneously in culture, and that the basal composition that would support ESC and TSC growth at the same time required RPMI medium, which is usually used for conventional TSC culture, to be mixed with Neurobasal-A and DMEM-F/12 media, which is used to make N2B27 for conventional ESC culture. If one or several of these basal components were missing, one or both cell types failed to grow and/or began to differentiate.

The medium we developed also included extra-nutrients to promote cell growth and survival in culture, including L-glutamine and sodium pyruvate (Gibco Life-Technologies). We supplemented this medium with FGF4 and heparin, to promote TSC self-renewal *in vitro*, but excluded the inhibitors PD0325901, CHIR99021, and Leukaemia Inhibitory Factor (LIF), which maintain ground state pluripotency in ESCs[28]. This was because we wanted ESCs to be able to differentiate in co-culture. This medium also included 10% (vol/vol) FBS. Serum contains undefined factors which promote cell

proliferation and differentiation, and is required for mouse blastocysts to develop into egg cylinders *in vitro*[29,30]. We hypothesised that serum would provide essential signalling components to ESCs and TSCs in co-culture that might promote self-assembly of embryo-like structures, but we kept serum content low to minimise the possibility of precocious differentiation. If higher concentrations of serum were added to the culture, such as 20% or 30%, ETS-embryo morphology was compromised and the structures failed to develop a cavity at their centre.

By keeping the serum concentration low in the medium, we found that this medium composition supported the proliferation and formation of rosette-like structures of both ESCs and TSCs in Matrigel, which later self-assembled into 3D structures that resembled the egg cylinder (Fig. 1). These structures more faithfully mimicked the structure of the embryo than embryoid bodies and had similar cell number and tissue volume to the mouse egg cylinder[17].

Cells. The use of different cell lines does not affect the efficiency of ETS embryo generation. We have routinely used transgenic reporter ESCs in the co-culture system to report different aspects of ETS-embryo development including mesoderm specification[31], PGC-like cell specification[32] or to demarcate the ESC compartment by possession of fluorescent membranes[33] (STEP 1). Cells can also be derived de novo from a transgenic mouse line-of-interest and used in this protocol. Additional information on the derivation and culture techniques of stem cells from the mouse embryo can be found in: 'Manipulating the Mouse Embryo: a Laboratory Manual'[34]. Notably, ESCs and TSCs can still form embryo-like structures even when derived from different mouse strains.

ECM support. We recommend the use of Growth Factor Reduced Matrigel Matrix, (STEP 13) however every batch of matrix must be individually tested in the system to ensure reproducibility. Matrigel lots and batches can be tested before ETS-embryo generation by embedding ESCs and TSCs separately and culturing for 72h to ensure the cells grow as expected (STEPS 14-15). For ESCs embedded in Matrigel, a single lumen should develop at the centre of the structure within 48h[14] and the cells should express the epiblast markers Sox2 or Otx2. TSCs embedded in Matrigel should continue to express ExE markers such as Eomes or Tfap2C as described below (Fig.2).

Imaging. ETS-embryos can be fixed, stained and imaged *in situ* at any time-point after plating, (STEPS 16-22) but mesodermal lineages appear only after 4-5 days in culture (Fig.2) (see ANTICIPATED RESULTS). If live-imaging is required, we recommend plating all structures in a single plane using the '3D on top' method (see BOX 1). The '3D on top' method can be used with various dish sizes, depending on the requirements of the experiment. Plating volumes for example dishes (Mattek Glass Bottomed culture dishes and Ibidi angiogenesis μ-plates) are included in Supplementary Table 1.

MATERIALS

REAGENTS

- Mitotically inactivated mouse embryonic fibroblasts (MEFs) ready to use (R&D systems, PSC001) or prepared from mouse embryos as described in **Box 1.**
- Mouse trophoblast stem cells (derived as described in **Box 2**).
- Mouse ESCs derived from the mouse embryo at blastocyst stages (as described in **Box 3**) or obtained commercially from suppliers such as The Jackson Laboratory.
- DMEM/F-12 (Gibco- Life Technologies, Cat No 21331-020)
- DMEM (+ L-glutamine) (Gibco- Life Technologies Cat. No. 41966052)
- Neurobasal A liquid (Gibco- Life Technologies Cat. No. 10888-022)
- MegaCell RPMI-1640 medium (Sigma Aldrich Cat. No. M3817)
- N2 supplement (Made as described in RSU)

CRITICAL: Home-made N2 supplement yields better resuts than commercial N2 (Thermo Fisher Scientific Cat. No. 17502048). The use of some brands of commercial N2 can result in ETS-embryos with smaller or no cavities. Before using commercial N2 in this protocol, batch test the commercial product by using it to make N2B27 medium (described in 'Reagent setup') and use this to culture ESCs embedded in Matrigel. ESC cysts should make lumens within 48h.

