Human embryonic organoids to recapitulate periodic somitogenesis *in vitro*

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Method Article

Keywords: Induced pluripotent stem cell (iPSC), Organoid, Self-organization, Presomitic mesoderm (PSM), Somites, Somitogenesis, Epithelialization

Posted Date: April 29th, 2022

DOI: https://doi.org/10.21203/rs.3.pex-1852/v1

Abstract

Somites are epithelial structures that are periodically formed on both sides of the neural tube during vertebrate embryogenesis. Here we describe a protocol to generate human embryonic organoids, somitoids, that recapitulate periodic somitogenesis from human induced pluripotent stem cells (iPSCs) in one week (Sanaki-Matsumiya et al., 2022). Somitoids display the oscillations and waves of the segmentation clock, and the presomitic mesoderm (PSM) undergoes epithelialization and forms pairs of somite-like structures according to the segmentation clock.

Introduction

The formation of bilateral somites out of the PSM tissue, somitogenesis, is regulated by the molecular oscillator called the segmentation clock (Hubaud et al., 2014), and the formed somites display organized epithelial structures. Somites are precursor tissues of the vertebrate, rib cage, and part of the skin, and thus abnormalities in somitogenesis can cause congenital disorders in axial skeletons (Turnpenny et al., 2007). As studying somitogenesis in human embryos is ethically challenging, modeling the process *in vitro* should contribute to a better understanding of human somitogenesis in health and disease.

Mouse somitogenesis has recently been modeled with embryonic organoids from mouse embryonic stem cells (Matsumiya et al., 2018; van den Brink et al., 2020; Veenvliet et al., 2020). Regarding human models, PSM-like cells that display the segmentation clock as well as somite-like tissues have been induced from human pluripotent stem cells (Chu et al., 2019; Matsuda et al., 2020; Diaz-Cuadros et al., 2020; Budjan et al., 2022). However, there is no human organoid to recapitulate both periodic somitogenesis coupled with the segmentation clock and the maturation into epithelial structures.

We have developed and characterized novel human embryonic organoids, termed somitoids, that periodically form epithelial somites (Sanaki-Matsumiya et al., 2022). Here we describe the step-by-step protocol of human somitoids. Somitoids are generated as aggregates of human iPSCs and cultured in the presence of a WNT activator, BMP inhibitor, TGFb inhibitor, and FGF for 2 days. Subsequently, the somitoids start elongating and displaying the oscillatory expression of the segmentation clock in the PSM. When Matrigel is added on day 4, the PSM of somitoids undergoes epithelialization and periodically forms somite-like structures.

Reagents

Note: Several materials in the somitoid protocol are irreplaceable. The incompatible materials we tested so far are listed in Table 1.

For maintenance of human iPSCs

Human iPSCs (201B7 line, #HPS0063; Takahashi et al., 2007)

Recombinant Laminin iMatrix-511silk E8 (Laminin; Amsbio, AMS.892 021)

StemFit Basic04 Complete Type (StemFit04CT; Ajinomoto, B4BASIC04CT)

0.5x CTS™ TrypLE™ Select Enzyme (0.5x TrypLE; Gibco, A12859-01; diluted by 0.5 mM EDTA / PBS)

Y-27632 (Sigma, Y0503)

1x Phosphate buffered saline (PBS; without Mg^{2+} and Ca^{2+})

0.5 mM EDTA pH8 (in PBS)

Cell Lifter (STEMCELL, 38067)

Note: Penicillin-streptomycin should not be used during maintenance as it slows down cell proliferation.

For induction of somitoids

DMEM/F12 (Gibco, 21331020)

N2 supplement (R&D, AR009)

Neurobasal medium (Gibco, 21103049)

B27 supplement (Gibco, 17504-044)

Glutamax (Gibco, 35050-038)

Nonessential amino acids (NEAA; Gibco, 11140-035)

Sodium pyruvate (Gibco, 11360-039)

Penicillin-streptomycin (Gibco, 15140122)

Note: The basal medium of somitoids (N2B27 medium) is a mixture of DMEM/F12 with 1x N2 supplement and Neurobasal medium with 1x B27 supplement at a 1:1 ratio. The N2B27 medium was also supplemented with 2 mM Glutamax, 0.1 mM NEAA, 1 mM sodium pyruvate, and penicillin-streptomycin. The medium should be stored at 4 °C and used within a month. Alternatively, the medium can be prepared without the N2 and B27 supplements and stored at 4 °C for a few months, and the supplements can be added to the medium immediately before use.

