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Takashi Tsuji *Editor*

Organ Regeneration

3D Stem Cell Culture & Manipulation

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Edited by

Takashi Tsuji

Laboratory for Organ Regeneration, RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan

Organ Technologies Inc., Tokyo, Japan

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Editor
Takashi Tsuji
Laboratory for Organ Regeneration
RIKEN Center for Developmental Biology
Kobe, Hyogo, Japan

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Dedication

This book is dedicated to the memory of Yoshiki Sasai, a scientist who made a great contribution to the advancement of developmental biology.

Preface

Organogenesis is a complex process that involves tissue self-organization, cell-cell interactions, regulations of cell signaling molecules, and cell movements. During embryonic development, organ-forming fields are organized in a process depending on the body plan. Various lineages of stem cells are produced and play central roles in organ development. In recent years, stem cell researchers have made advances in various aspects of three-dimensional organogenesis including cell growth, differentiation, and morphogenesis. Studies using multipotent stem cells have provided knowledge of the complex pattern formation and tissue self-organization during embryogenesis.

Stem cell research not only promotes basic biology but also can aid the development of regenerative medicine as a potential future clinical application. The current approaches to developing future regenerative therapies are influenced by our understanding of embryonic development, stem cell biology, and tissue engineering technology. To restore the partial loss of organ function, stem cell transplantation therapies were developed for several diseases such as hematopoietic malignancies, Parkinson's disease, myocardial infarction, and hepatic insufficiency. The next generation of regenerative therapy will be the development of fully functioning bioengineered organs that can replace lost or damaged organs following disease, injury, or aging. It is expected that bioengineering technology will be developed to reconstruct fully functional organs in vitro through the precise arrangement of several different cell types.

In recent years, significant advances in techniques for organ regeneration have been made using three-dimensional stem cell culture in vitro. Several groups recently reported the generation of neuroectodermal and endodermal organs via the regulation of complex patterning signals during embryogenesis and self-formation of pluripotent stem cells in three-dimensional (3D) stem cell culture. Other groups attempted to generate functional organs that develop by reciprocal epithelial and mesenchymal interactions using embryonic organ inductive stem cells. Several groups reported the generation of three-dimensional mini-organs/tissues by the reproduction of stem cells and their niches. These studies provide a better understanding of organogenesis in developmental biology and open possibilities for methodologies to be used in next-generation organ regenerative therapy.

Here, we focus on recent studies of organ regeneration from stem cells using in vitro three-dimensional cell culture and manipulation. These protocols have led both basic and clinical researchers to face new challenges in the investigation of organogenesis in developmental biology in order to develop applications for next-generation regenerative therapies.

I sincerely thank all of the authors for their contributions. I am also grateful to Dr. John Walker, the Editor in Chief of the MIMB series, for his continued support. I also thank Patrick Martin and Yasutaka Okazaki, Editors of the Springer Protocol series.

Kobe, Hyogo, Japan *Takashi Tsuji*

Contents

Contributors

Francesco Antonica • *Institute of Interdisciplinary Research in Molecular Human Biology (IRIBHM), Université Libre de Bruxelles, Brussels, Belgium; Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, UK*

Kyosuke Asakawa • *Laboratory for Organ Regeneration, RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan*

Nicole E. Brown • *Department of Pediatric General and Thoracic Surgery, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA*

Sabine Costagliola • *Institute of Interdisciplinary Research in Molecular Human Biology (IRIBHM), Université Libre de Bruxelles, Brussels, Belgium*

Mototsugu Eiraku • *In Vitro Histogenesis team, RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan*

Eri Hashino • *Department of Otolaryngology—Head and Neck Surgery, and Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, IN, USA*

Masatoshi Hirayama • *Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan*

Keisuke Ishihara • *DFG Research Center for Regenerative Therapies Dresden, Technische Universität Dresden, Dresden, Germany*

Taisuke Kadoshima • *Cell Asymmetry team, RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan; Asubio Pharma Co., Ltd., Kobe, Hyogo, Japan*

Dominika Figini Kasprzyk • *Institute of Interdisciplinary Research in Molecular Human Biology (IRIBHM), Université Libre de Bruxelles, Brussels, Belgium*

Karl R. Koehler • *Department of Otolaryngology—Head and Neck Surgery, and Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, IN, USA*

Akira Kurisaki • *Graduate School of Life and Environmental Sciences, The University of Tsukuba, Tsukuba, Ibaraki, Japan; Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki, Japan*

Atsushi Kuwahara • *Laboratory for In Vitro Histogenesis, RIKEN Center for Developmental Biology, Chuo, Kobe, Japan; Regenerative and Cellular Medicine Office, Sumitomo Dainippon Pharma Co., Ltd., Chuo, Kobe, Japan; Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd., Osaka, Japan*

Melissa H. Little • *Murdoch Children's Research Institute, Parkville, VIC, Australia; Department of Pediatrics, University of Melbourne, Parkville, VIC, Australia*

Matthias P. Lutolf • *Laboratory of Stem Cell Bioengineering, Institute of Bioengineering, School of Life Sciences and School of Engineering, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland; Institute of Chemical Sciences and Engineering, School of Basic Science, EPFL, Lausanne, Switzerland*

Mahe M. Maxime • *Department of Pediatric General and Thoracic Surgery, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA; Department of Pediatrics, University of Cincinnati, Cincinnati, OH, USA*

Andrea Meinhardt • *DFG Research Center for Regenerative Therapies Dresden, Technische Universität Dresden, Dresden, Germany*
Helmrath A. Michael • *Department of Pediatric General and Thoracic Surgery, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA; Department of Pediatrics, University of Cincinnati, Cincinnati, OH, USA*
Keiko Muguruma • *Laboratory for Cell Asymmetry, RIKEN Center for Developmental Biology, Chuo, Kobe, Japan*
Jorge O. Múnera • *Division of Developmental Biology, Cincinnati Children's Hospital, Cincinnati, OH, USA*
Tokushige Nakano • *Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd., Osaka, Japan*
Jing Nie • *Department of Otolaryngology—Head and Neck Surgery, and Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, IN, USA*
Ryuichi Nishinakamura • *Department of Kidney Development, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan*
Taka-aki K. Noguchi • *Graduate School of Life and Environmental Sciences, The University of Tsukuba, Tsukuba, Ibaraki, Japan*
Miho Ogawa • *Laboratory for Organ Regeneration, RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan; Organ Technologies Inc., Tokyo, Japan*
Masamitsu Oshima • *Department of Oral Rehabilitation and Regenerative Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan; Laboratory for Organ Regeneration, RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan*
Chikafumi Ozone • *Department of Endocrinology and Diabetes, Graduate School of Medicine, Nagoya University, Nagoya, Aichi, Japan; Laboratory for Organ Regeneration, RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan*
Holly M. Poling • *Department of Pediatric General and Thoracic Surgery, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA*
Adrian Ranga • *Laboratory of Stem Cell Bioengineering, Institute of Bioengineering, School of Life Sciences and School of Engineering, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland; Department of Mechanical Engineering, KU Leuven, Belgium*
Mírian Romitti • *Institute of Interdisciplinary Research in Molecular Human Biology (IRIBHM), Université Libre de Bruxelles, Brussels, Belgium*
Hideya Sakaguchi • *In Vitro Histogenesis team, RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan; Department of Clinical Application, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan*
Andrea Alex Schiavo • *Institute of Interdisciplinary Research in Molecular Human Biology (IRIBHM), Université Libre de Bruxelles, Brussels, Belgium*
Keisuke Sekine • *Department of Regenerative Medicine, Yokohama City University Graduate School of Medicine, Yokohama, Kanagawa, Japan*
Hidetaka Suga • *Department of Endocrinology and Diabetes, Nagoya University Hospital, Nagoya, Aichi, Japan*
Atsuhiro Taguchi • *Department of Kidney Development, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan*

Minoru Takasato • *Murdoch Children's Research Institute, Parkville, VIC, Australia; RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan*

Takanori Takebe • *Department of Regenerative Medicine, Yokohama City University Graduate School of Medicine, Yokohama, Kanagawa, Japan; Advanced Medical Research Center, Yokohama City University, Yokohama, Kanagawa, Japan; PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan; Department of Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, OH, USA*

Elly M. Tanaka • *Research Institute of Molecular Pathology, Vienna, Austria*

Hideki Taniguchi • *Department of Regenerative Medicine, Yokohama City University Graduate School of Medicine, Yokohama, Kanagawa, Japan; Advanced Medical Research Center, Yokohama City University, Yokohama, Kanagawa, Japan*

Koh-ei Toyoshima • *Laboratory for Organ Regeneration, RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan; Organ Technologies Inc., Tokyo, Japan*

Kazuo Tsubota • *Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan*

Takashi Tsuji • *Laboratory for Organ Regeneration, RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan; Organ Technologies Inc., Tokyo, Japan*

James M. Wells • *Division of Developmental Biology, Cincinnati Children's Hospital Research Foundation, Cincinnati, OH, USA*

Yasuhiro Yoshimura • *Department of Kidney Development, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan*

Chapter 1

Generation of Various Telencephalic Regions from Human Embryonic Stem Cells in Three-Dimensional Culture

Taisuke Kadoshima*, Hideya Sakaguchi*, and Mototsugu Eiraku

Abstract

In the developing embryo, telencephalon arises from the rostral portion of the neural tube. The telencephalon further subdivides into distinct brain regions along the dorsal-ventral (DV) axis by exogenous patterning signals. Here, we describe a protocol for in vitro generation of various telencephalic regions from human embryonic stem cells (ESCs). Dissociated human ESCs are reaggregated in a low-cell-adhesion 96-well plate and cultured as floating aggregates. Telencephalic neural progenitors are efficiently generated when ESC aggregates are cultured in serum-free medium containing TGFβ inhibitor and Wnt inhibitor. In long-term culture, the telencephalic neural progenitors acquire cortical identities and self-organize a stratified cortical structure as seen in human fetal cortex. By treatment with Shh signal, the telencephalic progenitors acquire ventral (subpallial) identities and generate lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE). In contrast, by treatment with Wnt and BMP signals, their regional identities shift to more dorsal side that generates choroid plexus and medial palllium (hippocampal primordium).

Key words SFEBq culture, Human ESCs, Telencephalon, Cerebral cortex, Ganglionic eminence, Medial pallium, Hippocampus

1 Introduction

Telencephalon has been one of the most interesting regions of the brain for many researchers, in part by their complex function and beautiful structure. The telencephalon includes cerebral cortex, hippocampus, ganglionic eminences, and choroid plexus [1–4]. The cerebral cortex is the center of integral neural activity and has a six-layered laminar structure. It plays key roles for movement, sensory, language, intention, cognition, and so on [5]. The hippocampus is the basement of memory formation (especially for episodic memory) and learning, and it has beautiful structure containing dentate gyrus (DG) and cornu ammontis (CA) area [6]. The three ganglionic eminences give rise to ventral telencephalic

Taisuke Kadoshima and Hideya Sakaguchi contributed equally to this work

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tissues such as striatum and globus pallidus, and it also generates GABAergic interneuron that tangentially migrates into cerebral cortex [7]. The choroid plexus has essential roles for development and homeostasis of central nervous system by the generation of cerebro spinal fluid (CSF) and formation of blood-CSF barrier [4].

Dysfunction of each of these tissues causes several neurological or neuropsychiatrical disorders such as dementia, autism, mood disorders, and schizophrenia [8]. To explore these diseases, there has been one difficulty that the target is "human." In vitro neural-induction technology using pluripotent stem cells (PSCs), however, can complement this [9–11]. Because human PSCs (ES and induced pluripotent stem (iPS) cells) derived neural tissues reflect human nature, significant progress of this technology enables modeling of human-specific neural diseases [12–16]. Furthermore, three-dimensional (3D) tissue induction from human PSCs can recapitulate neural developmental step with characteristic structure of human neural tissues, and it enables examination of human embryogenesis and disease mechanisms [10, 11]. These technologies, thus, will be useful for future translational researches.

As a 3D induction method, SFEBq (serum-free floating culture of embryoid body-like aggregates with quick reaggregation) is a versatile method, and using this culture method, we have previously reported the induction of several telencephalic tissues from mouse/human ESCs [12, 14, 16–21]. In this culture, several thousands of dissociated mouse and human ESCs are reaggregated using low-cell-adhesion 96-well culture plate. The floating aggregates cultured in serum-free medium that contains no or minimal growth factors can efficiently differentiate into neural progenitors with 3D structure. In the presence of a low level of growth factor signal, the neuroectoderm is efficiently specified into cortical progenitors positive for Foxg1, Emx1, and Pax6. Once the cortical fate is determined, the anterior-posterior (AP) and dorsoventral (DV) pattern of telencephalon can be modified by patterning signals, such as Shh for ventral differentiation and Wnts and BMPs for dorsal differentiation [22, 23]. Based on this strategy, we have succeeded in the generation of cerebral cortex, ganglionic eminences and its derivatives, choroid plexus, and hippocampus, in 3D order [12, 14, 16, 20, 21].

In this chapter, we describe a detailed protocol for the generation of each telencephalic tissue from human ESCs and show its technical points. First, we describe the induction of cerebral cortex and its long-term culture techniques, and then focus on how to modulate DV axis in SFEBq culture (*see* Fig. 1).

Fig. 1 (continued) treatment with 0.5 nM BMP4 and 3 μM CHIR 99021 (GSK3 inhibitor, also known as Wnt agonist) from day 18 to 42. (**d**) Timetable of medial pallium tissue induction from human ESCs. Transient exposure of 0.5 nM BMP4 and 3 μM CHIR 99021 from days 18 to 21 partially dorsalizes the telencephalic progenitors and induces medial pallium tissue. Approximate periods of each event and the medium used are indicated

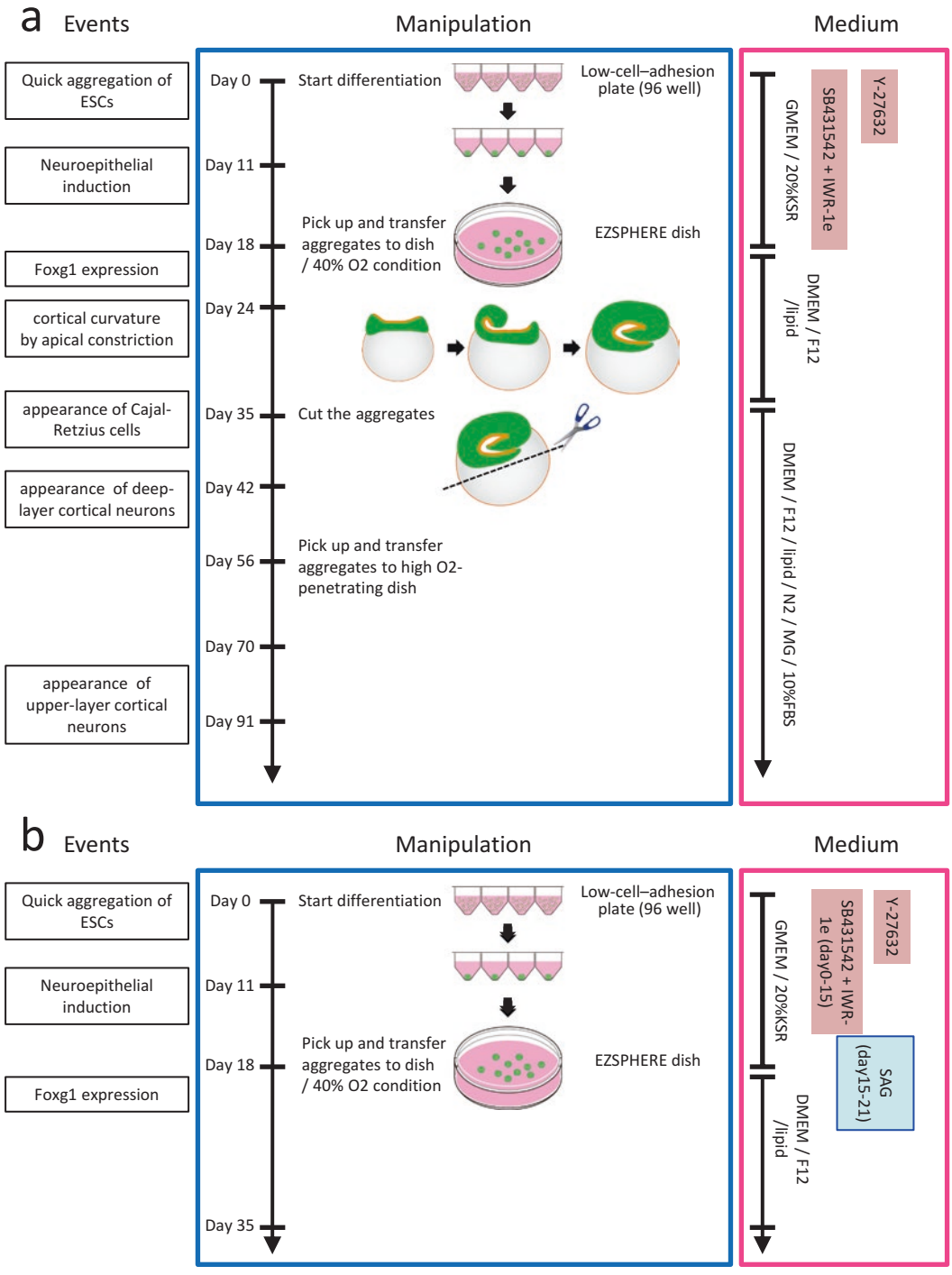

Fig. 1 Schematic diagram of SFEBq methods for various telencephalic regions differentiation from human ESCs. (**a**) Timetable of cortical tissue induction from human ESCs. (**b**) Timetable of LGE and MGE induction from human ESCs. The telencephalic progenitors are ventralized and generate LGE or MGE tissues by treatment with smoothened agonist SAG (30 nM for LGE induction and 500 nM for MGE induction). (**c**) Timetable of choroid plexus tissue induction from human ESCs. The telencephalic progenitors are dorsalized and generate choroid plexus tissue by

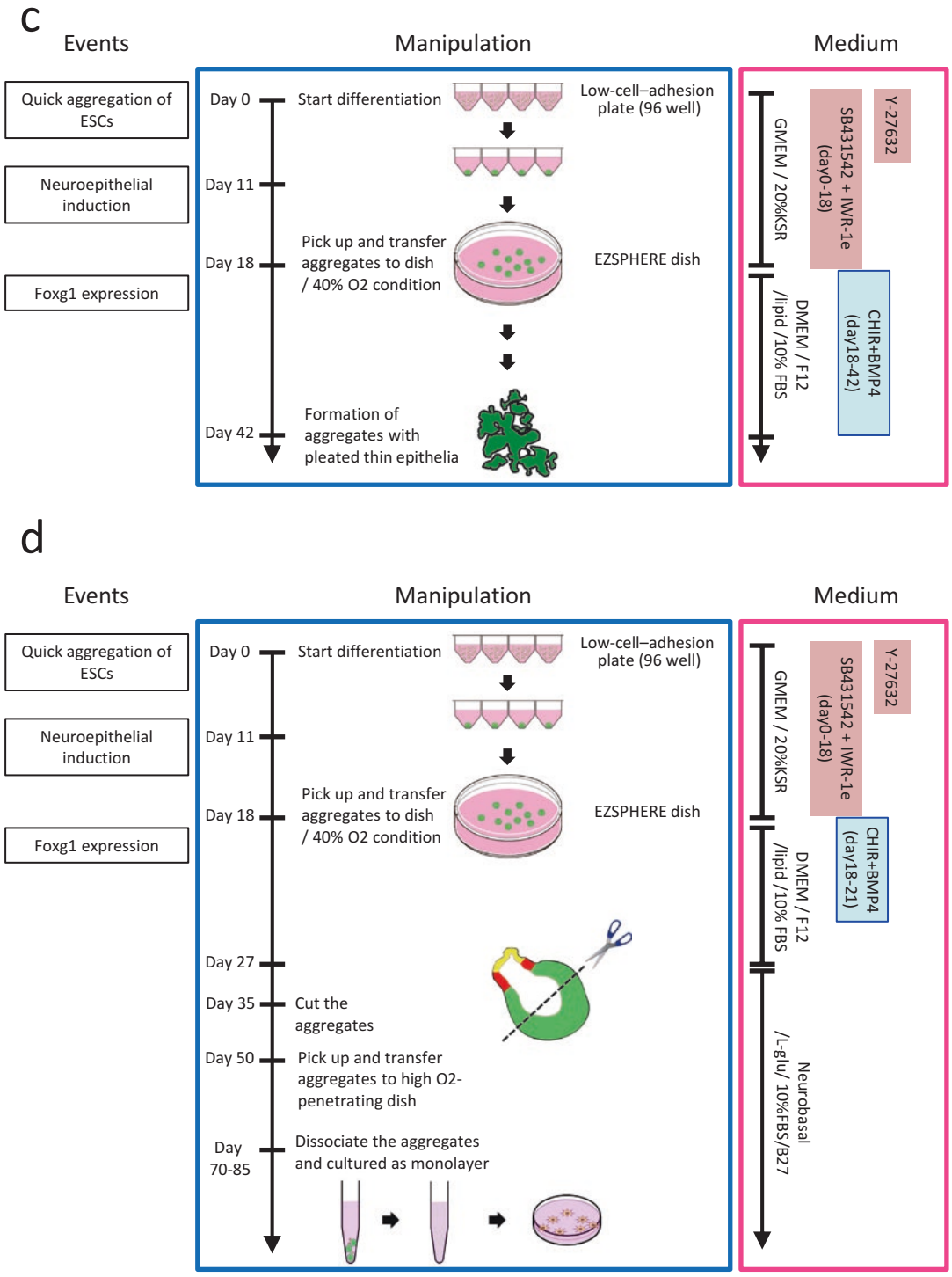

Fig. 1 (continued)

2 Materials

1. Heparin: To prepare a stock solution at 5 mg/mL, dissolve 5 mg of Heparin in 1 mL PBS. Store at 4 °C for several months.
2. Knockout Serum Replacement (KSR) (*see* **Note 1**).
3. Matrigel (growth factor-reduced): Thaw Matrigel overnight at 4 °C. Keep Matrigel on ice and make 1 mL aliquots in 1.5 mL tubes using precool P1000 tips. Store small aliquots at −20 °C for several months (*see* **Note 2**).
4. Gelatin solution (0.1%, wt/vol): To prepare gelatin solution (0.1%, wt/vol), dissolve 0.5 g of gelatin in 500 mL of water by autoclaving. The solution can be stored at 4 °C for up to 3 months.
5. DNase I: To prepare a stock solution at 10 mg/mL, dissolve 100 mg of DNase I in 10 mL of PBS. Store small aliquots at −20 °C for several months.
6. Recombinant human BMP4: Reconstitute 10 μg of BMP4 in 100 μL of 4 mM HCl containing 0.1% BSA to make a 100 μg/mL stock. Store small aliquots at −20 °C for 3 months.
7. Y-27632 (ROCK inhibitor): To prepare a stock solution at 10 mM, reconstitute 10 mg of Y-27632 in 3.1 mL of H_2O. Store small aliquots at −20 °C for several months.
8. IWR-1-endo (Wnt inhibitor): To prepare a stock solution at 30 mM, reconstitute 10 mg of IWR-1-endo in 814 μL of DMSO. Store small aliquots at −20 °C for several months.
9. SB431542 (TGFβ inhibitor): To prepare a stock solution at 10 mM, reconstitute 10 mg of SB431542 in 2.4 mL of ethanol. Store small aliquots at −20 °C for several months.
10. Smoothened agonist (SAG): To prepare a stock solution at 10 mM, reconstitute 1 mg of SAG in 204 μL of DMSO. Store small aliquots at −20 °C for several months. To prepare the working solution (1 mM), dilute the 10 mM stock 1:10 in H_2O. Store the working solution at 4 °C for 1 month.
11. CHIR 99021 (GSK3 inhibitor): To prepare a stock solution at 30 mM, reconstitute 5 mg of CHIR 99021 in 358 μL of DMSO. Store small aliquots at −20 °C for several months.
12. ESC maintenance medium: DMEM/F-12 supplemented with 20% (vol/vol) KSR, 2 mM glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-ME, 1% (vol/vol) penicillin-streptomycin. Filter the solution with a 0.2 μm filter bottle, store at 4 °C and use within 1 month. Add 5 ng/mL bFGF freshly on the day of use.
13. ESC dissociation solution: 0.25% (wt/vol) trypsin and 1 mg/mL collagenase IV in PBS containing 20% (vol/vol) KSR and

1 mM $CaCl_2$. Sterilize the solution by filtering through a 0.2-μm bottle-top filter. Store small aliquots at −20 °C for several months.

14. Neural induction medium: GMEM supplemented with 20% (vol/vol) KSR, 0.1 mM nonessential amino acids, 1 mM pyruvate, 0.1 mM 2-ME, 1% (vol/vol) penicillin-streptomycin. Filter the solution with a 0.2 μm filter bottle, store at 4 °C, and use within 1 month.
15. Neural differentiation medium: DMEM/F-12-GlutaMAX medium supplemented with 1% Chemically Defined Lipid Concentrate, 1% (vol/vol) penicillin-streptomycin, and 0.1% (vol/vol) fungizone. Filter the solution with a 0.2-μm filter bottle, store at 4 °C, and use within 1 month. Add 1% (vol/vol) N2 supplement freshly on the day of use.
16. Cortical maturation medium: Prepare cortical maturation medium by adding 5 μg/mL Heparin and 10% (vol/vol) FBS to neural differentiation medium. Filter the solution with a 0.2-μm filter bottle, store at 4 °C and use within 1 month. Add 1% (vol/vol) N2 supplement and 1% (vol/vol) Matrigel freshly on the day of use.
17. Hippocampal maturation medium: Neurobasal medium supplemented with 2-mM L-glutamine, 1% (vol/vol) penicillin-streptomycin, 0.1% (vol/vol) fungizone, and 10% (vol/vol) FBS. Filter the solution with a 0.2-μm filter bottle, store at 4 °C and use within 1 month. Add 2% (vol/vol) B27 without vit.A supplement freshly on the day of use.
18. Poly-D-Lysine (PDL) solution (0.2 mg/mL): To prepare 0.2 mg/mL PDL solution, dissolve 5 mg of PDL in 25 mL of water. The solution can be stored at 4 °C for up to 3 months.
19. Laminin/Fibronectin solution: Laminin/Fibronectin solution is prepared by adding 200 μL Laminin (1 mg/mL) and 96 μL Fibronectin (1 mg/mL) to 11.7 mL PBS.

3 Methods

3.1 Maintenance Culture of Human ESCs

Human ESCs are maintained on a feeder layer of mouse embryonic fibroblasts (MEF) inactivated by mitomycin C treatment in ESC maintenance medium under 2% CO_2.

1. Aspirate ESC maintenance medium from a tissue culture dish, wash twice with 10 mL PBS, and then aspirate.
2. Add 1.5 mL ESC dissociation solution and incubate for 7–8 min at 37 °C.
3. Add ESC maintenance medium (w/o bFGF) and detached en bloc from the feeder layer by pipetting with a wide-bore P1000 tip.

4. Transfer the cell suspension into a 15 mL conical tube and centrifuge at 180 × *g* for 3 min at room temperature.
5. Remove the supernatant and resuspend the cell in 2 mL ESC maintenance medium (w/o bFGF).
6. Break the ESC clumps into smaller pieces (several dozens of cells) by gentle pipetting with a P1000 tip.
7. Transfer the cell suspension into a 15 mL conical tube containing 10 mL of ESC maintenance medium (1:4–1:6 split ratio).
8. Transfer the cell suspension onto fresh feeder-layer dish and incubate at 37 °C under 2% CO_2. From the next day, change 10 mL ESC maintenance medium once daily and passage the cells every 5–6 days (70–80% confluent).

3.2 Cortical Tissue Differentiation from Human ESCs and Long-Term Culture (see Fig. 1a)

Prepare one 10 cm culture dish of human ESCs on feeder layers grown to 70–80% of confluency (*see* **Note 3**).

1. Aspirate ESC maintenance medium from a tissue culture dish, wash twice with 10 mL PBS, and then aspirate.
2. Add 1.5 mL ESC dissociation solution and incubate for 7–8 min at 37 °C.
3. Add ESC maintenance medium (w/o bFGF) and detached en bloc from the feeder layer by pipetting with a wide-bore P1000 tip.
4. Transfer the cell suspension into a 15 mL conical tube and centrifuge at 180 × *g* for 3 min at room temperature.
5. Remove the supernatant and resuspend the cell in 10 mL ESC maintenance medium (w/o bFGF) containing 20 μM Y-27632.
6. Transfer the ESC clumps to a gelatin-coated dish and incubate at 37 °C for 1.5 h to adhere MEF cells onto the dish bottom (this prevents contamination of MEF cells).
7. Collect the medium containing the floating ESC clumps from the dish into a 15 mL conical tube and centrifuge at 180 × *g* for 3 min at room temperature.
8. Remove the supernatant and wash once with 10 mL of PBS.
9. Add 2 mL TrypLE Express containing 0.05 mg/mL DNase I and 10 μM Y-27632 and incubate at 37 °C for 5 min.
10. Dissociate the ESC clumps into single cells by gentle pipetting with a P1000 tip.
11. Add 10 mL neural induction medium and centrifuge at 180 × *g* for 5 min at room temperature.
12. Remove the supernatant and resuspend the cells in neural induction medium.
13. Count the number of cells using a cell counter.

14. Adjust the concentration to 9×10^4 cells/mL with neural induction medium containing 20 μM Y-27632, 3 μM IWR-1-endo, and 5 μM SB431542.
15. Plate ESCs into a 96-well low-adhesion plate (9000 cells per 100 μL per well) (*see* **Note 4**).
16. Incubate the plate at 37 °C under 5% CO_2.

Define the day on which the SFEBq culture is started as day 0.

17. On culture day 3, add 100 μL neural induction medium containing 10–20 μM Y-27632, 3 μM IWR-1-endo, and 5 μM SB431542 to each well. From days 6 to 18, change the medium containing 3 μM IWR-1-endo and 5 μM SB431542 once every 3–4 days (*see* Fig. 2 and **Note 5**).
18. On culture day 18, transfer the floating aggregates to a 10-cm EZ-SPHERE dish. Add 12 mL neural differentiation medium and further culture in suspension under the 40% O_2/5% CO_2 condition (*see* **Notes 6,** 7). From days 21 to 35, change the neural differentiation medium once every 3–4 days (*see* Fig 2. and **Note 8**).
19. On culture day 35, transfer the aggregates to a plastic dish and cut the aggregates into half-size with fine forceps and scissors under a dissecting microscope. Return the cut aggregates to the 10 cm EZ-SPHERE dish containing of 15 mL fresh cortical maturation medium. From days 35, change the cortical maturation medium once every 3–4 days. To prevent cell death in the central portions of large aggregates, the aggregates are cut into half-size every 2 weeks.
20. On culture day 56, transfer the aggregates to a plastic dish and cut the aggregates into half-size with fine forceps and scissors under a dissecting microscope. Transfer the cut aggregates onto 6-cm dishes with high O_2-penetrating bottoms (Lumox dish) dish containing of 6 mL fresh cortical maturation medium. Change the cortical maturation medium every 3 days. To prevent cell death in the central portions of large aggregates, the aggregates are cut into half-size every 2 weeks. From culture day 70, the concentration of Matrigel is increased (2% (vol/vol)), and B27 without vit.A supplement is also added to the cortical maturation medium (*see* Fig 3 and **Note 9**).

3.3 Ventralizing the Telencephalic Tissues (see Fig. 1b)

In this SFEBq culture, the regional identities of the human ESCs-derived telencephalic progenitors along the DV axis can be modified by patterning signals. By treatment with Shh signaling, the telencephalic progenitors can acquire ventral (subpallial) identities and generate lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE). We describe a protocol for LGE and MGE differentiation from the telencephalic progenitors below.

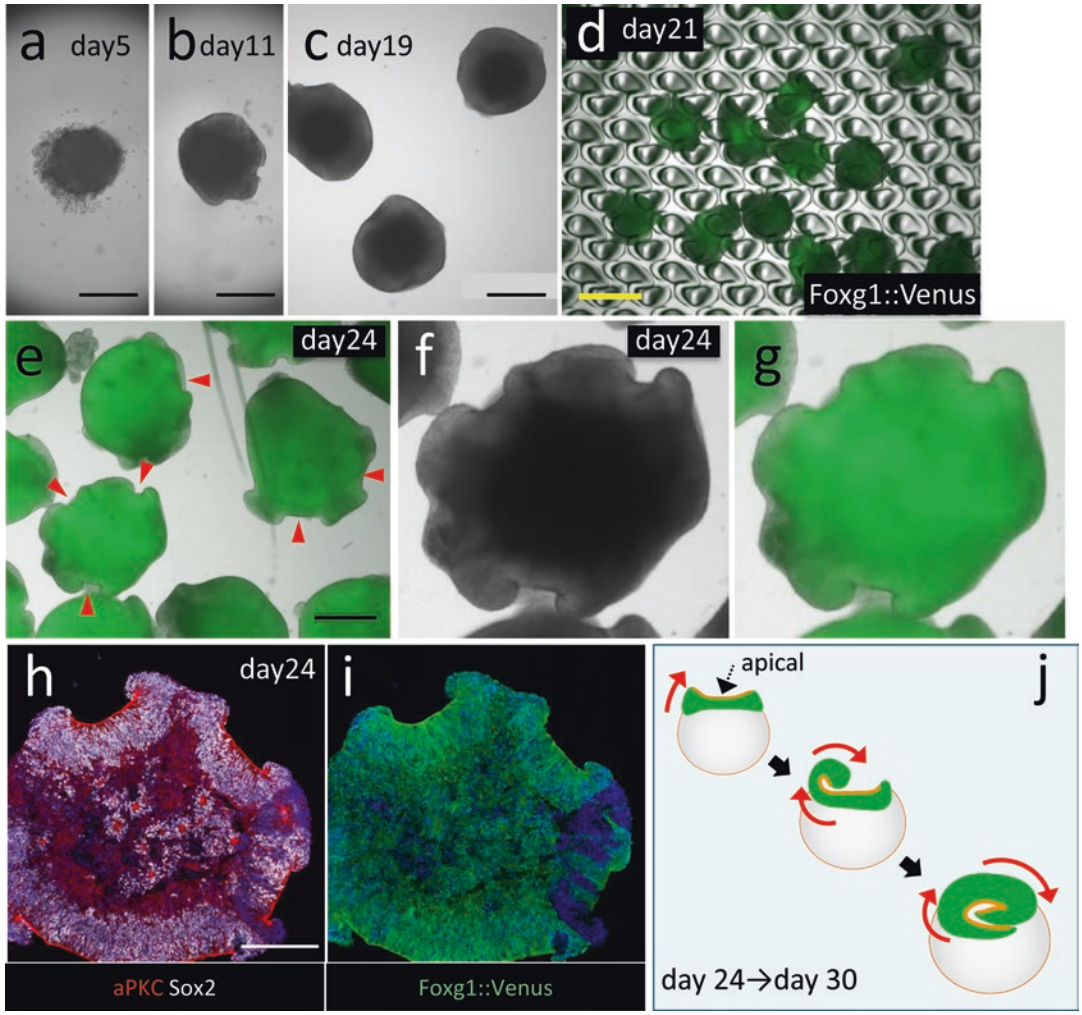

Fig. 2 Progression of the telencephalic neuroepithelial development in human SFEBq culture. (**a**) Dissociated human ESCs are quickly reaggregated and form almost uniformly in a few days. (**b, c**) A continuous translucent neuroepithelia is seen in every aggregate around day 10 and it grows into more thick and tight structure. (**d**) Human ESCs-derived aggregates are transferred and cultured using EZ-SPHERE dishes to prevent the adhesion to each other. (**e, f**) From around day 24, the surface of the Foxg1::Venus+ aggregates starts to become apically concave (*arrowheads*). (**h, i**) Immunostaining with Foxg1::Venus (*green*), aPKC (*red*), Sox2 (*white*), and DAPI (*blue*) in a cross-section of day 24 aggregates. (**j**) Schematic of dynamic rolling morphogenesis of cortical neuroepithelium. Scale bars: 500 μm (**a–c, e**), 1 mm (**d**), and 200 μm (**h**)

The telencephalic progenitors on day 15 are obtained by the same culture condition as the cortical tissue differentiation.

1. On culture day 15, change medium by removing half of the supernatant and replacing it with the same volume of fresh neural induction medium containing SAG to obtain a final concentration of 30 nM (LGE induction) or 500 nM (MGE induction), respectively.

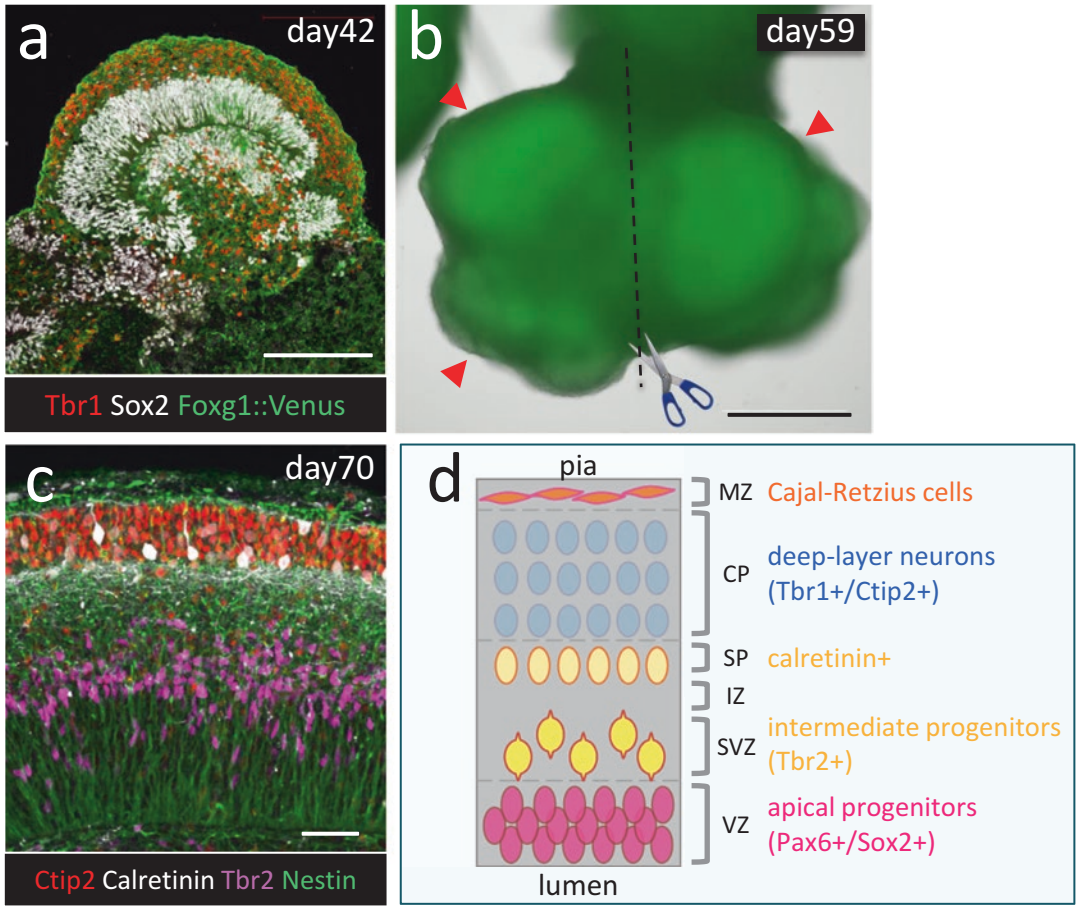

Fig. 3 Self-organized stratified cortical tissue in human SFEBq culture. (**a**) Self-formation of axial polarity seen in human ESCs-derived cortical neuroepithelium on day 42. (**b**) Human ESCs-derived aggregate containing cortical neuroepithelium (*arrowheads*) visualized Foxg1::Venus on day 59. The aggregate is cut into half-size (*dashed line*) every 2 weeks to prevent cell death in the central portions of aggregate. (**c**) Immunostaining of day 70 human ESCs-derived cortical neuroepithelium with zone-specific markers. (**d**) Schematic of the stratified structure of human ESCs-derived cortical tissue on day 70. MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone, VZ, ventricular zone. Scale bars: 200 μm (**a**), 500 μm (**b**), and 50 μm (**c**)

2. On culture day 18, transfer the floating aggregates to a 10 cm EZ-SPHERE dish. Add 15 mL neural differentiation medium containing 30 nM (LGE induction) or 500 nM (MGE induction) SAG and further culture in suspension under the 40% O_2/5% CO_2 condition.
3. On culture day 21, change medium completely to 15 mL neural differentiation medium (w/o SAG). From day 24, change neural differentiation medium once every 3–4 days (*see* Fig. 4).

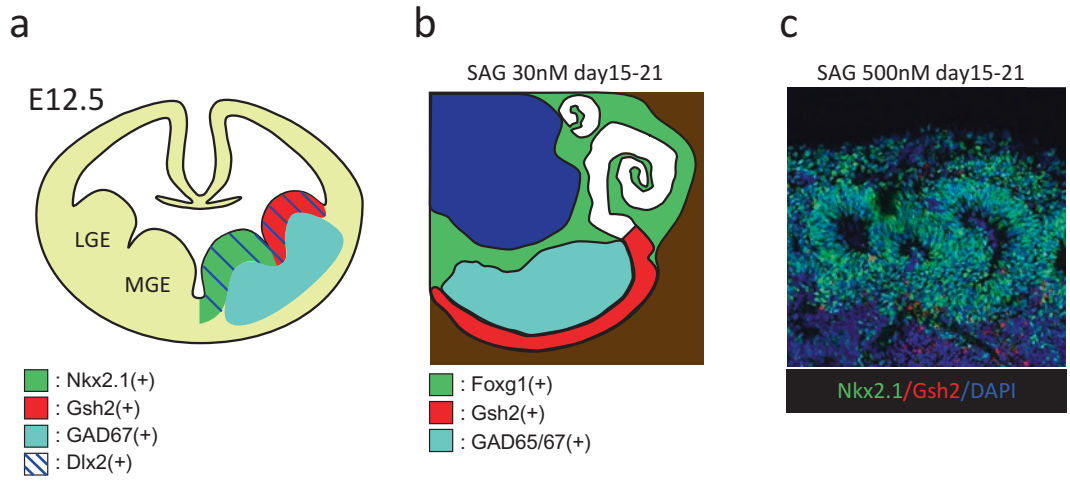

Fig. 4 Induction of ventral telencephalon. (**a**) Developmental process of ventral telencephalon in mice. Shh is first observed at an early developmental phase (E8-E9.5) in the diencephalon and mesendoderm adjacent to the ventral telencephalon, and Shh induces Nkx2.1 in the MGE area. Then, Shh is expressed in the MGE and preoptic area by E12.5, and Gsh2 is expressed between Nkx2.1 and Pax6 domains, and $Gsh2^+$/Nkx2.1- domain possesses LGE identity. (**b**) Schematic of human ES cell-derived cortex-LGE tissues induced by a moderate treatment with SAG. Continuous tissue including cortical ($Pax6^+$) and LGE ($Gsh2^+$) domains was generated in a sequential order, as seen in vivo. A mass of $GAD65^+$ GABAergic neurons was generated underneath the $Gsh2^+$ LGE NE, whereas the rest of the telencephalic NE was largely positive for the cortical NE marker Pax6. (**c**) Higher concentrations of SAG (500 nM, days 15–21) induced the medial ganglionic eminence (MGE) marker Nkx2.1 at the cost of Pax6 and Gsh2 expression (day 42). Scale bar: 100 μm

3.4 Dorsalizing the Telencephalic Tissues

By treatment with Wnt and BMP signal, the regional identities of the telencephalic progenitors can be shifted to more dorsal portion. We describe protocols for choroid plexus and medial pallium differentiation from the telencephalic progenitors below.

3.4.1 Choroid Plexus Tissue Generation (see Fig. 1c)

The telencephalic progenitors on day 18 are obtained by the same culture condition as the cortical tissue differentiation.

1. On culture day 18, transfer the floating aggregates to a 10-cm EZ-SPHERE dish. Add 15 mL neural differentiation medium containing CHIR 3 μM, 0.5 nM BMP4, and 10% (vol/vol) FBS and further culture in suspension under the 40% O_2/5% CO_2 condition (*see* **Note 10**). From days 18 to 42, change the medium once every 3–4 days (*see* Fig. 5).

3.4.2 Medial Pallium Induction (see Fig. 1d)

The telencephalic progenitors on day 21 are obtained by the same culture condition as the choroid plexus induction.

1. On culture days 21 and 24, change the medium completely to 15 mL neural differentiation medium containing 10% (vol/vol) FBS (w/o CHIR and BMP4) and further culture in suspension under the 40% O_2/5% CO_2 condition.

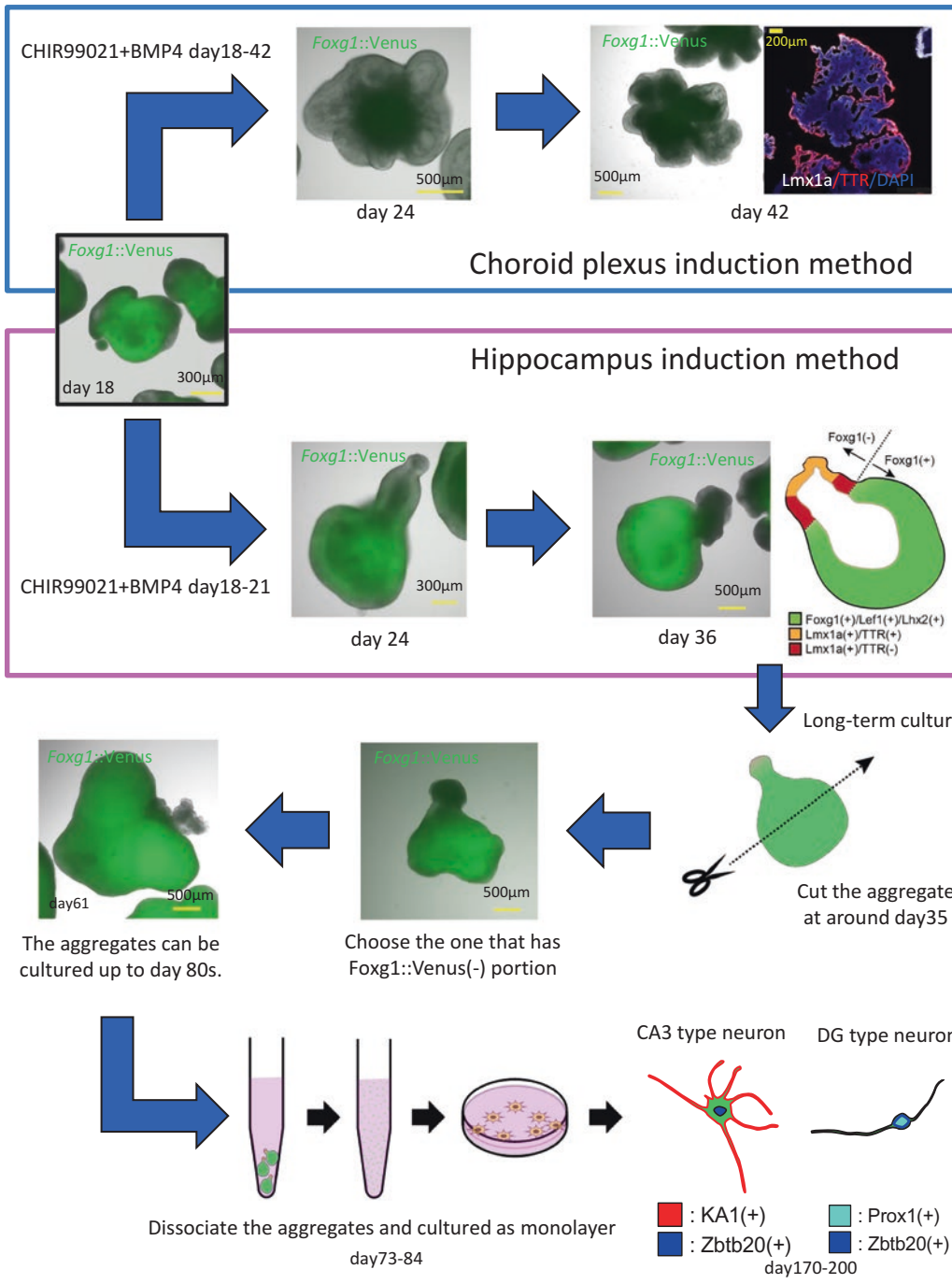

Fig. 5 Schematic of the induction method for dorsomedial telencephalic tissues. Continuous addition of dorsalizing factors induces choroid plexus (the most dorsomedial portion of telencephalin), whereas transient addition of dorsalizing factors induces dorsomedial telencephalic tissues that include future hippocampal region. For long-term culture, aggregates are cut into half size, and the one that has Foxg1::Venus(–) portion is further cultured for induction of hippocampal tissues. Human ES cell-derived dorsomedial telencephalic tissues are cultured up to day 80. To examine neural population, the aggregates are dissociated to single cell, and cultured as monolayer. After 100 days from dissociation, KA1-positive neurons (CA type) and Prox1-positive neurons (DG type) can be detected

2. On culture day 27, change the medium to 15 mL hippocampal maturation medium. From day 27, change the medium once every 3–4 days. At day 35, cut the aggregates into half-size with fine forceps and scissors under a dissecting microscope to prevent cell death in the central portions of large aggregates (*see* **Note 11**).
3. On culture day 50, transfer the aggregates to a plastic dish. Transfer the aggregates onto 6-cm dishes with high O_2-penetrating bottoms (Lumox dish) dish containing 6 mL fresh hippocampal maturation medium. Change the medium once every 3–4 days (*see* Fig. 5).

3.4.3 Dissociation Culture of Hippocampal Neurons

1. Prepare glass or plastic slide dish.
2. Coat the dish with PDL solution, and incubate at 4 °C overnight.
3. Wash by distilled water three times.
4. Coat with Laminin/Fibronectine solution.
5. Incubate at 37 °C for 3 h or overnight.
6. Wash by PBS twice, then put hippocampal differentiation medium, and preserve at 37 °C.
7. On culture days 73–84, pick up the aggregates to 15 mL Falcon tube, and wash by PBS twice.
8. Add 1–2 mL papain enzyme solution (Neural Tissue Dissociation Kit), and incubate for 30 min at 37 °C water bath.
9. Add 1 mL hippocampal maturation medium, and dissociate by pipetting 30–40 times.
10. The dissociated cells are filtered with a 40-μm cell strainer.
11. The cells are plated onto poly-D-lysine/laminin/fibronectin-coated dishes at a density of 300,000–500,000 cells/cm^2 in hippocampal maturation medium.
12. The medium was changed every 3–4 days (*see* Figs. 1d and Fig. 5).

3.5 Immunohistochemistry for SFEBq Aggregates

1. Transfer the SFEBq aggregates into a 15 mL conical tube, and wash twice with PBS at room temperature.
2. Fix aggregates with 4% (wt/vol) PFA for 10–30 min at 4 °C.
3. Wash twice with PBS at room temperature.
4. Cryoprotect with 15% (wt/vol) sucrose overnight at 4 °C.
5. Take several (up to ten) aggregates in a small amount of 15% (wt/vol) sucrose using a wide-bore P1000 tip and settle down aggregates to the bottom of a cryomold.
6. Remove excess liquid around the settled aggregates using a pipette.

Table 1
Antibodies required

Antibody	Host	Supplier	Cat. No.	Dilution
aPKC	Rabbit	Santa Cruz	sc-216	1:100
Nestin	Rabbit	Covance	PRB-315C	1:200
Foxg1	Rabbit	TakaRa	M227	1:1000
Pax6	Rabbit	Covance	PRB-278P	1:250
Sox2	Goat	Santa Cruz	sc-17320	1:250
Tbr1	Rabbit	Abcam	ab31940	1:500
Ctip2	Rat	Abcam	ab18465	1:5000
Calretinin	Rabbit	Chemicon	AB5054	1:2000
Tbr2	Rabbit	Abcam	ab23345	1:500
Gsh2	Rabbit	*See* ref. [17]		1:10,000
Nkx2.1	Mouse	Novocastra	NCL-L-TTF-1	1:500
Lef1	Rabbit	Cell Signalling	2230S	1:500
Ttr	Rabbit	DAKO	A0002	1:1000
Prox1	Mouse	Millipore	MAB5654	1:200
Zbtb20	Rabbit	Sigma Ardrich	HPA016815	1:200
Neuropillin2	Goat	R&D	AF2215	1:40
Lmx1a	Guinea pug	*See* ref. [14]		1:10,000–20,000
Otx2	Rabbit	Abcam	ab21990	1:1000

7. Embed aggregates with O.C.T. compound and freeze them at −20 °C in the cryostat chamber (*see* **Note 12**).
8. Make serial sections using a cryostat (*see* **Note 13**).
9. Carry out immunostaining using the antibodies listed in Table 1.

4 Notes

1. As the activity of KSR in terms of supporting telencephalic/cortical differentiation varies from lot to lot, several different lots of KSR should be tested to find optimal ones for the differentiation. In some KSR lots, a lower concentration (e.g., 10%, vol/vol) may work better.
2. The lot-to-lot concentration variability in commercial Matrigel products can affect the ability to maintain the continuous neuroepithelial structure; we preferentially use one of relatively high concentration (>9.5 mg/mL).

3. The quality of human ESCs culture is quit critical for the cortical tissue formation. Examine aggregates under a microscope to confirm the undifferentiated phenotype and the confluency (70–80%).
4. We typically seed 9000 cells per well. However, depending on cell line, the number of cells to seed may have to be optimized.
5. If there are considerable numbers of dead cells present in the cultures, 1 h pretreatment with Y-27632 and/or higher concentration of Y-27632 (up to 50 μM) on day 0 may reduce cell death.
6. Check the floating aggregates under microscope to ensure that a continuous translucent neuroepithelial structure is formed on the outer periphery of the aggregates. The formation of continuous neuroepithelial structure is critical for the cortical tissue formation. We always check the telencephalic differentiation by the fluorescence of Foxg1::Venus. The Foxg1 expression is also detectable by RT-qPCR and immunohistochemistry on day 18. Our protocol for cortical tissue differentiation typically produces Foxg1::venus$^+$ cells in 70–80% of total cells on day 34 as evaluated by FACS. If the Foxg1 expression is very low, check the Wnt inhibitor and/or reduce the concentration of KSR.
7. We always use EZ-SPHERE dish to prevent the adhesion of aggregates to each other (*see* Fig. 2d).
8. Check the floating aggregates under microscope to ensure that the surface of the aggregates starts to become apically concave around on day 24. The curving morphogenesis continues until around day 30, and generates semispherical structures with a lumen inside (*see* Fig. 2e–j).
9. Cortical regions can be recognized by Foxg1::Venus$^+$ dome-like and translucent neuroepithelial structures. Cut the aggregates into half-size not to damage the cortical neuroepithelium with fine forceps and scissors under a dissecting microscope (*see* Fig. 3b).
10. Choroid plexus tissue can be induced by other BMP proteins such as BMP2 or BMP7. However, higher concentration is needed than BMP4 (BMP2: 200 ng/mL, BMP7: 600 ng/mL).
11. Cut aggregates to retain Foxg1::Venus$^-$ protrusion and Foxg1::Venus$^+$ epithelium in one, and use this for further long-term culture (we do not use another Foxg1::Venus$^+$ remaining part).
12. If desired, store aggregates in a deep freezer (−80 °C) for up to several months.
13. We typically make 10–16 μm serial sections for immunostaining.

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Chapter 2

Generation of a Three-Dimensional Retinal Tissue from Self-Organizing Human ESC Culture

Atsushi Kuwahara, Tokushige Nakano, and Mototsugu Eiraku

Abstract

A three-dimensional (3D) tissue generated in vitro is a promising source to study developmental biology and regenerative medicine. In the last decade, Yoshiki Sasai's group have developed a 3D stem cell culture technique known as SFEBq and demonstrated that embryonic stem cells (ESCs) have an ability to self-organize stratified neural tissue including 3D-retina. Furthermore, we have reported that ESC-derived retinal tissue can form an optic cup and a ciliary margin, which are unique structures in the developing retina. In this review, we focus on self-organizing culture technique to generate 3D-retina from human ESCs.

Key words SFEBq culture, Human ESCs, Retina, Neural retina, RPE, Optic cup, Ciliary margin

1 Introduction

The retina is the main visual sensory tissue in mammals. During retinal development, the optic cup derived from the rostral diencephalon is composed of the inner and outer walls that differentiate into neural retina (NR) and retinal pigment epithelium (RPE), respectively. Yoshiki Sasai's group pioneered methodology for inducing 3D neural tissues from embryonic stem cells (ESCs) (for review *see* [1, 2]). We have previously reported that embryonic stem cells (ESCs) have an ability to self-organize stratified 3D-retina by using a self-organizing stem cell culture technique known as SFEBq [3]. In this study, we also demonstrated the emergence of an optic cup, a unique structure in the developing retina. We then applied this mouse ESC culture technique to human ESCs and generated human 3D-retina and an optic cup [4]. We further developed a hESC-differentiation culture technique, named induction-reversal culture method, to generate human ciliary margin-like retinal stem cell niche [5]. Importantly, 3D-retina generated by these culture methods is now studying to apply in regenerative medicine field [6–8]. Since we have published a

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detailed protocol for mESC differentiation [9], in this review we focus on our recent advances in the retinal differentiation culture method of hESCs.

2 Materials

1. Low-cell adhesion 96-well plates with V-bottomed conical wells (96-well V-bottomed plate).
2. 90-mm Petri Dishes for suspension cell culture (Floating culture dish).
3. Knockout Serum Replacement (KSR; *see* **Note 1**).
4. hESC maintenance medium: DMEM/F-12 (suitable for 2% CO_2 culture) supplemented with 20% (vol/vol) KSR, 2 mM glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-ME, 100 U/mL penicillin, and 100 μg/mL streptomycin. Filter the medium with a 0.2-μm filter bottle, store at 4 °C, and use within 2 weeks. Add 7.5 ng/mL bFGF freshly on the day of use.
5. Basic fibroblast growth factor (bFGF): To prepare a stock solution at 100 μg/mL, reconstitute 50 μg of bFGF in 500 μL of hESC maintenance medium. Store small aliquots at −20 °C for 3 months. To prepare the working solution (0.75 μg/mL), dilute in the hESC maintenance medium. Store the working solution at 4 °C for 3 weeks. Avoid freeze thaw cycle.
6. hESC dissociation solution: 0.25% (wt/vol) trypsin and 1 mg/mL collagenase IV in PBS containing 20% (vol/vol) KSR and 1 mM $CaCl_2$. Sterilize the solution by filtering through a 0.2-μm filter. Store small aliquots at −20 °C for several months.
7. GMEM differentiation medium: GMEM supplemented with 20% (vol/vol) KSR, 0.1 mM nonessential amino acids, 1 mM pyruvate, 0.1 mM 2-ME, 100 U/mL penicillin, and 100 μg/mL streptomycin. Filter the solution with a 0.2-μm filter bottle, store at 4 °C, and use within 3 weeks.
8. gfCDM + KSR medium: growth-factor-free CDM (gfCDM) supplemented with 10% KSR medium, while gfCDM contains 45% Iscove's modified Dulbecco's medium (IMDM), 45% Ham's F12 (F12), Glutamax, 1% chemically defined lipid concentrate, monothioglycerol (450 μM), 100 U/mL penicillin, and 100 μg/mL streptomycin [10].
9. RPE-induction medium: DMEM/F-12-Glutamax medium supplemented with 1% (vol/vol) N2 supplement, 100 U/mL penicillin, and 100 μg/mL streptomycin. Filter the solution with a 0.2-μm filter bottle, store at 4 °C, and use within 2 weeks. Add CHIR99021 (3 μM) and SU5402 (5 μM) freshly on the day of use.

10. Retina maturation medium: DMEM/F-12-Glutamax medium supplemented with 1% (vol/vol) N2 supplement, 10% (vol/vol) FBS, 0.5 μM retinoic acid, 0.1 mM taurine, 0.25 μg/mL Fungizone, 100 U/mL penicillin, and 100 μg/mL streptomycin. Filter the solution with a 0.2-μm filter bottle, store at 4 °C, and use within 2 weeks.
11. Gelatin solution: To prepare gelatin solution (0.1%, wt/vol), dissolve 0.5 g of gelatin in 500 mL of water by autoclaving. The solution can be stored at 4 °C for up to 3 months.
12. DNase I: To prepare a stock solution at 10 mg/mL, dissolve DNase I in PBS. Store small aliquots at −20 °C for several months.
13. Y-27632 (ROCK inhibitor) [11]: To prepare a stock solution at 10 mM, reconstitute Y-27632 in H_2O. Store small aliquots at −20 °C for several months.
14. Matrigel (growth factor-reduced): Thaw Matrigel overnight at 4 °C. Keep Matrigel on ice and make aliquots in 2 mL tubes using precool P1000 tips. Store small aliquots at −20 °C for several months (*see* **Note 2**).
15. IWR-1-endo (Wnt inhibitor): To prepare a stock solution at 10 mM, reconstitute IWR-1-endo in DMSO. Store small aliquots at −20 °C for several months.
16. Smoothened agonist (SAG): To prepare a stock solution at 10 mM, reconstitute SAG in DMSO. Store small aliquots at −20 °C for several months. To prepare the working solution (100 μM), dilute the stock in PBS. Store the working solution at 4 °C for 1 month.
17. Recombinant human BMP4 (BMP4): To prepare a stock solution at 1 μM, reconstitute 50 μg of BMP4 in 1375 μL of 0.1% BSA/PBS. Store small aliquots at −20 °C for 3 months. Store aliquots at 4 °C for 3 weeks. Avoid freeze thaw cycle.
18. CHIR99021 (GSK3 inhibitor; CHIR): To prepare a stock solution at 10 mM, reconstitute CHIR99021 in DMSO. Store small aliquots at −20 °C for several months.
19. SU5402 (FGFR inhibitor): To prepare a stock solution at 10 mM, reconstitute SU5402 in DMSO. Store small aliquots at −20 °C for several months.
20. All trans retinoic acid (RA): Prepare 100 mM stock solution in DMSO. Store small aliquots at −80 °C for several months. To prepare the working solution (3.3 mM), dilute the 100 mM stock in EtOH. Store the working solution at −20 °C for several months.
21. Taurine: Prepare 50 mM stock solution in PBS. Store small aliquots at −20 °C for several months.

3 Methods

3.1 Maintenance of Human ESCs

Undifferentiated hESCs are maintained on a feeder layer of mouse embryonic fibroblasts (MEF) inactivated by mitomycin C treatment in ESC maintenance medium under 2%-CO_2 conditions. For passaging, hESC colonies are detached with hESC dissociation solution and broken into smaller pieces by gentle pipetting. The passages are performed at a 1:3–1:5 split ratio every third or fourth days [5, 12].

3.1.1 Preparation of MEF Feeder-Layer Dish

1. Add 6 mL Gelatin solution (0.1%, wt/vol) in a tissue culture dish (100 mm).
2. Stand for 0.5–2 h at 37 °C.
3. Thaw the inactivated MEF stock and centrifuge it in a centrifuge tube.
4. Add 8 mL MEF medium and plate on gelatin-coated culture dish.
5. Incubate for 4–48 h at 37 °C.

3.1.2 Passage

1. Wash twice MEF feeder-layer dish with 10 mL PBS and incubate in hESC maintenance medium (w/o bFGF) at 37 °C.
2. Prepare 70% confluent hESCs cultured on a MEF feeder-layer dish (100 mm).
3. Aspirate hESC maintenance medium from 70% confluent hESCs, wash twice with 10 mL PBS, and then aspirate.
4. Add 1.5 mL ESC dissociation solution and incubate for 7–8 min at 37 °C.
5. Add hESC maintenance medium (w/o bFGF) and detach en bloc from the feeder layer by pipetting.
6. Break hESC clumps into smaller pieces (several dozens of cells) by gentle pipetting.
7. Plate hESC clumps onto fresh feeder-layer dish (1:3–1:5 split ratio).
8. Culture in hESC maintenance medium supplemented with bFGF (7.5 ng/mL) at 37 °C under 2%-CO_2 conditions.

From the next day, change 10 mL hESC maintenance medium (+bFGF) every day and passage the cells every third or fourth days (60–70% confluent).

3.2 Generation of Retinal Progenitors from hESCs by Using "ECM-Addition Method" (Days 0–18)

Prepare hESCs on feeder layers grown to ~70% of confluency (Subheading 3.1, *see* **Note 3**). Undifferentiated hESCs can differentiate into retinal progenitors by using "extracellular matrix (ECM)-addition method" as described previously [4]. On culture day 18, aggregates contain retinal epithelium. The percentage of retinal progenitor marker Rx is typically around 60% as determined by FACS [4, 5].

Day 0: Plating

1. Prepare 70% confluent hESCs cultured on a MEF feeder-layer dish (100 mm).
2. Prepare gelatin-coated culture dish (100 mm).
3. Aspirate hESC maintenance medium from 70% confluent hESCs, wash twice with 10 mL PBS, and then aspirate.
4. Add 1.5 mL ESC dissociation solution and incubate for 7–8 min at 37 °C.
5. Add hESC maintenance medium (w/o bFGF) and detach en bloc from the feeder layer by pipetting.
6. Plate hESC clumps in 6 mL hESC maintenance medium (w/o bFGF) supplemented with 10 μM Y-27632 on a gelatin-coated dish.
7. Incubate at 37 °C for 1.0–1.5 h to adhere contaminated MEF cells to the dish bottom.
8. Collect the medium containing the floating ESC clumps from the dish and transfer into a 15 mL conical tube.
9. Centrifuge at 180 × *g* for 3 min at 25 °C, remove the supernatant, and suspend with 10 mL of PBS.
10. Centrifuge at 180 × *g* for 3 min at 25 °C and remove the supernatant.
11. Dissociate hESC clumps into single cells by using TrypLE Express supplemented with 20 μM Y-27632 and 0.05 mg/mL DNase I.
12. Centrifuge at 180 × *g* for 3 min at 25 °C and remove the supernatant.
13. Resuspend the cells in the GMEM differentiation medium.
14. Count the number of cells using a cell counter.
15. Adjust the concentration to 9.0×10^4 cells per mL in the GMEM differentiation medium supplemented with 20 μM Y-27632 and 3 μM IWR-1-endo.
16. Plate hESCs into a 96-well low-adhesion V-bottomed plate (9000 cells per 100 μL per well) (*see* **Note 4**).
17. Culture at 37 °C under 5%-CO_2 conditions.

    Define the day on which the SFEBq culture is started as day 0.

Day 2

18. On culture day 2, add 50 μL GMEM differentiation medium supplemented with 3 μM IWR-1-endo and 3% Matrigel to each well (3 μM IWR-1-endo and 1% Matrigel at final concentration).

From days 6 to 12, change the medium containing 3 μM IWR-1-endo and 1% Matrigel every 3–4 days.

Day 12

19. On culture day 12, transfer the floating aggregates to a 90-mm floating culture dish. Culture in suspension in the GMEM differentiation medium supplemented with 10% FBS, 100 nM SAG, and 1% Matrigel at 37 °C under 5%-CO_2 conditions.

From days 12 to 18, change the medium containing 10% FBS, 100 nM SAG, and 1% Matrigel every 3–4 days.

Day 18

20. On culture day 18, transfer the floating aggregates to a 90-mm floating culture dish and further culture in NR-selective culture, RPE-selective culture or induction-reversal culture (*see* below).

3.3 Generation of Optic Cups from hESCs by Using "ECM-Addition Method" (Days 12–30)

Prepare hESCs on feeder layers grown to ~70% of confluency (Subheading 3.1, *see* **Note 3**). Undifferentiated hESCs can differentiate into optic cups by using "ECM-addition method" as described previously [4]. Prepare the floating aggregates on culture day 12 as described (**steps 1–18** in Subheading 3.2).

Days 12–30

1. On culture day 12, transfer the floating aggregates to a 90-mm floating culture dish. Culture in suspension in GMEM differentiation medium supplemented with 10% FBS and 1% Matrigel at 37 °C under 5%-CO_2 conditions.
2. On culture day 15, culture the aggregates in the GMEM differentiation medium supplemented with 3 μM CHIR, 100 nM SAG, 10% FBS, and 1% Matrigel at 37 °C under 5%-CO_2 conditions.
3. On culture day 18, culture the aggregates in the DMEM/F--12-Glutamax medium supplemented with 1% (vol/vol) N2 supplement, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under 5%-CO_2 conditions.

   From days 12 to 30, change the medium every 3–4 days.

3.4 Generation of Retinal Progenitors from hESCs by "BMP Method" (Days 0–18)

Prepare hESCs on feeder layers grown to ~70% of confluency (Subheading 3.1, *see* **Note 3**). Undifferentiated hESCs can differentiate into retinal progenitors by using "BMP method" as described previously [5]. On culture day 18, aggregates contain retinal epithelium. The percentage of retinal progenitor marker Rx is typically around 80% as determined by FACS.

Day 0: Plating

1. Prepare 70% confluent hESCs cultured on a MEF feeder-layer dish (100 mm).
2. Prepare gelatin-coated culture dish (100 mm).
3. Aspirate hESC maintenance medium from 70% confluent hESCs, wash twice with 10 mL PBS, and then aspirate.
4. Add 1.5 mL ESC dissociation solution and incubate for 7–8 min at 37 °C.
5. Add hESC maintenance medium (w/o bFGF) and detach en bloc from the feeder layer by pipetting.
6. Plate hESC clumps in 6 mL hESC maintenance medium (w/o bFGF) supplemented with 10 μM Y-27632 on a gelatin-coated dish.
7. Incubate at 37 °C for 1.0–1.5 h to adhere contaminated MEF cells to the dish bottom.
8. Collect the medium containing the floating ESC clumps from the dish and transfer into a 15 mL conical tube.
9. Centrifuge at 180 × *g* for 3 min at 25 °C, remove the supernatant, and suspend with 10 mL of PBS.
10. Centrifuge at 180 × *g* for 3 min at 25 °C and remove the supernatant.
11. Dissociate hESC clumps into single cells by using TrypLE Express supplemented with 20 μM Y-27632 and 0.05 mg/mL DNase I.
12. Centrifuge at 180 × *g* for 3 min at 25 °C and remove the supernatant.
13. Resuspend the cells in gfCDM + KSR medium.
14. Count the number of cells using a cell counter.
15. Adjust the concentration to 1.2×10^5 cells per mL in gfCDM + KSR medium supplemented with 20 μM Y-27632. Concentration of Y-27632 is diluted into half by half medium change every 3–4 days.
16. Plate hESCs into a 96-well low-adhesion V-bottomed plate (12,000 cells per 100 μL per well) (*see* **Note 4**).
17. Culture at 37 °C under 5%-CO_2 conditions.

Define the day on which the SFEBq culture is started as day 0. Add 50 μL gfCDM + KSR medium on day 2 or 3 (150 μL per well at final volume).

Day 6

18. On culture day 6, change medium with gfCDM + KSR medium supplemented with 3 nM BMP4 to each well (1.5 nM (55 ng/

mL) BMP4 at final concentration) (*see* **Note 5**). From days 6 to 18, change the medium with gfCDM + KSR medium every 3–4 days. Concentration of BMP4 is diluted into half by half medium change every 3–4 days.

Day 18

19. On culture day 18, transfer the floating aggregates to a 90-mm floating culture dish and further culture in NR-selective culture, RPE-selective culture, or induction-reversal culture (*see* below).

BMP addition promotes differentiation of hESCs to Rx^+/$Chx10^+$ retinal progenitors (Fig. 1).

3.5 Generation of Multilayered NR-Tissue from hESCs in NR-Selective Culture Condition (Days 18–60)

Prepare hESC-derived retinal progenitors by using ECM-addition method (Subheading 3.2) or BMP method (Subheading 3.4). On culture day 18, retinal progenitors form retinal epithelium and can differentiate into multilayered NR-tissue on day 35 by culturing in Retina maturation medium, which is DMEM/F-12-Glutamax medium supplemented with 1% N2 supplement, 10% FBS, 0.5 μM retinoic acid, 0.1 mM taurine, 0.25 μg/mL Fungizone, 100 U/mL penicillin, and 100 μg/mL streptomycin [4, 5].

Day 18

1. On culture day 18, transfer the floating aggregates to 90-mm floating culture dish and further culture in Retina maturation medium under 40%-O_2/5%-CO_2 conditions.
   From days 18 to 60, change the medium every 3–4 days.
2. (optional) On culture days 18–35, transfer the aggregates to Cell culture dish and dissect the NR-like tissue with fine forceps and scissors under a stereo microscope [4]. Return dissected aggregates to the 90-mm floating culture dish with fresh Retina maturation medium.

3.6 Generation of RPE-Like Tissue from hESCs in RPE-Selective Culture Condition (Days 18–35)

Prepare hESC-derived retinal progenitors by using the ECM-addition method (Subheading 3.2) or the BMP method (Subheading 3.4). On culture day 18, retinal progenitors form retinal epithelium and can differentiate into RPE-tissue on day 35 by culturing in the RPE-induction medium, which is DMEM/F--12-Glutamax medium supplemented with 1% N2 supplement, 100 U/mL penicillin, 100 μg/mL streptomycin, 3 μM CHIR99021, and 5 μM SU5402 [4, 5].

Day 18

1. (optional) On culture day 18, transfer the aggregates to Cell culture dish and dissect the NR-like tissue with fine forceps and scissors under a stereo microscope. Return dissected aggregates to the 90-mm floating culture dish with fresh RPE-induction medium.

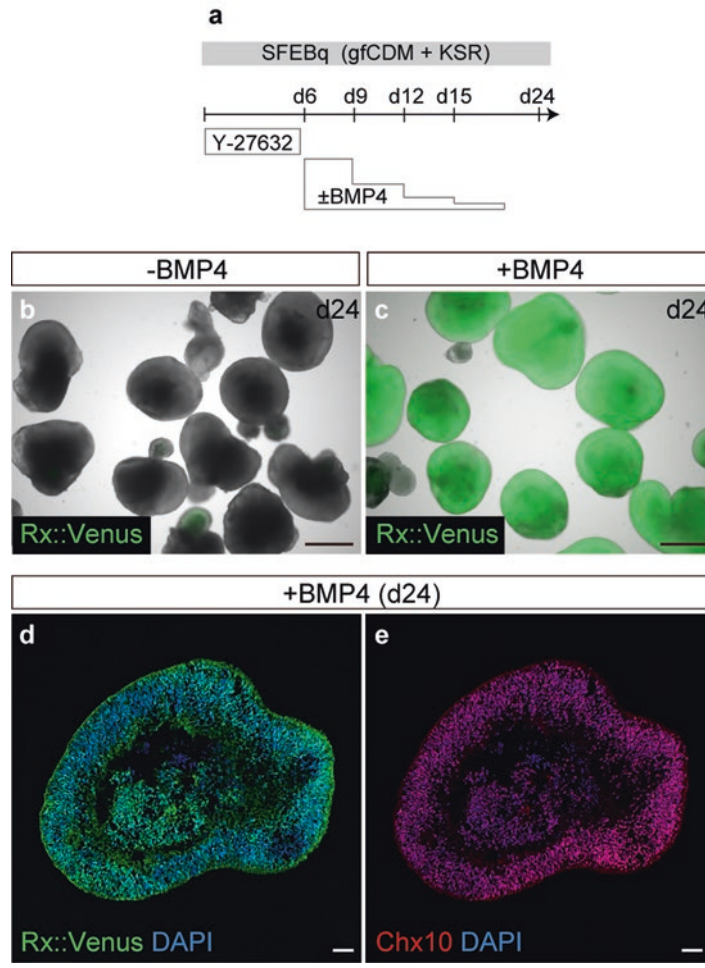

Fig. 1 Selective NR generation in self-organizing hESC culture by BMP method. (**a**) Timing of BMP4 treatment. BMP4 (1.5 nM) was added to medium on day 6, while its concentration was diluted into half by half medium change on days 9, 12, and 15. (**b**, **c**) Induction of Rx::Venus by transient BMP4 treatment (c; b, untreated control) in hESC aggregates. (**d**, **e**) Immunostaining of NR tissue (day 24) generated by BMP method with antibodies for Venus (**d**) and Chx10 (**e**). *Blue*, nuclear staining with DAPI. Modified from Kuwahara et al. *Nat Commun* 2015

2. On culture day 18, transfer the floating aggregates to 90-mm floating cell culture dish and further culture in RPE-induction medium under 5%-CO_2 conditions.

From days 18 to 24, change the medium every 3–4 days with RPE-induction medium.

Day 24

3. On culture day 24, transfer the floating aggregates to 90-mm floating cell culture dish and further culture under 5%-CO_2 conditions in RPE-differentiation medium, which is DMEM/

F-12-Glutamax medium supplemented with 1% N2 supplement, 100 U/mL penicillin, 100 μg/mL streptomycin, 3 μM CHIR99021, and 1% FBS. From days 24 to 35, change the medium every 3–4 days with RPE-differentiation medium.

3.7 Generation of Ciliary Margin-Like Tissue from hESCs by "Induction-Reversal Culture Method" (Days 18–150)

Prepare hESC-derived retinal epithelium progenitors by using ECM-addition method (Subheading 3.2) or BMP method (Subheading 3.4). Culturing retinal epithelium in RPE-induction medium from days 18 to 24 induces transition from NR-fate into RPE-fate. Then, culturing in retina maturation medium from days 24 to 35 facilitates reversion of RPE-biased epithelium back to NR-fate. This step-wise "induction-reversal culture method" generates both RPE and NR in the same aggregate (turnip-shaped aggregate, Fig. 2d). Then, NR-RPE tissue boundary in turnip-shaped aggregate self-forms a ciliary margin-like tissue on culture day 63 [5].

