

Generation of functional heart organoids from mouse embryonic stem cells.

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Method Article

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Abstract

For the understanding of the spatiotemporal regulation of cardiogenesis, it is important to generate *in vitro* model of hearts. This protocol introduces how to generate heart organoids from mouse embryonic stem cell-derived embryoid bodies (EB) in the presence of the laminin-entactin (LN/ET) complex and fibroblast growth factor 4 (FGF4) by three consecutive steps, 1) the culture of ESCs, 2) *in vitro* differentiation of ESCs into EBs and 3) *in vitro* culture of EBs for the generation of heart organoids. The generated heart organoids possess structural and functional capacity similar to atrial and ventricular parts of *in vivo* embryonic heart. This simplified protocol also provides a promising research tool with a broad range of applications, including drug testing.

Introduction

Mammalian heart development involves differentiation of diverse cell types, including atrial and ventricular muscle cells known as cardiomyocytes (CMs), specific myocytes constituting conducting tissue such as Purkinje fibers, smooth muscle (SM) cells, and non-muscle cells including endothelial cells (ECs) and neuronal cells. Many researchers have developed an *in vitro* system that mimics *in vivo* cardiogenesis in order to better understand the function of heart, and provide technical advantages for drug testing and model for cardiac pathology. However, three-dimensional (3D) heart organoid generation itself has been hampered by the structural complexity of the heart.

For 3D culture methods using embryonic stem cells (ESCs) or adult stem cells, ECMs including Matrigel and laminin were widely used for *in vitro* organogenesis, including the development of the brain¹, stomach², kidney³, and intestinal tissues⁴. In case of 3D heart modeling, FGF can be thought as a good candidate given that FGF and FGFR1 are required for cardiac gene expression.

This heart organoid model system using the laminin-entactin (LN/ET) complex⁵ in the ECM and FGF4⁶ generates heart organoids with atrium- and ventricle-like structures containing CM, conducting tissues, SM and ECs that exhibited myocardial contraction and action potentials. Also, these heart organoids exhibited the ultrastructural, histochemical and gene expression profile similarities to embryonic hearts via consecutive morphological changes like self-organization and, therefore, without the requirement of complex differentiation protocols.

These heart organoids can be used to study mouse heart development and can also provide an effective tool with applications, such as drug safety testing for hearts.

Reagents

□ Gelatin (Nacalai Tesque, 16631-05)

□ DMEM medium (Sigma, D5796)

□PBS (-) (Wako, 166-23555)

□Knockout DMEM (Gibco, 10829-018)

□100 × NEAA (Wako, 139-15651)

□EmbryoMax 100 × Nucleoside for ES cells (Millipore, ES-008-D)

□Penicillin-streptomycin/glutamine (Sigma-Aldrich, G6784)

□CHIR99021 (GSK-3βinhibitor; Axon, 1386)

□PD0325901 (MAPK inhibitor; Wako, 162-25951)

□ESGRO Mouse LIF medium Supplement (LIF) (Millipore, ESG1107)

□Trypsin-EDTA solution (Sigma, T4049)

□Fetal Bovine Serum (FBS) (Gibco, 10437-028)

□Laminin/Entactin Complex (BD, 354259)

□DMEM/F12 medium (Gibco, 11330-032)

□Penicillin/Streptomycin (Sigma, P4333)

□Knockout Serum Replacement (KSR) (Gibco, 10828-028)

□iMatrix-411 (Nippi, 892 042)

□Sodium pyruvate (Sigma, S8636)

□β-Mercaptoethanol (Gibco, 21985)

□L-glutamine (Sigma, G7513)

□Progesterone (Sigma, P6149)

□β-estradiol (Sigma, E2257)

□Insulin (Sigma, I6634)

□Transferrin (Sigma, T8158)

□Sodium selenite (Sigma, S9133)

□Recombinant human FGF4 (R&D systems, 235-F4)

□Recombinant human BMP4 (R&D systems, 314-BP-010)