SB431542 hydrate (Sigma, S4317)

CHIR99021 (Sigma, SML1046)

DMH1 (Sigma, D8946)

Recombinant Human FGF-basic (bFGF; PeproTech, AF-100-18B)

Note: The somitoid induction medium is the N2B27 medium containing 10 μM SB431542, 10 μM CHIR99021, 2 μM DMH1, and 20 ng/ml bFGF. Prepare it immediately before use.

U-Shaped-Bottom, 96-well-plate (96U-well plate; Thermo Scientific, 174925)

Growth factor reduced Matrigel (Matrigel; Corning, 356231)

Equipment

Procedure

Culturing human iPSCs

Note: Prewarm all media to room temperature (RT) before use.

1. Coat a 3.5-cm culture dish with 2 ml of 2.4 μg/ml laminin in PBS at 37 °C for 1 hr.

2. Replace the laminin solution with 1.5 ml StemFit04CT medium and keep the dish at 37 °C and 5% CO_2.

3. When human iPSCs reach 70-80% confluency, remove the StemFit04CT medium and wash with 2 ml PBS twice.

4. Add 300 μl of 0.5x TrypLE and incubate at 37 °C for 3-4 min.

Note: Too long incubation is harmful to cell growth and colony morphology.

5. Remove 0.5x TrypLE and wash with 2 ml PBS twice.

6. Add 1 ml StemFit04CT medium containing 10 μM Y-27632 and collect cells with a scraper.

7. Dissociate into single cells by pipetting.

8. Transfer the cell suspension into a 1.5 ml tube.

9. Count the cell number using a counting chamber.

10. Seed 1.2-1.5 x 10^4 cells to the laminin-coated dish.

11. Culture the cells at 37 °C and 5% CO2.

12. Next day, change the medium to the fresh StemFit04CT medium without Y-27632.

Note: An ideal passage frequency is every 6 days.

Generation of human somitoids

Note: Before making somitoids, make sure that the human iPSCs have a 'good colony' morphology (Fig. 1).

Induction Day -4: Preculture of human iPSCs

1. Collect cells according to steps 1 through 9 of 'Culturing human iPSCs'.

2. Seed 2.0-2.4 x 10^4 cells to the 3.5-cm laminin-coated dish.

3. Culture the cells at 37 °C and 5% CO2.

Induction Day -3 and Day -1: Medium change

1. Change the medium to the fresh StemFit04CT medium without Y-27632.

Induction Day 0: PSM induction from human iPSCs

Note: The cells should be 50-70% confluent on Day 0.

1. Prepare the somitoid induction medium (the N2B27 medium containing SB431542, CHIR99021, DMH1, and bFGF) at RT immediately before use.

2. Remove the StemFit04CT medium from the 3.5-cm dish of iPSCs and wash with PBS twice.

3. Add 2 ml of 0.5 mM EDTA and incubate at 37 °C for 6-7 min.

4. Dissociate into single cells by pipetting.

5. Transfer the cell suspension into a 15 ml tube containing 8 ml of the N2B27 medium.

6. Centrifuge at 152 ×g for 3 min at RT and remove the supernatant.

7. Resuspend the cell pellet in 10 ml of the N2B27 medium.

8. Repeat 6-7

9. Centrifuge at 152 ×g for 3 min at RT and remove the supernatant completely.

10. Resuspend into 150-500 µl of the somitoid induction medium containing 10 µM Y-27632.

11. Count the cell number.

Note: 6.0-10.0 x 10^4 cells should be collected from a 3.5-cm dish.

12. Aliquot 50 µl of the cell resuspension (350 cells) to each well of a 96U-well plate.

13. Centrifuge at 152 ×g for 2 min at RT.

14. Incubate at 37 °C and 5% CO2.

Induction Day 1: Medium change

1. Add 150 µl of the somitoid induction medium without Y-27632.

2. Incubate at 37 °C and 5% CO2.

Induction Day 2 and Day 3: Medium change to N2B27

1. Remove 150 µl of the medium and add 150 µl of the fresh N2B27 medium (without SB431542, CHIR99021, DMH1, or bFGF).

2. Incubate at 37 °C and 5% CO2.

Note: Somitoids should start showing the oscillations of the segmentation clock on Day 2-3, and the shape of somitoids should become oval on Day 3-4.

