Stem cell-derived mouse embryos develop within an extra-embryonic yolk sac to form anterior brain regions and a beating heart-like structure.

Gianluca Amadei
University of Cambridge https://orcid.org/0000-0001-5405-968X
Charlotte E. Handford
University of Cambridge https://orcid.org/0000-0002-5245-8027
David Glover
Caltech
Magdalena Zernicka-Goetz (magdaz@caltech.edu)
Caltech

Method Article

Keywords: mouse embryos, gastrulation, neurulation, organogenesis, stem cells, ETiX-embryoids, embryo-like structures, synthetic embryos

Posted Date: December 5th, 2022

DOI: https://doi.org/10.21203/rs.3.pex-2006/v2

Abstract

Mouse embryos are an invaluable tool to study mammalian development but establishment of novel transgenic mouse lines is a costly and time-consuming endeavour. Stem cell models, on the other hand, lend themselves to quick genetic manipulation and are easily scalable, yet they do not successfully capture morphogenesis.

Here, we report stem cell-based structures called ETiX-embryoids that can recapitulate mouse post-implantation morphogenesis. ETiX-embryoids form by aggregating embryonic and trophoblast stem cells to embryonic stem cells transiently expressing Gata4. Over the course of 4 days, these structures develop like mouse post-implantation egg cylinders; they then undertake and complete gastrulation on day 5 and 6, respectively. On day 7, ETiX-embryoids undergo neural induction and form the mouse anterior-posterior axis from neural folds to tail bud by movements of convergent extension. On day 8 they form a brain, establish a beating heart-like structure and a gut tube, as they develop inside extraembryonic membranes equivalent to amnion and yolk sac. We detail here the experimental set up and procedure required to generate ETiX-embryoids, and anticipate that they will establish themselves as a novel, powerful tool to study mammalian development without generating a novel mouse lines.

Introduction

In natural development, the zygote develops into the epiblast, forming the organism; the extraembryonic visceral endoderm (VE) instead contributes to the yolk sac; and the extraembryonic ectoderm (ExE), gives rise to the embryonic portion of the placenta. Since it is possible to generate stem cells from each of these lineages, this raises the possibility that the mammalian organism could be completely regenerated from multiple components, instead of using a single totipotent zygote.

Embryonic stem cells (ESCs), which derive from the epiblast, have been reported to form embryo like-structures upon aggregation, and when they are embedded in Matrigel they give rise to trunk-like structures with somites, a neural tube and a gut[1–5]. Although aspects of neural development can be induced in such “gastruloids” by inhibiting the initial burst of WNT activity, they ultimately do not capture gastrulation movements, and they do not replicate the complete anatomy of natural embryos.

Here, we report a stem cell-based procedure to generate structures, called ETiX-embryoids, that develop like a mouse embryo up to E8.5 days of development. ETiX-embryoids comprise a combination of embryonic stem cells, trophoblast stem cells and embryonic stem cells transiently expressing Gata4[6]. Over the course of 8 days, ETiX-embryoids undertake and complete gastrulation, undergo neural induction and develop neural folds and a beating heart-like structure. Importantly, their stem cell, modular nature makes them amenable to genetic manipulation, thus poising them to be a novel and powerful tool to study mouse development.

Reagents

2-β-mercaptoethanol (Gibco 31350010)

- Advanced DMEM/F12 (Gibco, 21331-020)
- AggreWell rinsing solution (StemCell Technology 07010)
- B27 (Gibco 10889038)
- β-estradiol (Tocris 50-28-2, stock: 10uM in DMSO)
- CHIR99021 (Cambridge Stem Cell Institute or StemCell Technology 72052)
- DMEM (Gibco 41966052)
- DMEM, bicarbonate-buffered, without glutamine (Gibco 11054)
- DMEM low-glucose, with no bicarbonate buffer (Gibco 11880)
- DMEM/F-12 (Gibco 21331020)
- Doxycycline (Sigma-Aldrich D9891-5G, stock: 1mg/mL in water)
- Foetal bovine serum (Cambridge Stem Cell Institute or Gibco 10270106)
- FGF4 (R&D Systems 7486-F4-025, stock: 100 ug/mL)
- Gelatine type A (Sigma-Aldrich G1890)
- D-(+)-Glucose (G8644-100ML)
- GlutaMAX (Gibco 35050038)
- Heparin (Sigma-Aldrich H3149-25KU, stock: 1 mg/mL in PBS)
- HEPES (Thermo Fisher Scientific 15630056)
- Human cord serum (Cambridge Blood and Stem cell Biobank, UK)
- Human adult blood serum (Sigma-Aldrich H3667-100ML)
- ITS-X (Thermo Fisher Scientific 51500-056)
- Leukaemia inhibitory factor LIF (Cambridge Stem Cell Institute or StemCell Technologies 78056)
- MEM non-essential amino acids (Gibco 11140035)
- N-acetyl-L-cysteine (Sigma-Aldrich A7250, stock: 50 mM in water)