□BIO (Cayman chemical, 13123)

Reagent setup

□MEF medium: 10% FBS, 2mM L-glutamine, penicillin/Streptomycin (1/100 dilution) in DMEM medium

□ES medium: 20% KSR, 100 µM β-mercaptoethanol, 1× NEAA, 1 × EmbryoMax Nucleoside, penicillin-streptomycin/glutamine (1/100 dilution) in Knockout DMEM

□FBS medium: 20% FBS, 100 µM β-mercaptoethanol, 1× NEAA, 1 × EmbryoMax Nucleoside, penicillin-streptomycin/glutamine (1/100 dilution) in Knockout DMEM

□Heart organoid medium: 20% KSR, 1 mM sodium pyruvate, 100 µM β-mercaptoethanol, 2 mM L-glutamine, penicillin/streptomycin (1/100 dilution), 60 ng/ml progesterone, 30 ng/ml β-estradiol, 5 µg/ml insulin, 20 µg/ml transferrin, and 30 nM selenite in DMEM/F12 medium.

Equipment

□Bio clean bench (Sanyo, MCV-B91F)

□Biological safety cabinet (Sanyo, MHE-130AJ)

□Nitrile, power free exam gloves (Kimberly-Clark, KC100)

□Cell culture humidified CO2 incubator 37°C, 5% CO2

(Sanyo, MCO-19A1C (UV), MCO-19A1C)

□Water bath set at 37°C (TAITEC, Thermo minder SD mini)

□Phase contrast microscope (Olympus, CKX41)

□Table top centrifuge (TOMY, Low speed centrifuge LCX-100)

□Microcentrifuge, cooling (TOMY, MX-150)

□12-well cell culture plates (IWAKI, 3815-012)

□6-well cell culture plates (IWAKI, 3810-006)

□Prime surface 96-well U U-bottom 96-well culture plates (Sumiron, MS-9096U)

□8-well chamber slides (Falcon, 35418)

- 15 ml Centrifuge Tubes, sterile (STAR, RSV-15CT2)
- 50 ml Centrifuge Tubes, sterile (STAR, RSV-50CT2)
- 1.5ml microtubes (Axygen, MCT-175-C)
- Disposable serological 2 ml pipettes, sterile (True line, TR3006)
- Disposable serological 5 ml pipettes, sterile (True line, TR3007)
- Disposable serological 10 ml pipettes, sterile (True line, TR3008)
- Disposable serological 25 ml pipettes, sterile (True line, TR3009)
- 8-channel pipettor (PipetPAL)
- Sterile reagent reservoirs (Integra, #4312)
- Wide bore 200 ml pipette tips (RAININ, BioClean Ultra, RT-UNV-A-200 ml)
- Dissection forceps (FST by DUMONT, #5 INOX)
- Stereomicroscope (Leica, MZ16F)

Procedure

Culture of ESCs

1. Coat 12-well plates with 1 ml of 0.2% gelatin solution per well and place at room temperature (RT) for over 30 minutes.
2. Remove 0.2% gelatin solution by aspirating.
3. Seed mitomycin treated mouse embryonic fibroblast (MEF) feeder cells (100,000 cells per well, 12-well plate) in 1 ml of MEF medium and incubate at 37°C, 5% CO₂ incubator for 1 day.
4. Wash MEF plates with 1 ml of PBS (-) and remove PBS (-).
5. Seed murine ESCs (100,000 cells per well, 12-well plate) on MEF plates in 1 ml of KSR based ESC medium (ES medium) supplemented with CHIR99021 (final 3mM), PD0325901(final 1mM), and LIF (1000 Unit/ml) and incubate at 37°C, 5% CO₂ incubator.
6. Change ES medium every day.
7. After 2 days, aspirate culture medium and wash the cells with 1 ml of PBS (-).
8. Add 0.4 ml of 0.25% Trypsin-EDTA solution and incubate at 37°C for 3 minutes.

