

Creation of bladder assembloids by reconstituting tissue stem cell/tumour cell-derived organoids with multiple stromal components

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Method Article

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Abstract

Current organoid models are limited by their inability to mimic mature organ architecture and associated tissue microenvironments^{1,2}. Here, we create multi-layered normal bladder assembloids and tumour assembloids by reconstituting tissue stem cell/tumour cell-derived organoids with multiple stromal components to represent an organized tissue architecture and functionality of adult bladder, and to mimic *in vivo* pathophysiological features of patient-derived urothelial carcinoma, respectively. Our assembloid model will facilitate development of an innovative model system for studying tissue regeneration and tumorigenesis at molecular and cellular levels, and also provide a unique experimental tool for establishing novel personalized therapeutic options, such as new drug screening strategies that are customized for different stages and types of cancer in individual patients.

Introduction

Organoids are derived from three major cell types: tissue-restricted adult stem cells, pluripotent stem cells and, in the case of tumour organoids, tumour cells (reviewed in^{3,4}). These organoids are widely used for modelling key characteristics of organs and tissues to better understand various aspects of human disease such as cancer. However, current organoids are still limited because such model systems fail to account for a large number of factors, including native tissue architecture and surrounding microenvironment^{1,2,5}.

In this study, we report the 3D reconstitution of normal bladder and patient-specific bladder tumour “assembloids” - organoids derived from tissue stem/tumour cells reconstituted with other components of the tissue stroma/microenvironment such as stromal fibroblasts, endothelial cells, and muscle cells.

Reagents

Cells

- Long-term cultured (> 6 months) mouse bladder organoids
- Mouse embryonic fibroblasts (MEF)
- HULEC
- Mouse bladder smooth muscle cells (BSMC)
- Human bladder tumor organoids
- Cancer-associated fibroblasts (CAF)

Reagents

- DMEM (Welgene, # LM001-07)
- FBS (Merck, # ES009B-KC)
- Penicillin/streptomycin (Gibco, # 15140122)
- Advanced DMEM/F-12 (Gibco, # 12634028)
- HEPES (Sigma, # H3375)
- Nicotinamide (Sigma, # 3376)
- N-acetyl-L-cysteine (Sigma, #A9165)
- GlutaMAX™ Supplement (Gibco, # 35050061)
- Recombinant mouse EGF (Peprotech, # 315-09)
- 50X B-27 supplement (Gibco, #17505-044)
- A8301 (Tocris, # 2939)
- Growth factor reduced, Matrigel (Corning, # FAL-354230)

Equipment

- 12-well Not treated culture plates (Corning, # 351143)
- Hemocytometer (Marienfeld, # 0640010)
- Parafilm (Sigma, # P7543)
- Microcentrifuge tube (Axygen, # MCT-150-C)
- 15 ml tube (SPL, # 50015)
- 35 mm petri-dish (SPL, # 10035)
- 100 mm petri-dish (SPL, # 10090)
- Spray bottle containing 70% (vol/vol) ethanol
- Sterilized scissors
- Fine forceps
- Centrifuge

- 12-well version of spinning reactor or Orbital shaker
- Biosafety cabinet
- CO₂ incubator

Procedure

*** Perform every step in the biosafety cabinet**

Generation of normal mouse bladder assembloids

- * This protocol is standardized to a preparation of culture in 2 wells of 12-well plate (= 10 assembloids).
- * A day before, thaw 60 µl of Matrigel overnight at 4 °C.
- * Prepare long-term (> 6 months) bladder organoids, mouse embryonic fibroblasts (MEF), HULEC, bladder smooth muscle cells (BSMC) cultures in advance.

1. Prepare two sheets of Parafilm at the size of 1.5 cm x 1.5 cm, put them in a 100 mm petri dish.

* Spray your gloves and the Parafilm with 70 % (vol/vol) ethanol.