- Commercial ETS-embryo Medium (Cell Guidance Systems Cat. No. M13-25)
- Human Apo-tansferrin, cell culture tested (Sigma Aldrich Cat. No. T1147-100MG)
- Bovine Albumin Fraction V (7.5% solution, (wt/vol)) (Gibco- Life Technologies Cat. No. 15260-037)
- Chemically defined Human insulin solution (Sigma Cat. No. I9278-5ML)
- Putrescine dihydrochloride powder, cell culture tested (Sigma Aldrich Cat. No. P5780-5G)
- Sodium selenite powder, cell culture tested (Sigma Aldrich Cat. No. S5261-10G)
- Progesterone powder, cell culture tested (Sigma Aldrich Cat. No. P8783-1G)
- B27 supplement minus AO (Gibco- Life Technologies Cat. No. 10889-038)
- Embryonic stem cell grade foetal bovine serum (FBS) (Gibco- Life Technologies Cat. No. 10439024)

  CRITICAL: FBS must be tested in ESC culture before use in the ETS embryo system. To do this, make up FC2iLIF medium (described in 'Reagent setup') and culture ESCs in adherent culture on gelatin in this medium for two weeks. Assess ESC growth and proliferation during this time, colony morphology, and expression of pluripotency markers. If the FBS is of appropriate quality, then ESCs should become confluent within 48h of culture, and colonies should be rounded and raised, with tightly packed cells. Expression of pluripotency markers can be

tested by immunofluorescence staining to detect expression of Oct4, Nanog, and Sox2 transcription factors, which should be localised to the nucleus. To check for differentiation, cells can also be immunostained for T/Brachyury protein, which should not be expressed in naïve pluripotent ESCs. The gold standard test for naïve pluripotent ESCs is to perform a chimera assay in which ESCs are introduced into the pre-implantation embryo and their contribution is checked. This is recommended for cell-lines which have been derived but not previously validated in this way.

- L-glutamine (Gibco- Life Technologies Cat. No. 25030-024) or Glutamax (Gibco- Life Technologies Cat. No. 35050-038)

- 2-Mercaptoethanol (2 ME) solution (50 mM) (Gibco- Life Technologies Cat. No. 31350-010) CAUTION: 2-Mercaptoethanol is toxic. Use personal protective equipment and discard waste appropriately.

- MEM Non-Essential Amino Acid Solution (NEAA) (Gibco- Life Technologies Cat. No. 11140035)

- Sodium pyruvate 100mM solution (Gibco-Life Technologies Cat. No. 11360039)

- Penicillin-streptomycin solution (Gibco-Life Technologies Cat. No. 15140122)

- PD0325901 (Stemgent, 04-0006)

- CHIR99021 (Stemgent, Cat. No. 04-0004)

- Recombinant mouse Leukaemia Inhibitory Factor (LIF) (Stemfactor, Cat. No. 03-0011)

- Recombinant Human Fibroblast Growth Factor 4 (FGF-4) (Peprotech Cat. No. 100-31)

- Heparin (Sigma Cat. No. H3149)

- Sterile Phosphate Buffered Saline (PBS) (Gibco-Life Technologies Cat. No. 10010056)

- Trypsin-EDTA (Gibco-Life Technologies Cat. No. 25300054)

- Growth Factor Reduced Matrigel matrix (BD, Corning Cat. No. 356234)

- Autoclaved water

- Ethanol solution (Sigma Aldrich Cat. No. 02860-4x25ml)

- Gelatin from cold water fish skin (Sigma Aldrich Cat. No. G7765)

- Bovine serum albumin (BSA) (Sigma Aldrich Cat. No. A3311-50G)

- Triton-X-100 (Sigma Aldrich Cat. No. T9284-100ML)

- 16% Paraformaldehyde (Electron Microscopy Sciences, Cat. No. 15710) CAUTION: Paraformaldehyde is toxic. Use personal protective equipment and discard waste appropriately.

- Antibodies specific to Tfap2C (1:200; Santa Cruz, Cat. No. sc-8977), Brachyury/T (1:400; R&D systems, Cat. No.AF2085), anti-GFP (1:1000; Nacalai Biochemicals, cat. No. 04404-84) Eomes

(1:400; Abcam, Cat. No. ab183991), Sox2 (1:200; Santa Crux, Cat. No. sc-365823), Otx2 (1:200; R&D systems, Cat. No. AF1979).

- Fluorophore conjugated secondary antibodies (1:500): donkey anti-goat Alexa Fluor 488 (Life Technologies, Thermofisher Scientific A11055), donkey anti-goat Alexa Fluor 568 (Life Technologies, Thermofisher Scientific A21447), donkey anti-rat Alexa Fluor 488 (Life Technologies, Thermofisher Scientific A21208), donkey anti-mouse Alexa Fluor 568 (Life Technologies, Thermofisher Scientific A10037), donkey anti-rabbit Alexa Fluor 647 (Life Technologies, Thermofisher Scientific A31573).

