Biochemical and Biophysical Research Communications 391 (2010) 38–42

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Generation of functional gut-like organ from mouse induced pluripotent stem cells ☆

Takeshi Ueda [a], Takatsugu Yamada [a,*], Daisuke Hokuto [a], Fumikazu Koyama [a], Shogo Kasuda [b], Hiromichi Kanehiro [a], Yoshiyuki Nakajima [a]

[a] Department of Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan
[b] Department of Legal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan

ARTICLE INFO

Article history:
Received 27 October 2009
Available online 4 November 2009

Keywords:
Induced pluripotent stem (iPS) cells
Induced gut (iGut)
Organ regeneration
Motor function
Peristalsis
Hanging drop culture

ABSTRACT

Induced pluripotent stem (iPS) cells have the pluripotency to differentiate into broad spectrum derivatives of all three embryonic germ layers. However, the in vitro organ differentiation potential of iPS cells to organize a complex and functional "organ" has not yet been demonstrated. Here, we demonstrate that mouse iPS cells have the ability to organize a gut-like organ with motor function in vitro by a hanging drop culture system. This "induced gut (iGut)" exhibited spontaneous contraction and highly coordinated peristalsis accompanied by a transportation of contents. Ultrastructural analysis identified that the iGut had large lumens surrounded by three distinct layers (epithelium, connective tissue and musculature). Immunoreactivity for c-Kit, a marker of interstitial cells of Cajal (ICCs, enteric pacemaker cells), was observed in the wall of the lumen and formed a distinct and dense network. The neurofilament immunoreactivity was identified to form large ganglion-like structures and dense neuronal networks. The iGut was composed of all the enteric components of three germ layers: epithelial cells (endoderm), smooth muscle cells (mesoderm), ICCs (mesoderm), and enteric neurons (ectoderm). This is the first report to demonstrate the in vitro differentiation potential of iPS cells into particular types of functional "organs." This work not only contributes to understanding the mechanisms of incurable gut disease through disease-specific iPS cells, but also facilitates the clinical application of patient-specific iPS cells for novel therapeutic strategies such as patient-specific "organ" regenerative medicine in the future.

Introduction

Induced pluripotent stem (iPS) cells have been established from mouse and human adult somatic cell cultures through reprogramming by transduction of four defined transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) [1–4]. The iPS cells have the pluripotency to differentiate into broad spectrum derivatives of all three embryonic germ layers. Recent studies have reported that mouse and human iPS cells can differentiate into particular types of "cells," such as hematopoietic cells (dendritic cells and macrophages), cardiomyocytes, vascular cells, motor neurons, and insulin-producing cells under appropriate conditions [5–14]. However, the pluripotent ability of iPS cells to organize a complex and functional "organ," which is composed of a variety of cell types in an orderly manner and exhibits systematic function, has not yet been demonstrated.

To induce an in vitro organ differentiation potential of iPS cells, we used a three-dimensional hanging drop culture system to form embryoid bodies (EBs) [15,16]. In the present study, we demonstrate that mouse iPS cells have the ability to organize a gut-like organ with motor function in vitro. This functional gut-like organ induced from mouse iPS cells, which we designated "iGut (induced gut)," is organized by enteric components of all three germ layers, including epithelial cells (endoderm), smooth muscle cells (mesoderm), interstitial cells of Cajal (ICCs) (mesoderm), and enteric neurons (ectoderm). Notably, iGut represents a three-dimensional structure with lumen and exhibits mechanical activity such as spontaneous contraction and highly coordinated peristalsis accompanied by transportation of contents. This is the first report to demonstrate the in vitro differentiation potential of iPS cells into particular types of "organs," which consist of derivatives of all three embryonic germ layers. This work not only contributes to our understanding of the mechanisms of gut development and disease, but also facilitates the clinical application of patient-specific

Abbreviations: EB, embryoid body; ES cell, embryonic stem cell; ICCs, interstitial cells of Cajal; iGut, induced gut; iPS cell, induced pluripotent stem cell; LIF, leukemia inhibitory factor; NF, neurofilament.

☆ T.U., T.Y. and D.H.: study concept and design, acquisition of data, analysis and interpretation of data; F.K., S.K., and H.K.: technical support; N.Y.: study supervision.

* Corresponding author. Fax: +81 744 24 6866.
E-mail address: highnet@naramed-u.ac.jp (T. Yamada).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.10.157

iPS cells for novel therapeutic strategies such as patient-specific organ transplantation.