2. Prepare long-term bladder organoids.

a. Release the bladder organoids from Matrigel drop by physically pipetting the organoid medium and collect them in a new 15 ml tube.

b. Centrifuge at 1,500 rpm, 4 °C, for 5 min.

c. Remove the supernatant, wash the organoid pellets with 1 ml of Advanced DMEM/F12 medium.

d. Centrifuge at 1,500 rpm, 4 °C, for 5 min.

e. Remove the supernatant, add 1-2 ml of DMEM containing 10 % FBS and 1 % penicillin/streptomycin.

f. Transfer the organoids with medium to the 35 mm petri dish, plate the dish on ice.

3. Prepare MEF and HULEC mixture.

a. Prepare 1.5×10^6 MEF and 2×10^5 HULEC, put together in one microcentrifuge tube.

b. Centrifuge at 1,500 rpm, 4 °C, for 3 min.

- c. Remove the supernatant, add 40 μ l Matrigel to cell pellets (perform on ice), plate on ice
4. Set 20p pipette at 4 μ l, take one organoid only.
 - * Take < 1 μ l of organoid (+ medium that follows when picking the organoid) while holding it.
5. Take up to 4 μ l of MEF and HULEC mixture.
 - * Mix the MEF and HULEC mixture well before taking the mixture.
6. Make the Matrigel droplet (containing an organoid, MEF, and HULEC) on a sheet of Parafilm.
7. Place the organoid in the center of the droplet by gently pipetting the Matrigel droplet.
8. Repeat the procedure 6-7 to generate 10 droplets.
 - * Make 5 droplets on each sheet. This generates 10 droplets on 2 sheets.
9. Incubate at 37 °C for 5-10 min to solidify the gel.
10. Remove the droplets from the Parafilm using a fine forceps, put them in a 12-well version of spinning bioreactor containing 2 ml of pre-warmed assembloid medium.
 - * Put 5-7 droplets in each well of 12-well plate.
11. Culture the droplets (= organoid with stroma) in a spinning bioreactor in 37 °C incubator with 5.0 % CO₂ for two days.
 - * If you don't have a 12-well version of spinning bioreactor, orbital shaker (95 rpm) can be alternatively used.
12. Prepare two sheets of Parafilm at the size of 1.5 cm x 1.5 cm, put them in a 100 mm petri dish.
 - * Spray your gloves and the Parafilm with 70 % (vol/vol) ethanol.
13. Prepare BSMC and HULEC mixture.
 - a. Prepare 6 x 10⁵ BSMC and 6 x 10⁵ HULEC, put together in one microcentrifuge tube.
 - b. Centrifuge at 1,500 rpm, 4 °C, for 3 min.
 - c. Remove the supernatant, add 20 μ l Matrigel to cell pellets (perform on ice), plate on ice
14. Take out the cultures of bladder organoid with stroma from the incubator, take each of them (+ medium that follows when picking the organoid with stroma) using 20p pipette, and put on a sheet of Parafilm.

15. After removing the residual medium from the Parafilm, take 2 µl of BSMC and HULEC mixture using 20p pipette, put it on each of organoid with stroma to make the layered Matrigel droplet.

* Place the organoid with stroma in the center of the droplet by gently pipetting the Matrigel droplet.

16. Repeat the procedure 14-15 to generate 10 droplets.

* Make 5 droplets on each sheet. This generates 10 droplets on 2 sheets.

17. Incubate at 37 °C for 5-10 min to solidify the gel.

18. Remove the droplets from the Parafilm using a fine forceps, put them in a 12-well version of spinning bioreactor containing 2 ml of pre-warmed assembloid medium.

* Put 5-7 droplets in each well of 12-well plate.

19. Culture the droplets (= assembloids) in a spinning bioreactor in 37 °C incubator with 5.0 % CO₂ for seven days

* Medium change: every other day

Generation of human bladder tumour assembloids

* This protocol is standardized to a preparation of culture in 6 wells of 12-well plate (= 30 assembloids)

* A day before, thaw 150 µl of Matrigel overnight at 4 °C.