Induction Day 4: Matrigel addition

1. Remove 150 µl of the medium and add 150 µl of the fresh N2B27 medium containing 10% Matrigel.

2. Incubate at 37 °C and 5% CO2.

Note: The medium is not changed after Matrigel addition. Matrigel solution should not be reused.

Note: Somitoids start forming somites after Matrigel addition on Day 4. By Day 7, approximately 10 rows of somites are formed. Example images of somitoids are shown in Fig. 2 and 3.

Troubleshooting

iPSCs do not grow well

The morphology of iPSC colonies is critical (Fig. 1). To remove ‘bad colonies’, culture cells at low densities. Or pick a ‘good colony’ and expand the cells.

Modulate the concentration of laminin coating and/or the incubation period with 0.5x TrypLE when passaging.

Cell collection rate is low on Day 0

Modulate the incubation period with 0.5 mM EDTA.

Using the Essential 6 (E6) medium for centrifugation instead of N2B27 may improve the collection rate.

Cells do not aggregate on Day 1

Use iPSCs with a ‘good colony’ morphology (Fig. 1).

Modulate the incubation period with 0.5 mM EDTA on Day 0; it can also be changed to 2.5 mM EDTA for 6-7 min or 0.5x TrypLE for 3-4 min.

The N2B27 medium is old; use a fresh medium.

The initial cell number is not appropriate; it can be modulated within the range of 350-1000 cells, depending on the cell line.

The procedures on Day 0 take too long; they should be done within 30-45 min.

Aggregates do not grow on Day 2-4

Use iPSCs with a ‘good colony’ morphology (Fig. 1).

Modulate the initial cell number.

Somites are not formed on Day 5-7

Matrigel is old; use a freshly thawed one.

Modulate the concentration of Matrigel within the range of 5 -10%.

Time Taken

Anticipated Results

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Acknowledgements

We would like to thank all members of the Ebisuya laboratory and EMBL Barcelona for support and advice.

This work was supported by EMBL; Takeda Science Foundation (to M.E.); the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No. 101002564) (to M.E.); Project PGC2018-097872-A-I00 (MCIU/AEI/FEDER, UE) funded by the Spanish Ministry of Science, Innovation and Universities (MCIU) and co-funded by the European Regional Development Fund (ERDF, EU) (to M.E.); and fellowships from the Japan Society for the Promotion of Science (JSPS) and Daiichi Sankyo Co., Ltd. (to M.S-M.).