- N2 Supplement (Life Technologies 17502048)
- Neurobasal-A (Gibco 10888022)
- PBS (Life Technologies 10010056)
- PD0325901 (Cambridge Stem Cell Institute)
- Penicillin/streptomycin (Gibco 15140122)
- Progesterone (Sigma-Aldrich P0130-25G, stock: 1 mg/mL in DMSO)
- Rat serum (adult rats, Charles River)
- RPMI 1640, no Glutamine (Thermo Fisher Scientific 21870076)
- Sodium pyruvate (Gibco 11360039)
- Trypsin-EDTA 0.05% (Life Technologies 25300054)

Media Recipes:

N2B27 medium (for 50 mL):

- 25 mL of Neurobasal-A
- 25 mL of DMEM/F12
- 500 uL of P/S
- 500 uL of GlutaMAX
- 100 uL of 2-β-mercaptoethanol
- 500 uL of B27
- 250 uL of N2
- Supplement with LIF, Chiron and PD to make N2B27/2iLIF

Feeder Cell medium (FC, for 1 full bottle):

- 1 bottle of DMEM (Gibco 41966052)
- 90 mL of heat-inactivated FBS
- 5 mL of P/S
- 5 mL of GlutaMAX
- 5 mL of MEM non-essential amino acids
- 5 mL of Sodium pyruvate
- 1.2 mL of 2-β-mercaptoethanol

Trophoblast Stem Cell medium (TS, for 50 mL)

- 50 mL of RPMI 1640
- 10.4 mL of FBS
- 520 uL of P/S
- 520 uL of GlutaMAX
- 520 uL of Sodium pyruvate
- 104 uL of 2-β-mercaptoethanol
- For 10 mL of TSF4H medium, add 10 uL of Heparin stock and 2.5 uL of FGF4 stock

In vitro culture medium (IVC)[7]

Base for IVC (ADF+++)

- 50 mL of Advanced DMEM/F12
- 500 uL of GlutaMAX
- 500 uL of ITS-X
- 125 uL of P/S

IVC-20% FBS (10 mL)

- 8 mL of ADF+++
- 2 mL of FBS
- 8 uL of β-estradiol
- 2 uL of Progesterone
- 5 uL of N-acetyl-L-cysteine

IVC-30% FBS (10 mL)

- 7 mL of ADF+++
- 3 mL of FBS
- 8 uL of β-estradiol
- 2 uL of Progesterone
- 5 uL of N-acetyl-L-cysteine

DMEM/Rat serum medium/Human cord serum (DRH, for 1 mL)[8]

- 250 uL of DMEM, bicarbonate-buffered, without glutamine (Gibco 11054)
- 500 uL of rat serum
- 250 uL of human cord serum
- 10 uL of GlutaMAX
- 10 uL of P/S

On day 7 of ETiX-embryoid culture, this medium was supplemented with D-(+)-Glucose at a concentration of 3.0 mg/mL (Step 45); on day 8, the concentration of D-(+)-Glucose was increased to 3.5 mg/mL (Step 46). Human and rat serum were heat-inactivated for 35 minutes and filter-sterilised prior to use.