* Prepare human bladder tumour organoids (~ 1000 organoids at day 10), patient-derived cancer-associated fibroblasts (CAF), HULEC cultures in advance.

1. Prepare six sheets of Parafilm at the size of 1.5 cm x 1.5 cm, put them in a 100 mm petri dish.

* Spray your gloves and the Parafilm with 70 % (vol/vol) ethanol.

2. Prepare patient-derived human bladder tumour organoids.

a. Release the tumour organoids at day 10 from a Matrigel drop (generally contains 1,000 organoids) by physically pipetting the organoid medium and collect them in a new 15 ml tube.

b. Centrifuge at 1,500 rpm, 4 °C, for 5 min.

- c. Remove the supernatant, add 1 ml of Advanced DMEM/F12 medium and transfer to a new microcentrifuge tube.
 - d. Centrifuge at 1,500 rpm, 4 °C, for 5 min.
 - e. Remove the supernatant, add 200 µl of Advanced DMEM/F12 medium, place on ice.
3. Prepare CAF and HULEC.
 - a. Prepare 7.5×10^6 CAF and 6×10^5 HULEC.
 4. Put CAF and HULEC together in the microcentrifuge tube containing tumour organoids prepared above.
 5. Centrifuge at 1,500 rpm, 4 °C, for 3 min.
 6. Remove the supernatant, add 150 µl Matrigel and mix well (perform on ice)
 7. Take 5 µl of mixture containing tumour organoids, CAF, and HULEC and make the Matrigel droplets on a sheet of Parafilm.
 8. Repeat the procedure 7 to generate 30 droplets.
 - * Make 5 droplets on each sheet. This generates 30 droplets on 6 sheets.
 9. Incubate at 37 °C for 5-10 min to solidify the gel.
 10. Remove the droplets from the Parafilm using a fine forceps, put them in a 12-well version of spinning bioreactor containing 2 ml of pre-warmed assembloid medium.
 - * Put 5-7 droplets in each well of 12-well plate.
 11. Culture the droplets (= tumour assembloids) in a spinning bioreactor in 37 °C incubator with 5.0 % CO₂ for seven days.
 - * If you don't have a 12-well version of spinning bioreactor, orbital shaker (95 rpm) can be alternatively used.
 - * Medium change: every other day

Troubleshooting

- Failed formation of central lumen and epithelial folds in normal bladder assembloids: Use big (> 200 µm) long-term bladder organoids with clear lumen and thick epithelial layers.
- Failed formation of interconnected fibroblasts and endothelial cell networks which surround epithelial organoids in normal/tumour assembloids: Check cell viability of organoids, fibroblasts, and endothelial

cell cultures. Prepare fresh medium. Increase the spinning speed (make sure that the assembloids are cultured with shaking in enough culture medium).

Time Taken

Generation of normal bladder assembloids

- Day -2. Generation of organoid with stroma: 1 hr
- Day 0. Generation of bladder assembloid: 1 hr
- Day 0-7. Medium change: 10 min
- Total time to generate mature normal bladder assembloids: ~9 days

Generation of bladder tumour assembloids

- Day 0. Generation of bladder tumour assembloid: 1 hr
- Day 0-7. Medium change: 10 min
- Total time to generate mature bladder tumour assembloids: ~7 days

Anticipated Results

If performed correctly, the size of bladder organoid with stroma droplets cultured for two days should be reduced. Also, after ~7 days culture of bladder assembloids, assembloids should form tightly organized three distinct layers with a central lumen, including an epithelial layer with multiple folds, middle stromal layer, and outer muscle layer.

In case of tumour assembloids, after ~7 days culture, tumour assembloids should show well-grown epithelial tumor cells surrounded/intervened by CAF and endothelial cells, mimicking histopathology of parental tumour.

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