Day 18

1. (optional) On culture day 18, transfer the aggregates to Cell culture dish and dissect the NR-like tissue with fine forceps and scissors under a stereo microscope.
2. For RPE-induction culture, transfer the floating aggregates (day 18) to a 90-mm floating culture dish and further culture in RPE-induction medium (Subheading 3.6) under 5%-CO_2 conditions (*see* **Note 6**).

From days 18 to 24, change the medium with RPE-induction medium every 3–4 days.

Day 24

3. For NR-reversal culture, transfer the floating aggregates (day 24) to a 90-mm floating culture dish and further culture in Retina maturation medium under 40%-O_2/5%-CO_2 conditions (Subheading 3.5).

From days 24 to 150, change the medium with Retina maturation medium every 3–4 days.

Multilayered stratified NR is often formed near the ciliary margin-like tissue (Fig. 2e).

3.8 Neurosphere Culture from hESC-Derived Ciliary Margin-Like Tissue (Day 60)

Prepare hESC-derived ciliary margin-like tissue by using the induction-reversal culture method (Subheading 3.7). Cells in ciliary margin-like tissue can form neurospheres after culturing in retinosphere medium, which is DMEM/F12-Glutamax medium supplemented with 2% B27 supplement (without vitamin A), 20 ng/mL human bFGF, 20 ng/mL human EGF, 5 μg/mL Heparin, 0.25 μg/mL Fungizone, 100 U/mL penicillin, and 100 μg/mL streptomycin [5].

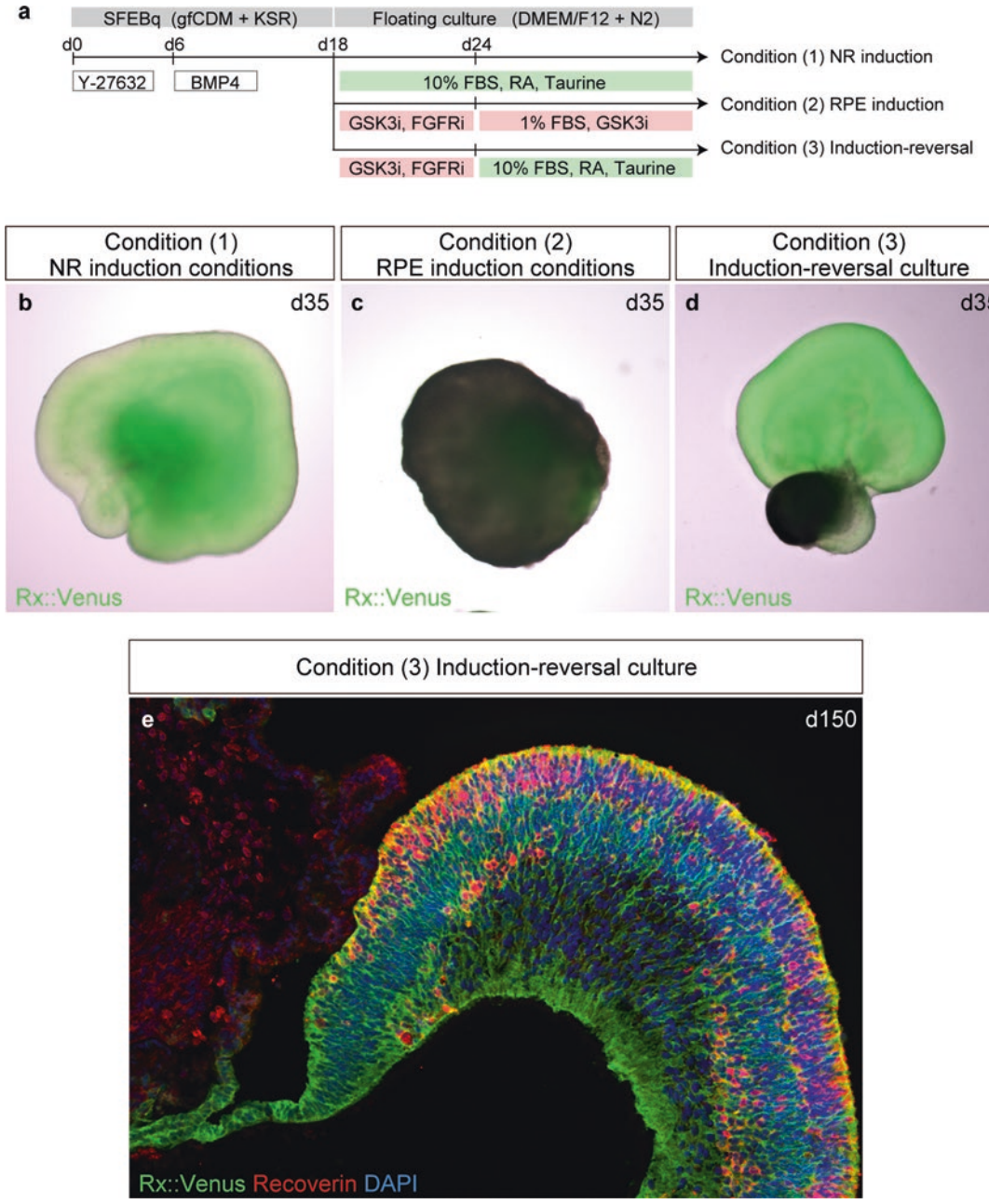

Fig. 2 Generation of turnip-shaped NR-RPE conjugated aggregates in induction-reversal culture. (**a**) Time table for three culture conditions. (**b**–**d**) External appearance of self-formed NR and RPE structures under different conditions on day 35. (**e**) Immunostaining of NR tissues in the turnip-shaped NR-RPE conjugated aggregates on day 150 sectioned along the central-peripheral axis. Modified from Kuwahara et al. *Nat Commun* 2015

1. (optional) Transfer the aggregates to Cell culture dish and dissect the ciliary margin-like tissue with fine forceps and scissors under a stereo microscope.

2. Digest cells at 37 °C for 30 min by using papain (Neural Tissue Dissociation Kit) and gently dissociate into single cells by pipetting.
3. Plate single cells on 96-well flat-bottom plates (MPC coated) at the density of 1000–3000 cells/well ($0.5–1.5 \times 10^4$ cells/mL) and culture for 7–14 days in retinosphere medium.
4. (optional) For secondary sphere formation assay, dissociate primary spheres by using papain (Neural Tissue Dissociation Kit) and culture in retinosphere medium.

3.9 Immunostaining of Aggregates

Prepare cell aggregates with retinal tissue. Cells are fixed in paraformaldehyde (PFA) and immunostained as described previously [5].

1. Transfer the SFEBq aggregates into 1.5 mL microtube or 15 mL conical tube and wash with PBS at 25 °C.
2. Fix aggregates with 4% (wt/vol) PFA at 4 °C for 15 min.
3. Wash with PBS.
4. Cryoprotect with 20% (wt/vol) sucrose in PBS at 4 °C for 12–72 h.
5. Transfer aggregates into a cryomold.
6. Embed aggregates with O.C.T. compound and freeze them.
7. Cut 10–15 μm thick frozen sections using a cryostat.
8. (optional) Treat frozen sections with heat-based antigen retrieval in Target Retrieval solution (15 min at 105 °C).

Both ECM-addition method and BMP method show a similar time course of differentiation: $Brn3b^+$ cells (~d28), Crx^+ cells (~d35), $Recoverin^+$ (~d45), $RXRG^+$ cells (~d60), NRL^+ cells (~d100), $S\text{-}opsin^+$ cells (~d130), and $Rhodopsin^+$ cells (~d130). When we applied the "induction-reversal" methods, differentiation of these markers tended to be delayed by several days (corresponding to the time for RPE induction phase).

4 Notes

1. It is important to choose KSR lot suitable for retinal differentiation.
2. It is important to choose Matrigel lot suitable for ECM-addition method.
3. The quality of human ESCs is critical for retinal differentiation.
4. The number of plating hESCs should be optimized for each hESC/iPSC line, because it affects retinal differentiation efficacy. We typically seed 6000–15,000 cells per well in a 96-well V-bottomed plate.

5. BMP4 was added to culture to final 1.5 nM on day 6, and its concentration was diluted into half by half medium change every third day. The addition of BMP4, started on day 3 and day 6, induced Rx^+ NR epithelium at day 18 with similar efficiency.
6. The addition of 5 μM SU5402 treatment to RPE-induction medium increased efficiency and reproducibility of RPE induction and formation of ciliary margin-like tissues after the reversal culture, while CHIR99021 treatment without SU5402 tended to give higher variations in the level of RPE induction.

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Competing financial interests
A.K. is employed by Sumitomo Dainippon Pharma Co., Ltd. T.N. is employed by Sumitomo Chemical Co., Ltd. The authors are inventors on patent applications.

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Chapter 3

3D Culture for Self-Formation of the Cerebellum from Human Pluripotent Stem Cells Through Induction of the Isthmic Organizer

Keiko Muguruma

Abstract

Pluripotent stem cells (PSCs) possess self-organizing abilities in 3D culture. This property has been demonstrated in recent studies, including the generation of various neuroectodermal and endodermal tissues. For example, PSCs are able to differentiate into specific type of neural tissues, such as the neocortex and the optic cup, in response to local positional information brought about by signals during embryogenesis. In contrast, the generation of cerebellar tissue from PSCs requires a secondary induction by a signaling center, called the isthmic organizer, which first appears in the cell aggregate in 3D culture. Such developmental complexity of cerebellum has hampered establishment of effective differentiation culture system from PSCs, thus far.

We recently reported that cerebellar neurons are generated from human PSCs (hPSCs). In this chapter, we describe an efficient protocol for differentiation of 3D cerebellar neuroepithelium from hPSCs. We also describe the protocols for further differentiation into specific neurons in the cerebellar cortex, such as Purkinje cells and the granule cells.

Key words Purkinje cells, Cerebellum, Pluripotent stem cells, Self-organization, Neural differentiation, Cerebellar development, Isthmic organizer

1 Introduction

Over the past decade, numerous studies have demonstrated steered differentiation of PSCs into various tissues by mimicking the signaling environments during embryogenesis. These expanding techniques offer multiple directions of the research. From a scientific viewpoint, these techniques would provide valuable information in understanding the mechanisms of embryogenesis and organogenesis during development. From a straightforward application perspective, these techniques are expected to be used for regenerative medicine in replacement therapy with iPSC-derived tissues. Besides, the techniques to differentiate the tissues with high functionality in vitro, especially from patient-specific iPSCs,

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can be used in the construction of in vitro disease models, which would be powerful tools for the understanding of the disease mechanisms and the discovery of drugs.

The cerebellum has been considered a major component of the motor system. It sends outputs to a variety of brain regions that are related to motor functions and receives feedback inputs from them. It is well known that the dysfunction of the cerebellum caused by its damage or the cerebellar diseases leads to impairments in motor and postural control. Among cerebellar diseases, spinocerebellar ataxias (SCAs) are a heterogeneous group of dominantly inherited neurological disorders associated with degeneration of neurons in the cerebellum and its associated pathways. Although a vast number of causative genes have been identified, several common pathophysiological pathways appear to underlie the neurodegeneration observed in these conditions. While disease modeling with patient-specific iPSCs has been extensively investigated for many diseases including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and Huntington's disease (HD), only a few studies on iPSC-derived models of SCA have been reported [1–5]. Success in the development of the models appears to depend primarily on the presence of efficient differentiation protocols [6, 7].

In contrast to other brain regions, the cerebellum forms in a much more complicated way during the ontogenesis. The cerebellar anlage arises in the dorsal region of the rostral hindbrain, under the strong inductive influence of the neighboring signaling center at the junction of the midbrain and the hindbrain, called the isthmus [8, 9]. The isthmic organizer secretes patterning molecules such as Fgf8 and Wnt1. We recently reported that cerebellar neurons could be efficiently generated from PSCs by recapitulating the self-inductive signaling microenvironment in 3D culture [10, 11]. In this culture, the isthmic organizer tissue is first induced from PSCs in the cell aggregate by self-forming manner, and subsequently it in turn generates cerebellar neurons. While the analyses with patient-derived iPSCs or neurons differentiated from them may provide some insights into the pathogenesis of the cerebellar diseases, they cannot directly assay the functions and dysfunctions that are specific to mature cerebellar neurons. Recapitulation of accurate phenotypes of patients in vitro with patient-derived cerebellar neurons would provide much more information and be crucial for understanding the cerebellar diseases. It is important to generate specific neuronal subtypes for the construction of the disease models [6, 7]. In this chapter, we provide a detailed description of a protocol for 3D culture of cerebellar neurons and tissues. We also describe methods for analyses of cell-specific characterization utilized for the in vitro cerebellar development.

2 Materials

2.1 Reagents

1. Human ESCs: ESCs are cultured on Mouse embryonic fibroblasts (MEF) as feeder cells.
2. Animals: A closed colony strain ICR. Noon of the day on which the vaginal plug was detected is designed as embryonic day (E) 0.5.
3. 0.1 w/v% Gelatin solution: Dissolve 0.5 g of gelatin in 500 mL of dH_2O by autoclaving. The solution is stored at 4 °C.
4. 0.1 M 2-Mercaptoehtanol: 2-Mercaptoehtanol is diluted with PBS (−).
5. Maintenance medium for hESCs: Combine 500 mL of DMEM/F12, 125 mL of KnockOut™ Serum Replacement, 6.25 mL of MEM Non-Essential Amino Acids Solution, 6.25 mL of L Glutamine 200 mM Solution, 625 μL of 0.1 M 2-Mercaptoehtanol. Sterilize the solution by filtering through a 0.2-μm bottle-top filter, store at 4 °C and use within 1 month.
6. Mytomycin-treated MEF: MEF is available for purchase. Expose Mytomycin at 10 μg/mL for 3 h to inactivate mitotic activity of MEF. Store an aliquot of Mytomycin-treated MEFs at −80 °C until use.
7. Maintenance medium for MEF: Combine 500 mL of DMEM, 55 mL of Fetal Bovine Serum (FBS), 2.7 mL of penicillin-streptomycin. Sterilize the solution by filtering through a 0.2-μm bottle-top filter. Store at 4 °C and use within 1 month.
8. 100 μg/mL basic FGF (bFGF): Dissolve 100 μg of recombinant human bFGF protein in 1 mL of maintenance medium for hESCs. Store an aliquot at −20 °C.
9. 500 ng/mL bFGF: The concentration of 100 μg/mL bFGF solution is adjusted to 500 ng/mL with maintenance medium for hESCs.
10. 10 mg/mL Collagenase Type IV: The concentration of collagenase IV is adjusted to 10 mg/mL with PBS.
11. 1 M $CaCl_2$: The concentration of $CaCl_2/2H_2O$ is adjusted to 1 M with dH_2O. The Solution is autoclaved to sterile.
12. Dissociation solution for hESCs (CTK solution): Combine 10 mL of 10 mg/mL Collagenase Type IV, 10 mL of 2.5% Trypsin solution, 100 μL of 0.1 M $CaCl_2$, 20 mL of KSR, and 60 mL of PBS (−). Sterilize the solution by filtering through a 0.2-μm bottle-top filter. Store an aliquot at −20 °C.
13. Y-27632 dihydrochloride: Dissolve in dH_2O to make 10-mM solution. Sterilize the solution by filtering through a 0.2-μm filter. Store at −20 °C.
14. SB431542: Dissolve in DMSO to make 100-mM solution.

15. DNase I: Dissolve sterile DNase I in culture grade water to make 10 mg/mL. Store at −20 °C.
16. 200 ng/μL bFGF: Dissolve 25 μg of recombinant human bFGF protein in 125 μL 0.1% BSA/PBS.
17. 250 mg/mL Bovine Serum Albumin (BSA): Dissolve 5 g of BSA in 20 mL of culture grade water. Store an aliquot at −20 °C.
18. 10 mg/mL apo-transferrin: Dissolve 100 mg of apo-transferrin in 10 mL of dH_2O. Sterilize the solution by filtering through a 0.2-μm filter. Store at −20 °C.
19. 10 mg/mL insulin: Dissolve 100 mg of insulin in 10 mL of dH_2O (*see* **Note 1**). Sterilize the solution by filtering through a 0.2-μm filter. Store at −20 °C.
20. Cerebellar differentiation medium: Combine 100 mL of IMDM, GlutaMAX™ Supplement, 100 mL of Ham's F-12 Nutrient Mix, GlutaMAX™, 4 mL of 250 mg/mL BSA, 2 mL of Chemically Defined Lipid Concentrate, 300 μL of 10 mg/mL apo-transferrin, 140 μL of 10 mg/mL insulin, 7.8 μL of 1-thioglycerol. Sterilize the solution by filtering through a 0.2-μm bottle-top filter. Store an aliquot at 4 °C and use within 1 month.
21. Cerebellar maturation medium 1: Combine 500 mL of Neurobasal® Medium, 5 mL of GlutaMAX Supplement, 5 mL of N2 Supplement, 2.5 mL of penicillin-streptomycin.
22. 200 ng/μL FGF19: Dissolve 25 μg of recombinant human FGF19 protein 125 μL 0.1% BSA/PBS.
23. 100 ng/μL CXCL12/SDF-1: Dissolve 10 μg of recombinant human CXCL12/SDF-1.
24. FACS buffer: Combine 1 mM of EDTA/2Na, 1 v/v% of FBS in DMEM/F12.
25. 0.1 mg/mL Poly-D-lysine (PDL): Dissolve 5 mg PDL in 50 mL culture grade water.
26. 40 μg/mL Laminin solution: Dilute 100× 1 mg/mL of Laminin.
27. Cerebellar maturation medium 2: Dissolve 3.85 g of glucose, DMEM/F12, 1.2 g of $NaHCO_3$ in 980 mL dH_2O. Adjust pH to 7.6 with NaOH. Make volume to 1 L with dH_2O. Sterilize the solution by filtering through a 0.2-μm bottle-top filter. Store an aliquot at 4 °C and use within 1 month. Add 100× penicillin-streptomycin.
28. Cerebellar maturation medium 3: Combine 10 v/v% FBS in Cerebellar maturation medium 2.
29. 200 ng/mL Tri-iodothyronine (T3): Dissolve T3 in Ethanol to make 200 mg/mL. Add 10 μL of 200 mg/mL solution to 10 mL of PBS (−). Sterilize the solution by filtering through a 0.2-μm filter. Store an aliquot at −20 °C.
30. 40 mg/mL BSA: Dissolve 5 g of BSA in 125 mL of culture grade water. Store an aliquot at −20 °C.

31. 1.6-mM Cytosine ß-D-arabinofuranoside hydrochloride (Ara-C): Dissolve of 4.5 mg of Ara-C in 10 mL of PBS (–). Sterilize the solution by filtering through a 0.2-μm filter. Store an aliquot at −20 °C.
32. Cerebellar maturation medium 4: Combine 4 mL of N_2 supplement, 100 μL of 200 ng/mL T3, 100 μL of 40 mg/mL BSA to Cerebellar maturation medium 2 before use.
33. Antibodies (*See* Table 1)

Equipment

Table 1
List of primary antibodies and their dilutions used for immunostaining

Antibody	Company	Cat. no.	Host species	Dilution
Barhl1	Atlas	HPA004809	rabbit	1/200
Calbindin	Swant	300	mouse	1/1000
Calbindin	Synaptic Systems	214004	guinea pig	1/300
En2	SantCruz	sc-8111	goat	1/100
GAD65	BD Pharmingen	559931	mouse	1/200
GABA	Sigma	A2052	rabbit	1/10,000
GluRδ2	SantCruz	sc-26118	goat	1/100
Kirrel2	R&D	AF2930	goat	1/500
Kirrel2	R&D	MAB2564	mouse	1/50
Lhx5	SantCruz	sc−19347	goat	1/100
Lim1+2	DSHB	4F7	mouse	1/50
Lmx1a	SantCruz	sc-54273	goat	1/100
L7	Takara/Clontech	M202	rabbit	1/2000
MAP2	Sigma	M1406	mouse	1/500
N-cadherin	BD Transduction	610920	mouse	1/1000
Nestin	Atlas	AMAb90556	mouse	1/1000
Nestin	Covance	PRB-315C	rabbit	1/1000
Olig2	Millipore	AB9610	rabbit	1/1000
Olig2	R&D	AF2418	goat	1/100
Otx2	SantCruz	sc-30659	goat	1/100
Otx2	Abcam	ab21990	rabbit	1/100
Otx2	R&D	MAB1979	mouse	1/1000
Skor2	Atlas	HPA046206	rabbit	1/100
SMI-32	Covance	SMI32R	mouse	1/200
Tbr1	Abcam	ab31940	rabbit	1/1000
TuJ	Covance	PRB-435P	rabbit	1/2000
TuJ	Covance	MMS-435P	mouse	1/500

34. 96-well low-cell adhesion V-bottomed culture plate: Prime Surface.
35. 96-well low-cell adhesion U-bottomed culture plate: NunclonSphera.

3 Methods

3.1 Differentiation for Cerebellar Progenitors from hESC

1. Day 0: hESC plating. Prepare sub-confluent maintained hESCs. Since hESCs are usually maintained on feeder cells, a panning step is necessary to minimize carryover of feeder cells in differentiation culture.
2. Aspirate hESC maintenance medium from a tissue culture dish, wash twice with PBS (−), and then aspirate.
3. Add 1 mL of pre-warmed CTK solution and incubate for 5 min at 37 °C.
4. Add 5 mL of maintenance medium and transfer the hESC colonies into a 15 mL conical tube.
5. Centrifuge at 180 × *g* for 5 min at room temperature (RT).
6. Remove the supernatant and resuspend the hESC colonies in 5 mL of maintenance medium.
7. Transfer the hESC colonies to a 100-mm gelatin-coated dish and incubate at 37 °C for 60–120 min to make feeder cells adhere to the dish (*see* **Note 2**).
8. Collect the medium containing floating hESCs from the dish into a 5 mL conical tube.
9. Centrifuge at 180 × *g* for 5 min at RT.
10. Remove the supernatant and resuspend the cells in 5 mL of PBS (−).
11. Centrifuge at 180 × *g* for 5 min at RT.
12. Remove the supernatant and add 1.5 mL of TrypLE Express and incubate for 5 min in 37 °C water bath.
13. Add 2 mL of cerebellar differentiation medium and suspend hESC colonies by pipetting with a P1000 tip.
14. Centrifuge at 180 × *g* for 5 min at RT.
15. Remove the supernatant and resuspend the cells in 3 mL of cerebellar differentiation medium.
16. Count the number of cells using a hematocytometer.
17. Adjust the number of dissociated cells to 4×10^4 cells/mL with cerebellar differentiation medium.
18. Seed dissociated hESCs into a 96-well V-bottomed culture plate at a density of 6000 cells/150 μL/well.
19. Incubate the plate at 37 °C under 5% CO_2.

20. Day 2: On the culture day 2, add bFGF2 to each well to a final concentration of 50 ng/mL. To do this, prepare 800 ng/mL of bFGF solution (250-fold dilution of 200 ng/μL bFGF) and then add 10 μL of diluted bFGF solution to each well. Continue to incubate cells for a further 5 day.
21. Day7: Change medium. On the culture day 7, change the medium by removing a third part of the supernatant (50 μL) from each well and replacing it with the same volume of fresh cerebellar differentiation medium. Continue to incubate aggregates for a further 7 day.
22. Day 14: Medium change. On culture day 14, change medium by moving the aggregates to a 100 mmφbacterial grade petri dish containing 15 mL of the cerebellar differentiation medium and transfer an aggregate to an each well of U-bottomed culture plate with 150 μL of the medium from the petri dish (*see* **Note 3**).
23. Day 14: If you need for a stratified cerebellar plate neuroepithelium, add FGF19 to a final concentration of 100 ng/mL. If you need cerebellar neurons, such as Purkinje cells and granule cells, the addition of FGF19 is not necessary.
24. Incubate the cells at 37 °C under 5% CO_2 for 7 days.
25. Day 21: Transfer and medium change. On culture day 21, transfer the aggregates to a 100-mm φbacterial grade petri dish containing 15 mL of Neurobasal® Medium. And then transfer the aggregates to a new 100-mm φbacterial grade petri dish containing 15 mL of Cerebellar maturation medium 1. Continue to incubate the cells for more 14 days.
26. Day 28: If you need for a stratified cerebellar plate neuroepithelium, add SDF-1 to a final concentration of 300 ng/mL (*see* **Note 4**). If you need mature cerebellar neurons, such as Purkinje cells and granule cells, the addition of SDF-1 is not necessary.
27. Days 28, 35, 42, and 49. Change medium once a week with cerebellar maturation medium 1 (*see* **Note 5**).

3.2 Immunohistochemistry fo hESC Aggregates

1. Fix aggregates with 4% PFA for 10–20 min at RT.
2. Wash twice with PBS (−) at RT.
3. Cryoprotect with 20 w/v% sucrose overnight at 4 °C.
4. Take several aggregates in a small amount of sucrose using a wide-bore yellow tip and settle down aggregates to the bottom of a mold by leaving them undisturbed for 5 min.
5. Remove excess liquid around the settled aggregates using a pipette.
6. Embed aggregates with O.C.T. compound and freeze them at −20 °C in the cryostat chamber (*see* **Note 6**).

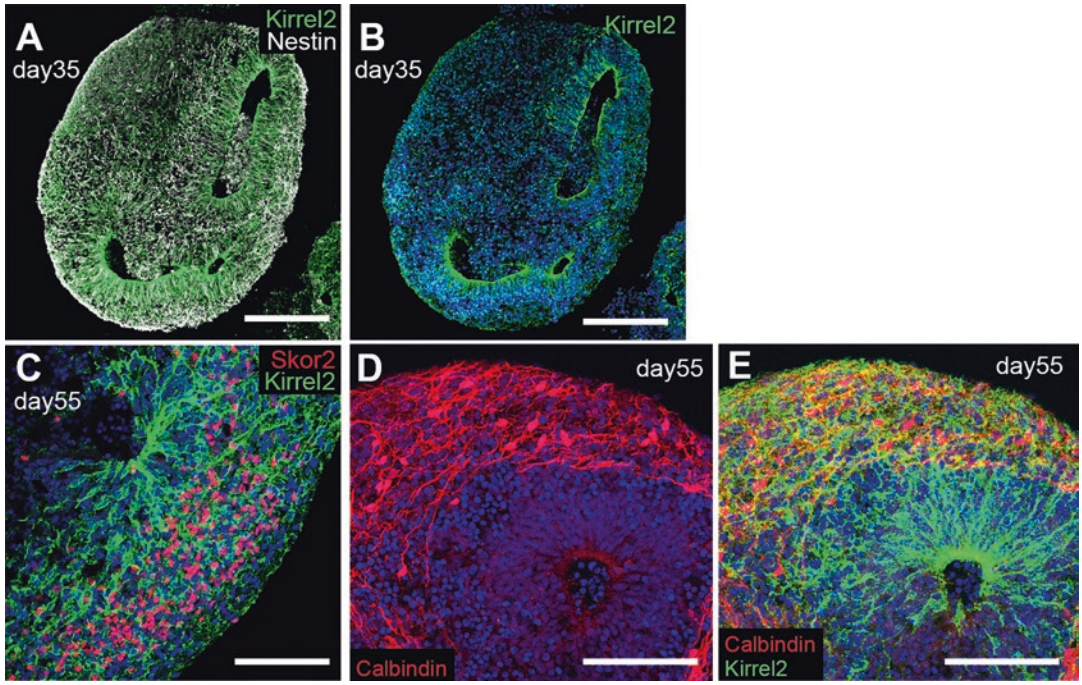

Fig. 1 Self-organization of cerebellar plate neuroepithelium from hESCs. (**a** and **b**) Immunostaining for Kirrel2, and Nestin of hESC aggregates (day 35). (**c**–**e**) Immunostaining of the aggregate on day 55. Skor2 and Calbindin were expressed in the periphery of Kirrel2$^+$ cerebellar plate neuroepithelium. The scale bars represent 200 μm (**a** and **b**), 100 μm (**c**–**e**)

7. Make serial sections (10-μm thickness) using a cryostat.
8. Carry out immunostaining using the antibodies listed in Table 1, Fig. 1.

3.3 Purification of hESC-Derived Purkinje Cell Progenitors

1. Collect the cell aggregates into a 15 mL conical tube on culture day 35.
2. Wash twice with 5 mL of HBSS (−) or PBS (−).
3. Add 1.5 mL of TrypLE Express and incubate for 5–10 min in 37 °C water bath.
4. Add 50 μL of DNase I and 2 mL of FACS buffer.
5. Suspend the cells by pipetting with a wide-bore blue tip.
6. Centrifuge at 180 × *g* for 5 min at 4 °C.
7. Remove the supernatant and resuspend the cells in 2 mL of FACS buffer.
8. Pour the cell suspension to a 5 mL round-bottom tube with cell strainer snap cap.
9. Count the number of cells using a hematocytometer.
10. Adjust the number of dissociated cells to 1 × 10^6 cells/100 μL with FACS buffer.
11. Add anti-Kirrel2 antibody (R&D AF2930) and incubate with agitation for 30 min at 4 °C.

12. Centrifuge at 180 × *g* for 5 min at 4 °C.
13. Wash with FACS buffer and centrifuge at 180 × *g* for 5 min at 4 °C (repeat three times).
14. Add secondary antibody and incubate with agitation for 30 min at 4 °C.
15. Centrifuge at 180 × *g* for 5 min at 4 °C.
16. Wash with FACS buffer and centrifuge at 180 × *g* for 5 min at 4 °C (repeat three times).
17. Suspend with FACS buffer.
18. Add 1/10 volume of 7-AAD.
19. Collect the sorted cells chilled cerebellar maturation medium 3.
20. Adjust the number of cells to 1×10^6 cells/mL.

3.4 Dissection of Mouse Rhombic Lip (RL)

Purified Kirrel2$^+$ Purkinje cells gradually die and the surviving neurons fail to express late Purkinje cell markers such as L7 and Calbindin even 2 weeks later in a conventional neuronal culture without appropriate trophic microenvironment. Cerebellar granule cells derived from mouse RL promote the maturation and survival of purified hESC-Purkinje cells in vitro. Dissecting manipulation of embryos and cerebellar tissues should be performed in chilled medium to prevent drying.

1. Sacrifice the pregnant mouse (embryonic day 14.5) by cervical dislocation and cut the abdomen along the midline with iris scissors. Resect the uterus and wash it with chilled Cerebellar maturation medium 2.
2. Dissect the embryos from the uterus. Amputate the fetal head from the body and resect the brain from the head.
3. Separate the cerebellar plate from the brain and isolate upper RL with spring scissors and fine forceps (Fig. 2).

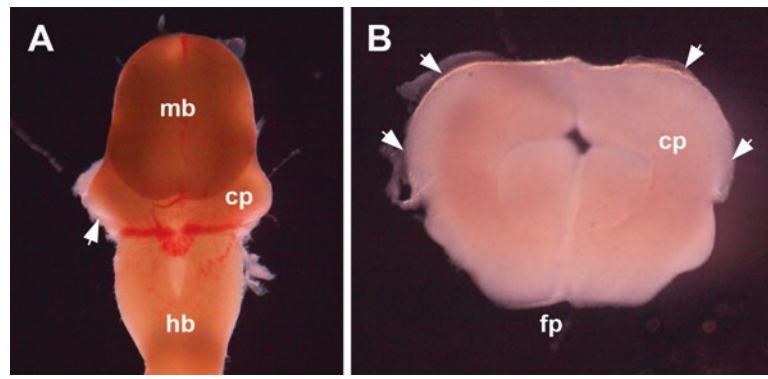

Fig. 2 Dissection of rhombic lip from mouse brain (E14.5) (**a**) Dorsal view of brain. (**b**) Cerebellar plate with rhombic lip (*Arrows*), midbrain (mb), cerebellar plate (cp), hindbrain (hb), floor plate (fp)

3.5 Coculture with Mouse Cerebellar Granule Cells for Maturation of hESC-Purkinje Cells

1. Transfer the mouse upper RL to 15 mL tube with chilled PBS (−).
2. Centrifuge at 180 × *g* for 2 min at 4 °C.
3. Remove the supernatant.
4. Add 1 mL of TrypLE Express and incubate for 5–10 min in 37 °C water bath.
5. Add 10 μL of DNase I and 2 mL of cerebellar maturation medium 3.
6. Suspend the cells by pipetting with P1000 tip.
7. Centrifuge at 180 × *g* for 5 min at 4 °C.
8. Remove the supernatant and add 2 mL of cerebellar maturation medium 3 and suspend.
9. Count the number of cells using a hematocytometer.
10. Adjust the number of cells to 1×10^6 cells/mL with cerebellar maturation medium 3.
11. Combine sorted hESC-derived Kirrel2$^+$ cells with dissociated mouse RL-derived cells at a ratio of 1:10.
12. Plate the mixed cells onto PDL/Laminin-coated round cover glasses (12 mmφ) at a density of 0.8×10^5 cells/80 μL in cerebellar maturation medium 3.
13. Incubate the cells at 37 °C under 5% CO_2 for 6 h and then add 15-fold volume of cerebellar maturation medium 4 (*see* **Notes 7** and **8**).
14. Feed the cells by replacing a half of the old medium with a fresh cerebellar maturation medium 4 with Ara-C (Fig. 3).

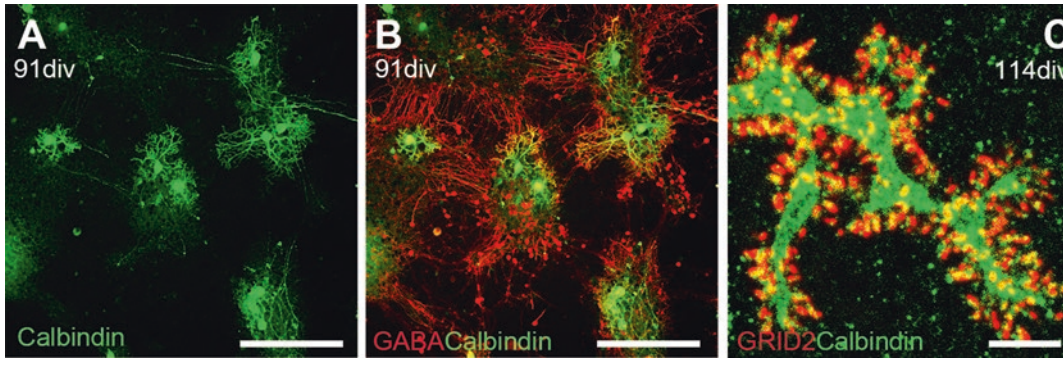

Fig. 3 Immunostaining of dissociated coculture of hESC-derived progenitors with mouse granule cells. (**a** and **b**) Calbindin and GABA are expressed in hESC-derived Purkinje cells on culture day 91. (**c**) High-power view of the hESC-derived Purkinje cells on culture day 114. Calbindin$^+$ dendritic spines express GRID2 (GluRδ2). The scale bars represent 100 μm (**a** and **b**), 5 μm (**c**)

4 Notes

1. Insulin has low solubility at neutral pH. It can be solubilized in dilute acetic or hydrochloric acid, pH 2–3.
2. The majority of feeder cells adhere to gelatin-coated dish. In order to minimize carryover feeder cells in differentiation culture, this panning step is necessary. To avoid cell death of hESCs, add 5 μL of 10 mM Y-27632.
3. This step is helpful for minimize carryover old medium in fresh medium.
4. FGF19 should be added on culture day 14 prior to the addition of SDF-1.
5. Check cells to appear the neuroepithelial-like structure in aggregates under bright-field microscopy.
6. If needed, store aggregates in a deep freezer (−80 °C) for up to several months.
7. Check the cell attaching to the cover glasses prior to the addition of medium.
8. The addition of medium reduces the concentration of FBS, which promotes glial differentiation.

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Chapter 4

Reconstitution of a Patterned Neural Tube from Single Mouse Embryonic Stem Cells

Keisuke Ishihara, Adrian Ranga, Matthias P. Lutolf, Elly M. Tanaka, and Andrea Meinhardt

Abstract

The recapitulation of tissue development and patterning in three-dimensional (3D) culture is an important dimension of stem cell research. Here, we describe a 3D culture protocol in which single mouse ES cells embedded in Matrigel under neural induction conditions clonally form a lumen containing, oval-shaped epithelial structure within 3 days. By Day 7 an apicobasally polarized neuroepithelium with uniformly dorsal cell identity forms. Treatment with retinoic acid at Day 2 results in posteriorization and self-organization of dorsal–ventral neural tube patterning. Neural tube organoid growth is also supported by pure laminin gels as well as poly(ethylene glycol) (PEG)-based artificial extracellular matrix hydrogels, which can be fine-tuned for key microenvironment characteristics. The rapid generation of a simple, patterned tissue in well-defined culture conditions makes the neural tube organoid a tractable model for studying neural stem cell self-organization.

Key words Mouse embryonic stem cells, Organoid, Neural tube, Neuroepithelium, Cyst, Lumen, PEG hydrogel, Artificial extracellular matrix, Patterning

1 Introduction

In recent years, a class of 3D cell culture technology termed "organoids" has enabled the study of organ development and function by bottom–up reconstruction. Combining technologies from tissue engineering and stem cell biology, the organoid field is anticipated to provide unique insights into the self-organizing principles of cells undergoing differentiation in a 3D environment [1], as well as to accelerate translational research and drug discovery. Some of the largest challenges faced by this emerging field are the high variability and low throughput of the protocols reported. So far, self-organization in the nervous system organoids has been achieved by forming large, pre-aggregated cultures consisting of 3000–10,000 embryonic stem cells (reviewed in [2]), which may already be a heterogeneous population before induction to

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different lineages. Another common issue is the use of Matrigel (or similar 3D matrices derived from tumor extracellular matrix), a poorly defined material in terms of chemical composition and physical properties, as the 3D environment. Development of defined culture conditions is anticipated to reduce experimental variability and advance organoid research for quantitative biology and disease modeling.

In this article, we describe the protocol for neuroepithelial organoid formation originally described in [3]. Compared to other 3D culture methods, the neural tube organoid possesses the following advantages: (1) rapid formation of lumen containing epithelium in 3 days, (2) patterned neural tube-like structures in 7 days, (3) small ~200 μm spherical tissues with relatively simple architecture and single lumen, (4) clonal growth from single cell suspensions, (5) serum-free, chemically defined culture medium, (6) fine-tuning of the physical environment (e.g., elasticity, degradability, and chemical composition) with synthetic PEG hydrogels [4].

Here, we outline the major steps for our neuroepithelial cyst generation protocol. At Day 0, mouse embryonic stem (mES) cells are embedded into Matrigel as a single cell suspension and cultured in neural induction medium (Fig. 1a). Cells proliferate, differentiate, and self-organize into a structure that possesses a single lumen and shows apicobasal polarity at Day 3 and commit to neural lineage at Day 5. The morphology resembles an embryonic neural tube. As the presence of a single lumen makes these structures more similar to epithelial cysts as described for kidney epithelial cells and clearly distinguishes them from embryoid bodies or floating aggregates, we refer to them as neuroepithelial cyst. As the cysts grow, the inner apical surfaces separate further, marking a clear open lumen. The resulting tissue is a spherical, pseudostratified neuroepithelium residing in the midbrain/hindbrain region with cells exhibiting interkinetic nuclear migration and harboring a uniformly dorsal identity. By Day 7, post-mitotic neurons at the basal surface project axons into the Matrigel.

Treatment with retinoic acid at Day 2–3 posteriorizes the cells to the cervical level of the neural tube and simultaneously yields spontaneous dorsal–ventral (DV) patterning of the tissue via induction of a Shh expressing floor plate that patterns the neuroepithelium (Fig. 2). Based on the neuroepithelial tissue architecture and the potential for dorsal–ventral patterning, these structures were termed neural tube organoids. We have also explored the optimal parameters of the 3D environment via combinatorial synthesis of custom PEG hydrogels [4, 5]. This study has already helped us reduce the variability of the tissue size and morphology (Fig. 1c) and understand which physical aspects of the 3D environment are important for the efficient establishment of apicobasal polarity and dorsal–ventral patterning.

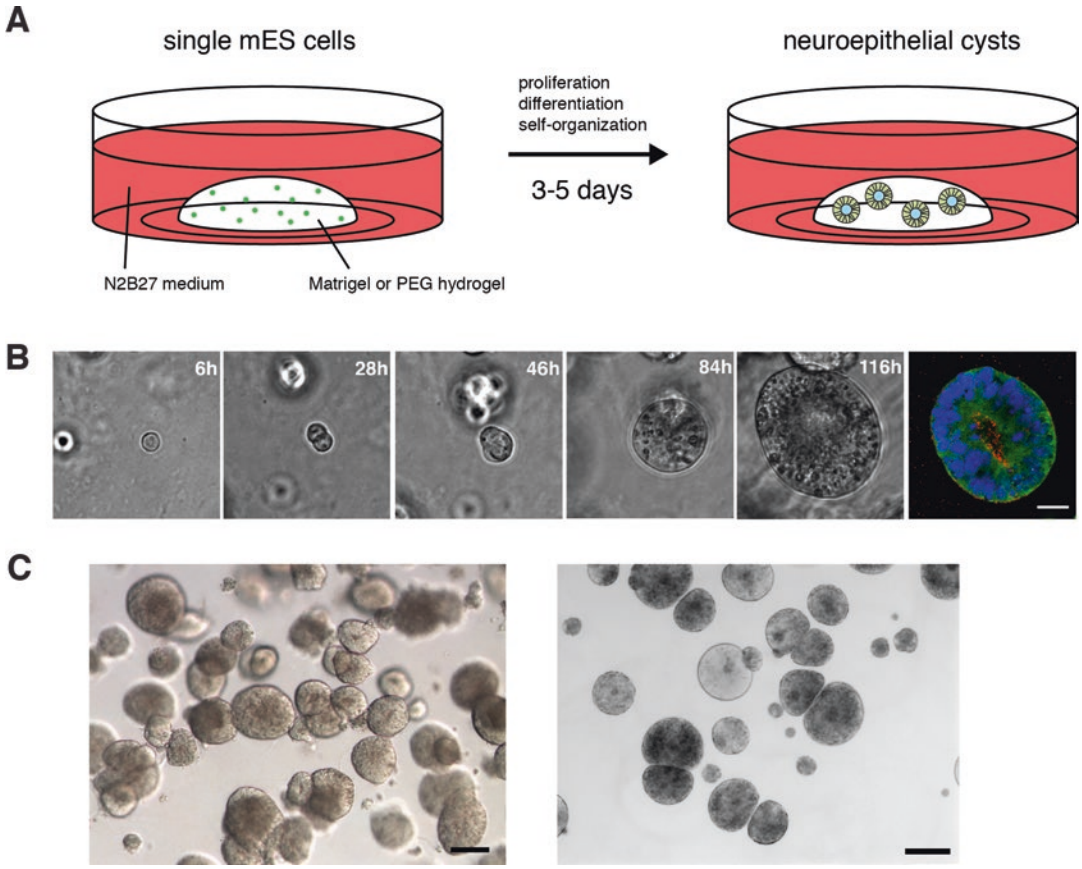

Fig. 1 Rapid neuroepithelial cyst formation from single mES cells. (**a**) Schematic of neuroepithelial cyst formation. Single mES cells embedded in a 3D environment and cultured in neural induction medium. (**b**) Phase-contrast images showing the clonal growth of a cyst over 5 days. The same cyst, fixed and visualized for PROMININ-1 (*red*), Sox1::GFP (*green*), nuclei (*blue*). The tissue exhibits an apicobasal polarity with the apical lumen in the interior. Reprinted from "Meinhardt A, Eberle D, Tazaki A, Ranga A, Niesche M, Wilsch-Bräuninger M, Stec A, Schackert G, Lutolf M, Tanaka EM 3D reconstitution of the patterned neural tube from embryonic stem cells. Stem Cell Rep 3(6):987–999, Copyright (2014), with permission from Elsevier. (**c**) Bright field images of Day 6 cysts grown in Matrigel (*left*) and PEG hydrogel with Laminin (*right*). Scale bars 100 μm

There are important differences between the neural tube morphogenesis that happens in vitro and in vivo. The organoid does not recapitulate the epithelial folding process as observed in neural plate invagination in vivo. During development, the notochord and the dorsal ectoderm provide the positional information for DV patterning, but these external tissues are absent in the organoid system. An interesting aspect of this organoid system is the opportunity to study: (1) Collective cell polarization concomitant with the pluripotent to epiblast differentiation, (2) Environmental influences on the neuroepithelium and neural differentiation, and (3) A retinoic acid induced intrinsic circuitry that achieves localized SHH expression presumably through a Turing-type autoactivation/inhibition mechanism.

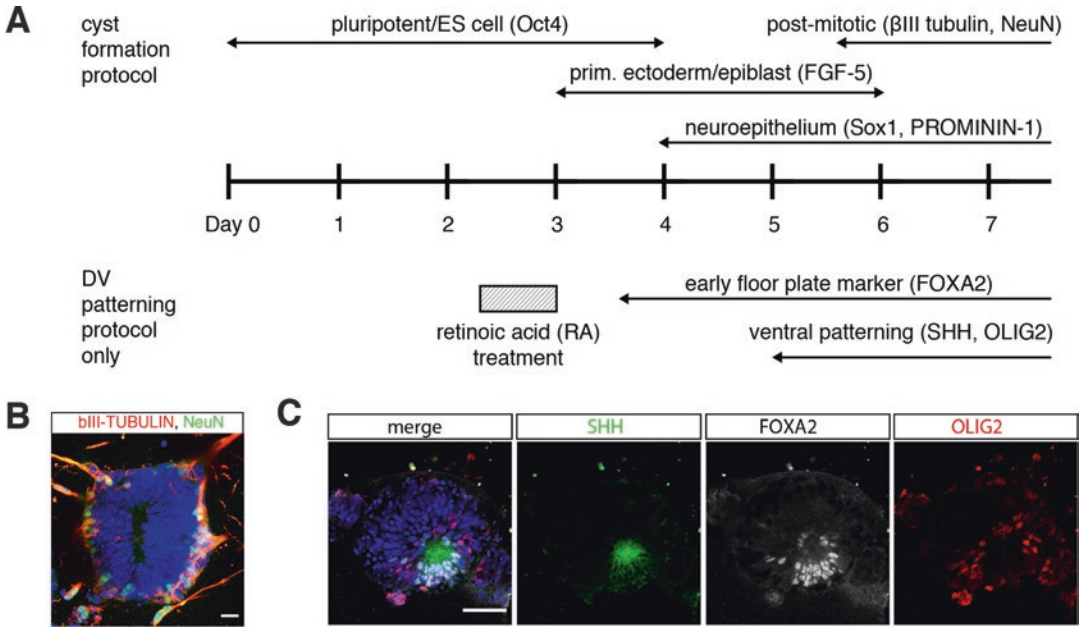

Fig. 2 Retinoic acid treatment induces dorsal–ventral patterning of the neuroepithelium. (**a**) Timeline summarizing the dynamic transition of cellular identity during the neuroepithelial cyst formation protocol. The default identity of the tissue is anterior and dorsal. When 250 nM retinoic acid (RA) is added to the media at Days 2–3, the neuroepithelium is converted to posterior identity, and dorsal–ventral (DV) patterning is observed simultaneously. (**b**) Around Day 7, post-mitotic neurons extend its axons as visualized by NeuN and βIII tubulin staining. (**c**) RA-treated cysts at Day 7, showing dorsal–ventral patterning as evidenced by the graded distribution of Shh, FOXA2, and OLIG2. Reprinted from "Meinhardt A, Eberle D, Tazaki A, Ranga A, Niesche M, Wilsch-Bräuninger M, Stec A, Schackert G, Lutolf M, Tanaka EM 3D reconstitution of the patterned neural tube from embryonic stem cells. Stem Cell Reports 3(6):987–999, Copyright (2014), with permission from Elsevier

2 Materials

Prepare all solutions at room temperature and store under indicated conditions.

2.1 Mouse ES Cell Culture

1. Mouse feeder-independent ES cells (*see* **Note 1**).
2. mES growth medium. Store at 4 °C up to 4 weeks.
3. Accutase cell detachment solution. Store in 10 mL aliquots at −20 °C. Thawed aliquots are kept at 4 °C up to two weeks (*see* **Note 2**).
4. PBS: filtered D-PBS.
5. 10 cm Falcon Optilux dish.

2.2 Neuroepithelial Cyst Formation in Matrigel

1. N2B27 medium. Store at 4 °C up to 1 week.
2. Modified N2 supplement (*see* **Note 3**). Store at −20 °C up to 3 weeks.
3. B27 supplement (containing Vitamin A). Aliquoted and stored at −20 °C.

4. All-trans retinoic acid: Prepare 10 mM stocks in DMSO. Store in liquid nitrogen and protect from light.
5. Matrigel: Standard Matrigel matrix for general cell culture. Aliquoted and stored at −20 °C (*see* **Note 4**).
6. Glass bottom dishes, 35 mm diameter (*see* **Note 5**).

2.3 Media Preparation

1. Modified N2-supplement. Add 335 µL 25 mg/mL BSA solution (*see* **Note 6**), 500 µL 100 mg/mL Apo-Transferrin (*see* **Note 7**), 625 µL 20 mg/mL Insulin (*see* **Note 8**), 16.5 µL 0.6 mg/mL Progesterone (*see* **Note 9**), 50 µL 160 mg/mL Putrescine (*see* **Note 10**), 5 µL 3 mM Sodium selenite (*see* **Note 11**), into 3.47 mL DMEM/F12 to make a 5 mL modified N2-supplement. Mix well. Store 500 µL aliquots at −20 °C.
2. mES cell growth medium. DMEM (high glucose, GlutaMax), 15% fetal calf serum (*see* **Note 12**), 2 mM L-glutamine 1 mM sodium pyruvate 1× penicillin–streptomycin 1× nonessential amino acids, 0.1 mM β-mercaptoethanol, 1000 U/mL Leukemia Inhibitory Factor. Mix all components and pass through 0.22 µm. Store at 4 °C.
3. N2B27 medium. 50 mL DMEM/F12, 50 mL Neurobasal medium, 0.5 mL modified N2 supplement, 1 mL B27 supplement, 1% penicillin–streptomycin, 100 µL 0.1 mM β-mercaptoethanol, 250 µL Pyruvate + Glutamate (*see* **Note 13**). Store at 4 °C.

2.4 Components for Neuroepithelial Cyst Formation in PEG Hydrogel

1. 10% (w/v) PEG hydrogel precursor solution. Stochiometrically balanced PEG precursor components (comprising glutamine peptide NQEQVSPLERCG and MMP-insensitive lysine peptide FKGGGDQGIAGFERCG), as prepared in [4, 6, 7].
2. 200 U/mL Activated Factor XIII (*see* **Note 14**).
3. 1 mg/mL Laminin (*see* **Note 15**).
4. 10× hydrogel buffer: 500 mM Tris, 500 mM calcium chloride, pH 7.6. Filter and store at room temperature.

2.5 Solutions for Sample Fixation and Immunofluorescence

1. 4% paraformaldehyde solution: Add 4 g of paraformaldehyde to 50 mL of water. Stir and heat to 60–70 °C. Add one drop of 10 N NaOH to dissolve. Add 50 mL of 200 mM sodium phosphate buffer pH 7.2. Store at 4 °C and use within 2 weeks.
2. Citrate solution: 10 mM citrate buffer pH 6.1. Store at 4 °C.
3. Quench solution: 150 mg Glycine dissolved in 10 mL PBS + 0.3% Triton X-100.
4. Blocking solution: 100 mg BSA dissolved in 20 mL PBS + 0.3% Triton X-100.

2.6 Solution for Recovering Cysts from Matrigel

1. Cell recovery solution. Store at 4 °C (*see* **Note 16**).

3 Methods

3.1 Maintenance of mES Cells

Culture mES cells in mES growth medium on 10 cm Falcon Optilux dish without any coating. Cells are cultured at 37 °C and 5% CO_2.

The detailed protocol for passaging is described below:

1. Pre-warm the mES growth medium, PBS, and Accutase at 37 °C.
2. Check the cells before you start (*see* **Note 17**).
3. Discard the medium carefully by aspiration (*see* **Note 18**).
4. Wash off the cells with 10 mL of PBS (*see* **Note 19**).
5. Transfer the cells to a 15 mL Falcon tube. Centrifuge at 300 × *g* for 3 min. Discard supernatant.
6. Flick the tube several times to loosen the cell pellet.
7. Add 1.5–2 mL Accutase to the cells in the Falcon tube. Mix by pipetting cells up and down. Incubate for 2 min at 37 °C.
8. Add 8 mL mES cell growth medium and mix.
9. Take up the solution in a pipette. Put the pipette tip in the middle of a clean 10 cm dish. Press tip against the bottom of the dish but slightly off the perpendicular axis and push out the cells. Repeat ten times to triturate the cell clumps (*see* **Note 20**).
10. Transfer the cell solution to a fresh 15 mL Falcon tube and centrifuge at 300 × *g* for 3 min.
11. Discard the supernatant and resuspend the pellet in 10 mL of mES cell growth medium.
12. Count the cells.
13. Transfer 600,000–700,000 cells to a new 10 cm dish containing 10 mL of fresh mES cell growth medium. Disperse the cells by moving the plate in side-to-side and back-and-forth motions.
14. Culture the cells at 37 °C.
15. Replace medium with fresh medium every day. Cells are usually ready to passage after two days.

For storage, mES cells should be suspended at a density of 2×10^6 cells/mL in mES growth medium + 10% DMSO, frozen in an isopropanol freezing container at −80 °C overnight and transferred to liquid nitrogen the following day.

3.2 Differentiation of mES Cells to Neuroepithelial Cysts in Matrigel

When the mES cells are ready to passage, it is time to start the cyst formation protocol.

1. Pre-warm the N2B27 medium to 37 °C. Thaw the Matrigel on ice (*see* **Note 21**).
2. Transfer 1,000,000 mES cells (from **step 11** in Subheading 3.1) into a 15 mL Falcon tube. Centrifuge at 300 × *g* for 3 min.
3. Discard supernatant. Add 3 mL PBS and resuspend cells. Centrifuge at 300 × *g* for 3 min.
4. Discard supernatant. Add 3 mL N2B27 and resuspend cells. Centrifuge at 300 × *g* for 3 min.
5. Discard supernatant. Add 200 μL N2B27 to resuspend cells at the desired density of 5,000,000 cells/mL.
6. Add 10 μL of the just prepared cell solution into 150 μL of freshly thawed Matrigel. Mix thoroughly. Avoid bubbles (*see* **Note 22**).
7. Equally distribute the mixture to five glass bottom dishes. Flatten the drops by smacking the dishes.
8. Incubate the dishes in the incubator at 37 °C for 15 min to solidify the Matrigel.
9. Take the dishes out from the incubator. Carefully add 2 mL N2B27 and culture at 37 °C. This is defined as Day 0.
10. Exchange 2 mL of fresh N2B27 medium every two days (*see* **Note 23**).
11. For dorsal–ventral patterning, add 250 nM retinoic acid to medium at Day 2 and incubate for 18 h (*see* **Note 24**).

3.3 Differentiation of mES Cells to Neuroepithelial Cysts in Synthetic PEG Hydrogels

1. Prepare a single cell suspension of mES cells in N2B27 medium in a similar manner to Subheading 3.2, **steps 1–5**, but at 2,000,000 cells/mL.
2. To make a 30 μL 1.5% (w/v final) PEG droplet containing 4,000 cells, add components in a sterile Eppendorf tube in the following order: 80 μL sterile water, 15 μL 10× hydrogel buffer, 22.5 μL 10% PEG hydrogel precursor solution, 15 μL Laminin, 10 μL cells. Mix well (*see* **Note 25**).
3. Add 7.5 μL of activated Factor XIII (*see* **Note 26**) to start the crosslinking reaction. Mix well for 1 min and dispense the solution equally to five glass bottom dishes (*see* **Note 27**).
4. Wait for 15 min at RT.
5. Add 2 mL N2B27 and culture at 37 °C (*see* **Note 28**). This is defined as Day 0.
6. Exchange 2 mL of fresh N2B27 medium every two days.
7. For dorsal–ventral patterning, add 250 nM retinoic acid to medium at Day 2 and incubate for 18 h.

3.4 Recovering Cells from Matrigel for Prolonged Culture

The Matrigel or the PEG hydrogel may be enzymatically digested to recover the neuroepithelial cysts. This is useful for downstream preparations such as RNA extraction and re-embedding in a new 3D environment for prolonged culture.

1. Carefully loosen coverslips from glass bottom dishes using a scalpel. Scrape off remaining glue on the coverslip.
2. Slide the Matrigel drop off the coverslip with scalpel, and transfer it to a 15 mL Falcon tube.
3. Add 1.5 mL Cell Recovery Solution.
4. Incubate on ice until the gel dissolves (30 min–1 h). Flick occasionally.
5. Dilute with PBS to 10 mL.
6. Spin at 30 × *g* for 1 min (*see* **Note 29**).
7. Carefully aspirate and discard the supernatant (*see* **Note 30**).
8. Add 10 μL of N2B27 medium and resuspend the cysts. Transfer all released cysts into 150 μL Matrigel. Make droplets and culture cysts in a similar manner to the cyst formation protocol.

3.5 Immunofluorescence and Microscopy

1. To fix cells, discard media from dish containing cysts. Apply 2 mL of ice cold 4% paraformaldehyde solution for 20 min at room temperature.
2. Discard solution. Add 2 mL of PBS (*see* **Note 31**).
3. Discard solution. Add 2 mL of quench solution, and incubate at room temperature for 25 min.
4. Discard solution. Add 2 mL of 1× Citrate buffer and incubate at 70 °C for 25 min. Skip **steps 4** and **5** if the citrate buffer incubation is unnecessary (*see* **Note 32**).
5. Let the dish cool down to room temperature for 25 min.
6. Discard solution. Block nonspecific sites by incubating cysts for 1 h at RT in blocking solution.
7. Discard solution and dry the plastic dish with a small paper. Mark a circle around the glass window with a hydrophobic marker (*see* **Note 33**).
8. Add primary antibodies in blocking solution, incubate overnight at 4 °C in the dark in a humidified chamber.
9. The next day, wash cysts 3 × 10′–3 × 15′ with PBS + 0.3% Triton X-100.
10. Dilute secondary antibodies in blocking solution. To stain nuclei, add 5 μg/mL Hoechst dye. Apply solution sample. Incubate overnight 4 °C in the dark in a humidified chamber.
11. Discard solution and add 2 mL of PBS.
12. Image the cysts on an inverted microscope (*see* **Note 34**).

4 Notes

1. Efficient cyst formation was confirmed with the following mES cell lines: R1, IB10, and 46C Sox1::GFP. 46C Sox1::GFP is a knock-in cell line derived from the E14Tg2a ES cell line [8]. All ES cell lines used are derived from the mouse strain 129. We experienced difficulty with JM8A3.N1 ES cell line (derived from C57Bl/6/N animals) and ES cells derived from the mouse strain shh-tm6Amc-/J (heterozygous and homozygous cells tested).
2. Trypsin/EDTA may also be used to passage mES cells.
3. The modified N2 formulation is described in [3, 9]. It has increased concentrations of Insulin and BSA.
4. Matrigel should be thawed overnight in the cold room before aliquoting. As Matrigel composition varies from lot to lot, it is advisable to test for cyst formation and DV patterning before purchasing a large batch.
5. Glass bottom dishes allow confocal imaging on an inverted microscope.
6. To make 75 mg/mL BSA solution, weigh 375 mg BSA, and make it to 5 mL with PBS. Filter sterilize it through 0.22 μm filters. Store 350 μL aliquots at −20 °C.
7. To make 100 mg/mL Transferrin, dissolve 500 mg Transferrin in 5 mL sterile water. Store 500 μL aliquots at −20 °C.
8. To make 20 mg/mL Insulin, 100 mg Insulin from bovine pancreas is dissolved in 5 mL 0.01 M HCl. Filter sterilize it. Prepare 630 μL aliquots and store at −20 °C. When preparing N2-supplement, we usually add Insulin as the last component. After adding Insulin, the solution may initially turn cloudy but should be clear before aliquoting.
9. To make 0.6 mg/mL Progesterone, dissolve 1.2 mg Progesterone in 2 mL ethanol. Store 50 μL aliquots at −20 °C.
10. To make 160 mg/mL Putrescine, dissolve 160 mg Putrescine in 1 mL sterile water. Filter sterilize it through 0.22 μm filters. Store 50 μL aliquots at −20 °C.
11. To make 3 mM Sodium selenite, weigh 2.59 mg Sodium selenite powder and dissolve it in 5 mL sterile water. Filter sterilize it through 0.22 μm filters. Prepare 200 μL aliquots and store at −20 °C.
12. Fetal calf serum needs to be heat inactivated at 56 °C for 30 min prior to aliquoting. To control for serum variability, different lots should be tested for mES cell growth and maintenance of pluripotency.

13. Pyruvate + Glutamate stock solutions are 100 mM sodium pyruvate and 200 mM L-Glutamine. Mix 5.5 mL of L-Glutamine and 8.25 mL of sodium pyruvate and store in aliquots at −20 °C.
14. To make 200 U/mL activated Factor XIII, reconstitute Factor XIII in water at 200 U/mL. To 1 ml of this solution, add 100 μL of thrombin (from human plasma, reconstituted at 20 U/mL in 10 mM Tris, 150 mM NaCl, 25 mM CaCl2, pH 7.4). Calcium ions activate thrombin, which in turn activates Factor XIII. Incubate at 37 °C for 30 min. Store aliquots at −80 °C.
15. Other extracellular matrix proteins such as Entactin, Collagen, Fibronectin may be used [4, 5].
16. TrypLE Express has also been used for recovering cysts from PEG hydrogels (*see* [4, 5]).
17. The morphology of cultured mES cells can vary between tight round colonies and flattened round colonies. Tight round colonies attach less tightly to the plastic than the flattened colonies and can therefore be washed off the plate more easily. Abnormal morphology is sign of differentiation, and such cultures should be discarded. Nutrient starvation and high confluence (>80%) are common factors that cause differentiation.
18. Floating cells will be discarded but there should still be plenty of cells left on the dish.
19. You will see the cells come off as you wash the surface with PBS. Take up the cell solution and repeat the rinse couple of times. In case of more flattened mES cells you might need to use Accutase or Trypsin/EDTA to lift them from the plate.
20. The idea is to dissociate the larger clumps into single cells. Do not hold the pipette completely perpendicular as this may damage the cells.
21. Do not thaw Matrigel with your hands as this may alter its physical properties.
22. Sufficient cell density is critical for efficient cyst formation after embedding in the 3D matrix. 50,000 cells per 150 μL Matrigel, divided into five dishes, is a suggested density for first timers.
23. From Day 5 medium is changed daily due to higher medium consumption of the cells. We observe the highest proportion of healthy cysts (judged by morphology) in a ring shaped region of intermediate radial distance from the center to the outer border.
24. The Day 2–3 time window of retinoic acid exposure is critical for efficient dorsal–ventral patterning. Retinoic acid is light

sensitive; protect it from light. The proportion of successful DV patterning as judged by localized Shh staining varies from 40 to 50% of cysts at Day 7 depending on the matrix and cell lines used [3, 5].

25. To control the elasticity of the PEG hydrogel, vary the amount of PEG hydrogel precursor solution and adjust the volume of water accordingly. Final PEG concentrations of 1, 1.5, 2, 3% result in elasticities of 0.5, 2, 4, 8 kPa as measured by indentation assay [4]. We found that an intermediate elasticity, 2–4 kPa, was necessary for the efficient cyst formation [5]. The small amount of Laminin (relative to PEG crosslinking sites) does not significantly affect the elasticity.
26. The most common cause of inconsistent PEG hydrogel solidification is due to inactive Factor XIII. Enzyme activity and cross-linking time significantly affects the mechanical properties of the resulting gel, and we recommend only one freeze thaw cycle activated Factor XIII solution. To test Factor XIII activation, mix PEG precursor solution, hydrogel buffer, water, and Factor XIII in a 10 μL reaction. A 1.5% PEG solution should solidify after 2–5 min as judged by poking the surface with a pipette tip. We routinely perform such tests before encapsulating cells.
27. Cells sink to the bottom of the solution before solidification. The experimenter may wish to tune the duration of mixing before dispensing the solution.
28. Do not wash gels in PBS, phosphate will precipitate in the presence of active Factor XIII, thereby rendering the gel opaque. PBS can be used at later time points, after the gel is completely polymerized and the enzyme is inactive.
29. The low speed spin preserves the integrity of the cysts. This is particularly important when re-embedding the cysts in Matrigel for prolonged cultures.
30. For RNA extraction, add lysis buffer at this step and proceed.
31. Samples are stored at 4 °C in PBS for up to one week. Seal with Parafilm to prevent evaporation.
32. Citrate buffer incubation exposes antigenic sites for antibody binding, but may have adverse effects depending on the antibody. The antibodies listed in Table 1 have all been tested to be compatible with citrate buffer incubation.
33. The hydrophobic circle contains the solution to the center of the dish. Smaller volumes of antibody solution can be used in this way.
34. Due to the thickness of the Matrigel droplet, it is easier to image cysts near the edges of droplet, closer to the cover slip.

Table 1
Table of primary antibodies used for immunofluorescence of neuroepithelial cysts

Target	Host/type	Dilution for IF	Source	Comments
NANOG	Rabbit monoclonal	1:500	Cell signaling (D2A3)	Pluripotency marker
OCT4	Rabbit polyclonal	1:500	Abcam	Pluripotency marker
MUSASHI			H. Okano	Neural stem cell marker
NESTIN	Mouse monoclonal	1:50	DSHB (Rat-401)	Neural stem cell marker
ECADHERIN	Mouse	1:200	BD Biosciences	Embryonic cell adhesion protein
NCADHERIN	Rat monoclonal	1:50	DSHB (MNCD2)	Neuroepithelium, apical
SOX1	Goat polyclonal	1:5000	R&D	Neuroepithelium
PROMININ-1	Rat monoclonal		W. Huttner (13A4)	Neuroepithelium, apical
ZO-1	Rat monoclonal	1:50	DSHB (R26.4C)	Neuroepithelium, apical
LIM1+2	Mouse monoclonal	1:100	DSHB (4F2)	DV patterning interneuron marker
Nkx6.1	Mouse monoclonal	1:100	DSHB (F55A10)	DV patterning motor neuron marker
BRN3a	Goat polyclonal	1:100	Santa Cruz (C-20)	DV patterning, dorsal
PAX3	Mouse monoclonal	1:50	DSHB	DV patterning, dorsal
FOXA2	Mouse monoclonal	1:50	DSHB (4C7)	DV patterning, early floor plate marker
SHH	Rabbit polyclonal	1:200	Santa Cruz	DV patterning, floor plate marker
ARX	Sheep polyclonal	1:200	R&D	DV patterning, late floor plate marker
ISL1/2	Mouse monoclonal	1:50	DSHB (39.4D5)	DV patterning, ventral, motor neuron marker
pSMAD	Rabbit polyclonal	1:200	Cell signaling	DV patterning, ventral, phospho-SMAD1/5/8
OLIG2	Goat polyclonal	1:200	R&D	DV patterning, ventral, motor neuron marker
OLIG2	Rabbit polyclonal	1:200	Millipore	DV patterning, ventral, motor neuron marker
EN1	Mouse monoclonal	1:50	DSHB (4G11)	Midbrain marker
OTX2	Rabbit polyclonal	1:200	Abcam	Midbrain marker, neural plate
HOXB4	Rat monoclonal	1:50	DSHB (I12)	Posterior neural plate marker
pH3	Rabbit polyclonal	1:200	Chemicon	Mitotic cells, phospho-histone H3
PAX7	Mouse monoclonal	1:50	DSHB	Neural plate progenitor marker
betaIIITUBULIN	Mouse monoclonal	1:200	Chemicon (TU-20)	Post-mitotic neuron, axonal
Map2a	Mouse	1:200	Sigma	Post-mitotic neuron, axonal
NeuN	Mouse monoclonal	1:200	Chemicon (A60)	Post-mitotic neuron, nuclear

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Chapter 5

Functional Pituitary Tissue Formation

Chikafumi Ozone and Hidetaka Suga

Abstract

The adenohypophysis, which mainly consists of anterior pituitary, plays important roles for endocrine systems by secreting several hormones indispensable for maintaining homeostasis. During early mouse development, the pituitary primordium (called Rathke's pouch) develops from oral ectoderm adjacent to ventral hypothalamus by interaction between these two tissues. By using mouse embryonic stem cells (ESCs), we recapitulated this in vivo micro-environment of the pituitary development and demonstrated that Rathke's pouch-like structures were self-formed from three-dimensional (3D) floating culture. The mouse ESC-derived Rathke's pouch-like structures subsequently differentiated into hormone-producing cells such as corticotrophs and somatotrophs. We have modified this technique for human pluripotent stem cells and recently reported that pituitary placodes can also be generated from human ESCs through a similar process. Here, we describe a protocol for human ESC culture for in vitro generation of 3D pituitary tissue.

Key words Pituitary gland, Hypothalamus, Embryonic stem cell, Pluripotent stem cell, Development, Regenerative medicine, Self-organization, 3D culture, Aggregate

1 Introduction

The anterior pituitary is a critical component of the endocrine system and secretes several important systemic hormones, such as adrenocorticotropic hormone (ACTH) and growth hormone (GH) [1, 2]. Some disorders of the pituitary called hypopituitarism can cause various maladies, some of which are life-threatening. The current therapy for hypopituitarism consists of the hormone-replacement therapy. However, this approach has some disadvantages. For example, it requires life-long treatment, because it is not a curative therapy. Moreover, exogenous hormone administration does not emulate the dynamic secretion changes of hormones in response to circadian rhythms or stress. For these reasons, the generation of functional pituitary tissue would be an advance forward developing a curative effective therapy—regenerative medicine—for pituitary diseases.

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Toward this aim, we previously reported efficient self-formation of 3D pituitary tissue from mouse ESCs in vitro [3]. We have achieved in vitro pituitary induction by using stem cell cultures to recapitulate in vivo pituitary development. During early mouse embryogenesis, the anterior pituitary arises from the dorsal region of the oral ectoderm upon receiving inductive signals from the juxtaposing ventral hypothalamus [4–9]. In response to inductive signals from the hypothalamus, the oral ectoderm forms a thickened placode, and subsequently invaginates to form a hollowed epithelial vesicle called Rathke's pouch—a primordium structure to the pituitary (Fig. 1a). Then, this vesicle differentiates into hormone-producing cells such as ACTH-producing cells (corticotrophs) and GH-producing cells (somatotrophs). In accordance with the pituitary developmental processes in vivo, we induced hypothalamus and oral ectoderm by using mouse ESC culture [3]. In floating aggregation culture of mouse ESCs (called SFEBq culture; *see* Chapter 1) suitable for hypothalamic induction [10], hypothalamic differentiation and oral ectoderm differentiation are simultaneously co-induced within a single aggregate by optimized treatments with hedgehog signals and by increasing the initial plating cell number from 3,000 to 10,000 per aggregate [3]. These two tissues form juxtaposing ectodermal layers; the outer layer is oral ectodermal tissue, and the inner is a hypothalamic neuroectoderm. In the co-presence of these two tissues, a part of the thickened oral ectoderm invaginates to self-form Rathke's pouch-like structures that have the ability to differentiate into pituitary hormone-producing cells after long-term culture. We demonstrated that mouse ESC-derived corticotrophs secreted ACTH in response to both positive and negative regulators that work in vivo, and when engrafted into hypopituitary mice, they rescued not only hormone levels but also survival of the hosts [3].

By modifying this mouse ESC culture method, we have recently reported the human pituitary differentiation method [11] (Fig. 1B). Here, we describe the detailed protocol for the generation of 3D pituitary tissue from human ESCs. We also discuss some differences between human and mouse ESC floating aggregate culture protocol for the differentiation of pituitary tissue.

2 Materials

2.1 Reagent Setup

0.1% (wt/vol) Gelatin solution. To prepare 0.1% (wt/vol) gelatin solution, dissolve 0.5 g of gelatin in 500 mL of water by autoclaving. The solution can be stored at 4 °C for up to 3 months.

0.1 M 2-ME. To prepare 2-ME, 0.1 M, dissolve 0.1 mL of 2-ME in 14.1 mL of PBS(−). The solution can be stored at 4 °C for up to 1 month.

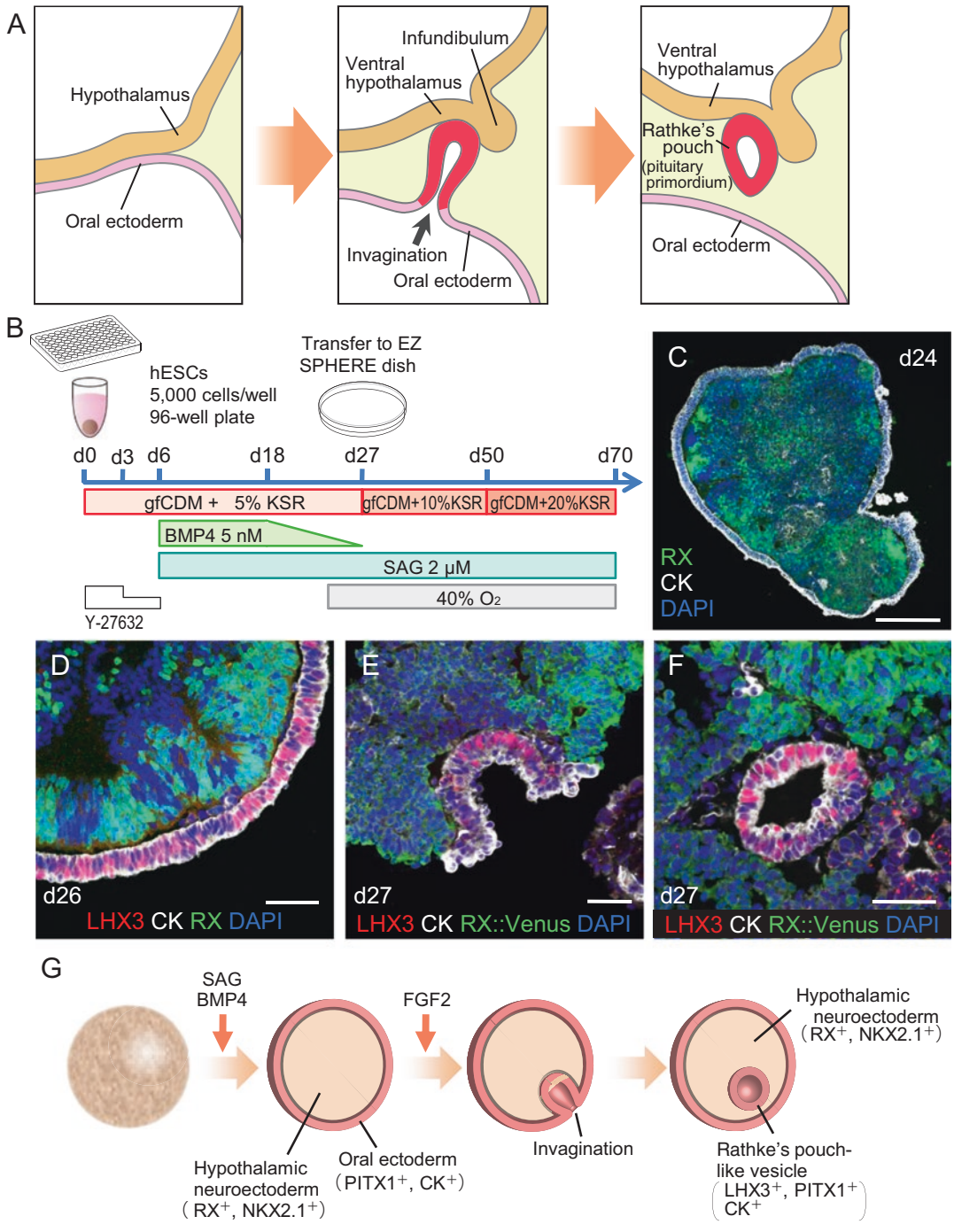

Fig. 1 Generation of pituitary placodes in floating hESC culture. (**A**) Schematic diagram of mouse pituitary development (embryonic days 10–13), sagittal view. (**B**) Culture protocol ACTH-producing cells. *d*, day. (**C**) Adjacent formation of oral ectoderm (pan-Cytokeratin+; *white*) and hypothalamic neuroectoderm (RX+; *green*) in 3D hESC culture. (**D**–**F**) Morphogenesis of Rathke's pouch-like structure in vitro. LHX3; *red*, pan-Cytokeratin; *white*, RX; *green*, DAPI; *blue*. (**G**) Schematic of generation of hESC-derived Rathke's pouch-like 3D structure in vitro. Scale bars, 200 μm (**C**) and 50 μm (**D**–**F**)

bFGF working solution for hESC maintenance. Reconstitute 100 μg of bFGF with 1 mL of hESC maintenance medium to a concentration of 100 ng/mL; next, divide the solution (50 μL per tube) into aliquots and store them at −20 °C until use. These aliquots can be stored for up to 3 months.

bFGF working solution for hESC maintenance. Retrieve 50 μL of bFGF stock solution from storage at −20 °C. Dilute it with 10 mL of hESC maintenance medium to a final concentration of 500 ng/mL. This solution can be stored at 4 °C for up to 2 weeks.

bFGF, for pituitary differentiation. Dissolve 25 μg of bFGF in 250 μL of 0.1% (wt/vol) BSA/PBS to yield a 100 ng/mL stock solution. This solution can be stored at 4 °C for up to two weeks or at −20 °C for up to 3 months.

BMP4. Reconstitute at 1 μM in 0.1% (wt/vol) BSA/PBS. This solution can be stored at 4 °C for up to two weeks or at −20 °C for up to 3 months.

Y-27632. Reconstitute at 10 mM in distilled water. This solution can be stored at 4 °C for up to two weeks or at −20 °C for up to 3 months.

DNase I. Reconstitute at 10 mg/mL in PBS. This solution can be stored at 4 °C for up to two weeks or at −20 °C for up to 3 months.