Materials and methods

Growth and differentiation of iPS cells. Mouse iPS cell line (20D-17: RIKEN BRC, Tsukuba, Japan) was prepared for our study. This iPS cells was produced from mouse skin (fibroblast cells) by Dr. Shinya Yamanaka (Center for iPS Cell Research and Application, Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto, Japan) and was carrying Nanog promoter-driven GFP/IRES/puromycin-resistant gene (Nanog-iPS cells) [3,4]. The iPS cells were maintained on feeder layers of radiation-treated mouse embryonic fibroblast (MEF: Millipore; Temecula, CA; http://www.Millipore.com) cells in Dulbecco's modified Eagle's medium (DMEM: Wako; Osaka, Japan; http://www.wako-chem.co.jp/) containing 10% fetal bovine serum (FBS: EmbryoMax® ES Cell Qualified FBS, Millipore), 0.1 mM nonessential amino acids (NEAA: Invitrogen, Carlsbad, CA; http://www.invitrogen.co.jp/), 0.1 mM 2-mercaptoethanol (2-ME: Wako), 1 mM sodium pyruvate (Lonza; Walkersville, ML), and 50 U/ml penicillin and 50 μg/ml streptomycin (Penicillin–Streptmycin liquid: Invitrogen) supplemented with 1000 U/ml of leukemia inhibitory factor (LIF: ESGRO®; Millipore). To eliminate contaminating MEF cells, maintained iPS cells were dissociated with 0.25% trypsin/EDTA (Mediatech; Manassas, VA; http://www.cellgro.com) and passaged twice (two days for first passage and one day for second passage) on gelatin-coated dishes without feeder cells. To induce embryoid body (EB) formation, iPS cells were dissociated to a single cell suspension with 0.05% trypsin/EDTA (Mediatech), and then cultured in hanging drops, as previously described [15,16]. Briefly, the medium for EB formation is the same medium as for iPS-cell maintained culture in the absence of LIF. The cell density of one drop was 500 iPS cells per 15 μl of medium. After 6-days in a hanging drop culture, iPS cells aggregate and form a sphere structure, so called EB. The resulting EBs were plated onto plastic 100-mm gelatin-coated dishes in the same medium as for EB formation and allowed to attach for the outgrowth culture. Cultures were maintained in a humidified chamber in a 5% CO_2/air mixture at 37 °C.

Immunohistochemistry. For immunohistochemical detection of c-Kit, a transmembrane receptor that has tyrosine kinase activity, whole-mount preparations of iGut were fixed in acetone (4 °C, 5 min), and for detection of smooth muscle actin and neurofilament (NF), the preparations were fixed in 4% paraformaldehyde (4 °C, 10 min). After fixation, the preparations were washed for 30 min in phosphate-buffered saline (PBS) (0.01 M, pH 7.4), and then incubated for 3 h at room temperature in 10% normal goat serum (Dako; Carpinteria, CA; http://www.dako.com) in PBS containing 0.3% (vol/vol) Triton X-100 (Calbiochem; san Diego, CA; http://www.emdbiosciences.com) to reduce non-specific antibody binding. Tissues were then incubated at 4 °C for 1 day with a rat monoclonal antibody raised against c-Kit protein (ACK2: 5 μg/ml in PBS; eBioscience, San Diego, CA; http://www.ebioscience.com), a rabbit monoclonal antibody against α-smooth muscle actin (1:250; Epitomics; Burlingame, CA; http://www.epitomics.com) and a rabbit polyclonal antiserum cocktail against primate and bovine low-(68–70 kDa), medium-(150 kDa), and high-molecular-weight NF (200–210 kDa) (1:500; Affiniti Research Products; Devon, UK; http://www.affiniti-res.com). This antiserum cocktail reacts with neuronal cell bodies, dendrites, and thick and thin axons. The c-Kit immunoreactivity was detected using an Alexa Flour 594-conjugated secondary antibody (1:200; Alexa Flour 594 goat anti-rat: Molecular Probes; Eugene, OR; http://probes.invitrogen.com) for 2 h in the dark at room temperature, whereas smooth muscle actin and NF immunoreactivity was detected using a Texas Red-conjugated secondary antibody (1:100; Texas Red goat anti-rabbit: Invitrogen) for 1 h in the dark at room temperature. Tissues were then examined using a laser confocal microscope (FV1000: Olympus; Tokyo, Japan; http://www.olympus.co.jp), which yielded confocal micrographs that were digital composites of Z-series scans of 10–15 optical sections through a depth of 10–20 μm. Final images were constructed using software (FV10-ASW Version 1.7: Olympus).