***Ex utero* culture medium**[9]

EUCM base:

· 969 uL of DMEM low-glucose, with no bicarbonate buffer (Gibco 11880)

· 11 uL of HEPES

· 10 uL of GlutaMAX

· 10 uL of P/S

EUCM (1mL)

· 250 uL of base

· 500 uL of rat serum

· 250 uL of human cord serum

On day 7 of ETiX-embryoid culture, this medium was supplemented with D-(+)-Glucose at a concentration of 3.0 mg/mL (Step 45); on day 8, the concentration of D-(+)-Glucose was increased to 3.5 mg/mL (Step 46). Human and rat serum were heat-inactivated for 35 minutes and filter-sterilised prior to use.

Note: in both DRH and EUCM medium the human cord serum can be replaced by human adult blood serum.

Cell lines

· Mouse embryonic stem cells (ESCs) – CD1 wildtype background (a gift of Prof. Jenny Nichols)

· Mouse trophoblast stem cells (TSCs) - CD1 wildtype background (generated in-house)

· Mouse tetO-Gata4 embryonic stem cells (tetO-Gata4 ESCs) - CD1 wildtype background (generated in-house)

· Mouse embryonic fibroblasts (MEFs, Insight Biotechnology, ASF-1201)

Additionally, we have been able to grow ETiX-embryoids comparable to the ones with a CD1 background with the following ESC lines:

· Sox2-Venus/Brachyury-mCherry/Oct4-ECFP ESCs (a gift from Dr. Jesse Veenvliet and Prof. Bernhard G. Hermann)

· CAG-GFP/tetO-mCherry ESCs (generated in-house)

· Blimp1-GFP ESCs (a gift from Prof. Azim Surani)

· BVSC ESCs (a gift from Prof. Wolf Reik)

With the following lines, instead, we have been able to obtain ETiX-embryoids that develop until day 6 but not beyond:

· Lfng reporter (LuVeLu) ESCs (a gift from Dr. Alexander Aulelha and Dr. Ina Sonnen)

· Msgn1-Venus ESCs (a gift from Prof. Olivier Pourquié)

· Hes7-Achilles ESCs (a gift from Prof. Olivier Pourquié)

· Sox1-GFP ESCs (a gift from Prof. Austin Smith)

· mTmG ESCs (generated by us in-house)

Plasticware

· Tissue culture treated 6-well plates (e.g. Thermo Fisher Scientific 140675)

· Suspension culture 6-well plate (CELLSTAR, Greiner Bio-One 657185)

· Suspension culture 48-well plate (CELLSTAR, Greiner Bio-One 677102)

· AggreWells (24-well format, StemCell Technology 34415)

Equipment

· Tissue culture grade incubator able to maintain 37 °C and 5% CO_2

· *Precision* rotating bottle culture apparatus (BTC Engineering / Cullum Starr Precision Engineering Limited, Cambridge)

· Gas mixer and pressurising chamber (Arad Technologies Ltd)[9]

We also used an alternative rotating bottle system for embryo culture that substitutes the device from Arad Technologies with one from BioSpherix. It has the following commercially available components-

1. *Precision* rotating bottle culture apparatus as above. http://www.cullumstarr.com/btc-engineering/rotating-bottle-culture-unit

2. OxyStreamer O2 and CO2 Live Cell Microscopy Controller (BioSpherix, Ltd., RRID: SCR_021176) https://biospherix.com/oxystreamer/

3. Gas cylinders (O2, N2 and CO2)

The gas mixer has input from all three gas cylinders (O2, N2 and CO2). The input pressure for each of the gases is maintained at 10 psi. The gases were mixed in the desired ratio (for example, 21% O2 and 5% CO2) and fed into the rotary bottle system. The final gas mixture was humidified before feeding into the rotatory bottles. The pressure of the gas (mixed + humidified) before at the entrance of the rotary bottle was 0.5 psi. The flow rate at the exit of the rotatory bottle system was 8-10 bubbles/sec. The bottles were rotated at 3 rpm. Media was changed every 24 hours with minimum disturbance to the embryos.

Procedure

Culture of mouse embryonic stem cells and mouse embryonic stem cells transiently expressing Gata4

1. We cultured mouse ESCs in N2B27/2iLIF on gelatinised, tissue culture-grade plates in an incubator kept at 37°C with 5% CO2 and 21% O2.

2. When the culture was 70/80% confluent, or the colonies had begun to touch and merge, or they had started flattening, we passaged the cells.

