et al., 2005). However, it is important to investigate the long-term effect of MPTP-induced depletion of dopaminergic neurons because it has been known that the fish has a strong regenerative capacity of the brain structures (Zupanc, 2008). To this end, we exposed 10-dpf larvae to 0.2 μg/ml MPTP for 2 days, and measured the amounts of both dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC), a metabolite of dopamine, in the whole brain at 3 months after the MPTP treatment. We verified that both dopamine and DOPAC of the whole brain significantly decreased in MPTP-treated fish in comparison with non-treated control fish (Fig. 6A and B). DOPAC/dopamine ration was increased significantly and this was consistent with other MPTP models (Fig. 6D) (Irwin et al., 1990). The reduction of dopamine and DOPAC may not reflect general toxicity of MPTP towards the whole neurons, because MPTP did not diminish the level of norepinephrine (Fig. 6C).

To confirm whether MPTP injured the cluster of the diencephalic TH^+ neurons, leading to the decline of dopamine content in the brain, we counted the number of TH^+ cells in the brain. The number of TH^+ dopaminergic neurons in the middle diencephalon was significantly less in the MPTP-treated group than that in the non-treated control group (Fig. 7A–C). In contrast to the dopaminergic neurons in the middle diencephalon, the TH^+ signal intensity of dopaminergic fibers in the telencephalon were scarcely affected by MPTP possibly reflecting the recovery of these fibers after MPTP exposure at their larval stage (data not shown). The number of TH^+ neurons in the rostro-ventral and the caudal part of the diencephalon, and in the medulla oblongata also did not show statistically significant differences (Fig. 7C). Western blot analysis of whole brain extract disclosed a slight, albeit not statistically significant, decrease in the level of TH protein in the MPTP-treated groups (Fig. 7D). These data suggest that MPTP imposed on the