Electron microscopy. The iGut were fixed with 2% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) at 4 °C. They were post-fixed with 2% OsO_4 in a 0.1 M phosphate buffer, and subsequently stained with uranyl acetate in a 0.05 M maleic acid buffer. Then, the specimens were dehydrated in a graded ethanol and embedded in the epoxy resin. Ultrathin sections stained with uranyl acetate and modified Sato's lead solution were submitted to TEM observation (H-7600: Hitachi; Brisbane, CA; http://www.hitachi.com).

Results

iPS cell differentiation and mechanical activity

iPS cells were cultured for 6 days in a hanging drop culture system and allowed to form spherical multicellular aggregates, embryoid bodies (EBs). After 3–5 days in subsequent outgrowth culture, various types of cell clusters, including cardiac muscle-like beating cell populations, emerged from the outgrowths of the attached EBs, as shown in video format (see *Nara Medical University* home page http://www.naramed-u.ac.jp/~1sur/iHeart2.wmv). On approximately Day 7, the gut-like structure, iGut, which was different from the beating cells, began to elevate on the outgrowth of EBs and contract spontaneously with an irregular rhythm (Fig. 1A). On approximately Day 10, the contracting iGut underwent a morphological transformation, which showed different size and shape including dome-like type and tubular type. The dome-like type iGut showed a hemispherical structure with a cavity that contained fluid and solids (see http://www.naramed-u.ac.jp/~1sur/iGut2.wmv) (Fig. 1B). The tubular type iGut proliferated to form more prominent three-dimensional tubular structures with lumen that extended its longitudinal axis in parallel to the dish bottom (Fig. 1C). On approximately Day 14, the iGut began rhythmic contractions (Fig. 1D). On approximately Day 21, the iGut showed distinct and highly coordinated contraction patterns with regular rhythms. This mechanical activity was composed of periodic contraction and relaxation (see http://www.naramed-u.ac.jp/~1sur/iGut1.wmv) (Fig. 1E–H). It was very similar to GI motility, i.e., peristalsis. The peristalsis-like contractions were accompanied by a transportation of contents (see http://www.naramed-u.ac.jp/~1sur/iGut3.wmv) (Fig. 1I–L). The iGut with peristalsis-like contractions exhibited the periodic movements of back and force in the closed lumen (see http://www.naramed-u.ac.jp/~1sur/iGut4.wmv). The frequency of contraction was 6.9 ± 0.3 cycles per minutes at 37 °C (n = 14 iGut). The frequency was strongly dependent on the temperature and decreased to 3.2 ± 0.5 cycles per minutes at 20 °C (n = 14 iGut), which is one of the characteristics of mature GI tract.

Immunohistochemistry

Immunohistochemistry was used to identify the cellular components of the contracting iGut. Immunoreactivity for smooth muscle actin was observed in the wall surrounding the lumen and spreading out from the iGut (Fig. 2A). Immunoreactivity for c-Kit, a marker for ICC [17], was observed in the iGut that showed spontaneous contractions. The c-Kit immunopositive (c-Kit+) cells were mostly multipolar, and formed a distinct and dense network