3. 1 mL of gelatine was added to a clean well of a tissue culture dish in preparation for replating. We incubated the plate at room temperature in the biosafety cabinet until needed (at least 5 min).

4. To passage, we washed the culture once with 1 mL of 1x PBS and then added 500 uL-1ml of Trypsin; we incubated the plate in the incubator for 4 minutes.

5. We stopped the trypsinisation reaction by adding 1-2 volumes of FC medium and then we resuspended the cell suspension 2-3 times by gentle pipetting with a P1000 to break the cells colonies.

6. We transferred the cell suspension to a 15 mL Falcon tube and centrifuged it at 0.2 x g (RCF) for 4 minutes.

7. Following centrifugation, we carefully aspirated the medium as not to disturb the cell pellet and then washed the cells with 1 mL of PBS.

8. To wash, we gently resuspended the culture twice with the P1000 and then centrifuged them one more time at 0.2 x g.

9. After centrifugation, we carefully aspirated the PBS and gently resuspended the cell culture in 1 mL of N2B27/2iLIF by pipetting twice with the P1000.

10. We aspirated the gelatine from the plate and added 2 mL of N2B27/2iLIF to the well. We plated the cells at a dilution of either 1:5, 1:10, 1:20. In our experience, we found that a confluent culture plated at 1:5 will need to be re-passaged on the following day; a culture plated at 1:10 will be ready after 2 days and a culture plated at 1:20 will be confluent after 3 days.

11. We mixed the cells evenly by gently moving the plate side to side and front-back three times (or in a figure of 8 motion) and placing the plate in the incubator.

Culture and passaging of TSCs

We passaged TSCs similarly to ESCs but with the following differences:

12. Six hours before passaging, we replaced the FC medium of a well coated with inactive MEFs with 2 mL of TSF4H medium. We find that medium conditioning prevents differentiation of TSCs.

13. After 6 hours, we aspirated the medium from TSCs and washed them twice with PBS.

14. We added 500 uL-1ml of Trypsin and incubated the plate at 37°C for 4 minutes.

15. We stopped the trypsinization reaction with 2 mL of TS medium.

16. We dissociated TS cells by gentle pipetting with the P1000 for 3-4 times.

17. We transferred the cell suspension to a 15 mL Falcon tube and centrifuged it for 4 minutes at 0.2 x g (RCF).

18. Following centrifugation, the supernatant was carefully aspirated without disturbing the pellet.

19. We added 1 mL of TSF4H and resuspended the cells by gentle pipetting with the P1000.

20. We plated TS cells either at 1:10 or 1:20. Denser or sparser dilutions may cause differentiation of the cells. Cells plated at 1:20 will general be confluent again after 4 days; cells plated at 1:10 will generally be confluent after 3 days.

21. After the passage, we changed media on the cells on the following day; after that, the medium was topped up by adding 2 mL of TSF4H.

Preparation and seeding of cells for ETiX-embryoid formation

22. On the day before the experiment, tetO-Gata4 ESCs were passaged as described earlier and plated at a dilution of 1:5. This was to ensure and homogenous response to doxycycline.

23. On the day of the experiment, we provided fresh media to the ESCs and tetO-Gata4 ESCs. Six hours before the start of the experiment, we added Dox (1:1000 dilution) to the tetO-Gata4 ESCs and we topped up the medium on TSCs. We also changed the medium on one plate of MEFs to TSF4H to allow them to condition it. This was then used for propagation of the TSC line.

24. After six hours of induction with Dox, we began the experiment.

25. 500 mL of rinsing solution was added to the desired number of AggreWells, the plate was then centrifuged at 2000 x g (RCF) for 5min and incubated at room temperature in the biosafety cabinet until the cell suspensions were prepared and quantified. The plate was checked for bubbles and if present, the plate was centrifuged again for an additional 5 min.

26. We placed the appropriate amount of FC medium to equilibrate in a Falcon tube with a loose cap in the incubator. Each AggreWell requires 1.5 mL of medium in total. 0.5ml of this is placed in the AggreWell first and the remaining 1ml is supplemented with Rock inhibitor and used for the resuspension of cells (see later steps 33 and 34).

