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Reconstructing human somitogenesis with somitoid and segmentoid

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Abstract

The metameric organization of vertebrates is first implemented when somites, which contain the precursors of skeletal muscles and vertebrae, are rhythmically generated from the presomitic mesoderm[1]. This process of somitogenesis is vital for body plan development[2], yet very little is known about human somitogenesis given limited access to early embryos and ethical concerns. Here we develop two novel 3D culture systems of human pluripotent stem cells (PSCs), called somitoids and segmentoids, which can recapitulate the formation of epithelial somite-like structures[3]. In contrast to gastruloids[4,5] or trunk-like structures[6] which harbor cell lineages derived from the three germ layers, our two models contain almost exclusively paraxial mesoderm. Somitoids recapitulate the temporal sequence of somitogenesis, with all cells undergoing differentiation and morphogenesis in a synchronous manner. This system can provide unlimited amounts of cells precisely synchronized in their differentiation and will allow exploring these patterning processes at an unprecedented level of detail. On the other hand, segmentoids reconstruct the spatio-temporal features of somitogenesis, including gene expression dynamics, tissue elongation, sequential somite morphogenesis, and AP polarity patterning. They therefore provide an excellent proxy to study human somitogenesis. Together, these two complementary models provide a valuable platform to decode general principles of somitogenesis and advance knowledge of human development.

Introduction

Reagents

Matrigel (Corning 35277) – *for iPS cell culture*

Matrigel (Corning CB-40234A) – *for segmentoid embedding*

mTeSR1 medium (StemCell Technologies 05851)

Accutase (Corning 25058CI)

Y-27362 dihydrochloride (Tocris Bioscience1254)

DMEM/F12 GlutaMAX (Gibco10565042)

Insulin-transferrin-selenium (Gibco 41400045)

CHIR99021 (Tocris 4423)

LDN193189 (Stemgent 04-0074)

Laminin-521 (Stemcell Technologies 77003)

0.1% Gelatin in water (Stemcell Technologies 07903)

Fetal Bovine Serum (Sigma-Aldrich EmbryoMax ES-009-B)

Neurobasal medium (Gibco 21103049)

N2 Supplement-B (Stemcell Technologies 07156)

B-27 Supplement (Gibco 17504044)

2-Mercaptoethanol (Gibco 21985023)

DPBS (Gibco 14190-250)

Equipment

6-well TC cell culture plate (Falcon 353046)

96-well U-bottom non-treated plate (Greiner bio-one 650185 or Falcon 351177) – *for somitoid*

96-well cell-repellent U-bottom plate (Greiner bio-one 650970) – *for segmentoid*

Automatic cell counter (Nexcelom Cellometer)

Cell counting chamber (Nexcelom CHT4-SD100-002)

Centrifuge

Pipettes and tips

Conical Centrifuge Tubes

Stereo microscope in the cell culture hood

Incubator with 37℃, 5.5% CO2, and humidity

Procedure

Human iPS cell culture

1. Add 1ml ice cold Matrigel solution (diluted with DMEM/F12; dilution fold as suggested by the manufacturer) into one well of a 6-well cell culture plate and incubate at 37℃ for 20 min.

2. Remove cell culture medium, wash twice with 1ml PBS, add 1ml Accutase, and incubate at 37℃ for 5min.

3. Gently pipette mix and transfer into a conical tube with 4 ml DMEM/F12. Centrifuge at 300rcf for 4 min.

4. Remove supernatant and gently resuspend cell pellet using 2ml mTeSR1 + 10μM Y-27362. Load 20 μl cell mix into the cell counting chamber, determine cell density using the automatic cell counter, and calculate the volume needed for 500,000 cells.

5. Remove all medium in the Matrigel-coated plate and add 1.5ml mTeSR1 + 10μM Y-27362. Add 500,000 cells into the well, shake the plate well to mix evenly, and put it into the 37℃ incubator with 5.5% CO2 and humidity.