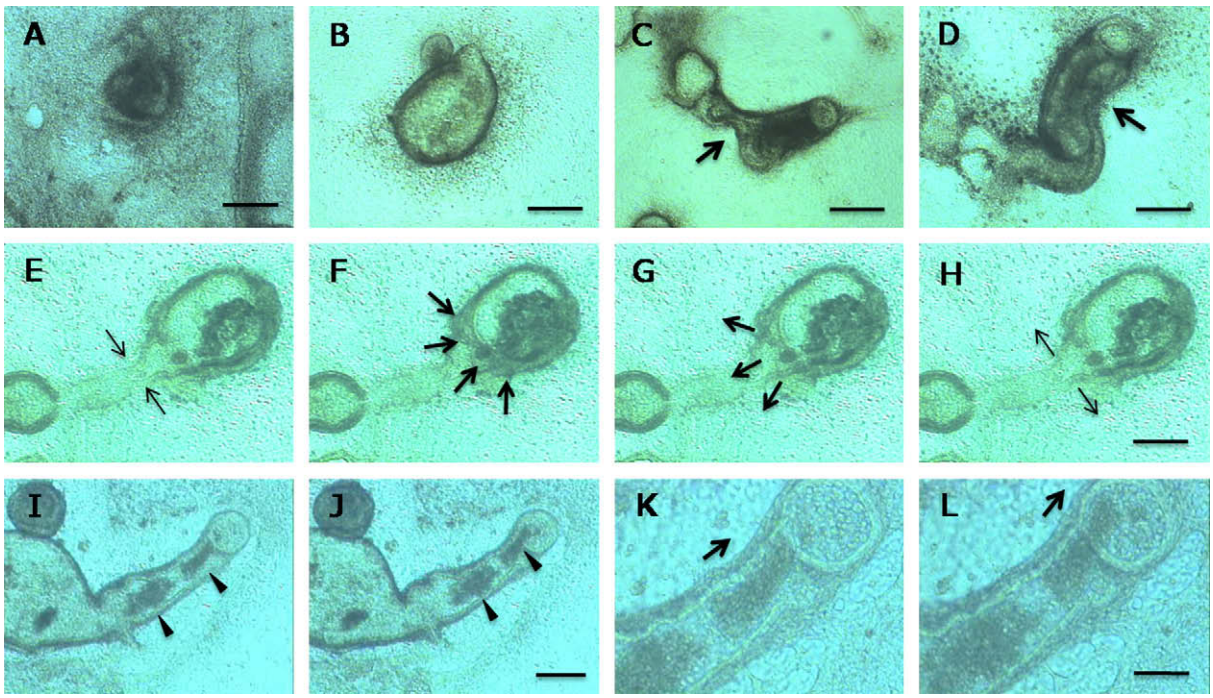

Fig. 1. The iGut began to elevate on the outgrowth of EBs and contract spontaneously with an irregular rhythm after 7 days in outgrowth culture (A). Dome-like type iGut on Day 10. They showed a hemispherical dome-like structure with a cavity that contained fluid and solids (B). Tubular type iGut on Day 10. They proliferated to form more prominent three-dimensional tubular structures with lumen that extended its longitudinal axis in parallel to the dish bottom (C). iGut on Day 14. They began rhythmic contractions (D). iGut on Day 21. They showed distinct and highly coordinated peristalsis-like contraction patterns with regular rhythms (E–H) This mechanical activity was composed of periodic contraction (E, F) and relaxation (G, H). The peristalsis-like contractions were accompanied by transportation of contents (arrowhead) (I–L). Scale bars represent the following sizes: 300 μm, (A); 500 μm, (B–J); and 200 μm, (K, L).