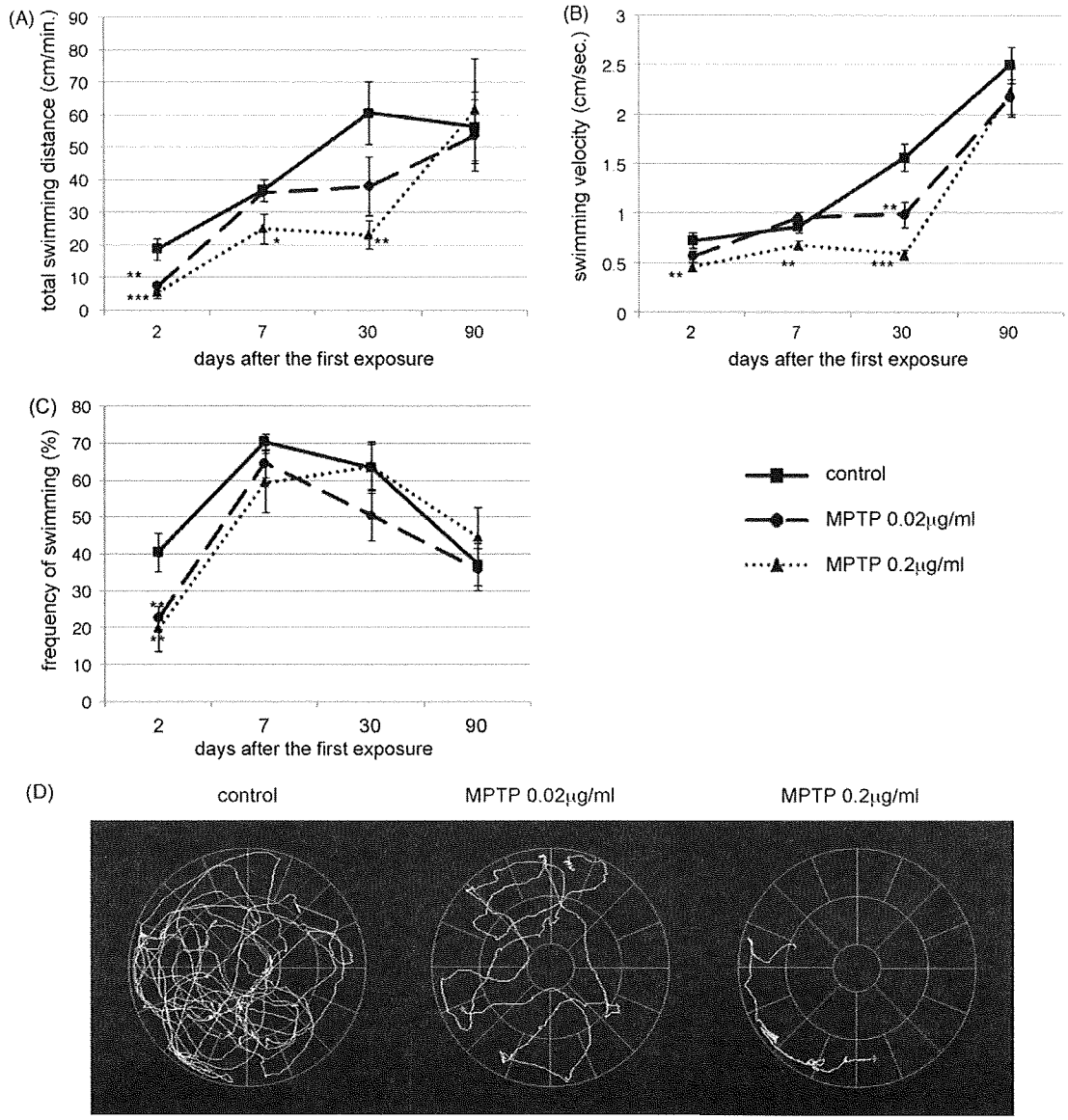

Fig. 5. Spontaneous movement analysis of MPTP-treated medaka. (A) Total swimming distance (cm/min), (B) swimming velocity (cm/s), (C) frequency of swimming movement (%), (D) representative swimming track immediately after the 2 days exposure of MPTP. Total swimming distance and swimming velocity of 0.2 μg/ml MPTP-treated fish decreased significantly 2, 7 and 30 days after the first exposure (12, 17 and 40-dpf) (A, B and D). The frequency of swimming movement also decreased 2 days after the first exposure (12-dpf) (C). The MPTP-treated fish showed gradual recovery from the defective movement and all the parameters did not differ among the groups 90 days after the first exposure (100-dpf) (A–C). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control. $n = 10$ for each group.

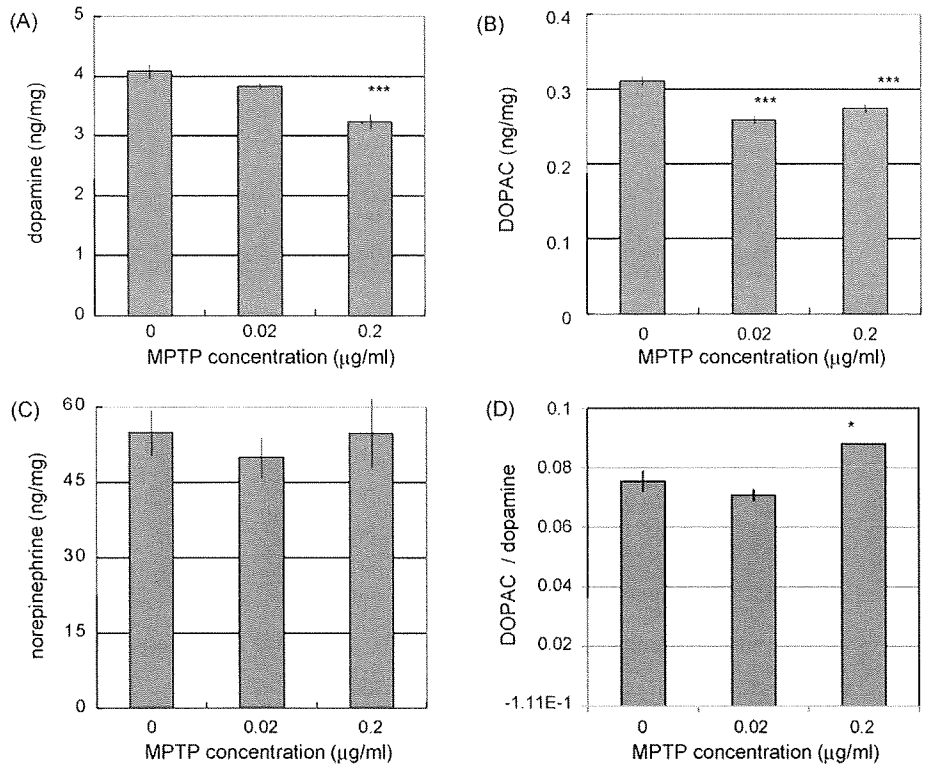

Fig. 6. HPLC analysis of neuro-transmitters in MPTP-treated medaka. Dopamine (A), DOPAC (B) and norepinephrine (C) amount in the whole brain of adult (100-dpf) medaka treated with water (control), 0.02 μg/ml MPTP and 0.2 μg/ml MPTP at the larval stage. All values are presented as the amount (ng) per protein weight (mg). Dopamine and DOPAC were decreased in 0.2 μg/ml MPTP-treated fish (A and B). Norepinephrine did not differ among the groups (C). (D) Showed DOPAC/dopamine ratio. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control. $n = 8$ for each group.

larval stage causes selective and persistent loss of the middle diencephalic TH$^+$ neurons after 3 months of exposure.