MEF culture medium. Mix 500 mL of DMEM and 50 mL of FBS. This medium can be stored at 4 °C for up to a month.

Human ESC maintenance medium. Combine 500 mL of DMEM/F-12, 125 mL of KSR, 5 mL of NEAA, 6.25 mL of L-glutamine, 500 μL of 0.1 M 2-ME. Sterilize the solution by filtering through a 0.2-μm bottle-top filter, store at 4 °C, and use within a month. Basic FGF should be freshly added to the culture medium each time at medium change. For 10 mL of maintenance medium, add 100 μL of bFGF working solution so that the final concentrations become 5 ng/mL.

Growth factor-free chemically defined medium (gfCDM). Prepare by combining 250 mL of IMDM (containing GlutaMAX), 250 mL of F-12 (containing GlutaMAX), 5 mL of CDLC, 10 mL of 250 mg/mL BSA, and 19.5 μL of 1-thioglycerol. Sterilize the solution by filtering through a 0.2-μm bottle-top filter, store at 4 °C and use within 2 weeks.

Pituitary differentiation medium (gfCDM + 5% KSR/gfCDM + 10% KSR). Prepare gfCDM supplemented with 5 or 10% (vol/vol) KSR. Sterilize the solution by filtering through a 0.2-μm bottle-top filter, store at 4 °C, and use within 2 weeks.

Pituitary differentiation medium (gfCDM + 20 % KSR). Combine 400 mL of gfCDM, 100 mL of KSR, and 500 μL of fungisone. Sterilize the solution by filtering through a 0.2-μm bottle-top filter, store at 4 °C, and use within 2 weeks.

3 Methods

3.1 Human ESC Maintenance

1. *Plating MEFs on a coated dish to generate a feeder layer for hESCs*. Coat a 100-mm dish with an appropriate volume of 0.1% (wt/vol) gelatin. For a 100-mm dish, use 7 mL of 0.1% gelatin.
2. Incubate the plates at 37 °C for an hour.
3. After incubation, remove the coating solution and add the mitomycin C-treated MEF suspension in MEF culture medium. Optimized MEF cell numbers are 12×10^5 for a 100-mm dish and 4×10^5 for a 60-mm dish. It is important to seed the feeder cells at the appropriate density to avoid hESC differentiation. MEFs seeded at this density can usually be used for human ESC culture 1–2 days after seeding.
4. Incubate MEFs at 37 °C and 5% CO_2 overnight.
5. *Maintenance of human ESCson the feeder layer*. We routinely use hESC lines such as KhES-1. Passage the ESCs onto fresh MEF plates from **step 4** once the ESCs have reached 20% confluency (usually after 4 days). Healthy human ESCs should appear with clear colony edges and high cell density in the center of the colony. The suitable colony size for passaging should be between 1 and 2 mm in diameter.
6. Take human ESCs at 20% confluency in a 100-mm dish, aspirate the medium and wash it twice with 10 mL of PBS. Remove the PBS. Add 1.2 mL of dissociation solution and ensure that the solution covers all the cells.
7. Incubate the cells at 37 °C, 2% CO_2, for 10 min.
8. Shake the plate to facilitate cell detachment. When all the colonies are detached, add pre-warmed 10 mL of hESC maintenance medium. Resuspend and collect the colonies in a tube for centrifugation.
9. Centrifuge at 180 × *g* for 10 s at room temperature.
10. Remove the supernatant and resuspend the colonies in 1 mL of hESC maintenance medium.
11. Gently pipette the colonies into fragments sized 100–200 μm by using a P-1000 tip and resuspend them in 30–50 mL of hESC maintenance medium containing 300–500 μL of bFGF working solution.

12. Take a fresh MEF feeder dish from **step 4**, discard the MEF medium, wash it with 10 mL of PBS twice, and aspirate and discard the PBS.
13. Plate 10 mL of the medium containing the dissected colonies from **step 11** into the MEF feeder dish from **step 12**.
14. Incubate the cells with daily medium change (10 mL of hESC maintenance medium containing 100 μL of bFGF working solution) at 37 °C, 2% CO_2, for 3–5 days.

3.2 Human ESC Differentiation, In Vitro Pituitary Corticotroph Differentiation

1. *Day 0*: Add 7 mL of 0.1% (wt/vol) gelatin solution to a culture dish and incubate the dish at 37 °C for more than 30 min.
2. Take human ESCs at 20% confluency in a 100-mm dish, aspirate the medium, and wash it twice with 10 mL of PBS. Remove the PBS. Add 1.2 mL of dissociation solution and ensure that the solution covers all the cells.
3. Incubate the cells at 37 °C, 2% CO_2, for 10 min.
4. Shake the plate to facilitate cell detachment. When all the colonies are detached, add pre-warmed 10 mL of hESC maintenance medium containing 2 μL of DNase I. Resuspend and collect the colonies in a tube for centrifugation.
5. Centrifuge at 180 × *g* for 10 s at room temperature.
6. Remove the supernatant and resuspend the colonies in 7 mL of hESC maintenance medium with 10 μM Y-27632.
7. Remove the gelatin solution from the other culture dish setup in **step 15**.
8. Pour 7 mL of the medium containing the detached colonies and Y-27632 from **step 20** into the gelatin-coated dish (*see*-**Note 1**).
9. Incubate the cells at 37 °C, 2% CO_2, for 90 min to make MEF feeder cells adhere to the dish bottom.
10. Gently transfer the detached colonies into a 15 mL conical tube.
11. Centrifuge at 180 × *g* for 10 s at room temperature.
12. Remove the supernatant and resuspend the cells gently in 10 mL PBS.
13. Centrifuge at 180 × *g* for 10 s at room temperature.
14. Remove the supernatant and resupend the cells in 1.6 mL of TrypLE Express containing 30 μg/mL DNase I and 10 μM Y-27632.
15. Incubate the cells in water bath at 37 °C for 4 min.
16. Pipette the colonies very gently into single cells by using a P-1000 tip and resuspend them in 10 mL of gfCDM + 5%

(vol/vol) KSR containing 30 μg/mL DNase I and 10 μM Y-27632.

17. Centrifuge at 180 × *g* for 3 min at room temperature.
18. Remove the supernatant and resupend the cells in 1 mL of gfCDM + 5% KSR containing 20 μM Y-27632.
19. Count the number of cells using a cell counter.
20. Adjust the concentration to 5×10^4 cells/mL with gfCDM + 5% KSR containing 20 μM Y-27632.
21. Plate hESCs into a 96-well low-cell-adhesion plate with V-bottomed conical wells (5000 cells per 100 μL/well; *see* **Note 2**).
22. Incubate the plate at 37 °C under 5% CO_2 and 21% O_2.
23. *Day 3* (*see* **Note 3**). Add gfCDM + 5% KSR medium (100 μL/well) without Y-27632 to each well.
24. *Day 6: addition of SAG and BMP4.* Remove half of the supernatant and replacing it with the same volume of gfCDM + 5% KSR with 4 μM SAG and 10 nM recombinant human BMP4 (2 μM and 5 nM final concentrations, respectively) (*see* **Note 4**).
25. *Days 9, 12, and 15: medium change*. Change medium by removing half of the supernatant and replacing it with the same volume of gfCDM + 5% KSR supplemented with 2 μM SAG and 5 nM BMP4 (*see* **Note 5**).
26. *Days 18, 21, and 24: medium change*. Change medium by removing half of the supernatant and replacing it with the same volume of gfCDM + 5% KSR supplemented with 2 μM SAG (without BMP4). Incubate the plate under 40% O_2. By day 24, Cytokeratin$^+$ non-neural ectoderm is observed surrounding RX$^+$ neuroectoderm in the aggregates (Fig. 1c).
27. *Day 27* (*see* **Note 6**)*: Transfer*. Transfer the aggregates from a 96-well plate to a 100-mm EZ SPHERE dish (at a density of ~60 aggregates per dish) containing 13 mL of gfCDM + 10% (vol/vol) KSR supplemented with 2 μM SAG. Incubate the plate at 37 °C under 5% CO_2 and 40% O_2. By days 27–30, Rathke's like-structures (LHX3$^+$) are observed in the aggregates (Fig. 1d–g).
28. *Medium change*. From day 30, perform a full medium change every third day. Increase the concentration of KSR of the medium to 20% (vol/vol) from day 50. SAG (2 μM) is needed throughout the culture from day 6. ACTH-positive cells can be detected by immunohistochemistry by day 65 (Fig. 2).

3.3 Immunohistochemistry for ESC Aggregates

See Chapter 1.

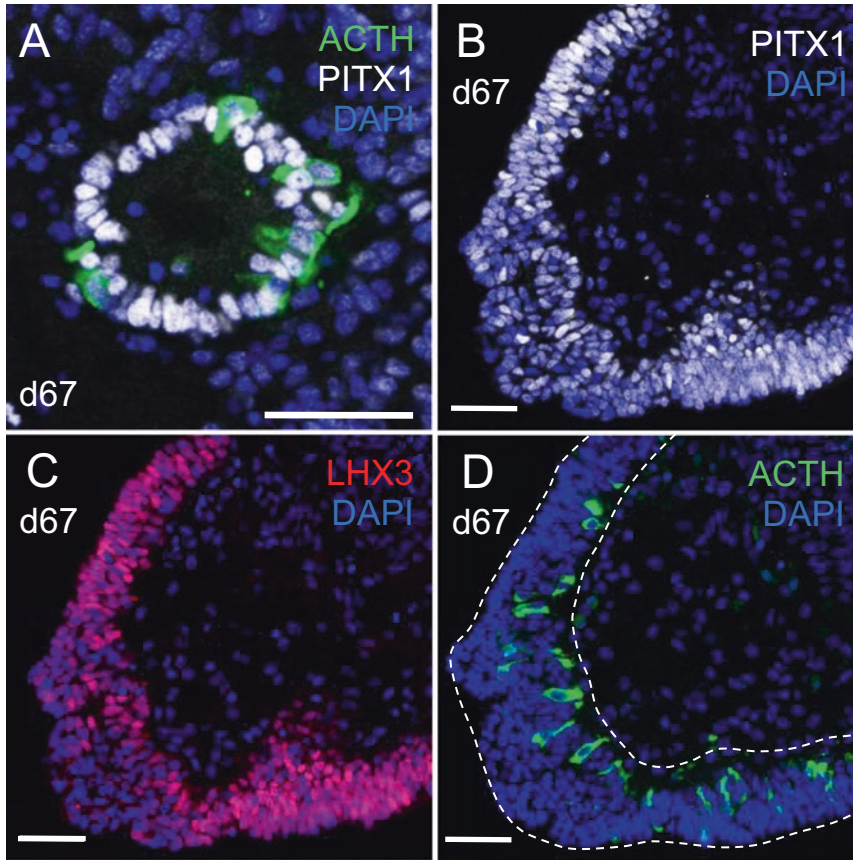

Fig. 2 Human ESC-derived corticotrophs in SFEBq culture. (**A**) PITX1 + pouch-forming ectoderm (*white*) generated ACTH$^+$ (*green*) cells in day-67 aggregates. (**B**–**D**) Immunostaining of day-67 non-pouch-forming pituitary placode for PITX1 (*white*; **B**), LHX3 (*red*; **C**), ACTH (*green*; **D**)

4 Notes

1. It takes about 20 min from **steps 1** to **8**.
2. It takes about 30 min from **steps 10** to **21**.
3. If hESCs do not aggregate well by day 3, they do not differentiate into pituitary placodes efficiently.
4. In mouse ESC culture, treatment with exogenous BMP4 is not necessarily needed for pituitary induction, because endogenous BMP expression is elevated in a large cell aggregate (10,000 cells/well); enabling the differentiation of non-neural ectoderm [3, 12]. On the other hand, in hESC culture, endogenous BMP signals are not enough to induce oral ectoderm. Thus, exogenous BMP is required for human pituitary differentiation in vitro [11].

5. 20 ng/mL bFGF treatment during days 15–27 facilitates the formation of Rathke's pouch-like structures. This FGF2 treatment, however, causes no substantial changes in the induction of hormone-producing cells, because non-invaginated portion of oral ectodermal tissue also expresses LHX3 after long-term culture and differentiates into pituitary endocrine cells. Thus, the morphological formation of pouch structures is not necessarily required for inducing pituitary hormonal cells.
6. Non-invaginated pituitary placodes are observed surrounding neuro-epithelium under stereomicroscope and phase-contrast microscope by day 27.

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Chapter 6

Directed Differentiation of Mouse Embryonic Stem Cells Into Inner Ear Sensory Epithelia in 3D Culture

Jing Nie, Karl R. Koehler, and Eri Hashino

Abstract

The inner ear sensory epithelium harbors mechanosensory hair cells responsible for detecting sound and maintaining balance. This protocol describes a three-dimensional (3D) culture system that efficiently generates inner ear sensory epithelia from aggregates of mouse embryonic stem (mES) cells. By mimicking the activations and repressions of key signaling pathways during in vivo inner ear development, mES cell aggregates are sequentially treated with recombinant proteins and small molecule inhibitors for activating or inhibiting the Bmp, TGFβ, Fgf, and Wnt signaling pathways. These stepwise treatments promote mES cells to sequentially differentiate into epithelia representing the non-neural ectoderm, preplacodal ectoderm, otic placodal ectoderm, and ultimately, the hair cell-containing sensory epithelia. The derived hair cells are surrounded by a layer of supporting cells and are innervated by sensory neurons. This in vitro inner ear organoid culture system may serve as a valuable tool in developmental and physiological research, disease modeling, drug testing, and potential cell-based therapies.

Key words Inner ear, Hair cells, Sensory epithelium, Vestibular, Mouse pluripotent stem cells, Organoid, Three-dimensional culture

1 Introduction

Inner ear hair cells are mechanosensitive receptors that detect sound, gravity, and movement, and convert these signals into our sense of hearing and balance [1]. These sensory hair cells do not regenerate to any clinically relevant degree in mammalian inner ears, resulting in permanent hearing loss or vestibular impairments affecting millions of people worldwide [2].

Studies of mouse, chick, zebrafish, and *Xenopus* inner ear development have accumulated evidence that bone morphogenetic protein (Bmp) signaling, fibroblast growth factor (Fgf) signaling, and Wnt signaling are important in the development of the inner ear. During embryonic development in vivo, Bmp signaling activates a region of the definitive ectoderm, resulting in the formation of non-neural ectoderm [3–9]. Subsequent Bmp inhibition, along

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with Fgf signaling activation, induces the formation of the preplacodal region from the non-neural ectoderm [6, 10, 11–15]. The otic placode, along with other cranial placodes (e.g., olfactory, lens, epibranchial, etc.), is derived from the preplacodal region [3, 16]. Wnt is known to be a signaling cue that promotes the formation of the otic placode [17, 18]. Following induction, the otic placode invaginates to form the otic vesicle [19], which is the source of nearly all cell types of the mature inner ear, including the sensory hair cells [1] (Fig. 1).

We recently developed a method to derive inner ear sensory epithelia harboring functional hair cells from mouse embryonic stem cells (mESCs) in a three-dimensional (3D) culture, using stepwise treatment of signaling molecules, such as BMP-4 and FGF-2, that mimic those present in inner ear development in vivo [20–22] (Figs. 1 and 2). Our method was built upon recent

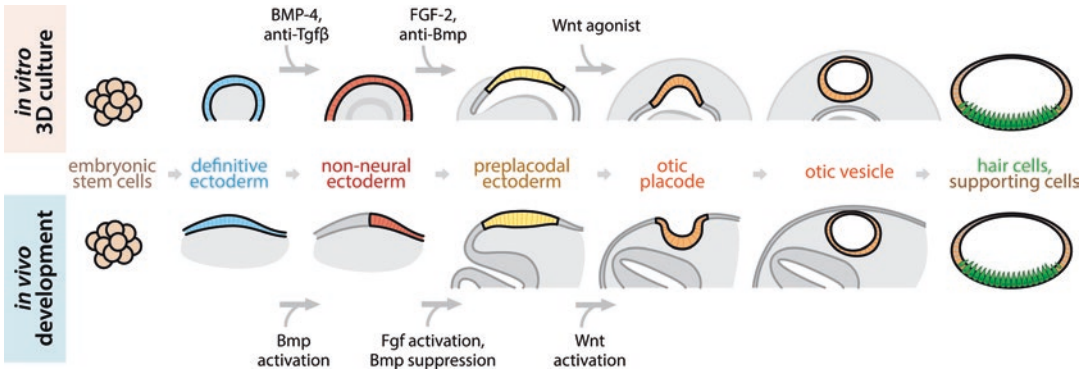

Fig. 1 In vitro differentiation of inner ear sensory epithelium in 3D culture (*top row*) is achieved through manipulation of signaling pathways that are known to be essential in in vivo inner ear differentiation (*bottom row*)

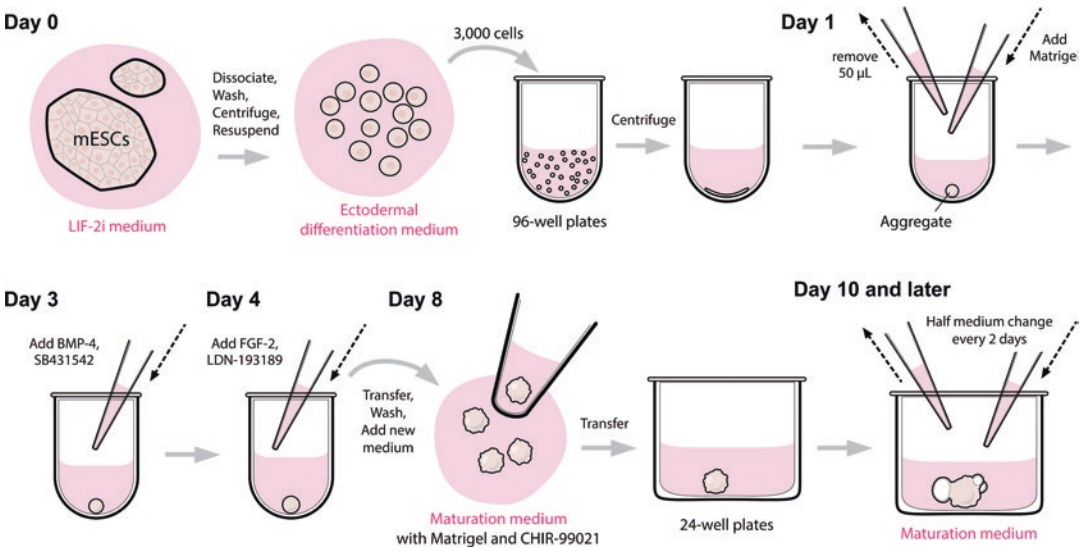

Fig. 2 Experimental procedures of 3D inner ear organoid culture

advances in cerebral and retinal tissue generation protocols in 3D culture [23–25]. In these culture systems, the definitive ectoderm, a common precursor for inner ear epithelia, retinal, and cerebral tissues, was successfully generated. The key techniques for the definitive ectoderm generation are to aggregate the dissociated pluripotent stem cells into spheroids in low-cell-adhesion U-bottom 96-well plates in a KnockOut serum replacement (KSR)-containing medium, followed by treatment with Matrigel that promotes the formation of a basement membrane. To guide the definitive ectoderm to develop into the non-neural ectoderm, we treat the mouse ES cells-derived aggregates with a recombinant human BMP-4 protein on differentiation day 3. To suppress undesirable mesoderm tissues from arising upon BMP-4 activation, the transforming growth factor-β (TGF-β) inhibitor SB-431542 [26] is added along with BMP-4. Following the induction of the non-neural ectoderm, Fgf signaling activation and Bmp inhibition are achieved through the addition of a recombinant human FGF-2 protein and the Bmp inhibitor LDN-193189 on differentiation day 4. The combined signaling cues result in the formation of the preplacodal region, which later develops into the otic placode between day 6 and day 8. On differentiation day 8, the aggregates are transferred to a minimum defined medium for a long-term culture. Similar to the in vivo morphogenesis events, cells of the otic placode invaginate and form the otic vesicles during days 9–12.

Sensory epithelium harboring inner ear hair cells positive for vestibular hair cell markers, such as Myo7a, Brn3c, calretinin, Sox2, and Pax2, begin to arise on day 14 (Fig. 4b–d″). Like hair cells in vivo, hair cells generated in 3D organoids produce stereocilia bundles with a protruding kinocilium. Moreover, these in vitro derived hair cells are fully functional based on FM1–43 dye uptake assays and electrophysiology studies [20, 27]. In addition to functional hair cells, a layer of Sox2-positive supporting cells as well as sensory neuron-like cells also arise in the differentiation culture [20].

In our previous studies, after the preplacodal region is derived through stepwise BMP-4/SB-431542/FGF-2/LDN-193189 treatment during the 3D differentiation culture, the aggregates undergo a self-guided development starting on day 8. We have demonstrated that the endogenous Wnt signaling is critical in otic placodal derivation from the preplacodal region during the self-guided development, as treatment with the Wnt inhibitor XAV939 during days 8–10 significantly reduced the formation of the otic vesicles [20]. In addition, in vivo studies in mice and zebrafish have also shown that Wnt signaling promotes the derivation of the otic placode from the preplacodal region at the expense of other placodal lineages [17, 18]. In an effort to maximize the production of the otic hair cells for high-throughput assays, we have recently

incorporated a treatment with the potent Wnt agonist CHIR-99021 [28, 29] on day 8 of culture (Figs. 1 and 2), and have found a significant increase in hair cell formation [30].

The genetic context of the organoid cultures is no longer limited to wild-type or transgenic mice derivatives, thanks to the recent breakthroughs in genome engineering technologies. Specific mutants, reporter cell lines, and cell lines integrated with complex genetic modules can now be easily created in ES cells. This opens up exciting opportunities in applying the inner ear organoid culture system in research and therapies, such as elucidating mechanisms of inner ear development and physiology, disease modeling, high-throughput drug efficacy and toxicity testing, and cell-based therapeutics.

2 Materials

2.1 Reagents and Reagent Setup

2.1.1 Reagents

1. Mouse ES cells acclimated to growth in LIF-2i medium [31] in feeder-free condition. We primarily use a R1 mES cell line (derived from R1 mice), R1/E mES cell line (ATCC), and Atoh1-nGFP mES cell line [32, 33] (a generous gift from Dr. Stefan Heller, Stanford University) for differentiation culture, but this protocol has been tested with a mouse iPS cell line and several transgenic mES cell lines with comparable differentiation results.
2. 0.1% Gelatin.
3. PBS (phosphate-buffered saline), pH 7.4.
4. Accutase cell dissociation reagent.

2.1.2 Solubilization and/or Aliquoting of Reagents (See **Note 1***)*

1. 10-mM PD-0325901 stock solution: Add 207 μL of DMSO (Dimethyl sulfoxide) to 1 mg of PD-0325901 powder and mix thoroughly. Store the resulting 10-mM PD-0325901 solution in 5.5 μL aliquots at −20 °C for up to 6 months.
2. 100 ng/μL human recombinant BMP-4 stock solution: Add 100 μL of 4 mM HCl to 10 μg of lyophilized BMP-4 and mix thoroughly. Store the resulting 100 ng/μL BMP-4 solution in 2 μL aliquots at −20 °C for up to 3 months, or at −80 °C for up to 6 months.
3. 200 ng/μL human recombinant FGF-2 stock solution: Add 250 μL of sterile PBS with 0.1% BSA to 50 μg of lyophilized FGF-2 and mix thoroughly. Store the resulting 200 ng/μL FGF-2 solution in 2.5 μL aliquots at −80 °C for up to 3 months.
4. KnockOut serum replacement (KSR): Store in ~760 μL aliquots at −20 °C for up to 18 months. Protect from light.

5. 10-mM CHIR-99021 stock solution: Store the 10-mM CHIR-99021 solution in 15.5 μL aliquots at −20 °C for up to 6 months.
6. 10-mM SB-431542 stock solution: Store the 10-mM SB-431542 solution in 2 μL aliquots at −20 °C for up to 6 months.
7. 10-mM LDN-193189 stock solution: Store the 10-mM LDN-193189 solution in 2 μL aliquots at −20 °C for up to 6 months.
8. Matrigel: Aliquot 250 μL of ice-cold Matrigel into ice-cold microcentrifuge tubes sitting on ice using a prechilled pipet tip. Store aliquots at −20 °C for up to 2 years. Thaw aliquots overnight at 4 °C or for at least 2 h at 4 °C before use (*see* **Note 2**).

2.1.3 Preparation of Culture Media (See **Note 1***)*

1. LIF-2i mES cell maintenance medium: For every 10 mL of LIF-2i medium, combine and mix the following reagents: 4.8 mL of Advanced DMEM/F12 medium, 4.8 mL of Neurobasal medium, 100 μL of GlutaMAX supplement, 50 μL of N-2 supplement, 100 μL of B-27 supplement minus vitamin A (*see* **Note 3**), 20 μL of Normocin (*see* **Note 4**), 10 μL of 1×10^6 units/mL LIF, 3 μL of 10 mM CHIR-99021, and 1 μL of 10-mM PD-0325901. Store the complete LIF-2i medium at 4 °C until use. It is best to finish using the complete medium within 1 week.
2. Ectodermal differentiation medium: For every 10 mL of ectodermal differentiation medium, combine and mix the following reagents: 9.65 mL of G-MEM, 100 μL of 100 mM sodium pyruvate, 100 μL of MEM nonessential amino acids solution, 150 μL of KnockOut serum replacement, 20 μL of Normocin, and 18 μL of 2-mercaptoethanol. Store the complete ectodermal differentiation medium at 4 °C until use. For every 96-well plate of differentiation culture, 25–30 mL of complete ectodermal differentiation medium should be made. It is best to finish using the complete medium within 1 week.
3. Maturation medium: For every 10 mL of maturation medium, combine and mix the following reagents: 9.8 mL of Advanced DMEM/F12, 100 μL of GlutaMAX supplement, 100 μL of N-2 supplement, and 20 μL of Normocin (*see* **Note 4**). Store the complete maturation medium at 4 °C until use. It is best to finish using the complete medium within 2 weeks.
4. Maturation medium with Matrigel and CHIR-99021: For every 10 mL of Matrigel (1% final concentration) and CHIR-99021 (3-μM final concentration) supplemented maturation medium, first add 100 μL of ice-cold Matrigel into 9.7 mL of ice-cold Advanced DMEM/F12 using a prechilled pipet tip. Immediately mix well by inverting the container (we usually use a 50 mL conical tube) several times (*see* **Note 2**). After the

ice-cold Matrigel is diluted in the ice-cold Advanced DMEM/F12 and mixed well, the Matrigel will no longer gel at higher temperatures (RT or 37 °C); thus, it is no longer necessary to keep the mixture ice-cold. Then continue to add the following reagents: 100 μL of GlutaMAX supplement, 100 μL of N-2 supplement, 20 μL of Normocin (*see* **Note 4**), and 3 μL of 10 mM CHIR-99021. It is best to make this Matrigel and CHIR-99021 supplemented maturation medium right before the day 8 treatment. At least 12.5 mL of this supplemented medium should be made for each 24-well plate to be seeded with aggregates on differentiation day 8.

2.2 Equipment

1. Cell culture dishes and plates: 35 mm, 60 mm, or 100 mm dishes, depending on how many 96-well plates of differentiation culture to start. At least two 96-well plates of differentiation culture can be started using ≥50% confluent mES cells cultured on a 35 mm dish. 6-well plates can also be used to culture mES cells, and the surface area of a well of a 6-well plate is equal to that of a 35-mm dish.
2. Low cell adhesion U-bottom 96-well plates: Nunclon Sphera coated (Thermo Fisher Scientific).
3. Low cell adhesion 24-well flat-bottom plates: Nunclon Sphera coated (Thermo Fisher Scientific).
4. 37 °C/5% CO_2 humidified incubator.
5. Biosafety cabinet.
6. Vacuum aspirator and disposable glass Pasteur pipets.
7. Water bath.
8. A centrifuge that is capable of centrifuging 2 mL microcentrifuge tubes, and a centrifuge that is capable of centrifuging 15 mL conical tubes and 96-well plates.
9. Automated cell counter or hemocytometer for manual cell counting.
10. Inverted microscope.
11. Pipet tips (20 μL, 200 μL, and 1 mL), microcentrifuge tubes (0.5 mL, 1.5 mL, and 2 mL), and conical tubes (15 mL and 50 mL).
12. Single channel (2, 20, 200 μL, and 1 mL) and multi-channel (200 μL) pipets.
13. Pipette basins.
14. Scissors for cutting the pipet tips to make wide-mouth pipet tips.
15. Sprayer filled with 70% ethanol.

3 Methods

3.1 Thawing Cryopreserved mES Cells and Plating in a Feeder-Free Condition

1. In a biosafety cabinet (*see* **Note 1**), coat a 60-mm cell culture dish (*see* **Note 5**) with 2 mL of 0.1% gelatin for at least 20 min in room temperature (RT).
2. Warm at least 15 mL of LIF-2i medium in a 37 °C water bath.
3. Take out a vial of cryopreserved mESCs from the liquid nitrogen tank.
4. Thaw the vial of mESCs by gentle agitation in a 37 °C water bath. Remove the vial from the water bath when only a few ice crystals are remaining. To reduce the likelihood of contamination, keep the O-ring and cap out of the water.
5. Remove the vial from the water bath, and decontaminate the vial by spraying with 70% ethanol.
6. In a biosafety cabinet, transfer the cells from the cryogenic vial into a 15 mL conical tube by gentle pipetting.
7. Gently rinse the vial with an additional 1 mL of LIF-2i medium and then slowly transfer contents to the 15 mL tube.
8. Slowly add LIF-2i medium dropwise to the 15 mL tube to bring the total volume to 10 mL.
9. Centrifuge the 15 mL tube at 180 × *g* for 10 min at RT.
10. Aspirate gelatin from the 60-mm dish. Leave the dish sitting open in the biosafety cabinet until dry.
11. When centrifuging is finished, carefully aspirate the supernatant from the 15 mL conical tube and resuspend the cell pellet in 3 mL of pre-warmed LIF-2i medium.
12. Add the cell suspension to the dried gelatin-coated dish. It is recommended that the plating density should not exceed 50,000 cells/cm^2.
13. Move the dish back and forth and side to side several times to evenly distribute cells across the cell culture dish. Incubate the dish in a humidified 37 °C/5% CO_2 incubator.

3.2 mES Cell Maintenance and Passaging in a Feeder-Free Condition

1. 1 day after plating, change the medium by replacing spent medium with fresh pre-warmed LIF-2i to remove any cell debris. Additional medium change is not required unless cells exceed 50% confluency or when the color of the culture medium is turning from red to orange or yellow.
2. Cells should be passaged when they are 50%–80% confluent. If the colonies are sparse but becoming too large, the cells should be passaged even when the overall confluency is less than 50%. mESCs are usually passaged twice a week, and the

frequency of passaging can be controlled by adjusting the split ratio (usually between 1:10 and 1:50) during each passaging.

3. To passage mESCs, first coat a 60-mm cell culture dish (*see* **Note 5**) with 2 mL of 0.1% gelatin for at least 20 min in a biosafety cabinet at RT.
4. Warm a tube of LIF-2i medium in a 37 °C water bath. Also warm an aliquot of accutase (400 μL or more) at RT.
5. Aspirate the spent LIF-2i medium from the culture dish.
6. Slowly add 400 μL of accutase and incubate the cells in the 37 °C incubator for 1–3 min until cells start to detach from plate. Confirm the detachment of cells from dish under a microscope.
7. While cells are being dissociated in the incubator, aspirate gelatin from the 60 mm dish and leave the dish sitting open in the biosafety cabinet until dry.
8. Wash the dissociated cells off the dish by gently pipetting 1 mL of pre-warmed LIF-2i across the dish. Repeat the pipetting across the dish several times to completely wash off the cells and to break up the cell clumps. Avoid creating bubbles.
9. Collect cells into a 2 mL microcentrifuge tube. Gently pipet up and down several times using a p1000 tip to further break up cell clumps into single cells. Avoid creating bubbles.
10. Centrifuge cells for 2.5 min at 180 × *g* at RT.
11. Carefully remove supernatant completely by aspiration (*see* **Note 6**) and resuspend cell pellet with 1 mL of LIF-2i.
12. Add 20 μL (1:50 split ratio)—100 μL (1:10 split ratio) of cells and 3 mL of LIF-2i to the dried gelatin-coated 60-mm dish. Select split ratio according to when you next wish to split the cells or to use the cells for a differentiation culture.
13. Move the dish back and forth and side to side a few times to evenly disperse cells across the surface of the dish. Incubate the dish in a humidified 37 °C/5% CO_2 incubator.

3.3 Differentiation Day 0: mESC Dissociation and Seeding in 96-well Plate

1. Differentiation culture can be started when mESCs are ~50–80% confluent.
2. Prepare a tube of ectodermal differentiation medium (25–30 mL for each 96-well plate culture), mix well, and warm medium in a 37 °C water bath. Warm an aliquot of accutase (400 μL or more) at RT.
3. Aspirate spent LIF-2i medium from the mESC dish, and wash the cells three times with PBS (*see* **Note 7**).
4. To dissociate cells, slowly add 400 μL of accutase and incubate the dish in the 37 °C incubator for 1–3 min until cells start to detach from plate. Confirm the detachment of cells from dish under a microscope.

5. Wash the dissociated cells off the dish by gently pipetting 1 mL of ectodermal differentiation medium across the plate. Repeat the pipetting across the dish several times to completely wash off the cells and to break up the cell clumps. Avoid creating bubbles.
6. Collet cells into a 2 mL microcentrifuge tube. Gently pipet up and down several times using a p1000 tip to further break up cell clumps into single cells (*see* **Note 8**). Avoid creating bubbles.
7. Centrifuge cells for 2.5 min at 180 × *g* at RT.
8. Carefully remove supernatant by aspiration (*see* **Note 6**). Resuspend cell pellet in 1 mL of ectodermal differentiation medium and mix well.
9. Determine the concentration of cells with an automatic cell counter or a hemocytometer.
10. Pipet appropriate volume of cells and mix with fresh pre-warmed ectodermal differentiation medium in a conical tube to acquire a final concentration of 30,000 cells per mL. The final volume should be 11 mL (i.e., 3.3×10^5 total cells) for each 96-well plate to be seeded (*see* **Note 9**).
11. Invert the tube several times to mix the cells. Pour cells into a multichannel pipet basin and gently pipet 100 μL of cells into each well (i.e., 3000 cells/well) of a low-cell-adhesion U-bottom 96-well plate using a multichannel pipet.
12. Centrifuge the 96-well plate at 90 × *g* for 5 min to assist the aggregation of mESCs.
13. Incubate the plate in a humidified 37 °C/5% CO_2 incubator for 24 h. Overnight the mESCs at the bottom of the low-cell-binding wells will aggregate to form spheres (Figs. 2 and 3a–e).
14. Thaw an aliquot of Matrigel from a − 20 °C freezer (one 250 μL aliquot for each 96-well plate differentiation culture) by placing it in a 4 °C fridge overnight.

3.4 Differentiation Day 1: Addition of Matrigel

1. Add 220 μL of cold Matrigel to 5.28 mL of ice-cold ectodermal differentiation medium in a cold 15 mL conical tube using a prechilled pipet tip to make 5.5 mL of complete medium (4% Matrigel concentration). Immediately mix by inverting the tube several times (*see* **Note 2**).
2. Warm the complete medium to 37 °C in a water bath.
3. By holding a multichannel pipet at an angle, carefully remove 50 μL of medium from each well of the 96-well plate and make sure the aggregates at the bottom of the wells are not disturbed.
4. Add 50 μL of the complete medium (4% Matrigel in ectodermal differentiation medium) to each well using a multichannel pipet. Carefully pipet up and down several times to mix, while

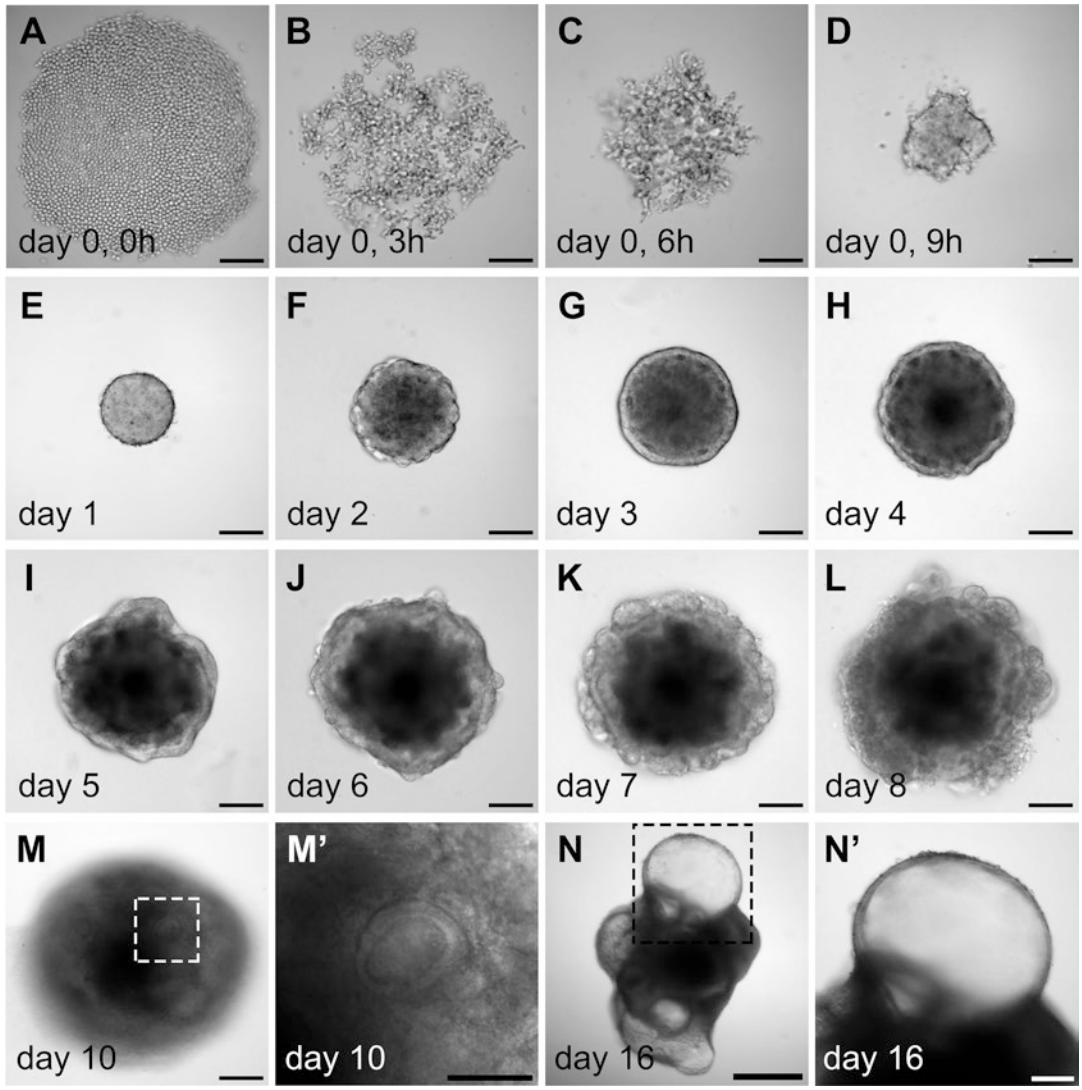

Fig. 3 Morphology of the aggregates during in vitro differentiation. (**a–d**) Aggregation of the mouse ES cells during day 0, which is likely mediated by cell surface adhesion proteins. (**e–l**) Morphological changes of the aggregates during day 1 to day 8 culture. Note that after Matrigel addition on day 1, an epithelium develops on the surface of the aggregates on day 3 (**g**). In a self-guided manner, some of the interior cell mass breaches out during day 6 to day 8. Concurrently, the epithelium is rearranged from the outer surface to the interior of the aggregates (**j–l**). (**m–m′**) Vesicles embedded inside the aggregates become apparent on day 10. (**n–n′**) A day 16 aggregate with multiple protruding vesicles. (**m′**) and (**N′**) are higher magnification images of (**m**) and (**n**), respectively. *Scale bars*, 100 μm (**a–m, n′**); 50 μm (**m′**); 500 μm (**n**)

making sure that the aggregates are not sucked into the pipet tips as this will damage the aggregates. The final concentration of Matrigel is now 2%.

5. Incubate the plate in a humidified 37 °C/5% CO_2 incubator for 48 h (Figs. 2 and 3e).

3.5 Differentiation Day 3: Addition of BMP-4 and SB-431542

1. In a 15 mL conical tube, add 1.5 μL of 100 ng/μL BMP-4 and 1.5 μL of 10 mM SB-431542 to 3 mL of ectodermal differentiation medium. Mix by inverting the tube several times.
2. Warm the complete medium to 37 °C in a water bath.
3. Pour the complete medium into a multichannel pipet basin.
4. Pipet 25 μL of the BMP-4/SB-431542 containing ectodermal differentiation medium to each well of the 96-well plate using a multichannel pipet. Carefully pipet up and down several times to mix well, while making sure that the aggregates are not sucked into the pipet tips. The final concentration of BMP-4 is now 10 ng/mL, and that of SB-431542 is 1 μM.
5. Incubate the plate in a humidified 37 °C/5% CO_2 incubator for 24 h (Figs. 2 and 3g).

3.6 Differentiation Day 4: Addition of FGF-2 and LDN-193189 (See Note 10)

1. In a 15 mL conical tube, add 2.25 μL of 200 ng/μL FGF-2 and 1.8 μL of 10 mM LDN-193189 to 3 mL of ectodermal differentiation medium. Mix by inverting the tube several times.
2. Warm the complete medium to 37 °C in a water bath.
3. Pipet 25 μL of the FGF-2/LDN-193189 containing ectodermal differentiation medium to each well of the 96-well plate using a multichannel pipet. Carefully pipet up and down several times to mix well, while making sure that the aggregates are not sucked into the pipet tips. The final concentration of FGF-2 is now 25 ng/mL, and that of LDN-193189 is 1 μM.
4. Incubate the plate in a humidified 37 °C/5% CO_2 incubator for 4 days (Figs. 2 and 3h).

3.7 Differentiation Day 8: Transfer Aggregates to Matrigel and CHIR-99021 Supplemented Maturation Medium for Long-Term Culture

1. On day 7, thaw an aliquot of Matrigel from a −20 °C freezer by placing it in a 4 °C fridge overnight.
2. On day 8, make Matrigel (1% final concentration) and CHIR-99021 (3-μM final concentration) supplemented maturation medium (*see* **Note 2**). The volume of the complete medium prepared should be at least 12.5 mL for each 24-well plate to be seeded. Up to four 24-well plates can be seeded with aggregates from each 96-well plate.
3. Warm the Matrigel and CHIR-99021 supplemented maturation medium to 37 °C in a water bath.
4. Using a wide-mouth pipet tip on a 200 μL single channel pipet, carefully transfer individual aggregates from the 96-well plate to a 15 mL conical tube (*see* **Note 11**).
5. After all aggregates are settled at the bottom of the conical tube, carefully remove the spent medium and wash three times with at least 5 mL of PBS. Carefully remove excess PBS after the washes (*see* **Note 12**).

6. Add a few mL of the pre-warmed Matrigel and CHIR-99021 supplemented maturation medium to the 15 mL conical tube, and quickly pour the medium along with the aggregates to a 60 mm dish. If there are remaining aggregates in the conical tube, repeat this step to transfer the remaining aggregates to the 60-mm dish.
7. At this stage the aggregates are big enough to be visible by eyes. Using a wide-mouth pipet tip on a 200 μL single channel pipet, carefully transfer each aggregate along with 50 μL of medium from the 60-mm dish to each well of a low-cell-adhesion 24-well flat-bottom plate. Aggregates in one 96-well plate can be transferred to up to four 24-well plates.
8. Gently add 450 μL of the Matrigel and CHIR-99021 supplemented maturation medium to each well of the 24-well plate to make a final volume of 500 μL of medium in each well.
9. Incubate the plate in a humidified 37 °C/5% CO_2 incubator for 48 h (Figs. 2, 3l and 4a).

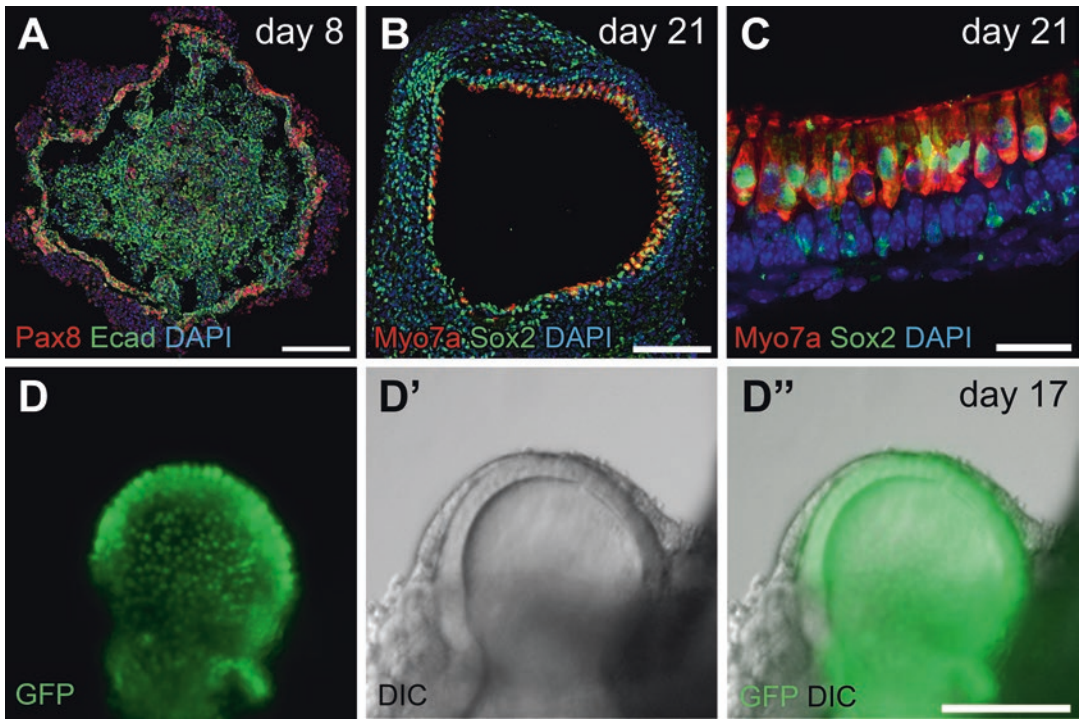

Fig. 4 Preplacodal ectoderm on a day 8 aggregate and inner ear sensory epithelium on later staged aggregates. (**a**) Pax8 and E-cadherin (Ecad) label the preplacodal regions on a day 8 aggregate. (**b–c**) Inner ear hair cells expressing Myo7a and Sox2 tightly organized at the interior surface of vesicles on day 21. (**d–d″**) Live Imaging of a Atoh1-nGFP aggregate. GFP with nuclear localization signal (nGFP) is expressed under an Atoh1 promoter, thus marking the inner ear hair cells. GFP signals in (**d**) and (**d″**) are overlaid from two focal planes. *Scale bars*, 100 μm (**a–b, d–d″**); 20 μm (**c**)

3.8 Differentiation Day 10 and later: Long-Term Culture in Maturation Medium

1. Perform a half medium change every other day starting on day 10 (i.e., days 10, 12, 14, and so on), by removing 250 µL of spent medium and gently adding 250 µL of pre-warmed maturation medium (without Matrigel or CHIR-99021).
2. Return the plate in a humidified 37 °C/5% CO_2 incubator. Aggregates can be cultured for up to 30 days under these conditions. For longer culture durations, different medium compositions or culture formats are likely to be required. Vesicles can be observed starting from day 9 or day 10 (Figs. 3m, m′), and inner ear hair cells should first arise within otic vesicle epithelia between day 14 and day 16 (Figs. 3n, n′; 4b–d″). Aggregates can be used for analysis/experiments (e.g., immunofluorescence, electrophysiology recording, flow cytometry, qPCR, Western blot, etc.) at any point during the differentiation culture depending on the experimental needs.

4 Notes

1. All reagents and culture media coming in contact with living cells or aggregates must be prepared in a biosafety cabinet with sterile tubes and pipet tips, etc. All experiments/treatments on living cells or aggregates must be carried out in a biosafety cabinet, except for viewing/imaging under a microscope, centrifuging, thawing cells in a water bath, and fixing aggregates for immunofluorescence staining.
2. It is extremely important that Matrigel and all pipet tips, conical tubes, and medium coming in contact with Matrigel should be ice-cold, since Matrigel starts to gel above 10 °C. After the ice-cold Matrigel is diluted in culture media and well mixed, the Matrigel will no longer gel at higher temperatures (RT or 37 °C); thus, it is no longer necessary to keep it cold.
3. It is important to use B-27 supplement without vitamin A rather than using the regular B-27 supplement in the LIF-2i medium for mES cell culture. Vitamin A (retinol) can be converted to retinoic acid in culture, which can induce spontaneous neuronal differentiation of the mES cells [34–36].
4. Normocin is used as an anti-microbial reagent in all culture and differentiation media in this protocol. It is especially important to not use aminoglycosides-containing anti-microbial reagents like the widely used penicillin-streptomycin in the maturation media, as aminoglycosides are known to be toxic to inner ear hair cells [37].
5. This protocol uses 60-mm dishes (Surface area = 2827 mm^2) to culture mES cells. To reduce the amount of LIF-2i culture

medium and reagents (e.g., gelatin, accutase, etc.) being used, mES cells can also be cultured in 35-mm dishes or 6-well plates (Surface area = 962 mm^2 per dish/well). If using the latter two types of culture dishes/plates, use 1 mL of gelatin to coat the dish/well, use 300 μL accutase for cell dissociation, use 1 mL LIF-2i to wash the accutase dissociated cells from the dish/well, and use 2 mL of LIF-2i medium to feed the cells. Though the surface area of a 35-mm dish or a well of a 6-well plate is ~70% smaller than that of a 60-mm dish, mES cells cultured in one dish/well are still enough to start at least two 96-well plates in a differentiation experiment when the cells are more than 50% confluent.

6. To completely remove the medium without the risk of aspirating away the cell pellet, put the glass Pasteur pipet tip against the interior lateral wall of the round-bottom 2 mL microcentrifuge tube near the opening area of the tube. Slowly tilt the 2 mL tube from an up-right position to a horizontal position, which allows the liquid to be slowly aspirated at the opening area of the tube. The cell pellet should stay at the bottom of the 2 mL tube. This technique only works with the round-bottom 2 mL tubes but not the 1.5 mL or 0.5 mL microcentrifuge tubes or the 15 mL conical tubes, as in the latter three types of tubes the bottoms are narrow, thus some liquid will remain at the bottom when the tubes are tilted to horizontal or even to up-side-down positions. It is essential to use this technique to remove as much liquid from the cell pellet as possible, as excessive reagents in the remaining liquid may negatively affect the downstream experiments.
7. It is important to thoroughly wash the ES cells to completely remove LIF-2i, as excessive residual inhibitory components in the LIF-2i medium (i.e., LIF, CHIR-99021, and PD-0325901) may repress the differentiation of the ES cells in the 3D differentiation culture.
8. After the 1–3 min of accutase digestion followed by washing and pipetting, nearly all the cells should be dissociated into single cells. This can be confirmed using an automated cell counter or under the microscope on a hemocytometer during cell counting. Therefore, there is no need to forcefully pipet cells through a cell strainer tube to ensure single cell dissociation.
9. For example, if the concentration of the cell suspension is 1×10^6 cells/mL and one 96-well plate is to be seeded, add 330 μL of cell suspension (3.3×10^5 cells ÷ 1×10^6 cells/mL = 0.33 mL) in 10.67 mL of ectodermal differentiation medium to make 11 mL of cells with a 30,000 cells/mL concentration.

10. The timing of FGF-2/LDN-193189 treatment may need to be optimized depending on the mES cell line being used. Day 4 appears optimal for most cell lines, including R1 and R1/E. However, treatment between days 3.5 and 4.5 may be more suitable for other cell lines.
11. Make wide-mouth pipet tips by cutting the end of the pipet tip using a sterile scissor in a biosafety cabinet. Make sure the resulting wide-mouth opening of the pipet tip is bigger than the aggregates to be transferred.
12. Thoroughly wash the aggregates to avoid small molecules and recombinant proteins from previous treatments (i.e., BMP-4, SB-431542, FGF-2, and LDN-193189) from affecting aggregates in the maturation stages.

Acknowledgments

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Chapter 7

Generation of Functional Thyroid Tissue Using 3D-Based Culture of Embryonic Stem Cells

Francesco Antonica, Dominika Figini Kasprzyk, Andrea Alex Schiavo, Mírian Romitti, and Sabine Costagliola

Abstract

During the last decade three-dimensional (3D) cultures of pluripotent stem cells have been intensively used to understand morphogenesis and molecular signaling important for the embryonic development of many tissues. In addition, pluripotent stem cells have been shown to be a valid tool for the in vitro modeling of several congenital or chronic human diseases, opening new possibilities to study their physiopathology without using animal models. Even more interestingly, 3D culture has proved to be a powerful and versatile tool to successfully generate functional tissues ex vivo. Using similar approaches, we here describe a protocol for the generation of functional thyroid tissue using mouse embryonic stem cells and give all the details and references for its characterization and analysis both in vitro and in vivo. This model is a valid approach to study the expression and the function of genes involved in the correct morphogenesis of thyroid gland, to elucidate the mechanisms of production and secretion of thyroid hormones and to test anti-thyroid drugs.

Key words Thyroid development, Embryonic stem cells, Regenerative medicine, Hypothyroidism

1 Introduction

Embryonic stem cells (ESCs) have been thoroughly described as an efficient complementary tool used to uncover molecular and morphogenetic mechanisms occurring during embryogenesis of several tissues such as optic-cup [1], pituitary gland [2], and retina [3]. Many elegant studies have demonstrated how ESCs can be used to recapitulate in vitro many phases of embryonic development of several tissues, including also the thyroid gland [4]. Before 2012 no efficient in vitro protocol of ESCs differentiation into thyroid follicular cells was established. Some independent studies proposed various approaches to differentiate embryonic stem cells into thyroid follicular cells [5, 6], but none of them showed convincing

[$]Those authors have equally contributed to the preparation of the manuscript.

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data on morphological and functional thyroid hallmarks of the differentiated cells. Due to the fact that there was no known extrinsic or morphogenic factor capable to prime the differentiation of thyroid progenitors, we have thought to commit ESCs to thyroid fate by inducing the expression of genes already identified as being important for thyroid development. During the last decade it has been demonstrated how the induced expression of lineage-specific transcription factors can direct or enhance the differentiation of ESCs into specific cell types, such as hematopoietic [7], adipose [8], and pancreatic cells [9]. *Nkx2-1* [10] and *Pax8* [11] have been already described as important transcription factors playing a pivotal role in both correct thyroid development and physiology, by controlling the expression of many genes belonging to the complex machinery of thyroid hormones synthesis [12, 13]. In fact, studies using knockout mouse models for *Nkx2-1* [10] or *Pax8* [11] have demonstrated how their lack triggers an abnormal thyroid organogenesis characterized by agenesis [12]. We have recently shown how the inducible co-expression of *Nkx2-1* and *Pax8* in ESCs-derived embryoid bodies (EBs) can commit the cells into thyroid fate; their subsequent treatment with thyroid-stimulating hormone (TSH) leads the cells to form fully functional thyroid follicles [4]. We devised a strategy to differentiate ESCs into functional thyroid follicles using a three-step differentiation protocol: (1) generation of EBs; (2) embedding of EBs into a 3D-matrigel support and induction of *Nkx2-1* and *Pax8* co-expression by doxycycline-TetOn system; (3) stimulation of folliculogenesis by rhTSH treatment (Fig. 1A). This in vitro protocol leads to the differentiation of cells showing proper thyroid hallmarks such as thyroid markers expression and an intrinsic capacity to metabolize iodide [4]. Moreover, ESCs-derived thyroid follicles are capable of restoring thyroid homeostasis when transplanted into athyroid mice [4]. Our protocol demonstrated for the first time that it is possible to achieve thyroid organogenesis in vitro by the aid of ESCs. Interestingly, in 2015 Kotton and colleagues showed how pluripotent stem cells can be differentiated into functional thyroid using a combination of 3D culture and chemically defined media [14]. Our model can be a powerful tool for those investigating molecular and morphological events occurring during thyroid development. Due to the fact these in vitro follicles are functional, they can be used for physiological and toxicity studies (chemical testing for thyroid disruption properties).

2 Materials

All solutions should be prepared under sterile conditions; media and reagents for cell culture should be filtered if prepared using nonsterile reagents. It is recommended that enzyme and serum reagents should be evaluated for enzyme reactivity and

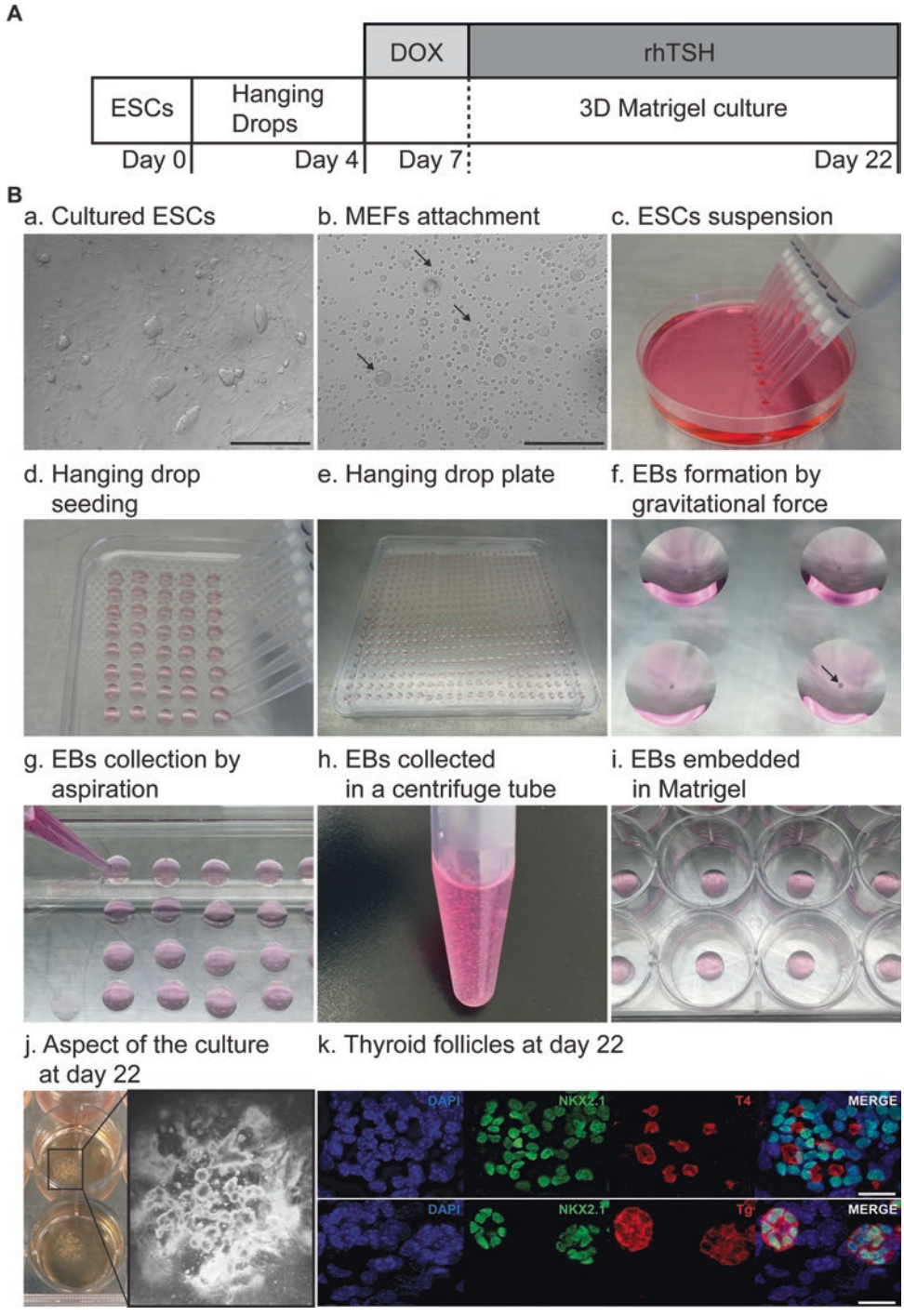

Fig. 1 In vitro thyroid differentiation protocol. (**A**) Schematic representation of the differentiation protocol. (**B**) Murine ESCs grown on a MEF-feeder (scale bar 500 μm) (*a*). Selective removal of MEFs: fibroblasts attach to the gelatin-coated dish (as indicated by *arrows*) (scale bar 500 μm) (*b*). Preparation of hanging drops: aspirate the single-cell suspension of ESCs with an 8-channel pipette (*c*), dispense it in small drops on the cover of a 245-mm dish (*d*) and then carefully turn it over (*e*). After 4 days of culture one EB will form in each drop (*f*). EBs recovery and embedding: recover EBs with a P1000 micropipette (*g*), move them to ice-cold Matrigel (*h*) and then dispense a droplet of EBs-Matrigel suspension in the center of each well of a 12-well plate (*i*). Macroscopic aspect of differentiated cells at the end of the differentiation protocol (*j*). Analysis of in vitro differentiated follicles at day 22: immunofluorescence staining for NKX2-1/T4 (*upper* pictures) or NKX2-1/Tg (*bottom* pictures) (scale bar 20 μm) (*k*)

embryonic stem cell culture efficiency, respectively, prior to experimental use.

1. Cell lines: A2Lox *Nkx2-1-Pax8* mouse ESC line (*see* **Note 1**).
2. ESCs medium: mouse ESCs are routinely cultured in DMEM, 15% FBS (ES Cell qualified FBS), supplemented with LIF (1000 U/mL final), nonessential amino acids (0.1 mM final), sodium pyruvate (1 mM final), penicillin and streptomycin (50 U/mL final), and 2-mercaptoethanol (0.1 mM final).
3. Differentiation medium: thyroid differentiation is performed in DMEM supplemented with 15% FBS, vitamin C (50 μg/mL final), nonessential amino acids (0.1 mM final), sodium pyruvate (1 mM final), penicillin and streptomycin (50 U/mL final), and 2-mercaptoethanol (0.1 mM final).
4. Ca2+/Mg2+-free PBS.
5. 2-mercaptoethanol at 100 mM: dilute 50 μL of 2-mercaptoethanol stock in 7 mL of PBS and filter sterilize.
6. Trypsin 0.05% (w/v)/EDTA 0.5 mM: dilute EDTA and Trypsin in PBS at their respective final concentration.
7. Gelatin 0.1% (w/v) solution: weigh 1 g of gelatin from porcine skin and put it in 1 L of distilled water, then autoclave it and store gelatin solution at 4 °C for up to 4 weeks.
8. Doxycycline: dissolve Doxycycline hyclate in PBS to a final concentration of 1 mg/mL. Aliquot and store at −20 °C for 3 months.
9. rhTSH: reconstitute Thyrogen® in 1.2 mL of sterile water for injections to obtain a final concentration of 0.9 mg/mL, corresponding to 3.6 U/mL. Dilute 1 mL of dissolved Thyrogen® to a final concentration of 1 U/mL. Store at −20 °C for up to 1 year.
10. Vitamin C: dissolve vitamin C in distilled water to a final concentration of 50 mg/mL. Aliquot and store at −20 °C for 3 months.
11. HBSS.
12. Digestion medium: HBSS containing Dispase II (10 U/mL final) and Collagenase type IA (125 U/mL final).
13. Growth factor reduced (GFR) Matrigel matrix (Becton Dickinson, Biosciences, 354230) (*see* **Note 2**).
14. 15-mm glass coverslips.
15. Coverslip processing: immerse 15-mm coverslips in 1 N HCl for 1 h. Rinse thrice with 100 mL of pure water and then rinse twice with 100 mL of absolute ethanol. Air-dry on a clean piece of paper, transfer to a clean baker, cover with foil, and sterilize in an oven at 180 °C. Put coverslips into 12-well

plates using flamed tweezers. Store at RT for an unlimited amount of time.

16. Acrodisc 32-mm syringe filter with 0.2-μm supor membrane.
17. 1.5 mL microcentrifuge tubes.
18. Disposable plastic serological pipettes 5, 10, and 25 mL.
19. Centrifuge conical tubes, 15 and 50 mL.
20. Micropipettes P200, P1000.
21. 8-channel pipette.
22. Sterile pipette tips with aerosol barrier.
23. Tissue culture dishes, 100 and 245-mm.
24. Multiwell culture plates, 12-well.
25. Hamilton TLC syringe, 10 μL, Model 701 RN (Hamilton 7635-01).
26. Small Hub Removable Needle, 30 G, 40 mm, Point Style 4, angle 30° (Hamilton 7803-07).
27. Surgical tools for transplantation under the kidney capsule: large surgical scissors and forceps to cut the dorsal skin, mayo scissors to separate the skin from the fascia, tweezers to hold the kidney.

3 Methods

3.1 A2LoxNkx2-1-Pax8 Cells Culture

A2Lox *Nkx2-1-Pax8* murine ESC line is routinely cultured on MEF feeders and passaged 1:8-1:10 every 2–3 days. For culture procedures, *see* Ref. 15.

3.2 In Vitro Thyroid Differentiation

3.2.1 Embryoid Bodies Formation and Embedding in 3D Matrigel

Day 1 (Differentiation day 0) (Fig. 1A)

1. Two hours before to passage ESCs (Fig. 1B-a), replace the medium with 10 mL of fresh ESCs medium (*see* **Note 3**).
2. In the meantime, prepare three gelatin-coated 100-mm dishes. Put 10 mL of gelatin solution into each dish and incubate for 45 min at RT. Aspirate off gelatin and let the dishes dry with the lid open for 15 min at RT.
3. Aspirate off ESCs medium from ESCs and rinse with 10 mL of PBS. Aspirate off PBS and add 2.5 mL of trypsin/EDTA solution.
4. Incubate for 3 min at 37 °C, 5% CO_2 (*see* **Note 4**).
5. Pipette up/down three to four times with a P1000 micropipette to break cell clumps and inactivate trypsin by adding 7.5 mL of ESCs medium. Resuspend thoroughly the cells by pipetting up and down with a 10 mL pipette several times.

6. Transfer the cell suspension into a 50 mL tube and centrifuge for 3 min at 290 × *g*, RT. Discard the supernatant.
7. Add 2 mL of differentiation medium to the cellular pellet and resuspend into a single-cell suspension using a P1000 micropipette. Add differentiation medium to a final volume of 30 mL and aliquot 10 mL in each of three gelatin-coated 100-mm dishes.
8. Incubate for up to 30 min at 37 °C, 5% CO_2 to selectively remove fibroblasts (Fig. 1B-b) (*see* **Note 5**).
9. Carefully aspirate the medium with cells in suspension and move it into a 50 mL tube.
10. Count the cells and adjust the concentration to 40,000 cells/mL with differentiation medium (*see* **Note 6**).
11. Fill a 100-mm dish with the diluted cell suspension (Fig. 1B-c).
12. Put 35 mL of PBS in a 245-mm dish (*see* **Note 7**). Using an 8-channel micropipette, aspirate cells from the 100-mm dish and dispense 25 μL of cell suspension (corresponding to 1000 cells) onto the cover of the 245-mm dish making small drops (Fig. 1B-c, d) (*see* **Note 8**).
13. Carefully turn over and close the cover, incubate the cells in hanging drops for 4 days in an incubator at 37 °C, 5% CO_2 (Fig. 1B-e) (*see* **Note 9**).

Day 5 (Differentiation day 4):

1. Thaw Matrigel aliquots on ice at least 2 h before starting EBs collection (*see* **Note 10**).
2. Gently open the cover of the 245-mm dish and turn it over. Recover EBs by aspirating them with a P1000 micropipette (Fig. 1B-f, g) and harvest 160 drops per 15 mL tube (one 245-mm dish into three tubes). Put the tubes back in the incubator and let EBs sink to the bottom (*see* **Note 11**).
3. For each tube, aspirate off the medium and leave the EBs in a volume of about 200 μL. Move the tube on ice. With a P200 micropipette set on 90 μL aspirate all the EBs from the 15 mL tube and move them in one ice-cold Matrigel aliquot. Mix gently to homogeneously disperse the EBs (Fig. 1B-h) (*see* **Note 12**).
4. Gently plate 55 μL of EBs-Matrigel suspension (*see* **Note 13**) on the center of each 15 mm coverslip inside a well of a 12-well plate (or directly on the well bottom, *see* **Note 14**) (Fig. 1B-i).
5. Incubate at 37 °C, 5% CO_2 for up to 30 min to allow Matrigel to gelatinize. Add then 1 mL of differentiation medium supplemented with Doxycycline (1 μg/mL final) to each well. Add 1 mL of differentiation medium without Doxycycline to the wells that will be used as controls. Incubate at 37 °C, 5% CO_2 for 48 h.

Day 7 (Differentiation day 6):

1. Carefully change the medium of each well and replace it with 1 mL of fresh differentiation medium supplemented or not with Doxycycline (1 μg/mL final) (*see* **Note 15**).

3.2.2 Thyroid Differentiation

Day 1 (Differentiation day 7):

1. Gently aspirate off the medium from all the wells. Wash twice with 1 mL of PBS only the cells previously cultured in the presence of Doxycycline (*see* **Note 16**).
2. Add 1 mL of differentiation medium supplemented with rhTSH (1 mU/mL final) to the cells previously cultured in the presence of Doxycycline. Add 1 mL of differentiation medium without rhTSH to the control cells. Incubate at 37 °C, 5% CO_2 for 48 h. Change the medium every other day (*see* **Note 17**).

Day 16 (Differentiation day 22):

1. After culture for 15 days, multiple functional thyroid follicles will have developed in each well treated with Doxycycline and rhTSH (Fig. 1B-j, k). Proper differentiation of the cells at day 22 can be assessed by immunofluorescence or qRT-PCR assays; iodide organification can be performed to assay the functionality of the follicles. Detailed protocols for immunofluorescence, qRT-PCR, and iodide uptake and organification are described by Antonica et al. [4].

3.3 Transplantation of ESCs-Derived Thyroid Follicles Under the Renal Capsule

3.3.1 Purification of an ESCs-Derived Thyroid-Follicles Enriched Cell Population

1. After 22 days of differentiation wash twice the wells with HBSS (Fig. 2A-a).
2. Add 1 mL per well of digestion medium.
3. Incubate for 30 min at 37 °C, 5% CO_2 (*see* **Note 18**).
4. Manually dissociate cell clumps with a P1000 micropipette by gently pipetting up and down the digestion medium containing cells inside (Fig. 2A-a, b).
5. Collect all the medium with the digested cells from 12 wells and put it into a 15 mL conical tube, centrifuge at 200 × *g* for 3 min (*see* **Note 19**).
6. Aspirate off the supernatant and resuspend the pellet in an equal volume of differentiation medium, proceed with a centrifugation at 200 × *g* for 3 min.
7. Repeat **step 6**.
8. Aspirate off the medium and resuspend gently the pellet in 65 μL of differentiation medium.
9. Use 10 μL for each kidney to transplant (*see* **Note 20**).

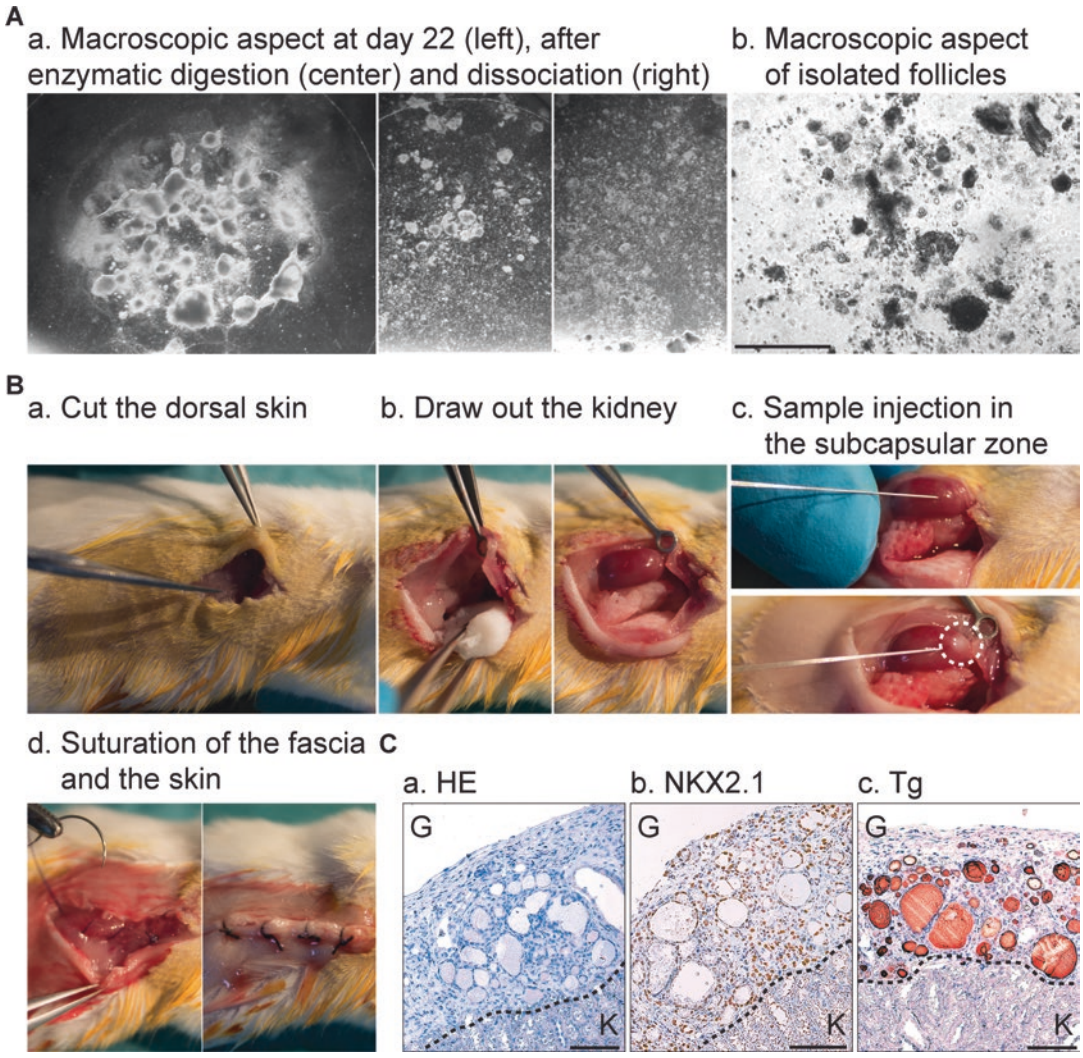

Fig. 2 Preparation of ESC-derived thyroid follicles for transplantation. (**A**) Differentiated murine ESCs at day 22 of the differentiation (*left*), after enzymatic digestion (*center*), and mechanical dissociation (*right*) (*a*). Microscopic aspect of the samples ready for transplantation (*b*). (**B**) Subrenal capsule transplantation of ESC-derived thyroid follicles. Make an incision on the dorsal skin (*a*) and exfoliate between the skin and the fascia. Insert wet tampons in the abdominal cavity and draw out the kidney (*b*). Inject thyroid follicles in the subcapsular zone of the kidney (*c*). Return the kidney into the peritoneal cavity, remove all tampons, and then suture the fascia and the skin (*d*). (**C**) Histological analysis of the kidney 4 weeks after grafting: hematoxylin and eosin staining (*a*); NKX2-1 (*b*) or Tg (*c*) staining show the incorporation of the grafted ESC-derived thyroid follicles (*G*) above the kidney tissue (*K*) (scale bar 100 μm)

3.3.2 Transplantation

1. Anesthetize the mouse to be grafted following your institution guidelines.
2. Place the mouse on its stomach and immobilize the hands and feet, apply aqueous-based gel on the eyes.

3. Shave the dorsal hair and properly disinfect the skin.
4. Dissect the dorsal skin longitudinally just under the costal margin for approximately 2 cm (Fig. 2B-a).
5. Separate the skin from the fascia.
6. Dissect the fascia and draw out the kidney. Help the kidney to stay out of the cavity by inserting some wet tampons or gauzes (Fig. 2B-b).
7. Aspirate follicles inside the TLC syringe (after mounting the needle).
8. Insert the needle at the limit between the kidney and the renal capsule.
9. Inject the cells and verify the place of injection by the appearance of visible liquid in the subcapsular zone (Fig. 2B-c) (*see* **Note 21**).
10. Gently return the kidney to the peritoneal cavity. Remove all tampons or gauzes.
11. Suture the fascia and skin (Fig. 2B-d).

3.3.3 Analysis of the Grafting

1. 4 weeks after transplantation mice can be sacrificed for the analysis of the grafted kidneys (Fig. 2C-a–c). Blood samples can also be collected for measuring thyroid hormone levels: in this case samples should be taken from hypothyroid mice before and 4 weeks after grafting. Detailed protocols for collection of kidneys and blood samples, histological, physiological, or serological analysis are described by Antonica et al. [4].

4 Notes

1. A2Lox *Nkx2-1-Pax8* mouse ESC line was generated from A2Lox.cre line (kind gift from M. Kyba) by electroporation with p2Lox targeting vector [15] (kind gift from M. Kyba) in which cDNA encoding for *Nkx2-1* and *Pax8* had been previously subcloned [4]. All the procedure and findings described in this manuscript have been successfully tested and reproduced also in different cell lines generated as described by Conklin and colleagues [16]. Briefly, cDNA encoding for the mouse isoforms of *Nxk2-1* and *Pax8* was cloned (separated by an Ires sequence) in the Entry vector pEnt R3L2 TetO(fl)-3. rtTA was subcloned into the Entry vector pEntL1L3 EF1α-tTA-2 substituting for the tTA in order to have a TetON instead of TetOFF system. Both entry vectors were recombined with the destination vector pRosa26 R1-ccdB-R2 RexNeo PI-SceI by Gateway cloning. The final plasmid was transfected in the mouse stem cell line G4. After neomycin selection positive clones were further characterized for the inducible co-expression of both transcription factors.

