Generation of self-assembling heart organoids from human pluripotent stem cells

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Method Article

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Abstract

Studying the development and disease etiology in the developing human heart is limited by the ability to recapitulate the complexity of the heart *in vitro*. This protocol introduces a method to generate complex individually contained heart organoids derived from human pluripotent stem cells (hPSCs). Embryoid bodies (EBs) are formed in a 96 well plate generating 1 EB per well, allowing for 96 individualized experiments with each plate. EBs are differentiated into human heart organoids in a 3-step Wnt pathway modulation strategy including an activation, inhibition and a second activation step corresponding to mesoderm, cardiac mesoderm and proepicardial organ inductions, respectively. The resulting human heart organoids demonstrated cellular, molecular, structural, and transcriptomic features that closely resemble the developing fetal heart.

Introduction

The human heart is a complex functional organ that is comprised of a plexus of cell and tissue types including the myocardium that is made up of atrial and ventricular cardiomyocytes, the epicardium which encapsulates the heart, the endocardium which lines the inner surface of the heart chambers, and other non-myocyte cells such as cardiac fibroblasts and endothelial cells. With cardiovascular disease leading in causes of mortality rates in much of the world, and with congenital heart defects affecting 1 in 1000 live births, the need for reliable models of the human heart is evident. The last two decades have seen great advances in stem cell technologies and differentiation techniques. Human pluripotent stem cells (hPSCs) have been successfully used to generate cardiac cells in vitro1,2 in 2D monolayers. These 2D models, however, lack the 3D complexity and the multi-cell lineages that are present in the human heart.

Organoids, which are 3D in vitro models of organs, can be used to study the development and onset of diseases that affect humans. Organoids demonstrating effective organogenesis and organ recapitulation has been shown for the brain3,4, kidney5,6, liver7,8, pancreas9,10, lungs11,12 and intestine13,14. Recently, methods to create heart organoids from mouse and human stem cells have emerged in attempt to model various aspects and tissues of the human heart15–21. The heart organoid derivation protocol described here employs the use of human pluripotent stem cells to generate highly complex and sophisticated fetal heart organoids in less than three weeks. The differentiation method is easy to implement, requiring only basic cell culture techniques in one easy to follow protocol, is cost-effective, utilizing few chemical inhibitors and growth factors is define media conditions, and is amenable to high-throughput techniques yielding a large number of self-contained samples per experiment.

After 15 days of differentiation, the resulting heart organoids self-organize into complex structures that include multiple internal chambers, endothelial vascular network, and tissues from all three heart layers. Robust beating can be observed as early as 6 days after differentiation begins, with all organoids exhibiting functional activity at day 15. These organoids also allow access to developmental stages of

the human heart that are inaccessible in clinical applications such as heart field formation and early atrioventricular specifications.

The protocol described here introduced a technique to generate sophisticated, self-assembling human heart organoids in a highly reproducible system that allows for high-content studies of heart development, disease etiology and pharmacological screening.

Reagents

Essential 8 Flex Medium Kit (Gibco, A2858501)

DMEM/F12 (Gibco, 10566016)

Penicillin-Streptomycin (Gibco, 15140122)

Thiazovivin (Millipore Sigma, SML1045)

Matrigel GFR (Corning, CB40230)

ReLeSR (Stem Cell Technologies, NC0729236)

Accutase (Innovative Cell Technologies, NC9464543)

Phosphate Buffer Solution (Gibco, 10010049)

RPMI 1640 (Gibco, 11875093)

B-27 Supplement (Gibco, 17504-044)

B-27 Supplement Minus Insulin (Gibco, A1895601)

Dimethyl Sulfoxide (Millipore Sigma, D2650)

CHIR-99021 (Selleck, 442310)

BMP-4 (Gibco, PHC9534)

Activin A (R&D Systems, 338AC010)

WNT-C59 (Selleck, NC0710557)

Paraformaldehyde (MP Biomedicals, IC15014601)

Glycine (Millipore Sigma, 410225)

Equipment

96-well Clear Ultra Low Attachment Microplates (Costar, 07201680)

Moxi Cell Counter (Orflo Technologies, MXZ001)

6-Well Flat Bottom Cell Culture Plates (Corning, 0720083)

15 ml Centrifuge Tubes, sterile (Fisher Scientific, 02682002)

Plate and tube Centrifuge

Biological safety cabinet

Cell culture humidified CO2 incubator

P300 multichannel pipette

P20 micropipette

P200 micropipette

P1000 micropipette

Procedure

PSC culture and passage

Coat 6 wells of a 6-well plate with Matrigel by thawing 0.5 mg of Matrigel in 12 mL of cold DMEM/F12 media and pipetting 2 ml to each well and incubate at 37°C for at least 2 hours before use.

Remove DMEM/F12 from the wells and seed thawed or passaged PSCs at a 1:2 ratio for cryovials and a 1:6 ratio for passages from another 6 well plate, in Essential 8 Flex (E8) media supplemented with 2 µM of Thiazovivin, for a final volume of 2 ml per well.

Incubate at 37°C, 5% CO2 for 24 hours before changing the media to fresh E8 media.

Change the media every 48 hours until cell colonies reach about 70% confluency.

To passage confluent wells, wash each well with 1 ml of 1x phosphate-buffered solution (PBS) for several seconds.