6. Next day, change medium with 2 ml mTeSR1.

7. Next day, change medium with 2.5 ml mTeSR1.

8. Next day, change medium with 3.5 ml mTeSR1.

9. Next day, start again from Step 1.

Somitoid protocol

(**Day -1**)

1. Remove the medium of iPS cell culture (~80% confluency), wash twice with PBS, add 1ml Accutase, and incubate at 37℃ for 5min. *More confluent culture tends to result in increased organoid death in this protocol.*

2. Gently pipette mix and transfer into a conical tube with 4 ml DMEM/F12. Centrifuge at 300rcf for 4 min.

3. Remove supernatant and gently resuspend cell pellet using 2ml mTeSR1 + 10μM Y-27362. Load 20 μl cell mix into the cell counting chamber, determine cell density using the automatic cell counter.

4. Calculate and dilute cell mix using mTeSR1 medium + 10 μM Y-27362, so that 100 μl diluted cell mix contain 3,000 cells. Dispense 100 μl into each well of the 96-well U-bottom non-treated plates (Greiner bio-one 650185 or Falcon 351177). *Do not use the cell-repellent plate as used in the segmentoid protocol.*

5. Centrifuge the plate at 300 rcf for 2 min and put it into the incubator without disturbing the pellet.

(**Day 0**)

1. Prepare DICL medium: DMEM/F12 supplemented with 1% insulin-transferrin-selenium (ITS; Gibco 41400045), 3 μM CHIR99021 (Tocris 4423) and 0.5 μM LDN193189 (Stemgent 04-0074).

2. Remove all medium from each well under a stereo dissection microscope in the cell culture hood. The aggregate often appears as a flat disk attached at the bottom, which makes it easy to remove as much

medium as possible. Sometimes several small aggregates are formed but they will fuse in subsequent culture.

3. Add 150 µl DICL medium. Gently pipette the aggregate under a stereo dissection microscope to make sure it is unattached from the well. Put the plate back into the incubator.

(**Day 1**)

1. The aggregate should appear as a sphere resting in suspension on the bottom of the well. Remove 130 µl medium from each well and add in 150 µl fresh DICL medium. This step is optional as we did not observe any difference when no medium change is performed on Day 1.

(**Day 2**)

1. Add 1ml 0.1% gelatin solution (Stemcell Technologies 07903) or other coating solution to a well of 6-well TC plate. Incubate at 37℃ for 1h.

2. Prepare somite-inducing medium (SIM): DMEM/F12 supplemented with 1% ITS, 1.5 µM CHIR99021, 0.5 µM LDN193189, and 5% Fetal Bovine Serum (FBS; Sigma-Aldrich EmbryoMax ES-009-B). *Note that the concentration of CHIR99021 is lowered by half from what is used in DICL medium.*

3. Remove all gelatin solution from the well and add in 2 ml SIM medium.

4. Transfer the organoids one by one from the U-bottom wells into the flat TC plate well, using the 200 µl pipette. Under the stereo microscope, position the organoid at the very tip so very small release with little carry-over medium is sufficient for the transfer. Up to 20 organoids can be put into one well.

5. Shake the plate to mix well the organoids and incubate without further medium change.

Segmentoid protocol

(**Day -1**)

1. Add 1ml ice cold Matrigel solution (diluted with DMEM/F12; dilution fold as suggested by the manufacturer) into one well of a 6-well cell culture plate and incubate at 37℃ for 20 min.

2. Remove the medium of iPS cell culture (~100% confluency), wash twice with PBS, add 1ml Accutase, and incubate at 37℃ for 5min.

3. Gently pipette mix and transfer into a conical tube with 4 ml DMEM/F12. Centrifuge at 300rcf for 4 min.

4. Remove supernatant and gently resuspend cell pellet using 2ml mTeSR1 + 10μM Y-27362. Load 20 μl cell mix into the cell counting chamber, determine cell density using the automatic cell counter, and calculate the volume needed for 200,000 cells.

5. Remove all medium in the Matrigel-coated plate and add 1.5ml mTeSR1 with 10μM Y-27362. Add 200,000 cells into the well, shake the plate well to mix evenly, and put it into the 37℃ incubator with 5.5% CO2 and humidity. *The cell number per well is the most important parameter throughout this protocol.*

(**Day 0**)

1. Prepare DICL medium: DMEM/F12 supplemented with 1% insulin-transferrin-selenium (ITS; Gibco 41400045), 3 μM CHIR99021 (Tocris 4423) and 0.5 μM LDN193189 (Stemgent 04-0074).