EQUIPMENT

- ibiTreat µ-plates, 8-well (Ibidi, Cat. No. 80826)
- Angiogenesis µ-plate (Ibidi, Cat. No. IB-81506)
- Glass-bottomed –culture dish (MatTek, Cat. No. P35G-1.5-14-C)
- Nunclon Tissue culture plates, six well, 12 well (Thermofisher Scientific Cat No. 140675 & 150628)
- Sterile Syringe Filters CA 0.2µm, 33mm (Gilson cat. No. ALG422A)
- Nikon inverted microscope for tissue culture
- Set of laboratory pipettes for tissue culture work
- P1000, P200, P20, P10, P2 filter pipette tips
- CO_2 incubator (New Brunswick, Galaxy 170R) set at 5% CO_2 and 37°C.
- Biological safety cabinet (Nuaire Cell Gard Class II)
- Vacuum pump (Integra Vacusafe)
- Ice and ice bucket
- Sterile 1.5 ml Eppendorf safelock tubes (Fisher Scientific Cat. No. 0030120086)
- Sterile 15ml falcon tubes (Corning 430791)

REAGENT SETUP

CRITICAL: All tissue culture reagents must be kept sterile and prepared in a Biological safety cabinet suitable for tissue culture (see EQUIPMENT).

FBS

Heat-inactivate the FBS by incubation for 30 minutes in a water bath at 56°C. Filter-aliquot the inactivated serum using sterile syringe filters (0.2µm, 33mm) and store in 40ml volumes at -20°C for up to six months.

Apo-transferrin solution

To prepare a stock apo-transferrin solution, dissolve 100mg of powder in 10ml of sterile autoclaved water. Spin the solution at 1100g for 3 minutes at room temperature, then transfer to a fresh sterile 1.5 ml Eppendorf tube. Filter-aliquot into 1ml volumes and store at -20°C for up to eight weeks.

Progesterone

To prepare a stock progesterone solution, dissolve 0.006g of powder in 10ml of Ethanol. Filter-aliquot this solution into sterile 1.5 ml Eppendorf tubes and cover lids of tubes with parafilm to prevent evaporation. Store at -20°C for up to six months.

Sodium selenite

To prepare a stock sodium selenite solution, dissolve 0.006g of powder in 10ml of sterile autoclaved water. Filter-aliquot this solution into sterile 1.5 ml Eppendorf tubes and store at -20°C for up to six months.

Putrescine dihydrochloride

To prepare a stock putrescine dihydrochloride solution, dissolve 0.8g of powder in 5ml of sterile autoclaved water. Filter-aliquot this solution into sterile 1.5 ml Eppendorf tubes and store at -20°C for up to six months.

FGF-4 solution reconstitution

Reconstitute the lipophilized FGF-4 according to the manufacturer's instructions. Prepare a 1000x (25ug/ml) stock solution by dilution using PBS 0.1% BSA (wt/vol). Aliquot into sterile 1.5 ml Eppendorf tubes and store at -80°C for up to eight weeks. Do not re-freeze after thawing.

Heparin solution reconstitution

Prepare a 1000x (1mg/ml) stock solution (wt/vol) of heparin solution by dissolving 10mg heparin in 10ml of PBS. Aliquot into sterile 1.5ml Eppendorf tubes and store at -80°C for up to six months. Do not re-freeze after thawing.

Gelatin solution

To prepare a 0.1% gelatin solution (wt/vol) dissolve 1g of gelatin in 100ml of water and autoclave the solution. Store at 4°C for 1 month.

Feeder Cell 'FC' Medium

Prepare a solution comprising the following: DMEM supplemented with 15% FBS (vol/vol), 2mM L-glutamine, 0.1mM 2-ME, 0.1mM NEAA, 1mM sodium pyruvate, and 1% penicillin-streptomycin (vol/vol). Store at 4°C and use within 1 month.

FC 2iLIF Medium

Supplement the FC medium solution with PD0325901 (1μM), CHIR99021 (3μM) and LIF (0.1mM). Store at 4°C and use within 1 week.

N2 supplement

Prepare a solution comprising the following: DMEM/F-12 supplemented with 10% human apo-transferrin solution (vol/vol), 10% Bovine Albumin Fraction V (vol/vol), 25% human insulin solution, putrescine dihydrochloride (1611 μg/ml), sodium selenite (0.6 μg/ml) and progesterone (0.6 μg/ml). Filter-aliquot this solution into sterile 0.5 ml Eppendorf tubes and store at -20°C for up to 1 month. Avoid repeated freeze-thaw cycles.

N2B27

Prepare a solution comprising the following: 50% DMEM/F-12 (vol/vol) and 50% Neurobasal A (vol/vol), supplemented with 2mM L-glutamine, 0.1mM 2ME, 0.5x N2 supplement, 1x B27 supplement. Store at 4°C and use within 2 weeks.

N2B27 2iLIF

Supplement the N2B27 solution with PD0325901 (1μM), CHIR99021 (3μM) and LIF (0.1mM). Store at 4°C and use within 1 week.

ETS-embryo medium

Prepare a solution comprising the following: 40% 'MegaCell' RPMI (vol/vol), 25% DMEM/F-12 (vol/vol) and 25% Neurobasal A (vol/vol) supplemented with 10% FBS (vol/vol), 2mM L-glutamine, 0.1mM 2ME, 0.5mM sodium pyruvate, 0.25x N2 supplement (made in house), 0.5x B27 supplement, plus 12.5 ng/ml FGF4 and 500ng/ml heparin. Also add 1% penicillin-streptomycin (vol/vol). Store at 4°C and use within 1 week. Alternatively, the complete formulation can be purchased as a ready-to-use formula from Cell Guidance Systems, (Cat. No: M13-25)

TS cell medium

Prepare a solution comprising the following: 'MegaCell' RPMI with 20% FBS (vol/vol), 2mM L-glutamine, 0.1mM 2-ME, 1mM sodium pyruvate, and 1% penicillin-streptomycin (vol/vol). Store at 4°C and use within 1 month.

