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Stepwise Protocol of Establishing Embryo Model from A Continuous Totipotent-Like Cell-Based Embryo Model Recapitulates Mouse Embryogenesis from Zygotic Genome Activation to Gastrulation

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Abstract

The development of stem-cell-derived models of mammalian embryogenesis has provided invaluable tools for investigating embryo development. However, constructing embryo models that can continuously recapitulate the developmental trajectory, from zygotic genome activation (ZGA) to gastrulation, remains challenging. Here we report the development of a chemical cocktail to induce totipotent-like cells with robust proliferation ability, and leveraged these cells to establish a stepwise protocol to generate a continuous embryo model. This model sequentially mimics mouse embryogenesis from embryonic day 1.5 to 7.5. It recapitulates key developmental milestones, including ZGA in 2-cell embryos, the diversification of embryonic and extraembryonic lineages from 4-cell to 64-cell stages, the formation of blastocysts, and the subsequent development into post-implantation egg cylinders. Notably, these structures undergo gastrulation, as evidenced by formation of a primitive streak-like structure and the subsequent emergence of several early organogenesis hallmarks. Our study opens avenues for modeling mammalian embryogenesis *in vitro*.

Materials

- 0.05% trypsin-EDTA (Gibco, 25300-062)
- Aggrewell (Stemcell Technologies, 34415)
- Elvitegravir (1 µM, MCE, HY-14740)
- CHIR-99021 (3 µM, Selleck, S1263)
- CD1530 (0.2 µM, MCE, HY-108527)
- PD0325901(0.5 µM, Selleck, S1036)
- RPMI Medium 1640 basic (Gibco, 22400-089)
- Knockout serum replacement (Gibco, 10828-028)
- N2 supplement (Gibco, 17502-048)
- B27 supplement (Gibco, 12587-010)
- GlutaMAX (Gibco, 35050-061)
- Nonessential amino acids (Gibco,11140-050)
- Sodium pyruvate (Gibco, 11360-070)
- Sodium DL-lactate solution (Sigma, L7900)
- Chemically Defined Lipid Concentrate (Gibco, 11905-031)
- Bovine Serum Albumin (Sigma, A1933)
- Penicillin-Streptomycin (Gibco, 15140163)
- 2-Mercaptoethanol (Gibco, 21985023)
- FBS (VISTECH, SE200-ES)
- KSR
- KSOM (LIFE-iLAB, CE002)
- N2B27 basal
- TSC basal
- GA-017 (10 µM, Selleck, E1145)
- Recombinant Human BMP-4 (20 ng/ml, Novoprotein, C093)
- Recombinant Human FGFb (157AA) (20 ng/ml, Novoprotein, C046)
- Recombinant Mouse FGF-4 (25 ng/ml, Novoprotein, CR66)
- Heparin (1 µg/ml, Macklin, H811552)
- Recombinant Human/Mouse/Rat Activin A (20 ng/ml, Novoprotein, C687)
- XAV-939 (2 µM, Selleck, S1180)
- Recombinant Human BMP-4 (5 ng/ml, Novoprotein, C093)
- Advanced DMEM/F12 (Gibco, 12634010)
- ITS-X supplement (Gibco, 51500056)
- β-estradiol (Sigma, E8875)
- Progesterone (Sigma, Y000699)
- N-acetyl-L-cysteine (Sigma-Aldrich, A9165)
- T3 (3,3',5-Triiodo-L-thyronine sodium) (MCE, HY-A0070)
- D(+)-Glucose (MCE, HY-B0389)
- CD female rat serum (Gibco, 11054)
- Human cord serum
- HEPES (Gibco, 15630056)

Formation of S1-S8 embryoids

1 To generate embryo models that sequentially recapitulate different stages, from zygotic genome activation to the gastrulation, we developed a stepwise protocol. At S1, mouse EPSCs were digested by 0.05% trypsin-EDTA (Gibco, 25300-062) into single cells, then, 9600 mouse EPSCs/well were plated in the pretreated Aggrewell (Stemcell Technologies, 34415). 1 ml totipotency medium was added to each well and treat for 2 days. Totipotency medium: 1640 basal medium supplemented with Elvitegravir (1 μM, MCE, HY-14740), CHIR-99021 (3 μM, Selleck, S1263), CD1530 (0.2 μM, MCE, HY-108527), PD0325901(0.5 μM, Selleck, S1036). 1640 basal medium: RPMI Medium 1640 basic (Gibco, 22400-089) supplemented with 5% knockout serum replacement (Gibco, 10828-028), 1% N2 supplement (Gibco, 17502-048), 2% B27 supplement (Gibco, 12587-010), 1% GlutaMAX (Gibco, 35050-061), 1% nonessential amino acids (Gibco,11140-050), 1% sodium pyruvate (Gibco, 11360-070), 0.14% sodium DL-lactate solution (Sigma, L7900) and 0.2% Chemically Defined Lipid Concentrate (Gibco, 11905-031), 50 μg/ml Bovine Serum Albumin (Sigma, A1933), 1% Penicillin-Streptomycin (Gibco, 15140163). To accommodate the varying proliferation rates of different cell lines, the addition and concentration titration of 2-Mercaptoethanol (Gibco, 21985023) could be considered.