2. Remove all medium from each well and add 2 ml DICL medium.

(**Day 1**)

1. Prepare N2B27 medium: 1:1 mix of DMEM/F12 and Neurobasal medium (Gibco 21103049), supplemented with 1% N2 Supplement-B (Stemcell Technologies 07156), 2% B-27 Supplement (Gibco 17504044), and 0.1% 2-Mercaptoethanol (Gibco 21985023).

2. Remove all DICL medium and wash once with PBS. Add 1ml Accutase and incubate at 37℃ for 5min.

3. Gently pipette mix and transfer into a conical tube with 4 ml DMEM/F12. Centrifuge at 300rcf for 4 min.

4. Remove supernatant and gently resuspend cell pellet using 300 μl N2B27 + 10μM Y-27362. Load 20 μl cell mix into the cell counting chamber, determine cell density using the automatic cell counter.

5. Add 150 μl N2B27 + 10μM Y-27362 into each well of the 96-well cell-repellent U-bottom plate (Greiner bio-one, 650970). *Note that the plate is different from what is used in the somitoid protocol.*

6. Calculate volume and add 6,000-8,000 cells into each well.

7. Centrifuge the plate at 300 rcf for 2 min and put it into the incubator without disturbing the pellet.

(**Day 2**)

1. Thaw Matrigel (Corning CB-40234A) on ice and prepare ice-cold 10% Matrigel solution using N2B27 media.

2. A single spheroid should be formed in each well. Remove medium under a dissection microscope and add 30 μl ice-cold 10% Matrigel. Gently tap the plate to facilitate each organoid to sink to the bottom.

3. Incubate the plate at 37°C for one hour.

4. Carefully add 150 µl N2B27 medium to each well. Lean the tip against the inside wall to avoid breaking the solidified Matrigel. Incubation and no further medium change is needed.

Troubleshooting

1. We found that CHIR99021 from different companies might have varied qualities, thus the concentration might need to be adjusted accordingly.

2. Note that different kinds of 96-well U-bottom plates are used for somitoid and segmentoid protocols. While we found that the cheap, non-treated plates work best for somitoid, the ultra-low adhesive, cell-repellent plates are important for segmentoid.

3. For somitoid, the aggregation size is important. Aggregates of <2,000 cells often lead to cell death, while aggregates of >4,000 cells often lead to poor differentiation in the center. Also, 100% confluent iPS culture for aggregation often results in unhealthy somitoids and for this protocol we dissociate cells when they are ~80% confluent.

4. For segmentoid, the cell number on Day -1 is the single most important parameter of this protocol. Higher cell number during the 2D differentiation step will lead to poorly elongated and poorly patterned structures with accelerated epithelization speed, which seem to be resulted from a faster exhaustion of the NMP population.

We want to point out that the exact cell number listed in our protocol is only meaningful in specific contexts. Many routines and habits of cell culture can introduce variations. For example, different labs grow iPS cells in different ways. In our cell culture method, dissociation of iPS cell culture at different confluency will lead to different cell survival rates next day; Thus seeding the same number of cells does not necessarily mean the same actual cell density next day. In addition, different counting methods and habits are adopted among labs. We suggest that to start with, a range of cell numbers (e.g. 180K, 210K, 240K, 270K) should be tried on Day -1. Once a working number is calibrated, keep cell culture routine consistent to lower variability: dissociating cell culture at the same confluency, counting cells in the same way, paying attention to the time between media change, etc.

Time Taken

Anticipated Results

Somitoid: The organoid should spread to the fullest by the end of Day 3, appearing as a flat disk attached on the surface. “Cracks” should be observed on Day 4 and a field of somite-like structures should be visible under a bright-field microscope on Day 5.

Segmentoid: The organoid should break symmetry on Day3 and start to elongate; On Day 4 the organoid should contain half segmented and half unsegmented regions, while on Day 5 the organoid should

appear as a segmented rod with somite-like structures clearly visible.

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