Figures

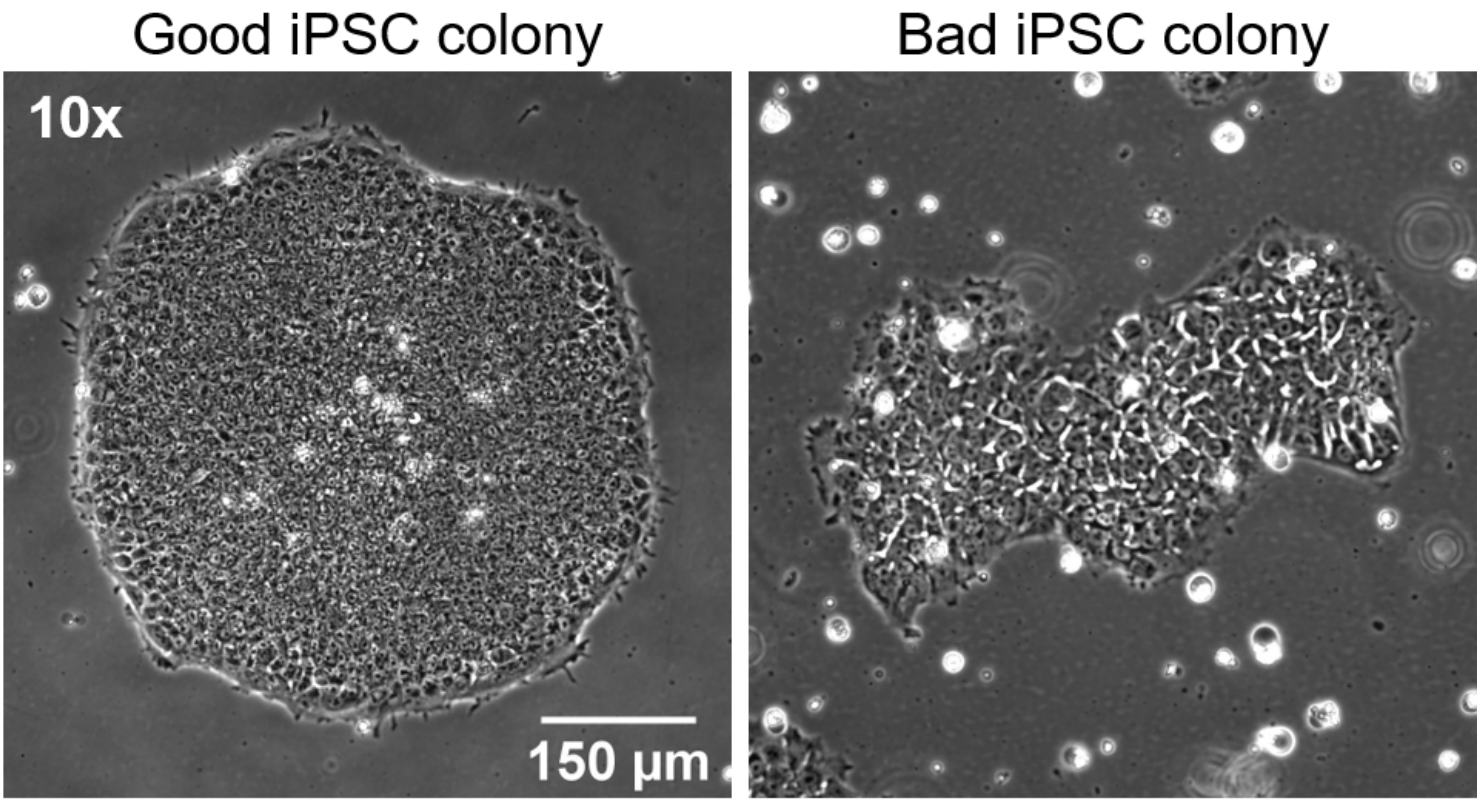

Figure 1

Figure 1

Morphology of human iPSC colonies. iPSCs with a ‘good’ colony morphology are suitable to make somitoids.