TSF4H medium

Supplement the TS cell medium solution with 25ng/ml of recombinant FGF4 and 500ng/ml of heparin. Store at 4°C and use within 1 week.

4% PFA solution

Dilute the 16% PFA solution four times in PBS to produce 4% PFA (vol/vol). Store at 4°C and use within 1 week.

Blocking buffer for antibody incubations

Prepare a solution of PBS with 3% BSA (wt/vol). Store at 4°C and use within 1 week.

Box 1: Preparation of mitotically inactivated mouse embryonic fibroblasts (MEFs)

A brief procedure follows, more detailed instructions can be be found in Nagy et al.[35].

Additional materials required:

CBA/C57Bl6 (F1) mice

CAUTION: Experiments using mice must conform to Institutional and National regulations. We obtained approval to use the procedure below from Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

Mitomycin-C (Sigma-Aldrich Cat. No. M4287)

DNaseI (New England Biolabs Cat. No. M0303S)

Procedure:

i) Mate CBA/C57Bl6 (F1) mice and sacrifice pregnant females after 13 or 14 days post coitum.
CRITICAL: Carry out all dissection steps in a tissue culture hood under aseptic conditions using sterile instruments.

ii) Dissect out uterine horns in sterile PBS, and remove embryos using fine forceps.

iii) Dissect away the brain and red organs from each embryo, then mince the tissue using a razor blade until it can be pipetted.

iv) Digest the minced tissue using 0.05% trypsin-EDTA and 100ul of DNaseI per embryo, and incubate for 15 minutes at 37°C.

v) Inactivate the trypsin using freshly prepared FC medium, and transfer the cell-suspension to a falcon tube.

vi) Centrifuge at 300g for 5 min, then subsequently remove the supernatant and resuspend the cells in fresh FC medium.

vii) Plate the cells on gelatine coated tissue culture dishes and culture overnight at 5% CO_2 and 37°C.

viii) Change the medium the following day to remove dead and unattached cells.

ix) Expand the cells until confluent, and then inactivate using Mitomycin-C at 10μg/ml suspended in PBS.

PAUSEPOINT: Once inactivated, MEFs can be kept in the incubator for up to 2 weeks until needed, if medium is changed regularly.

END BOX 1

Box 2: Derivation of Trophoblast stem cells (TSCs)

Trophoblast stem cells can be derived from the mouse embryo at blastocyst or early post-implantation stages. Steps for derivation from pre-implantation blastocysts are outlined below, but more detailed protocols are also available[36].

Additional materials required

Mice of the desired strain. We have used CBA x C57Bl/6 (F1 mice) and transgenic mice of mixed genetic background. Please note that ESCs and TSCs do not need to be derived from the same mouse strain to form ETS-embryos. Experiments using mice must conform to Institutional and National regulations. We obtained approval for these experiments from The Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

M2 medium (Sigma, M7167)

Procedure:

i)   Before starting, prepare 'conditioned medium' by culturing MEFs in TS medium for 3 days. Collect the medium and store for use during derivation.

ii)   Plate MEFs on 4-well tissue culture plates at a density of 4×10^4 cells per well and culture in TS cell medium overnight at 5% $CO2$ and 37°C.

iii)   Mate mice and sacrifice pregnant females after 4.5 days post coitum to obtain pre-implantation blastocysts.

iv)   Dissect out the uterus, flush uterine horns using M2 medium, and collect the blastocyst stage embryos.

v)   Replace the TS cell medium on the MEFs with TSF4H medium and seed one blastocyst per well in the four-well plates covered with MEFs.

vi)   Culture overnight at 5% $CO2$ and 37°C, and allow blastocysts to attach to the MEFs and form outgrowths.

vii)   Replace the medium each day.

viii)   Once the outgrowths have expanded, aspirate the medium and wash with PBS, then dissociate the outgrowths using 0.05% trypsin-EDTA. Incubate for 10 minutes at 5% CO_2 and 37°C.

ix)   Inactivate the trypsin using 70% conditioned medium, plus 1.5x TSF4H (TS medium containing 37.5 ng/ml FGF4 and 1.5 µg/ml heparin) medium then disaggregate the outgrowths by pipetting up and down.

x)   Incubate at 5% CO_2 and 37°C, and change the medium every other day until TSC colonies begin to appear. The number of days for cells to appear will vary (usually from 6-10 days), but epithelial colonies with clear boundaries should form. Cells other than TSCs may also appear, in which case, pick the colonies of TSCs using a pipette and re-plate in new four well-plates coated with MEFs and culture at 5% CO_2 and 37°C in TSF4H medium.