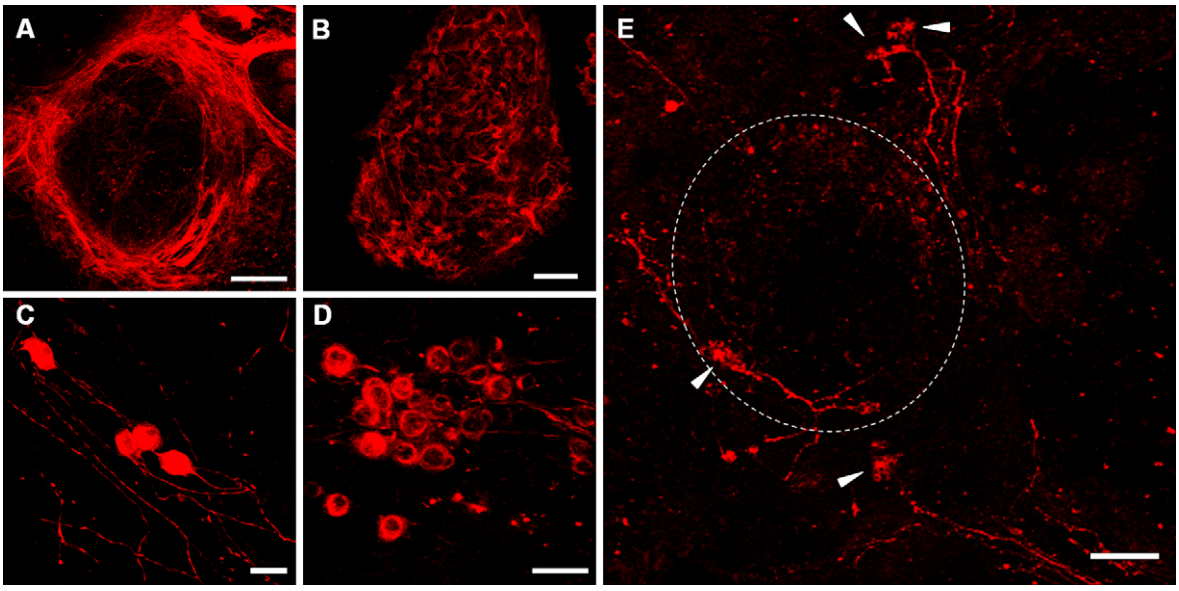

Fig. 2. Immunohistochemistry for iGut.: The contracting iGut showing α-smooth muscle actin immunoreactivity in the wall of the iGut surrounding the lumen and some immunopositive cells spreading out from the iGut (A). A large number of c-Kit+ cells were identified in the wall of the lumen, and formed a distinct and dense network. The c-Kit+ cells were mainly multipolar with prominent dendrites (B). NF+ cells located in the wall of iGut. They were mostly multipolar with prominent dendrites and axon-like processes (C). NF+ cells formed large ganglion-like structures outside the iGut (D). The neurofilament immunopositive (NF+) cells were observed in the wall and outside the iGut, which exhibited peristalsis-like contractions. They formed large ganglion-like structures inside the wall and outside the iGut (*arrowhead*). The NF fibers from ganglions located close to the iGut were often seen projecting into the contracting iGut (*broken line*) (E). Scale bars represent the following sizes: 200 μm, (A); 80 μm, (B); 30 μm, (C); 30 μm, (D) and 100 μm, (E).

(Fig. 2B). Immunoreactivity for neurofilament (NF) was detected in the iGut that exhibited peristalsis-like contractions. The NF immunopositive (NF+) cells were mostly multipolar with prominent dendrites and axon-like processes (Fig. 2C), and formed large ganglion-like structures (Fig. 2D). NF+ cells were distributed not only outside the iGut but also in the wall, and NF+ fibers from ganglions located close to the iGut were often seen projecting into the contracting iGut (Fig. 2E).

Ultrastructural characteristics of iGut

Ultrastructural analysis confirmed the cellular components of the iGut. The wall of the iGut was composed of three distinct layers: epithelium (EP), connective tissue (CT), and muscle layer (ML) (Fig. 3A). The innermost was a flat and single layer of epithelium, which did not form any plica, intestinal villus, or crypt. Most of the epithelial cells were columnar type, with fewer and shorter microvilli than those in epithelia in the mouse GI tract (Fig. 3B). Well-developed goblet cells and tuft cells were observed among the columnar cells (Fig. 3C and D). The second layer was connective tissue composed of fibroblasts and collagen fibers. Both blood and lymphatic vessels were absent. The outermost layer was composed of prominent smooth muscle cells, filled with myofilaments, caveolae, and dense bodies in their cytoplasm (Fig. 3E, 3F). The outer surface of the iGut was lined with a flat and thin monolayer, similar to the serosa.

Discussion

In the current study, we succeeded in inducing differentiation of mouse iPS cells into a functional gut-like organ from embryoid bodies (EBs). This functional gut-like organ induced from iPS cells, which we designated "iGut (induced gut)," was composed of the enteric derivatives of all three embryonic germ layers: epithelial cells (endoderm), smooth muscle cells and ICCs (mesoderm), and enteric neurons (ectoderm), and exhibited mechanical activity such as spontaneous contraction and peristalsis-like motion. The iPS cells are identified to have pluripotency by ES cell-marker gene expression, ES cell-specific surface antigens and teratoma formation after subcutaneous transplantation into immune-deficient mice [1–3]. However, the iPS cells are not completely identical to ES cells in the global gene-expression patterns and DNA methylation status [1–3]. Although the iPS cells were confirmed to differentiate into all three germ layers in teratomas, including neural tissue, cartilage, smooth muscle, adipose and gut-like epithelium, the differentiation potential of iPS cells to organize a complex and functional "organ" has not been demonstrated [1–4]. This is the first report to demonstrate the in vitro differentiation potential of iPS cells into particular types of "organs," which not only consist of a variety of derivatives of all three germ layers but also exhibit systematic functions.