2. A temperature below 4–6 °C is needed to keep Matrigel in a liquid state. Prepare all materials (1.5 mL tubes and tips) and put them in the freezer the day before preparing Matrigel aliquots. Thaw Matrigel bottles on ice in the refrigerator overnight and make 600 μL aliquots working on ice and quickly. Store them at −20 °C and strictly avoid multiple freeze-thaws.
3. Aspirate off old ESCs medium in order to remove dead cells and debris.
4. Check the cells under the microscope. Cells should detach in clumps.
5. During this step MEFs would attach to the gelatin-coated dish, while ESCs would stay in suspension. Check MEFs' attachment under the microscope.
6. When counting, some fibroblast (bigger and irregular cells compared to the others) could still be present so do not take them into consideration and count only the small round cells.
7. Adding PBS to the bottom of the plate will ensure the maintenance of proper humidity in the plate and prevent hanging drops from drying out.
8. Cells contained in each drop will generate one EB. Normally, 480 drops can be placed on one 245-mm dish cover, corresponding to seeding 60 times eight drops with the multichannel pipette.
9. Move the dishes carefully, since every abrupt movement might cause the drops to fall or merge. Do not move the plate throughout the 4 days of EB formation.
10. Matrigel can be otherwise thawed on ice in the refrigerator overnight the day before EBs collection. One 600 μL aliquot is sufficient to embed EBs in 12 drops.
11. During this step EBs should be kept at room temperature no longer than 10 min. 15 mL tubes have to be moved back to the incubator as soon as all EBs have been recovered from one dish.
12. Embedding step has to be performed on ice as well; P200 pipette tips have to be precooled before use. 30 μL is added as a safe volume to ensure enough material for the procedure.
13. Make sure to remix the EBs-Matrigel suspension frequently during this step, in order to plate a constant number of EBs (approximatively eight per well). This will ensure the reproducibility of the differentiation protocol.
14. Use coverslips if you want to perform an immunofluorescence assay at the end of the differentiation protocol.
15. When changing the medium pay attention not to perturb the attachment of the Matrigel drops, since they can detach easily.

16. Washing with PBS will ensure the maximum removal of residual Doxycycline.
17. It is desirable to exchange the entire volume of the cell culture medium. When getting close to the end of the differentiation protocol, it might be necessary to change the medium every day, since cells might have expanded a lot.
18. Check regularly the Matrigel drop dissolving under the microscope. Stop incubation when cells are free from the matrix.
19. Thyroid follicles will mainly compose the pellet and single cells of different origin will stay in the supernatant.
20. With these volumes six kidneys can be grafted from 12 initial wells; 5 μL is added as a safe volume to ensure enough material for all the procedures.
21. Pay attention to gently remove the needle, in order to prevent the exit of cells from the injection site.

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Chapter 8

Functional Tooth Regeneration

Masamitsu Oshima, Miho Ogawa, and Takashi Tsuji

Abstract

Three-dimensional organogenesis in vivo is principally regulated by the spatiotemporal developmental process that relies on the cellular behavior such as cell growth, migration, differentiation, and cell-to-cell interaction. Organ development and morphogenesis have been elucidated to be regulated by the proper transient expression of various signaling molecules including cytokines, extracellular matrix, and adhesion molecules based on the epithelial and mesenchymal interactions. Current bioengineering technology for regenerating three-dimensional organ has progressed to the replication of organogenesis, thereby enabling the development of fully functional bioengineered organs using bioengineered organ germs that are generated from immature stem cells via tissue engineering technology in vitro.

To achieve precise replication of organogenesis, we have developed a novel three-dimensional cell manipulation method designated the organ germ method, and enabled the generation of a structurally correct and fully functional bioengineered tooth in vivo. This method is also expected to be utilized for analyzing gene and protein functions during organogenesis. Here, we describe protocols for the tooth germ reconstitution by using the organ germ method and for the functional analysis of tooth development in vitro and in vivo.

Key words Tooth regeneration, Organ replacement regenerative therapy, Bioengineered tooth, Organ germ method, Cell manipulation, Transplantation

1 Introduction

The tooth is an ectodermal organ arising from a tooth germ, whose development is regulated by reciprocal epithelial-mesenchymal interactions [1, 2]. The tooth has a three-dimensional multicellular structure that includes enamel, dentin, cementum, pulp, and periodontal ligament (PDL) to establish functional cooperation with the maxillofacial region [3, 4]. In the tooth development, various dental-cell lineages, such as ameloblasts, odontoblasts, pulp cells, PDL cells, cementoblasts, and osteoblasts, occurred from the dental epithelium or mesenchyme [5]. These cells secrete a collagenous extracellular matrix to accumulate the enamel, dentin, cementum, PDL, and alveolar bone. In mature tooth after tooth development, immature cells seem to be maintained as dental stem cells that are

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thought to act as a self-repair system for dental tissues and supply a wide variety of each dental cell type after the dental tissue injury [5]. The different tooth types such as incisors, canines, premolars, and molars have characteristic morphological features that are programmed at predetermined sites in the oral cavity during tooth development [6]. Tooth morphology, including tooth size and tooth cusp number, is determined in the tooth-forming field by the expression of specific genes in the immature oral epithelium and the neural crest-derived mesenchyme of the embryonic jaw [2]. The macro-patterning of tooth size can be modeled by the reaction-diffusion mechanism that is regulated by the signaling molecules in the dental mesenchyme [6]. In addition, micro-patterning of the cusp number and position is thought to involve the specific FGF4 expression at secondary signaling centers [7, 8]. Thus, tooth development and morphogenesis have been elucidated to be regulated by the proper transient expression of various signaling molecules, such as cytokines, extracellular matrix, and adhesion molecules based on the epithelial and mesenchymal interactions [9].

Three-dimensional organogenesis in vivo is principally regulated by the spatiotemporal developmental events that rely on the cellular behavior such as cell growth, migration, differentiation, and cell-to-cell interaction [10]. In the research field of tooth organogenesis, many studies for developmental mechanisms based on epithelial-mesenchymal interaction have been revealed by using in vivo transplantation experiments and/or in vitro three-dimensional organ culture method, but not utilization of a two-dimensional cell culture [11]. Furthermore, current bioengineering technology for regenerating three-dimensional organs has progressed to the replication of organogenesis through epithelial-mesenchymal interactions that occur in the developing embryo, thereby enabling the development of fully functional bioengineered organs using bioengineered organ germs that are generated from immature stem cells via tissue engineering technology in vitro [12, 13]. To realize whole-tooth replacement, the first major issue is the development of a three-dimensional cell-manipulation technology using completely dissociated epithelial and mesenchymal stem cells in vitro. So far, two conventional approaches, the biodegradable scaffold method [14, 15] and the cell aggregation method [16–18], for generating bioengineered tooth germ have been attempted. These methods are able to partially replicate tooth organogenesis, although fundamental problems regarding the low frequency of tooth formation and the irregularity of the resulting tooth tissue structures have not been resolved.

To achieve precise replication of organogenesis, we have developed a novel three-dimensional cell manipulation method designated the “organ germ method.” This method involves compartmentalization of epithelial and mesenchymal stem cells at a high cell density to replicate the epithelial-mesenchymal interactions in

organogenesis [19–22]. The bioengineered tooth germ generates a structurally correct tooth in vitro, and erupted successfully with correct tooth structure when transplanted into the oral cavity [20]. In addition, we have generated a bioengineered tooth unit composed of mature tooth, PDL, and alveolar bone and demonstrated the successful engraftment of a bioengineered tooth unit with bone integration between the alveolar bone of the tooth unit and that of the recipient [21]. The bioengineered tooth germ replicated the developmental mechanism based on epithelial-mesenchymal interactions including gene expression profiles, signaling molecules, and transcription factor pathways [19, 22]. Therefore, our bioengineered organ technology can be employed to analyze in various studies on signaling networks during tooth development and screenings for potential tooth-inductive stem cells. This method is also available for the observation in three-dimensional organ morphogenesis and for cell behavior during organ developmental processes, thereby enabling functional analyses through the up-regulation or down-regulation of candidate gene expression and protein at the single cell level [19, 22].

In this chapter, we provide a detailed protocol for the three-dimensional bioengineered tooth germ reconstitution using tooth germ-derived epithelial and mesenchymal cells. Furthermore, we describe the unique methods in murine model for the generation of bioengineered tooth unit into subrenal capsule and the transplantation of bioengineered tooth germ/tooth unit into oral cavity.

2 Materials

Solutions should be prepared using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 M Ω cm at 25 °C) and analytical grade reagents. In order to prevent contamination, all surgical tools and instruments should be sterilized in the autoclave prior to each use. It is recommended that enzyme and serum reagents should be evaluated for enzymatic reactivity and primary cell culture efficiencies, respectively, prior to experimental use.

1. Animals: An inbred mouse strain (i.e., C57BL/6, Balb/c, C3H, etc.) should be available for these experiments. Mouse embryonic age is determined based on the day of appearance of a vaginal plug in a pregnant mouse (embryonic day 0; E0).
2. Ca^{2+}/Mg^{2+}-free, phosphate-buffered saline (PBS(−)): 137-mM sodium chloride (NaCl), 2.7-mM potassium chloride (KCl), 8.0-mM anhydrous disodium hydrogen orthophosphate (Na_2HPO_4), and 1.5-mM potassium phosphate monobasic (KH_2PO_4). Store at 4 °C (*see* **Note 1**).
3. Medium: Isolation of tooth germ and dissociation of single cells are performed in basal cell culture medium supplemented

with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 10-mM 1-4-(2-hydroxyethyl)-piperazineethanesulfonic acid (HEPES). In organ culture of bioengineered tooth germ, this medium is added with the above-mentioned supplements excluding HEPES. Store at 4 °C (*see* **Note 2**).

4. Dispase solution: The concentration of Dispase is adjusted to 50 U/mL using tenfold in Hanks'-balanced salt solutions (HBSS) and stored at −20 °C until use (*see* **Note 3**).
5. Collagenase solution: The concentration of collagenase I is adjusted to 27,300 U/mL using distilled water and stored at −20 °C until use (*see* **Note 4**).
6. Reagent A: 3.66 μL/mL Collagenase solution adjusted by PBS(−).
7. Reagent B: 0.25% Trypsin adjusted by PBS(−).
8. Reagent C: Mixture solution of 1.83 μL Collagenase solution and 0.25% Trypsin adjusted by PBS(−).
9. 70 U/mL Deoxyribonuclease I (DNase I) from bovine pancreas (*see* **Note 5**).
10. Collagen Gel: Component in 100 μL of tenfold concentrated α-Minimum Essential Medium (αMEM) and 100 μL reconstitution buffer (0.08 N sodium hydroxide and 200-mM HEPES) in 800 μL Cellmatrix Type I-A (*see* **Note 6**).
11. Methylene blue gel (*see* **Note 7**).
12. Surgical tools for the embryo dissection from the uterus: large surgical scissors and forceps to cut the abdominal skin and muscle, and small surgical scissors and forceps to dissect the uterus (*see* **Note 8**).
13. Surgical tools for the transplantation into subrenal capsule: large surgical scissors and forceps to cut the abdominal skin and muscle, and micro-scissors and forceps to transplant a bioengineered tooth germ for the generation of a bioengineered tooth unit (*see* **Note 9**).
14. Surgical tools for the transplantation into jawbone: disposable surgical knife to cut off the gingiva, and drills to prepare a transplant-hole in the jawbone for bioengineered tooth germ or bioengineered tooth unit implantation (*see* **Note 10**).
15. Dissecting microscope (*see* **Note 11**).
16. Sterile disposable 1 mL syringes and 25 g needles (5/8; 0.50 × 16 mm) for tooth germ extraction (*see* **Note 12**).
17. Sterile disposable 1.5 mL microtube (*see* **Note 13**).
18. Sterile disposable gel loader tips and pipette tips for the reconstitution of bioengineered tooth germ (*see* **Note 14**).
19. Culture at the medium-gas interface (cell culture insert/0.4-μm pore size membrane, *see* **Note 15**).

20. Sterile micropipette (*see* **Note 16**).
21. Size-control device (plastic ring-shaped structure, *see* **Note 17**).

3 Methods

During the tooth germ dissection from embryos, surgical manipulation should be performed in the culture medium for the prevention of tissue drying.

3.1 Extraction of Tooth Germ from Mouse Embryo

1. After sacrificing the mouse, cut the abdomen along the midline with small scissors. Dissect the uterus containing embryos and wash it by using PBS(−) (*see* **Note 18**).
2. Isolate the embryos from the uterus. Cut off the fetal head from the body and separate the lower jaw from the head. Immediately put the lower jaw into cold (4 °C) culture medium (*see* **Note 19**).
3. Resect the tongue from the lower jaw (Fig. 1a, b, and c, *see* **Note 20**) and separate a unilateral jaw (Fig. 1d and e).
4. Eliminate the Meckel's cartilage from the lower jaw (Fig. 1f, *see* **Notes 21** and **22**) and then resect the tissue of the opposite side from Meckel's cartilage.
5. Eliminate all surplus tissue from around an incisor and molar tooth germ (Fig. 1g, *see* **Note 23**).
6. During the dissection of all tooth germs, keep the isolated tooth germs at 4 °C in cold culture medium (Fig. 1h and i).

3.2 Separation of Epithelial and Mesenchymal Tissues from Tooth Germ

1. Wash the isolated tooth germs twice in PBS(−), and then add 50 U/mL Dispase solution at room temperature for 10.5 min (Fig. 2 *left column*, *see* **Notes 24** and **25**).
2. Stop the enzymatic reaction by adding culture medium. Thereafter, wash the tooth germs twice in the similar culture medium (*see* **Note 26**).
3. Add 1 μL DNase into 2 mL of culture medium and incubate at room temperature for a few seconds (*see* **Note 27**).
4. Separate the tooth germ epithelial and mesenchymal tissues under a microscope using 25 G needles (Fig. 2 *center column*, *see* **Note 28**).
5. Keep the each tissue at 4 °C in cold culture medium.

3.3 Enzymatic Dissociation to Single Cells from Tooth Germ-Derived Epithelial Tissue

1. Collect the epithelial tissues by pipetting operation into a 15 mL tube, and centrifuge at 590 × *g* for 3 min. Discard the medium and wash the tissue pellet twice with PBS(−).
2. Remove the PBS(−) and then add 2 mL of enzyme reactive Reagent A into the same tube. Incubate the epithelial tissues

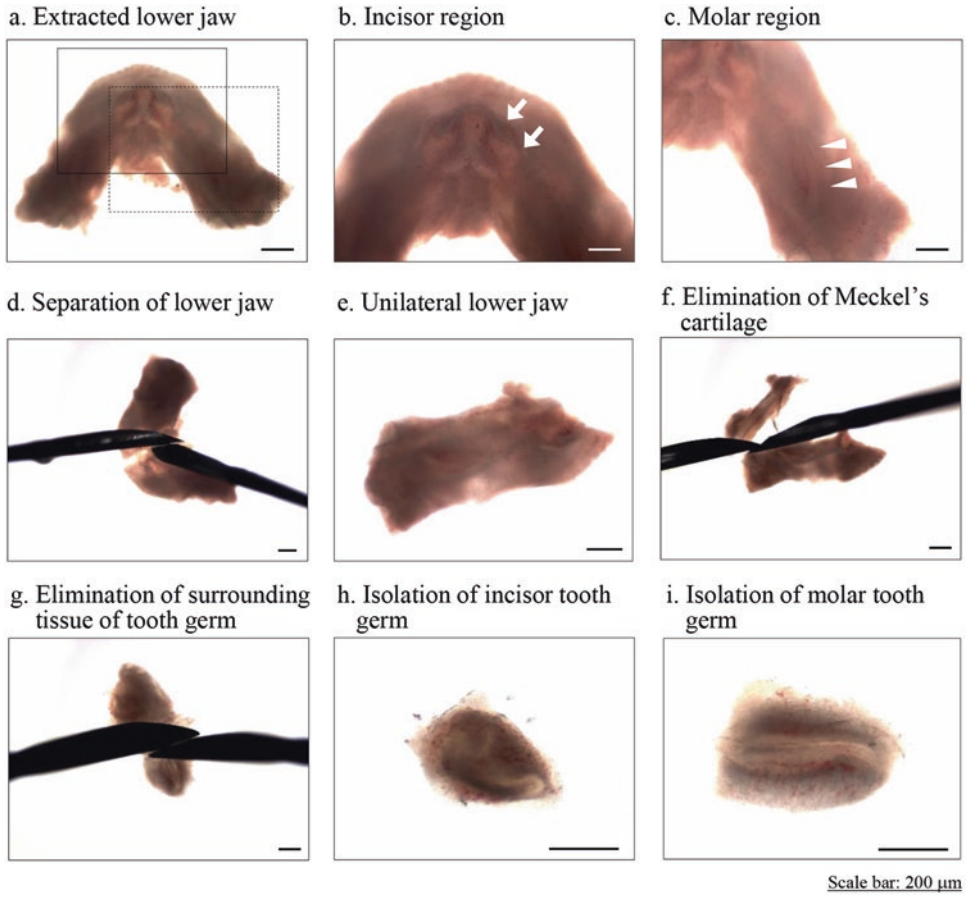

Fig. 1 Tooth Germ Dissection under the Microscopic Surgery. Extraction of the lower jaw from an embryonic day (ED) 14.5 mouse embryo (**a**). An extended image of the incisor tooth germ region (**b**) and the molar tooth germ region (**c**). Separation of left and right lower jaws (**d** and **e**). Removal of Meckel's cartilage (**f**) and elimination of surrounding tissue of tooth germs (**g**). Isolation of the incisor and molar tooth germ (**h** and **i**). The accuracy and quality of the tooth germ isolation are important for a successful development of bioengineered tooth germ. *Arrow*: Incisor tooth germ; *Arrowhead*: Molar tooth germ

for 10 min in a 37 °C water bath. Repeat this procedure twice (*see* **Note 29**).

3. Centrifuge the epithelial tissues at 590 × *g* for 3 min and discard all of Regent A. Add 2 mL of enzyme reactive Reagent B and incubate the epithelial tissues for 5 min in the 37 °C water bath.
4. Stop the enzyme reaction by adding 6 mL of culture medium. Centrifuge at 590 × *g* for 5 min.
5. Aspirate the supernatant to a residual volume of approximately 80 μL, and disperse the cell pellet by tapping (*see* **Note 30**).
6. Add 1 mL of culture medium in order to eliminate the residual enzymatic solution, and centrifuge at 590 × *g* for 3 min.

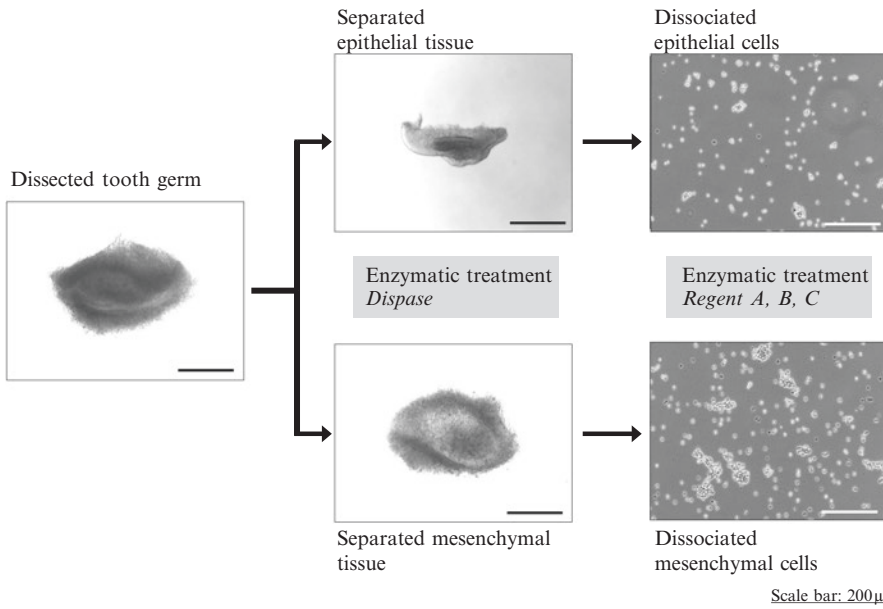

Fig. 2 Preparation of Epithelial and Mesenchymal Single Cells. Dissected tooth germs (*left*) treated with 50 U/mL Dispase solution. Separation of epithelial tissue (*upper-middle*) and mesenchymal tissue (*lower-middle*) from tooth germ under a microscope using 25 G needles. Isolation of single epithelial cells (*upper-right*) and single mesenchymal cells (*lower-right*) after enzymatic treatment using Regents A, B, and C

7. Aspirate the supernatant until the residual volume of approximately 200 μL and then add 1 μL of DNase solution to the residual medium. Create a single cell suspension by gently pipetting ten times by using a micropipette with P200 tip (*see* **Note 31**) and sift the suspension through a cell strainer to obtain a uniform single cell suspension (Fig. 2 *right column*).

3.4 Enzymatic Dissociation to Single Cells from Tooth Germ-Derived Mesenchymal Tissue

1. Collect the mesenchymal tissues by pipetting operation into a 15 mL tube and centrifuge at 590 × *g* for 3 min. Discard the medium and wash the tissue pellet twice with PBS(−).
2. Remove the PBS(−), and add 2 mL of enzyme reactive Reagent C. Incubate the mesenchymal tissues for 10 min in the 37 °C water bath.
3. Stop the enzyme reaction by adding 6 mL culture medium and centrifuge at 590 × *g* for 5 min.
4. Aspirate the supernatant to a residual volume of approximately 80 μL and disperse the cell pellet by tapping (*see* **Note 30**).
5. Add 1 mL of culture medium and centrifuge at 590 × *g* for 3 min.
6. Aspirate the supernatant until the residual volume of approximately 200 μL and thereafter add 1 μL of DNase solution to the residual medium. Create a single cell suspension by gently

pipetting ten times by using a micropipette with P200 tip (*see* **Note 31**) and sift the suspension through a cell strainer to obtain a uniform single cell suspension (Fig. 2 *right column*).

3.5 Reconstitution Procedure of the Bioengineered Tooth Germ

1. Prepare the siliconized 35–60 mm petri dish and 1.5 mL tube that thinly coats by using the sterile silicon grease (*see* **Note 32**).
2. Transfer the epithelial or mesenchymal single cell suspensions isolated from tooth germ into the siliconized 1.5 mL tube, respectively.
3. Centrifuge at 600 × *g* for 3 min and discard the supernatant by using a micropipette with P1000 or P200 tip.
4. Centrifuge at 600 × *g* for 3 min and discard the residual supernatant on the cell pellets using a micropipette and a gel-loading tip under the microscope manipulation (Fig. 3A-a, b, and c, *see* **Note 33**).

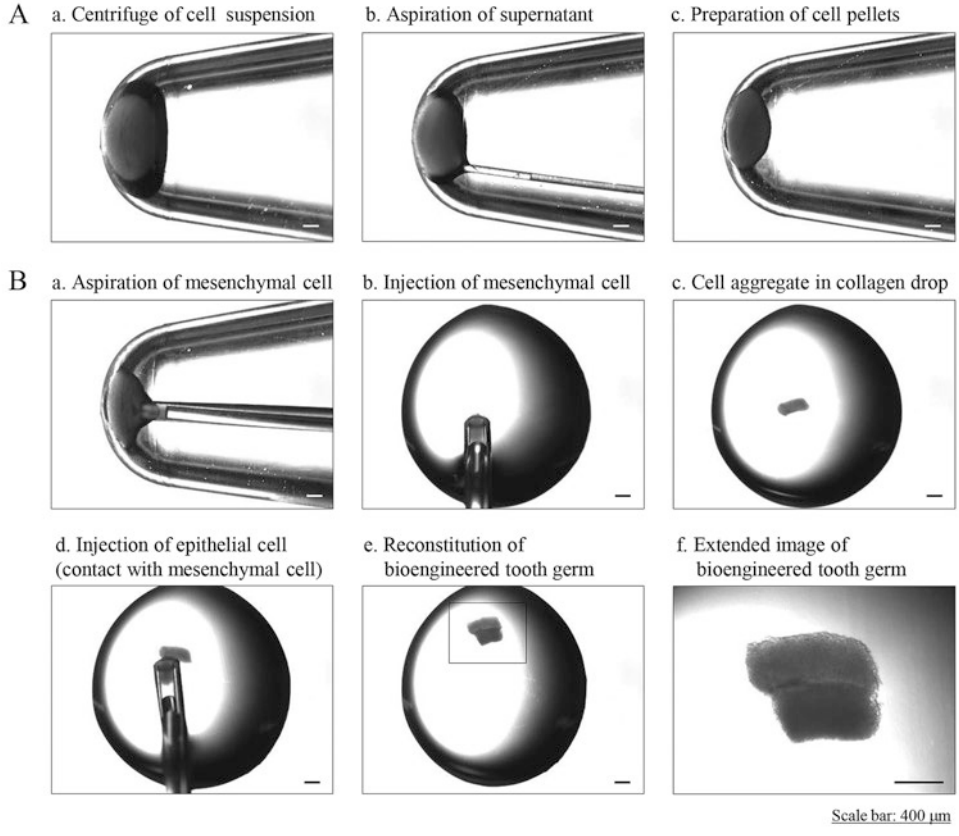

Fig. 3 Reconstitution of Bioengineered Tooth Germ using Organ Germ Method. (A) Centrifuge and remove the residual supernatant around the cell pellets (a, b, and c). (B) Aspirate (a) and inject the total volume of mesenchymal cells into the center of the collagen drop (b and c). Subsequently, aspirate and inject the epithelial cells into the same collagen drop, make contact with the mesenchymal cell aggregate (d, e, and f)

5. Prepare a droplet of 30 μL collagen gel on the siliconized petri dish (*see* **Note 34**).
6. Take approximately 0.3–0.4 μL volume of the mesenchymal cell pellet by using a micropipette with 0.1–10 μL pipette tip the microscope manipulation (*see* **Note 35**). Apply the cell pellet slowly into the collagen drop, and make a cell aggregate with high cell density (Fig. 3B-a, b and c, *see* **Note 36**).
7. Similarly, apply approximately 0.2–0.3 μL volume of the epithelial cell pellet into the same collagen drop, and make contact with the mesenchymal cell aggregate (Fig. 3B-d, e and f, *see* **Notes 37** and **38**).

3.6 In Vitro Organ Culture System of the Bioengineered Tooth Germ

1. Incubate the petri dish holding the collagen gel drop for 15 min at 37 °C under highly humidified 95% air and 5% CO_2 in order to solidify the collagen gel (Fig. 4A-a, *see* **Note 39**). Set the cell culture insert into a 12-well plate filled with culture medium (350–400 μL/well).
2. Pick up the collagen gel drop with fine tweezers and transfer the drop onto the cell culture insert (Fig. 4A-b and c, *see* **Note 40**).
3. Replace the culture medium supplemented with 10% FBS, 100 μg/mL ascorbic acid, and 2-mM L-glutamine. Remove the medium in each well, and replace with fresh medium every other day (*see* **Note 41**).
4. After organ culture for 14 days, multiple bioengineered teeth will have developed in the collagen gel. Each bioengineered tooth has the correct tooth tissue structure (Fig. 4B). When the bioengineered tooth germs are regulated by the three-dimensional contact area with the epithelial cell aggregate and the mesenchymal cell aggregate, they can develop normally in the same culture condition. (Fig. 4C, *see* **Note 42**).

3.7 Generation of a Bioengineered Tooth Unit by the Subrenal Capsule Transplantation

1. Manufacture a plastic ring-shaped structure designated as the size-control device to control the length and shape of the bioengineered tooth unit (Fig. 5A-a, *see* **Note 17**).
2. Place the single or multiple bioengineered tooth germ into the size-control devices (Fig. 5A-b and c). The bioengineered tooth germs are placed into these devices and fixed by the collagen gel (*see* **Note 43**).
3. Anesthetize the experimental mouse to be implanted and place the mouse at prone position. Fix the hands and feet of mouse on the operating table.
4. Shave the dorsal hair with a razor (*see* **Note 44**).
5. Cut off approximately 2 cm in the dorsal skin, and exfoliate between the skin and the fascia (Fig. 5B-a).
6. Dissect the fascia and draw out the kidney (Fig. 5B-b).

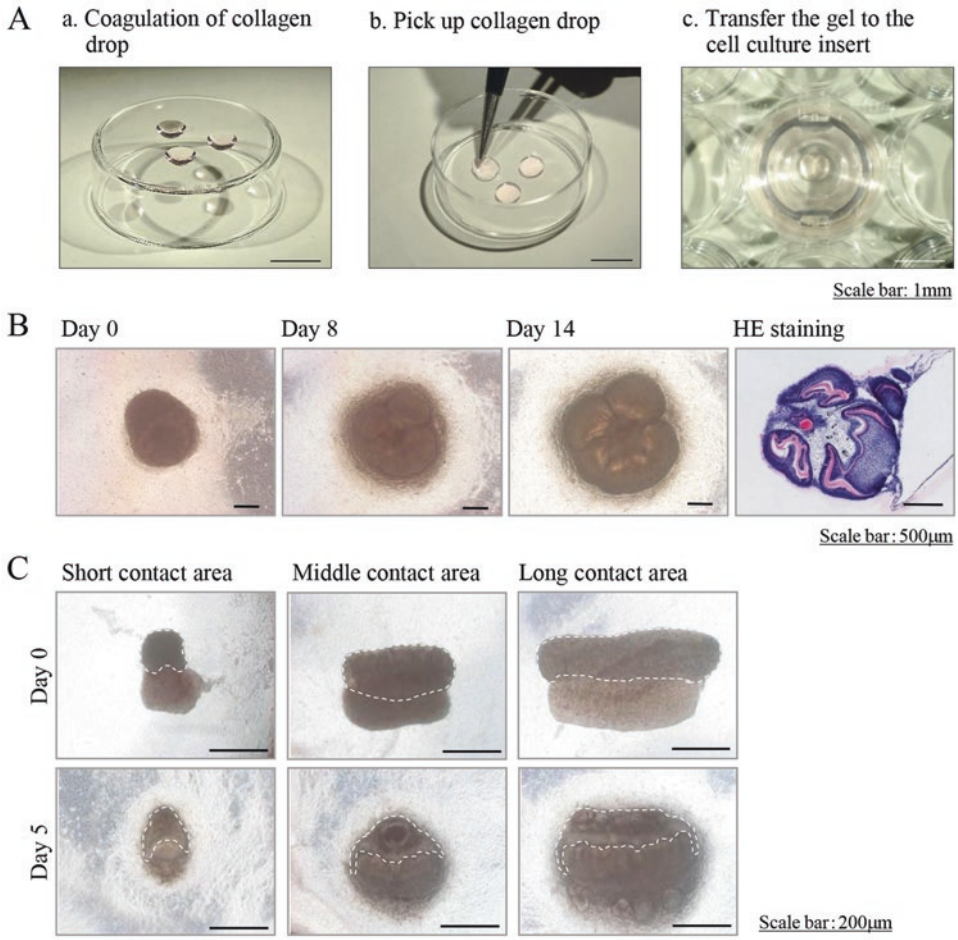

Fig. 4 In vitro Organ Culture of Bioengineered Tooth Germ. (A) Coagulate the collagen gel drop at 37 °C (a). Pick up the collagen drop (b) and transfer onto the cell culture insert in a 12-well culture plate (c). (B) Representative images of bioengineered tooth germ in in vitro organ culture. Phase-contrast image (*first, second, and third photographs from the left*) and Hematoxylin and Eosin (HE) staining (*fourth photograph from the left*). (C) Phase-contrast images of three-dimensional contact area groups (*left*, short contact; *center*, middle contact; *right*, long contact) of the bioengineered tooth germs after 0 and 5 days of organ culture. All of the bioengineered tooth germs develop to the early-bell stage at organ culture Day 5

7. Dissect approximately 2–3 mm of the subrenal capsule outer membrane (Fig. 5B-c, *see* **Note 45**).
8. Exfoliate between the kidney parenchyma and the outer membrane by the fine tweezers, and insert the size-control device including bioengineered tooth germ samples into the interspace (Fig. 5B-d, *see* **Note 46**).
9. Gently return the kidney to the peritoneal cavity. Suture the fascia and skin (Fig. 5B-e and f).
10. Approximately 30–60 days after transplantation, the single bioengineered tooth unit or the multiple bioengineered teeth unit with the correct tooth structure will have developed in the subrenal capsule (Fig. 5C-a, b, c).

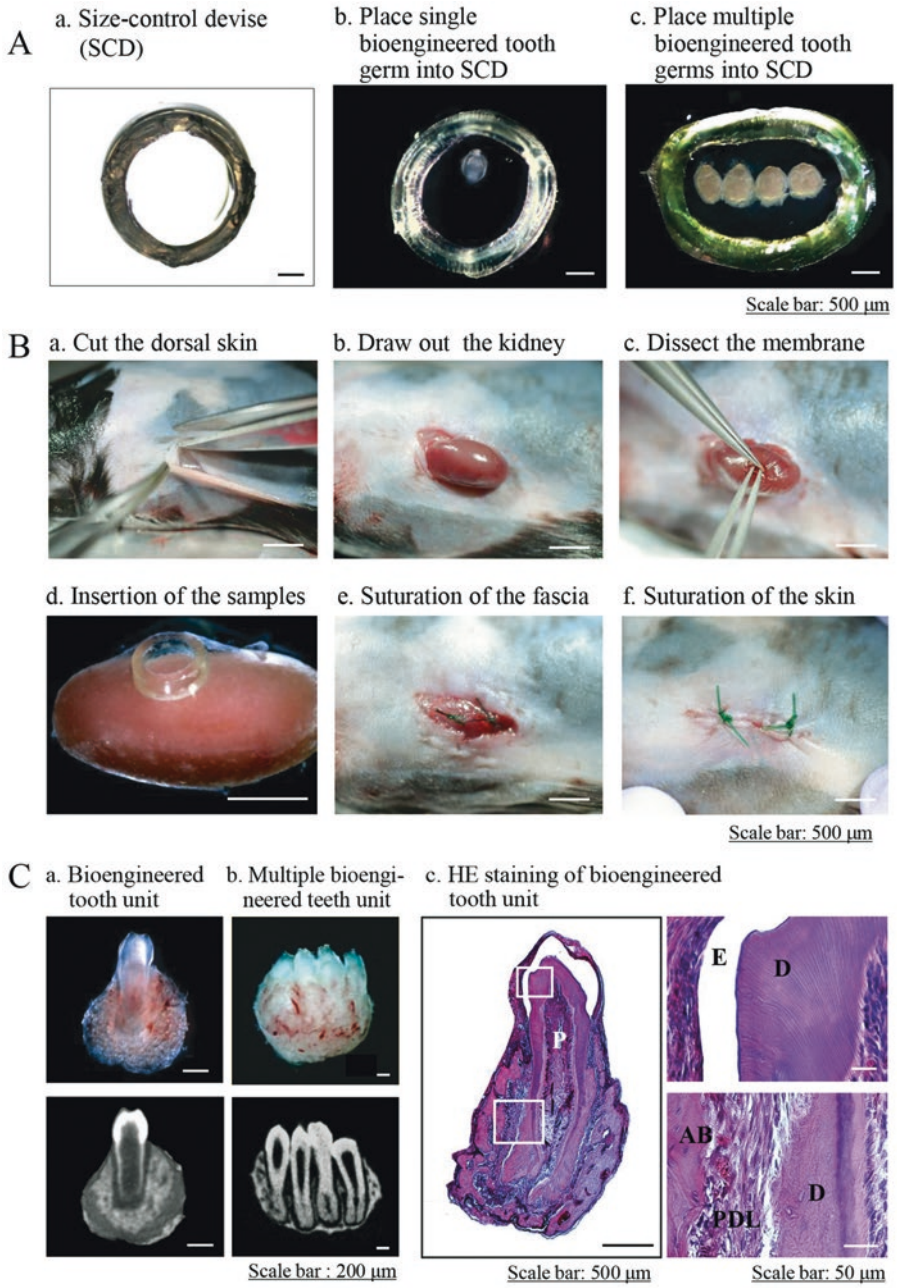

Fig. 5 Generation of Bioengineered Tooth Unit into the Subrenal Capsule Transplantation. (A) Manufacture a plastic ring-shaped structure designated as the size-control device (SCD; *left*). The device for the generation of a single bioengineered tooth unit is 2.5-mm inside diameter and 1.3-mm thickness, and the device for the generation of multiple bioengineered teeth unit is an elliptic structure at 2.5-mm height, 4.0-mm width, and 1.3-mm thickness (*center and right*). The bioengineered tooth germs are placed into these devices and fixed with the collagen gel (*center and right*). (B) Make an approximately 2.0 cm incision on the dorsal skin (a) and exfoliate between the skin and the fascia. Draw out the kidney (b) and dissect approximately 2–3 mm of the subrenal capsule outer membrane (c). Exfoliate between the kidney parenchyma and the outer membrane with thin tweezers and insert the SCD including bioengineered tooth unit into the interspace (d). Return the kidney into the peritoneal cavity and then suture the fascia and the skin (e and f). (C) Photographs and Micro-CT images of a bioengineered tooth unit (a) and multiple bioengineered teeth unit (b) at 30–60 days post-transplantation into the subrenal capsule. Hematoxylin and Eosin staining of a bioengineered tooth unit developed at 30 days post-transplantation into the subrenal capsule (c). The bioengineered tooth unit has the same tooth tissue structures as a natural tooth. Pulp tissue (P), enamel (E), dentin (D), periodontal ligaments (PDL), and alveolar bone (AB)

3.8 Transplantation of a Bioengineered Tooth Germ into the Upper Jaw

1. For the bioengineered tooth germ transplantation into the oral cavity, generate a bioengineered tooth germ by reconstitution manipulation, and then perform the organ culture for 5–6 days (Fig. 6A, *see* **Note 47**).
2. Anesthetize the experimental mouse to be implanted, and fix the body on the dissecting table. Subsequently, tie a string to an incisor tooth on the upper and lower jaw. Fix by pulling the cheek on both sides to preserve the visual field of surgical operation.
3. Cut the periodontal ligament of the upper first molar by using 25 G needle (*see* **Note 48**).
4. Extract the upper first molar using forceps (Fig. 6B-a, b and c, *see* **Note 49**).
5. Wait for healing the oral gingiva and alveolar bone socket during 3 weeks after tooth extraction (Fig. 6B-d). Under deep anesthesia of that mouse, fix the body on the dissecting table, and tie a string to an incisor tooth on the upper and lower mandibles.
6. Fix the bilateral buccal mucosa by pulling their cheek on both sides to preserve the visual field of surgical operation.
7. Using a scalpel, incise the oral gingiva at the upper tooth extraction site, and expose the alveolar bone surface (Fig. 6B-e, *see* **Note 50**).
8. Create a transplant-hole using a ϕ 0.4 mm drill, and expand the transplant-hole using a ϕ 0.8 mm drill (Fig. 6B-f, *see* **Note 51**).
9. Transplant a bioengineered tooth germ into the transplant-hole. That bioengineered tooth germ must place facing upward (toward the tooth crown direction) in the transplant-hole (Fig. 6B-g).
10. Suture the oral gingiva using 8–0 nylon (Fig. 6B-h, *see* **Note 52**).
11. Approximately 45 days after transplantation, the bioengineered tooth will reach the occlusal plane and erupt from the oral mucosa (Fig. 6C-a). The bioengineered tooth will achieve a proper occlusion with the natural lower teeth (Fig. 6C-b), and exhibit correct tooth tissue structures similar to the murine natural teeth (Fig. 6C-c).

3.9 Transplantation of a Bioengineered Tooth Unit into the Lower Jaw

1. For the identification of bioengineered tooth in the recipient's jawbone, administer the calcein regent at daily (1.6 mg/kg) via a subcutaneous dose to the transplanted bioengineered tooth germ in the subrenal capsule during the development of bioengineered tooth unit (Fig. 7A).
2. Anesthetize the experimental mouse (4-weeks-old) to be implanted, and fix the body on the dissecting table. Subsequently, tie a string to an incisor tooth on the upper and lower jaws. Fix

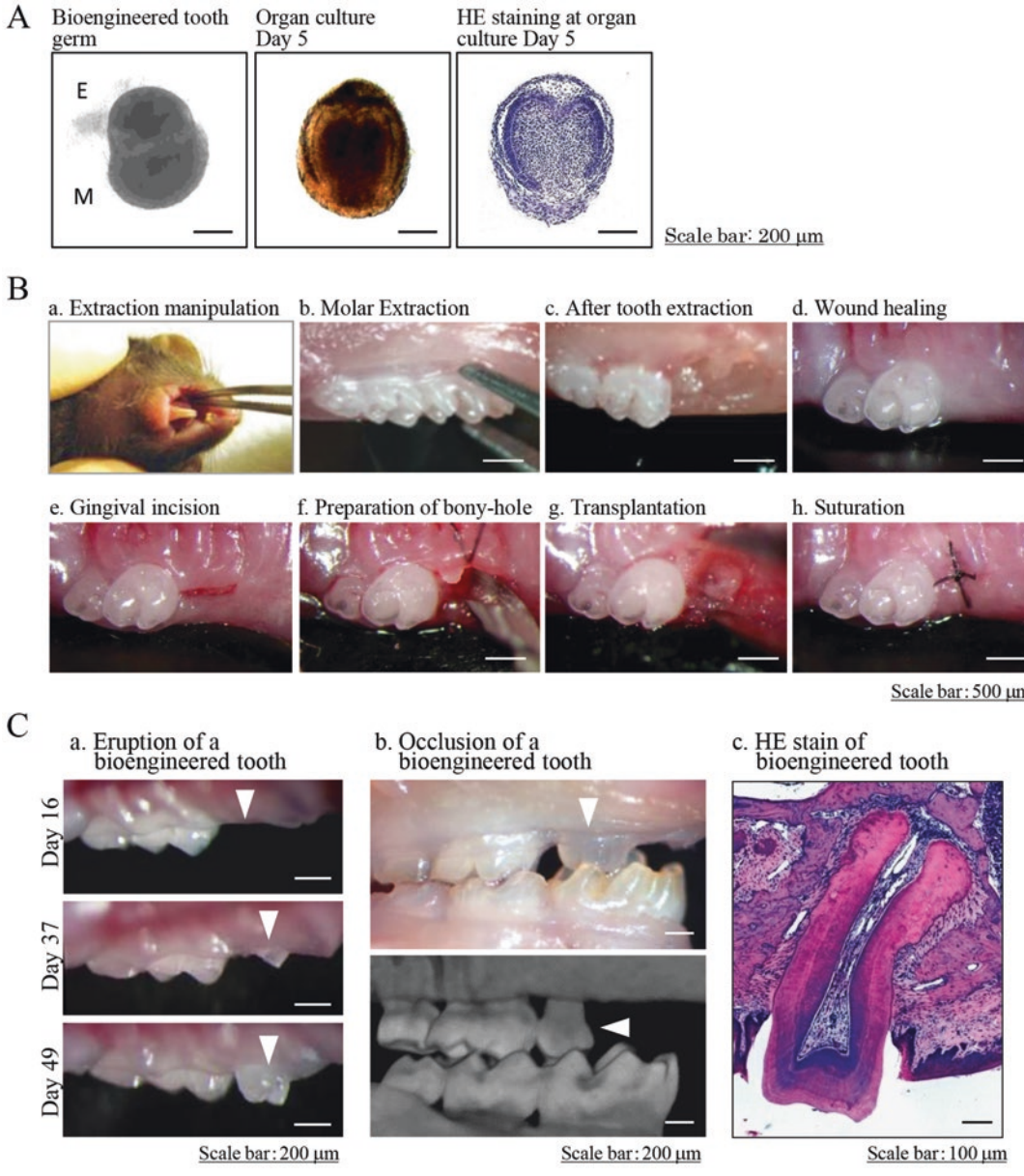

Fig. 6 Eruption and Occlusion of Bioengineered Tooth in the Oral Cavity. (A) Generation of a bioengineered tooth germ by using small-size reconstitution (*left*), and representation of developing bioengineered tooth germ at 5 days organ culture (*center*). The image shows Hematoxylin and Eosin staining of bioengineered tooth germ at 5 days organ culture (*right*). (B) Extract the upper first molars under anesthesia (a, b, and c). Mice were maintained for approximately 3 weeks to allow for natural bone healing of the tooth socket and oral gingiva (d). Following repair, an approximately 1.5-mm-long incision was made through the oral gingiva at the bone healing area (e). Preparation of a transplant hole of approximately 0.5–1.0 mm in diameter in the exposed alveolar bone surface (f). Transplantation of a bioengineered tooth germ into the bony-hole (g). Suture the oral gingiva using 8–0 nylon thread (h). (C) Photographs of the oral cavity showing a bioengineered tooth during the processes of eruption (*left column*). △; Bioengineered tooth. Photograph and micro-CT image of occlusal condition in the erupted bioengineered tooth (*center column*). Hematoxylin and Eosin staining of a bioengineered tooth developed in the mouse jaw bone at day 83 post-transplantation (*right*). The bioengineered tooth has the same tooth tissue structures as a natural tooth

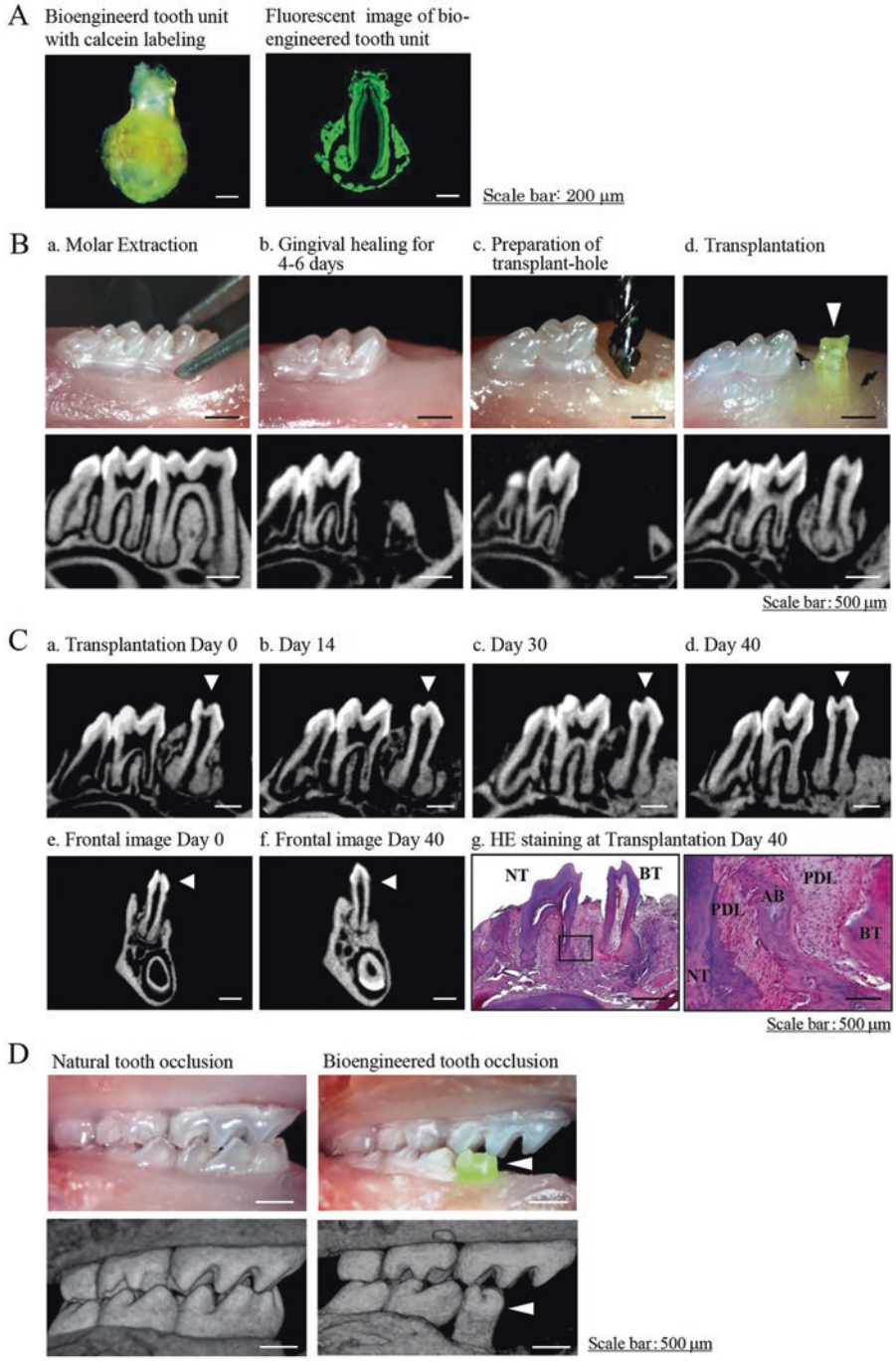

Fig. 7 Engraftment of Bioengineered Tooth Unit through the Bone Remodeling. (A) Preparation of a calcein-labeled bioengineered tooth unit via a subcutaneous dose of calcein reagent to the transplanted bioengineered tooth germ in the subrenal capsule (*left*). Tooth hard tissues including enamel, dentin, cementum, and alveolar bone are distinctively labeled by calcein (*right*). (B) Extract the lower first molars under anesthesia (a), and the resulting gingival wounds have been allowed to heal for 4–6 days (b). Following gingival repair, an approximately 1.5-mm long incision was made through the oral gingiva, and preparation of a transplant hole of

by pulling the cheek on both sides to preserve the visual field of surgical operation.

3. Cut the periodontal ligament of the lower first molar using 25 G needle (*see* **Note 48**).
4. Extract the lower first molar using forceps (Fig. 7B-a, *see* **Note 49**).
5. Wait for healing the oral gingiva during 4–6 days after tooth extraction (Fig. 7B-b). Under deep anesthesia of that mouse, fix the body on the dissecting table, and tie a string to an incisor tooth on the upper and lower mandibles.
6. Fix the bilateral buccal mucosa by pulling their cheek on both sides to preserve the visual field of surgical operation.
7. Using a scalpel, incise the oral gingiva at the lower tooth extraction site, and expose the extracted tooth socket.
8. Expand the extracted tooth socket (1.2 mm mesiodistally, 1.0 mm buccolingually, and 0.6 mm vertically) as a transplant-hole using a ϕ 0.8–1.0 mm drill (Fig. 7B-c).
9. Transplant a bioengineered tooth unit into the transplant-hole. When the bioengineered tooth unit is transplanted, it is located at a position reaching the occlusal plane with the opposing upper first molar (Fig. 7B-d, *see* **Note 53**).
10. Suture the oral gingiva using 8–0 nylon (Fig. 7B-d).
11. Partial bone integration can be observed at 14 days after transplantation, and full bone integration around a bioengineered tooth root is seen at 40 days after transplantation through natural bone remodeling in the recipient (Fig. 7C). The bioengineered tooth can achieve a functional occlusion with the natural upper teeth (Fig. 7D).

4 Notes

1. PBS(−) is used to wash the tissues and cells. Therefore, it should be an isotonic solution that does not cause the cell injury.
2. Select the basal medium after performing the examination (e.g., Dulbecco's Modified Eagle Medium). HEPES is added

Fig. 7 (continued) approximately 1.0–1.2 mm in diameter (c). Transplant a bioengineered tooth unit into the jawbone at a position reaching the occlusal plane. Suture the incision gingiva using 8–0 nylon thread (d). (C) Micro-CT images (*sagittal section;* a–d, *frontal section;* e and f) showing a bioengineered tooth unit during the functional engraftment process. △; Bioengineered tooth. Partial bone integration was observed at 14 days after transplantation, and full bone integration around a bioengineered tooth root was seen at 40 days after transplantation (a–d, e, and f). Hematoxylin and Eosin staining of the engrafted bioengineered tooth unit at day 40 post-transplantation (g). (D) Photograph and micro-CT image of occlusal condition in the natural tooth (*left*) and the engrafted bioengineered tooth (*right*)

to keep the pH constant, but HEPES is not added into the culture medium for organ culture to avoid any cytotoxicity during long culture period.

3. Dispase solution dissolves the basal membrane between the epithelial and the mesenchymal tissue of the tooth germ. Even when using the same reagents described in this protocol, we recommend strictly evaluating the conditions of temperature and reaction time because enzymatic activity is easily decreased at lower temperature.
4. Collagenase is an enzyme used to digest collagen matrix. Intercellular collagen molecules are resolved by collagenase and tissues are separated into single cells. Even when using the same reagents described in this protocol, we recommend evaluating the conditions of temperature and reaction time, since enzymatic activity is easily decreased at lower temperature.
5. In many cases, cells may be partially damaged by enzymatic treatment and release their DNA. The DNA released by enzymatic treatment cause the aggregation of cells, and the following cell-manipulation steps are difficult. DNase I can prevent this cell aggregation by digesting the DNA.
6. Collagen gel can be easily made using Cellmatrix Type I-A manufactured by Nitta Gelatin Inc.
7. When the reconstituted tooth germ is transplanted into a transplant-hole, a methylene blue gel is necessary to determine the direction of tooth eruption. Use of methylene blue, a biocompatible dye, dissolved in agarose gel to stain the epithelial tissue of the reconstituted tooth germ can facilitate orientation of the bioengineered tooth germ during intraoral transplantation.
8. Surgical instruments should be washed and sterilized by autoclaving prior to each use in order to prevent contamination.
9. When the bioengineered tooth germ is transplanted into subrenal capsule, the kidney injury must be prevented by using a ring-shaped forceps.
10. We recommend the use of minimum-size surgical tools including scalpel blades, forceps, and drills, which can be more easily manipulated in the murine oral cavity.
11. We recommend the use of a dissecting microscope for the tooth germ isolation that can have the objective lens with 6.5–50 times magnification and a light source with the transmitted beam.
12. A 25 G needle is suitable for most of the surgical manipulations including the tooth germ extraction, the isolation of tooth germ tissue, and the mature tooth extraction.
13. Round-bottom microtubes should be used, since the square-bottom microtubes are unsuitable for forming a cell pellet by centrifugation.

14. A 0.1–10 μL pipette tip is suitable for making a cell aggregate with high-cell density in the collagen gel.
15. A membrane should be used with a pore size that is sufficient for liquid components to pass through. We recommend the use of a cell culture insert/0.4 μm pore size membrane.
16. The PIPETMAN® P2 micropipette manufactured by Gilson Inc. is suitable for an easily creation of cell aggregates in the collagen gel.
17. The size-control device should be the non-degradable/biocompatible materials such as plastic and metal.
18. In order to avoid the risk of bacterial contamination, do not use the same tweezers and scissors for cutting skin and muscle.
19. Mouse embryos can be easily isolated by the surgical procedure of the uterine and amniotic membranes.
20. Before the isolation of tooth germ, you should find clearly an incisor tooth germ and a molar tooth germ in the unilateral jaw.
21. Dissecting manipulation of tooth germ should be performed using a pair of 25 G needles. One needle with the cutting surface turned toward the tooth germ is fixed on the sample, while the other needle cuts the tissue by sliding along the cutting surface of the first needle. Be careful not to press the tissues too strongly with the first needle to avoid a tissue destruction.
22. The incisor and molar tooth germ is adjacent to Meckel's cartilage. Surgical manipulation by using 25 G needles should be performed carefully when separating the Meckel's cartilage from jawbone.
23. A precise manipulation of the tooth germ dissection can affect the frequency of successful development of a bioengineered tooth germ.
24. Transfer the tooth germs into another petri-dish together with a small amount of culture medium. Do not directly transfer a tooth germ in order to avoid injury to the tooth germ.
25. Carefully follow the reaction time and the temperature of enzyme treatment, since long enzyme reactions cause the injury of tooth germ.
26. Quick and precise washing steps are required for the Dispase treatment.
27. Tissues and isolated single cells, which have been enzymatically treated, can be easy to aggregation due to the released DNA. The addition of DNase digests DNA and can prevent cell aggregation.
28. Needle manipulation should be performed carefully to avoid injury to the epithelial and mesenchymal tissues.

29. The separated tissues can be mixed in the enzymatic reagents in order to react equally with several tissues. You must be taken to avoid the tissues sticking to the tube wall.
30. When the cell aggregation cannot be dispersed by tapping, the cell-pellet can be manipulated into a single cell suspension by gently pipetting up and down.
31. Micropipette manipulation should be performed gently at a constant speed.
32. Be careful not to apply too much silicone grease and not to apply it to the inside of the 1.5 mL tube-cover.
33. Remove as much of the residual supernatant as possible after the centrifugation to create high-density cell pellets. If residual supernatant remains, the cell aggregate cannot be created into a collagen gel.
34. The cell manipulation that reconstitutes a bioengineered tooth germ should be performed quickly, since the collagen gel solidifies with a change at the room temperature and with the passage of time.
35. For the reconstitution of a bioengineered tooth germ, aspirate only the required amount of cells by using a pipette tip.
36. Insert the pipette tip into the collagen gel using a P2 micropipette. A cell aggregate is extruded slowly, and the pipette tip must be precisely operated so that cell aggregation becomes a spherical or a rod-like form. To avoid the contamination of air bubbles into the collagen gel, the cell insertion should be stopped precisely when all cells have been extruded from the pipette tip.
37. When bioengineering a tooth germ using both epithelial and/or mesenchymal tooth germ tissues (i.e., reconstitution of tissue and tissue, or tissue and cell), ensure that there is sufficient contact between the tissue and cell aggregate.
38. When you create a bioengineered tooth germ using this cell manipulation method, the number of developed bioengineered tooth germs can be reduced by creating a small volume for the cell aggregate.
39. Turn the siliconized dish upside down so that the reconstructed tooth germ does not sink to the bottom of the collagen gel.
40. Peel off the collagen gel from the bottom aspect of the siliconized dish. The edge of the collagen gel should be picked up with tweezers and carefully installed onto the cell culture insert.
41. It is desirable to exchange the entire volume of organ culture medium.
42. The width of bioengineered tooth crown can be regulated by controlling the contact area between the epithelial and mesenchymal cell layers. This manipulation is conducted using a

Hamilton syringe. Several three-dimensional contact area groups (short, middle, and long contacts) of the bioengineered tooth germs can develop normally to the early-bell stage at organ culture Day 5.

43. The width and thickness of size-control device are important for the insertion into subrenal capsule. As a guide, the device for the generation of a single bioengineered tooth unit is recommended to a 2.5-mm inside diameter and 1.3-mm thickness, and the device for the generation of multiple bioengineered teeth unit is recommended to an elliptic structure at 2.5-mm height, 4.0-mm width and 1.3-mm thickness.
44. Use soapy water for shaving. To avoid the risk of bacterial contamination, shave hair from the center part of the back to the root of the hind leg in a broad range.
45. When the dissection of the subrenal capsule outer membrane is too large, there is a risk that the outer membrane will peel off from the kidney parenchyma. Also, be careful not to dry out the kidney during the surgical operation.
46. When the size-control device is transplanted into the subrenal capsule, be careful not to tear the outer membrane. We recommend that this device inserts by holding down the kidney parenchyma.
47. The bioengineered tooth germ for oral transplantation is suitable for the developmental process at bell-shape stage that performed organ culture for 5–6 days.
48. A needle is inserted to cut off the periodontal ligament in the entire periphery of the upper first molar. Be careful not to bore to the maxillary sinus when the needle is inserted deeply.
49. There is a risk of tooth-root fracture when a tooth is pulled up forcibly. The tooth should be extracted by making the tooth vibrate slowly.
50. Carry out the incision and exfoliation that enable the creation of a gingival periosteal flap.
51. The transplant-hole should be formed with sufficient attention and in consideration of the direction of tooth eruption. The direction of the transplant-hole is influenced by the bioengineered tooth eruption.
52. Be careful not to injure a transplanted tooth germ by the suture needle. Also, the suturing should be closed completely to avoid leakage of the transplanted tooth germ.
53. When the bioengineered tooth unit is transplanted into the oral cavity, the transplant-hole should be adjusted appropriately in accordance with the alveolar bone size of a transplanted bioengineered tooth unit.

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Chapter 9

Functional Hair Follicle Regeneration by the Rearrangement of Stem Cells

Kyosuke Asakawa, Koh-ei Toyoshima, and Takashi Tsuji

Abstract

Hair follicles develop from the ectoderm in embryos and cyclically regenerate using proper spatiotemporal signaling molecules, which are conserved in organogenesis during adulthood. Previously, we demonstrated that bioengineered hair follicle germs could regenerate functional hair follicles via a three-dimensional cell manipulation technique, which we named the "organ germ method." We could also regulate the type of hair follicle and pigmentation with correct structures by rearranging the source of the cells. In this article, we describe a detailed protocol for the regeneration of functional hair follicles and their stem cell niches by the rearrangement of embryonic or adult hair follicle-derived epithelial and mesenchymal cells.

Key words Organ germ method, Bioengineered hair follicle, Regeneration, Organ culture, Transplantation

1 Introduction

The hair follicle is an ectodermal skin appendage that is conserved in vertebrates. The hair shafts produced from hair follicles have important roles in thermoregulation, physical insulation, sensitivity to noxious stimuli, and social communication [1]. During embryogenesis, the hair follicle develops through proliferation, morphogenesis, and differentiation through the interaction between immature epithelial and mesenchymal cells via signaling molecules [1, 2]. Adult hair follicles contain several pools of spatially distributed stem cells that are defined by unique molecular signatures [3–7]. Epithelial stem cells in the permanent region contribute not only to hair follicle regeneration but also to skin homeostasis [6–8]. The dermal papilla (DP), which is the mesenchymal component underlying the epithelium of the hair follicle, protects mesenchymal stem cells and prompts epithelial stem cells to undergo hair follicle differentiation [9, 10]. The differences in the type of hair follicles are responsible for the number or type of mesenchymal stem cells in the DP [11]. Transcription factor SOX2,

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which is expressed in DP cells, is one of the key factors that determines the morphological features of hair follicles by regulating the differentiation of epithelial cells [12, 13]. The hair follicle has long been used as a study tool for the elucidation of the mechanisms of organogenesis through the interaction between the epithelium and mesenchyme and stem cell biology.

Many patients with various types of alopecia experience a decrease in their quality of life (QOL), including intractable disease, such as hypotrichosis, injury and burns, and male pattern baldness owing to the denaturation of the hair follicle. To alleviate various types of alopecia, pharmacotherapy, substitution with artificial materials, such as wigs, and surgical autologous hair follicle unit transplantation (FUT) have been used [14]. FUT has clinically achieved the restoration of proper hair appearance by controlling hair type and density via the representation of the natural hair's orientation. However, it is highly invasive, and the number of hair follicles cannot be changed. To achieve hair follicle regeneration, many studies have attempted to develop novel technologies. De novo folliculogenesis can be achieved by the replacement of mesenchyme with DP cells, which prompts the self-organization of skin-derived epithelial and mesenchymal cells [15–19]. The development of a novel technique for the fundamental regeneration of hair follicles would allow for the control of the hair type, pigmentation, and direction of the hair shaft.

Recently, we demonstrated functional hair follicle regeneration from somatic stem cells and their niche cells using the organ germ method [20–22]. The regenerated hair follicle repeats cyclical regeneration, which is caused by reconstruction of the stem cell niche. Additionally, this bioengineered hair follicle germ showed reconstruction of another stem cell niche by the cooperative interaction between other types of stem cells. Furthermore, we were able to regenerate the hair follicles according to the original hair type, including the appropriate connections with hair type-specific surrounding tissues, such as nerves and muscular tissue. These results indicate the importance of the epithelial-mesenchymal cell interaction during the regeneration of the hair follicle. Additionally, the hair follicle is one of the ideal organs to realize three-dimensional organ regenerative therapy. This method is applicable to the fundamental study of both regenerative therapy for three-dimensional organs and organ developmental biology, such as morphogenesis and gene expression analyses induced by epithelial-mesenchymal interactions.

In this chapter, we describe a detailed protocol for the regeneration of functional hair follicles and their stem cell niches by the rearrangement of embryonic or adult hair follicle-derived epithelial and mesenchymal cells. We also describe methods for the intracutaneous transplantation of a bioengineered hair follicle germ. This protocol not only contributes to the realization of clinical applications of regenerative therapy but also provides important insight into stem cell biology.

2 Materials

1. Animals: An inbred mouse strain should be available for these experiments. Mouse embryonic age is determined based on the day of appearance of a vaginal plug in a pregnant mouse (embryonic day 0; ED0).
2. Ca^{2+}/Mg^{2+}-free, phosphate-buffered saline (PBS(−)): 137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 8.0 mM anhydrous disodium hydrogen orthophosphate (Na_2HPO_4), and 1.5 mM potassium phosphate monobasic (KH_2PO_4). Store at 4 °C.
3. Culture media (DMEM10): In organ culture of bioengineered hair germ, DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Store at 4 °C.
4. Dissection media (DMEM10/HEPES): For isolation and dissociation of primary single cells, 10 mM 1-4-(2-hydroxyethyl)-piperazineethanesulfonic acid (HEPES) is added to DMEM10 to avoid pH changes. Store at 4 °C.
5. Culture media for dermal papilla (DMEM10/b): In primary culture of dermal papilla, add the 10 ng/mL basic FGF (recombinant human) for culture media (DMEM10). Store at 4 °C.
6. Dispase reagent: The concentration of dispase is adjusted to 50 U/mL using tenfold in Hanks' balanced salt solutions (HBSS) and stored at −20 °C until use.
7. Collagenase solution: The concentration of collagenase I is adjusted to 27,300 U/mL using distilled water and stored at −20 °C until use.
8. Reagent A: PBS(−) containing 50 U/mL collagenase I.
9. Reagent B: PBS(−) containing 0.25% trypsin.
10. Collagenase reagent: DMEM10/HEPES containing 10,000 U/mL collagenase I.
11. Dispase/collagenase reagent: 50 U/mL dispase containing 100 U/mL collagenase I.
12. 70 U/mL deoxyribonuclease I (DNase I) from bovine pancreas.
13. Collagen gel
    (a) Blend A–C described below at a ratio of 8:1:1:
    (b) A: Cellmatrix Type I-A.
    (c) B: 10× a-Minimum Essential Medium (MEM).
    (d) C: Reconstitution buffer.
14. Trypsin/EDTA solution: PBS(−) containing 0.05% trypsin.
15. Surgical tools for dissection (*see* **Note 1**).

16. Surgical tools for the transplantation (*see* **Note 2**).
17. Dissecting microscope.
18. Sterile disposable 1.5 mL microtube (*see* **Note 3**).
19. Sterile disposable gel loader tips and pipette tips for the reconstitution of bioengineered hair germ (*see* **Note 4**).
20. Culture at the medium-gas interface (cell culture insert/0.4-μm pore size membrane, *see* **Note 5**).
21. Sterile micropipette (*see* **Note 6**).

3 Methods

The dissection of embryos, skin tissues, and hair germs should be performed in medium to prevent drying and maintained at 4 °C throughout the procedure, except for the enzyme reaction and in vitro organ culture. Surgical instruments should be washed and sterilized by autoclaving prior to each use to prevent contamination. Needle manipulation should be performed carefully to avoid injury to the epithelial and dermal papilla tissue.

3.1 Preparation of Mouse Embryo Dorsal Cells

3.1.1 Preparation of Mouse Embryo Dorsal Skin

1. Euthanize the pregnant mice (ED18) by cervical dislocation, and disinfect the abdominal skin with 70% ethanol.
2. Cut the abdominal skin along the midline with scissors and forceps, and remove the uterus.
3. Place the uterus in a plastic dish filled with cold PBS(−), and resect the embryo from the uterus (Fig. 1a).
4. Amputate the head, and separate the dorsal skin from the body using scissors and forceps (*see* **Note 7**).
5. Immediately place the dorsal skin into a 35-mm dish filled with cold DMEM10/HEPES, trimming the connecting tissues from the dermal component with forceps (Fig. 1b–d, *see* **Note 8**).

3.1.2 Separation of the Dorsal Skin Epithelium and Mesenchymal Tissues

1. Following dissection of the trimmed dorsal skin into 3–5-mm^2 sections, wash the dissected dorsal skin twice in PBS(−) (*see* **Note 9**).
2. Add dispase reagent, and incubate at 4 °C for 1 h with shaking (55 rpm, reciprocally, Fig. 1e).
3. Stop the enzyme reaction by washing out the dispase reagent twice with cold PBS(−), and add DMEM10/HEPES.
4. Add 1 μL of DNase reagent into 2 mL of DMEM10/HEPES, and incubate the reaction at room temperature for a few seconds.
5. Separate the dorsal skin epithelial and mesenchymal tissues under a stereomicroscope using 25-G needles (Fig. 1f and h).
6. Keep each tissue at 4 °C in cold DMEM10/HEPES.

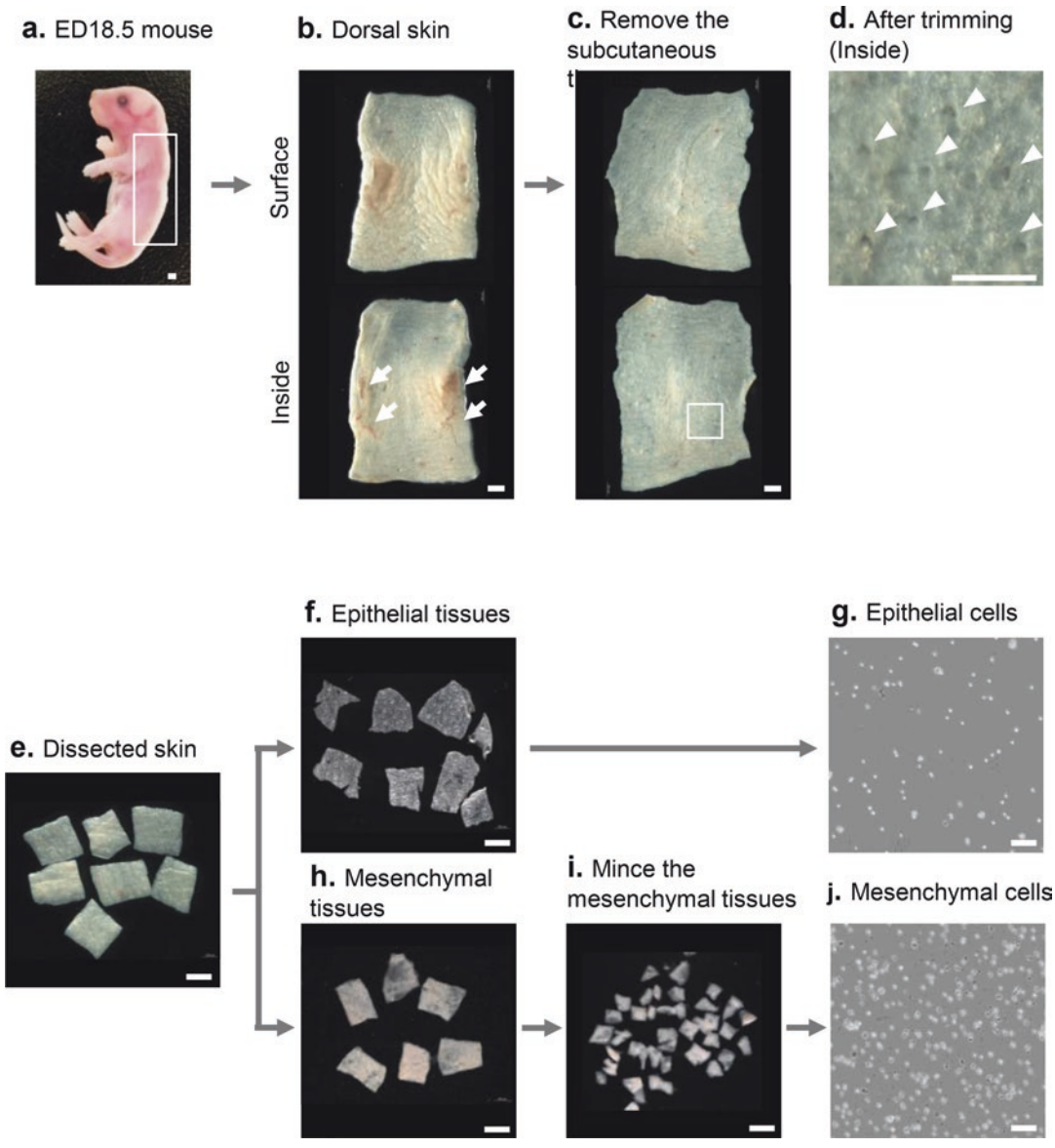

Fig. 1 Preparation of single epithelial and mesenchymal cells from the dorsal skin of embryonic mice. (**a**) Macroscopic image of the ED18.5 mouse embryo. (**b**) Surface and inside images of separated dorsal skin from the *white-boxed* area shown in (**a**). *White arrows* indicate the connecting tissues or blood vessels. (**c**) Surface and back images after trimming the dorsal skin. No connecting tissues or blood vessels are seen. (**d**) Highly magnified image of the trimmed dorsal skin. *White arrowheads* indicate the hair follicle germ. (**e**) Macroscopic image of the dissected dorsal skin. (**f** and **h**) Macroscopic image of the epithelial and mesenchymal tissues separated from dorsal skin by enzyme treatment and fine needles (approx. 3–5 mm^2). (**g**) Epithelial cell suspension obtained after trypsin treatment. (**i**) Mesenchymal tissues minced with a scalpel. (**j**) Mesenchymal cell suspension obtained after collagenase treatment. Scale bars, 200 μm

3.1.3 Dorsal Skin Epithelial Tissue Dissociation by Enzymatic Reaction

1. Wash the epithelial tissues twice in cold PBS(−).
2. Collect the epithelial tissues in a 15-mL tube and centrifuge at 600 × *g* for 3 min.
3. Aspirate the supernatant, add 2 mL of Reagent A, and incubate the enzyme reaction in a 37 °C water bath for 20 min.
4. Centrifuge the epithelial tissues at 600 × *g* for 3 min, and discard all of Reagent A.
5. Add 2 mL of Reagent A, and incubate the enzyme reaction in a 37 °C water bath for 20 min.
6. Centrifuge the epithelial tissues at 600 × *g* for 3 min, and discard all of Reagent A.
7. Add 2 mL of Reagent B, and incubate the epithelial tissues for 10 min in a 37 °C water bath.
8. Add 2 mL of DMEM10/HEPES to stop the enzyme activity, and centrifuge the tissue at 600 × *g* for 3 min.
9. Aspirate the supernatant (residual volume of approx. 50 μL), and add 1 mL of DMEM10/HEPES containing 0.5 μL of DNase reagent (*see* **Note 10**).
10. Suspend the cell pellet by pipetting, pass the cell suspension through a cell strainer, and collect in siliconized 1.5-mL tubes (Fig. 1g, *see* **Note 11**).

3.1.4 Dissociation of Dorsal Skin Mesenchymal Tissue by Enzymatic Reaction

1. Dissect the mesenchymal tissues into 1–2-mm^2 sections with a scalpel (Fig. 1i).
2. Collect the mesenchymal tissues in a 50-mL tube and centrifuge at 600 × *g* for 3 min.
3. Aspirate the supernatant, add 3 mL of collagenase reagent, and incubate at 37 °C for 1 h with shaking in a water bath (55 rpm, reciprocally).
4. Add 12 mL of cold DMEM10/HEPES and centrifuge at 600 × *g* for 5 min at 4 °C.
5. Aspirate the supernatant (residual volume of approx. 50 μL) to stop the enzymatic reaction, and add 1 mL of DMEM10/HEPES containing 0.5 μL of DNase reagent (*see* **Note 10**).
6. Disperse the cell pellet by pipetting five times with a 1000-μL micropipette and pass the cell suspension through a cell strainer to siliconized 1.5-mL tubes (Fig. 1j, *see* **Note 11**).

3.2 Preparation of Adult Mouse Vibrissae Follicle Cells

3.2.1 Preparation of Adult Mouse Vibrissae Follicles

1. After euthanizing the 8-week-old mice by cervical dislocation, dissect the whisker pad from the mice, and briefly disinfect with povidone-iodine disinfectant, 70% ethanol, PBS(−), and DMEM10/HEPES for 10–20 s at each step (Fig. 2a, b, *see* **Note 12**).