9. Add 1 ml of 10% FBS-DMEM medium and pipette cells with 1000 ml tip.
10. Count the number of cells by using hemacytometer.
11. Pool the cells into a 15 ml tube and centrifuge at 1,500 rpm for 5 minutes.
12. Aspirate medium and resuspend the cells with ES medium supplemented with CHIR99021 (final 3mM), PD0325901 (final 1mM), and LIF (1000 Unit/ml).
13. Seed murine ESCs (100,000 cells per well, 12-well plate) on MEF plates and incubate at 37°C, 5% CO₂ incubator.

EB formation

1. Prepare 6-well culture plates coated with 0.2% gelatin solution, before trypsinization of ESCs.
2. Aspirate culture medium from 2 days cultured ESCs and wash the cells with 1 ml of PBS (-).
3. Add 0.2 ml of 0.25% Trypsin-EDTA solution and incubate at 37°C for 2-3 minutes.
4. Add 1 ml of 10% FBS-DMEM medium and pipette cells with 1000 ml tip.
5. Pool the cells and transfer the cells into 6-well plates coated with 0.2% gelatin and incubated at 37°C for 45 minutes to remove feeder cells.
6. Collect floating ESCs gently without contamination of feeder cells and count the number of cells by using hemacytometer.
7. Centrifuge at 1,000 rpm for 5 minutes.
8. Aspirate medium and resuspend the cells with warm FBS-supplemented ES medium without LIF (FBS ES medium).
9. Seed 1,000-5,000 ESCs in 200 ml of FBS ES medium onto each well of a U-bottomed 96-well plate by using 8-channel pipettor.
10. Incubate at 37°C, 5% CO₂ incubator for 4 days without changing medium. The cells form aggregate spheres on the next day of seeding.

Generation of heart organoids

Plate coating with LN/ET gel (85.7 $\mu\text{g}/\text{cm}^2$).

1. Before coating plates, thaw 2 mg/ml of LN/ET complex on ice.
2. Add 30 ml of LN/ET complex into a well of 8-well chamber slides.
3. Incubate 8-well chamber slides at RT for 2 hours.

In vitro culture of EBs for the generation of heart organoids

6. Warm the heart organoid medium and FGF4.
7. Mix the heart organoid medium with FGF4 (30-60 ng/ml).
8. Add 200 ml of the medium in each well of LN/ET gel coated 8-well chamber slides.
9. Carefully transfer an EB (showing sphere morphology) cultured for 4 days by using 200 ml tips, into a well of LN/ET gel coated 8-well chamber slides with the medium.
10. Maintain cultures in 5% CO₂ incubator at 37°C.
11. Replace medium at day3, 5, 7, 9, 11,13,14, and 15.
12. From day9, use FGF4-heart organoid medium additionally supplemented BMP4 (50 ng/ml), BIO (2.5 µM), and LIF (1,000 units/ml). In case of long term culture of heart organoids, such as over 15 days culture, carefully transfer heart organoids into iMatrix-411-coated (10.7 µg/cm²) chamber slides in 200 µl of heart organoid medium.
13. Observe the morphogenesis of heart organoids under microscope. These heart organoids usually show the initial beating movement by 3 days, and continue to grow with morphogenetic changes.
14. Usually collect heart organoids over 9 days of culture after chamber formation. When transfer heart organoids for collecting or performing further analysis, use wide bore tip and/or fine dissection forceps under a stereomicroscope.

Troubleshooting

Time Taken

Day -6 : ES cell seeding (0.5 h)

Day -5 : Medium change (0.5 h)

Day -4 : EB formation (1.5 h)

Day 0 : chamber slide coating with LN/ET complex (> 2 h; depends on the time of thawing LN/ET complex), preparation medium (0.5 h), transfer EBs (0.5 h)

Day 1 to 15 : medium change (0.5 h)

Anticipated Results

This protocol can generate structural and functional heart organoids.

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