Remove PBS from wells and add 1 ml of ReLeSR followed by aspiration of all but a thin film of ReLeSR after 10 seconds, in accordance with manufacturer instructions. Incubate at room temperature for 2-5 minutes until gaps appear between the cells under a microscope.

Add 1 ml of E8 supplemented with 2 µM of Thiazovivin and gently tap the side of the plate to induce detachment of the cells from the well.

Pipette the detached cells in media a couple of times to break up any large colonies, and transfer to a 15 ml Falcon tube containing E8 supplemented with 2 µM, at a 1:6 ratio (1 well worth of cells into 12 ml or media).

Pipette 2 ml of media with cells to each well of a fresh 6-well plate coated with Matrigel.

Repeat steps 3 to 9.

EB formation (day -2 to day 0)

Day -2: Wash wells containing about 70% confluent PSCs with 1 ml of PBS for several seconds.

Add 1 ml of room temperature Accutase to each well for 3-6 minutes, gently tapping the side of the plate every minute, until most cells detach from the well. (2-3 wells of a 6 well plate yield enough cells for a single 96-well plate worth of EBs).

Add 1 ml of E8 supplemented with 2 µM of Thiazovivin to each well.

Pipette the media with cells several times to create a single cell suspension.

Transfer the media from all wells with detached cells to a 15 ml Falcon tube and centrifuge for 5 minutes at 300g.

Remove supernatant from tube and resuspend in 1 ml of E8 supplemented with 2 µM of Thiazovivin.

Count cells using a Moxi Cell Counter and dilute the cells to a 100,000 cells per ml solution.

Using a multichannel micropipette, distribute 100 µL to each well of a round bottom, ultra-low attachment 96-well plate, resulting in 10,000 cells per well.

Centrifuge the plate for 3 minutes at 100 g.

Incubate for 24 hours at 37°C, 5% CO2.

Day -1: Carefully remove 50 µL of spent media from each well using a multichannel micropipette.

Add 200 µL of fresh E8 (warmed to 37°C), for a final volume of 250 µL per well.

Incubate for 24 hours at 37°C, 5% CO2.

Day 0: initiate differentiation protocol

Heart organoid differentiation (day 0 to day 15 and onwards)

Day 0: Remove 2/3 (166 µL) of media from each well and add 166 µL of RPMI 1640 supplemented with B-27 minus insulin warmed to 37°C, containing 6 µM CHIR99021, 1.875 ng/mL BMP4 and 1.5 ng/mL Activin A (final well concentration of 4 µM CHIR99021, 1.25 ng/ml BMP4, and 1 ng/ml Activin A).

Incubate for 24 hours at 37°C, 5% CO2.

Day 1: Remove 166 µL of media from each well and add 166 µL of RPMI 1640 supplemented with B-27 minus insulin warmed to 37°C.

Incubate for 24 hours at 37°C, 5% CO2.

Day 2: Remove 166 µL of media from each well add 166 µL of RPMI/B27-ins containing 3 µM Wnt-C59 (final well concentration of 2 µM Wnt-C59).

Incubate for 48 hours at 37°C, 5% CO2.

Day 4: Remove 166 µL of media from each well and add 166 µL of RPMI 1640 supplemented with B-27 minus insulin warmed to 37°C.

Incubate for 48 hours at 37°C, 5% CO2.

Day 6: Remove 166 µL of media from each well and add 166 µL of RPMI 1640 supplemented with B-27 warmed to 37°C.

Incubate for 24 hours at 37°C, 5% CO2.

Day 7: Remove 166 µL of media from each well and add 166 µL of RPMI 1640 supplemented with B-27 warmed to 37°C containing 3 µM CHIR99021 (final well concentration of 2 µM CHIR99021).

Incubate for 1 hour at 37°C, 5% CO2.

Remove 166 µL of media from each well and add 166 µL of RPMI 1640 supplemented with B-27 warmed to 37°C.

Incubate for 48 hours at 37°C, 5% CO2.

Day 9 to 15: Remove 166 µL of media from each well and add 166 µL of RPMI 1640 supplemented with B-27 warmed to 37°C.

Incubate for 48 hours at 37°C, 5% CO2.

Continue changing media every 48 hours until organoids are ready for analysis or treatment.

Organoids can be fixed by incubating in 4% Paraformaldehyde for 30-45 minutes are room temperature.

Wash fixed organoid with PBS with 1.5 g/L glycine three times before proceeding to subsequent staining or analysis preparation.

Troubleshooting

Time Taken

Day -2: EB formation (1-1.5 hour)

Day -1: Media change (0.5 hour)

Day 0: Differentiation initiation (0.5-1 hour)

Day 1: Media change (0.5 hour)

Day 2: Wnt inhibition media change (0.5 hour)

Day 4: Media change (0.5 hour)

Day 6: Media change (0.5 hour)

Day 7: Epicardial induction (2 hours)

Day 9-15: Media change (0.5 hour)

Anticipated Results

Complex functional human heart organoids containing cardiomyocytes, endocardial cells, epicardial cells, cardiac fibroblasts, endothelial cells in a vessel-like network and internal chambers is expected. Robust beating should be observable as early as day 6, with all organoids beating by day 15. Organoids can be used to investigate steps of heart development including mesoderm formation (days 0-2), cardiac mesoderm formation (days 3-6), heart field specification (days 3-9), proepicardial tissue formation (days 7-15), endothelial network formation (days 11-13) and chamber formation (days 5-15).

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