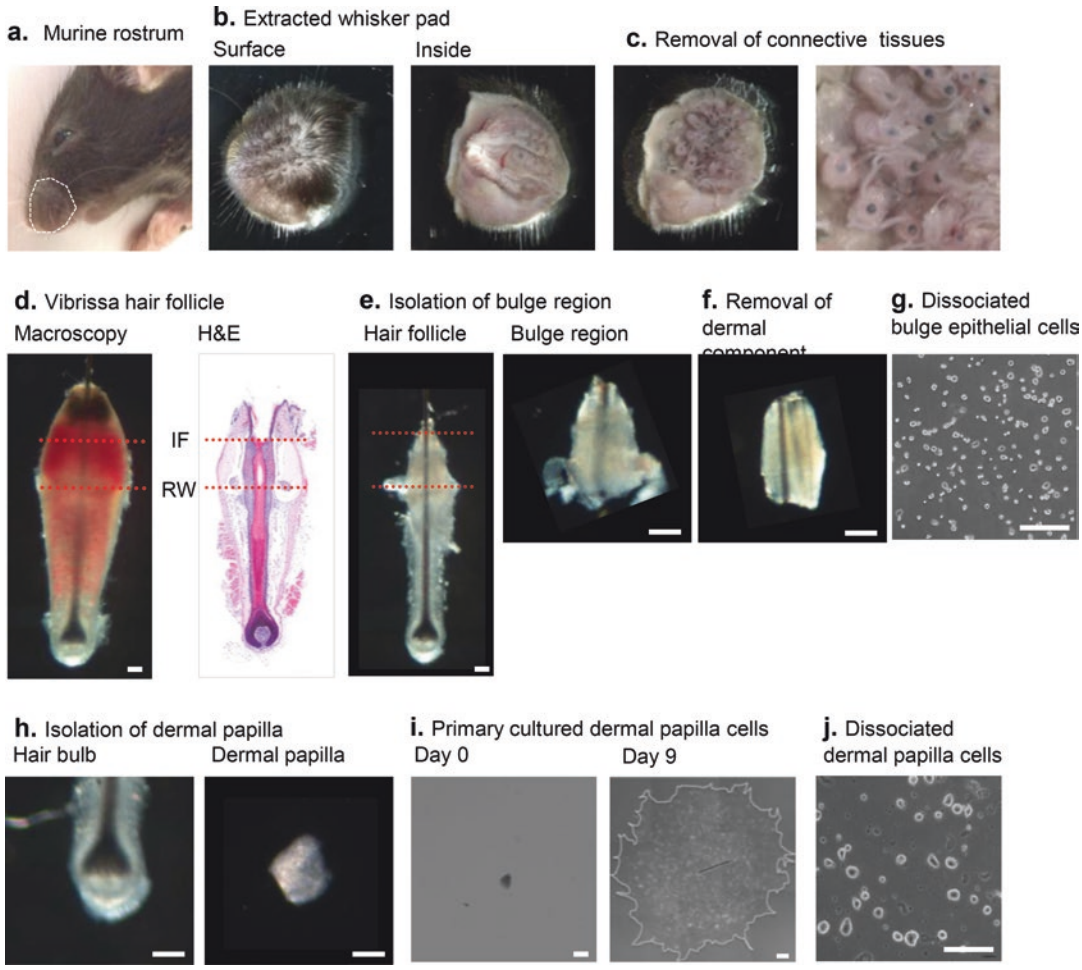

Fig. 2 Preparation of single bulge epithelial and dermal papilla cells from the vibrissae of adult mice. (**a**) Macroscopic images of the mice vibrissae hair follicle. Whisker pad in the murine rostrum area (*dotted line* area in (**a**)). (**b**) Extracted whisker pad area. (**c**) The whisker pad after removal of the connective tissues. A highly magnified image is shown on the *right*. (**d**) Macroscopic and histological images of the mouse whole vibrissae hair follicle. The *dashed lines* (*red*) via macro-morphological observations (*left*) and H&E staining (*right*) indicate the location of the dissection. (**e**) Macroscopy of a whole vibrissae hair follicle after removal of the collagen sheath (*left*) and the dissected bulge region (*right*). (**f**) Bulge epithelial tissue after removal of the dermal components. (**g**) Phase-contrast image of single cells from the bulge epithelium. (**h**) Microscopic images of the hair bulb region (*left*) and the dermal papilla (*right*) of an adult vibrissae at the anagen phase. (**i**) Phase-contrast image of the isolated dermal papilla (*left*, day 0) and outgrowth colony after 9 days of in vitro cell propagation (*right*). (**j**) Dissociated primary cultured dermal papilla cells 9 days after explantation. Scale bars, 100 μm. *IF* infundibulum, *RW* ringwulst

2. Remove the connective tissue and adipose tissues attached to the hair follicles from the mesenchymal component of each whisker pad to locate the individual vibrissae (Fig. 2c).
3. Isolate whole vibrissae follicles, including the collagen sheath, from the whisker pad with fine forceps (Fig. 2d, *see* **Note 13**).
4. Keep at 4 °C in cold DMEM10/HEPES.

3.2.2 Enzymatic Separations of Single Epithelial Cells from the Bulge Region of Adult Mouse Vibrissae Follicles

1. Remove the collagen sheath from the vibrissae hair follicles (Fig. 2e, left).
2. Separate the bulge region, defined as the area that extends from below the sebaceous gland to above the ringwulst, under a stereomicroscope using 25-G needles (Fig. 2e, right).
3. Wash the bulge region twice in PBS(−).
4. Remove the PBS(−), add dispase/collagenase reagent, and incubate at 37 °C for 5 min.
5. Stop the enzyme reaction by washing out the dispase reagent twice with cold PBS(−) and add DMEM10/HEPES.
6. Remove the connective tissue from the bulge region under a microscope with 25-G needles (Fig. 2f).
7. Collect the epithelial tissues in a 35-mm dish.
8. Wash the bulge epithelial tissues twice with PBS(−), and incubate the bulge epithelial tissues in 2 mL of trypsin reagent for 1 h at 37 °C.
9. Stop the enzymatic reaction by adding 2 mL of DMEM10/HEPES.
10. Add the 2 μL of DNase reagent, and dissociate the bulge epithelial cells by gently pipetting five times with a 1000-μL micropipette (*see* **Note 14**).
11. Pass the cell suspension through a cell strainer, collect in 15-mL tubes, and centrifuge at 600 × *g* for 3 min at 4 °C.
12. Aspirate the supernatant (residual volume of approx. 50 μL), and add 1 mL of DMEM10/HEPES containing 0.5 μL of DNase reagent (*see* **Note 10**).
13. Pass the cell suspension through a cell strainer, and collect the suspension in siliconized 1.5-mL tubes (Fig. 2g, *see* **Note 11**).

3.2.3 Primary Cultivation of Dermal Papilla Cells

(Prepare the day before the 9 days of reconstruction of bioengineered hair follicle germ derived from the adult vibrissae.)

1. Amputate the hair bulb region from anagen vibrissae follicles (Fig. 2h, *see* **Note 15**).
2. Isolate dermal papillae with 25-G fine needles and explant onto a 60-mm cell culture dish containing 10 ng/mL of FGF2 in culture medium (Fig. 2i, *see* **Note 16**).
3. Change the entire quantity of culture medium every 4 days (primary cultures for 9 days as described previously in 5, 12, and 24).
4. Wash the primary cultured dermal papilla cells twice in PBS(−).
5. Aspirate the PBS(−), add 0.5 mL of trypsin/EDTA solution, and incubate for 5 min at 37 °C.

6. Stop the enzymatic reaction by adding culture medium, and collect the dermal papilla cells into a 15-mL tube using a 1000-μL micropipette.
7. Centrifuge at 600 × *g* for 3 min, and aspirate the supernatant.
8. Add 1 mL of DMEM10/HEPES, and pass the cell suspension through a cell strainer into 15-mL tubes (Fig. 2j).

3.3 Reconstitution of the Bioengineered Hair Follicle Germ

1. Prepare siliconized 35-mm petri dishes using a cotton swab (*see* **Note 11**).
2. Centrifuge at 600 × *g* for 3 min at 4 °C, and aspirate the supernatant using a micropipette and a 1000-μL or 100-μL micropipette tip (*see* **Note 17**).
3. Centrifuge at 600 × *g* for 3 min at 4 °C, and remove the residual supernatant on the cell pellets using a micropipette and a gel-loading tip under a stereomicroscope (*see* **Note 18**).
4. Place 30 μL of collagen gel on a siliconized petri dish.
5. Aspirate a 0.2–0.3-μL volume of the mesenchymal cell pellet using a micropipette and a 0.1–10-μL micropipette tip under a stereomicroscope (*see* **Note 19**).
6. Add the cell pellet slowly into the collagen drop, and make a spherical cell aggregate (Fig. 3a, b, *see* **Note 20**).
7. Similarly, apply a 0.2–0.3-μL volume of the epithelial cell pellet into the same collagen drop, and make contact with the mesenchymal cell aggregate (Fig. 3c–e, *see* **Note 21**).
8. Incubate the dish for 15 min at 37 °C and solidify the gel.
9. To form intraepithelial tissue connections between the host skin and the bioengineered hair follicle, insert a nylon thread guide (8–0 nylon surgical suture) into a bioengineered hair germ through the thrusting epithelial and mesenchymal cell portions (Fig. 3f, *see* **Note 22**).
10. Pick up the drop with forceps, and place the drop onto the cell culture insert containing 1 mL of DMEM10 (Fig. 3g–i, *see* **Note 23**).

3.4 Intracutaneous Transplantation of a Bioengineered Hair Follicle Germ

1. Anesthetize and retain the recipient Balb/c nu/nu mouse (6 weeks old) (Fig. 4a).
2. Position the mouse on its side.
3. Briefly disinfect the skin surface of the recipient mouse with povidone-iodine disinfectant and 70% ethanol.
4. Create a shallow stab wound that is nearly parallel to the skin surface on the back of nude mice using a 20-G ophthalmic V-lance (Fig. 4b).

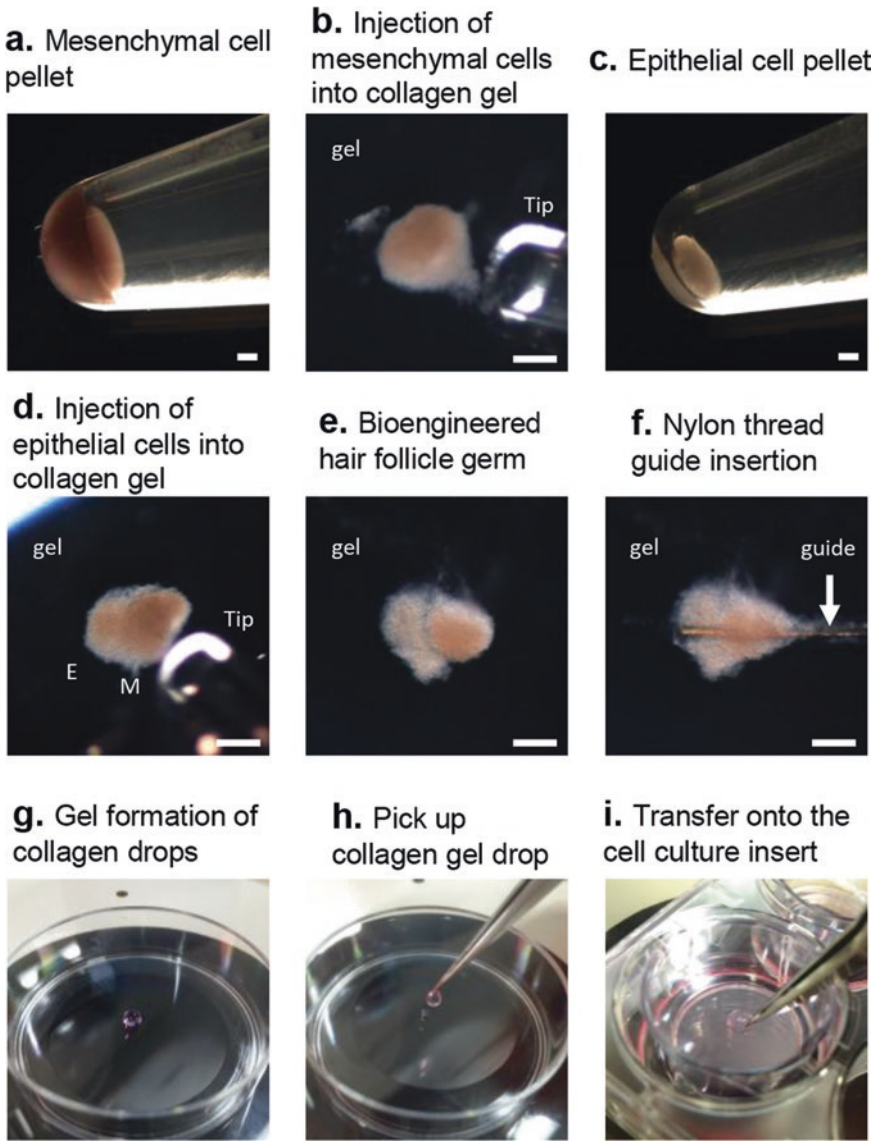

Fig. 3 Reconstruction of the bioengineered hair follicle germ. Reconstitution of the bioengineered hair follicle germ derived from mouse embryonic dorsal skin. (**a**, **b**) Aspirate and inject the total volume of epithelial cells into the center of the collagen drop. (**c**, **d**) Subsequently, aspirate and inject the mesenchymal cells into the same drop adjacent to the epithelial cell aggregate cells. (**e**, **f**) Insert a nylon thread guide into a bioengineered hair germ through the epithelial and mesenchymal cell portions. (**g**) Gel formation and organ culture of the bioengineered hair follicle germ. Coagulate the collagen gel drops at 37 °C. (**h** and **i**) Pick up the collagen gel drop and transfer onto a cell culture insert. Scale bars, 200 μm. *E* epithelial cells, *M* mesenchymal cells; guide, nylon thread guide

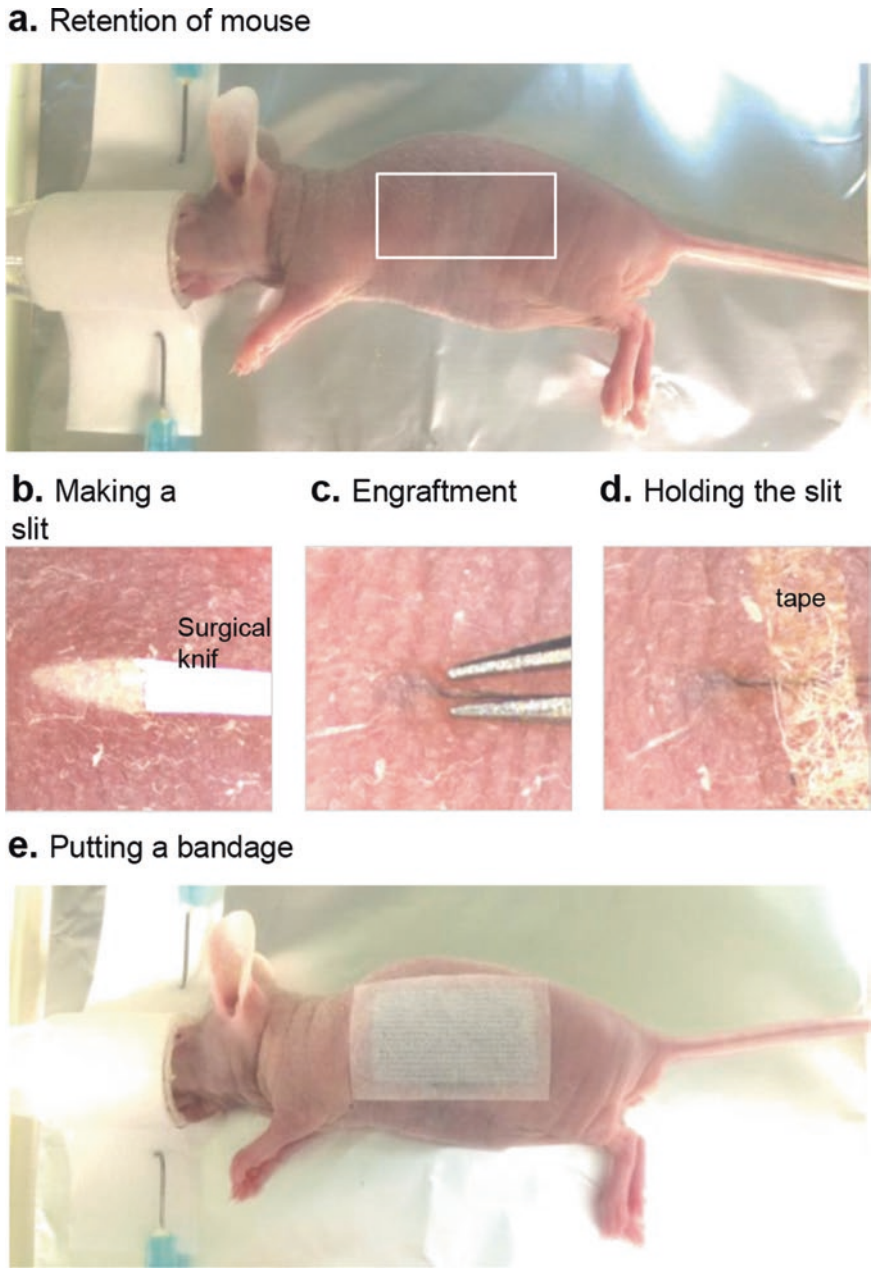

Fig. 4 Intracutaneous transplantation of the bioengineered hair follicle germ. (**a**) Retention of Balb/c nu/nu mice under the stereomicroscope. (**b**) Surgical formation of a slit as an engraftment site for the bioengineered hair follicle germ. Make a slit in the back skin of nude mice with a microsurgical knife. (**c**, **d**) Transplant the bioengineered hair follicle germ into the slit with forceps and hold the nylon thread with surgical tape. (**e**) Put a bandage on the transplantation site with surgical tape

5. Engraft a bioengineered hair follicle germ, which contains a nylon guide, and place in the epithelial portion and into the shallow stab wound (Fig. 4c, *see* **Note 23**).
6. Hold the nylon guide using surgical tape on the skin surface (Fig. 4d).
7. Completely dress the engraftment using surgical tape (Fig. 4e, *see* **Note 24**).
8. Approximately 14 or 25 days after transplantation, the bioengineered pelage or vibrissae will exhibit the correct tissue structures similar to the murine natural vibrissae follicle and erupt from the skin surface (Fig. 5a, b). The bioengineered hair follicle will reproduce the proper arrangement and connections with the cutaneous tissues of the recipient skin (Fig. 5c, *see* **Note 25**).

3.5 Regeneration of the Pigmented Bioengineered Hair Follicle

3.5.1 Preparation of Melanocyte Stem Cell Niche Cells (Proximal Hair Matrix, PHM)

1. Prepare the vibrissae following the methods described in Subheading 3.2.1.
2. Separate the hair bulb using 25-G needles under a stereomicroscope (Fig. 6a, b).
3. Wash the hair bulb region twice in PBS(−).
4. Peel off the collagen sheath from the hair bulb region under a stereomicroscope with 25-G needles and collect the epithelial tissues in 35-mm dish (Fig. 6c).
5. Remove the epithelial tissue from the upper Auber's line under a stereomicroscope with 25-G needles (Fig. 6d).
6. Detach the proximal hair matrix (PHM) and mesenchymal tissues with fine needles and collect the epithelial tissues in 35-mm dish (Fig. 6e).

Fig. 5 (continued) and vibrissae. (**a**) *Upper panels* indicate the area just after transplantation at day 0, the healing of the wound at day 3, and the eruption and growth of the hair shaft and growth at day 14 and 24 in the bioengineered pelage and at day 25 and 37 in vibrissae. (**b**) The *left* two panels indicate fluorescence microscopy and low-magnification H&E panels presenting the same bioengineered hair follicles. The boxed areas in the low-magnification H&E panels are shown at a higher magnification in the *right* panels. The *arrowhead* indicates a sebaceous gland. (**c**) The connections between the follicles of a natural and EGFP-labeled bioengineered pelage (*upper two lines*) and a natural and EGFP-labeled bioengineered vibrissae (*lower two lines*) to the arrector pili muscles and nerve fibers were analyzed by immunohistochemical staining using specific antibodies against calponin for smooth muscle (CNN, *red in the left three panels*), troponin for striated muscle (TNN, *red in the right three panels*), and neurofilament H (NF, *white*). The nuclei were stained using Hoechst 33258 (Nuc, *blue*). The boxed areas in the left panel in each data set are shown at a higher magnification in the right two panels. The *arrows* and *arrowheads* indicate the muscle and nerve fibers connected to the pelage follicles, respectively. The *broken lines* indicate the outermost limit of the hair follicle. Scale bars are 100 μm in low-magnification and 50 μm in high-magnification photographs. *ddp* deep dermal plexus, *dm* dermal muscle layer, *m* muscle. Scale bars are 1.0 mm in macroscopy and 100 μm in histological images

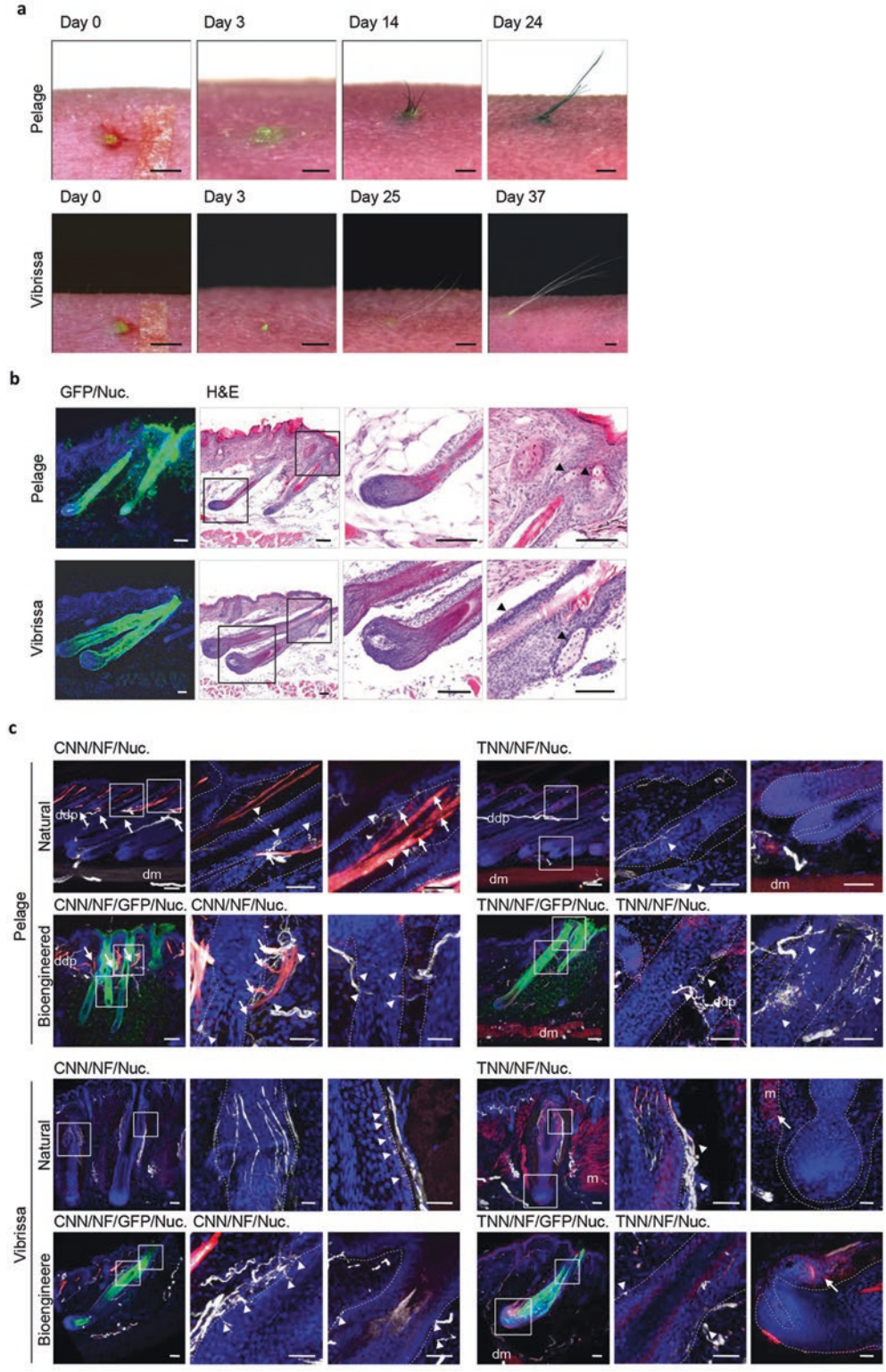

Fig. 5 Intracutaneous transplantation of the bioengineered hair follicle germ. (**a**, **b**) Macro-morphological and histological observations of the bioengineered hairs during the regeneration of the bioengineered pelage

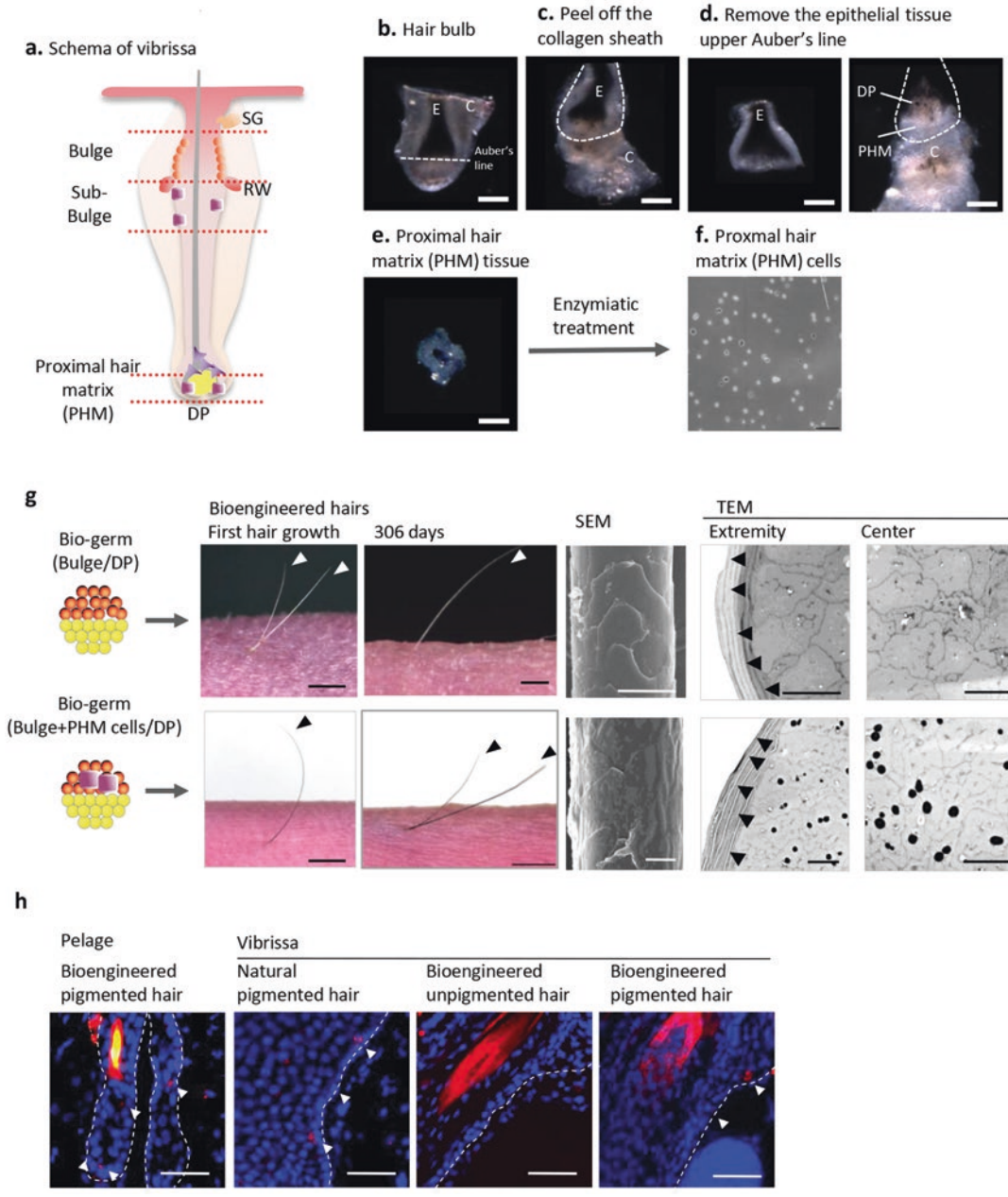

Fig. 6 Regeneration of the pigmented bioengineered hair follicle. (**a**) Schema of the vibrissae. (**b**, **c**) Cut off the hair bulb and peel off the collagen sheath. (**d**) Removal of the epithelial tissue above the Auber's line (*dotted line* area in (**b**)). (**e**) Detachment of the PHM from mesenchymal components. (**f**) PHM cells dissociated by enzymatic reaction. *E* epithelium, *C* collagen sheath, *DP* dermal papilla. (**g**) Analyses of hair pigmentation of the bioengineered vibrissae by various combinations with the bioengineered vibrissae follicle cell components. The bioengineered vibrissae follicle germ, which was reconstituted between the bulge region-derived epithelial stem cells and the primary cultured DP cells (*upper*), was combined with the proximal region of the hair matrix (*lower*). These bioengineered hair shafts were analyzed by macroscopic (*left*), SEM (*center*), and TEM (*right two panels*) observations. Scale bars are 1 mm for macroscopy, 20 μm for SEM, and 2 μm for TEM. The arrowheads in the TEM images indicate the cuticle layers. (**h**) In situ hybridization analysis of the sub-bulge region of natural and bioengineered hair follicles using a specific antisense probe for Dct mRNA. The nuclei were stained using Hoechst 33258 (Nuc, *blue*). The *arrowheads* indicate Dct mRNA expression in the cells. The *broken lines* indicate the outermost limit of the hair follicle. Scale bars, 50 μm

7. Wash the PHM tissues twice with PBS(−), and incubate the PHM tissues in 2 mL of 0.05% trypsin reagent for 1 h at 37 °C.
8. Stop the enzymatic reaction by adding 2 mL of DMEM10/HEPES.
9. Add 2 μL of DNase reagent, and dissociate the epithelial cells by gently pipetting five times with a 1000-μL micropipette.
10. Pass the cell suspension through a cell strainer, collect in 15-mL tubes, and centrifuge the epithelial tissues at 600 × *g* for 3 min at 4 °C.
11. Aspirate the supernatant to a residual volume of 50 μL, and add 1 mL of DMEM10/HEPES containing 0.5 μL of DNase reagent.
12. Pass the cell suspension through a cell strainer and collect in 15-mL tubes (Fig. 6f).

3.5.2 Reconstruction of the Hair Follicle Germ with the Addition of the PHM Cells

(For the preparation of bulge and cultured DP cells, *see* Subheadings 3.2.2and 3.2.3).

1. Prepare siliconized 35-mm petri dishes using a cotton swab (*see* **Note 11**).
2. Mix the bulge cells and PHM cells at the ratio of 50:1 in a siliconized 1.5-mL tube.
3. Centrifuge at 600 × *g* for 3 min at 4 °C, and aspirate the supernatant using a micropipette and a 1000-μL or 100-μL micropipette tip (*see* **Note 17**).
4. Centrifuge at 600 × *g* for 3 min at 4 °C, and remove the residual supernatant on the cell pellets using a micropipette and a gel-loading tip under a microscope (*see* **Note 18**).
5. Place 30 μL of collagen gel on a siliconized petri dish.
6. Aspirate a 0.2–0.3-μL volume of the DP cell pellet (approx. 3×10^3 cells) using a micropipette and a 0.1–10-μL micropipette tip under a microscope (*see* **Note 19**).
7. Apply the cell pellet slowly into the collagen drop, and make a spherical cell aggregate (Fig. 3a, b, *see* **Note 20**).
8. Similarly, apply a 0.2–0.3-μL volume of the epithelial cell pellet (approx. 1×10^4 cells) into the same collagen drop, and make contact with the DP cell aggregate (Fig. 3c–e, *see* **Note 21**).
9. Incubate the dish for 15 min at 37 °C and solidify the gel.
10. To form intraepithelial tissue connections between the host skin and the bioengineered hair follicle, insert a nylon thread guide (8–0 nylon surgical suture) into a bioengineered hair germ through the thrusting epithelial and dermal papilla cell portions (Fig. 3f).

11. Pick up the drop with forceps and place the drop onto the cell culture insert containing 1 mL of DMEM10 (Fig. 3g–i, *see* **Note 22**).
12. Intracutaneous transplantation should be performed as described in Subheading 3.4.

4 Notes

1. Surgical instruments should be washed and sterilized by autoclaving prior to each use in order to prevent contamination.
2. When the bioengineered hair germ is transplanted into recipient dorsal skin, 20-G ophthalmic V-lance (ALCON) is suitable for making a slit to prevent the excess damages.
3. For forming a cell pellet by centrifugation, round-bottom microtube is suitable.
4. A 0.1–10 μL pipette tip is suitable for making a cell aggregate with high-cell density in the collagen gel.
5. Cell culture insert/0.4 μm pore size membrane is suitable for organ culture.
6. The PIPETMAN® P2 micropipette manufactured by Gilson Inc. is suitable for reconstruction of cell aggregates in the collagen gel.
7. Cut off the dorsal skin from the forelimbs to hind limbs carefully to avoid injury.
8. Grasp the connective tissues, and remove them gently from the skin with one pair of forceps. The blood vessels are almost eliminated, and the transparency of the skin increases.
9. The tissue size is critical for efficient harvesting of dissociated skin cells. When the tissues are too big, the enzymatic reaction is not uniform.
10. Tissues and cells that have been enzymatically treated can easily aggregate owing to released DNA, making subsequent manipulations difficult. The addition of DNase digests DNA and can prevent cell aggregation.
11. 35-mm petri dishes and 1.5-mL tubes should be coated with silicone grease using a cotton swab. Remove excess silicone from the 1.5-mL tubes. Be careful not to apply too much silicone grease and not to apply it to the inside of the 1.5-mL tube cap. Round-bottom microtubes should be used because square-bottom microtubes are unsuitable for forming a cell pellet by centrifugation.
12. Do not perform each process for more than 20 s. Excessive exposure to 70% ethanol leads to the denaturation of the cell.

13. Pick up the upper end of the hair follicle with forceps, and pull it up from the inside of the whisker pad. Never grasp the bulge region or the hair bulb region.
14. Carefully follow the time and the temperature of enzyme reactions because long enzyme reactions can injure the hair follicle. Non-whole tissues are dissociated by gently pipetting.
15. Hair follicles continue regenerating in the adult. The stage of the hair regeneration cycle was defined in previous reports [1]. We recommend using anagen stages I–IV at the point of growth of the dermal papilla.
16. Outgrowth is confirmed by fixing the DP hanging on the dish.
17. In this step, it is not necessary to remove all of the supernatant.
18. Remove as much of the residual supernatant as possible to create a high-density cell pellet. If residual supernatant remains, the cell aggregate will not be made into a collagen gel.
19. A 0.1–10-μL micropipette tip is suitable for making a highly concentrated cell aggregate in the collagen gel.
20. Insert the micropipette tip into the collagen gel using a 2-μL micropipette. The cell aggregate is extruded slowly, and the micropipette tip must be precisely operated so that the cell aggregation becomes spherical. Cell insertion should be stopped precisely when all cells have been extruded from the micropipette tip in order to prevent air bubbles from entering the gel.
21. The cell manipulation should be performed quickly because the collagen gel solidifies with changes in temperature and the passage of time.
22. Remove the collagen gel from the bottom of the siliconized dish. The side of the collagen gel should be picked up with tweezers and carefully placed onto the cell culture insert.
23. The transplant depth is very important. When the bioengineered hair germ is transplanted too deep, the hair shaft does not erupt from the skin.
24. The stab wound heals 1 week later; take the bandage off after 1 week.
25. The density and area of the bioengineered hairs can also be regulated by repeatedly transplanting bioengineered hair follicle germs.

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Chapter 10

Functional Salivary Gland Regeneration

Miho Ogawa and Takashi Tsuji

Abstract

The salivary gland plays important roles in maintaining the health and homeostasis of the oral cavity by regulating functions, such as chewing, digestion, cleaning, and swallowing. Salivary gland dysfunction causes dry mouth syndrome, which includes many oral problems, such as dental caries, bacterial infection, mastication dysfunction, and swallowing dysfunction. Therefore, salivary gland regeneration is expected, and we attempted to establish a method by manual approaches. Previously, a novel three-dimensional cell manipulation technique, the "organ germ method," to create a bioengineered organ germ enabled the regeneration of structurally correct and fully functional bioengineered organs, including the teeth, hair follicles, and secretory glands, in vivo. Here, we describe the protocol for salivary gland germ regeneration using this method and the functional analysis of the regenerated salivary gland.

Key words Salivary gland, Bioengineered salivary gland germ, Organ germ method, Saliva secretion, Transplantation

1 Introduction

The salivary gland plays essential roles in maintaining oral health in cooperation with the teeth and other organs. The salivary glands arise from their germ that is induced by reciprocal oral epithelial and mesenchymal interactions during embryogenesis [1, 2]. The epithelial cells of the salivary glands differentiate into acinar cells, duct cells, and myoepithelial cells and form the secretory gland-specific structure [3, 4]. The salivary glands consist of three major salivary glands, the submandibular glands, sublingual and parotid glands, and minor glands [2]. The sublingual glands secrete mucous saliva, which is enriched with glycoproteins, such as mucin proteins. The submandibular and parotid glands secrete serous saliva, which mainly contains the amylase protein [5].

Many studies attempted to analyze the development and morphogenesis of the salivary gland by assessing the epithelial and mesenchymal interactions and epithelial morphogenesis and elucidating the roles and molecular mechanisms of cytokines and extra-

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cellular matrices [6, 7]. These analyses were performed in vitro using three-dimensional organ cultures of salivary gland germ and epithelial tissue. The epithelial cell aggregate culture showed the self-organization and branching morphogenesis that occur during salivary gland regeneration [8]. In addition, salivary gland epithelial and mesenchymal cell-mixed aggregation cultures promoted salivary gland development and increased branching morphogenesis [8]. The adult salivary gland stem cells also generated the three-dimensional epithelial tissue structure of the salivary gland, including duct and acinar cells, in collagen gel cultures [9]. These results indicate that the interactions between epithelial and mesenchymal cells are important for salivary gland germ development and morphogenesis. However, it was expected that a method for fully functional regeneration of the salivary gland in vivo would be developed.

Previously, we have developed a three-dimensional cell manipulation method, "the organ germ methods," which can reproduce the developmental process of organogenesis during embryogenesis [10]. Using this method, the bioengineered organ germs can regenerate ectodermal organs, such as the teeth, hair follicles, and exocrine gland germs [11–14]. We also showed that the bioengineered salivary gland germs could develop and form mature salivary glands, including the submandibular gland, sublingual gland, and parotid gland [15]. The bioengineered salivary glands exhibit complete functions, such as the secretion of saliva in response to taste stimulation, cleansing functions, and swallowing functions. Therefore, our three-dimensional organ technique will be applicable to the analyses of salivary gland development, morphogenesis, and functions. This technique can be useful for fundamental studies of the onset of Sjögren's syndrome and salivary gland cancer.

In this chapter, we provide a detailed description of the technical protocol used to regenerate the three-dimensional bioengineered salivary gland. We also describe the methods used for the orthotopic transplantation and analyses utilized for the in vivo study of salivary gland development.

2 Materials

All solutions used in this experiment should be prepared with ultrapure water (prepared by purifying deionized water to achieve a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. All surgical instruments should be washed and sterilized in an autoclave prior to use to prevent contamination. It is recommended that the enzyme and serum reagents should be evaluated for enzyme reactivity and primary cell culture efficiencies, respectively, prior to use in the experiments.

1. Animals: An inbred mouse strain (i.e., C57BL/6, Balb/c 3T3, etc.) should be used in these experiments. Mouse embryonic age is determined based on the day of appearance of a vaginal plug in a pregnant mouse (embryonic day 0 (E0)) (*see* **Note 1**).
2. Ca^{2+}/Mg^{2+}-free, phosphate-buffered saline (PBS(−)): 137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 8.0 mM anhydrous disodium hydrogen orthophosphate (Na_2HPO_4), and 1.5 mM potassium phosphate monobasic (KH_2PO_4). Store at 4 °C (*see* **Note 2**).
3. Medium: The salivary glands are isolated, and single cells are dissociated in a basal cell culture medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 10 mM 1-4-(2-hydroxyethyl)-piperazine ethanesulfonic acid (HEPES). For organ culture, the abovementioned supplements, with the exception of HEPES, are added to this medium (*see* **Note 3**).
4. Dispase solution: The concentration of dispase is adjusted to 50 U/mL and stored at −20 °C until use (*see* **Note 4**).
5. Collagenase solution: The concentration of collagenase I is adjusted to 27,300 U/mg using distilled water, and it is stored at −20 °C until use (*see* **Note 5**).
6. Reagent A: 3.66 μL/mL collagenase solution adjusted by PBS(−).
7. Reagent B: 0.25% trypsin adjusted by PBS(−).
8. Reagent C: 3.66 μL/mL collagenase and 0.25% trypsin adjusted by PBS(−).
9. 70 U/mL deoxyribonuclease I (DNase I) from bovine pancreas solution (*see* **Note 6**).
10. Collagen gel: Component in 100 μL tenfold concentrated α-minimum essential medium (αMEM) and 100 μL mixed buffer (0.08 N sodium hydroxide and 200 mM HEPES) in 800 μL Cellmatrix Type I-A (*see* **Note 7**).
11. Surgical tools used to dissect the embryos from the uterus: Large surgical scissors and forceps to cut the abdominal skin and muscle and small surgical scissors and forceps to dissect the uterus (*see* **Note 8**).
12. Surgical tools used for the resection of the salivary glands and transplantation (*see* **Note 9**).
13. Dissecting microscope (*see* **Note 10**).
14. Sterile disposable 1-mL syringes and 25-G needles (5/8; 0.50 × 16 mm) for salivary gland germ extraction (*see* **Note 11**).
15. Sterile disposable 1.5-mL microtubes (*see* **Note 12**).

16. Sterile disposable gel loader tips and pipette tips for reconstituting the bioengineered salivary gland germ (*see* **Note 13**).
17. Culture at the medium-gas interface (cell culture insert/0.4-μm pore-size membrane; *see* **Note 14**).
18. Sterile micropipette (*see* **Note 15**).
19. 9-0 polyglycolic acid (PGA) monofilament (9-0 PGA absorbable surgical suture).

3 Methods

3.1 Extraction of Mouse Embryo Salivary Gland Germ

The dissection and manipulation of embryos, salivary gland germs, and salivary gland tissues should be performed in medium to prevent drying.

1. Euthanize the pregnant mice by cervical dislocation and disinfect the abdominal skin with 70% ethanol.
2. Cut the abdomen skin along the midline with small scissors. Resect the uterus and wash it with PBS(−) (*see* **Note 16**).
3. Dissect the embryos from the uterus in a 100-mm culture dish containing cold (4 °C) PBS(−). Amputate the fetal head from the body and separate the lower and upper jaws. Immediately collect both jaws in a 35-mm dish containing cold culture medium (*see* **Note 17**).

3.2 Extraction of the Submandibular and Sublingual Glands from the Lower Jaw

1. Place the lower jaw in 200 μL culture medium in a 100-mm dish and remove the back of the throat.
2. Resect the tongue from the lower jaw, but do not injure the salivary glands (Fig. 1A-a, b, and c; *see* **Note 18**).
3. Cut between the resected Meckel's cartilage and salivary glands. Resect and discard all extra tissue from around the salivary glands (Fig. 1A-d, e, and f; *see* **Note 19**).
4. Isolate the submandibular and sublingual gland germs (Fig. 2).
5. Store the isolated salivary gland germs in cold culture medium on ice (*see* **Notes 20** and **21**).

3.3 Extraction of the Parotid Gland from the Upper Jaw

1. Cut the upper jaw with the backline of both eyes and cut it in half along the midline (Fig. 1B-a, b, and c).
2. Resect the jaw along the masseter muscle (Fig. 1B-d, e, and f).
3. Resect all extra tissue from around the parotid gland germ (Fig. 1B-g, h, and i).
4. Store the isolated parotid gland germs in cold culture medium on ice (*see* **Notes 20** and **21**).

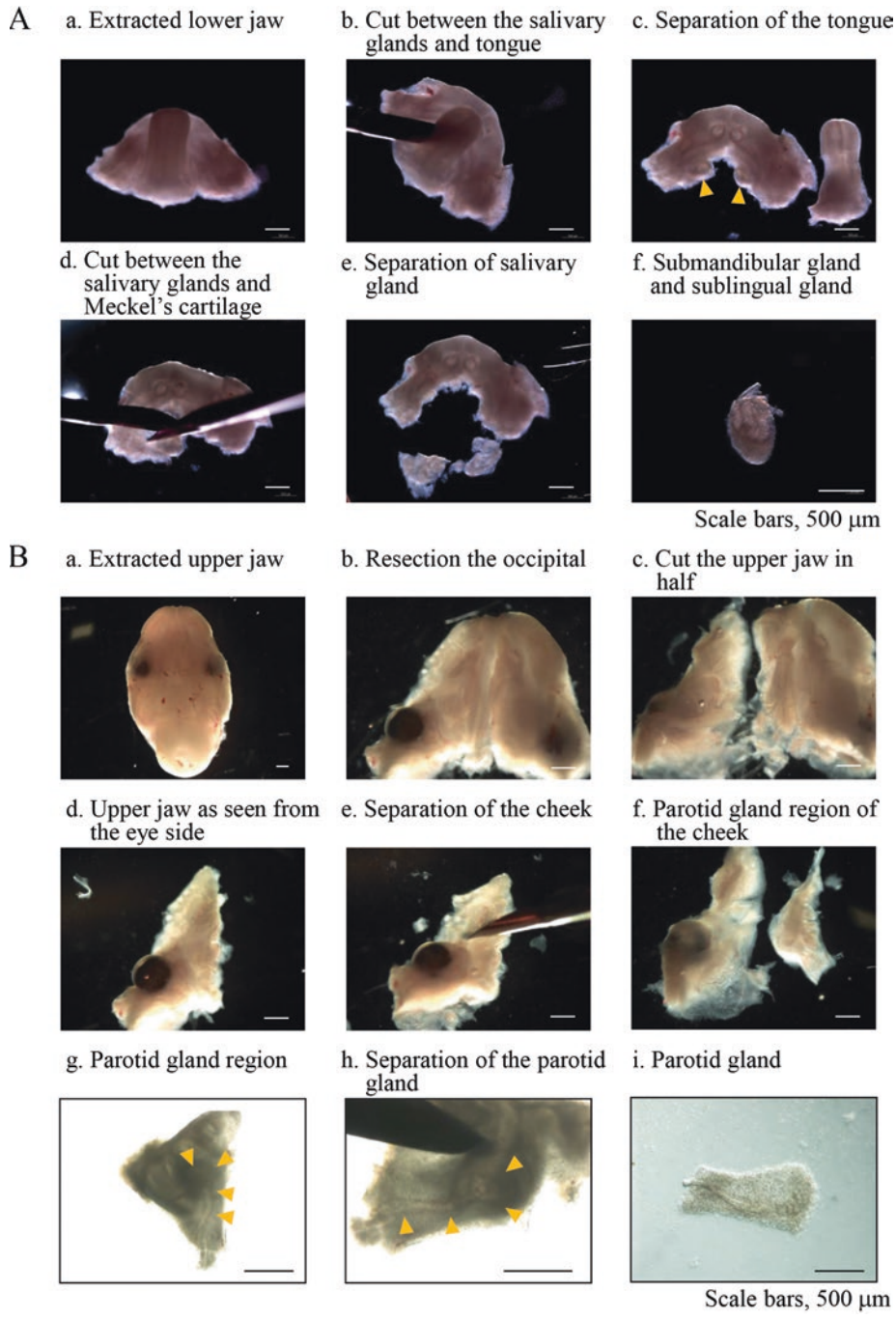

Fig. 1 Dissection of the E13.5 or 14.5 salivary gland germ. (**A**) Extraction of the lower jaw from an E13.5 or E14.5 mouse embryo (*a*). Removal of the tongue from the *lower jaw* (*b* and *c*). Separation of the salivary gland from the *lower jaw* (*d*) and removal of excess tissue (*e*). Extended image of the submandibular and sublingual gland germ (*f*). The quality of the isolated salivary gland germ is crucial for the successful development of bioengineered salivary gland germ. *Arrow*: salivary gland germ region. (**B**) Extraction of the *upper jaw* from an E14.5 mouse embryo (*a*). The *backline* of both eyes was removed (*b*) and the jaw was cut in half along the *midline* (*c*). Resection of the jaw along the masseter muscle (*d*, *e*, and *f*). Extended image of the parotid gland region (*g*). Removal of excess tissue (*h*) and isolation of the parotid gland (*i*)

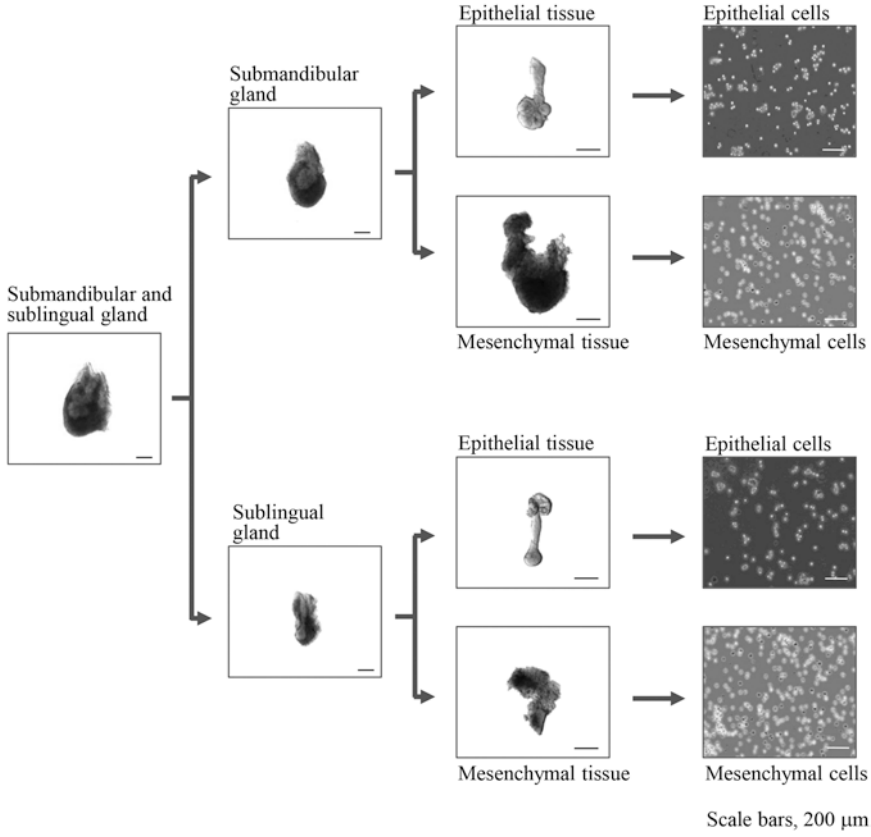

Fig. 2 Preparation of single epithelial and mesenchymal cells. Dissected (*left*) and separated submandibular gland germ (*upper, second photograph from the left*) and sublingual gland germ (*lower, second photograph from the left*). Separation of the epithelial tissue (*upper, third photograph from the left*) and mesenchymal tissue (*lower, third photograph from the left*) from each salivary gland germ. Preparation of single epithelial cells (*upper, fourth photograph from the left*) and single mesenchymal cells (*lower, fourth photograph from the left*) after the enzymatic treatments

3.4 Separation of the Salivary Gland Germ Epithelial and Mesenchymal Tissues

1. Wash the extracted salivary gland germs twice with PBS(−). Add 1 mL dispase solution, and conduct the enzyme reaction at room temperature for 1.5 min (Fig. 2; *see* **Note 22**).
2. Add cold culture medium to stop the enzyme reaction. Wash the salivary gland germs twice with culture medium (*see* **Note 23**).
3. Add 2 mL culture medium and 1 μL DNase and incubate the mixture at room temperature for a few seconds (Fig. 2; *see* **Note 24**).
4. Separate the salivary gland germ epithelial and mesenchymal tissues under a microscope using 25-G needles (Fig. 2; *see* **Note 25**).
5. Store each tissue in cold culture medium on ice.

3.5 Enzymatic Separation of Single Cells from the Salivary Gland Germ Epithelial Tissue

1. Collect the epithelial tissues for each type of salivary gland in a 15-mL tube and centrifuge the tissues at 590 × *g* for 3 min at 4 °C. Discard the supernatant and wash the cell pellet twice with PBS(−).
2. Aspirate the liquid to remove the PBS(−) and add 2-mL enzyme reactive Reagent A. Incubate the epithelial tissues for 10 min in a 37 °C water bath. Repeat this procedure twice (*see* **Note 26**).
3. Centrifuge the epithelial tissues at 590 × *g* for 3 min at 4 °C and discard all of Regent A. Add 2-mL enzyme reactive Reagent B and incubate the epithelial tissues in a 37 °C water bath for 5 min.
4. Stop the enzyme reaction by adding 6 mL culture medium. Centrifuge the sample at 590 × *g* for 5 min at 4 °C.
5. Aspirate the supernatant to a residual volume of 80 μL and resuspend the cell pellet by tapping (*see* **Note 27**).
6. Immediately add 1 mL culture medium and centrifuge at 590 × *g* for 3 min at 4 °C.
7. Aspirate the supernatant to a residual volume of 200 μL and add 1 μL of DNase solution to the residual volume. Create a single cell suspension by gently pipetting ten times with a micropipette and a P200 tip (*see* **Note 28**), and then shift the suspension through a cell strainer (Fig. 2).

3.6 Enzymatic Separation of Single Cells from the Salivary Gland Germ Mesenchymal Tissue

1. Collect the mesenchymal tissues for each type of salivary gland in a 15-mL tube and centrifuge at 590 × *g* for 3 min at 4 °C. Discard the supernatant and wash the cell pellet twice with PBS(−).
2. Aspirate the liquid to remove the PBS(−) and add 2-mL enzyme reactive Reagent C. Incubate the mesenchymal tissues in a 37 °C water bath for 10 min.
3. Stop the enzyme reaction by adding 6 mL culture medium and centrifuge the sample at 590 × *g* for 5 min at 4 °C.
4. Aspirate the supernatant to a residual volume of 80 μL and resuspend the cell pellet by tapping (*see* **Note 27**).
5. Immediately add 1 mL culture medium and centrifuge the sample at 590 × *g* for 3 min at 4 °C.
6. Aspirate the supernatant to a residual volume of 200 μL and add 1 μL of DNase solution to the residual volume. Create a single cell suspension by gently pipetting ten times with a micropipette and a P200 tip (*see* **Note 28**), and then shift the suspension through a cell strainer (Fig. 2).

3.7 Reconstitution of the Bioengineered Salivary Gland Germ

1. Prepare siliconized 35-mm Petri dishes and 1.5-mL tubes coated with silicon grease (*see* **Note 29**).

2. Transfer the epithelial or mesenchymal single cell suspensions isolated from the salivary gland germ into separate siliconized 1.5-mL tubes.
3. Centrifuge the samples at 600 × *g* rpm for 3 min at 4 °C and discard the supernatant using a micropipette and a P1000 or P200 tip.
4. Centrifuge the samples at 600 × *g* for 3 min at 4 °C and discard the residual supernatant on top of the cell pellets using a micropipette and a gel-loading tip under a microscope (Fig. 3a–c; *see* **Note 30**).
5. Prepare a droplet of 30-μL collagen gel on a siliconized Petri dish (*see* **Note 31**).
6. Aspirate a 0.3–0.4 μL volume of the mesenchymal cell pellet using a micropipette and a 0.1–10-μL pipette tip under a microscope (*see* **Note 32**). Slowly apply the cell pellet to the collagen drop to make a spherical cell aggregate (Fig. 3d–f; *see* **Note 33**).
7. Similarly, apply a 0.2–0.3 μL volume of the epithelial cell pellet to the same collagen drop such that it contacts the mesenchymal cell aggregate (Fig. 3g, h; *see* **Notes 33** and **34**).
8. String the 9-0 nylon thread from the epithelial to the mesenchymal cell aggregate to transplant the regenerated salivary gland germ (Fig. 3i) (*see* **Note 35**).

3.8 In Vitro Organ Culture of the Bioengineered Salivary Gland Germ

1. Incubate the Petri dish containing the collagen gel drop at 37 °C for 15 min to allow the collagen gel to coagulate (Fig. 4A; *see* **Note 36**). Set the cell culture insert into a 6-well plate filled with culture medium (1 mL/well).
2. Pick up the collagen gel drop with tweezers and transfer the drop onto the cell culture insert (Fig. 4A; *see* **Note 37**).
3. Exchange the culture medium every other day (*see* **Note 38**).
4. Three days after organ culture, a salivary gland structure is observed in the collagen gel. Five days after organ culture, a vacuole is formed by the liquid discharged from the bioengineered salivary gland (Fig. 4B).

3.9 Transplantation of a Bioengineered Salivary Gland Germ

1. Anesthetize the mouse and shave the hair around the cheek with a razor (Fig. 5C-a; *see* **Notes 39–41**).
2. Place the mouse on its side and immobilize the mouth and shoulder so that the face cannot move.
3. Cut the skin toward the throat from the bottom of the ear using scissors and forceps (Fig. 5C-b).
4. Expand the skin using clips to secure the operation field (Fig. 5C-b).

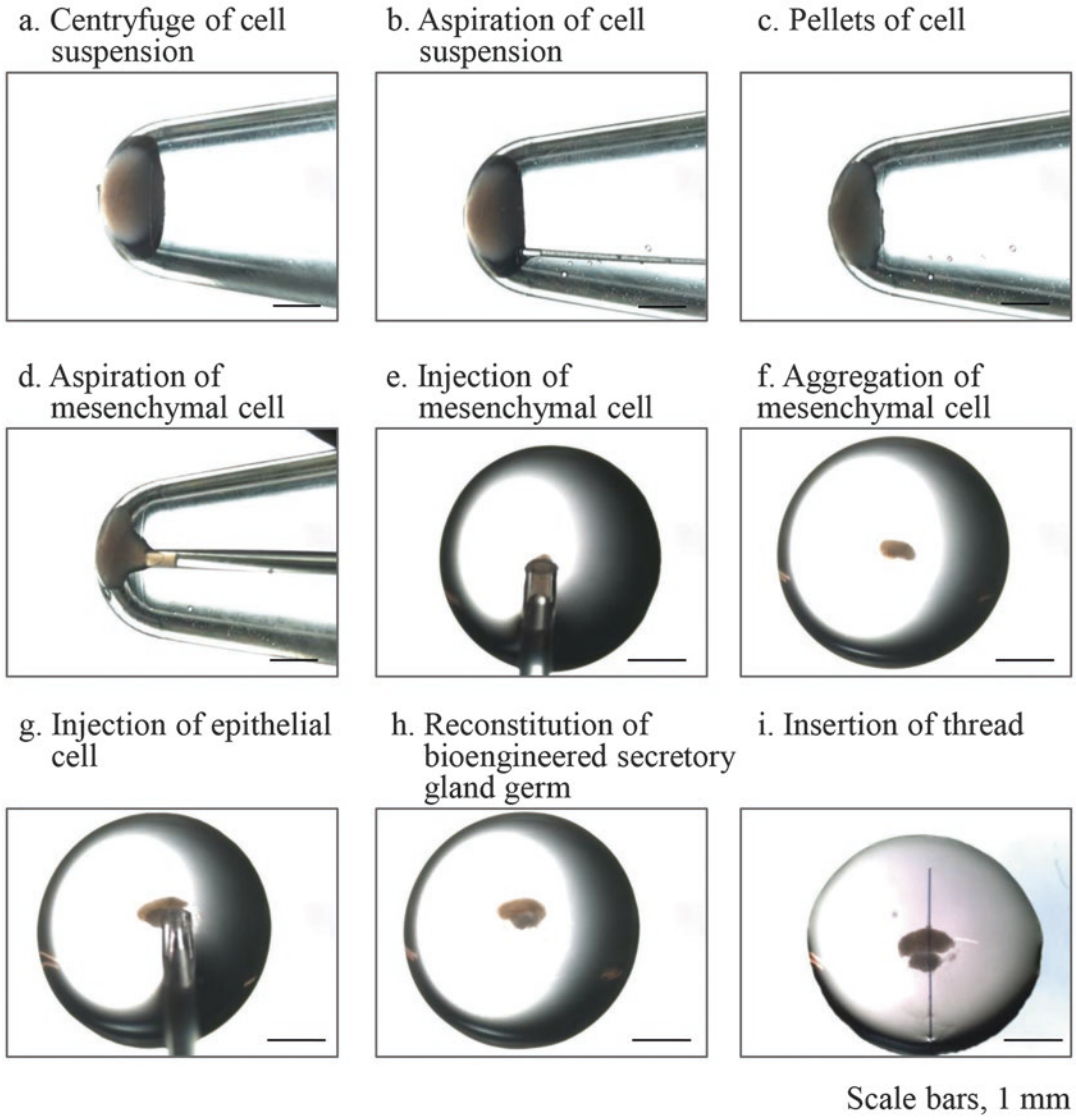

Fig. 3 Reconstitution of bioengineered salivary gland germ. Centrifuge and remove the residual supernatant from around the cell pellets (**a**–**c**). Aspirate (**d**) and inject the total volume of mesenchymal cells into the center of the collagen drop (**e** and **f**). Subsequently, aspirate and inject the epithelial cells into the same drop adjacent to the mesenchymal cell aggregate (**g** and **h**). The bioengineered salivary gland germ containing a PGA monofilament guide for transplantation (**i**)

5. Peel the parotid glands from the surrounding tissue without damaging the blood vessels. Place the wet paper on the parotid gland and duct to avoid drying (Fig. 5C-c; *see* **Note 42**).
6. Ligate the blood vessels and the ducts of both the submandibular and sublingual glands and cut them (Fig. 5C-d; *see* **Note 43**).
7. Cut the parotid gland duct approximately 2/3 of the distance from the front of the masseter muscle (Fig. 5C-e; *see* **Note 44**).

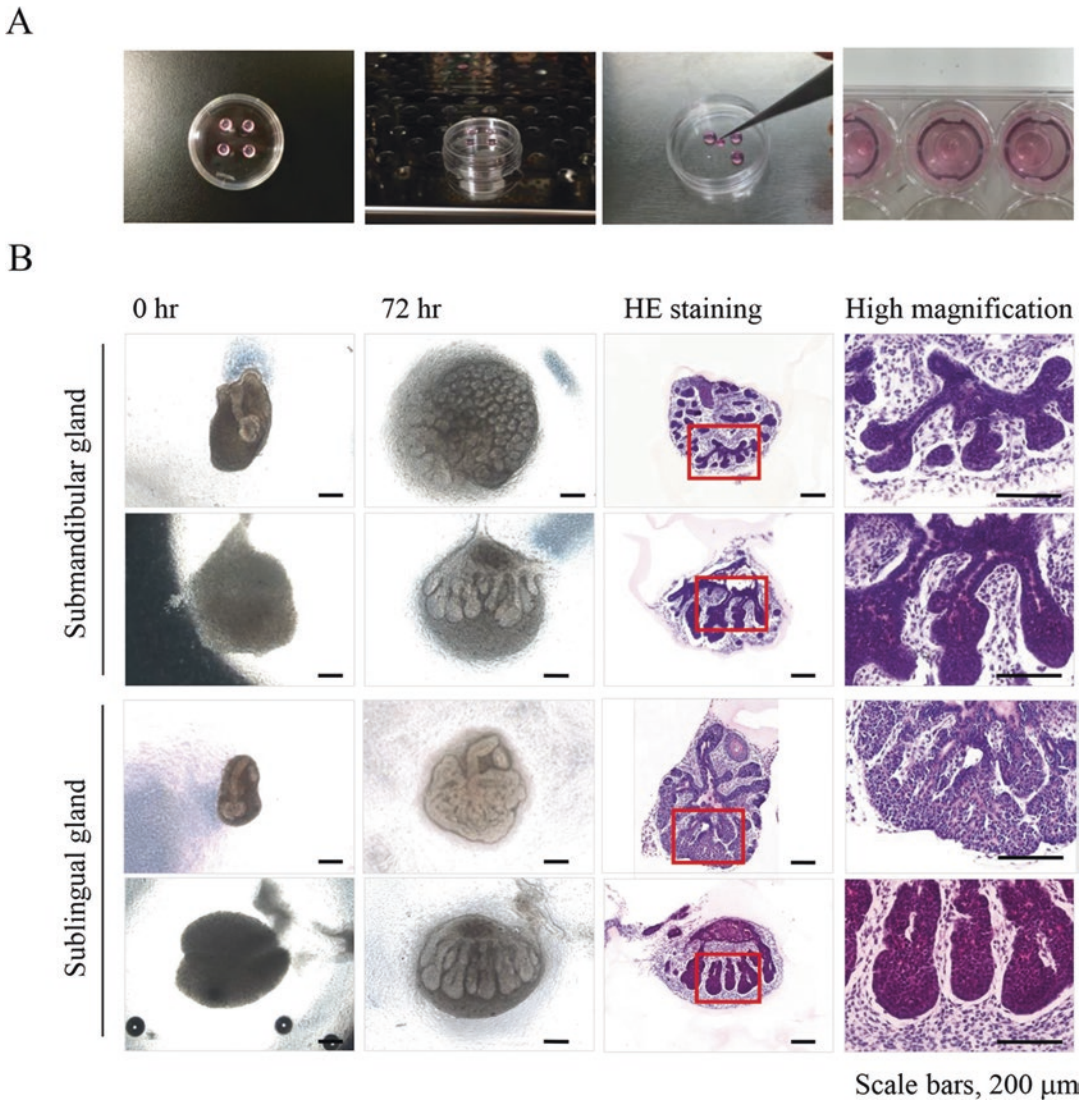

Fig. 4 In vitro organ culture of the bioengineered salivary gland germ. (**A**) Invert the dish (*left*) and coagulate the collagen gel drop at 37 °C (*second photograph from the left*). Pick up the collagen drop (*third photograph from the left*) and transfer it onto the cell culture insert (*fourth photograph from the left*). (**B**) Typical images of a bioengineered submandibular gland germ (*upper columns*) and submandibular gland germ (*lower columns*) after 0 and 72 h of organ culture. Phase contrast image (*first and second photographs from the left*) and hematoxylin and eosin staining (*third and fourth photographs from the left*)

8. After 2–3 days of organ culture, pick up the bioengineered salivary gland germ with the collagen gel and 9-0 nylon from the insert and place it on the masseter muscle (Fig. 5B; *see* **Note 45**).
9. Grasp the thread with tweezers and insert it into the parotid gland duct cavity.
10. Pull the gel and place the bioengineered salivary gland germ as close to the cut surface of the parotid gland duct as possible (Fig. 5A, C-f and g).

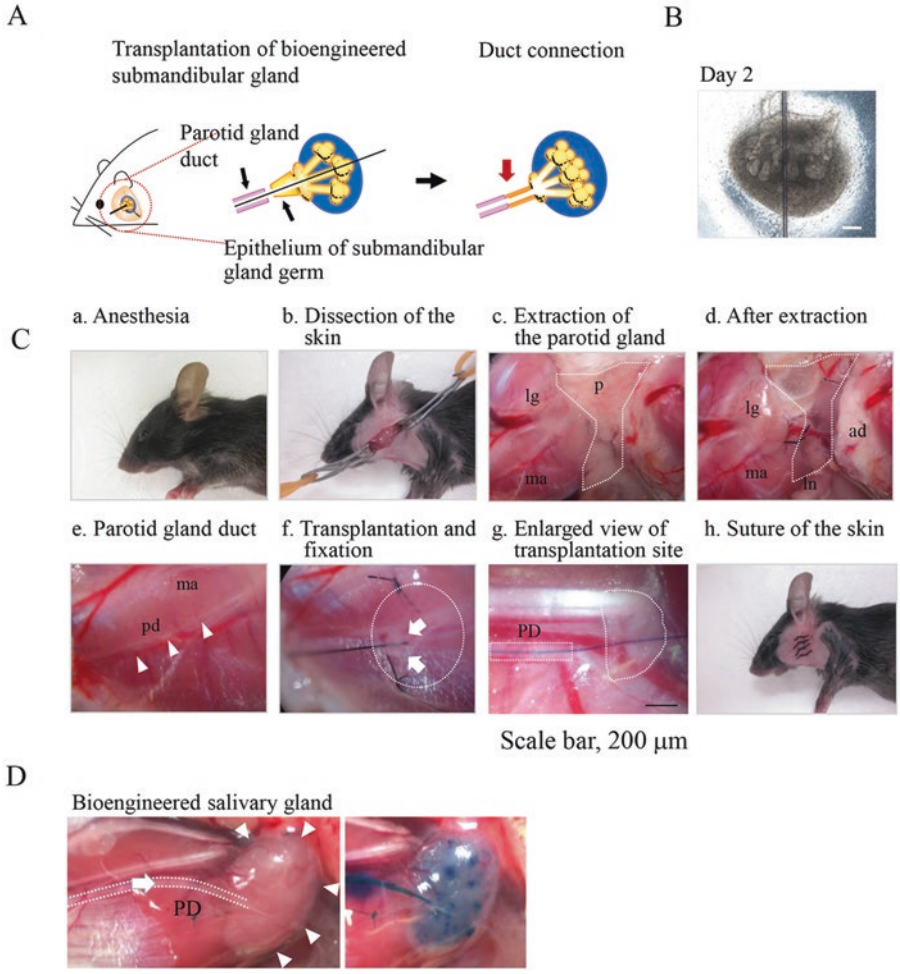

Fig. 5 Transplantation of the bioengineered salivary gland germ. (**A**) Schematic representation of the transplantation procedure. (**B**) Bioengineered salivary gland germ containing the 9-0 nylon thread after 2 days of culture. (**C**) Anesthetization (*a*) and dissection of the skin of 7-week-old female C57BL/6 mice (*b*). Extraction of the parotid, submandibular, and sublingual glands (*c* and *d*). Extended image of the parotid duct (*e*) and the transplantation of the bioengineered salivary gland germ (*f* and *g*). Suturing the skin (*h*). Lacrimal gland (lg), parotid gland (p), muscle (ma), lymph node (ln), adipose tissue (ad), and parotid gland duct (PD). (**D**) Photographs of a bioengineered salivary gland 30 days after orthotopic transplantation (*left*) and duct connection analysis after injecting Evans Blue into the parotid duct from the oral side. Parotid gland duct (PD). *Arrowhead*: bioengineered salivary gland. *Arrow*: injection site

11. Suture the gel into the masseter muscle with 8-0 nylon.
12. Suture the skin and transplant it into the other side in the same manner (Fig. 5C-h).
13. Thirty days after transplantation, the bioengineered salivary gland duct will connect with the host parotid gland (Fig. 5D). The structure of the bioengineered salivary gland is equivalent to its natural form (Fig. 6A; *see* **Note 46**).

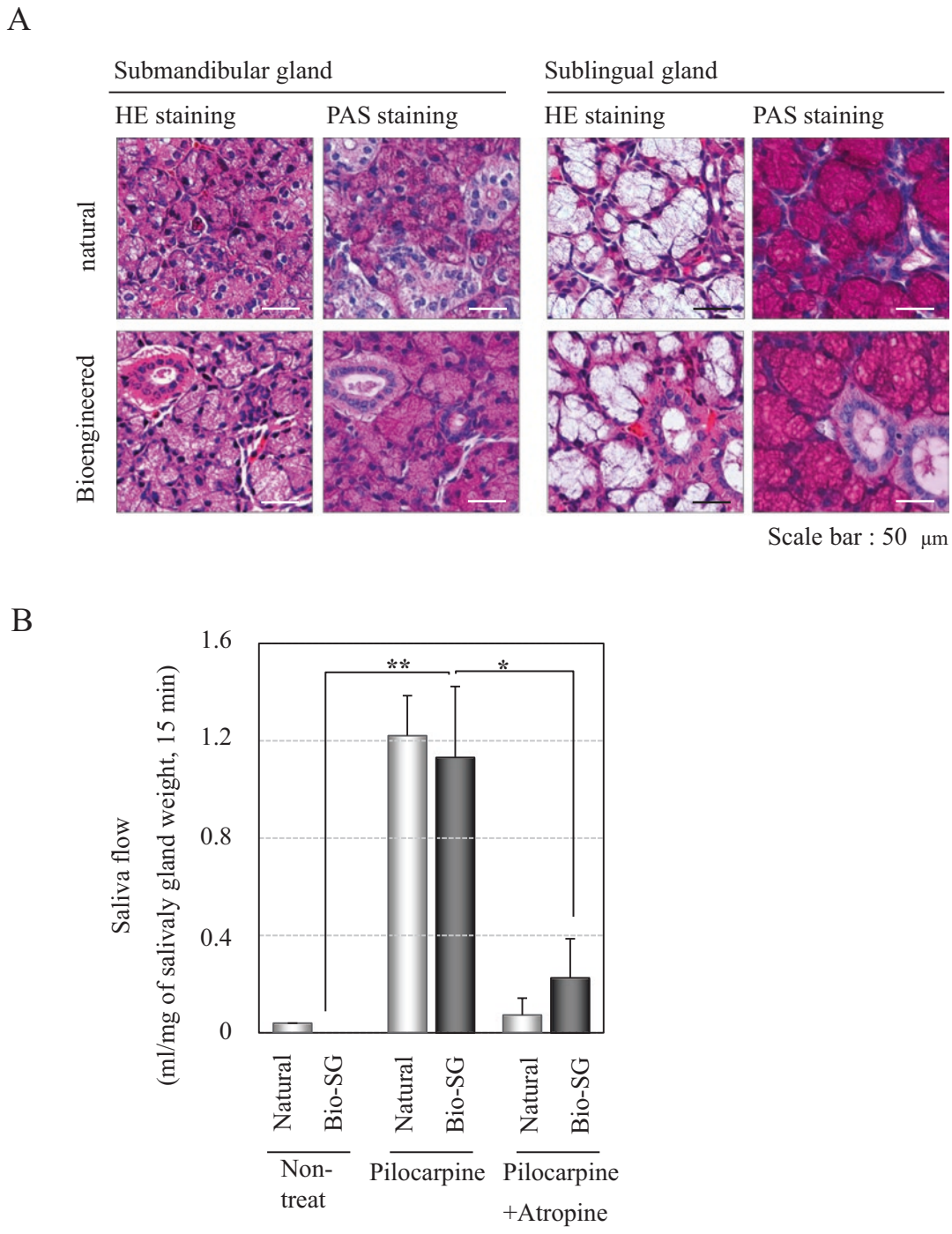

Fig. 6 Functional analysis of the bioengineered salivary gland. (**a**) Hematoxylin and eosin (H&E) staining (*first and third photographs from the left*) and periodic acid and Schiff (Pas) staining (*second and fourth photographs from the left*) of the submandibular gland (*left columns*) and the sublingual gland (*right columns*), including the natural (*upper*) and bioengineered (*lower*) salivary glands. (**b**) Assessment of the amount of saliva secretion in normal mice (*light bars*) and bioengineered submandibular gland-engrafted mice (*dark bars*) before and after the administration of pilocarpine without or with atropine. *$p < 0.05$, **$p < 0.001$ by Student's *t*-test

3.10 Measurement of the Secreted Saliva

1. Cut the filter paper into small pieces and measure the weight of each piece.
2. Measure the body weight and anesthetize the mouse.
3. After 4 min of anesthesia, inject of 300 μg pilocarpine/kg body weight into the intraperitoneal cavity.
4. Remove the water from the oral cavity using filter paper.
5. Six minutes after the injection, collect the saliva from the oral cavity using a small piece of filter paper at 1-min intervals for 25 min and immediately measure the weight of each piece of filter paper. The total amount of saliva can be calculated by determining the difference of the weight of the paper (Fig. 6B).

4 Notes

1. We recommend using the E13.5 embryos for submandibular and sublingual gland regeneration and E14.5 embryos for sublingual and parotid gland regeneration.
2. PBS(−) is used to wash the tissues and cells. Therefore, it should be an isotonic solution that does not cause cell injury.
3. Choose the basal medium after performing the examination experiment (e.g., Dulbecco's Modified Eagle's Medium). HEPES is added to maintain a constant pH, but HEPES is not added into the medium for organ culture to avoid any cytotoxicity during the extended culture period.
4. Dispase dissolves the basal membrane between the epithelial and mesenchymal tissues of the salivary gland germ. Even when using the same reagents described in this protocol, we recommend that the temperatures and reaction times should be evaluated because enzymatic activity is readily decreased at 4 °C.
5. Collagenase is an enzyme used to digest collagen. Intercellular collagen molecules are dissolved by collagenase, and the tissues are separated into single cells. Even when using the same reagents described in this protocol, we recommend that the temperatures and reaction times are evaluated because enzymatic activity is readily decreased at 4 °C.
6. Cells can be partially damaged by the enzymatic treatment and release their DNA. The DNA released by the enzymatic treatment can cause cells to aggregate, making the subsequent manipulations difficult. DNase can prevent this cell aggregation by digesting the DNA.
7. The collagen gel can easily be generated using Cellmatrix Type I-A manufactured by Nitta Gelatin Inc.

8. Surgical instruments should be washed and sterilized by autoclaving prior to each use to prevent contamination.
9. We recommend the use of minimum-sized surgical tools, such as scissors and tweezers, which can be more easily manipulated.
10. We recommend the use of a dissecting microscope capable of 6.5 to 50-fold magnification with a transmitted beam applied as the light source.
11. A 25-G needle is suitable for most of the manipulations performed during salivary gland germ extraction.
12. Round bottom microtubes should be used because square bottom microtubes are unsuitable for forming a cell pellet by centrifugation.
13. A 0.1–10-μL pipette tip is suitable to generate a highly concentrated cell aggregate in a collagen gel.
14. A membrane with a pore size that is sufficient for liquid components to pass through the membrane should be used. We use a cell culture insert/0.4-μm pore-size membrane.
15. The PIPETMAN® P2 micropipette manufactured by Gilson Inc. is recommended for facile creation of cell aggregates in the collagen gel.
16. Do not use the same tweezers and scissors to cut the skin and muscle to avoid the risk of bacterial contamination.
17. Mouse embryos can be easily isolated by surgically removing the uterine and amniotic membranes.
18. Dissections and manipulations of the salivary gland germ should be performed using a pair of 25-G needles. One needle with the cutting surface turned toward the salivary gland germ is fixed on the sample, and the other needle cuts the tissue by sliding along the cutting surface of the first needle. Care should be taken not to press too firmly on the tissue with the first needle, which can result in a tear in the tissue.
19. The submandibular and sublingual gland germ is adjacent to Meckel's cartilage. Careful needle manipulation should be used when separating both salivary gland germs from Meckel's cartilage.
20. Correct dissections and salivary gland germ manipulations can influence the frequency of successful development of a bioengineered salivary gland germ.
21. Transfer the salivary gland germ together with the medium. Do not directly transfer a salivary gland germ to avoid injury to the salivary gland germ.
22. Carefully follow the time and the temperature of enzyme reactions because long enzyme reactions can injure the salivary gland germ.