27. We also put 1 mL of gelatine in a well of a tissue culture treated 6 well plate, which we incubated at room temperature. This is required for MEF depletion from the TSC suspension.

28. We dissociated ESCs and TSCs as described earlier. At the end of the dissociation, TSCs were resuspended in 2 mL of TSF4H, and plated in the well that was coated with gelatine (in step 27).

29. We incubated the TSC suspension for 20-30 minutes to allow for the MEFs to stick to the plate and leave an enriched suspension of TSCs in the supernatant. After 20 minutes, the cell suspension was collected for the experiment. If needed, a fraction of this suspension was also replated onto inactive MEFs for propagation (see step 23).

30. At the end of the dissociation and after a wash in PBS, ESCs and tetO-Gata4 ESCs were resuspended in 1-2 mL of FC medium. 10ul of this suspension were collected for quantification and the remainder of cells were placed in the incubator until needed.

31. We quantified the cell suspensions using a haemocytometer.

32. We transferred the volumes yielding 6,000 ESCs, 6,000 tetO-Gata4 ESCs and 19,200 TSCs to a 15 mL Falcon tube and centrifuged them for 4 minutes at 0.2 x g (RCF).

33. The rinsing solution was aspirated from the AggreWell and we rinsed the wells with 1 mL of PBS twice and then twice with 1 mL of FC. After these washes, we added 500 uL/well FC that was equilibrated in step 26. We placed the AggreWell plate back in the incubator until the cells were ready to be plated.

34. We added Rock inhibitor at a final concentration of 0.5 nM to the remaining medium in the incubator and used it to resuspend the cells at the end of the centrifugation (for a 5uM stock we used Rock inhibitor at 1:10000). 1 mL of medium with Rock inhibitor is added for each well to be plated.

35. We added cells to the AggreWell dropwise on the centre of each well.

36. We centrifuged the plate for 3 minutes at 100 x g (RCF), and placed the plate in the incubator (Day 0).

37. On the next day (Day 1), media change was performed twice by removing 1ml of medium from each well and adding 1ml of fresh FC medium without ROCK inhibitor. The media changes need to be performed very gently exercising great care as not to disturb the structures forming within the wells. Media is removed and added slowly by placing the pipette tip against the side of the wells, avoiding formation of bubbles.

38. On D2, media change was performed once to replace 1ml of medium with 1ml of fresh FC medium.

39. On D3, 1 ml of medium was removed from each well and 1.5ml of IVC-20% FBS was added, following its equilibration for 20 minutes in the incubator.

40. On D4, ETiX-embryoids in the AggreWell were transferred to CELLSTAR 6 well multiwell plate for suspension culture (Greiner Bio-One 657185) with 5ml of IVC-30% FBS per well.

41. All ETiX-embryoids were collected from AggreWell for analysis at 4 days of development and analysed under a stereomicroscope. We selected ETiX-embryoids with cylindrical morphology and two clearly defined cellular compartments (an ESC compartment and TSC compartment) surrounded by an outer cell layer, the VE-like layer. We expect the ESC compartment to be epithelialized with a lumen. The TSC compartment is more variable in appearance and therefore, even though one would also want an epithelial-looking TSC compartment similar to the extra-embryonic ectoderm of natural embryos, we select a wider range of appearances for the TSC compartment. Since the majority of ETiX-embryoids were generated by using wild-type, unlabelled stem cell lines, the selection was based on morphology alone. ETiX-embryoids with the correct body plan of ESC and TSC compartments surrounded by a VE-like layer were then transferred to equilibrated media to continue their culture. At day 4, we were able to collect 10%-15% of the structures formed in the pyramidal microwells.

42. When selecting at D5, however, we included additional criteria: i) we expect the lumen of the ESC and TSC compartment to be merged; ii) ideally we can observe the beginning of gastrulation on one side of

the ETiX-embryoids; iii) we expect the AVE to have migrated to the ESC-TSC boundary and be opposite to the forming streak; iv) ETiX-embryoids with the AVE stuck at the tip of the structure or not at the boundary were excluded. From day 4 to day 5 we cultured 20% of the structures collected at day 4.

43. On Day 5 each ETiX-embryoid was transferred to a single well of 48-well, non-adherent dish with 250 uL of DRH or EUCM medium.