xi)   Continue to expand the TSCs. On average, cells need to be passaged every 3-4 days, or when wells are 80% confluent. Cultures should be split at a ratio of 1:10. To passage TSCs: incubate with 0.05% trypsin- EDTA for 3-5 minutes; neutralise with an equal volume of TSC medium; gently disaggregate cells with a pipette; spin down cells (1000g for 3 minutes); resuspend in TSC medium; and plate on a fresh plate of mitotically inactivated MEFs. [16]

xii)   TSCs can be cultured longer term on MEFs or can be cultured in MEF-free conditions on gelatin-coated tissue culture plates by keeping the cells in MEF-conditioned TS cell medium (more details can be found in Tanaka, 2006[36] or Rossant, 2006[37]) As a third alternative, TS cells can also be maintained in completely defined conditions detailed protocols to maintain TSCs in this way have been developed by Kubaczka et al, 2014[38] and by Ohinata et al, 2014[39]. TSCs maintained in all conditions are suitable for generating ETS-embryos.

        PAUSEPOINT TSCs can be frozen for storage by freezing at -80°C in TS freezing medium (10% DMSO, 70% TS cell medium, 20% FBS)

END BOX 2

Box 3: Derivation of ESCs

This is briefly described below. More detailed protocols for how to derive ESCs from mouse embryos are available should further guidance be required [34].

Additional materials required:

Female Mice of the desired strain. To date, we have used 129 ESCs, E14 ESCs, and have derived ESCs from transgenic mice of a mixed genetic background. All these strains are suitable to generate ETS-embryos. Experiments using mice must conform to Institutional and National regulations. We obtained approval for these experiments from the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

Procedure:

i)   Recover blastocysts from pregnant female mice and seed on inactivated MEF feeder cells in FC2iLIF medium.

ii)   Culture overnight at 5% CO2 and 37°C.

iii)   Allow the blastocysts to hatch, attach to the MEFs and form outgrowths. This usually takes 48 hours.

iv)   Once the outgrowths have expanded, dissociate these by washing with PBS and incubating with 0.05% Trypsin-EDTA for 10 minutes.

v)   Inactivate the trypsin with fresh FC2iLIF medium and disaggregate the outgrowths by pipetting up and down.

vi)   Culture at 5% CO2 and 37°C, changing the medium every other day. ESC colonies should appear within 1 week of culture.

PAUSEPOINT: ESCs can be frozen for storage by freezing at -80°C in FC freezing medium (10% DMSO, 70% FC medium, 20% FBS)

END BOX 3

PROCEDURE

Cell culture TIMING- minimum of 1 week

CAUTION: The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

1) Establish both TSC and ESC cultures by carrying out options A and B. Manage ESC and TSC culture and passaging so that passaging-days become synchronised. MEF Feeder cells need not be removed to begin the ETS-embryo experiment. Feeder-free conditions are preferable for ESC culture, but MEFs will not contaminate the culture if they are still present when you start the experiment. When ESCs and TSCs are confluent (80%) on the same day, proceed to the next step to generate ETS embryos.
    A) TSC culture

i. Plate mitotically inactivated mouse embryonic fibroblasts (MEFs) in a six-well tissue culture plate at half the density normally used for ESC culture (1×10^5 cells/ml). Culture in FC medium at 5% CO2 and 37°C for at least 24 hours, to allow the MEFs to attach to the plate.

ii. Thaw a vial of frozen TSCs as quickly as possible and re-suspend in TSF4H medium for culture. Remove the FC medium from the MEF plate and seed the TSC suspension on top of the MEFs (Typically, ~1-3× 10^5 TSCs can be plated per well of a six-well plate).

iii. Culture overnight at 5% CO2 and 37°C to allow the TSCs to attach, then replace the medium with fresh TSF4H medium.

? TROUBLESHOOTING

iv. Continue to culture the cells, changing the culture medium every two days. Passage the cells as described in Box 2. CRITICAL STEP TSCs can be passaged up to ~20 times for use in this protocol. Cells which have been passed a large number of times may have developed chromosomal abnormalities and should not be used in the culture.

v. Proceed to step 2 when you have plates of TSC and ESC cells (from option B) that are both 80% confluent.

B) ESC culture

i. Cover the wells of a 12-well tissue culture plate with 0.01% gelatin (wt/vol) solution overnight.

ii. Thaw a vial of frozen ESCs as quickly as possible and re-suspend in the medium in which the cells were derived (FC2iLIF or N2B27 2iLIF).

? TROUBLESHOOTING

iii. Remove the excess gelatin from one well of the 12-well plate and seed the ESCs. Typically, ~1.5×10^5 ESCs can be plated per well of the 12-well plate. Incubate the ESCs overnight to attach, then replace the medium with fresh FC2iLIF or N2B272iLIF.

iv. Culture cells at 5% CO2 and 37°C, changing the medium every other day, and inspecting the cultures each day to ensure cells are heathy. Healthy ESCs should form domed, bright colonies with clear boundaries. Passage the ESCs every two days, or when the well reaches confluency. To passage cells: incubate with 0.05% trypsin-EDTA for a maximum of 5 minutes; neutralise with an equal volume of FC medium ; gently disaggregate cells with a pipette; spin down cells (1000g for 5 minutes); resuspend in FC2iLIF or N2B272iLIF; and plate on a fresh gelatin coated plate[28]

CRITICAL STEP ESCs can be passaged up to ~20 times for use in this protocol. Cells which have been passed a large number of times may have developed chromosomal abnormalities and should not be used in the culture.

vi. Proceed to step 2 when you have plates of ESC and TSC cells (from option A) that are both 80% confluent.