23. Quick and precise washing is required for the dispase solution.
24. Tissues and cells that have been treated with enzymes can easily aggregate due to the released DNA, making the subsequent manipulations difficult. The addition of DNase digests the DNA and can prevent cell aggregation.
25. Needle manipulation should be performed carefully to avoid injuring the epithelial and mesenchymal tissues.
26. Tissues can be mixed to allow the enzymatic regents to react equally with several tissues. Care should be taken to prevent the tissues from sticking to the tube wall.
27. When cell aggregates are not dispersed by tapping, the cell pellet can be manipulated into a single cell suspension by gently pipetting up and down.
28. Micropipette manipulation should be performed gently at a constant speed.
29. Be careful to apply a sufficient, but not an excessive, amount of silicone grease, and do not apply it to the inside of the lid of the 1.5-mL tube.
30. Remove as much of the residual supernatant as possible to create high-density cell pellets. If residual supernatant remains, the cell aggregate will not be incorporated into the collagen gel.
31. The cell manipulation to reconstitute a bioengineered salivary gland germ should be performed quickly because the collagen gel solidifies with changes in temperature and with the passage of time.
32. Aspirate only the required amount of cells using a pipette tip to reconstitute the bioengineered salivary gland germ.
33. Insert the pipette tip into the collagen gel using a P2 micropipette. A cell aggregate is extruded slowly, and the pipette tip must be precisely operated so that the cell aggregate becomes spherical. The insertion should be stopped precisely when all cells have been extruded from the pipette tip to prevent introducing air bubbles into the gel.
34. When bioengineering a salivary gland germ using both epithelial and mesenchymal salivary gland germ tissues (i.e., reconstitution of tissue and tissue, or tissue and cell), ensure that there is sufficient contact between the tissue and cell aggregate.
35. For ease of use, cut the nylon thread to a length of approximately 600 mm. Use care in handling the thread because it is easy to lose the nylon thread.
36. Invert the siliconized dish to prevent the sample from sinking to the bottom of the collagen gel (Fig. 4A).
37. Tear off the collagen gel from the bottom aspect of the siliconized dish. The collagen gel should be picked up with tweezers

by its sides and carefully installed onto the cell culture insert (Fig. 4A).

38. It is desirable to exchange the entire volume of the organ culture medium.
39. Anesthetize the mouse using an intraperitoneal injection of 5 mg/mL pentobarbital.
40. Use soapy water for shaving to avoid the risk of bacterial contamination.
41. Transplantation of the bioengineered salivary glands is best when it is connected to the parotid gland duct. It is possible to facilitate an efficient connection by connecting the bioengineered salivary glands and parotid gland duct using nylon thread (Fig. 5A).
42. The parotid gland duct cavity is immediately deflated by the cut; therefore, the cut is made prior to transplantation.
43. A salivary gland defect model mouse in which all of the submandibular glands, sublingual glands, and parotid glands were removed will die within 5 days because it is difficult for the animal to drink water well. Viscous water is needed to increase the survival rate of this mouse.
44. When a bioengineered salivary gland germ is transplanted closer than 2/3 of the distance from the masseter muscle, its development deteriorates. In addition, it is difficult to transplant the bioengineered salivary gland germ at a position within 2/3 of this distance, so be careful where you cut the duct.
45. Use care to ensure that the bioengineered salivary gland germ does not fall off the thread.
46. Atropine should be intraperitoneally injected 10 min before the anesthesia injection to analyze the suppression of saliva secretion by atropine.

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Chapter 11

Generation of a Bioengineered Lacrimal Gland by Using the Organ Germ Method

Masatoshi Hirayama, Kazuo Tsubota, and Takashi Tsuji

Abstract

In organogenesis including lacrimal gland development, cell arrangement within a tissue plays an important role. The lacrimal gland develops from embryonic ocular surface epithelium through reciprocal epithelial and mesenchymal interaction, which is organized by interactive regulation of various pathways of signaling molecules. Current development of an in vitro three-dimensional cell manipulation procedure to generate a bioengineered organ germ, named as the organ germ method, has shown the regeneration of a histologically correct and fully functional bioengineered lacrimal gland after engraftment in vivo. This method demonstrated a possibility of lacrimal gland organ replacement to treat dry eye disease, which has been a public health problem leading reduction of visual function. Here, we describe protocols for lacrimal gland germ regeneration using the organ germ method and methods for analyzing the function of the bioengineered lacrimal gland after its transplantation in vivo.

Key words Bioengineered lacrimal gland, Organ germ method, Cell manipulation, Transplantation, Lacrimal gland germ

1 Introduction

The lacrimal glands are ectodermal secretory glands occurring from the lacrimal gland germ, which develop from embryonic ocular surface through epithelial and mesenchymal interaction in a process of embryonic organogenesis [1]. After an induction of initial epithelial bud, cells in the lacrimal gland germ progress differentiation into mature secretory gland structure such as acini, duct, and myoepithelial cells [1, 2]. The acini of the lacrimal gland secrets aqueous tear and tear proteins, which drain into ocular surface as tears via lacrimal duct [3]. Myoepithelial cells envelop the acini and help to secret tears by contraction [1]. Development of the lacrimal glands and its branching morphogenesis has been investigated to elucidate complex signaling pathways with transcription factors, small molecules, and growth factors as an important key to build up mature lacrimal gland structure [1, 4, 5].

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Regulation of growth and differentiation of various cell types during organogenesis governs correct 3D cell organization in tissue [6, 7]. In current research on lacrimal gland development, investigations on the molecular mechanisms of branching morphogenesis have been performed using in vitro and in vivo experiments [3, 4, 7, 8]. At the same time, attempts to identify tissue stem cells in the lacrimal glands, which have an ability to recover function of the lacrimal glands after injury, have been reported [9]. To make 3D structure of the lacrimal glands, decellularized lacrimal gland tissue models are reported to study natural scaffolds of extracellular matrix architecture [10]. The development of 3D assembly procedure with various cells has been required to realize the lacrimal gland organ regeneration.

Recently, we have demonstrated an in vitro 3D cell manipulation method, called as the bioengineered organ germ method, which reconstituted cell compartmentalization between epithelial and mesenchymal cells at a high cell density [11]. This method has been first applied to regeneration of tooth germ and hair follicle, which could develop into a structurally correct and fully functioning tooth and hair follicle in an adult mammal in vivo [12, 13]. We next applied this methodology to regenerate secretory glands such as salivary glands and lacrimal glands [14, 15]. Our bioengineered lacrimal gland germ could develop into mature lacrimal gland structure with proper tear secretion ability in vivo after transplantation which enables its duct to connect to recipient mouse. Thus, our 3D bioengineered organ regeneration technique can be employed for use in various studies on development of the lacrimal glands and screenings for potential of lacrimal gland organ regenerative therapy [16].

In this chapter, we provide a detailed description of a protocol for 3D bioengineered lacrimal gland germ reconstitution using embryonic day 16.5 lacrimal gland germ-derived epithelial and mesenchymal cells. We also describe methods for analyses utilized for the in vivo analysis of the bioengineered lacrimal gland function.

2 Materials

The basic procedures and clues of the organ germ methods to reconstitute bioengineered lacrimal gland germs are common with those of other ectodermal organs such as the teeth, hair follicle, and salivary gland. All fluid solutions should be prepared using ultrapure water (MilliQ) and analytical grade reagents. All surgical materials should be sterilized in an autoclave prior to each use in order to prevent contamination. It is recommended that enzyme and serum reagents should be evaluated for enzyme reactivity and primary cell culture efficiencies, respectively, prior to experimental use.

1. Animals: An inbred mouse strain (i.e., C57BL/6) is used in these experiments. Mouse embryonic age is determined based on the day of appearance of a vaginal plug in a pregnant mouse (embryonic day 0 (ED 0)).
2. Ca^{2+}/Mg^{2+} -free, phosphate-buffered saline (PBS(−)): 137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 8.0 mM anhydrous disodium hydrogen orthophosphate (Na_2HPO_4), and 1.5 mM potassium phosphate monobasic (KH_2PO_4). Store at 4 °C.
3. Medium: Isolation of lacrimal gland germ and dissociation of single cells are performed in basal cell culture medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and 10 mM 1-4-(2-hydroxyethyl)-piperazineethanesulfonic acid (HEPES). For organ culture, this medium is added with the abovementioned supplements, excluding HEPES to avoid any cytotoxicity.
4. Dispase solution: Dispase (50 U/mL) stored at −20 °C until use (*see* **Note 1**).
5. Collagenase solution: The concentration of collagenase I is adjusted to 100 U/mL using distilled water and stored at −20 °C until use (*see* **Note 2**).
6. Reagent A: 3.66 μL/mL collagenase solution adjusted by PBS(-).
7. Reagent B: 0.25% trypsin adjusted by PBS(-).
8. Reagent C: Mixture solution of 1.83 μL collagenase solution and 0.25% trypsin adjusted by PBS(-).
9. 70 U/mL deoxyribonuclease I (DNase) from bovine pancreas (*see* **Note 3**).
10. Collagen gel: Component in 100 μL of 10× concentrated α-minimum essential medium (α MEM) and 100 μL mixed buffer (0.08 N sodium hydroxide and 200 mM HEPES) in 800 μL Cellmatrix Type I-A manufactured by Nitta Gelatin Inc.
11. 9-0 polyglycolic acid (PGA) monofilament (9-0 PGA absorbable surgical suture) (*see* **Note 4**).
12. Surgical tools for the dissection of embryos from the uterus: Large surgical scissors and forceps to cut the abdominal skin and muscle and small surgical scissors and forceps to dissect the uterus (*see* **Note 5**).
13. Surgical tools for transplantation into lacrimal excretory duct of the extra-orbital lacrimal gland-removed mouse: surgical scissors to cut off the extra-orbital lacrimal glands and tweezers to transplant to remaining lacrimal gland excretory duct (*see* **Note 6**).
14. Dissecting microscope (*see* **Note 7**).

15. Sterile disposable 1 mL syringes and 25 G needles (5/8, 0.50 × 16 mm) for lacrimal gland germ extraction (*see* **Note 8**).
16. Sterile disposable 1.5 mL round-bottom microtube (*see* **Note 9**).
17. Sterile disposable gel-loading pipette tip and 0.1–10 μL pipette tips for reconstitution of bioengineered lacrimal gland germ (*see* **Note 10**).
18. Cell culture insert/0.4 μm pore-size membrane for organ culture (*see* **Note 11**).

3 Methods

3.1 Extraction of Mouse Embryo Lacrimal Gland Germ

Dissecting procedures of embryos, lacrimal gland germs, and lacrimal gland tissues should be performed in medium to prevent drying.

1. After sacrificing the mouse, cut the abdomen along the midline with small scissors. Resect the uterus and wash it with PBS(−) on ice. To avoid bacterial contamination, do not use the same surgical tools for cutting the skin.
2. Dissect the embryos from the uterus. Separate the fetal head from the body and separate the ocular area from the unilateral head. Immediately place the ocular area in medium on ice.
3. Dissect the lacrimal gland germ from the unilateral ocular area using a pair of 25 G needles.
4. Make sure to remove peripheral fibrous tissues from the lacrimal gland germ (Fig. 1a–c), and also isolate the harderian gland germ if needed (Fig. 1d) (*see* **Note 12**).
5. Keep the isolated lacrimal gland germs in cold medium on ice. Do transfer the lacrimal gland germ with the medium to avoid injury to the tissue.

3.2 Separation of Lacrimal Gland Germ Epithelial and Mesenchymal Tissues

1. Wash the extracted lacrimal gland germs twice in PBS(−), add 50 U/mL dispase solution, and conduct the enzyme reaction at room temperature for 1.5 min. Do not react more than 10 min to avoid injury to the germs.
2. Stop the enzyme reaction by adding culture medium. Quickly wash the lacrimal gland germs twice in the same culture medium.
3. Add 1 μL DNase into 2 mL of culture medium and incubate at room temperature for a few seconds (*see* **Note 13**).
4. Carefully separate the lacrimal gland germ epithelial and mesenchymal tissues under a microscope using 25 G needles.
5. Keep each tissue in cold culture medium on ice.

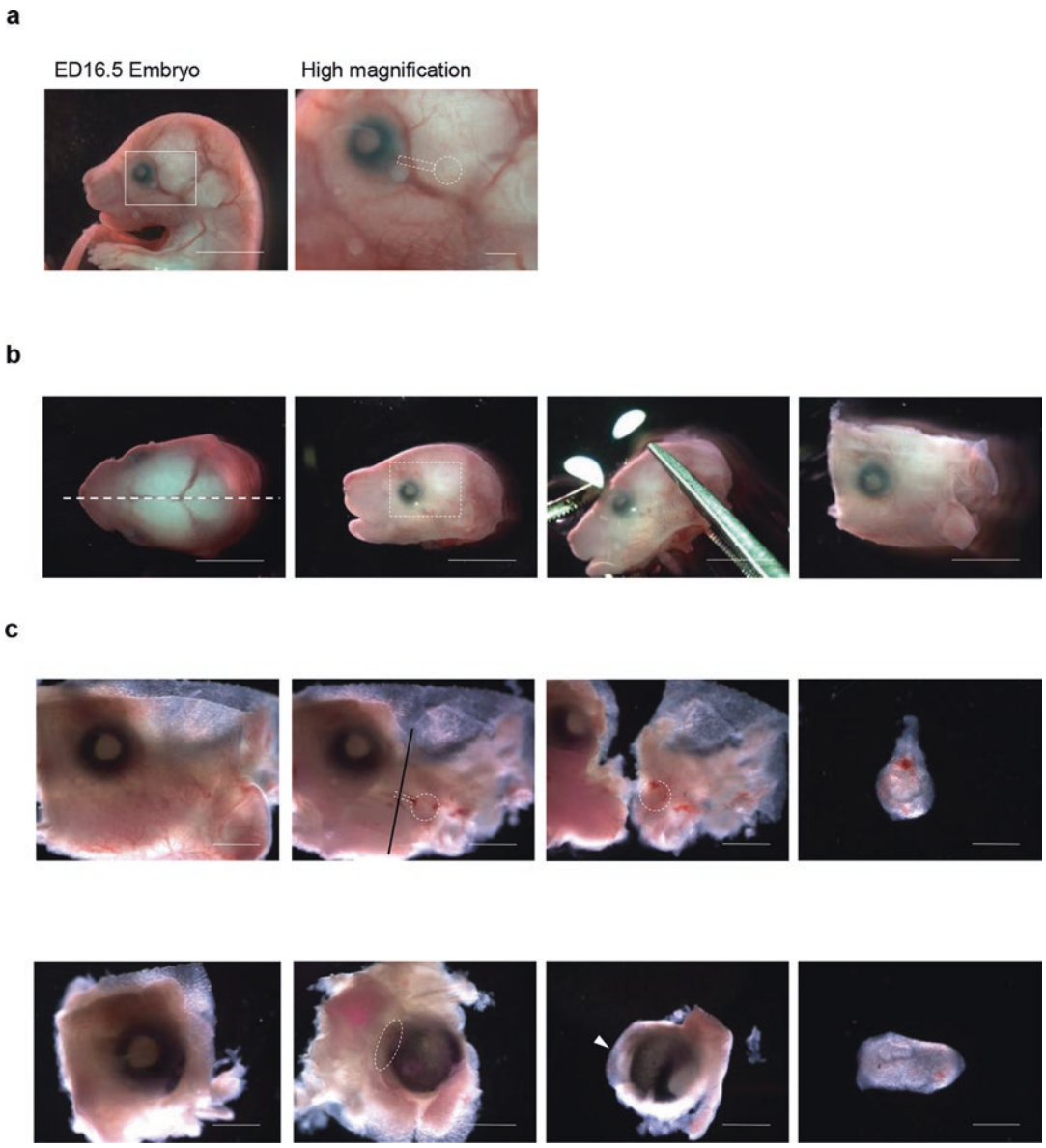

Fig. 1 Isolating the lacrimal gland germ and harderian gland germ from mouse embryo. (**a**) Photographs of the area where the lacrimal gland locates in ED16.5 mouse embryo (*left*). The *boxed area* in the *left panel* is the lacrimal gland site shown at a higher magnification in the *right panel*. The *white dotted line* indicates the lacrimal gland germ. The scale bar, 5 mm in the left and 2.5 mm in the *right panel*. (**b**) Photographs of the procedure of dissecting the lacrimal gland site from the embryo. After removing the body of the embryo, the head was cut along with the sagittal suture (*white dotted line, left*). Peripheral tissues of the lacrimal gland site (*white dotted box, center-left*) was removed by using a tweezer (*center-right* and *right*). Scale bar, 5 mm. (**c**) Photographs of the procedure to isolate the lacrimal gland germ. The face skin was removed to find the lacrimal gland germ (*white dotted line, center-left, left*) in the dissected area (*left*). After cutting the duct of the lacrimal gland germ along with the *black line* (*center-left*), the peripheral tissue of the lacrimal gland germ was carefully removed (*center-right*, *right*). Scale bar, 1 mm. (**d**) Photographs of the procedure to isolate the harderian gland germ. Phase-contrast images of the development and branching morphogenesis of the bioengineered lacrimal gland germ in organ culture at day 1 (*left*) and day 5 (*right*). Scale bar, 1 mm. Reprinted and modified from Hirayama et al. [14]

3.3 Enzymatic Separation of Single Cells from Lacrimal Gland Germ Epithelial Tissue

1. Collect the epithelial tissues in a 15 mL tube and centrifuge at 600 × *g* for 3 min. Discard the medium and wash the cell pellet twice in PBS(−).
2. Carefully aspirate to remove the PBS(−) and add 2 mL of enzyme reactive Reagent A. Incubate the epithelial tissues for 10 min in a 37 °C water bath. Repeat this procedure twice (*see* **Note 14**).
3. Centrifuge the epithelial tissues at 600 × *g* for 3 min and discard all of Regent A. Add 2 mL of enzyme reactive Reagent B and incubate the epithelial tissues for 5 min in a 37 °C water bath.
4. Stop the enzyme reaction by adding 6 mL of culture medium. Centrifuge at 600 × *g* for 5 min.
5. Carefully discard the supernatant to a residual volume of 80 μL and disperse the cell pellet by tapping. When cell aggregation is not dispersed well by tapping, the cell pellet can be suspended by gentle pipetting.
6. Immediately add 1 mL of culture medium and centrifuge at 600 × *g* for 3 min.
7. Carefully aspirate the supernatant to a residual volume of 200 μL and add 1 μL of DNase solution to the residual volume. Create a single cell suspension by gently pipetting ten times at constant speed with a micropipette and a P200 tip and shift the suspension through a cell strainer.

3.4 Enzymatic Separation of Single Cells from Lacrimal Gland Germ Mesenchymal Tissue

1. Collect the mesenchymal tissues in a 15 mL tube and centrifuge at 600 × *g* for 3 min. Discard the medium, and wash the cell pellet twice in PBS(−).
2. Carefully aspirate to remove the PBS(−), and add 2 mL of enzyme reactive Reagent C. Incubate the mesenchymal tissues for 10 min in a 37 °C water bath.
3. Stop the enzyme reaction by adding 6 mL culture medium and centrifuge at 600 × *g* for 5 min.
4. Carefully aspirate the supernatant to a residual volume of 80 μL and disperse the cell pellet by tapping as the procedure for epithelial tissue.
5. Immediately add 1 mL of culture medium and centrifuge at 600 × *g* for 3 min.
6. Carefully aspirate the supernatant to a residual volume of 200 μL and add 1 μL of DNase solution to the residual volume. Create a single cell suspension by gently pipetting ten times at a constant speed with a micropipette and a P200 tip and shift the suspension through a cell strainer.

3.5 Reconstitution of the Bioengineered Lacrimal Gland Germ

1. Prepare siliconized 35 mm petri dishes and 1.5 mL tubes coated with silicon grease (*see* **Note 15**).
2. Transfer the epithelial or mesenchymal single cell suspensions isolated from lacrimal gland germs into separate siliconized 1.5 mL tubes, respectively.
3. Centrifuge at 600 × *g* for 3 min and discard the supernatant using a micropipette and a P1000 or P200 tip.
4. Centrifuge at 600 × *g* for 3 min and discard the supernatant on the cell pellets completely using a micropipette and a gel-loading tip under a microscope (*see* **Note 16**).
5. Prepare a droplet of 30 μL collagen gel on a siliconized petri dish. Do quickly to avoid gel solidification.
6. Aspirate a 0.3–0.4 μL volume of the mesenchymal cell pellet using a micropipette and a 0.1–10 μL pipette tip under a microscope. Apply the cell pellet slowly into the collagen drop and make a spherical cell aggregate. Do not extrude air bubble to keep shape of cell aggregate in the gel.
7. Similarly, apply a 0.2–0.3 μL volume of the epithelial cell pellet into the same collagen drop and make contact with the mesenchymal cell aggregate. Ensure that there is sufficient contact between epithelial cells and mesenchymal cells (Fig. 2a, b).
8. For transplantation of the bioengineered lacrimal gland germ, insert a 9-0 PGA monofilament to the bioengineered lacrimal gland germ. Make sure to insert the thread on the center of the bioengineered germ (*see* **Note 17**) (Fig. 2c, 3a, b).

3.6 In Vitro Organ Culture of the Bioengineered Lacrimal Gland Germ

1. Turn the siliconized dish upside down and incubate the petri dish holding the collagen gel drop for 15 min at 37 °C to fix the collagen gel. Set the cell culture insert into a 12-well plate filled with culture medium (350 μL/well).
2. Pick up the collagen gel drop with tweezers carefully and transfer the drop onto the cell culture insert. Do not directly pick the bioengineered germ by tweezers to keep the shape and the location of the thread.
3. Replace the culture medium supplemented with 10% FBS. Remove the medium and replace with fresh medium every other day if needed.
4. After culture for 2 days, the branching epithelium with duct structure along with gel around thread will have developed in the collagen gel (Fig. 3b).

3.7 Transplantation of a Bioengineered Lacrimal Gland Germ into the Extra-Orbital Lacrimal Gland-Removed Mouse

1. Anesthetize the mouse using intraperitoneal injection of 5 mg/mL pentobarbital.
2. Place the mouse on its stomach and immobilize the hands, feet, and face.

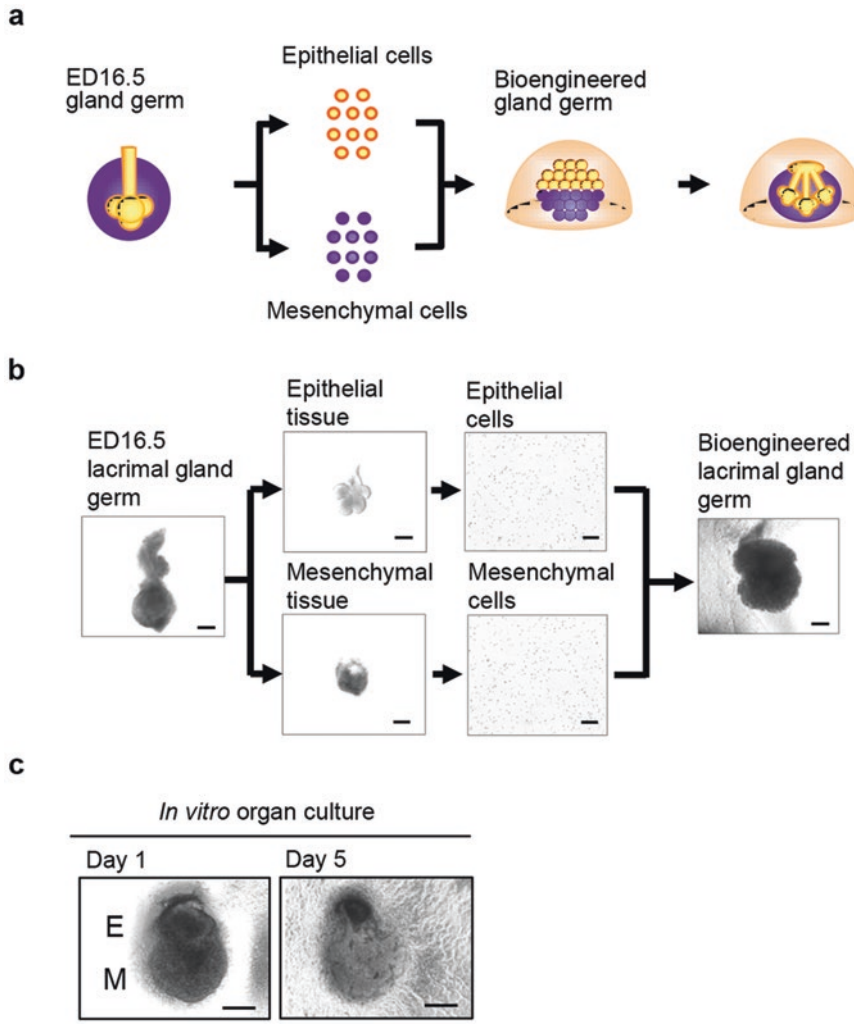

Fig. 2 Generation of the bioengineered lacrimal gland using the organ germ method. (**a**) Schematic representation of procedures of generation of the bioengineered lacrimal gland germ. Reprinted and modified from Hirayama et al. [14]. (**b**) Phase-contrast images of the dissected ED16.5 lacrimal gland germ (*left*), separated epithelium (*center-left*, *upper*) and mesenchyme (*center-left*, *lower*), single cells after enzyme reaction of the epithelium (*center-right, upper*) and the mesenchyme (*center-right*, *lower*) and reconstituted bioengineered lacrimal gland in collagen gel drop (*right*). Scale bar, 100 μm. Reprinted and modified from Hirayama et al. [14]. (**c**) Phase-contrast images of the development and branching morphogenesis of the bioengineered lacrimal gland germ in organ culture at day 1 (*left*) and day 5 (*right*). Scale bar, 100 μm. Reprinted and modified from Hirayama et al. [14]

3. Shave the hair on face cheek with a razor using soapy water for shaving.
4. Cut the facial skin in approximately 10 mm in length and exfoliate between the skin and the fascia, and expose the whole extra-orbital lacrimal gland and the lacrimal excretory duct.
5. Dissect the extra-orbital lacrimal gland on the base of the lacrimal excretory duct using scissors (*see* **Note 18**).

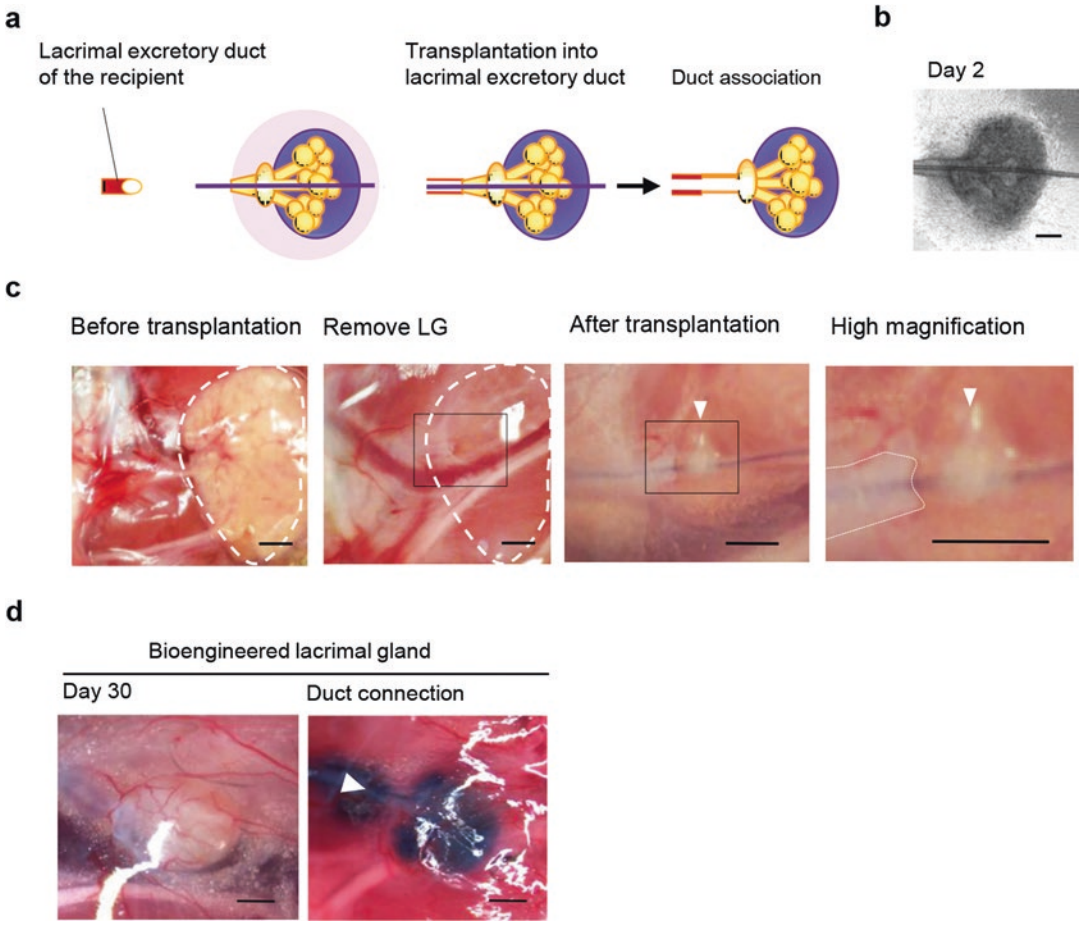

Fig. 3 Transplantation procedure of the bioengineered lacrimal gland. (**a**) Schematic representation of transplantation of the bioengineered lacrimal gland into an extra-orbital lacrimal gland-removed mouse. (**b**) Phase-contrast image of the bioengineered lacrimal gland in organ culture at day 2 with duct induction by using thread-guide procedure. Scale bar, 100 μm. Reprinted and modified from Hirayama et al. [14]. (**c**) Macroscopic photographs of procedures for bioengineered gland germ transplantation. The extra-orbital lacrimal gland was completely removed (*left*, *center-left*, *white dotted line*). The *boxed area* in the *center-left panel* is the transplantation area shown at a higher magnification in the *center-right panel*. The *boxed area* in the *center-right panel* is shown at a higher magnification in the *right panel*. The thread was inserted into the recipient lacrimal excretory duct (*center-right*, *right*). The *white dotted line* in the *right panel* indicates the host lacrimal duct. The *white arrowhead* in the *center-right* and *right panels* indicates the transplanted bioengineered lacrimal gland with a thread. Scale bar, 1 mm. Reprinted and modified from Hirayama et al. [14]. (**d**) Macroscopic photographs of bioengineered lacrimal glands at 30 days after transplantation (*left*). The duct connection between the bioengineered lacrimal gland and the recipient lacrimal excretory duct are visualized using Evans blue dye injection. The *arrowhead* indicates the injection site. Scale bar, 500 mm. Reprinted and modified from Hirayama et al. [14]

6. Pick up the cultured bioengineered lacrimal gland with thread by pinching the edge of the gel using tweezer, and transfer to the area of removed extra-orbital lacrimal gland (*see* **Note 19**). Drop an aliquot of cold PBS(-) gently to prevent drying the stuff.

7. Insert the PGA thread into the opening of the recipient's lacrimal excretory duct, and adjust the location of transplant to contact the epithelium of bioengineered gland germ and the recipient's lacrimal excretory duct (Fig. 3a, c).
8. Fix the location of the transplant by suturing the gel to masseter more than at two points.
9. Carefully close the scar by suturing the skin.
10. Thirty days after transplantation, the bioengineered lacrimal gland with the correct lacrimal gland structure and duct association will have developed in the transplanted area (Fig. 3d).

3.8 Tear Volume Measurement Using Pilocarpine Stimulation

1. Record weight of a mouse.
2. Anesthetize the mouse by using intraperitoneal injection of 5 mg/mL pentobarbital, and wait for 5 min.
3. Inject 300 mg/weight (g) of pilocarpine to intraperitoneal.
4. After 2 min of injection, remove the water on ocular surface completely by touching a tip of Kimwipe paper to lower lid tear meniscus.
5. After 5 min of injection, start to collect the tear for 5 min by using 0.5 mL micropipette, and record the amount of tear according to the time course (Fig. 4a) (*see* **Note 20**).

3.9 Tear Volume Measurement Using Menthol Stimulation to Ocular Surface

1. Place the mouse in a modified DecapiCone restraint with sufficient acclimation.
2. Collect the tear fluid from the edge of the eyelid margin without touching the eye using a 0.5 μL micropipette as a baseline tear volume. Following measurement using the method is recommended to be performed on another day if the micropipette touches the ocular surface, to avoid irregular tear secretion.
3. Following baseline measurements, directly apply 10 μL of 0.1 mM menthol to the ocular surface with a micropipette. After 2 min, wick the fluid away with a Kimwipe by lightly touching the tear meniscus at the lateral canthus.
4. Collect the tear for 15 min after removal of the menthol fluid according to the time course (Fig. 4b) (*see* **Note 20**).

4 Notes

1. Dispase dissociates the basal membrane between epithelial and the mesenchymal tissue of the lacrimal gland germ. It is recommended to evaluate the conditions of temperature and reaction time, since enzymatic activity is easily decreased at 4 °C.
2. Intercellular collagen molecules are resolved by collagenase and tissues are separated into single cells.

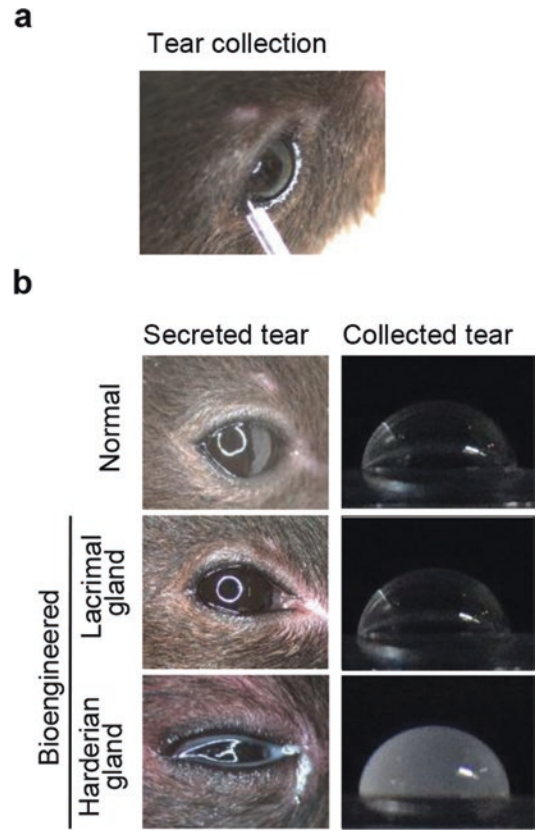

Fig. 4 Tear collection procedures using a micropipette. (**a**) Macroscopic photograph of the procedure of tear collection using a micropipette. Tip of the micropipette touches to only tear meniscus, not to ocular surface. (**b**) Photographs of eyes after stimulation of tear secretion (*left*) and the collection of tear fluid (*right*), including a normal lacrimal gland (*upper*), a bioengineered lacrimal gland-transplanted mouse (*center*), and a bioengineered harderian gland-transplanted mouse, which secretes white tear containing lipids (*lower*). Reprinted and modified from Hirayama et al. [14]

3. DNase can prevent this cell aggregation by digesting the DNA after enzymatic reaction.
4. Cut the PGA thread to be a fragment of 10 mm length before procedure.
5. Surgical instruments should be washed and sterilized by autoclaving prior to each use in order to prevent contamination.
6. We recommend the use of minimum-size surgical tools, which can be more easily manipulated on the surgical area in mouse.
7. We recommend the use of a dissecting microscope capable of 6.5–50× magnification with a transmitted beam applied as the light source.

8. A 25 G needle is suitable for most of the surgical manipulations including the lacrimal gland germ extraction and the isolation of the germ tissue.
9. A round-bottom microtube should be used, since a square-bottom microtube is unsuitable for forming a cell pellet by centrifugation.
10. A 0.1–10 μL pipette tip is suitable for making a cell aggregate with high cell density in the collagen gel.
11. A membrane should be used with a pore size that is sufficient for liquid components to pass through. We recommend the use of a cell culture insert/0.4 μm pore-size membrane.
12. Dissecting lacrimal gland germ manipulations can affect the successful rate of a bioengineered lacrimal gland germ development.
13. Tissues and cells after enzymatic treatment can easily aggregate due to released DNA, making subsequent manipulations difficult. The addition of DNase digests DNA and can prevent cell aggregation.
14. Care should be taken to avoid tissues sticking to the tube wall.
15. Take care not to apply too much silicon grease, and wipe the extra grease out after coating by a clean swab.
16. Remove the residual supernatant as possible to create high-density cell pellets.
17. It is recommended that the bioengineered lacrimal gland should be reconstituted at a position just before the center to induce duct in the gel.
18. Bevel the lacrimal gland duct slightly to maximize the area of opening of the duct for transplantation surgery.
19. Place the thread of bioengineered lacrimal gland along with the lacrimal excretory duct.
20. To avoid irregular tear secretion, do not touch eye surface by Kimwipe and collect micropipette.

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Chapter 12

Generation of Gastrointestinal Organoids from Human Pluripotent Stem Cells

Jorge O. Múnera and James M. Wells

Abstract

Over the past several decades, developmental biologists have discovered fundamental mechanisms by which organs form in developing embryos. With this information it is now possible to generate human "organoids" by the stepwise differentiation of human pluripotent stem cells using a process that recapitulates organ development. For the gastrointestinal tract, one of the first key steps is the formation of definitive endoderm and mesoderm, a process that relies on the TGFb molecule Nodal. Endoderm is then patterned along the anterior-posterior axis, with anterior endoderm forming the foregut and posterior endoderm forming the mid and hindgut. A-P patterning of the endoderm is accomplished by the combined activities of Wnt, BMP, and FGF. High Wnt and BMP promote a posterior fate, whereas repressing these pathways promotes an anterior endoderm fate. The stomach derives from the posterior foregut and retinoic acid signaling is required for promoting a posterior foregut fate. The small and large intestine derive from the mid and hindgut, respectively.

These stages of gastrointestinal development can be precisely manipulated through the temporal activation and repression of the pathways mentioned above. For example, stimulation of the Nodal pathway with the mimetic Activin A, another TGF-β superfamily member, can trigger the differentiation of pluripotent stem cells into definitive endoderm (D'Amour et al., Nat Biotechnol 23:1534–1541, 2005). Exposure of definitive endoderm to high levels of Wnt and FGF promotes the formation of posterior endoderm and mid/hindgut tissue that expresses CDX2. Mid-hindgut spheroids that are cultured in a three-dimensional matrix form human intestinal organoids (HIOs) that are small intestinal in nature Spence et al., Nature 2011. In contrast, activation of FGF and Wnt in the presence of the BMP inhibitor Noggin promotes the formation of anterior endoderm and foregut tissues that express SOX2. These SOX2-expressing foregut spheroids can be further patterned into posterior foregut by addition of retinoic acid. Once formed, these posterior foregut spheroids can be grown in three-dimensional human gastric organoids (HGOs) that have all of the cell types of antral part of the stomach (Mc Cracken et al. 2014).

Here, we describe the detailed methods for generating stomach/human gastric organoids (HGOs) and human intestinal organoids (HIOs) from human pluripotent stem cells. We first present a method for generating definitive endoderm from pluripotent stem cells followed by differentiation of definitive endoderm into either posterior foregut spheroids or mid-hindgut spheroids. We then describe how three-dimensional culturing of these spheroids results in the formation of HGOs and HIOs, respectively.

Key words Definitive endoderm, Foregut, Hindgut, Human pluripotent stem cells, Gastric organoids, Intestinal organoids

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1 Introduction

The ability to generate "mini-guts" from human embryonic stem cells and induced pluripotent stem cells (IPSCs) now allows researchers to study aspects of human gastrointestinal development in vitro [1–4]. These "mini-guts" recapitulate developmental transitions that occur in vivo and can mature into tissue with the main differentiated cell types found within the stomach and intestines. Here, we describe the detailed methods for generating stomach/human gastric organoids (HGOs) and human intestinal organoids (HIOs) from human pluripotent stem cells (Fig. 1). We first present a method for generating definitive endoderm [5] from pluripotent stem cells followed by differentiation of definitive endoderm into either posterior foregut spheroids or mid-hindgut spheroids. We then describe how three-dimensional culturing [6] of these spheroids results in the formation of HGOs and HIOs, respectively.

2 Materials

2.1 HES Cell Culture

1. H1 or H9 human embryonic stem cells (WiCell Research Institute). Use of human embryonic stem cells should adhere to ethical guidelines. Both of these lines are approved by the NIH.
2. Induced pluripotent stem cells (generated by the Pluripotent Stem Cell Facility, Cincinnati Children's Hospital Medical Center).
3. Resuspend dispase in Advanced DMEM-F12 to a final concentration of 1 mg/mL. Filter-sterilize solutions by vacuum filtration using a Millipore filter sterilization tube. Make 10-mL aliquots and store aliquots at −20 °C for up to 6 months.
4. To aliquot hPSC-qualified Matrigel for hPSC culture, thaw a tube on ice overnight at 4 °C. Chill sterile microcentrifuge tubes in a microcentrifuge rack at −80 °C for 1 h prior to aliquoting Matrigel. Aliquot Matrigel into cold microcentrifuge tubes and store at −80 °C for up to 6 months.

2.2 Differentiation of ES Cells to Definitive Endoderm

1. Day 1 media: RPMI 1640, 2 mM L-glutamine, nonessential amino acids, penicillin-streptomycin, 100 ng/mL Activin A, and 50 ng/mL of BMP4. Exclude BMP4 when generating mid-hindgut spheroids.
2. Day 2 media: RPMI 1640, 2 mM L-glutamine, 0.2% dFBS vol/vol, nonessential amino acids, penicillin-streptomycin, and 100 ng/mL Activin A.
3. Day 3 media: RPMI 1640, 2 mM L-glutamine, 2% dFBS vol/vol, nonessential amino acids, penicillin-streptomycin, and 100 ng/mL Activin A.

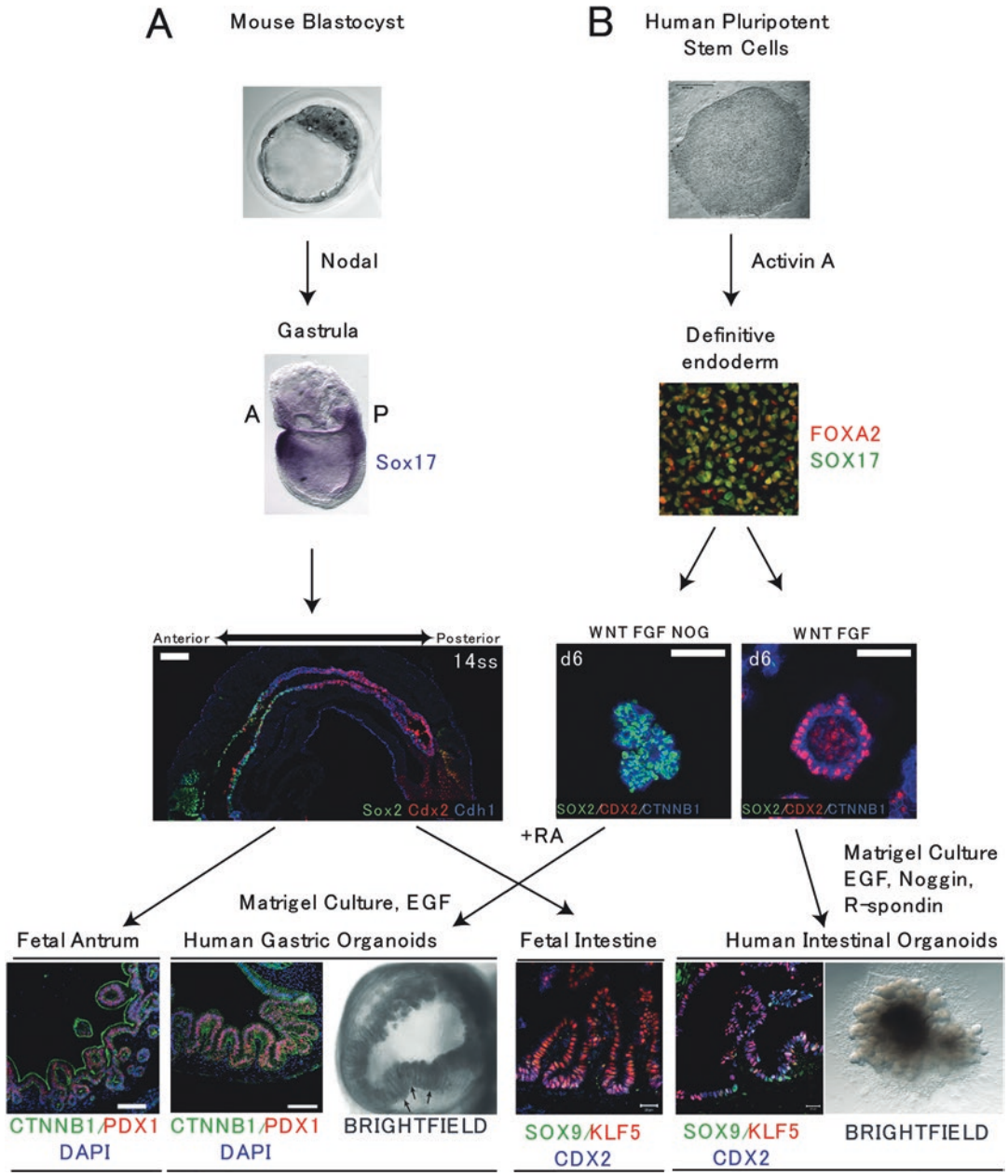

Fig. 1 Summary of methods. Mouse gastrointestinal development time-course (**a**). Time-course for generation of HIOs and HGOs (**b**)

2.3 Posterior Foregut Differentiation

1. Posterior foregut differentiation media: RPMI 1640, 2 mM L-glutamine, 2% dFBS vol/vol, penicillin-streptomycin, 500 ng/mL WNT3A, 500 ng/mL FGF4, 200 ng/mL Noggin. Add 2 μM retinoic acid on Day 3 of differentiation. WNT3a can be replaced with 2 μM CHIR99201.

2.4 Mid-Hindgut Differentiation

1. Mid-hindgut differentiation media: RPMI 1640, 2 mM L-glutamine, 2% dFBS vol/vol, penicillin-streptomycin, 500 ng/mL WNT3a, 500 ng/mL FGF4. WNT3a can be replaced with 3 μM CHIR99201.

2.5 Matrigel Culture

1. Gastrointestinal growth medium: Advanced DMEM/F-12, B27, N2, 15 mM HEPES, 2 mM L-glutamine, penicillin-streptomycin.
2. Antral specification media: Gastrointestinal growth medium, 100 ng/mL EGF, 100 ng/mL Noggin, 2 μM retinoic acid.
3. HGO growth media: Gastrointestinal growth medium, 100 ng/mL EGF.
4. HIO growth media: Gastrointestinal growth medium, 100 ng/mL EGF.

2.6 Immunostaining

1. Donkey serum (Jackson ImmunoResearch Laboratories).
2. Donkey anti-goat DyLight 488 (Jackson ImmunoResearch Laboratories).
3. Donkey anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories).
4. Donkey anti-mouse DyLight 488 (Jackson ImmunoResearch Laboratories).
5. Goat anti-SOX17 (R&D Systems).
6. Rabbit anti-FOXA2 (obtained as a kind gift from J. Whitsett).
7. Mouse anti-CDX2 (Thermo Scientific/Lab Vision).
8. SOX2 (Santa Cruz sc17320).
9. Draq5 (Cell Signaling Technology).
10. Fluoromount-G fluorescent mounting medium (Southern Biotech).
11. Immunostaining blocking buffer: 5% donkey serum in PBS, 0.5% vol/vol Triton X-100.

2.7 Equipment

1. Pipet-aid A-P cordless motorized serological pipettor (Drummond).
2. Pipetman single-channel pipettes (Gilson).
3. Purifier horizontal clean bench, 4 ft (Labconco).
4. Stereomicroscope.
5. Forceps and/or tungsten needle.
6. Nunclon delta surface tissue culture dish 6 wells (Nunc).
7. Nunclon delta surface tissue culture dish 24 wells (Nunc).
8. Nunclon delta surface tissue culture dish 4 wells (Nunc).
9. Disposable scalpel.

10. Serological pipettes 5, 10, and 25 mL.
11. Pasteur pipettes.
12. Sterilized filter pipette tips: 20, 200, and 1000 μL.
13. Microcentrifuge tubes, 1.5 mL.
14. Millipore Steriflip 0.22-μm sterilization filter conical tubes.
15. Nunc Thermanox plastic coverslips (Nunc).

3 Methods

3.1 ES Cell Culture

1. Thaw hESC-qualified Matrigel on ice overnight in a 4 °C refrigerator.
2. Resuspend Matrigel in 25 mL of ice-cold Advanced DMEM/F12.
3. Coat Nunc plates with resuspended Matrigel. For 6-well plates, use 1 mL per well. For 24-well plates, use 0.25 mL per well and add an additional 0.25 mL of Advanced DMEM-F12 per well.
4. Incubate plates at room temp for 1 h and store at 4 °C until use. Plates can be kept at 4 °C for up to 1 week.
5. Grow pluripotent stem cells in feeder-free conditions on hESC-qualified Matrigel. Culture hPSCs on hESC-qualified, Matrigel-coated 6-well Nunclon delta surface plates in a 5% CO_2 incubator at 37 °C.
6. Check cells daily for differentiation. Remove differentiated colonies.
7. Replace media daily and passage cells after 4–5 days.
8. Passage cells by aspirating medium and washing once with Advanced DMEM-F12.
9. Aspirate Advanced DMEM-F12 and add 1 mL of dispase solution per well to be split.
10. Place plates in a 5% CO_2 incubator at 37 °C for 5 min. After 5 min observe for folding of the edges of colonies.
11. Aspirate dispase and wash wells 3 times with Advanced DMEM-F12.
12. Aspirate Advanced DMEM-F12 and add 3 mL of mTESR1 per well.
13. Use a cell scraper to lift colonies off of the plate.
14. Break colonies into small chunks by pipetting up and down 3 times while holding pipette against the side of the well. Observe under microscope to ensure that no large clumps remain.
15. Plate cells into new 6-well plate by dispensing 0.5 mL of cells into each well containing 1.5 mL of mTESR1.

16. To ensure even dispersal of cells in the wells, shake three times clockwise, three times counter clockwise, three times back and forth, and three times side to side. Check cells the next day and repeat **steps 1–16** as needed.

3.2 Single-Cell Plating of ES Cells

1. Grow ES cells to 70–80% confluence (in 6-well plate), and remove differentiated cells before starting (*see* **Note 1**). Wash cells once with 2 mL of Advanced DMEM-F12.
2. Add 1 mL of Accutase to each well and place in incubator for 6–8 min (when cells detach).
3. Add 2 mL of Advanced DMEM-F12 per well and pipette up and down to detach adherent cells.
4. Pool cells from all wells into a 15-mL conical tube and mix by pipetting up and down to break cells into single cells.
5. Spin down cells at 300 × *g* for 3 min.
6. Remove supernatant without disturbing pellet and resuspend in mTesr1 with 10-μM Y-27632 ROCK inhibitor. Take a small aliquot for cell counting.
7. For generating foregut spheroids, plate 150,000 cells per well in a Nunclon surface 24-well plate. For generating mid-hindgut spheroids, plate 100,000 cells per well. Include the proper controls in the 24-well plate. Place coverslips on various wells to be used for the experiments.
8. To ensure even dispersal of cells in the wells, shake three times clockwise, three times counter clockwise, three times back and forth, and three times side to side. Check cells the next day. Proceed to Subheading 3.3.

3.3 Directed Differentiation of ES into Human DE

1. Replace media with Day 1 media for foregut differentiation. For mid-hindgut differentiation replace media with mTeSR1, incubate for 24 h, and then add Day 1 media. Note that Day 1 media differs depending on the type of tissue to be generated.
2. After 24 h in Day 1 media, cell death should be evident. The monolayer will appear sparse, but colonies of cells will have expanded. Aspirate Day 1 media and replace with Day 2 media (*see* **Note 2**). After 48 h cell death should still be evident. Cells will form a monolayer and should be 80–95% confluent. Aspirate Day 2 media and replace with Day 3 media.
3. After 72 h cell death should be minimal and the monolayer should be completely confluent. For convenience, Fig. 2 shows the cellular morphology expected for DE differentiation (DE to be used for mid-hindgut differentiation).

3.4 Differentiation of DE into Posterior Foregut Spheroids

1. Aspirate Day 3 media and replace with posterior foregut differentiation media. Change media every 24 h for a total 3 days and add 2 μM retinoic acid on the final day of posterior foregut differentiation.

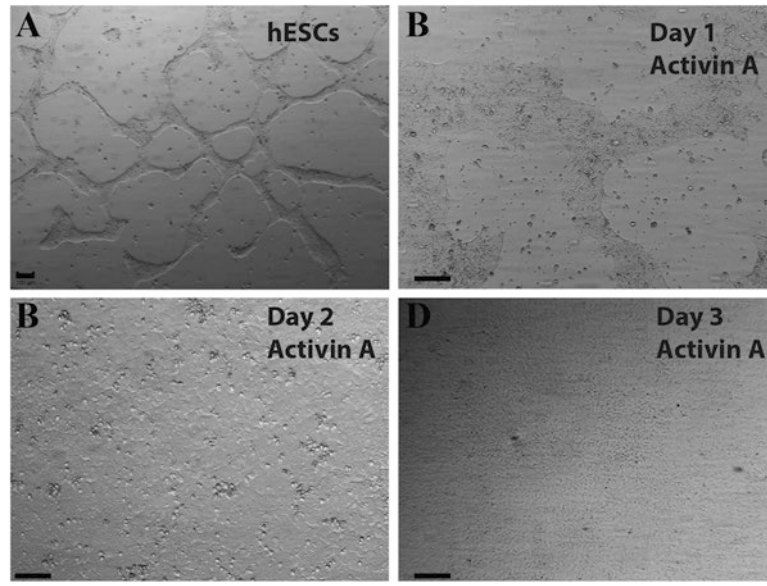

Fig. 2 Generation of definitive endoderm monolayer. Morphology of hESCs prior to starting DE differentiation (**a**). Morphology of cell following Day 1 of Activin A (**b**). Morphology of cell following Day 2 of Activin A (**c**). Morphology of cell following Day 3 of Activin A (**c**)

2. After 24 h, tubelike structures will begin to form in wells treated with Chiron and FGF4. Replace posterior foregut differentiation media.
3. After 48 h floating three-dimensional spheres will be present. Replace posterior foregut differentiation media.
4. After 72 h more floating spheres should be evident.
5. Proceed to Subheading 3.7.

3.5 Differentiation of DE into Mid-Hindgut Spheroids

1. Aspirate Day 3 media and replace with mid-hindgut differentiation media. Change media every 24 h for a total 4 days.
2. After 24 h, tubelike structures will begin to form in wells treated with Chiron and FGF4. Replace midgut-hindgut differentiation media.
3. After 48 h floating three-dimensional spheres will be present. Replace midgut-hindgut differentiation media.
4. After 72 h more floating spheres should be evident. Replace midgut-hindgut differentiation media. After 96 h hundreds of floating spheres should be evident. Fig. 3 shows the expected morphology during mid-hindgut differentiation.
5. Proceed to Subheading 3.7.

3.6 Immunostaining of DE Monolayer

1. Remove the plastic coverslip from 24-well dish containing cells and place it in a new 24-well dish.
2. Wash cells with PBS by carefully dispensing 0.5 mL into each well.

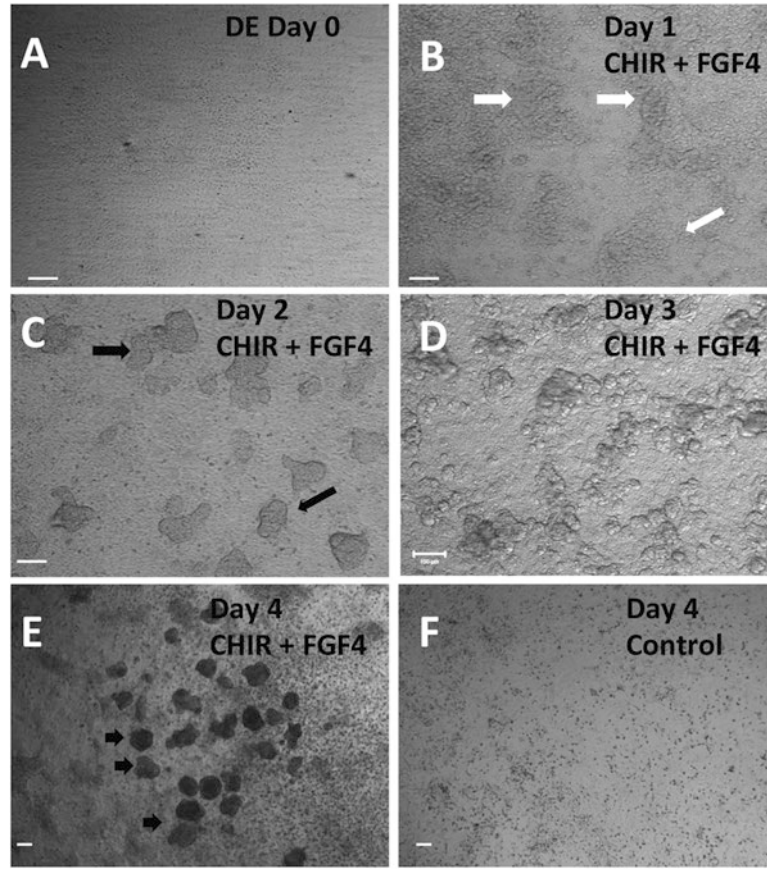

Fig. 3 Mid-hindgut spheroid formation. Spheroid formation from DE treated with mid-hindgut differentiation for 4 days with (**a–e**) and without (**a, f**) Chiron and FGF4. *White arrows* point to branching structures while *black arrows* point to budding structures

3. Aspirate PBS and add 0.5 mL of 4% (wt/vol) paraformaldehyde in PBS for 10 min at room temperature to fix cells.
4. Aspirate paraformaldehyde and perform three 5-min washes with PBS.
5. Aspirate PBS and block cells with 0.2 mL of immunostaining blocking buffer for 30 min at room temperature.
6. Aspirate blocking buffer.
7. Dilute primary antibodies listed in Subheading 2.6 in fresh blocking buffer (mouse anti-CDX2 1:300–500, goat anti-SOX17 1:500, rabbit anti-FOXA2, 1:1000). Vortex briefly to resuspend antibody solutions. Apply 0.2 mL of diluted antibody per well.
8. Incubate overnight at 4 °C in a rocking platform.
9. Aspirate primary antibodies and perform three 5-min washes with PBS. Aspirate the PBS.

10. Dilute secondary antibodies in fresh blocking buffer and add Draq5 nuclear stain (donkey anti-goat DyLight 488 1:500, donkey anti-rabbit Cy3 1:500, Draq5 1:1000). Vortex briefly to resuspend antibody solutions. Apply 0.2 mL of diluted antibody per well.
11. Incubate at room temperature for 2 h.
12. Aspirate secondary antibodies and perform three 5-min washes with PBS. Aspirate the PBS.
13. Use forceps to remove the coverslip with cells and mount on a microscope slide using Fluoromount-G.
14. Visualize cells under a fluorescent microscope. DE should stain positive for both FOXA2 and SOX17. Determine the percentage of DE present by quantitating the number of FOXA2/SOX17—double-positive cells divided by the total number of Draq5-positive cells. Use the same method to determine percentage of CDX2- and SOX2-positive cells.

3.7 Pro-Gastric Growth of Posterior Foregut Spheroids

1. Aliquot Matrigel into cold microcentrifuge tubes.
2. Pre-warm 24-well Nunclon surface plates by placing in 37 °C tissue culture incubator (*see* **Note 3**).
3. Transfer appropriate number of spheroids (50) per 50 μL of Matrigel into a 1.7-mL tube. For spheroids, wash once with 1 mL of mid-hindgut differentiation media without growth factors. Allow spheroids to settle by gravity and remove as much supernatant as possible.
4. Pipette spheroids or single-cell suspension into and aliquot of Matrigel and mix by slowly pipetting up and down 3–5 times. Be careful not to introduce bubbles into the Matrigel.
5. Plate spheroids carefully by touching the pipette tip to the bottom of the wells and slowly lifting as Matrigel mixture is dispensed (*see* **Note 4**).
6. Place plate back in 37 °C incubator being careful not to disturb the Matrigel droplets. Incubate for 10–15 min to allow Matrigel to solidify.
7. Add 0.5 mL per well of antral specification media.
8. Change media after 3 days and replace with gastrointestinal growth media with EGF.

3.8 Pro-Intestinal Growth of Mid-Hindgut Spheroids

1. Aliquot 750 μL of Matrigel into cold microcentrifuge tubes. This volume will be enough to plate 12 wells of spheroids.
2. Pre-warm 24-well Nunclon surface plates by placing in 37 °C tissue culture incubator.
3. Pipette 240 μL of spheroids into an aliquot of Matrigel and mix by slowly pipetting up and down 3–5 times. Be careful not to introduce bubbles into the Matrigel.

4. Plate spheroids carefully by touching the pipette tip to the bottom of the wells and slowly lifting as Matrigel mixture is dispensed (*see* **Note 4**).
5. Place plate back in 37 °C incubator being careful not to disturb the Matrigel droplets. After 5 min flip plate upside down to prevent spheroids from settling to the bottom of the well. Incubate for an additional 25 min to allow Matrigel to solidify.
6. Add 0.5 mL per well of intestine growth media with growth factors.

3.9 Splitting of HGOs and HIOs

1. After approximately 20 d for HGOs and 14 d for HIOs, organoids will have degraded the Matrigel droplet. To allow further expansion, split organoids and replate at a lower density (5–10 organoids per well) into fresh Matrigel.
2. Cut the tip of a 200-μL pipette to increase the bore size of the pipette. Make sure the bore size is large enough to allow organoids to pass through the tip without sustaining damage (*see* **Note 5**).
3. Dislodge the Matrigel bead containing organoids from each well using the cut 200 μL pipette.
4. Pipette the Matrigel bead up and down 3–5 times to dissociate organoids from the Matrigel.
5. Pipette mixture of organoids, Matrigel, and growth media into a 10 cm petri dish with 10 mL of warm Advanced DMEM-F12. For intestinal organoids proceed to **step 7**.
6. For HGOs, use a sterile tungsten needle or sterile fine forceps to dislodge any large pieces of Matrigel which are still attached to the organoids.
7. Using a cut 200-μL pipette tip, collect 5–10 organoids in as little medium as possible.
8. Replate HGOs as was done in Subheading 3.7, **step 4–7**. Replate HIOs as was done in Subheading 3.8, **step 2–6**.
9. Place plate containing organoids back into the tissue culture incubator at 37 °C and grow tissue for an additional 14 days.
10. Replace gastrointestinal growth medium every 4 d.
11. Once HGOs have reached 34 days of age, they will start to become necrotic, so we suggest using this stage as an end point. HIOs can be grown for several months.

4 Notes

1. Removing differentiation and establishing the optimal density of plating are crucial for proper DE induction. We suggest trying a few different plating densities to determine which works best.

2. Media should be dispensed on the side of the wells and not directly in the center of the well since this can disrupt the DE monolayer.
3. Pre-warming plates allows Matrigel to solidify faster.
4. Use care when dispensing Matrigel. Avoid touching the sides of the well as this will prevent formation of the Matrigel bead.
5. Cut pipette tip and minimize the amount of cracking on the tip. Matrigel, HGOs, and HIOs tend to get lodged in cracks.

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Chapter 13

Generation of a Three-Dimensional Kidney Structure from Pluripotent Stem Cells

Yasuhiro Yoshimura, Atsuhiro Taguchi, and Ryuichi Nishinakamura

Abstract

The kidney is a vital organ that has an important role in the maintenance of homeostasis by fluid volume regulation and waste product excretion. This role cannot be performed without the three-dimensional (3D) structure of the kidney. Therefore, it is important to generate the 3D structure of the kidney when inducing functional kidney tissue or the whole organ from pluripotent stem cells. In this chapter, we describe the detailed methods to induce kidney progenitor cells from pluripotent stem cells, which are based on embryological development. We also provide a method to generate 3D kidney tissue with vascularized glomeruli upon transplantation.

Key words Three-dimensional kidney structure, Nephron progenitor cells, Mouse embryonic stem cells, Human-induced pluripotent stem cells, Transplantation

1 Introduction

The kidney contains a large number of nephrons, functional units of the kidney, which consist of glomeruli and renal tubules. The main functions of the kidney, including filtration, reabsorption, and excretion, are performed within the nephrons. Patients with end-stage renal disease have lost these functions and need to be treated with dialysis or kidney transplantation. Research involving stem cell-induced kidneys has the potential to realize new therapies for patients.

The mammalian adult kidney is derived from the embryonic metanephros. It is generated from two different progenitor populations: the metanephric mesenchyme (MM) and ureteric bud (UB) [1]. The progenitor cells of the nephron epithelia exist in the MM and are induced to differentiate into glomerular epithelial cells and renal tubular epithelial cells by Wnt signaling from the UB [2]. Meanwhile, the UB differentiates into the epithelia of the collecting duct and ureter. To create a kidney structure from pluripotent stem

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cells based on embryological development, it is necessary to induce the progenitor cells including nephron progenitor cells.

Recently, we have shown that the mouse MM develops through *T* (Brachyury)-positive cells within the caudal portion of embryonic day (E) 8.5 embryos and posterior intermediate mesoderm of E9.5 embryos. These findings enabled us to establish an induction method for nephron progenitor cells from pluripotent stem cells (Fig. 1a) to reconstitute the three-dimensional (3D) structure of the kidney in vitro, including glomerular epithelial cells and renal tubules [3]. Moreover, when the induced nephron progenitor cells were transplanted into the subrenal capsule of immunodeficient mouse, the glomeruli were vascularized with host-derived endothelial cells [4]. These induced nephron structures are still immature compared with those in the adult. However, our methods are applicable to various experiments in the field of kidney research such as organ generation, disease modeling, and drug screening.

In this chapter, we provide a detailed protocol to induce a 3D kidney structure from pluripotent stem cells [mouse embryonic stem (ES) cells and human-induced pluripotent stem (iPS) cells] through nephron progenitor cells. Furthermore, we describe a method for transplantation of nephron progenitor spheres into the subrenal capsule of a host mouse.

2 Materials

1. Mouse ES cells (*see* **Note 1**).
2. Human iPS cells (*see* **Note 2**).
3. Pregnant mice: Mouse embryonic age is based on the day of the appearance of a vaginal plug in a pregnant mouse, which is defined as E0 (*see* **Note 3**).
4. Immunodeficient NOD/SCID/IL2R-γ chain null mice or NOD/SCID/JAK-3 null mice.
5. Mouse ES cell maintenance medium: Dulbecco's modified Eagle's medium (DMEM; High Glucose, Pyruvate) supplemented with 15% fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, and 1000 U/mL leukemia inhibitory factor (LIF; EMD Millipore, ESG1107).
6. 2i Medium: DMEM supplemented with 15% FBS (*see* **Note 4**), 0.1 mM 2-mercaptoethanol, 1000 U/mL LIF, 3 μM CHIR99021 (Axon Medchem, Cat# Axon1386), and 1 μM PD0325901 (Wako, Cat# 163-24001).
7. Serum-free differentiation medium for mouse ES cells: 75% Iscove's modified Dulbecco's medium (IMDM) and 25% Ham's F12 medium with 0.5× N2 (Thermo Fisher Scientific), 0.5× B27 without retinoic acid (Thermo Fisher Scientific),

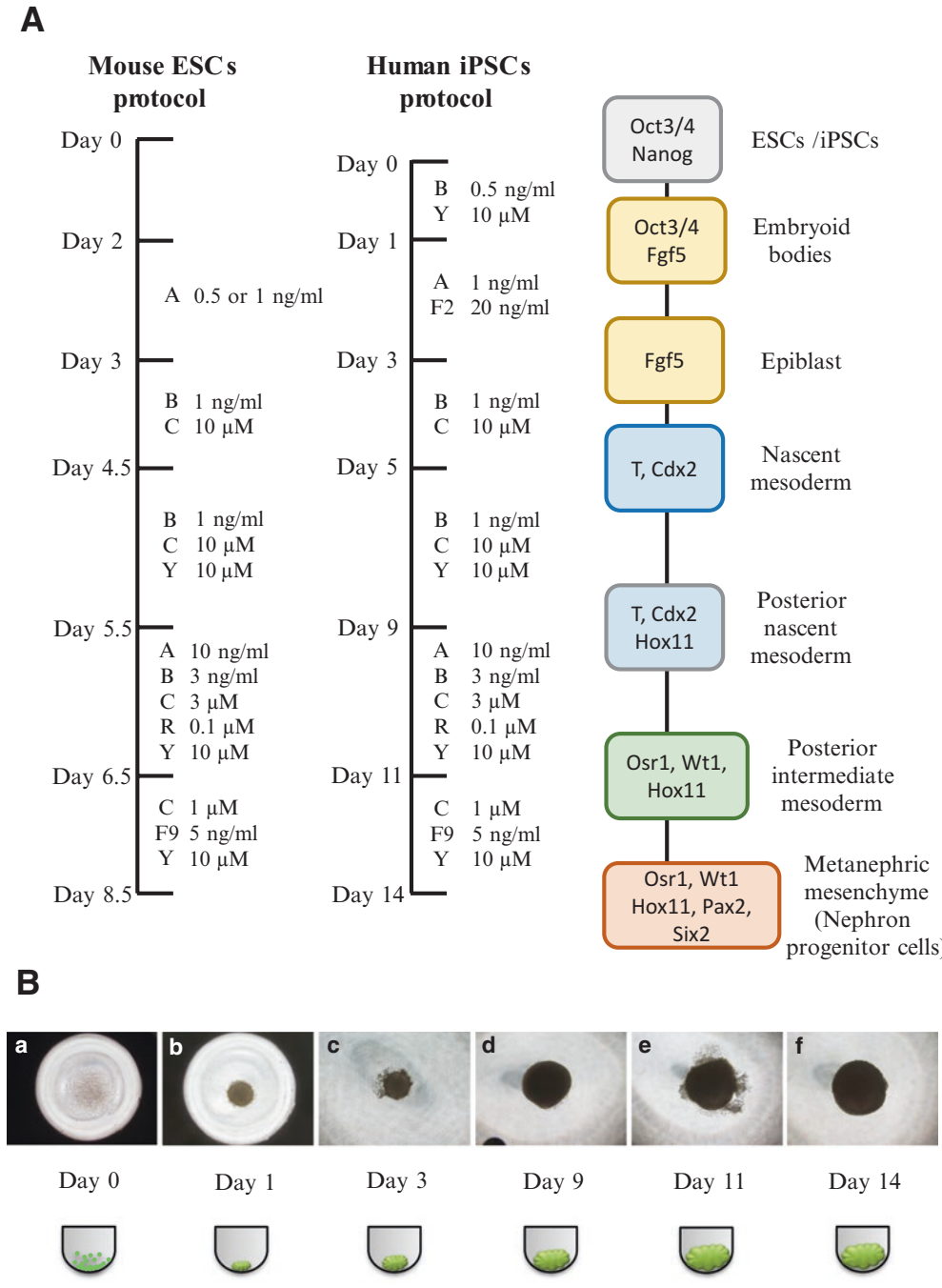

Fig. 1 Induction of nephron progenitor cells from mouse ES cells and human iPS cells. (**a**) Outline of the protocol. Induced cells differentiate into nephron progenitor cells at Day 8.5 of the mouse ES cell protocol or Day 14 of the human iPS cell protocol. *A* Activin, *B* BMP4, *C* CHIR99021, *F2* FGF2, *F9* FGF9, *R* retinoic acid, and *Y* Y27632. (**b**) Images and schematic diagrams of the time course of human iPS cell differentiation. The cells aggregate and form a small sphere at Day 1. The sphere gradually becomes larger over time

0.5× penicillin/streptomycin, 0.05% bovine serum albumin, 2 mM l-glutamine, 0.5 mM ascorbic acid, and 4.5×10^{-4} M 1-thioglycerol (*see* **Note 5**).

8. Primate ES medium (ReproCELL) and recombinant human basic fibroblast growth factor (FGF; Wako, Cat# 064-04541) for feeder-dependent human iPS cell maintenance.
9. StemFit AK02N (Ajinomoto) for feeder-free human iPS cell maintenance.
10. iMatrix-511 (Nippi).
11. Serum-free differentiation medium for human iPS cells: DMEM/F12 with 2% (vol/vol) B27 without retinoic acid, 2 mM l-glutamine, 1% (vol/vol) ITS (Thermo Fisher Scientific), 1% (vol/vol) nonessential amino acids (Thermo Fisher Scientific), 90 μM 2-mercaptoethanol, and 0.5% (vol/vol) penicillin/streptomycin.
12. Medium for mouse embryo dissection and spinal cord induction: DMEM containing 10% FBS.
13. 0.25% Trypsin–EDTA.
14. Dissociation Solution (ReproCELL).
15. Accutase (EMD Millipore).
16. Type IV collagenase diluted with 1× Hank's balanced salt solution at 10 mg/mL.
17. Growth factors: Recombinant human Activin A (R&D Systems, Cat# 338-AC), recombinant human bone morphogenetic protein (BMP) 4 (R&D Systems, Cat# 314-BP), CHIR99021, retinoic acid (Sigma-Aldrich, Cat# R2625), Y27632 (Wako, Cat# 257-00511), recombinant human basic FGF (R&D Systems, Cat# 233-FB), recombinant human FGF-9 (R&D Systems, Cat# 273-F9), and recombinant human vascular endothelial growth factor (VEGF; R&D Systems, Cat# 293-VE).
18. Agarose.
19. D-PBS(−).
20. Hematocrit tubes (Drummond Scientific Company, Cat# 1-000-7500-C/5).
21. 18-G indwelling needle.
22. Cell counter.
23. Surgical tools: Large surgical scissors, small surgical scissors, microdissecting spring scissors, tissue forceps, microdissecting forceps, razor, tungsten needle, 20-G plastic indwelling needle, surgical clip, bulldog artery spring clip, electric cautery, and heating plate.
24. Dissecting microscope.
25. Sterile disposable 35 and 60 mm Petri dishes.
26. Sterile disposable 15 mL polypropylene conical tubes.

27. Sterile disposable 1.5 mL microtubes.
28. Sterile disposable 24- and 96-well plates.
29. Sterile disposable 96-well U-bottom low cell binding plates (Thermo Fisher Scientific, Cat# 174929).
30. Sterile disposable 96-well V-bottom low cell binding plates (Sumitomo Bakelite, Cat# MS-9096V).
31. Sterile disposable polycarbonate membrane filter, 13 mm and 0.8 μm (Whatman, Cat# 110409).
32. Sterile micropipette.
33. Combination anesthetic: Normal saline with 0.75 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol..

3 Methods

3.1 Nephron Induction from Mouse ES Cells

3.1.1 Maintenance Culture of Mouse ES Cells

1. Seed mitomycin C-treated mouse embryonic fibroblasts (MEFs) at 1 day before passaging (1.2×10^6 cells per 0.1% gelatin-coated 60 mm dish).
2. Thaw mouse ES cells in mouse ES cell maintenance medium.
3. Passage mouse ES cells every 2 or 3 days using 0.25% Trypsin-EDTA for dissociation.

3.1.2 Maintenance in 2i Medium Before Differentiation

1. Before the induction of differentiation, passage mouse ES cells onto feeder-free gelatin-coated dishes in 2i medium (3×10^5 cells for 6-well plate or 35 mm dish in 2 mL of medium) (*see* **Notes 6** and 7).
2. Culture mouse ES cells in 2i medium for 2 days.

3.1.3 Differentiation into Nephron Progenitor Cells from Mouse ES Cells

(Day 0)

1. Aspirate the medium, and wash the cultured mouse ES cells once with 2 mL of PBS(−). Aspirate to remove the PBS(−).
2. Add 300 μL of Accutase, and incubate for 5 min at 37 °C in a 5% CO_2 incubator.
3. Stop the enzymatic reaction by adding 1.7 mL of differentiation medium, and collect the cell suspension in a 15 mL tube using a micropipette and P1000 tip.
4. Take a sample (10 μL) of the cell suspension, and count the cells.
5. Centrifuge the remaining cell suspension at $210 \times g$ for 4 min, and then discard the supernatant.
6. Add differentiation medium to the cell pellet to obtain a cell density of 2×10^4 cells/mL (1000 cells/50 μL), and pipette using a micropipette and P1000 tip (*see* **Note 8**).

7. Seed 50 μL of the cell suspension into each well of a 96-well U-bottom low cell binding plate.

(Day 2)

8. After 48 h, harvest the spheres using a micropipette with a P200 tip in a 15 mL tube, and wash with 2 mL of PBS(−).
9. Centrifuge the spheres at 210 × *g* for 1 min, and then discard the supernatant.
10. Add 500 μL of Accutase, and incubate for 5 min in a 37 °C water bath.
11. Stop the enzymatic reaction by adding 1.5 mL of differentiation medium, and pipette five times to dissociate into single cells using a micropipette and P1000 tip.
12. Take a sample (10 μL) of the cell suspension, and count the cells.
13. Centrifuge the remaining cell suspension at 210 × *g* for 4 min, and then discard the supernatant.
14. Add differentiation medium containing 0.5 or 1 ng/mL human Activin A to obtain a cell density of 1×10^5 cells/mL (1×10^4 cells/100 μL).
15. Seed 100 μL of the cell suspension into each well of a 96-well low cell binding plate (*see* **Note 9**).