We previously established an in vitro organ differentiation system that can reproduce a contracting gut-like organ from mouse ES cells by a three-dimensional hanging drop culture [15,16]. We applied this system to iPS cells and investigated the organ differentiation properties of iPS cells into a gut-like organ. Ultrastructural analysis revealed that the iGut had large lumens surrounded by three layers (i.e., epithelium, connective tissue and musculature). Immunoreactivity for c-Kit, a marker for ICC (enteric pacemaker cell), was distributed to form a distinct and dense network in the wall of the iGut that showed spontaneous contractions. This network structure was similar to that of ICC at the level of the myenteric plexus in a murine embryo or neonate [18]. Notably, the neurofilament (NF) immunopositive cells formed large ganglion-like structures and dense neuronal networks not only in the wall of the lumen but also outside the iGut showing highly coordinated peristalsis, suggesting that innervations to the iGut governs the peristalsis-like contractions. Interestingly, the neuronal fibers from ganglions located close to the iGut were often seen projecting into the wall of the iGut. These findings raise the possibility that the in vitro neuronal differentiation process in the iGut

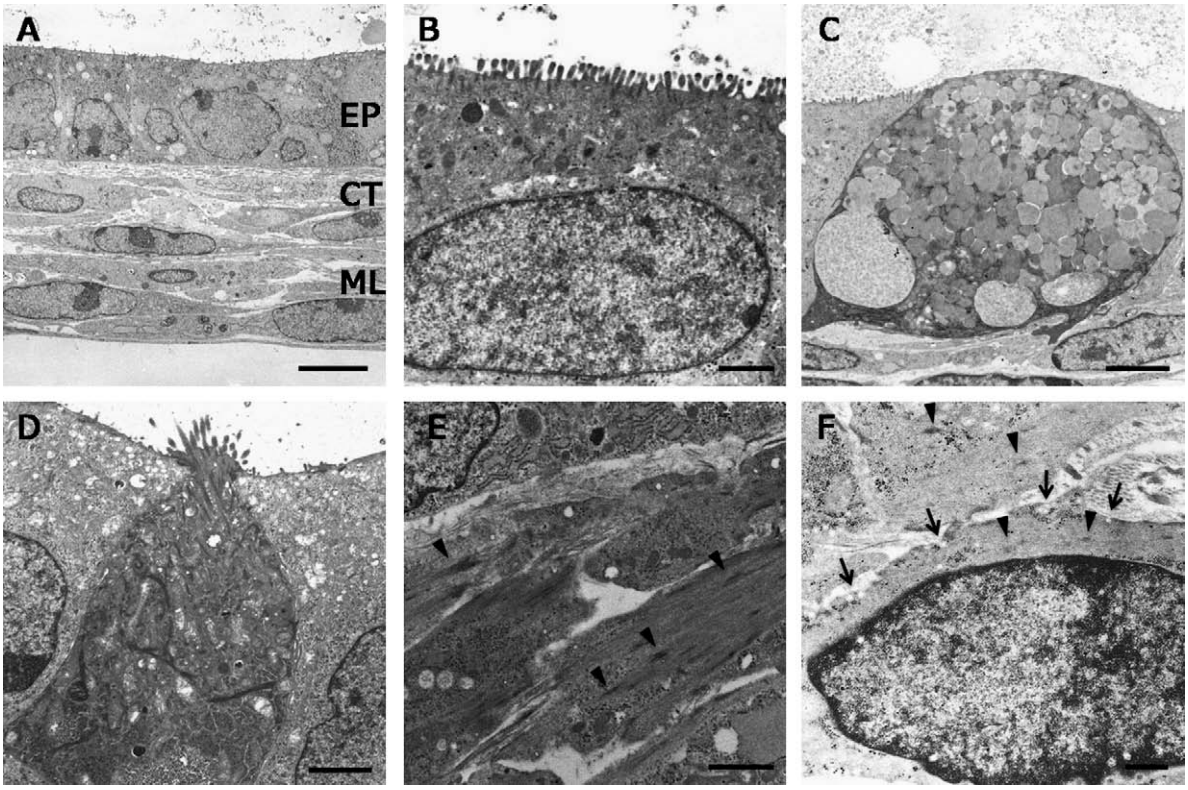

Fig. 3. Electron micrographs of contracting iGut.: A cross section of the wall clearly showing three layers: EP (epithelium), CT (connective tissue), and ML (muscle layer) (A). Most of the epithelial cells were columnar type, with fewer and shorter microvilli than those in epithelia in the mouse GI tract (B). A goblet cell with many mucous globules in the apical cytoplasm is prominent in the EP (C). Tuft cells were observed among columnar cells in the EP, and were characterized by microvilli with long bundles of straight filaments extending from the core of the microvilli and small vesicles in the apical cytoplasm (D). Smooth muscle cells in the muscle layer showing typical features of filament-like structures (i.e., thick, thin, and intermediate filaments), and dense bodies (*arrowheads*) (E). Smooth muscle cells filled with myofilaments, caveolae (*arrows*) and dense bodies (*arrowheads*) in their cytoplasm (F). Scale bars represent the following sizes: 6 μm, (A); 1 μm, (B); 3 μm, (C); 2 μm, (D); 1 μm, (E); and 500 nm, (F).

may mimic the enteric nervous system formation originating from neural crest cell migration during embryonic gut development in vivo [19,20].