3D embedding of ESCs and TSCs TIMING ~ 1 hour

CRITICAL To minimise time out-of-the-incubator, it is recommended to process ESCs and TSCs in parallel (steps 2-7). This can be done by a single researcher however if this is not possible, then it is recommended to trypsinize and re-suspend the ESCs first, then keep them in PBS under the tissue culture hood whilst working with the TSCs.

CRITICAL If it is useful to have all the cells in the same plane (for example when imaging multiple structures) a '3D on top' approach can be used, as described in Box 1.

2) Wash ESCs from step 1 with Phosphate Buffered Saline (PBS) and treat with 0.05% trypsin-EDTA for 3 – 4 minutes at 37°C.

3) Inactivate the trypsin using FC medium to obtain a single-cell suspension, and collect in a 15ml falcon tube.

   CRITICAL STEP: ESCs should be dissociated to single cells by pipetting. The ESCs can be left in suspension at room temperature under the tissue culture hood whilst working with the TSCs.

4) Wash TSCs from step 1 with PBS and then treat with 0.05% trypsin-EDTA for 4 minutes at 37°C.

5) Inactivate the trypsin using TS medium to obtain small clumps of TSCs in suspension. Collect the suspension in a 15ml falcon tube.

   CRITICAL STEP: TSCs should be pipetted gently 2-3 times using a wide pipette tip just as a P1000 to preserve small clumps.

   ? TROUBLESHOOTING

6) If two centrifuges are available, one centrifuge can be used to work with ESCs while another can be used for TSCs in parallel. Centrifuge ESCs for 5 minutes at 1000g at room temperature. and centrifuge the TSC suspension for 3 – 4 minutes at 1000g at room temperature.

7) After centrifugation of ESCs, resuspend the cells in PBS and centrifuge a second time to 'wash' the PD0325901, CHIR99021 and LIF from the ESCs.

   CRITICAL STEP: Washing of ESCs is important to remove 2iLIF.

8) **After centrifugation of TSCs,** remove the supernatant, and resuspend the pellet gently in RPMI/FBS media.

   CRITICAL STEP The handling of TSCs should be gentle to preserve small clumps of 2 to 8 cells. The size and number of cells in TSC clumps can be checked under the tissue-culture

microscope at each stage of the procedure. Over-washing can reduce viability of TSCs, so avoid this.

9) After the second centrifugation of ESCs is complete, resuspend once again in PBS and centrifuge a third time. TSCs can be left at room temperature in the tissue culture hood while ESCs are centrifuged for the third time.

10) After the third centrifugation of ESC, remove the supernatant and resuspend the cell pellet in PBS to obtain a single cell suspension.

11) Using a haemocytometer, count ~3-5,000 ESCs and 3-5,000 TSC clumps and mix in a 1:1 suspension in a fresh 15ml falcon tube.

12) Centrifuge the mix for 3-4 minutes at 1000g at room temperature, then remove the supernatant carefully using a P200 pipette, without disturbing the cell pellet.

3D embedding of ESC and TSC TIMING

13) Resuspend the pellet in liquid Matrigel (~100ul for all 8 wells of the μ-plate (Ibidi)).
   CRITICAL STEP: The use of growth-factor reduced Matrigel is recommended. This should be batch tested on cells before generating ETS embryos.
   CRITICAL STEP: Thaw Matrigel on ice. Matrigel must be kept on ice in order to prevent solidification during the cell preparation.
   CRITICAL STEP: Pipette tips for resuspending the cells should also be kept at 4 ^{0}C in order to prevent solidification of Matrigel.

14) Plate the Matrigel-cell suspension in small (10-20μl) drops on μ-plates (Ibidi) and allow the matrix to solidify for 2 minutes at 37°C. Plate one drop per well of the plate.
   Once the Matrigel is solidified, fill each culture well with ETS-embryo medium and culture in 5% CO2 at 37°C. **? TROUBLESHOOTING**

Co-culture of ESCs and TSCs to generate ETS embryos TIMING- maximum 6 days

15) Culture cells in ETS-embryo medium for up to six days after plating. Inspect cultures every day to monitor ETS-embryo formation. Change the medium every 48h or when cells deplete nutrients, as indicated by the phenol red present in the medium. When medium requires changing, the medium will change colour from a red-pink colour to a 'straw-like' colour. Proceed to next step at appropriate time point for your experiment (4, 5 or 6 of culture).
   ? TROUBLESHOOTING

Immunofluorescence analysis of ETS embryos TIMING- 2-3 days

16) Remove the medium from ETS-embryo cultures and rinse with PBS, then fix for 15 minutes at room temperature in 4% ice-cold paraformaldehyde (PFA) in PBS (vol/vol). The PFA will lead

to the collapse of the Matrigel droplets, and structures will stick to the optical plastic/ glass of the culture dish. **? TROUBLESHOOTING**

17) After fixation, rinse samples twice in PBS for 5 minutes each, then permeabilise at room temperature for 10 minutes in 0.3% Triton-X-100, 0.1% glycin in PBS (vol/vol).