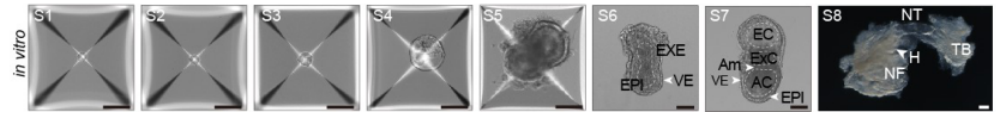
2 At stage 2, fresh 1 ml 4C-64C medium to each well for 1 day. At Stage 3, cells are obtained after an additional day of treatment under the same conditions. 4C-64C medium: 1640 basal medium supplemented with CHIR-99021 (10 μM, Selleck, S1263), Birabresib (0.05 μM, Selleck, S7360), CD1530 (0.2 μM, MCE, HY-108527), 8-Br-cAMP (1 mM, Selleck, S7857), 0.05% 2-Mercaptoethanol (Gibco, 21985023). To accommodate different cell lines, the KSR in 1640 basal medium could be replaced by FBS (VISTECH, SE200-ES), and the concentration of 2-Mercaptoethanol (Gibco, 21985023) could be titrated.

3 At stage 4, fresh 1 ml blastoids medium to each well for 3 days to generate blastoid. Blastoid medium: 50% KSOM (LIFE-iLAB, CE002), 25% N2B27 basal, 25% TSC basal, supplemented with CHIR-99021 (3 μM, Selleck, S1263), GA-017 (10 μM, Selleck, E1145), Recombinant Human BMP-4 (20 ng/ml, Novoprotein, C093), and Recombinant Human FGFb (157AA) (20 ng/ml, Novoprotein, C046). TSC basal: 80% RPMI Medium 1640 basic (Gibco, 22400-089), 20% FBS (VISTECH, SE200-ES), supplemented with 1% GlutaMAX (Gibco, 35050061), 1% Sodium Pyruvate (Gibco, 11360070). To accelerate the kinetics of blastoids formation, cell aggregates could be transferred to 6-well non-adherent suspension culturing plates (BEAVER, 40406). For the acquisition of post-implantation embryoids, early blastoids of 1-day treatment should be retained in the Aggrewell (Stemcell Technologies, 34415) for subsequent processing.

4 At stage 5, gently fresh 1 ml peri-implantation medium to each well. Following 3-day treatment, structures with the presence of early egg cylinder-like structures surrounded by a layer of visceral endoderm (VE)-like cells would emerge. Peri-implantation medium:

50% KSOM (LIFE-iLAB, CE002), 25% N2B27 basal, 25% TSC basal, supplemented with Recombinant Mouse FGF-4 (25 ng/ml, Novoprotein, CR66), Heparin (1 µg/ml, Macklin, H811552), Recombinant Human/Mouse/Rat Activin A (20 ng/ml, Novoprotein, C687), XAV-939 (2 µM, Selleck, S1180) and Recombinant Human BMP-4 (5 ng/ml, Novoprotein, C093).

5 At stage 6 and stage 7, S5 embryoids were transferred to 6-well non-adherent suspension culturing plates (BEAVER, 40406). Add 3-4 ml post-implantation medium/modified IVC1 medium to each well. Following 1-day treatment, structures morphologically resembling E6.5 embryos with two lumens would emerge. After an additional day of treatment, the structures would increase in size, and sub of them would develop into three cavities, which was similar to E7.5 embryos. Post-implantation medium/modified IVC1 medium: Advanced DMEM/F12 (Gibco, 12634010) supplemented with 20% FBS (VISTECH, SE200-ES), 1% mM GlutaMAX (Gibco, 35050061), 1% ITS-X supplement (Gibco, 51500056), 1% Penicillin-Streptomycin (Gibco, 15140163), 8 nM β-estradiol (Sigma, E8875), 200 ng/ml progesterone (Sigma, V9006969), 25 mM N-acetyl-L-cysteine (Sigma-Aldrich, A9165), 100 nM T3 (3,3',5-Triiodo-L-thyronine sodium) (MCE, HY-A0070), 1 mg/mL D(+)-Glucose (MCE, HY-B0389).

6 

At stage 8, S7 embryoids were selected under dissection microscope for further culture with roller culture system. We selected elongated egg cylinder with a thick EPI-like layer that resembled the EPI in natural mouse embryos, and transfer them to post-implantation medium/modified IVC2 medium. Post-implantation medium/modified IVC2 medium: 25% DMEM (Gibco, 11054) supplemented with 50% CD female rat serum, 25% human cord serum, 1% mM GlutaMAX (Gibco, 35050061), 1% Penicillin-Streptomycin (Gibco, 15140163), 1% Sodium Pyruvate (Gibco, 11360070) 11 mM HEPES (Gibco, 15630056) and 4 mg/mL D(+)-Glucose (MCE, HY-B0389). Rat serum and human cord serum were thawed at room temperature and heat-inactivated for 30 min at 56 °C. Up to 50 S7 embryoid were cultured in each bottle, filled with 2 ml post-implantation medium/modified IVC2 medium.