Next, we investigated a long-term effect of MPTP on neurological function by monitoring the spontaneous swimming movement of medaka over time. One month after the exposure, the frequency of swimming movement in MPTP-treated group completely recovered to the level of non-treated fish (Fig. 5C), although the total swimming distance and the velocity of swimming still decreased in MPTP-treated group (Fig. 5A and B). Three months after the exposure, the MPTP-treated group displayed the complete recovery of all the parameters of swimming movement tested (Fig. 5A–C). In summary, although MPTP treatment at the larval stage may irreversibly damage specific cluster of diencephalic TH$^+$ neurons and thereby decrease the amount of dopamine in the whole brain, the suppressive effect of MPTP on the spontaneous movement is observed only transiently and disappears 3 months after the exposure to MPTP.

4. Discussion

We here identified TH$^+$ dopaminergic neurons and noradrenergic neurons in the medaka brain. The larvae exposed to MPTP showed dopaminergic cell loss and reduced spontaneous movement. When these fish reached to an adult stage, they still displayed the loss of dopaminergic neurons associated with reduced amounts of dopamine in the whole brain, although the movement deficit gradually recovered to the normal level. Remarkably, MPTP-induced neuronal loss was restricted to the middle diencephalic clusters, which may include substantia nigra-like structure in teleosts. Therefore, we reasoned that the MPTP treatment at the larval stage allows the establishment of a medaka model of PD.

We demonstrated the specific toxicity of MPTP towards TH$^+$ neurons in the middle diencephalon. Other TH$^+$ neurons including diencephalic neurons outside this region and the neurons in medulla oblongata did not show the reduction in number. The vulnerability of the neurons in the paraventricular area of the middle diencephalon to MPTP, together with their anatomical features, supports the idea that these cells are the bona fide an equivalent of the substantia nigra in mammals. Toxic effect of MPTP specific to diencephalic TH$^+$ neurons was also reported in zebrafish (McKinley et al., 2005; Wen et al., 2008; Bretaud et al., 2004). By contrast, several other reports showed the reduction of not only dopaminergic but also norepinephrinergic neurons in MPTP-treated zebrafish and goldfish (Pollard et al., 1992; Anichtchik et al., 2004). The differential toxic effects of MPTP on norepinephrinergic neurons may depend on the routes of drug administration, as we noticed that in these reports the injection of MPTP into adult fish led to the injury of norepinephrinergic neurons, whereas submerging the fish in the water containing MPTP affected only dopaminergic neurons. We speculate that the pharmacodynamics is quite different between these two methods, with injection leading to a very high concentration in various tissues. Recent report showed the broad toxicity of MPTP not only on dopaminergic neurons but also on noradrenergic and histaminergic neurons even though they exposed zebrafish to the water containing MPTP (Sallinen et al., 2009). Because the amount of MPTP they used is much higher than our report, we speculate that this may be due to the broader spectrum injury of MPTP.

It is also an intriguing question whether the peripheral tissue is damaged by MPP$^+$ generated by peripheral MAO-B as suggested by MPTP-induced skin color change (Fig. 3A and B). The metabolic pathway and distribution of MPTP and MPP$^+$ in medaka should be investigated in the future.