Takahashi and Yamanaka [1] reported that mouse iPS cells differentiate into only sheet-like enteric smooth muscles within the adhesive outgrowth of EBs that are cultured in hanging drop for 3 days. In contrast, using our system with EBs cultured in a hanging drop for 6 days, we demonstrated that iPS cells can give rise to a three-dimensional gut-like organ, which consists not only of smooth muscle cells but also of epithelial cells, ICCs and enteric neuronal networks. These results suggest that the amount of time for EB formation in a hanging drop culture may play an important role in the directed differentiation of iPS cells into the three-dimensional organ. The EBs with egg-cylinder-like structures at the 6-day hanging drop culture may have the potential to regulate developmental programs associated with cell lineage commitment, and provide an appropriate microenvironment to differentiate iPS cells into enteric derivatives of all three embryonic germ layers and to reproduce the gut organization process in vitro [21,22].

We succeeded in inducing in vitro organization of a functional iGut from the iPS cells derived from mouse adult skin fibroblast cells through reprogramming by transduction of four defined transcription factors. This iGut had large lumens surrounded by three layers (epithelium, connective tissue and musculature) and exhibited spontaneous contractions and highly coordinated peristalsis, which was involved in the differentiation of ICCs (pacemaker cells) and enteric neuronal networks, respectively. Therefore, this work not only contributes to understanding the mechanisms of gut development and incurable disease, but also provides a powerful tool to develop effective and safe drugs for GI disorders through disease-specific iPS cells. Our success may facilitate the clinical application of patient-specific iPS cells for novel therapeutic strategies such as patient-specific organ transplantation. Our findings contribute to the scientific revolution that patient or disease-specific iPS cells may represent a novel and valuable cell population for clinical applications in "organ" regenerative medicine in the future.

Conflicts of interest

The authors disclose no conflicts.

Acknowledgment

We thank Mr. Yoshiaki Hanaichi and Mr. Hiroshi Takase (Hanaichi Ultrastructure Research Institute, Okazaki, Japan) for their technical assistance for electron microscopy. This work was supported by Research Grants-in-Aid from the Ministry of Education, Science and Culture of Japan (Nos. 21591649 and 21592280).