44. On day 6 each ETiX-embryoid was fed with an additional 250 uL of DRH/EUCM.

45. A supplement of 3.0 mg/mL of D-(+)-Glucose (Sigma G8644) was added to DRH/EUCM medium on Day7 when the ETiX-embryoids were moved to the rotating bottle culture chamber apparatus. Each rotating bottle contained 2 mL of medium and 3 ETiX-embryoids.

46. On day 8 DRH/EUCM medium was further supplemented with 3.5 mg/mL of D-(+)-Glucose. In each rotating bottle 2 ETiX-embryoids were cultured with 3 mL of medium.

47. At the end of day 8, we dissected and fixed the structures in 4% PFA for 20 minutes at room temperature in preparation for downstream analyses.

Troubleshooting

Time Taken

When healthy stocks of cells are frozen, it usually takes them 1-5 days to be confluent enough for the first passage. After the cells have been passaged once, they can be used for experiments, so usually one week after thawing the cells experiments can be set up. The time for each experiment is 8 days.

Anticipated Results

Following this protocol, it is possible to obtain stem cell-based structures that develop up to day 8 like natural embryos at the equivalent stage of E8.5. The parental stem cell lines can be genetically modified to probe tissue-specific phenotypes in any of the three lineages.

References

1. ten Berge, D. *et al.* Wnt Signaling Mediates Self-Organization and Axis Formation in Embryoid Bodies. *Cell Stem Cell* **3**, 508–518 (2008).

2. van den Brink, S. C. *et al.* Symmetry breaking, germ layer specification and axial organisation in aggregates of mouse embryonic stem cells. *Development* **141**, 4231–4242 (2014).

3. Xu, P.-F. *et al.* Construction of a mammalian embryo model from stem cells organized by a morphogen signalling centre. *Nature Communications* **12**, 3277 (2021).

4. Beccari, L. *et al.* Multi-axial self-organization properties of mouse embryonic stem cells into gastruloids. *Nature* **562**, 272–276 (2018).

5. Veenvliet, J. *et al.* Mouse embryonic stem cells self-organize into trunk-like structures with neural tube and somites. *Science (1979)* **370**, eaba4937 (2020).

6. Amadei, G. *et al.* Inducible Stem-Cell-Derived Embryos Capture Mouse Morphogenetic Events In Vitro. *Developmental Cell* **56**, 366-382.e9 (2021).

7. Bedzhov, I., Leung, C. Y., Bialecka, M. & Zernicka-Goetz, M. In vitro culture of mouse blastocysts beyond the implantation stages. *Nature Protocols* **9**, 2732–2739 (2014).

8. Sturm, K. & Tam, P. P. Isolation and culture of whole postimplantation embryos and germ layer derivatives. *Methods Enzymol* **225**, 164–190 (1993).

9. Aguilera-Castrejon, A. *et al.* Ex utero mouse embryogenesis from pre-gastrulation to late organogenesis. *Nature* **593**, 119–124 (2021).

Acknowledgements

We are very grateful to Jacob Hanna and Alejandro Aguilera-Castrejon for their kind provision of a device to regulate gas pressure and gas mixing on the roller bottles as described[9]. The device was modified by the Engineering Department (University of Cambridge) to enable pressure generation but not gas mixing and we therefore utilized only the pressure generating component of this device to enable development of embryoids to Day 8. We also built our own device that delivers defined gas mixtures but at 0.5 psi rather than 6.5 psi, as described[9]. The alternative arrangement utilises an instrument from BioSpherix and is described above. We found that delivery of gas mixtures at this lower pressure was effective in promoting development either when 21% oxygen was continuously supplied during culture or when the oxygen concentration was incrementally increased from 5% to 13% to 18% to 21% at daily intervals.

We are thankful to the Cambridge Blood and Stem Cell Biobank for providing human cord serum to us. The Cambridge Blood and Stem Cell Biobank is supported by the Cambridge NIHR Biomedical Research

Centre, Wellcome Trust – MRC Stem Cell Institute and the Cambridge Experimental Cancer Medicine Centre, UK.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Troubleshootingtableprotocolexchange.docx