PAUSE POINT: Following fixation samples can be processed immediately for immunofluorescence staining or left in PBS up to 2 weeks at 4°C.

18) Rinse again in PBS, then perform primary antibody incubation at 4°C overnight in blocking buffer. Examples of primary antibodies we have used successfully and recommended dilutions are given in the materials.

19) The following day, remove the primary antibody in blocking buffer and wash samples three times for 5 minutes each in PBS at room temperature to remove unbound antibody.

20) Perform secondary antibody incubation with an appropriate antibody in blocking buffer at room temperature for 2 hours.

PAUSE POINT: Incubation with secondary antibody can also be performed at 4°C overnight.

21) After incubation, wash the samples three times in PBS for 5 minutes each at room temperature, then incubate in DAPI plus PBS for a further 1 hour at room temperature.

22) Remove the DAPI/PBS solution, add PBS into plates and image ETS-embryo structures *in* situ in the optical plates using a confocal microscope (Leica or equivalent). **? TROUBLESHOOTING**

BOX 4: '3D on top' protocol for ETS-embryo generation

For imaging a high number of structures, it is useful to have all the cells in the same plane .For this purpose, a '3D on top' approach (modified from Lee et al., [40]) can be used. This method is amenable to the use of different optical plates of different sizes, depending on the purpose of the experiment. The modifications to the main procedure required to establish the '3D on top' approach for 8-well μ-plates (Ibidi) is described below and coating, seeding and media volumes for plates of different sizes is given in Supplementary Table 1.

1) Thaw Matrigel on ice before beginning the experiment.
2) Cover all 8-wells of μ-plates (Ibidi) evenly with 35ul of liquid Matrigel to cover the surface.
3) Place the plate in the incubator at 37°C for two minutes, or until the Matrigel has solidified.

4) Add 100ul of PBS to each well of the Matrigel-coated plate and place in the incubator until ready to use. This step prevents the Matrigel from drying out.

5) Trypsinize and wash cells as described in steps 2-11 of the main procedure to yield a 1:1 mixture of ESCs and TSC clumps in PBS.

6) Remove the PBS from each well of the plate and seed the 1:1 mix of ESCs and TSCs in a 25ul volume and allow them to attach for 10-15 minutes in the incubator.

   CRITICAL STEP: The cells must be allowed to attach in the incubator before proceeding. If cells do not attach, they will be removed during later steps and ETS embryos will not be generated.

7) Prepare a mixture of ETS medium and 10% liquid Matrigel (vol/vol), and keep this on ice until ready to use.

8) Once the cells have attached, remove the extra PBS with a vacuum pump or carefully using a pipette and fill each well of the plate with a mixture of ETS medium and 10% (vol/vol) Matrigel.

9) Culture cells in 5% CO_2 at 37°C for up to six days after plating. Inspect cultures every day to monitor ETS-embryo formation.

   END BOX

? TROUBLESHOOTING

See Table 1 for troubleshooting guidance.

Table 1: Troubleshooting

Step	Problem	Possible Reason	Possible solution
Step 1Aiii, 1Bii	Low rate and density of growth of structure in ECM	Low rate of cell survival/ recovery after thawing	Keep cells in adherent culture for at least one passage after thawing to increase the number of cells before attempting an ETS-embryo experiment
Step 1Aiii, 1Bii	Formation of disorganised structures which do	Use of differentiated ESCs and TSCs in the protocol	Maintain cells in conditions to preserve stem cell character and prevent differentiation. Inspect plates for morphological evidence of differentiation (altered colony morphology in ESC cultures and presence of trophoblast giant cells in TSC

			cultures) and do not use cells in the protocol that are differentiated.
Step 5	Low rate of TSC survival in ECM	TSC viability can be compromised if dissociated to single cells	Preserve small clumps by dissociating pellets gently after centrifugation. If TSCs are prone to dissociating into single cells, suspend cells using a wide P1000 filter tip to avoid complete dissociation of clumps.
Step 14	Low rate of ETS embryo generation and survival	If ESCs and TSCs remain out of culture for prolonged time than recommended, ETS-embryo generation can be compromised due to the decreased survival of the cells.	It is recommended to work with ESCs and TSCs in parallel to generate the mixed cell suspension in order to minimise the time that cells are handled in suspension. If this is not possible keeping the cells in buffered-based media can be used.
Step 15	High rate of cell death among ESCs in the first 48h during ETS embryo generation and culture	Medium change is too infrequent	Change the medium every day if high levels of cell death is observed and the media pH (indicated by phenol red) changes rapidly.