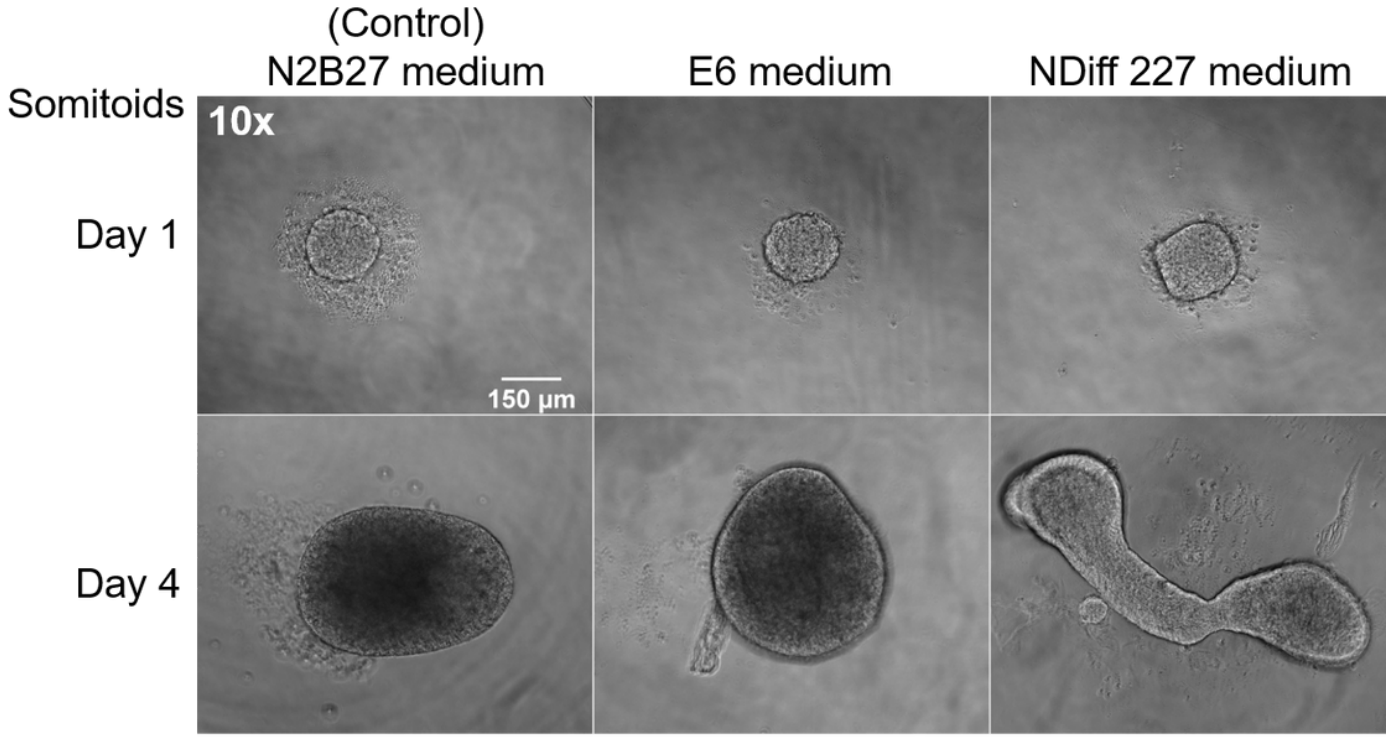

Figure 2

Figure 2

Day 1 and Day 4 somitoids with different basal media. The original protocol uses N2B27 medium.

Figure 3

Day 7 somitoids with N2B27 medium with 10% Matrigel (left) or E6 medium with 10% Matrigel (right). Matrigel was added on Day 4. The original protocol uses N2B27 medium.

Figure 4

Day 1 somitoids with different 96U-well plates. The original protocol uses Thermo 96U-well plate (left).

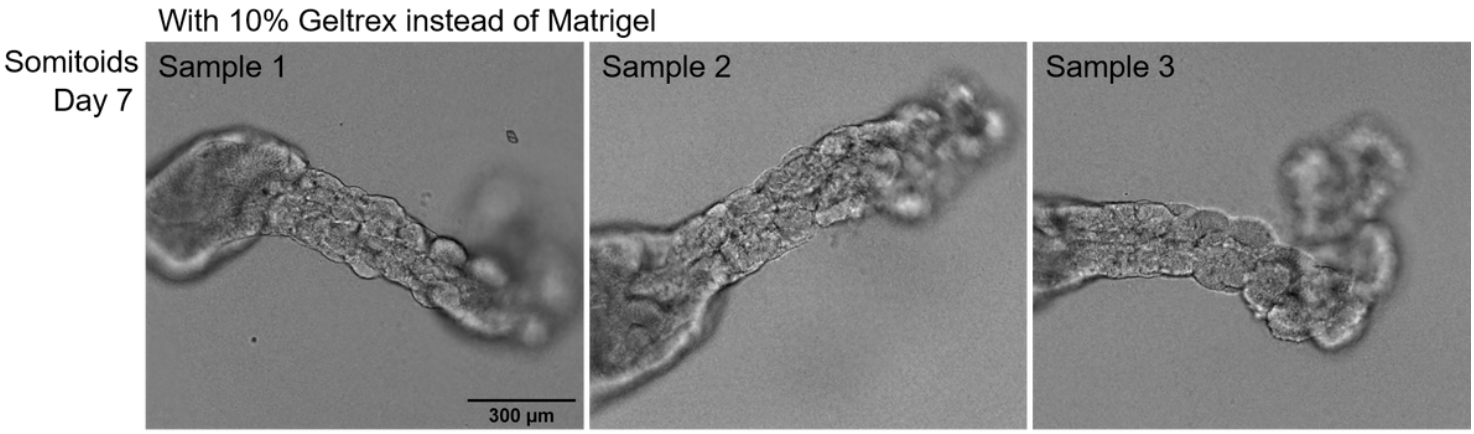

Figure 5

Three individual samples of Day 7 somitoids with 10% Geltrex instead of Matrigel. Geltrex was added on Day 4.

Supplementary Files

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

- Table1.xlsx