(Day 3)

16. After 24 h, transfer the spheres into another 96-well U-bottom low cell binding plate. Fill each well with 100 μL of BC10 medium containing 1 ng/mL human BMP4 and 10 μM CHIR99021 (*see* **Note 10**).

(Day 4.5)

17. Transfer the spheres into 100 μL of BC10Y medium containing 1 ng/mL human BMP4, 10 μM CHIR99021, and 10 μM Y27632 (*see* **Notes 11** and **12**).

(Day 5.5)

18. Transfer the spheres into 150 μL of ABC3RY medium containing 10 ng/mL Activin A, 3 ng/mL BMP4, 3 μM CHIR99021, 0.1 μM retinoic acid, and 10 μM Y27632 (*see* **Note 13**).

(Day 6.5)

19. Transfer the spheres into 200 μL of C1F9Y medium containing 1 μM CHIR99021, 5 ng/mL human FGF9, and 10 μM Y27632.
20. Culture the spheres for 2 days to induce nephron progenitor cells.

3.1.4 Harvesting a Mouse Embryonic Spinal Cord

(Day 8.5)

1. Euthanize a pregnant mouse (E12.5) by cervical dislocation.
2. Wash the abdomen with 70% EtOH, and make an incision along the midline. Cut the peritoneum (Fig. 2A-a), and resect the uterus from the abdominal cavity (*see* **Note 14**). Place the uterus in a 60 mm Petri dish with 5 mL of cold DMEM containing 10% FBS.
3. Cut the uterus between each embryo, and dissect the embryos from the uterus (Fig. 2A-b,c).
4. Amputate the fetal head and tail from the body, and lay the embryo in a spine position (Fig. 2A-d; *see* **Note 15**).
5. Remove the viscera inside the chest and abdomen, such as the heart, lungs, liver, and intestines (Fig. 2A-e).
6. Remove the urogenital system (mesonephros and metanephros) and aorta (Fig. 2A-f). After removing them, you can observe the appearance of the spinal cord (Fig. 2A-g).
7. Set the embryo in the prone position. Resect both sides of the somites from the spinal cord using a curved tungsten needle (Fig. 2A-h).
8. Resect the notochord from the ventral side of the spinal cord. Transfer the naked spinal cord into a 35 mm Petri dish with 2 mL of cold DMEM containing 10% FBS (Fig. 2A-i).
9. Cut the spinal cord into small pieces using small scissors (Fig. 2A-j).

   (Move onto the next step immediately).

3.1.5 3D Nephron Induction Using the Mouse Embryonic Spinal Cord

1. Prewarm 1 mL of DMEM containing 10% FBS with 1% penicillin/streptomycin in each well of a 24-well plate at 37 °C in a 5% CO_2 incubator.
2. Collect the induced spheres of nephron progenitor cells in a 60 mm dish with 4 mL of DMEM containing 10% FBS using a cut P20 tip (Fig. 2B–a).
3. Place a small drop of 2 μL DMEM onto the cover of the 96-well plate (Fig. 2B-b). Place a 0.8 μm polycarbonate filter onto each drop (Fig. 2B-c; *see* **Notes 16** and **17**).
4. Transfer a piece of spinal cord onto the filter using a cut P20 tip (Fig. 2B-d).
5. Place the induced spheres adjacent to the cut edge of the spinal cord (Fig. 2B-e). Attach the sphere directly to the spinal cord by aspirating residual medium using a micropipette and P10 tip (*see* **Note 18**).
6. Set the filter afloat on the air-fluid interface of the prewarmed medium (Fig. 2B-f).

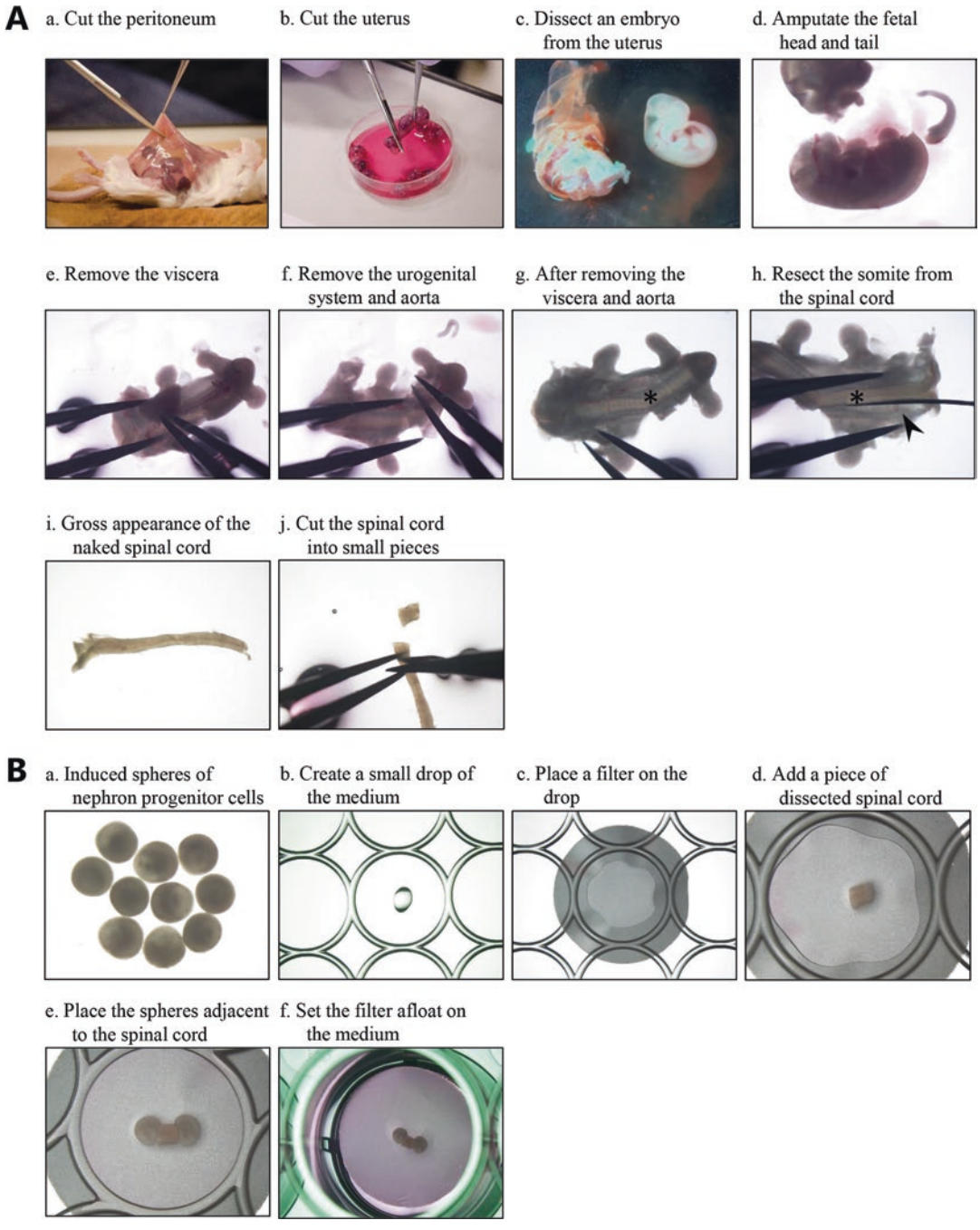

Fig. 2 3D kidney induction from nephron progenitor spheres by combining with the mouse embryonic spinal cord. (**A**) Procedure for isolation of the mouse embryonic spinal cord. An E12.5 embryo was used for dissection. *Asterisks*, spinal cord (*g, h*) and *arrowhead*, somite being removed (*h*). (**B**) Procedure for starting the culture of nephron progenitor spheres with a piece of dissected spinal cord

7. Change half of the medium (0.5 mL) every 3 days.
8. If you have succeeded to induce nephron progenitors, you will observe an epithelializing nephron in some parts of the sphere from 3 days and a well-epithelialized sphere from 6 days after induction.

3.2 Nephron Induction from Human iPS Cells

1. Seed mitomycin C-treated MEFs at 1 day before passaging (0.4×10^6 cells per 0.1% gelatin-coated 60 mm dish).
2. Thaw human iPS cells in Primate ES medium supplemented with 5 ng/mL recombinant human basic FGF and 1% penicillin/streptomycin. Change the medium every 24 h.
3. Passage the human iPS cells every 3 days using Dissociation Solution.

3.2.1 Maintenance Culture of Feeder-Dependent Human iPS Cells

3.2.2 Harvesting Human iPS Cells from MEFs (Feeder-Dependent Culture)

1. Add type IV collagenase to the culture medium at a final concentration of 1 mg/mL. Wait 1.5–2 h until 70–80% of the clumps have detached from the MEFs (*see* **Note 19**).
2. Detach the attached colonies by gently pipetting the medium. Harvest the colonies in a 15 mL tube.
3. Keep the harvested cell suspension still for 10 min. Remove the supernatant to deplete floating feeder cells.
4. Add 2 mL of PBS(−), and centrifuge at 210 × *g* for 4 min. Discard the supernatant.
5. Add 500 μL of Accutase, and incubate the cells for 5 min in a 37 °C water bath.
6. Stop the enzymatic reaction by adding 1.5 mL of differentiation medium. Gently pipette several times to dissociate into single cells using a micropipette and p1000 tip.
7. Take a sample (10 μL) of the cell suspension, and count the cells.
8. Centrifuge the remaining cell suspension at 210 × *g* for 4 min, and then discard the supernatant.

   (Move on to Subheading 3.2.5 immediately).

3.2.3 Maintenance Culture of Feeder-Free Human iPS Cells

1. Maintain human iPS cells in StemFit supplemented with 1% penicillin/streptomycin on an iMatrix-511-coated culture dish. Change the medium every 48 h.
2. Passage the human iPS cells every 6–8 days (2.8×10^4 cells per 60 mm dish in 4 mL of StemFit medium). Add 10 μM of Y27632 to the maintenance medium during 1 or 2 days after passaging.

3.2.4 Harvesting Human iPS Cells (Feeder-Free Culture)

1. Add Y27632 to the culture medium at a final concentration of 10 μM. Incubate the dish at 37 °C in a 5% CO_2 incubator for 60 min.

2. Aspirate the medium, and wash the cultured iPS cells twice with 4 mL of PBS(−). Aspirate to remove the PBS(−).
3. Add 500 μL of Accutase, and incubate for 5–10 min at 37 °C in a 5% CO_2 incubator (*see* **Note 20**).
4. Aspirate the Accutase.
5. Add 2 mL of PBS(−) (1 mL for a 35-mm dish), and detach the iPS cells by gently flushing with PBS(−).
6. Harvest the detached cells in a 15 mL tube with 6 mL of maintenance medium for iPS cell culture.
7. Repeat **steps 5** and **6** twice.
8. Gently pipette using a 10 mL pipette. Take a sample (10 μL) of the cell suspension, and count the cells.
9. Centrifuge the remaining cell suspension at 210 × *g* for 4 min, and then discard the supernatant.

   (Move on to the next step immediately).

3.2.5 Differentiation into Nephron Progenitor Cells from Human iPS Cells

(Day 0)

1. Add differentiation medium containing 0.5 ng/mL human BMP4 and 10 μM Y27632 to obtain a cell density of 1×10^5 cells/mL (1×10^4 cells/100 μL) (*see* **Notes 21** and **22**).
2. Seed 100 μL of the cell suspension into each well of a 96-well V-bottom low cell binding plate.
3. Centrifuge the plate at 210 × *g* for 4 min, and culture at 37 °C in a 5% CO_2 incubator (Fig. 1B–a).

(Day 1)

4. After 24 h, transfer the spheres to 150 μL of AF2 medium containing 1 ng/mL Activin A and 20 ng/mL human FGF2 (Fig. 1B-b; *see* **Note 23**).

(Day 3)

5. After 48 h, transfer the spheres to 200 μL of BC10 medium containing 1 ng/mL BMP4 and 10 μM CHIR99021 (Fig. 1B-c; *see* **Notes 24** and **25**).

(Days 5 and 7)

6. Replace half (100 μL) of the medium with BC10Y medium containing 1 ng/mL BMP4, 10 μM CHIR99021, and 10 μM Y27632 (*see* **Notes 12**, **26**, and **27**).

(Day 9)

7. Transfer the spheres to 200 μL of ABC3RY medium containing 10 ng/mL Activin A, 3 ng/mL BMP4, 3 μM CHIR99021, 0.1 μM retinoic acid, and 10 μM Y27632 (Fig. 1B-d; *see* **Note 28**).

(Day 11)

8. Transfer the spheres to 200 μL of C1F9Y medium containing 1 μM CHIR99021, 5 ng/mL human FGF9, and 10 μM Y27632 (Fig. 1B-e; *see* **Note 29**).

3.2.6 Extraction of a Mouse Embryonic Spinal Cord

(*See* Subheading 3.1.3)

3.2.7 3D Nephron Induction Using the Mouse Embryonic Spinal Cord

(Day 14)

(*See* Subheading 3.1.4)

If you have succeeded to induce nephron progenitors, you will observe an epithelializing nephron in some parts of the sphere from 3 days and well-epithelialized spheres from 9 days after induction (Fig. 3a–e).

3.3 Transplantation of Nephron Progenitor Cells

1. Induce nephron progenitor spheres (*see* Subheadings 3.1 and 3.2). (1 day before transplantation).
2. Attach induced spheres to a mouse embryonic spinal cord, and culture for 1 day to initiate tubulogenesis (*see* Subheadings 3.1.3 and 3.1.4).
3. Dissolve agarose powder in PBS(−) to prepare 4% agarose (*see* **Note 30**).

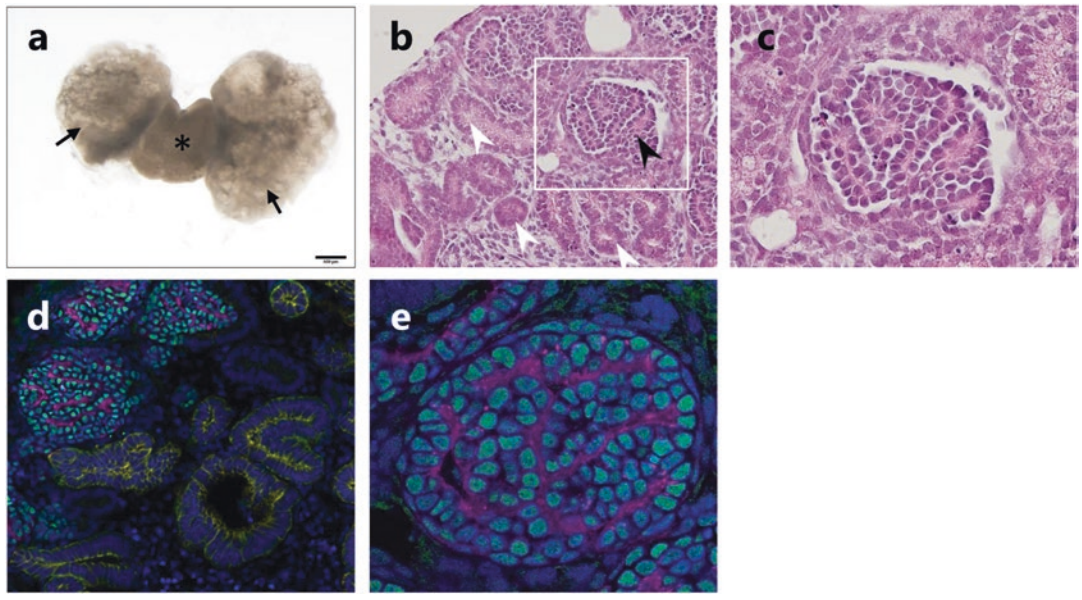

Fig. 3 Induced 3D kidney tissue from human iPS cells. (**a**) Image of an induced kidney (*black arrows*) at Day 9 after initiation of coculture with the mouse embryonic spinal cord (*asterisk*). (**b**, **c**) Hematoxylin and eosin staining of induced kidney tissue at Day 9. The white square in the lower magnification image (**b**) indicates the higher magnification image (**c**). The *black arrowhead* indicates the glomerulus; *white arrowheads* indicate renal tubules. (**d**, **e**) Immunohistochemistry of induced kidney tissue at Day 9. *Magenta*, nephrin (glomerulus marker); *green*, Wt1 (glomerulus marker); and *yellow*, E-cadherin (distal renal tubule marker)

4. Aspirate the 4% agarose solution into hematocrit tubes using a syringe (Fig. 4a; *see* **Note 31**). Incubate them at room temperature to gelatinize the agarose.
5. Push out the gelatinized agarose rod from the tubes.
6. Soak the rods in DMEM containing 10% FBS and 5 ng/mL recombinant VEGF overnight at 4 °C (*see* **Note 32**).

   (The day of transplantation).
7. Anesthetize the host mouse (immunodeficient NOD/SCID/IL2R-γ chain null mice or NOD/SCID/JAK-3 null mice) by an injection of the combination anesthetic (0.75 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol). Place the mouse in the prone position, and immobilize the hands and feet with tape.
8. Shave the back hair around the kidney with a razor.
9. Make an 8–10 mm incision in the dorsal skin with microscissors.
10. Incise the fascia, and draw out the kidney. Clamp the edge of the incised skin with bulldog spring clamp to keep the kidney out of the abdomen (Fig. 4b; *see* **Note 33**).
11. Incise the outer membrane of the subrenal capsule using a 24-G needle at approximately 2 mm at the caudal region of the kidney (Fig. 4c).
12. Flush the subcapsular space with approximately 500 μL of DMEM containing 10% FBS using a 24-G plastic indwelling needle connected to a 1 mL syringe (*see* **Note 34**).
13. Insert the rods carefully using forceps to place in a V-shaped position (Fig. 4d, e).
14. Cauterize the inserted rods to the renal capsule membrane briefly with an electric cautery (Fig. 4f; *see* **Note 35**).
15. Insert the induced nephron progenitor spheres with mouse embryonic spinal cords from the incised window with a 20-G plastic indwelling needle connected to a P-200 micropipette (Fig. 4g; *see* **Note 36**).
16. Return the host kidney to the retroperitoneal space. Suture the fascia, and close the skin with a wound closure clip (Fig. 4h).
17. After 20 days, vascularized glomeruli and renal tubules will be developed in the subrenal capsule.

4 Notes

1. Differentiation efficiency may differ between cell lines and/or maintenance culture conditions, both of which affect the endogenous signaling levels. Thus, if your cells do not differentiate properly, fine-tune the concentrations of several growth factors as described below.

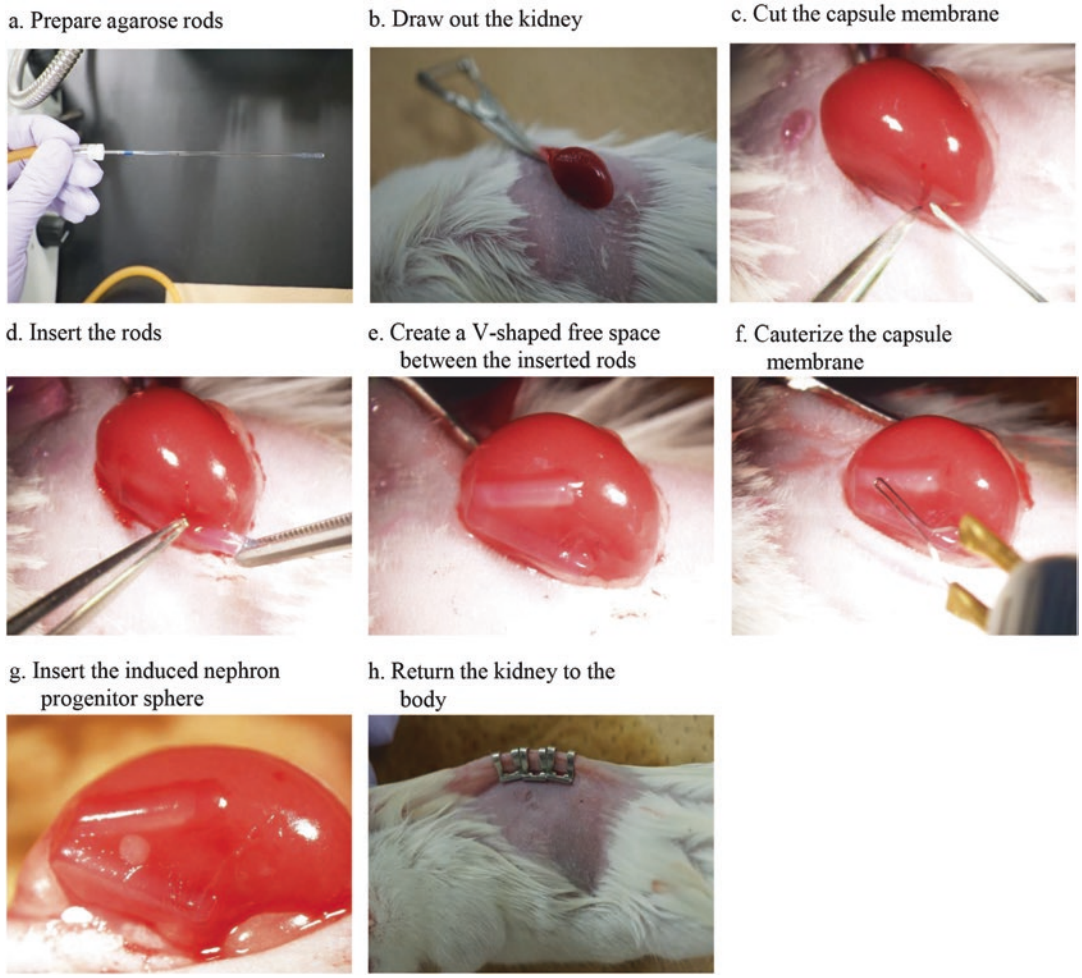

Fig. 4 Procedure for subrenal capsule transplantation to generate vascularized glomeruli. Agarose rods prepared in the hematocrit tube (**a**) are inserted into the subrenal capsule space (**b**-**d**) to release the tension of capsule membrane. The rods are arranged to form a V-shape (**e**), followed by cauterization with renal capsule membrane (**f**). The induced nephron progenitor sphere is inserted to the space between the rods (**g**). After transplantation, the host kidney is returned to the body (**h**)

2. This protocol was originally optimized for the 201B7 cell line. Differentiation efficiency may differ between cell lines and/or maintenance culture conditions, both of which affect endogenous signaling levels. Although we have confirmed that our protocol works with several human iPS cell lines, if your cells do not differentiate properly, fine-tune the concentrations of several growth factors as described below.
3. We generally use E12.5 or E11.5 pregnant ICR mice to harvest embryonic spinal cords.
4. We use serum-containing 2i medium, while commonly used LIF + 2i medium does not contain serum, because we only use this medium to remove feeder cells.

5. IMDM appears to deteriorate over time. Use IMDM within the expiration date, and use the mixed differentiation medium within 2 weeks.
6. Gelatin coating should be performed for at least 15 min at room temperature.
7. For certain cell lines, this condition may not be suitable to maintain mouse ES cells for a long period of time. Thus, we maintain mouse ES cells on MEFs and transfer to this condition just once before the differentiation.
8. The initial cell number affects the differentiation efficiency. If your cell line does not differentiate properly, decrease or increase the initial cell number.
9. If the differentiation efficiency is not sufficient using your cell line, try combinations of 0.5, 1, or 2 ng/mL Activin A without or with 0.1–0.3 ng/mL BMP4.
10. Transfer the spheres using a micropipette and P20 tip with minimal carry-over of the previous medium.
11. The size of the spheres increases gradually. You should cut the P20 tip to an appropriate size to avoid disrupting the spheres. Transfer the spheres to the other wells using a micropipette and cut P-20 tip.
12. Addition of Y27632 in this step is the result of induction method development using dissociated mouse embryos.
13. During this culture condition, you may observe dying cells, especially in the peripheral of the sphere. However, it is not problematic as long as most cells of the sphere are viable.
14. When you incise the peritoneum, you should be careful not to injure the intestines.
15. Other mice should be preserved in cold medium on ice.
16. This drop is used to immobilize the filter.
17. If manipulation of the floating membrane is difficult, you can use Transwell inserts.
18. Take care not to wet the edge of the filter before floating. Otherwise, the filter may sink.
19. Over time, most of the individual colonies begin to float as a clump. Observe every 15 min after 1 h of incubation.
20. Check the dissociation state by pipetting a small amount of Accutase over the colonies. If the colonies are smoothly detached from the dish, you can stop the reaction. If not, continue the incubation for 2-min intervals.
21. The initial cell number affects the differentiation efficiency. If your cell line does not differentiate properly, decrease or increase the initial cell number.

22. If the differentiation efficiency is not sufficient for your cell line, adjust the BMP4 concentration (0, 1, and 2 ng/mL).
23. Hereafter, transfer the spheres to another well filled with new medium in the 96-well U-bottom low cell binding plate. Use a micropipette and P20 tip to transfer spheres with minimal carry-over of the previous medium.
24. The size of the spheres increases gradually. You should observe the size of spheres and cut the P20 tip to an appropriate size. Transfer the spheres to other wells using a micropipette, and cut P-20 tip.
25. If the differentiation efficiency is not sufficient for your cell line, adjust the BMP4 concentration (0.1, 0.3, and 3 ng/mL).
26. In this step, you do not have to transfer the spheres to new wells. Discard half of the volume of the medium, and add the same volume of fresh medium.
27. Avoid aspirating the spheres, or they will break.
28. During this culture condition, you may observe dying cells, especially in the periphery of the sphere. However, it is not a problem as long as most of the cells in the sphere are viable.
29. If the procedure was performed successfully, the induced sphere should appear to be somewhat transparent rather than dark brown at Day 14.
30. You can use an autoclave (95 °C for 20 min) if the agarose does not dissolve by microwave boiling.
31. An 18-G indwelling needle is also available instead of the hematocrit tube if the size of the sphere is less than 1 mm.
32. These rods are essential to release the tension of host kidney capsules and secure the space for the transplanted spheres.
33. Avoid clumping the host kidney or renal artery and vein.
34. This step is intended to stretch the subcapsular space.
35. This step is intended to prevent the rods shifting from their initial positions.
36. Cut the plastic needle tip diagonally if the spheres are too large to aspirate.

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Chapter 14

Making a Kidney Organoid Using the Directed Differentiation of Human Pluripotent Stem Cells

Minoru Takasato and Melissa H. Little

Abstract

An organoid can be defined as a three-dimensional organ-like structure formed from organ-specific progenitor cells. Organ progenitor cells were empirically found to self-organize three-dimensional tissues when they were aggregated and cultivated in vitro. While this nature power of progenitor cells has an amazing potential to recreate artificial organs in vitro, there had been difficulty to apply this technology to human organs due to the inaccessibility to human progenitor cells until human-induced pluripotent stem cell (hiPSC) was invented by Takahashi and Yamanaka in 2007. As embryonic stem cells do, hiPSCs also have pluripotency to give rise to any organs/tissues cell types, including the kidney, via directed differentiation. Here, we provide a detailed protocol for generating kidney organoids using human pluripotent stem cells. The protocol differentiates human pluripotent stem cells into the posterior primitive streak. This is followed by the simultaneous induction of posterior and anterior intermediate mesoderm that are subsequently aggregated and undergo self-organization into the kidney organoid. Such kidney organoids are comprised of all anticipated kidney cell types including nephrons segmented into the glomerulus, proximal tubule, loop of Henle, and distal tubule as well as the collecting duct, endothelial network, and renal interstitium.

Key words Kidney organoid, Kidney development, Directed differentiation, Human pluripotent stem cells, Early embryogenesis, Primitive streak, Intermediate mesoderm, Metanephric mesenchyme, Ureteric bud

1 Introduction

The kidney is the organ responsible for filtering blood; regulating fluid, pH, and electrolyte; balancing metabolites; and controlling blood pressure. These functions are controlled by the nephron, the basic functional unit of the kidney, via urine production from blood, with this urine then passing through the collecting duct system. In adult humans, there are approximately 1 million nephrons comprising the normal kidney [1]. This basic kidney unit develops from two progenitor lineages, the metanephric mesenchyme and the Wolffian duct. The Wolffian duct first arises within

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the anterior intermediate mesoderm and extends rostrally concurrent with the elongation of the embryo body [2]. In contrast, the posterior intermediate mesoderm lies next to the anterior intermediate mesoderm and gives rise to the caudal mesonephric mesenchyme and metanephric mesenchyme [3]. In mouse development, these two progenitor lineages meet at embryonic day 10 (E10) to begin their reciprocal interaction, generating the metanephros, the kidney.

The Wolffian duct sprouts a bud, called the ureteric bud, in response to glial cell line-derived neurotrophic factor (Gdnf) secreted from the metanephric mesenchyme [4]. The ureteric bud invades the metanephric mesenchyme and branches to grow into a ureteric tree consisting the ureteric stalk and a number of collecting ducts. During this ureteric bud invasion, the metanephric mesenchyme receives signals back from the ureteric bud, such as Wnt11, Wnt9b, and Fgf9, that support the growth and stemness of the metanephric mesenchyme or induce nephrogenesis [5–7]. Induced metanephric mesenchyme undergoes a mesenchymal-to-epithelial transition (MET) to form a renal vesicle, which is polarized proximodistally, further developing into the glomerulus in the proximal end and the proximal and distal tubules at the other end.

Together with the nephrons and collecting ducts, the kidney also contains blood vessels, which supply the blood to the nephron and retrieve substances from renal tubules, and renal interstitium, which produces hormones and supports blood vessel formation as pericytes. *Foxd1*, a renal interstitial marker, is expressed in the entire intermediate mesoderm and paraxial mesoderm at E8.5 [8], and a lineage tracing study demonstrated that the *Osr1*-positive intermediate mesoderm and/or lateral plate mesoderm contribute to renal interstitium [9]. This *Osr1* lineage tracing study also showed that renal endothelial cells are derived from relatively anterior intermediate mesoderm marked by the *Osr1* between E7.5 and E8.5 [9]. Taken together, all four types of kidney progenitor derive from within the anterior or posterior intermediate mesoderm.

Based upon the above understanding of kidney developmental processes in the mouse, our protocol of generating kidney organoids took the approach of directing the differentiation of human pluripotent stem cells (hPSCs) into the anterior and posterior intermediate mesoderm, the origin of all four renal progenitor populations that self-organize renal structures under appropriate three-dimensional culture conditions (Fig. 1) [10]. The intermediate mesoderm is derived from the posterior primitive streak, which forms in the posterior end of the epiblast during early embryogenesis [11]. As many reports, including ours, showed previously, the posterior primitive streak can be differentiated from hPSCs by small molecules such as canonical WNT agonists, CHIR99021, or a combination of high BMP4 and low Activin A [12–14]. In this protocol, we use 8 μM CHIR99021 in a xeno-free basal medium,

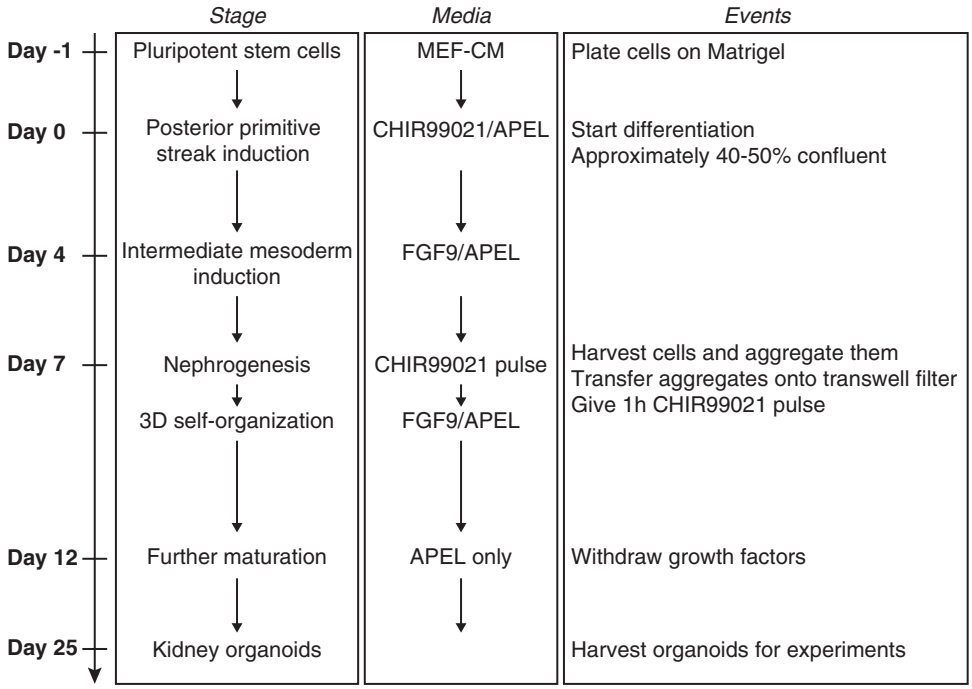

Fig. 1 A protocol outline for directing the differentiation of hPSCs into kidney organoids. A *down arrow* shown on the *left* represents time line of the differentiation that takes 25 days from initial plating of hPSCs to obtaining kidney organoids. "*Stage*" shows differentiation processes of hPCS during the protocol. "*Media*" shows changes of culture media. "*Events*" explains experimental manipulations in each step of the protocol

APEL, to induce *MIXL1*$^+$*T*$^+$ posterior primitive streak. APEL basal medium was originally developed for human embryonic stem cells differentiation into blood lineage via the primitive streak [15]. We demonstrated that at least 2 days of 8 μM CHIR99021 was required to obtain *MIXL1*$^+$ cells at 90% in efficiency [12]. Such induced posterior primitive streak spontaneously develops into mesoderm, especially to *OSR1*$^+$*FOXF1*$^+$ lateral plate mesoderm cells [12]. This propensity, however, can be directed to the *PAX2*$^+$*LHX1*$^+$ anterior intermediate mesoderm lineage by administration of 200 ng/mL FGF9, which is specifically expressed in the intermediate mesoderm in embryos [12, 16]. In the follow-up study, we found that a length of the initial CHIR99021 period decides anteroposterior regionalization of the intermediate mesoderm development in vitro [10, 17]. In other words, less days (2 days) of CHIR99021 administration induces anterior intermediate mesoderm, whereas more days (5 days) generates posterior intermediate mesoderm, whereas an intermediate number of days (3–4 days) forms both anterior and posterior intermediate mesoderm simultaneously [10]. To obtain *GATA3*$^+$ anterior intermediate mesoderm together with *HOXD11*$^+$ posterior intermediate mesoderm, we employ 4 days of 8 μM CHIR99021 followed by 3 days of 200 ng/mL FGF9.

To promote self-organization events generating renal structures under three-dimensional conditions, induced intermediate mesoderm cells are aggregated. Mouse embryonic kidney is typically cultured in vitro at an air–media interface in which explants are placed onto a filter membrane contacting on the culture medium [18–20]. This has been known to result in better growth and viability of an explanted kidney; hence, we also use this conventional method to culture cell aggregates. Within the aggregates, progenitors of the metanephric mesenchyme and the Wolffian duct interact with each other to initiate nephrogenesis. However, to maximize the number of nephrons formed, aggregates are exposed to a high concentration (5 μM) of CHIR99021 for 1 h, which stimulates the mesenchyme to undergo MET. Culture medium still includes 200 ng/mL FGF9 to support nephron formation for 5 days after the CHIR99021 pulse. Then, all growth factors are withdrawn from the APEL basal medium until the aggregates develop into kidney organoids.

2 Materials

1. Mouse embryonic fibroblast (MEF) culture medium (500 mL): 442.5 mL of DMEM high glucose, 50 mL of fetal bovine serum (FBS), 5 mL of GlutaMAX-1 (Thermo Fisher Scientific, Cat# 35050-061), and 2.5 mL of penicillin/streptomycin. Filter sterilize the medium through a polyethersulfone (PES) 0.22 μm vacuum-driven filter unit, and store it at 4 °C for up to 2 weeks.
2. hESC medium (500 mL): 386.5 mL of DMEM/F-12 (Thermo Fisher Scientific, Cat# 11320-082), 100 mL of knockout serum replacement (Thermo Fisher Scientific, Cat# 10828-028), 5 mL of GlutaMAX-1 (Thermo Fisher Scientific, Cat# 35050-061), 5 mL of nonessential amino acids (Thermo Fisher Scientific, Cat# 11140-050), 2.5 mL of penicillin/streptomycin, and 1 mL of 2-mercaptoethanol (55 mM) (Thermo Fisher Scientific, Cat# 21985-023). Filter sterilize media through a polyethersulfone (PES) 0.22 μm vacuum-driven filter unit and store it at 4 °C for up to 2 weeks (*see* **Note 1**).
3. MEF-conditioned hESC medium: Seed 1×10^7 mitotically inactivated MEFs onto a gelatin-coated 175 cm^2 tissue culture flask containing 40 mL of MEF culture medium (*see* Subheading 2, **item 1**). On the next day, change the culture medium to 40 mL of hESC medium without bFGF. After overnight culture, collect the conditioned medium into a 500 mL bottle and store it at 4 °C. Feed MEFs again with 40 mL of fresh hESC medium. Repeat this cycle another six times to pool

280 mL of the conditioned medium in total. Filter sterilize the medium through a polyethersulfone (PES) 0.22 μm vacuum-driven filter unit and aliquot it into 50 mL tubes. Store tubes at −20 °C for up to 6 months. MEF-conditioned hESC medium can be stored at 4 °C for up to 2 weeks once it is thawed (*see* **Note 1**).

4. APEL medium (100 mL): Once a bottle of STEMdiff APEL (100 mL volume: STEMCELL Technologies, Cat# 05210) is opened, add 0.5–1 mL of Antibiotic–Antimycotic (Thermo Fisher Scientific, Cat# 15240-062) and store it at 4 °C for up to 2 weeks.
5. Frozen stock of human pluripotent stem cells (hPSCs): For expanding hPSCs before cryopreserving them, cells are cultured in hESC medium supplemented with 10 ng/mL bFGF on mitotically inactivated MEF feeder layer by a single-cell culture method in which cells are passaged using TrypLE Select. Once hPSCs reach the desired number, harvest cells using TrypLE Select centrifuge (400 × *g* for 3 min) and resuspend them in 10% DMSO/90% FBS. Split cell suspension into cryo-vials for 1 mL per vial. Typically, hPSCs of 100% confluent in a 75 cm^2 tissue culture flask are split into nine cryo-vials, so that each cryo-vial contains 1.5–2.0 × 10^6 cells. Freeze vials in a freezing container at −80 °C overnight, and subsequently store them in liquid nitrogen (*see* **Note 2**).
6. 0.1% gelatin solution (500 mL): 0.5 g of gelatin in 500 mL of ultrapure water or DPBS and autoclave it. The solution can be stored at 4 °C for up to 3 months.
7. Matrigel-coated 25 cm^2 tissue culture flask: To prepare Matrigel-coated 25 cm^2 tissue culture flask, add 30 μL of hESC-qualified Matrigel into a 15 mL tube containing 3 mL of chilled DMEM/F-12. Mix it well and transfer it into a 25 cm^2 tissue culture flask. Keep the flask at room temperature for at least 30 min to allow Matrigel to coat the surface. Aspirate Matrigel solution and add 3 mL of DMEM/F-12 to the flask until use. Use the flask within a day (*see* **Note 3**).
8. bFGF stock solution (10 μg/mL): Centrifuge the tube briefly before opening. Reconstitute bFGF to 10 μg/mL in a filtered solution of 0.5% (wt/vol) BSA, 1 mM DTT, and 10% (vol/vol) glycerol in DPBS. Aliquot it into appropriate amounts and store them at −80 °C for up to 6 months. bFGF can be stored at 4 °C for up to 2 weeks once it is thawed.
9. FGF9 stock solution (100 μg/mL): Centrifuge the tube briefly before opening. Reconstitute FGF9 to 100 μg/mL in filtered DPBS containing 0.1% (wt/vol) human serum albumin. Aliquot it into appropriate amounts and store them at −80 °C for up to 6 months. FGF9 can be stored at 4 °C for up to 2 weeks once it is thawed.

10. Heparin stock solution (1 mg/mL): Reconstitute heparin sodium salt to 1 mg/mL in ultrapure water, and filter sterilize it through a polyethersulfone (PES) 0.22 μm syringe-driven filter unit. Heparin solution can be stored at 4 °C for more than 12 months.
11. CHIR99021 stock solution (10 mM): Centrifuge the tube briefly before opening. Reconstitute 10 mg of CHIR99021 into 2.149 mL of DMSO to make 10 mM stock. Aliquot it into appropriate amounts and store them at −20 °C.

3 Methods

All cell culture media should be pre-warmed to the room temperature before use.

3.1 Thawing Cryopreserved MEFs

This step should be performed 1 day prior to the Subheading 3.2.

1. Coat a 25 cm^2 tissue culture flask with 3 mL of 0.1% gelatin solution. Incubate the flask in a 37 °C CO_2 incubator for 1 h.
2. Prepare 10 mL of pre-warmed MEF culture medium in a 15 mL conical tube.
3. Thaw a frozen vial of mitotically inactivated mouse embryonic fibroblasts (MEFs) in 37 °C water bath until a small ice pellet remains. Transfer MEFs into a 15 mL conical tube containing pre-warmed MEF culture medium in a dropwise manner, and centrifuge at 400 × *g* for 3 min.
4. Remove supernatant and resuspend MEFs in MEF culture medium. Count cell number using a hemocytometer. Make cell suspension of 3×10^5 cells in 5 mL of MEF culture medium. Remove gelatin from (Subheading 3.1, **step 1**). Onto this gelatin-coated 25 cm^2 tissue culture flask, seed cells to obtain the density at 12×10^3 cells/cm^2 and incubate it overnight in a 37 °C CO_2 incubator.

3.2 Thawing Cryopreserved hPSCs

1. Prepare 10 mL of pre-warmed hESC medium in a 15 mL conical tube.
2. Thaw a frozen vial containing $1.5–2.0 \times 10^6$ hPSCs in 37 °C water bath until a small ice pellet remains. Transfer hPSCs into a 15 mL conical tube containing pre-warmed DMEM/F-12 in a dropwise manner and centrifuge at 400 × *g* for 3 min.
3. Remove supernatant and resuspend cells in 5 mL of hESC medium supplemented with 10 ng/mL bFGF.
4. Remove MEF culture medium from (Subheading 3.1, **step 4**) and plate above hPSCs suspension. Incubate it overnight in a 37 °C CO_2 incubator.

5. Change 5 mL of hESC medium supplemented with 10 ng/mL bFGF daily for 4–5 days.

3.3 Adapting hPSCs to a Matrigel-Coated Plate

Before starting this step, check the cell density from (Subheading 3.2, **step 5**). For optimal results, cells should be approximately 80–100% confluent on this day. If cells do not reach this confluency, allow them to grow for another day.

1. Prepare Matrigel-coated 25 cm^2 tissue culture flask (*see* Subheading 2, **item 9**).
2. To passage hPSCs onto Matrigel, wash hPSCs on MEF feeder layer in 25 cm^2 tissue culture flask (Subheading 3.2, **step 5**) with 3 mL DPBS twice. Aspirate DPBS.
3. Add 1 mL of TrypLE Select to cells and incubate the flask at 37 °C for 3 min.
4. Pipette 11 mL of DMEM/F-12 to cells, mix, and ensure cells have lifted off from the plastic surface (*see* **Note 4**).
5. Collect 4 mL of cell suspension in a 15 mL tube to obtain a 1:3 split ratio and centrifuge it at 400 × *g* for 3 min. A 1:2 split ratio can be chosen to obtain 70–100% confluency at (Subheading 3.4, **step 1**).
6. Remove supernatant and add 5 mL of MEF-conditioned hESC medium supplemented with 10 ng/mL bFGF to cells. Mix it gently.
7. Aspirate Matrigel-containing DMEM/F-12 from a prepared Matrigel-coated 25 cm^2 tissue culture flask (Subheading 3.3, **step 1**) and seed cells. Culture them in a 37 °C CO_2 incubator for 2 days with daily changing MEF-conditioned hESC medium supplemented with 10 ng/mL bFGF.

3.4 Plating hPSCs for the Differentiation

1. Wash hPSCs on Matrigel in 25 cm^2 tissue culture flask (Subheading 3.3, **step 7**) with 3 mL of DPBS twice. Aspirate DPBS.
2. Add 1 mL of TrypLE Select to cells and incubate at 37 °C for 3 min.
3. Pipette 10 mL of DMEM/F-12 to cells, mix, and ensure cells have lifted off from the plastic surface (*see* **Note 4**).
4. Collect cell suspension into 15 mL tube. Count cell number using a hemocytometer.
5. Calculate cell suspension volume to achieve 375,000 cells.
6. Aliquot cells into a 15 mL tube. Centrifuge the tube at 400 × *g* for 3 min.
7. Remove supernatant and resuspend cells in 4 mL of MEF-conditioned hESC medium supplemented with 10 ng/mL bFGF. Seed cells onto a prepared Matrigel-coated 25 cm^2 tissue culture flask to obtain the density at 15 × 10^3 cells/cm^2. Culture them overnight in a 37 °C CO_2 incubator.

3.5 Induction to Intermediate Mesoderm

1. Aspirate MEF-conditioned hESC medium from the 25 cm^2 tissue culture flask (Subheading 3.4, **step 7**) (Fig. 2, *Day 0*) (*see* **Note 5**).
2. Add 4 mL of APEL medium containing 8 μM CHIR99021 to hPSCs.
3. Culture them in a 37 °C CO_2 incubator for 2–5 days, refreshing APEL medium containing 8 μM CHIR99021 every 2 days. By day 2, pluripotent colonies break into single cells with spiky morphology (Fig. 2, *Day 2*). Duration of CHIR99021 determines the ratio of collecting duct/nephron in the organoid. A shorter CHIR99021 phase induces more anterior intermediate mesoderm, whereas a longer CHIR99021 phase results in

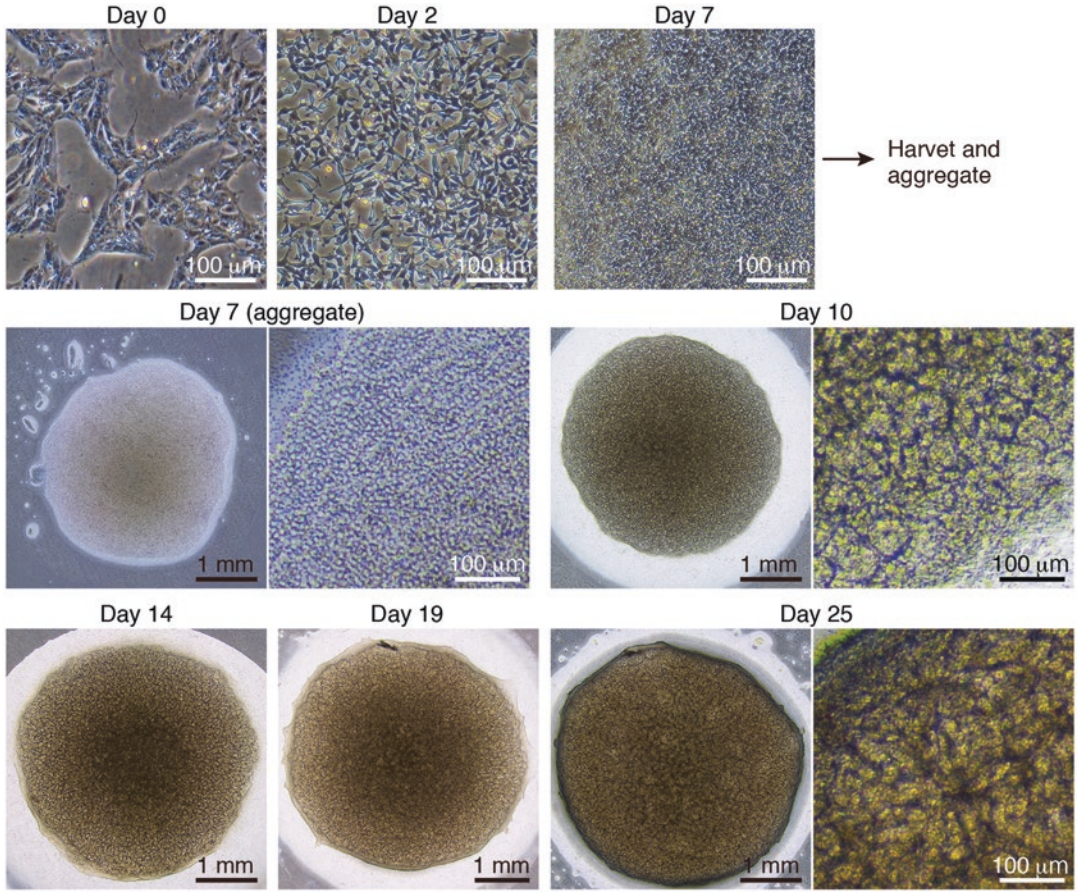

Fig. 2 Time course of the protocol from day 0–25. Representative bright field images showing morphological change from pluripotent stage to the kidney organoid. The morphology of hPSCs changes to a spiky shape by day 2 in response to CHIR99021 (Day 2). The cells reach confluence by day 4, and their surface looks hilly at day 7 (Day 7). Cells are dissociated and aggregated in a tube by centrifugation. Aggregates are placed on a transwell filter (Day7 aggregate). Within 3 days, cells start forming renal vesicles which are donut or coffee bean shaped (Day 10 high power). Aggregates undergo self-organization to develop kidney organoids with further complicated structures (Day 25)

more posterior intermediate mesoderm. To obtain both compartments, 4 days of CHIR99021 is typically recommended.

4. After the CHIR99021 phase, change culture medium to 8 mL of APEL medium supplemented with 200 ng/mL FGF9 and 1 μg/mL heparin (*see* **Note 6**).
5. Culture them in a 37 °C CO_2 incubator, refreshing medium every 2 days until day 7 of the differentiation including both CHIR99021 phase and FGF9 phase (Fig. 2, *Day 7*).

3.6 Transferring the Cells to 3D Culture Format

1. Aspirate the culturing medium from (Subheading 3.5, **step 5**) and wash with 3 mL of DPBS twice. Aspirate DPBS.
2. Add 1 mL of trypsin EDTA (0.05%) to cells and incubate them at 37 °C for 3 min.
3. Monitor under the microscope to make sure all cells have lift off from the surfaces after 2 min. If the cells are still attached to the surfaces, gently pipette the cells with trypsin and place back into the incubator for further 1 min.
4. Transfer cell suspension to a 15 mL tube containing 9 mL of MEF culture medium to neutralize trypsin. Centrifuge the tube at 400 × *g* for 3 min.
5. Aspirate the supernatant and resuspend the cells with 3 mL of APEL medium.
6. Take out 10 μL of cell suspension and perform a cell count with a hemocytometer.
7. Each organoid will have roughly 5×10^5 cells. Aliquot the required amount of cell suspension into a 1.5 mL microcentrifuge tube. Centrifuge the tube at 400 × *g* for 2 min to make a cell pellet.
8. Aliquot 1.2 mL of APEL containing 5 μM CHIR99021 into a 6-well transwell cell culture plate (Corning, Cat# 3450). The transwell filter attaches on the surface of the medium.
9. Pick a pellet up by using a P1000 or P200 wide bore tip (Fig. 3) (*see* **Note 7**).
10. Carefully place pellets onto the 6-well transwell filter of (Subheading 3.6,, **step 8**) with minimal APEL medium carry over. Incubate pellets at 37 °C for 1 h (Fig. 2, *Day 7 aggregate*).

3.7 Kidney Organoid Culture

1. After 1 h incubation, remove the medium of APEL containing 5 μM CHIR99021, and use 1.2 mL of APEL medium supplemented with 200 ng/mL FGF9 and 1 μg/mL heparin.
2. Culture pellets for 5 days with refreshing FGF9 and heparin-containing APEL medium every 2 days. Renal vesicle formation is typically observed within 3 days (Fig. 2, *Day 10*).
3. After 5 days, change the culture medium to APEL medium without FGF9 and heparin supplemented.

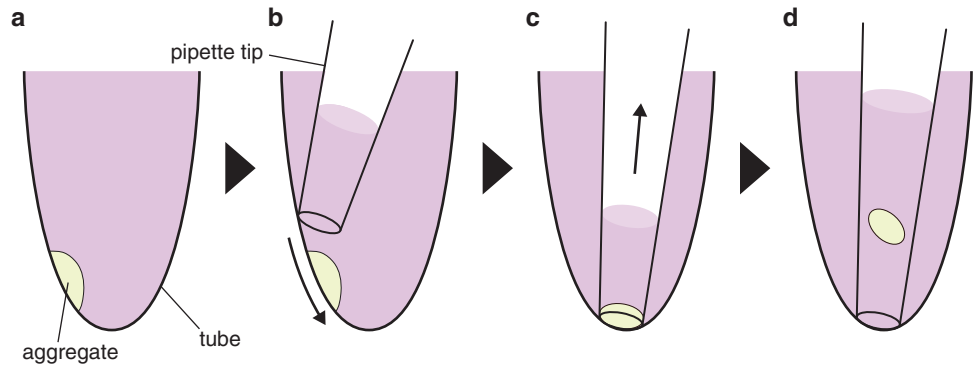

Fig. 3 A guide to pick up a cell aggregate using a pipette tip. (**a**) After centrifugation, a cell aggregate is formed on one side in a tube. (**b**) Set volume of a pipette to 100 μL and sack the medium in a half way (50 μL). (**c**) Scrape a cell aggregate by the pipette tip to bottom of the tube. (**d**) Sack another 50 μL of the medium to lift the aggregate up into the pipette tip. Before transfer onto a transwell filter, wait for 10 s until the aggregate comes down, and push out the aggregate with minimum volume of the media to carry over

4. Culture the organoids for another 6–13 days with refreshing APEL medium every 2 days.
5. Harvest kidney organoids for further experiments (Fig. 2, *Day 25*). Kidney organoids are typically 3–5 mm in diameter (Fig. 4a) and include nephrons segmented into four, glomerulus, proximal tubule, distal tubule, and collecting duct (Fig. 4b–e), as well as endothelial networks (Fig. 4f) and renal interstitial cells (Fig. 4g).

4 Notes

1. To ensure bFGF is fresh, only supplement the medium with 10 ng/mL bFGF just before using for hPSC maintenance.
2. We have experienced a huge variability in differentiation success rate between experiments when cells were obtained from a continuously maintained cell culture pool. To minimize this variation, hPSCs should be thawed one by one from a cryopreserved stock for each experiment.
3. Handle Matrigel on ice as it solidifies when warmed over 16 °C. P200 pipette tips should be also pre-chilled before use.
4. Pipette cells no more than twice as hPSCs are very sensitive.
5. Cells should reach to 40–50% confluent on this day. If not, adjust cell number of plating at (Subheading 3.4, **step 5**).
6. A shortage of FGF9 concentration and/or media volume causes an inefficient induction of the intermediate mesoderm.
7. If pellets break up during a transfer, perform multiple sequential centrifugations of 400 × *g* for 2 min. Rotate the tube 180° after each spin. Up to four sequential centrifugations can be performed.

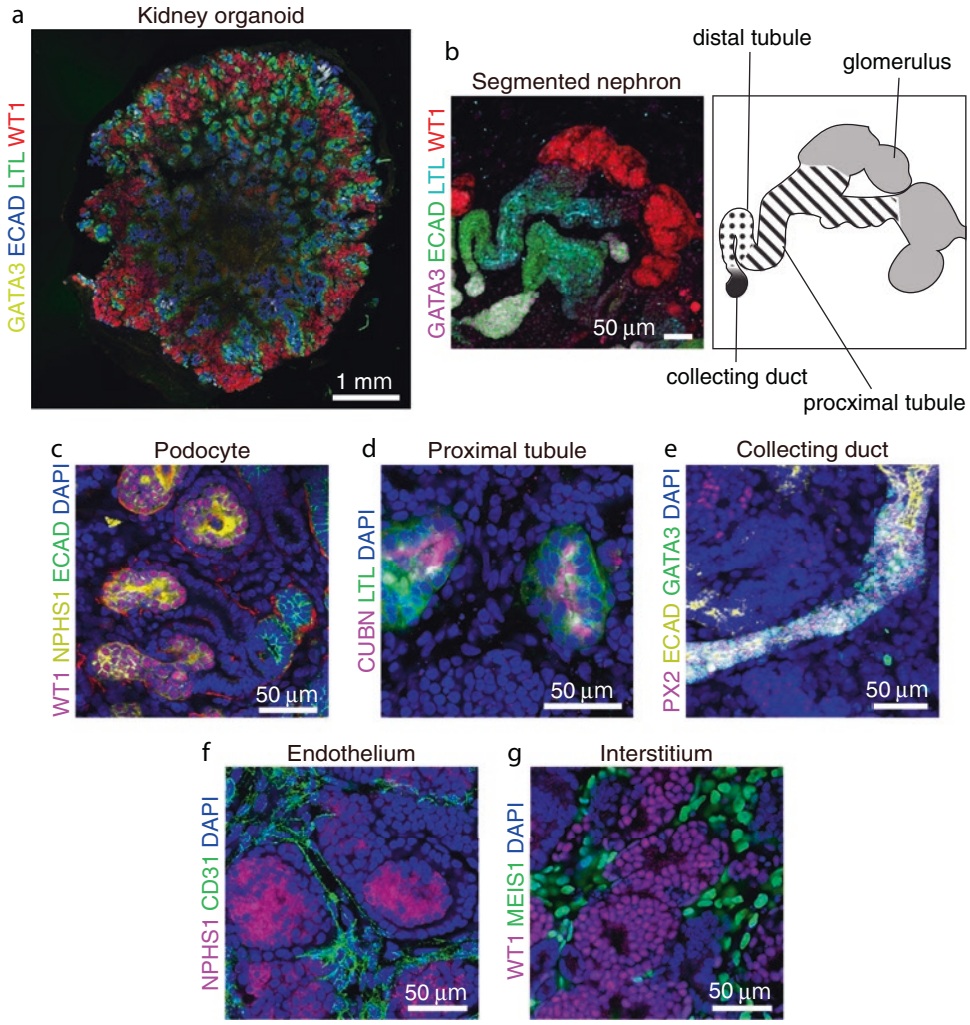

Fig. 4 Immunofluorescence images of kidney organoids. (**a**) A whole kidney organoid, approximately 5 mm in a diameter. (**b**) A segmented nephron containing glomerulus (*WT1*$^{+}$), proximal tubule (*LTL*$^{+}$), distal tubule (*ECAD*$^{+}$), and collecting duct (*GATA3*$^{+}$*ECAD*$^{+}$). (**c**) Podocytes marked by *WT1* and *NPHS1*. (**d**) Proximal tubules marked by *CUBN* and *LTL*. (**e**) Collecting duct marked by *PAX2*, *ECAD*, and *GATA3*. (**f**) Endothelium, marked by *CD31*, develops between glomeruli structures (*NPHS1*$^{+}$). (**g**) Renal interstitium marked by *MEIS1*. DAPI stains cell nuclei

Acknowledgments

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Chapter 15

Liver Regeneration Using Cultured Liver Bud

Keisuke Sekine, Takanori Takebe, and Hideki Taniguchi

Abstract

Here, we describe a protocol to develop a three-dimensional (3D) liver bud-like tissue from human iPSCs in vitro. This method mainly consists of two parts: (1) hepatic endoderm (HE) differentiation from human iPSCs in 2D culture and (2) co-culturing iPSC-HE with endothelial and mesenchymal cells. First, iPSCs were differentiated into definitive endoderm (DE) cells, and the DE cells were differentiated into HE cells, which were then co-cultured with endothelial cells and mesenchymal cells on Matrigel-coated plastic plates or micropattern plates. The cells rapidly condensed to generate 3D tissue masses. We named these iPSC liver buds (iPSC-LBs) because they resemble the developing liver bud from the perspective of gene expression, cell proliferation, and cell proportion. This liver bud culture system provides a novel approach for future clinical applications, for drug development, and as a tool for studying human development.

Key words Human-induced pluripotent stem cells, Liver bud, Three-dimensional culture, Self-organization, Multicellular interaction, Regenerative medicine, Drug development

1 Introduction

The development of human-induced pluripotent stem cells (iPSCs) has enabled us to access human pluripotent stem cells (PSCs) without ethical and/or political issues [1–3]. Furthermore, iPSC can be generated from cells with various genetic backgrounds [4]. The easy access to human PSC has led to remarkable progress in the development of differentiation methods toward hepatocytes [5, 6]. Hepatocytes or liver parenchymal cells are needed for regenerative medicine and drug discovery testing. However, the current conventional two-dimensional (2D) culture conditions are limiting in terms of maturation and efficient engraftment.

Insight into the differentiation of PSC toward hepatocytes has been mostly derived from in-depth study of mouse development [7–11]. Before the development of human iPSCs, mouse embryonic stem (ES) cells were used in most studies on hepatic differentiation from PSC. The efficiency of differentiation from mouse ES cells into endoderm is quite low, and the improvement of this efficiency progressed slowly. On the other hand, an efficient

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method for growing human endoderm from pluripotency was quickly developed soon after the development of human iPSCs [12]. These cells can be differentiated into definitive endoderm with over 95% efficiency [13–15]. However, differentiation and maturation thereafter have not reached satisfactory levels in terms of hepatocyte function [16].

We recently developed a three-dimensional cell culture system by co-culturing with iPSC-derived primitive hepatic cells, endothelial cells, and mesenchymal cells, in which cells self-organized to form a tissue mass. This tissue mass resembled a liver bud; liver primordia arise as a first sign of liver tissue development in developing embryo (iPSC-LB) [17–20]. Here, we describe a protocol to develop a three-dimensional (3D) liver bud-like tissue from human iPSC in vitro.

2 Materials

2.1 Reagents

2.1.1 Human Stromal Cell Maintenance

HUVECs were maintained with EGM BulletKit. hMSCs were maintained with Mesenchymal Stem Cell Growth Medium BulletKit.

2.1.2 Human iPSC Maintenance

1. We used several human iPSC lines, most of which were generated with non-genome-integrative episomal vectors.
   For visualization, EGFP knock-in reporters for the expression of adeno-associated virus integration site 1 (AAVS1::GFP) were used.
2. Dish coating: Laminin511-E8 (iMatrix).
3. iPSC culture media: mTeSR1.
4. Rock Inhibitor Y-27632 (×1000), final: 10 μM.
5. Cell detachment solution: Accutase.
6. Sterile PBS, room temperature.
7. iPSC cell freezing media: STEM-CELLBANKER.

2.1.3 Human iPSC Differentiation into iPSC-DE Cells

1. RPMI-B27: RPMI 1640 with 1% of B-27™ and 1% penicillin–streptomycin stored at 4 °C.
2. 100 μg/mL recombinant human Activin A; store at −30 °C (×1000), final: 100 ng/mL.
3. 50 μg/mL Wnt3a; stock at −30 °C (×1000), final: 50 ng/mL.
4. Dish coating: Laminin511-E8 (iMatrix).
5. 10 μM Rock Inhibitor Y-27632; store at −30 °C.

2.1.4 iPSC-HE Differentiation into iPSC-HE and iPSC-IH Cells

1. Base medium: KO-DMEM supplemented with 1% penicillin–streptomycin stored at 4 °C.
2. L-glutamine 200 mM ×200, final: 1 mM.

3. MEM NEAA ×100, final: 1%.
4. 2ME 55 mM ×550, final: 0.1 mM.
5. DMSO ×100, final: 1%.
6. Knockout serum replacement 20%.

2.1.5 iPSC-IH Differentiation into iPSC-MH Cells

1. Base medium: hepatocyte culture medium (HCM) composed of HBM basal medium supplemented with HCM SingleQuot Kit without EGF.
2. 5% FCS.
3. Dexamethasone ×1000.
4. Oncostatin M ×1000, final: 20 ng/mL.
5. HGF ×250, final: 10 ng/mL.

2.1.6 Generation of Human iPSC Liver Bud In Vitro

1. Matrigel matrix.
2. Liver bud media (HCM/EGM = 1:1): HCM composed of HBM basal medium supplemented with HCM SingleQuot Kit without EGF and EGM BulletKit.

2.1.7 Validation

APC antihuman CD184 (CXCR4) antibody (clone: 12G5) (Fig. 1).

RNA preparation kit: PureLink RNA Mini Kit or RNeasy Mini Kit (Qiagen).

Reverse transcription kit: High-Capacity cDNA Reverse Transcription Kit.

Universal Probe Library.

ELISA kit: Human Albumin ELISA Kit.

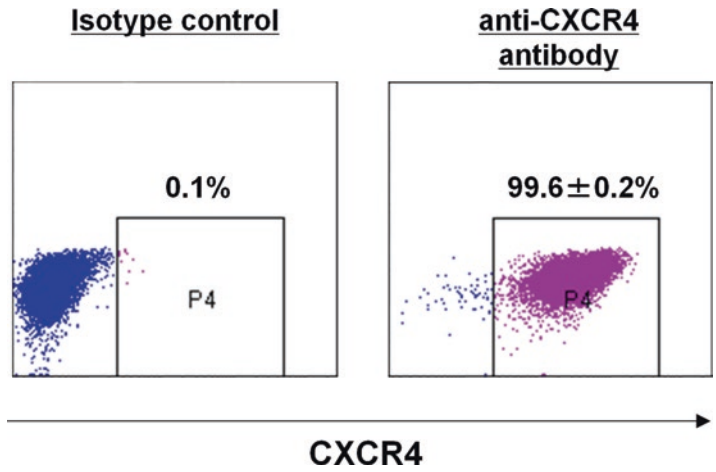

Fig. 1 Flow cytometric analysis to evaluate differentiation into iPSC-DE. Evaluation of CXCR4 protein expression on cell surface analyzed with isotype control (*left*) and anti-CXCR4 antibody (*right*). Over 99% of cells express CXCR4 and represent efficient differentiation of human iPSC into iPSC-DE

2.2 Equipment

1. Biosafety cabinet.
2. 37 °C, 5% CO_2 incubator.
3. Liquid nitrogen storage tank.
4. 4 °C refrigerator.
5. −20 °C freezer.
6. −80 °C freezer.
7. Pipet-Aid.
8. 37 °C water bath.
9. Phase-contrast microscope.
10. Fluorescence microscope.
11. Flow cytometry.
12. Real-time PCR machine.

3 Methods

All procedures were conducted at room temperature unless otherwise specified.

3.1 Precoating Culture Dish with Laminin-511-E8-Fragment (iMatrix511)

1. Coat a 60-mm dish, 100-mm dish, or six-well plate with diluted iMatrix511 at 0.1–1.5 μg/cm^2. Dishes and plates must be coated with laminin based on their area and not on the solution concentration. We routinely used 0.4–0.8 μg/cm^2 in 4 mL of PBS for 60-mm dishes, 8 mL of PBS for 1000-mm dishes, and 2 mL of PBS for six-well plates. Leave the dish or plate at 37 °C for approximately 1 h.

3.2 Thawing Human iPSC Vial (for 100-mm Dish) (See Notes 1 and 2)

1. Prepare a 15-mL conical tube with 5–10 mL of cold iPSC media.
2. Partially thaw the frozen vial of iPSC with water bath at 37 °C, until there is a small piece of ice remaining. Spray the vial with 70% ethanol to sterilize it.
3. Transfer the liquid content containing the cells into a 15-mL tube containing iPSC media using a PIPETMAN.
4. Centrifuge at 800 rpm (approximately 120 × *g*) for 3 min.
5. Meanwhile, aspirate the coating solution from the dish, and add 8 mL of human iPSC media with Rock Inhibitor Y-27632 (*see* **Notes 3** and **4**).
6. After centrifugation, aspirate the supernatant from the tube, and gently resuspend the pellet with 1 mL of human iPSC media. Pipette slowly and try to avoid excessive pipetting; count the cells and transfer approximately 1 × 10^5 cells to the dish.

7. Incubate at 37 °C, 5% CO_2 overnight, and change the media every 24 h, with human iPSC media without Rock Inhibitor.
8. Colonies should emerge anywhere from 3 to 6 days afterward.

3.3 Passaging Human iPSCs (for 100-mm Dish) (See Note 5)

1. Take the confluent iPSCs from the incubator, and wash them with 4 mL of PBS for the 100-mm dish.
2. Add 2 mL of Accutase; incubate in a 37 °C incubator for 5 min. Check cell detachment under a microscope.
3. Add 2 mL of pre-warmed human iPSC media to the dish.
4. Pipette and sprinkle the added iPSC media to the whole surface of the dish to detach the remaining undetached colonies.
5. Count the cells and dilute at the appropriate concentration (see below).
6. For passaging, seed approximately 1×10^5 cells into a laminin-precoated 100-mm dish with 9 mL of human iPSC media with Rock Inhibitor Y-27632.
7. Culture in a 37 °C, 5% CO_2 incubator, and change the media every 24 h, with human iPSC media without Rock Inhibitor.

3.4 Human iPSC Differentiation Toward iPSC-DE

1. Prepare laminin-coated six-well plates.
2. Take detached cell suspension in iPSC media from Subheading 3.3 (**step 5**).
3. Resuspend cells at approximately 1×10^6 cells/mL in iPSC-DE differentiation media with cytokines with Rock Inhibitor Y-27632.
4. Seed cell suspension at approximately $1 \times 10^5/cm^2$, i.e., approximately 1×10^6/well of a six-well plate on day 0.
5. Incubate the cells in a 37 °C, 5% CO_2 incubator for growth.
6. Change media every 2 days with iPSC-DE differentiation media with cytokines without Rock Inhibitor.
7. On day 6, when the cells should be differentiated into iPSC-DE, proceed to the next step. Prepare RNA from cells to analyze the gene expression by quantitative RT-PCR.

3.5 Differentiation of Human iPSC-DE to iPSC-HE and iPSC-IH

1. On day 6, change the media with iPSC-HE and iPSC-IH differentiation media.
2. Change the media every 2 days.
3. On day 10, the cells should have differentiated into iPSC-HE. Prepare RNA from the cells to analyze the gene expression by quantitative RT-PCR.
4. On day 13, the cells should have differentiated into iPSC-IH; proceed to the next step. Prepare RNA from the cells to analyze the gene expression by quantitative RT-PCR.

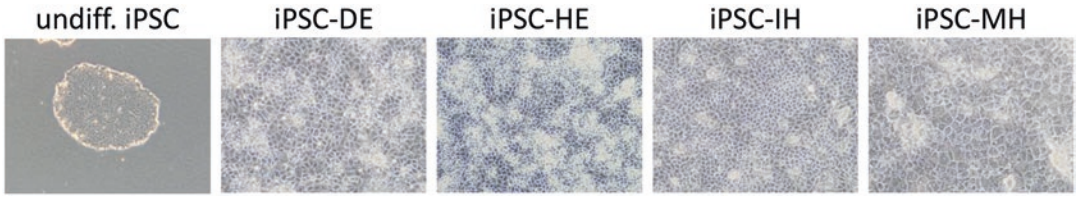

Fig. 2 Morphogenic changes observed during hepatic specification and differentiation of human iPSC under 2D culture. Undifferentiated iPSC colony, human iPSC-derived definitive endoderm (iPSC-DE), hepatic endoderm (iPSC-HE), immature hepatocytes (iPSC-IH), and mature hepatocytes (iPSC-MH)

3.6 Differentiation of Human iPSC-IH to iPSC-MH (Fig. 2)

1. On day 13, change the media with iPSC-MH differentiation media.
2. Change the media every 4 days.
3. Change the media on day 19, 24 h prior to collecting the media on day 20, to analyze the secreted human albumin protein in the media (optional).
4. On day 20, the cells should be differentiated into iPSC-MH; collect media for ELISA and prepare RNA from the cells to analyze the gene expression by quantitative RT-PCR.

3.7 Generation of iPSC-LBs

1. For generating iPSC-LB, prepare iPSC-HEs (see Subheading 3.5, **step 3**).
2. Prepare an appropriate amount of HUVEC and MSC for generating liver buds.

3.7.1 Preparation of iPSC-HE, HUVEC, and MSC (See Note 6)

3.7.2 Preparation of Matrigel-Coated Plate

1. Dilute Matrigel with ice-cold LB media at 1:1.
2. Coat a precooled 24-well plate with 400 μL of diluted Matrigel. Leave the plate in a 37 °C, 5% CO_2 incubator for at least 15 min to solidify the Matrigel.

3.7.3 Generation of iPSC-LB on a Matrigel-Coated Plate (Fig. 3)

1. Dissociate iPSC-HE with Accutase (*see* **Note 7**).
2. Dissociate HUVEC and MSC with Accutase or 0.05% trypsin.
3. Count cells independently and resuspend in LB media.
4. Mix iPSC-HE, HUVEC, and MSC at a proportion of 10:7:1. For one well of a 24-well plate, mix 1×10^6 cells of iPSC-HE, 7×10^5 cells of HUVEC, and 1×10^5 cells of MSC in LB media with Rock Inhibitor Y-27632.
5. Seed mixed cells gently on Matrigel, and add LB media to a total volume of 500 μL.

3.7.4 Cultivation of Generated iPSC-LB

1. LB should be generated on day 1. Add 500 μL of LB media without Rock Inhibitor.

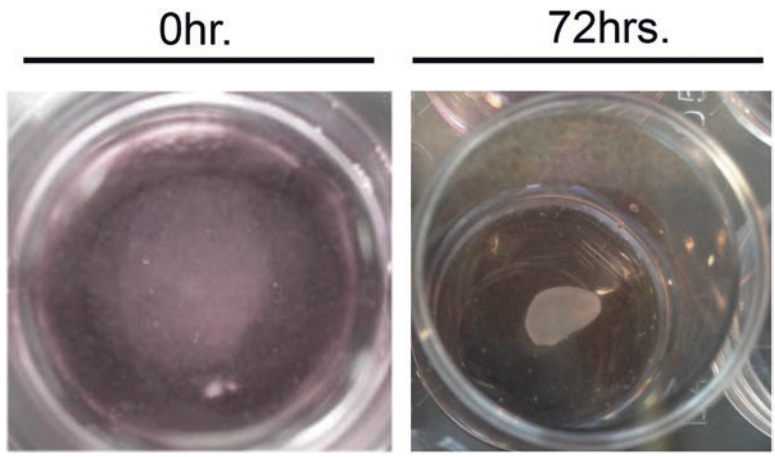

Fig. 3 Self-organized lived bud on Matrigel. Mixture of cells seeded on Matrigel. 0 h after seeding (*left*) and 72 h after seeding (*right*)

2. Cell condensation should have become firmly by day 2. Change half (500 μL) of the media every day until analysis (*see* **Note 8**).
3. On days 1–3, LB should be used for the transplantation experiment.

3.8 Evaluation of 2D Differentiated Cells and Generated iPSC-LB

3.8.1 Gene Expression Analysis of 2D Differentiated Cells and Generated iPSC-LB

1. Lyse cells with a standard RNA preparation kit. To lyse iPSC-LB, pipette up and down vigorously to thoroughly dissociate the cells.
2. Prepare total RNA according to the manufacturer's instructions.
3. Perform reverse transcription using 1 μg of total RNA with the Reverse Transcription Kit.
4. Perform qPCR using 10–50 ng cDNA. For the qPCR target genes, primers, and probe, *see* (Table 1, Fig. 4).

3.8.2 Albumin ELISA

1. Change the media 24 h prior to collecting the supernatant to measure the level of albumin secretion into the media. For the 2D culture, human albumin should be detected from day 16 onward.
2. Follow the manufacturer's instructions.

3.8.3 Other Evaluation Methods

It is also valid to evaluate the functional maturity of iPSC-MH by periodic acid–Schiff (PAS) staining to assess glycogen storage or by indocyanine green (ICG) uptake and release to assess the active uptake of the dye.