Step 16	Matrigel drops do not collapse upon fixation	PFA is too dilute and not kept ice-cold. Or very few cells have survived in the Matrigel.	Ensure the PFA is kept on ice throughout the procedure and that 4% PFA in PBS is used.
Step 22	High background and non-specific fluorescence is detected upon imaging	Non-specific antibody is retained in the samples due to insufficient washing	Lengthen the time of washing steps to 10min to ensure all unbound primary and secondary antibody is removed.

TIMING

- Step 1 Cell culture (A,B) Culture of ESCs and TSCs for experiment ~ 1 week
- Steps 2-14 Embedding of ESCs and TSCs in ECM for co-culture ~ 1 hour
- Step 15 Culture, monitoring and media changes ~ 6 days
- Steps 16-22 Analysis of ETS-embryos by immunofluorescence ~ 2-3 days

BOX 1 Steps 1-8 '3D on top' plating ~ 1 hour

BOX 1 Step 9 Co-culture to generate ETS-embryos ~ 6 days

ANTICIPATED RESULTS

ESCs and TSCs in close proximity should generate rosette-like structures that contact each other within 48h. After 72h compound structures with a lumen in the ESC compartment should be present, and after 96h, the two tissue compartments should share a common cavity (Fig. 1). The efficiency of ETS-embryo formation is variable, with an average of 22% of structures per well forming embryo-like structures[17] within 4 days of culture (400 structures were analysed in culture to calculate

this value for efficiency, with 88 structures forming compound structures). At this stage, ETS-embryos vary slightly in the number of cells which make up the ESC and TSC compartments respectively, but are consistent in sharing a continuous cavity at the centre of the structure by 96h. Compound structures can be considered as representative ETS embryos therefore if they develop this continuous cavity and if ESC and if ESCs and TSC compartments occupy approximately 50% of total tissue volume each[17].

It is important that ESCs are cultured to preserve the naïve pluripotent state before use in the protocol. If ESCs are allowed to differentiate in culture, ETS-embryo generation can be compromised. Differentiated ESCs can give rise to ETS-embryos with disorganised ESC compartments, which fail to organise a columnar epithelium, or a continuous pro-amniotic cavity. Similar structure morphology can be seen when too much serum is used in the ETS-embryo medium. If differentiated TSCs are used in this protocol, compound structures fail to grow in culture.

If cell viability is good, 100% of ETS-embryos will express lineage markers determining the cell identity in discrete embryonic and extraembryonic compartments (Fig. 2). However, mesodermal specification among ETS-embryos is variable. We found that 66% of the structures in culture that can be considered as ETS-embryos will specify some cells as mesoderm (by the detection of T/Brachury expression) somewhere in the ESC compartment of the structure. 43% of ETS embryos will specify mesoderm which is 'regionalised' in that it is localised to one side of the long axis of the structure within 4-5 days. 34% of ETS-embryos fail to specify any T/Brachyury positive cells in the ESC compartment.

ACKNOWLEDGEMENTS

AUTHOR CONTRIBUTIONS

S.E.H, B.S & M.Z.G designed the protocol, carried out the work, analysed the results and prepared the manuscript.

COMPETING INTERESTS

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Figure legends

Figure 1: Generation of polarised embryo-like structures from embryonic and extra-embryonic stem cells to form ETS-embryos. Schematic of the method used to generate ETS-embryos from ESCs and TSCs. (a) When 80% confluent, ESCs and TSCs are dissociated into single cells and small clumps respectively (Steps 2-10). Images from the haemocytometer square (0.25 x 0.25 mm) to count single ESCs and TSC clumps (Step 11) Scale bar= 50µm. (b) Mixing and resuspension of cells in liquid Matrigel (Steps 12, 13) (c) Plate cell-Matrigel suspension in small drops, allow to solidify and culture in ETS medium (Step 14) (d)&(e) The '3D on top' variation of culture (described in Box 4.) (f) Low-magnification of confocal images of the culture plate at indicated time points showing the co-culture of CAG-GFP labelled ESCs and wild-type TSCs on Matrigel. Single ESCs and TSC clumps (3h, inset) grow to produce single spheroid of ESC or TSC colonies (24h, inset) which later come together (48-72h) and finally merge to generate embryo-like structures, ETS-embryos (96h). Scale bar= 50µm. (g) Time scheme of ETS-embryo generation. (h) High-magnification of confocal snapshots of an ETS-embryo developing over 96h from clumps of cells. Within 12h, the single ESC divides to give rise to a doublet and later a spheroid of polarised cells, which later merges with the TSCs to generate an ETS-embryo. The ESC compartment is labelled with a CAG-GFP membrane marker to demarcate it from the TSC compartment. Yellow dashed lines outline the cavity. Scale bar= 10µm.

Figure 2: Molecular markers ascertaining to the lineage identity of the cell types in the embryonic and extra-embryonic compartments of polarised embryo-like structures, ETS-embryos. ETS-embryos cultured for 110h were analysed for ExE markers (EOMES, grey (a); TFAP2C, grey (b)); epiblast markers (SOX2, red (a); OTX2, red (b)) and mesoderm marker (T/Brachyury, green) by immunofluorescence. DAPI, blue; Scale bar= 20µm.

Supplementary table 1: Table listing the approximate volumes of medium and Matrigel used with this method for different sizes and types of optical culture dish.