References

[1] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126 (2006) 663–676.
[2] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors, Cell 131 (2007) 861–872.
[3] K. Okita, T. Ichisaka, S. Yamanaka, Generation of germline-competent induced pluripotent stem cells, Nature 448 (2007) 313–317.
[4] K. Takahashi, K. Okita, M. Nakagawa, S. Yamanaka, Induction of pluripotent stem cells from fibroblast cultures, Nat. Protoc. 2 (2007) 3081–3089.
[5] S. Senju, M. Haruta, Y. Matsunaga, S. Fukushima, T. Ikeda, K. Takahashi, K. Okita, S. Yamanaka, Y. Nishimura, Characterization of dendritic cells and macrophages generated by directed differentiation from mouse induced pluripotent stem cells, Stem Cells 27 (2009) 1021–1031.
[6] G. Narazaki, H. Uosaki, M. Teranishi, K. Okita, B. Kim, S. Matsuoka, S. Yamanaka, J.K. Yamashita, Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells, Circulation 118 (2008) 498–506.
[7] C. Mauritz, K. Schwanke, M. Reppel, S. Neef, K. Katsirntaki, L.S. Maier, F. Nguemo, S. Menke, M. Haustein, J. Hescheler, G. Hasenfuss, U. Martin, Generation of functional murine cardiac myocytes from induced pluripotent stem cells, Circulation 118 (2008) 507–517.
[8] J. Zhang, G.F. Wilson, A.G. Soerens, C.H. Koonce, J. Yu, S.P. Palecek, J.A. Thomson, T.J. Kamp, Functional cardiomyocytes derived from human induced pluripotent stem cells, Circ. Res. 104 (2009) e30–e41.
[9] C. Freund, C.L. Mummery, Prospects for pluripotent stem cell-derived cardiomyocytes in cardiac cell therapy and as disease models, J. Cell Biochem. 107 (2009) 592–599.
[10] D. Taura, M. Sone, K. Homma, N. Oyamada, K. Takahashi, N. Tamura, S. Yamanaka, K. Nakao, Induction and isolation of vascular cells from human-induced pluripotent stem cells, Arterioscler. Thromb. Vasc. Biol. 29 (2009) 1100–1103.
[11] S. Karumbayaram, B.G. Novitch, M. Patterson, J.A. Umbach, L. Richter, A. Lindgren, A.E. Conway, A.T. Clark, S.A. Goldman, K. Plath, M. Wiedau-Pazos, H.I. Kornblum, W.E. Lowry, Directed differentiation of human-induced pluripotent stem cells generates active motor neurons, Stem Cells 27 (2009) 806–811.
[12] M. Wernig, J.P. Zhao, J. Pruszak, E. Hedlund, D. Fu, F. Soldner, V. Broccoli, M. Constantine-Paton, O. Isacson, R. Jaenisch, Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease, Proc. Natl. Acad. Sci. USA 105 (2008) 5856–5861.
[13] D. Zhang, W. Jiang, M. Liu, X. Sui, X. Yin, S. Chen, Y. Shi, H. Deng, Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells, Cell Res. 19 (2009) 429–438.
[14] N. Yokoo, S. Baba, S. Kaichi, A. Niwa, T. Mima, H. Doi, S. Yamanaka, T. Nakahata, T. Heike, The effects of cardioactive drugs on cardiomyocytes derived from human induced pluripotent stem cells, Biochem. Biophys. Res. Commun. 387 (2009) 482–488.
[15] T. Yamada, M. Yoshikawa, M. Takaki, S. Torihashi, Y. Kato, Y. Nakajima, S. Ishizaka, Y. Tsunoda, In vitro function gut-like organ formation from mouse embryonic stem cells, Stem Cells 20 (2002) 41–49.
[16] T. Yamada, Y. Nakajima, Derivation and characterization of gut-like structures from embryonic stem cells, in: Kursad Turksen (Ed.), Methods in Molecular Biology, Embryonic Stem Cell Protocols, vol. II, Differentiation Models, vol. 330, Humana Press Inc., New Jersey, 2006, pp. 263–278.
[17] L. Thomsen, T.L. Robinson, J.C. Lee, L.A. Farraway, M.J. Hughes, D.W. Andrews, J.D. Huizinga, Interstitial cells of Cajal generate a rhythmic pacemaker current, Nat. Med. 4 (1998) 848–851.
[18] M. Kuwahara, T. Ogaeri, R. Matsuura, H. Kogo, T. Fujimoto, S. Torihashi, In vitro organogenesis of gut-like structures from mouse embryonic stem cells, Neurogastroenterol. Motil. 16 (Suppl. 1) (2004) 14–18.
[19] T.A. Heanue, V. Pachnis, Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies, Nat. Rev. Neurosci. 8 (2007) 466–479.
[20] A.J. Burns, Migration of neural crest-derived enteric nervous system precursor cells to and within the gastrointestinal tract, Int. J. Dev. Biol. 49 (2005) 143–150.
[21] G.M. Keller, In vitro differentiation of embryonic stem cells, Curr. Opin. Cell Biol. 7 (1995) 862–869.
[22] K. Abe, H. Niwa, K. Iwase, M. Takiguchi, M. Mori, S.I. Abé, K. Abe, K.I. Yamamura, Endoderm-specific gene expression in embryonic stem cells differentiated to embryoid bodies, Exp. Cell Res. 229 (1996) 27–34.