Table 1
Target genes, primers, and probes for qPCR using Roche Universal Probe Library

Target gene	UPL probe number	Primer sequence	Stage
hOCT4_Left hOCT_Right	q#60 q#60	cttcgcaagccctcatttc gagaaggcgaaatccgaag	Undiff. iPSC
hNANOG_Left hNANOG_Right	q#31 q#31	agatgcctcacacggagact tttgcgacactcttctctgc	Undiff. iPSC
hCer1_Left hCer1_Right	q#41 q#41	gccatgaagtacattgggaga cacagccttcgtgggttatag	iPSC-DE
hCXCR4_Left hCXCR4_Right	q#79 q#79	attgggatcagcatcgactc caaactcacacccttgcttg	iPSC-DE
hFOXA2_Left hFOXA2_Right	q#7 q#7	ggagacggtgttgcagaga actgctgtcttgggggtgt	iPSC-DE to iPSC-MH
hHNF4a_Left hHNF4a_Right	q#27 q#27	agcaacggacagatgtgtga tcagaccctgagccacct	iPSC-DE to iPSC-MH
hAFP_Left hAFP_Right	q#61 q#61	tgtactgcagagataagtttagctgac tccttgtaagtggcttcttgaac	iPSC-HE to iPSC-MH
hALB_Left hALB_Right	q#27 q#27	aatgttgccaagctgctga cttcccttcatcccgaagtt	iPSC-MH

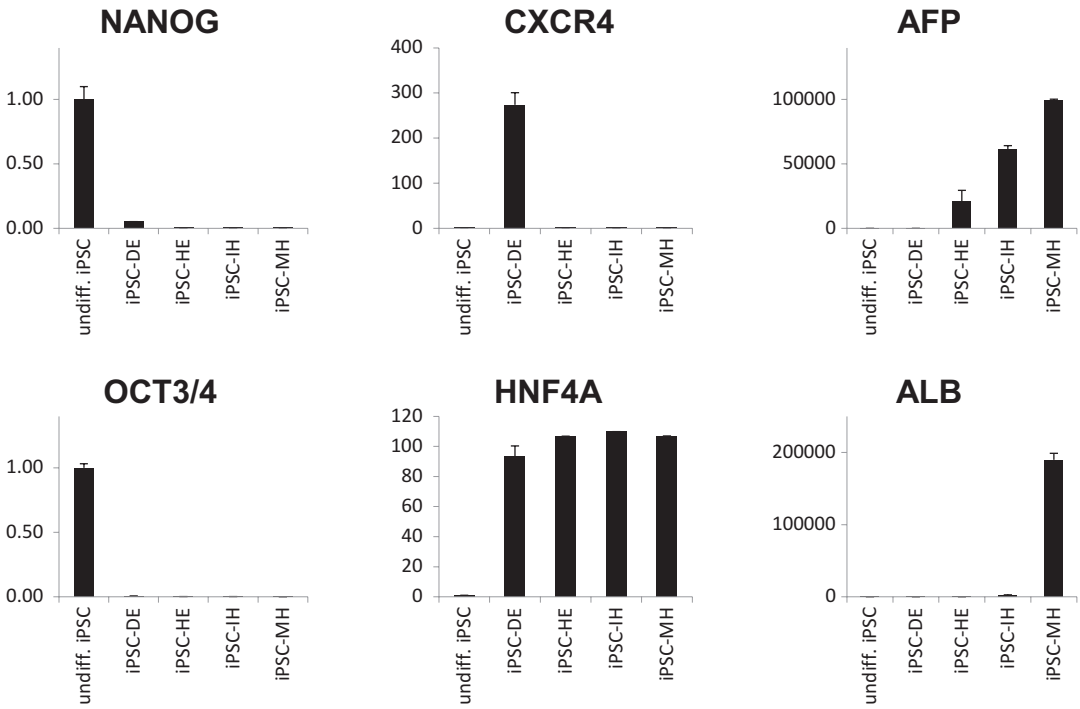

Fig. 4 qPCR evaluation of the differentiation cells. Gene expression profiles change in the course of hepatic differentiation of human iPSC under 2D culture (*see* also Table 1)

4 Notes

1. Methods for thawing iPSCs depend on how they were cryopreserved. The method described here is a canonical, slow-freezing method, and iPSCs can be thawed in a similar manner as that for other cell lines. If iPSCs were frozen following the vitrification method, which is used for cryopreserving biological materials such as fertilized eggs or embryos, follow the appropriate method for thawing frozen cells with vitrification.
2. We checked several iPSC lines on our hand and obtained relatively robust differentiation efficiency. However, it has been reported that the efficacy of the hepatic differentiation of iPSCs depends on the iPSC line; if the efficiency of differentiation is poor, then a change in the iPSC line should be considered.
3. iMatrix coating dries rapidly, and drying of the plates should be prevented.
4. Rock Inhibitor Y-27632 (10 nM) should be added to the media on the day of thawing or passage to improve survival efficiency.
5. Generally, human iPSCs need to be passaged once or twice a week. The cells are ready for passage once they have reached approximately 60–80% confluency in the entire dish. An appropriate split ratio and plating density ensure that culture cells remain in a healthy and undifferentiated state before the next passage.
6. Passage number is important for both HUVEC and MSC. Cells should be used before passage 10. If cell proliferation slows after passaging, these cells should be abandoned.
7. The three types of cells should dissociate simultaneously. If one person prepares all the cells, HUVEC and MSC should be dissociated first, followed by iPSC-derived HE.
8. Take care when changing media because cell-generated spheroids do not attach solidly; gently remove the old media without aspirating cell spheroids.

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Chapter 16

Formation of Stomach Tissue by Organoid Culture Using Mouse Embryonic Stem Cells

Taka-aki K. Noguchi and Akira Kurisaki

Abstract

In this chapter, we describe a method for the induction of stomach organoids from mouse embryonic stem (ES) cells. We used an embryoid body-based differentiation method to induce gastric primordial epithelium covered with mesenchyme and further differentiate it in Matrigel by 3D culture. The differentiated organoid contains both corpus- and antrum-specific mature gastric tissue cells. This protocol may be useful for a variety of studies in developmental biology and disease modeling of the stomach.

Key words Stomach organoids, Embryonic stem cells, Tissue formation, Disease model, Differentiation, Shh, Dkk-1, Barx1

1 Introduction

Generation of various tissue cells from pluripotent stem cells is important not only for the understanding of the mechanisms of differentiation but also for their application in regenerative medicine and disease modeling for incurable diseases. A number of differentiation methods have been reported for tissue cells derived from the endoderm, including pancreatic β cells [1] and hepatocytes [2]. Recently, other anterior foregut endoderm-derived tissue cells such as lung epithelial cells [3], thyroid cells [4], and antrum epithelial cells of the stomach [5] have been differentiated from embryonic stem (ES) cells. However, differentiation of the whole stomach tissue including both corpus and antrum from ES cells has not been established yet.

Therefore, we investigated a novel differentiation method for the whole stomach tissue from pluripotent stem cells. One of the essential factors for stomach tissue development is a transcription factor *Barx1*, which is specifically expressed in the mesenchymal layer of the whole gastric primordium and is required for specification of stomach epithelium during early development [6]. Barx1 induces secretion of sFRP1 and sFRP2 proteins, which antagonize

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Wnt and inhibit lineage commitment of foregut endoderm to the intestine. *Barx1* null mouse embryos show intestinalization of the stomach by expansion of intestinal *Cdx2* expression in the stomach epithelium [6]. Furthermore, overexpression of *Barx1* induces gastric genes in the early intestinal region [7]. Therefore, we used this functional marker gene for the stomach specification of foregut endoderm differentiated from mouse ES cells.

In this chapter, we describe the protocol for lineage specification of stomach primordium from mouse ES cells. We also explain the method for the generation of stomach organoids using 3D culture of the primordium in vitro [8].

2 Materials

All reagents and laboratory materials should be sterilized and prepared prior to experimental use. All solutions should be prepared using Milli-Q water. Recombinant proteins should be reconstituted according to the manufacturer's protocols and stored at −30 °C as frozen stocks. All aliquoted stocks should be used within 1 month.

2.1 Reagents

1. Cells: E14 mouse ES cells are available from American Type Culture Collection (ATCC). Mouse embryonic fibroblasts (MEFs) were used as a feeder cells for ES cell culture (prepared from ICR mouse embryos, *see* Subheading. 3.1).
2. Dulbecco's phosphate-buffered saline (PBS).
3. Stock solution of mitomycin C (MMC): Add 1 mL of autoclaved PBS to 2 mg of sterile MMC powder, and mix to completely dissolve MMC. Store at −80 °C for up to 6 months. The stock tubes should be protected from light.
4. Matrigel: Thaw one bottle of Matrigel at 4 °C overnight. Dispense 100 μL of Matrigel to autoclaved 1.5 mL tubes on ice. Store dispensed Matrigel tubes at −30 °C as frozen stocks. Upon usage, thaw the aliquoted Matrigel at 4 °C overnight. Matrigel should always be handled on ice or at 4 °C, as it quickly solidifies at room temperature.
5. Stock solution of gelatin (0.1% [w/v]): Dissolve 0.5 g porcine skin gelatin in 500 mL Milli-Q water, and sterilize by autoclaving. Store at room temperature for up to 1 month.
6. MEF medium: Dulbecco's modified Eagle's medium (DMEM; low glucose, containing 1000 mg/L glucose, 584 mg/L l-glutamine, and 110 mg/L sodium pyruvate) supplemented with 15% fetal bovine serum (FBS) and 100 U/mL penicillin/100 μg/mL streptomycin. For the preparation of 500 mL MEF medium, mix 420 mL DMEM (low glucose) with 75 mL

FBS and 5 mL penicillin/streptomycin (100× concentrated stock). Store at 4 °C for up to 1 month.

7. Mouse ES medium: DMEM (high glucose, containing 4500 mg/L glucose, 584 mg/L l-glutamine, and 110 mg/L sodium pyruvate) supplemented with 15% FBS, 0.1 mM non-essential amino acids (NEAA), 0.1 mM β-mercaptoethanol, 100 U/mL penicillin/100 μg/mL streptomycin, and 1000 U/mL human recombinant leukemia inhibitory factor (LIF). For the preparation of 500 mL ES medium, mix 415 mL DMEM (high glucose) with 75 mL FBS, 5 mL penicillin/streptomycin (100× concentrated stock), 5 mL NEAA (100× concentrated stock), 3.6 μL β-mercaptoethanol, and 500 μL human recombinant LIF (1000× concentrated stock). Store at 4 °C for up to 1 month.
8. Differentiation medium: DMEM (high glucose) supplemented with 15% FBS, 0.1 mM NEAA, 0.1 mM β-mercaptoethanol, and 100 U/mL penicillin/100 μg/mL streptomycin. Do not add human recombinant LIF. For the preparation of 500 mL differentiation medium, mix 415 mL DMEM (high glucose) with 75 mL FBS, 5 mL penicillin/streptomycin (100× concentrated stock), 5 mL NEAA (100× concentrated stock), and 3.6 μL β-mercaptoethanol. Store at 4 °C for up to 1 month.
9. KSR medium: DMEM (high glucose) supplemented with 15% knockout serum replacement (KSR), 0.1 mM NEAA, and 100 U/mL penicillin/100 μg/mL streptomycin. For the preparation of 500 mL KSR medium, mix 415 mL DMEM (high glucose) with 75 mL KSR, 5 mL penicillin/streptomycin (100× concentrated stock), and 5 mL NEAA (100× concentrated stock). Store at 4 °C for up to 1 month.
10. DS medium: KSR medium supplemented with 500 ng/mL recombinant human Dkk-1 and 500 ng/mL SHH. DS medium with growth factors can be stored at 4 °C for up to 1 week. However, addition of the growth factors just before use is recommended.
11. FGF medium: DMEM/F12 supplemented with 1% KSR, 0.1 mM NEAA, 100 ng/mL recombinant human fibroblast growth factor 10 (FGF10), 100 ng/mL WNT3A, 100 ng/mL NOGGIN, 250 ng/mL R-SPONDIN1, 50 ng/mL epidermal growth factor (EGF), N2, and B27. For the preparation of 500 mL FGF medium, mix 475 mL DMEM/F12 with 5 mL KSR, 5 mL NEAA, 5 mL N2 supplement (100× concentrated stock), and 10 mL B27 supplement (50× concentrated stock). Add growth factors on the day of use at the indicated concentrations. FGF medium with growth factors can be stored at 4 °C for up to 1 week.

12. Dissociation solutions: Trypsin (0.25% w/v)/ethylenediaminetetraacetic acid (EDTA) solution was used for passaging of ES cells, and dispase was used for digestion of Matrigel.
13. Frozen stock solutions: Cell Banker 1 was used for MEFs, and Stem Cell Banker was used for ES cells.

3 Methods

3.1 Preparation of MEFs

1. Add 10 mL of 0.1% (v/w) gelatin to 100 mm culture dishes, and incubate at 37 °C for 2 h or overnight for gelatin coating. Forceps and scissors for dissection of mouse embryos should be sterilized by autoclaving.
2. Collect E13.5 mouse embryos from pregnant female ICR mice. Isolate embryos from the placenta with forceps. Using fine forceps, remove the head, visceral tissues, and gonads from the embryos, and collect remaining stromal tissue. Rinse the tissue with PBS more than twice.
3. Transfer the tissue to a 50 mL centrifuge tube with a small volume of PBS, and mince the tissue into small fragments with a pair of scissors. Add 20 mL PBS and centrifuge the tube at 160 × *g* for 5 min.
4. Aspirate the supernatant, and resuspend the pellet with 0.25% (w/v) trypsin/EDTA solution by pipetting. Incubate at 37 °C for 15 min to dissociate MEFs from tissue (*see* **Note 1**).
5. Add 10 mL of MEF medium, and repeat pipetting 5–10 times. Remove debris by passing through a 100 μm cell strainer.
6. Centrifuge at 160 × *g* for 5 min, aspirate off the supernatant, and resuspend the pellet in MEF medium.
7. Transfer MEFs to the gelatin-coated 100 mm culture dishes. Use one gelatin-coated dish for each embryo, and culture at 37 °C with 5% CO_2 overnight.
8. The next day, remove the old medium by aspiration, add 10 mL fresh MEF medium, and culture at 37 °C with 5% CO_2 overnight. After 3–4 days of culture, split the MEFs to new gelatin-coated 150 mm culture dishes (1:4 dilution) using 0.25% (w/v) trypsin/EDTA (*see* **Note 2**).
9. After 2–3 passages, expand the MEFs until confluent in 150 mm culture dishes. Aspirate off the medium, and add 12.5 mL MEF medium supplemented with 70 μL of 2 mg/mL MMC stock to each 150 mm dish. Culture MEFs at 37 °C with 5% CO_2 for 2.5–3 h to induce cell cycle arrest.
10. Aspirate off the MMC medium, wash MEFs with 25 mL PBS twice, and dissociate the MEFs by incubating with 0.25% (w/v) trypsin/EDTA at 37 °C for 5 min. Add MEF medium, and centrifuge at 160 × *g* for 5 min.

11. Resuspend the pellet with MEF medium, and transfer the MMC-treated MEFs to gelatin-coated 60 mm culture dishes. Generally, four confluent 60 mm dishes of MEFs can be obtained from one confluent 150 mm dish of MEFs after MMC treatment. Then, culture the MMC-treated MEFs overnight (*see* **Note 3**).
12. The next day, renew the MEF medium, and culture the MMC-treated MEFs (MMC-MEFs) at 37 °C with 5% CO_2. The MMC-MEFs should be used as feeder cells for mouse ES cells within 2–3 days.

3.2 Mouse ES Cell Culture

1. Seed the MMC-MEFs in a 60 mm dish the day before starting ES cell culture (*see* Subheading 3.1).
2. Prepare 10 mL of prewarmed ES medium in a 15 mL centrifuge tube. Quickly thaw the frozen stock of E14 ES cells in a 37 °C water bath (less than 1 min). As soon as the stock is partially thawed, transfer the cells to the prewarmed ES medium in the 15 mL tube. Centrifuge at 160 × *g* for 5 min, and resuspend the pellet in 5 mL ES medium by pipetting 7–10 times. Aspirate off MEF medium from MMC-MEFs, transfer the ES cell suspension onto MMC-MEFs, and culture them at 37 °C with 5% CO_2 overnight.
3. The next day, aspirate off the medium, and add 5 mL fresh ES medium. Culture the cells at 37 °C with 5% CO_2 for another 2 days.
4. Renew the medium again. Colonies of ES cells will appear on the feeder layer (Fig. 1a). Continue to culture the cells at 37 °C with 5% CO_2 for 1 day. Prepare new MMC-MEF plates from the frozen stock (*see* **Note 3**).
5. The next day, wash ES cells with PBS twice, add 500 μL of 0.25% (w/v) trypsin/EDTA solution to ES cells, and incubate at 37 °C for 5 min. Add 5 mL ES medium, and dissociate ES cells to single cells by vigorous pipetting 7–10 times (*see* **Note 4**). Change the medium of the feeder cells with ES medium (**not MEF medium**). Transfer 50 μL of the single-ES-cell suspension to the feeder cells (*see* **Note 5**). Culture the ES cells at 37 °C with 5% CO_2 overnight.
6. The next day, renew the ES medium. Culture the ES cells at 37 °C with 5% CO_2 for another 2 days.

3.3 Differentiation of ES Cells by Embryoid Body Formation

1. Prepare gelatin-coated 100 mm dishes (*see* Subheading 3.1, **step 1**).
2. Wash ES cells cultured as detailed in Subheading 3.2 with PBS twice, and treat with 0.25% (w/v) trypsin/EDTA at 37 °C for 5 min. Add 5 mL differentiation medium (**do not add ES medium**; also *see* **Note 6**), and dissociate the colonies of ES cells to single cells by vigorous pipetting 7–10 times.

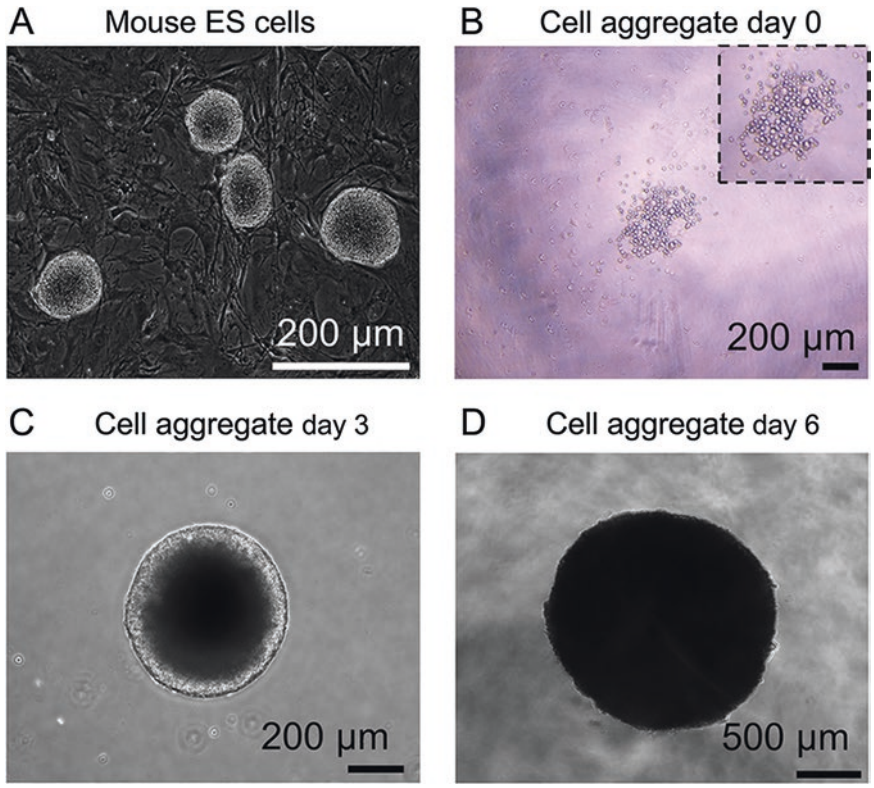

Fig. 1 Formation of ES cell aggregates in the early phase of differentiation. (**a**) Image of mouse ES cell colonies. (**b**) Image of single-cell dissociation of ES cells. Each single ES cell starts to aggregate soon after transferring to floating culture (day 0). (**c, d**) Images of ES cell aggregates at day 3 (**c**) and day 6 (**d**)

Confirm complete dissociation of all ES cell colonies to single cells by microscopy. Centrifuge the ES-cell suspension at 160 × *g* for 5 min, and resuspend the pellet in 5 mL differentiation medium by pipetting 7–10 times. Seed the ES cells on a gelatin-coated 100 mm dish, and incubate at 37 °C for 45 min to remove feeder cells.

3. Most of the feeder cells should attach to the gelatin-coated dishes in 45 min. However, ES cells will still be floating in the medium. Transfer the supernatant to a 15 mL tube, and centrifuge at 160 × *g* for 5 min. Resuspend the pellet in differentiation medium, and measure the cell density using a hemocytometer. Transfer 5.0×10^4 ES cells to a reservoir containing 10 mL differentiation medium. Dispense 100 μL of the ES cells suspension to each well of an ultra-low attachment 96-well plate. Each well will contain approximately 500 ES cells (Fig. 1b). The starting number of ES cells is important for successful formation of cell aggregates (embryoid bodies) (*see* **Note 7**). Culture the cells at 37 °C with 5% CO_2 for 6 days. Medium changes are not necessary during the 6 days of floating culture (*see* **Note 8**). The day of seeding ES cells in the 96-well plate is designated as day 0.

4. Days 1–5: Confirm embryoid body formation by microscopy (Fig. 1c, d).
5. Day 5: Prepare gelatin-coated 6-well plates (*see* Subheading 3.1, **step 1**).
6. Day 6: Transfer all embryoid bodies one by one from each well of the 96-well plate to 5 mL of KSR medium in a 15 mL tube using 200 μL wide-bore filter tips. Wait for 5 min at room temperature to allow embryoid bodies to settle at the bottom of the tube. Aspirate off the medium, add 5 mL KSR medium, and gently rock the tube for washing (*see* **Note 9**). Wait for 5 min again to let the embryoid bodies settle at the bottom of the tube. Aspirate off the medium, add DS medium, and transfer ten embryoid bodies to a gelatin-coated 6-well dish filled with 2 mL DS medium. Culture at 37 °C with 5% CO_2 overnight.

3.4 Induction of the Stomach Primordium from ES Cells

1. Day 7: All embryoid bodies should be attached on the gelatin-coated dishes. Aspirate off the old medium, and add 2 mL fresh DS medium to each well. Culture at 37 °C with 5% CO_2 for 3 days.
2. Day 10: Renew the medium with DS medium. Culture at 37 °C with 5% CO_2 for 3 days.
3. Day 11: Multiple small dome-like structures (stomach primordium) should be formed from the attached embryoid bodies (Fig. 2a). Confirm the formation of stomach primordium by immunofluorescent staining with antibodies against Sox2 (epithelium) and Barx1 (mesenchyme), as shown in Fig. 3a [8].
4. Day 13: Renew the medium with DS medium. Culture at 37 °C with 5% CO_2 for 3 days.
5. Day 16: Renew the medium with DS medium. Culture cells at 37 °C with 5% CO_2 for 3 days. Proliferation of stomach primordium-like balloons should be observed in the culture (Fig. 2b).
6. Day 19: Renew the medium with KSR medium, and culture at 37 °C with 5% CO_2 for 3 days. Stomach primordium-like cells may show peristaltic motion [8]. Culture at 37 °C with 5% CO_2 for 3 days.
7. Day 22: Renew the medium with KSR medium. Culture at 37 °C with 5% CO_2 for 3 days.
8. Day 25: Renew the medium with KSR medium. Culture at 37 °C with 5% CO_2 for 3 days.

3.5 Differentiation of the Stomach Tissue from the Primordium

1. Day 27: Prepare the Matrigel solution (*see* Subheading 2.1, **item 4**).
2. Day 28: Gently mix 75–100 μL liquid Matrigel with growth factors (100 ng/mL FGF10, 100 ng/mL WNT3A, 100 ng/mL NOGGIN, and 250 ng/mL R-SPONDIN1) by pipetting

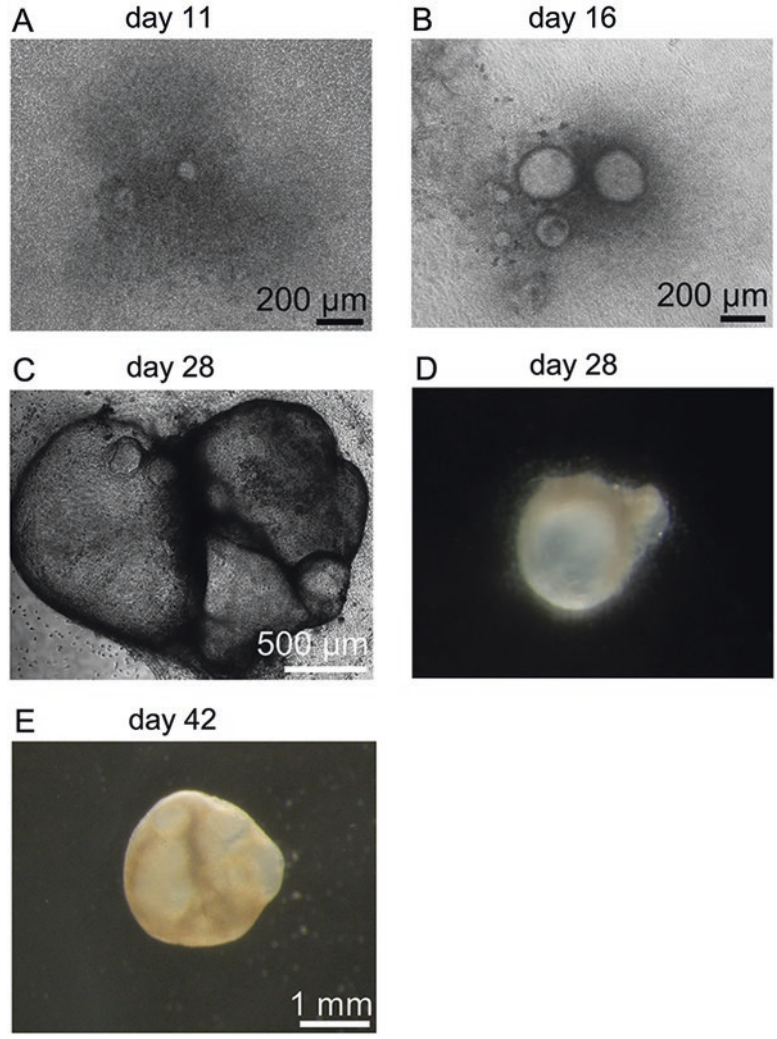

Fig. 2 Differentiation of embryoid bodies. (**a–c**) Images of embryoid bodies on attachment culture at day 11 (**a**), day 16 (**b**), and day 28 (**c**, **d**) Image of dissected stomach primordium at day 28 in PBS solution at × 6.8 magnification (**e**). Image of stomach organoid at day 42

on ice. The Matrigel solution should always be kept on ice until use. Harvest the stomach primordium-like spheroids. Wash the stomach primordium with 2 mL PBS twice, and add 2 mL PBS to each well (Fig. 2c). Carefully dissect only the stomach primordium-like balloon using sterile forceps under a microscope (Fig. 2d). Transfer the primordium to Matrigel supplemented with growth factors on ice, and mix gently. Then, transfer the Matrigel-containing primordium-like balloons to a 12-well culture plate or a 24-well plate as droplets using 200 μL wide-bore filter tips, and incubate them at 37 °C

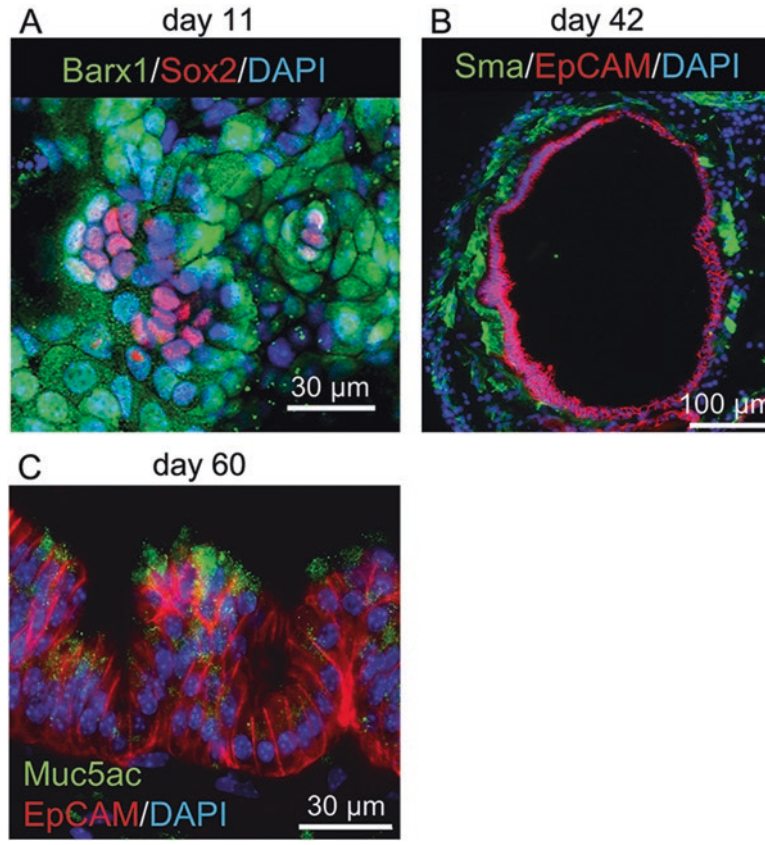

Fig. 3 Analysis of stomach organoids by immunofluorescence staining. Immunofluorescence staining of (**a**) Barx1 and Sox2, (**b**) smooth muscle actin (Sma) and EpCAM, and (**c**) Muc5ac and EpCAM in stomach organoids. Nuclei were stained with DAPI

for 30 min to polymerize Matrigel (Fig. 4). Add 1 mL of FGF medium to each well, and culture at 37 °C with 5% CO_2 for 14 days. Renew the medium every 4 days. Proliferation of stomach-like organoids should be observed after 14 days of culture. Stomach tissue formation can be observed by immunofluorescence staining for epithelial cell adhesion molecule (EpCAM) and smooth muscle actin (Fig. 3b).

3. Day 42 to day 60: After 42-day culture with FGF medium, the organoids can be further cultured in FGF medium from day 46 to around day 60. For immunofluorescence staining of stomach organoids, wash the organoids with PBS twice, and add dispase solution for digestion of Matrigel at 37 °C for 15–30 min. After digestion, gently wash the organoids with PBS three times, and pick up the organoids, using sterile forceps. More developed stomach organoids with mature gastric glands will be observed by immunofluorescence staining (Fig. 3c).

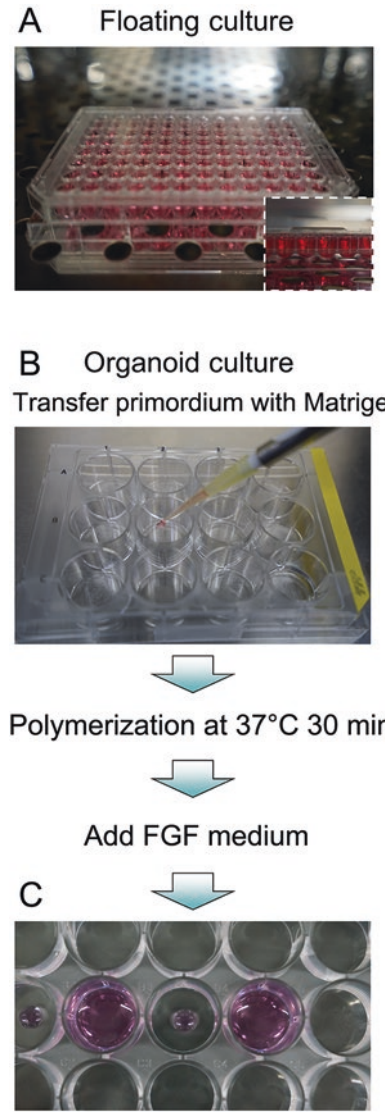

Fig. 4 Images of floating culture and 3D organoid culture. (**a**) Floating culture of embryoid bodies on low attachment cell culture dish. (**b–c**) Procedures for 3D–organoid culture in Matrigel

4 Notes

1. If the solution becomes viscous, add DNase I solution to digest the genomic DNA derived from dead cells. Digestion with DNase improves the handling of the cell suspension, and the recovery of MEFs may be increased because most of the dissociated cells pass through the cell strainer.

2. Expanded MEFs can be stored in the freezer any time after the first passage. Dissociate MEFs using 0.25% (w/v) trypsin/EDTA treatment, centrifuge at 160 × *g* for 5 min, and resuspend the pellet in Cell Banker 1. The MEFs can be stored in Cell Banker 1 freezing medium at −80 °C for up to 6 months.
3. Frozen stocks of MMC-treated MEFs can also be prepared. After dissociation of MMC-MEFs by trypsin/EDTA treatment and precipitation by centrifugation, resuspend the cells in Cell Banker 1. Dispense 1 mL of the cell suspension into a cryovial, and transfer to −80 °C. These frozen stocks can be stored for up to 1 month. When the feeder cells are needed, transfer the frozen stock from −80 °C to a 37 °C water bath, incubate until half of the frozen stock is thawed, and immediately transfer MEFs to 10 mL prewarmed MEF medium in a 15 mL tube. Centrifuge at 160 × *g* for 5 min, aspirate off the supernatant, resuspend in MEF medium, and transfer to a gelatin-coated 60 mm dish. Culture MMC-MEFs overnight at 37 °C with 5% CO_2. The next day, the feeder cells will be ready for ES cell culture.
4. Colonies of mouse ES cells should be completely dissociated into single cells to maintain pluripotent ES cells under good conditions.
5. Culture of ES cells can be stopped on the day of passage for cryopreservation. Wash the cells with PBS twice, and digest the ES cells with 0.25% (w/v) trypsin/EDTA at 37 °C for 5 min. Add ES medium, dissociate cells by vigorous pipetting, and centrifuge the cells at 160 × *g* for 5 min. Aspirate off the supernatant and gently resuspend the ES cells in freezing medium (Stem Cell Banker). Dispense the ES cell suspension into cryovials, and transfer them to −80 °C. These frozen stocks can be stored at −80 °C for up to 1 month or in liquid nitrogen for 1 year.
6. Do not add ES medium when the ES cells are harvested. LIF in the ES medium inhibits the differentiation of ES cells and results in poor differentiation.
7. Calculate the number of starting cells exactly (500 cells per well in a 96-well plate). If there are too many or too few starting ES cells, high-quality embryoid bodies may not be formed during the 6-day suspension culture.
8. Do not change the medium during the 6-day culture for embryoid body formation. ES cell aggregates generate both mesoderm and endoderm using growth factors secreted in an autocrine fashion.
9. Wash the embryoid bodies with KSR medium at least twice; if the aggregate medium is not washed well, stomach lineage specification will be inhibited by factors from the contaminating FBS in the differentiation medium.

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Chapter 17

In Vivo Model of Small Intestine

Mahe M. Maxime, Nicole E. Brown, Holly M. Poling, and Helmrath A. Michael

Abstract

The utilization of human pluripotent stem cells (hPSCs) offers new avenues in the generation of organs and opportunities to understand development and diseases. The hPSC-derived human intestinal organoids (HIOs) provide a new tool to gain insights in small intestinal development, physiology, and associated diseases. Herein, we provide a method for orthotropic transplantation of HIOs in immunocompromised mice. This method highlights the specific steps to successful engraftment and provides insight into the study of bioengineered human small intestine.

Key words Bioengineered small intestine, Human intestinal organoids, Pluripotent stem cells, Transplantation, In vivo model, Mesentery, Kidney capsule

1 Introduction

1.1 The Small Intestine

The small intestine is a complex hollow organ with several histological and functional layers that facilitate efficient digestive functions. This dynamic tube is composed of an outer layer of smooth muscle, a central layer of connective tissue, and an inner absorptive mucosa [1]. The inner lining of the small intestine is an epithelial monolayer consisting of pits called crypts that encircle and provide epithelial cells to protruding fingerlike structures called villi. The base of the crypt contains the proliferative compartment where stem cells and transit-amplifying cells divide and differentiate toward the apex of the villi. The intestinal epithelium contains four main differentiated cell lineages: absorptive, enterocytes; secretory, enteroendocrine cells; Paneth cells; and Goblet cells [2, 3]. The subjacent layers of the small intestine regroup connective tissues, smooth muscle, and an enteric nervous system. The smooth muscular layers are critical for the motor activities in the gut, including segmentation and peristalsis. They are arranged in a double layer: circular and longitudinal with intercalated enteric nerves regulating their contractions [4]. Understanding the complex interaction

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of the entire laminated intestine is required to appreciate the mechanisms underlying small intestinal development, physiology, and associated diseases thereof.

1.2 Model System of the Small Intestine

Limited in vivo models of human intestine exist, and the study of human intestinal biology has mostly relied on primary or established epithelial cell cultures [5]. Yet, in vitro cell culture systems do not faithfully mimic the intestinal physiology. Recent breakthroughs in the intestinal epithelial stem cell field allowed the research community to overcome these problems. A couple groups described long-term culture conditions of three-dimensional tissue-derived epithelial organoids that uniquely reassemble intestinal epithelial dynamics and physiology [6–8]. These experimental model systems constitute useful tools for studying the regulation of gastrointestinal stem cells as well as the intestinal epithelial cell fate determination throughout the digestive tract [9]. Although the in vivo transplantation using this model has been reported, thus far, their in vivo potential to address biological questions is limited [10, 11]. Other approaches have been developed to generate tissue-engineered small intestines or TESI. Using specific bio-scaffoldings and digested tissue from patients, these organoid units transplanted in murine recipients formed functional intestinal-like tissues resembling most of the intestinal lineages and functions [12, 13]. Emerging technologies utilizing fluidic devices like the "gut on a chip" have allowed for the in vitro mimicry of additional features to the system such as the inclusion of microbiota, shear stress, and tension [14, 15].

1.3 Human Intestinal Organoid Model

Applying the concepts from developmental biology to human pluripotent stem cells (hPSCs) has served as the basis for generating organoid models. Using a stepwise differentiation process, the hPSCs can be driven toward a mesendodermal fate, thus resulting in the generation of three-dimensional epithelial structure surrounded by mesenchyme called human intestinal organoids (HIOs) [16, 17]. Recently, we demonstrated that HIOs produced entirely in vitro can be transplanted under the kidney capsule of immunocompromised mice. After 6–8 weeks post-transplantation, the HIOs engrafted in vivo and formed a laminated intestinal tissue with an epithelium supported by the host vasculature. While expressing all the intestinal lineages in a crypt-villus architecture, the transplanted HIO resulted in significant expansion and maturation of the epithelium and mesenchyme and displayed digestive and absorptive functions [18, 19].

The transplanted HIOs exhibit key features of the human small intestine thus offering opportunities to broaden our knowledge from intestinal developmental processes to disease mechanisms [20]. In this chapter, we provide a detailed methodology to transplant hPSC-derived HIOs in a murine model. In addition, we also describe a method to study the small intestine's physiology using HIOs joined to the host intestinal tract.

2 Materials

2.1 Human Intestinal Organoid (HIO) Generation and Maintenance

All solutions should be prepared fresh using sterile cell culture grade reagents.

1. Human intestinal spheroids derived from human pluripotent stem cell lines (*see* Chapter 13).
2. Matrigel® Growth Factor Reduced; phenol red-free.
3. Intestinal growth medium: Advanced DMEM/F12 medium supplemented with 2 mM glutamine, 10 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 1× N2 supplement, 1x B27 supplement and filter sterilized with 0.22 μm filter (*see* **Note 1**).
4. Human recombinant epidermal growth factor (EGF) (10,000× stock; 500 mg/mL in sterile PBS/0.1% bovine serum albumin).
5. Collagen solution: Rat tail type I collagen is made fresh prior to embedding as per the manufacturer's instructions. 30 μL type I collagen gel is typically used to embed one HIO.

2.2 Murine Recipients, Surgical Equipment, and Reagents

Aseptic technique is essential for any survival surgery and requires that all surgical instruments and supplies be sterile. All surgical instruments should be washed and sterilized in an autoclave prior to use. All surgeries are performed under a HEPA-filtered laminar flow bioBubble to prevent microbial contamination of the surgical site.

1. Mice: Female or male immunocompromised NSG mice, NOD-scid IL2R gamma^{null}, are housed in microisolator systems in a barrier facility. The mice are used between 6 and 14 weeks of age (*see* **Note 2**).
2. Antibiotic diet: A modified chow diet (PicoLab Rodent Diet 20; LabDiet, St. Louis, MO, USA) is supplemented with 275 ppm sulfamethoxazole and 1365 ppm trimethoprim (LabDiet) (*see* **Note 3**).
3. Liquid diet is used for the side-to-side anastomosis surgery (Jevity® 1 Cal; Abbott Nutrition, Tipp City, OH, USA).
4. Antibiotics in water: 0.3 mg/mL of 275 ppm sulfamethoxazole and 1365 ppm trimethoprim are diluted in sterile water and are given ad libitum after the side-to-side anastomosis surgery (Bactrim®; Hi-Tech Pharmacal, Amityville, NY, USA).
5. Antibacterial drugs: 100 mg/kg of piperacillin and tazobactam are diluted in sterile saline solution and used for any surgeries (ZOSYN®; Pfizer, New York, NY, USA).
6. Buprenorphine.
7. Surgical suite (Fig. 1; *see* **Note 4**).
8. Surgical instruments (*see* Table 1).

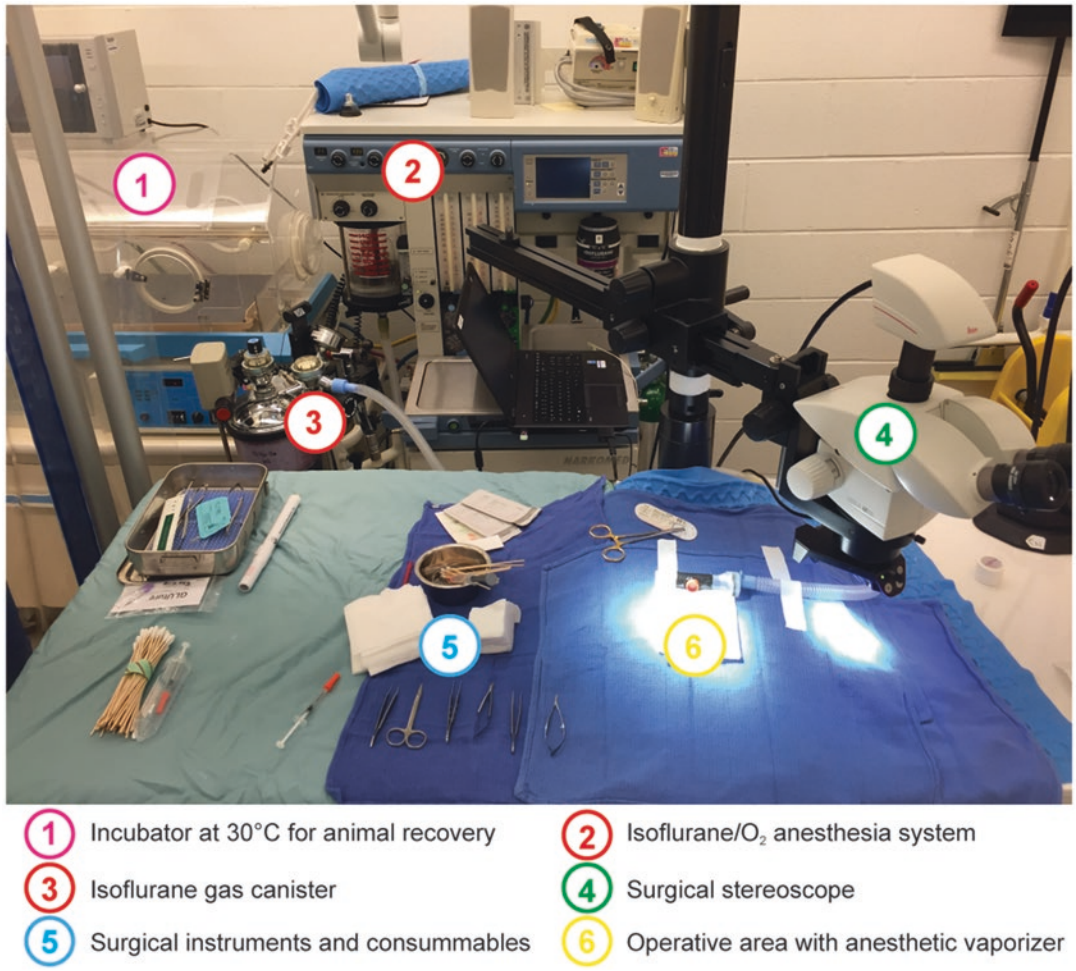

Fig. 1 Example of a surgical suite with an anesthetic system

Table 1
List of surgical instruments used for the different surgical procedures

Procedures	Instruments	Functions
All surgeries	Suture-tying forceps	Suture tying, HIO, and tissue handling
All surgeries	Ring forceps	Handling of the organs
All surgeries	Dissecting scissors	Muscle and skin incisions
All surgeries	Bishop-Harmon forceps	Muscle and skin handling
All surgeries	Halsey needle holder	Muscle and skin suturing
All surgeries	Sterilization tray	Gas sterilization for surgical instruments
Anastomosis	Microneedle holder	HIO and small intestine suturing
Anastomosis	Round handled suture-tying forceps	HIO and small intestine suturing
Subcapsular transplantation, anastomosis	Vannas spring scissors	Subcapsular pocket creation, HIO, and small intestine incisions

9. Isoflurane and anesthesia system (Fig. 1; *see* **Note 5**).
10. Sterile 7–0 nonabsorbable silk suture (PERMA-HAND®; Ethicon, Cincinnati, OH, USA).
11. Sterile 4–0 coated absorbable suture (VICRYL RAPIDE™; Ethicon, Cincinnati, OH, USA).
12. Sterile 9–0 nonabsorbable nylon suture with taper cut needle (ETHILON®; Ethicon, Cincinnati, OH, USA).
13. Octyl/butyl cyanoacrylate topical tissue adhesive (GLUture; WPI, Sarasota, FL, USA).
14. Sterile 18 G blunt fill needles.

3 Methodology

3.1 Human Intestinal Organoid Generation and Maintenance

The generation of human intestinal organoids (HIOs) has been described in detail in Chapter 13 by *Munera and colleagues* based on original protocols [16, 17]. In this section, we highlight important steps from the spheroid generation prior to the transplantation of the HIOs.

From subsequent intestinal spheroid generation in Chapter 13:

1. Collect the floating spheroids under a stereoscope, and transfer them in a 2 mL microtube filled with 1 mL intestinal growth medium (Fig. 2a,b; *see* **Notes 6** and 7).
2. Let the spheroids settle out in the microtube and discard the media.
3. Mix the pelleted spheroids with ice-cold Matrigel® to a concentration of 15–20 spheroids per 40 μL of Matrigel®.
4. Apply 50 μL of spheroid suspension in Matrigel® per well on a pre-warmed 24-well plate. Slowly eject the Matrigel®-embedded spheroids in the center of the well (Fig. 2c; *see* **Note 8**).
5. Place the 24-well plate in a 37 °C, 5% CO_2 incubator for 30 min to allow a complete polymerization of the Matrigel®.
6. Overlay the embedded spheroids with 500 μL of intestinal growth medium supplemented with 50 ng/mL of human recombinant EGF (1:10,000 dilution of 500 μg/mL stock).
7. Change intestinal growth media supplemented with 50 ng/mL of human recombinant EGF every 4 days (*see* **Note 9**).
8. At 14 days after spheroid plating (Fig. 2d), under a stereoscope, manually excise the HIOs out of the Matrigel®.
9. Collect the excised HIOs into a 24-well plate filled with intestinal growth media.
10. Place one HIO per center of each well of a new 24-well plate.
11. Overlay one HIO with 40 μL of fresh ice-cold Matrigel® per well.

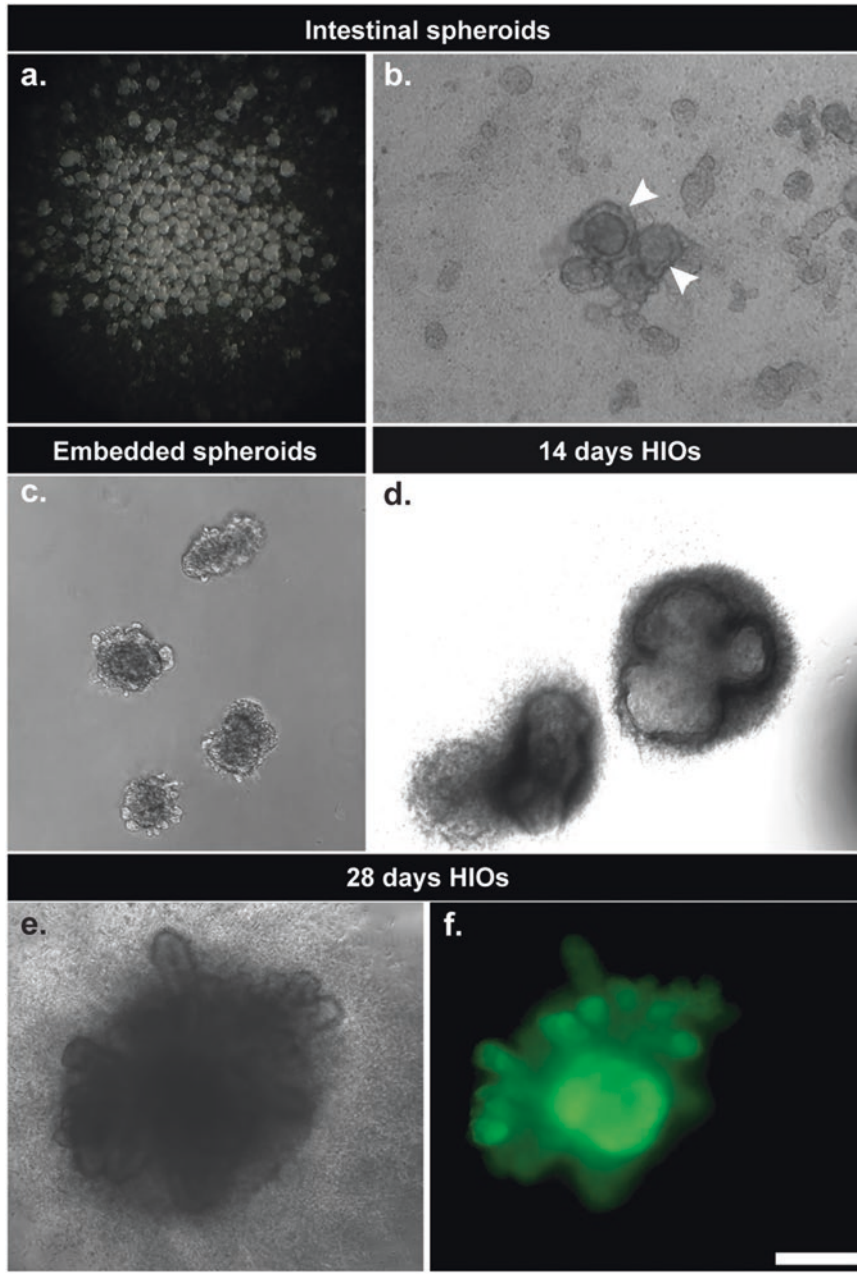

Fig. 2 Human intestinal organoid generation and maintenance prior to transplantation. (**a**) Intestinal spheroids generated after definitive endoderm differentiation and patterning from human pluripotent stem cells (data from H9 embryonic stem cell are shown). (**b**) Close-up picture on representative intestinal spheroids picked up before embedding in Matrigel®. (**c**) Embedded intestinal spheroids in Matrigel®. (**d**) Human intestinal organoids after 14 days in Matrigel®. (**e**–**f**) Human intestinal organoids after 28 days in Matrigel®, expressing green fluorescent protein (GFP) (*scale bar*, 250 μm)

12. Place the 24-well plate in a 37 °C, 5% CO_2 incubator for 30 min to allow a complete polymerization of the Matrigel®. Overlay the HIOs with 500 μL of intestinal growth medium supplemented with 50 ng/mL of human recombinant EGF (1:10,000 dilution of 500 μg/mL stock).
13. Change intestinal growth media supplemented with 50 ng/mL of human recombinant EGF every 4 days until transplantation.
14. At the day of transplantation, day 28, remove the HIO from Matrigel® using sterile tips and overlay with 1 mL of ice-cold DPBS (Fig. 2e–f; *see* **Notes 10** and **11**).
15. Pipet back and forth using a 1000 μL micropipette to remove the Matrigel® from the HIO. Repeat the procedure using ice-cold DPBS twice.
16. Embed one HIO per 30 μL drop of rat tail collagen type I (*see* **Note 12**).
17. Place the 24-well plate in a 37 °C, 5% CO_2 incubator for 30 min to allow a complete polymerization of the collagen plug. Overlay the HIOs with 500 μL of intestinal growth medium supplemented with 50 ng/mL of human recombinant EGF.
18. Incubate HIOs at 37 °C, 5% CO_2 until use (*see* **Note 13**).

3.2 Orthotropic Transplantation of Human Intestinal Organoids

Proper surgical technique must be practiced, that is, asepsis, gentle tissue handling, minimal dissection of tissue, appropriate use of instruments, effective hemostasis, and correct use of suture materials and patterns. The person performing the procedures must be appropriately trained and work under a mentor or veterinarian to perform the procedure. During a surgical procedure, the person performing the procedures must wear clean scrubs, sterile surgical gown, mask, cap, and sterile gloves. Sterile surgical gown and gloves must be donned and maintained in an aseptic manner. All NSG mice are maintained on regular chow supplemented with antibiotics prior to transplantation.

3.2.1 Renal Subcapsular Transplantation of HIOs

1. Bring the mice to the operating suite where weighting and assessment of health status are performed.
2. Anesthetize the mouse in an anesthetic gas vaporizer delivering an isoflurane/O_2 mixture (*see* **Note 14**).
3. Shave the left flank of the abdomen between the last rib and the iliac crest and the spine and the lower third of the abdominal wall. Remove loose fur (Fig. 3a; *see* **Note 15**).
4. Prepare the surgical site using povidone-iodine with a cotton swab. Repeat the procedure with new cotton swabs three times.
5. Repeat the procedure using 70% isopropyl alcohol with cotton swabs. Repeat the procedures with new cotton swabs three times.

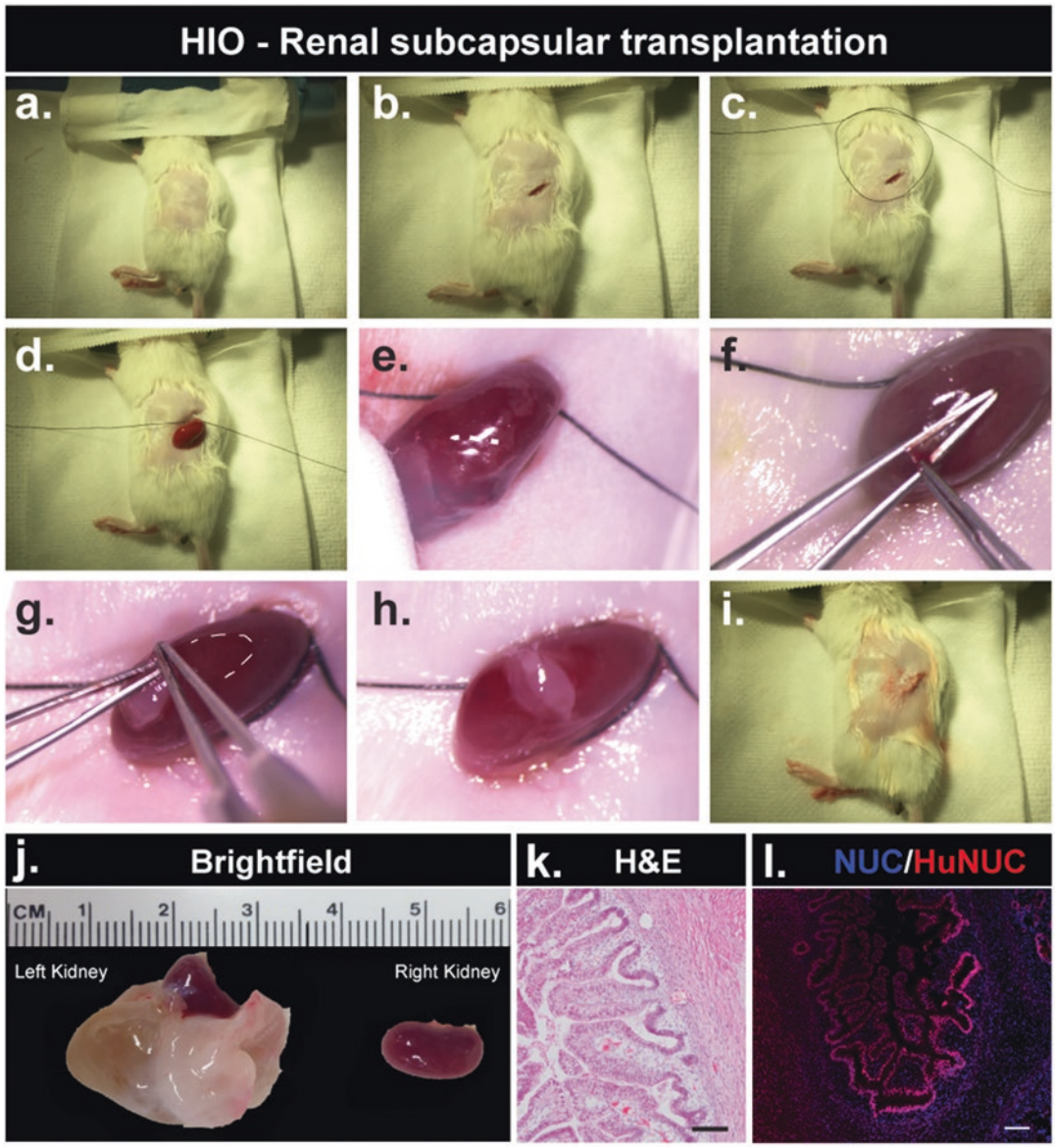

Fig. 3 Human intestinal organoid transplantation under the kidney capsule. (**a**–**b**) A left incision is performed in the skin and the subjacent muscle layer to access the left kidney. (**c**–**d**) A silk suture is placed to secure the kidney in the wound. (**e**–**h**) A small incision is made on the lateral aspect of the kidney, and a pocket is created under the capsule to introduce the collagen-embedded human intestinal organoid. (**i**) The kidney is returned to the abdominal cavity, and both abdominal and skin layers are sutured. (**j**) Photograph of a human intestinal organoid after 10 weeks posttransplantation. Transplanted organoid size ranges from 1 to 3 cm at the most. (**k**) Hematoxylin and eosin staining showing crypt and villus structures of the epithelium along with a laminated submucosal layer. (**l**) Human nuclear staining (HuNUC, *red*) shows the human origin of the transplanted organoid (*scale bars*, 100 μm)

6. Place ophthalmic ointment on the eyes to prevent drying of the cornea and administer buprenorphine (0.05 mg/kg) subcutaneously (*see* **Note 16**).

7. Restrain the mouse on lateral recumbency, the left kidney facing upward, and secure the mouse to a nose cone vaporizing isoflurane/O_2 mixture (*see* **Note 17**).
8. Monitor respiratory rate and effort, along with the surgical plane of anesthesia. Confirm the loss of pedal reflex by pinching the toe with forceps.
9. Use straight forceps and fine scissors to make an 8–10 mm left posterior subcostal skin incision just below the last rib.
10. Use fine scissors to make a subsequent 8–10 mm retroperitoneal muscle incision (Fig. 3b).
11. Identify the kidney using ring forceps and mobilize it into the wound.
12. Stabilize the kidney in the wound by placing a 7–0 silk suture loop with an untied square knot around the incision. Secure one ear of the suture with a needle holder to hold the knot and leave the other ear free (Fig. 3c).
13. Lift the kidney caudal pole through the abdominal incision and tie the silk suture by gently pulling the free ear until the kidney remains still (Fig. 3d; *see* **Note 18**).
14. Use a cotton swab to dry out the renal capsule.
15. Grasp the capsule under a surgical stereoscope with fine forceps and make a 2–3 mm incision with Vannas spring scissors in the capsule over the lateral aspect of the kidney (Fig. 3e).
16. Create a subcapsular pocket by gently sliding back and forth straight suture-tying forceps under the kidney capsule (*see* **Note 19**). Allowing the forceps to gently open when inside the capsule helps create enough space for the HIO (Fig. 3f).
17. Grab one collagen-embedded HIO using straight suture-tying forceps and insert it in the subcapsular pocket (Fig. 3g–h; *see* **Note 20**).
18. Cut the 7–0 silk suture and return the kidney within the abdominal cavity.
19. Flush the abdominal cavity with 2–3 mL of piperacillin/tazobactam solution to help prevent bacterial infection.
20. Close the incision in double layers with continuous over-and-over sutures using 4–0 VICRYL RAPIDE® suture (Fig. 3i; *see* **Note 21**).
21. Allow mice to recover in a warm and dry incubator (30 °C) and monitor at least every 15 min until they resume activity and are able to maintain a sternal or sitting position.
22. After recovery, place mice back into cages with regular bedding and provided ad lib Bactrim diet and water.
23. Evaluate animals 12 h later and then daily throughout the remainder of the experiment. Appetite, attitude, and hydration

should be noted as an indication of recovery from the surgery. Supplemental fluids and/or analgesics should be administered postoperatively as needed.

24. Sacrifice the mice at a desired time point (Fig. 3j–l; *see* **Note 22**).

3.3 Mesenteric Transplantation of the HIO

1. Bring the mice to the operating suite where weighting and assessment of health status are performed.
2. Anesthetize the mouse in an anesthetic gas vaporizer delivering an isoflurane/O_2 mixture.
3. Shave the abdomen between the last rib and the iliac crest. Remove loose fur (Fig. 4a).
4. Prepare the surgical site using povidone-iodine with a cotton swab. Repeat the procedure with new cotton swabs three times.
5. Repeat the procedure using 70% isopropyl alcohol with cotton swabs. Repeat the procedure with new cotton swabs three times.
6. Place ophthalmic ointment on the eyes to prevent drying of the cornea and administer buprenorphine (0.05 mg/kg) subcutaneously.
7. Restrain the mouse on dorsal recumbency and secure the mouse to a nose cone vaporizing isoflurane/O_2 mixture.
8. Monitor respiratory rate and effort, along with the surgical plane of anesthesia. Confirm the loss of pedal reflex by pinching the toe with forceps.
9. Use straight forceps and fine scissors to make an 8–10 mm midline abdominal skin incision parallel to the spine and in between the iliac crest and the last rib (Fig. 4b).
10. Use fine scissors to make a subsequent 8–10 mm retroperitoneal incision in the *linea alba* (muscle sparing) to gain access to the peritoneum.
11. Identify the cecum in the right upper quadrant, and use ring forceps and cotton swabs to expose delicately the small intestine and the mesentery (Fig. 4c; *see* **Note 23**).
12. Use a cotton swab to dry out the mesentery.
13. Place a collagen-embedded HIO on the mesentery using straight suture-tying forceps.
14. Apply an octyl/butyl cyanoacrylate glue in between the mesentery and the HIO (Fig. 4d–f; *see* **Note 24**).
15. Wait for 5 min to allow the complete curing of the glue.
16. Return the small intestine within the abdominal cavity. Carefully replace the organ avoiding any torsion of the gut or its blood supply.
17. Flush the abdominal cavity with 2–3 mL of piperacillin/tazobactam solution to help prevent bacterial infection.

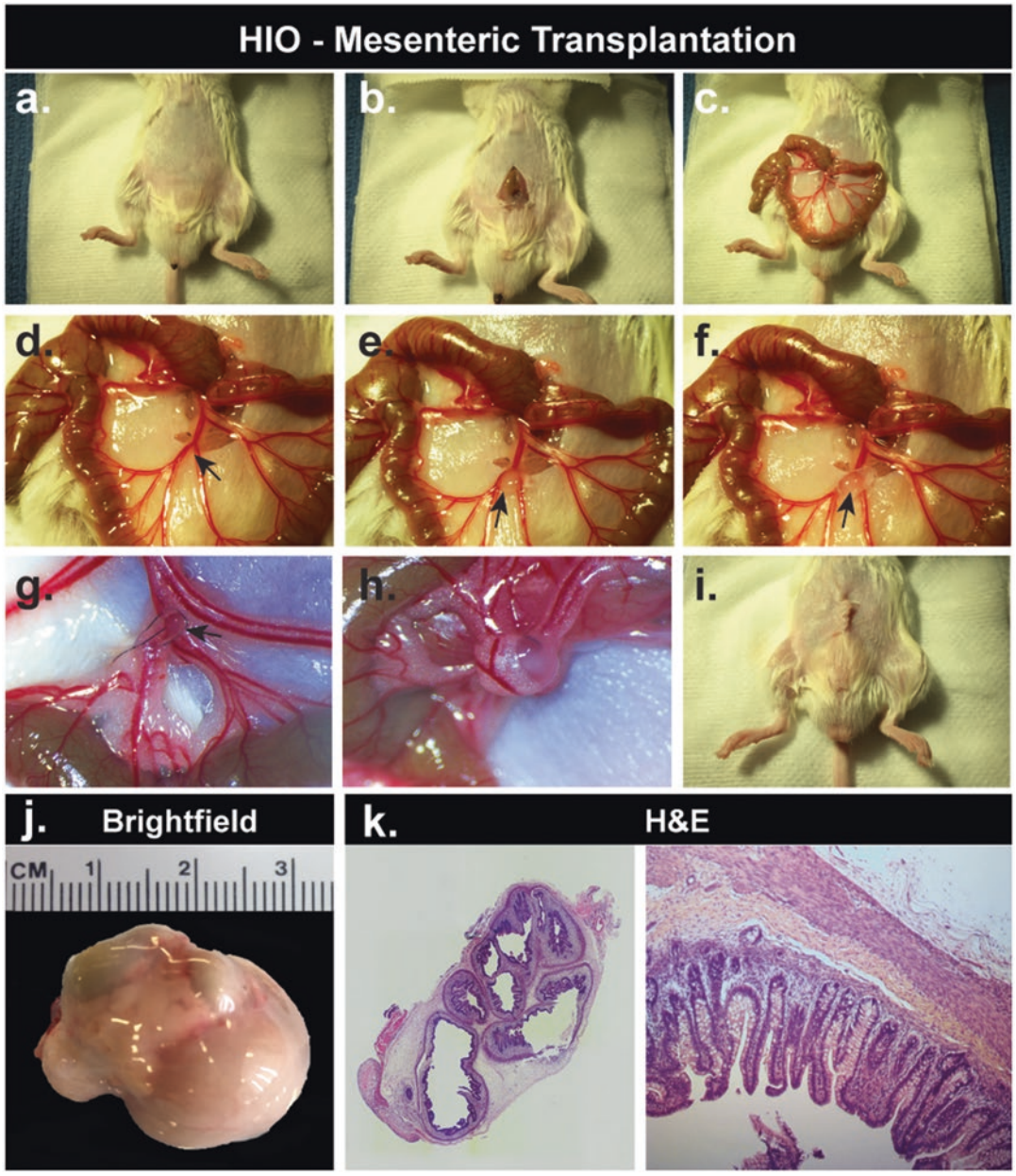

Fig. 4 Human intestinal organoid transplantation in the mesentery. (**a**–**c**) A midline incision is performed in the skin and the subjacent muscle layer to access the mesentery. (**d**–**f**) A collagen-embedded human intestinal organoid is placed and glued adjacent to the mesenteric vasculature (*black arrows*). (**g**–**h**) Alternatively, a string-purse suture can be performed to fold the organoid within the mesentery (*black arrows*). (**i**) The mesentery is returned to the abdominal cavity, and both abdominal and skin layers are sutured. (**j**) Photograph of a human intestinal organoid after 10 weeks posttransplantation. (**k**) Hematoxylin and eosin staining of the transplant showing multilobular cavities. Morphology of the transplant showing crypt and villus structures of the epithelium along with a laminated submucosal layer

18. Close the incision in double layers with continuous over-and-over sutures using 4–0 VICRYL® suture (Fig. 4i).
19. Allow mice to recover in a warm and dry incubator (30 °C) and monitor at least every 15 min until they resume activity and are able to maintain a sternal or sitting position.
20. After recovery, place mice back into cages with regular bedding and provided ad lib Bactrim diet and water.
21. Evaluate animals 12 h later and then daily throughout the remainder of the experiment. Appetite, attitude, and hydration should be noted as an indication of recovery from the surgery. Supplemental fluids and/or analgesics should be administered postoperatively as needed.
22. Utilize the mouse for side-to-side anastomosis or sacrifice the mice at a desired time point (Fig. 4j, k; *see* **Note 25**).

3.4 Side-To-Side HIO Mouse Small Intestine Anastomosis

All mice undergoing intestinal side-to-side anastomosis are provided with ad libitum liquid diet for 24–48 h prior to surgery and housed in cages with nonedible bedding (*see* **Note 26**).

1. Bring the mice to the operating suite where weighting and assessment of health status are performed.
2. Anesthetize the mouse in an anesthetic gas vaporizer delivering an isoflurane/O_2 mixture.
3. Shave the abdomen between the last rib and the iliac crest. Remove loose fur.
4. Prepare the surgical site using povidone-iodine with a cotton swab. Repeat the procedure with new cotton swabs three times.
5. Repeat the procedure using 70% isopropyl alcohol with cotton swabs. Repeat the procedures with new cotton swabs three times.
6. Place ophthalmic ointment on the eyes to prevent drying of the cornea and administer buprenorphine (0.05 mg/kg) subcutaneously.
7. Restrain the mouse on dorsal recumbency and secure the mouse to a nose cone vaporizing isoflurane/O_2 mixture.
8. Monitor respiratory rate and effort, along with the surgical plane of anesthesia. Confirm the loss of pedal reflex by pinching the toe with forceps.
9. Use straight forceps and fine scissors to make an 8–10 mm midline abdominal skin incision in the midline.
10. Use fine scissors to make a subsequent 8–10 mm incision in the *linea alba* to gain access to the peritoneum.
11. Identify the transplanted HIO, and use ring forceps and cotton swabs to expose it outside the abdominal cavity (Fig. 5a).
12. Orient the HIO and the murine small intestine to perform a side-to-side anastomosis. The HIOs are very mobile and should

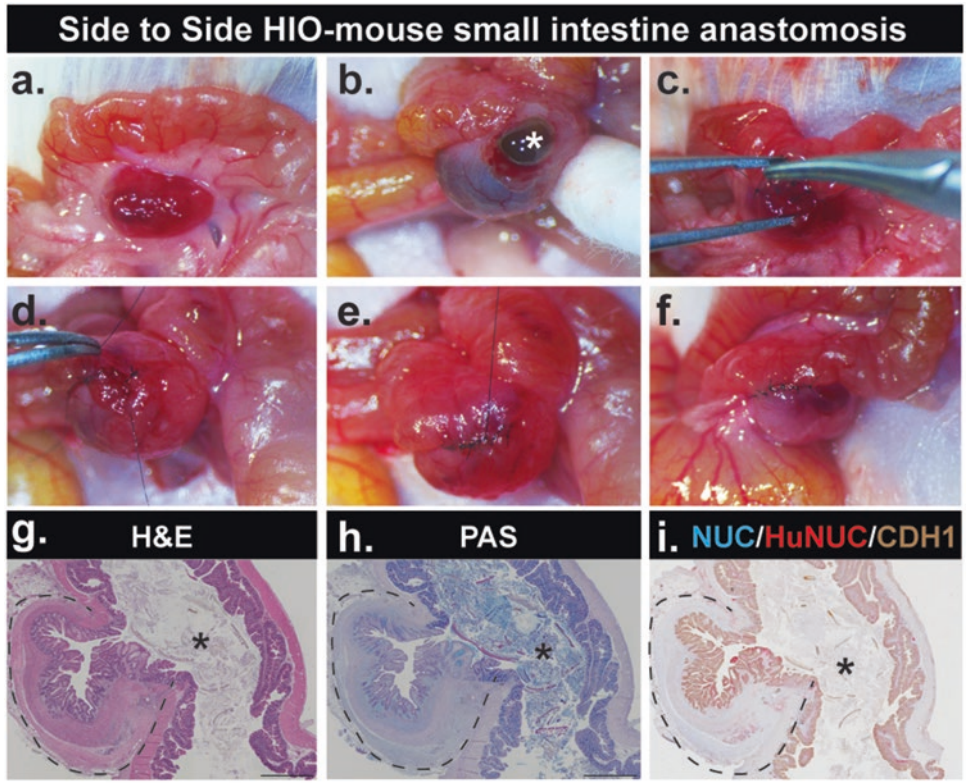

Fig. 5 Side-to-side anastomosis of the human intestinal organoid with the murine small intestine. (**a**–**b**) A 6–8 weeks transplanted human intestinal organoid is identified and irrigated to remove debris. A 5–6 mm incision on the antimesenteric side of the small intestine is performed to provide a bypass of around 5–6 mm. (**c**) Initial sutures are placed on the posterior sides of the HIO and small intestine with interrupted 9–0 nylon over-and-over suture under a stereoscope microscope. The first stitch should be place at 6 o'clock (middle of the posterior wall) and left long to help exposure for future posterior sutures. (**d**) Retracting on the middle suture, the posterior wall is exposed, and additional sutures are placed to from a nice mucosa to mucosa anastomosis. (**e**–**f**) The continuity of the intestine is restored by applying interrupted stitches on the anterior sides. (**g**–**h**) Hematoxylin and eosin and PAS stainings demonstrate the continuity between the murine small intestine (*black asterisk*) and the transplant (*black dotted line*). (**i**) Human nuclear staining (HuNUC, red) shows the human origin of the transplant. The cadherin 1 (CDH1) staining demonstrates the continuity between the murine and human epithelia

be oriented in a manner that will not obstruct the intestine after the anastomosis is performed.

13. Perform an incision of 5–6 mm using Vannas spring scissors on the HIO, and irrigate out the luminal debris with saline (Fig. 5b).
14. Perform a 5–6 mm incision on the antimesenteric side of the small intestine to provide a bypass of around 5–6 mm.
15. Place the initial sutures on the posterior sides of the HIO and small intestine with interrupted 9–0 nylon over-and-over suture under a stereoscope microscope. The first stitch should be place at 6 o'clock (middle of the posterior wall) and left long to help exposure for future posterior sutures. The two corner sutures

are placed next. Retracting on the middle suture, the posterior wall is exposed, and additional sutures are placed from a nice mucosa to mucosa anastomosis (Fig. 5c–d).

16. Restore the continuity of the intestine by applying interrupted stitches on the anterior sides (Fig. 5e–f).
17. Check for any leakage using a cotton swab.
18. Return the small intestine within the abdominal cavity. Carefully replace the organ avoiding any torsion of the gut or its blood supply.
19. Inject 2–3 mL of piperacillin/tazobactam solution into the abdominal cavity to help prevent bacterial infection.
20. Close the incision in double layers with continuous over-and-over sutures using 4–0 VICRYL® suture.
21. Allow mice to recover in a warm and dry incubator (30 °C), and monitor at least every 15 min until they resume activity and are able to maintain a sternal or sitting position.
22. After recovery, place mice back into cages with nonedible bedding and provided ad libitum antibiotic diet and water.
23. Evaluate animals 12 h later and then daily throughout the remainder of the experiment. Appetite, attitude, and hydration should be noted as an indication of recovery from the surgery. Supplemental fluids and/or analgesics should be administered postoperatively as needed. Evaluate mice and weigh and provide new liquid diet daily. In addition, change bedding as needed (usually every other day).
24. Utilize the mouse at a desired time point and analyze the transplant (Fig. 5g–i).

4 Notes

1. Divide intestinal growth medium into 10 mL aliquots in 15 mL conical tubes, and freeze at −20 °C for up to 3 months. Store thawed aliquots up to 5 days at 4 °C without loss of activity.
2. Males are preferably used for the kidney subcapsular transplantation as their kidneys are bigger and easier to work with.
3. The chow diet is supplemented with antibiotics and given to the mice at least 14 days prior to any surgeries. The antibiotics decrease inflammation and risk of infection.
4. The surgical suite consists of an operating table and a surgical scope placed under a sterile vertical laminar flow. The table is heated to 30 °C, and, additionally, a 30 °C water-heated pad is used to further control animal temperature.
5. The anesthesia system delivers an isoflurane and oxygen mixture that can be controlled and monitored to maintain the

anesthesia during surgery. The extra anesthetic gas is collected and evacuated into a canister.

6. The stereoscope is placed under a vertical laminar flow hood to prevent any contamination.
7. Collect the spheroids using a 200 μL sterile tip, which is cut in its extremity. Make sure to collect rounded spheroids with an average size of 20–50 μm. Avoid smaller or broken spheroids that would result in a low transplantation yield.
8. Eject the Matrigel®-embedded spheroids in the center of a pre-warmed 24-well plate where the center of each well has been pre-coated with a 10 μL Matrigel® bed. This will maintain the Matrigel® in the center of the well during polymerization.
9. Intestinal growth medium aliquots are thawed and can be kept up to 5 days at 4 °C without loss of activity. Add the human recombinant EGF prior to media change (1:10,000 stock dilution).
10. Prepare HIOs 1 to 2 h prior to surgery.
11. HIOs can be transplanted from day 26 to day 42 of culture. Before or beyond these time points, success of engraftment is not guaranteed. Control the quality of the HIOs using specific gene and protein expression to assess correct intestinal differentiation, i.e., CDX2, CDH1, and SOX2.
12. Collagen embedding can be facultative, but improve the handling of the HIOs during transplantation.
13. For early morning surgeries, HIOs can be embedded in the evening preceding the transplantation.
14. Final anesthetic gas concentration is achieved by delivering 2% isoflurane with 2.5–3 L/min O_2.
15. The left kidney is used for ease of access.
16. Analgesia provisions are most effective at reducing the intensity of painful stimulation when given prior to the surgery. Any animal showing evidence of pain should be provided with analgesia. Other opioids like buprenorphine can be used, i.e., butorphanol (0.2–2 mg/kg subcutaneous or intraperitoneal) or oxymorphone (0.2–0.5 mg/kg subcutaneous).
17. Keep the animal warm using a 37 °C heating pad. Adjust anesthetic gas concentration to 1.5–1.75% isoflurane with 2–3 L/min O_2.
18. This technique allows you to hold the kidney outside the abdominal cavity. Do not completely tie the knot to avoid renal vascular ligation and permanent kidney damage. Alternatively, curved forceps can be used to lift the kidney.
19. Slide the closed straight suture-tying forceps under the capsule and open the forceps while pulling it back. Repeat the motion until an appropriate size of the subcapsular pocket is achieved.

20. Inserted HIOs will not dislodge from under the subcapsular pocket.
21. VICRYL RAPIDE® sutures are synthetic coated absorbable sutures, and the animals will not chew them. Alternatively, skin staplers can be used.
22. In our experience, 6–8 weeks post-transplanted HIOs provide us with a fully laminated small intestinal tissue that can be further used for downstream applications ranging from physiological to molecular assays. HIOs transplanted beyond a year do not exhibit common intestinal epithelial features probably due to the accumulation of mucus and debris within the lumen.
23. Multiple mesenteric sites can be utilized for transplantation as long as they provide blood supplies and are not in area that would create volvulus and therefore intestinal obstruction.
24. Topical adhesive glue can be used to glue the HIOs to the mesentery. Alternatively, a purse-string suture can be created using 9–0 nonabsorbable nylon sutures with taper cut needles to form a mesenteric pocket for the HIO (Fig. 4g–h).
25. Similar to the subcapsular transplantation, 6–8 weeks post-transplanted HIOs provide us with a developed small intestinal tissue that can be further used for downstream applications or subsequent surgeries.
26. Liquid diet is well tolerated and isocaloric when compared to regular chow. The use of this diet prior to surgery prevents obstruction at the anastomosis site increasing the survival of the animals.

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