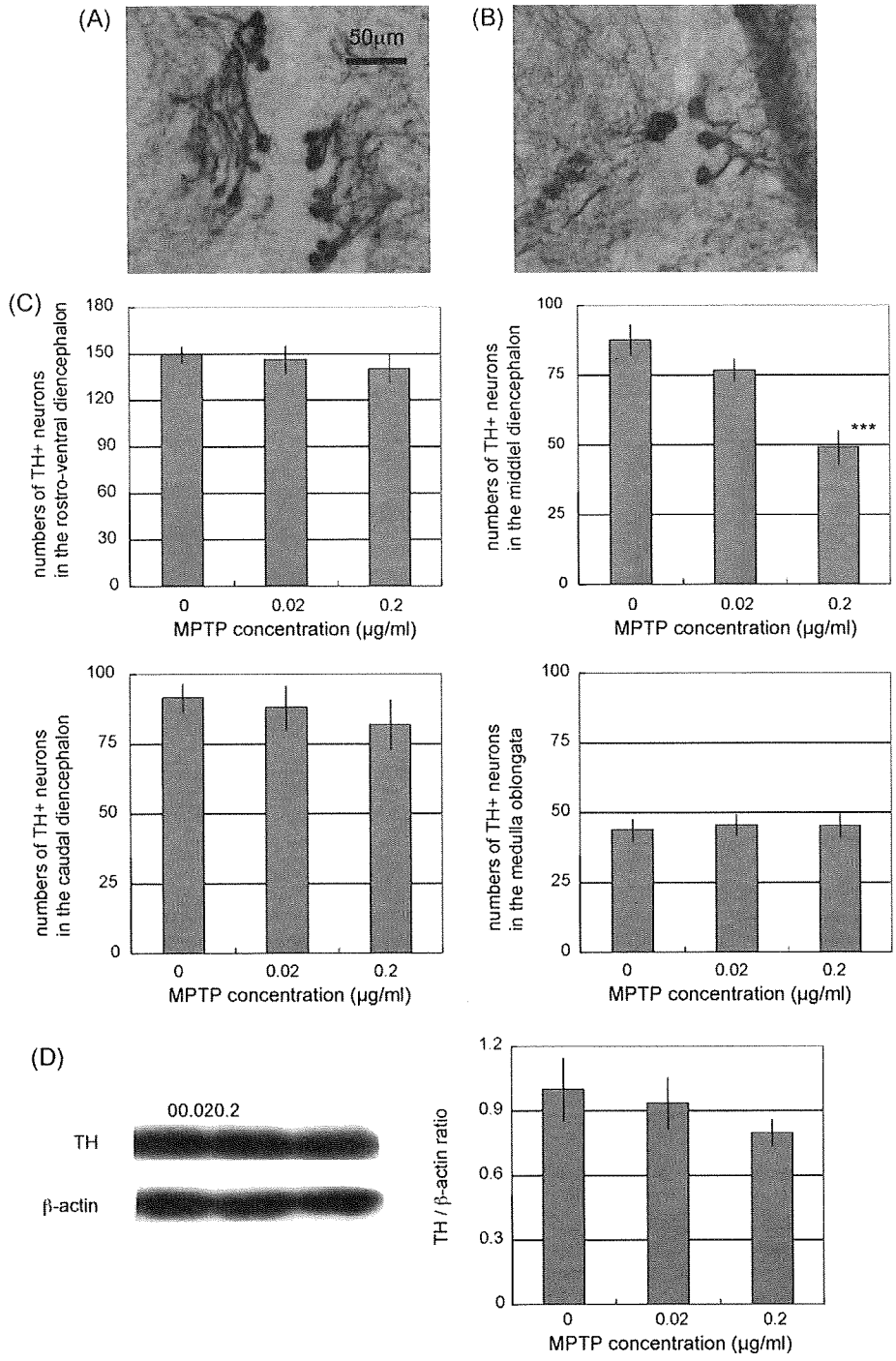

Fig. 7. TH immunohistochemistry and western blotting of adult medaka treated with MPTP at their larval stage. Samples were fixed 90 days after the first exposure (100-dpf). Coronal sections taken from the diencephalon level showed TH^+ neurons in control fish (A) and 0.2 μg/ml MPTP-treated fish (B). The numbers of TH^+ neurons in the middle diencephalon decreased significantly in MPTP-treated medaka (C). The numbers of TH^+ neurons in the rostro-ventral and caudal diencephalon, and the medulla oblongata did not show significant differences (C). $^{***}p < 0.001$ vs. control. $n = 8$ for each group. (D) The protein amount of TH in the whole brain of the control, 0.02 μg/ml MPTP-treated and 0.2 μg/ml MPTP-treated fish is examined by western blotting and then normalized by β-actin (loading control). The graph shows the ratio of TH/β-actin amount in each genotype (the average amount of control fish = 1). The amount of TH protein did not differ significantly among the groups. $n = 4$ for each group.

Previous studies of zebrafish and goldfish did not follow the long time course of MPTP toxicity, but continuous observation is important for the model animal because of the following reasons. First, PD is a late-onset and long-lasting neurodegenerative disorder. Second, the brains of teleost fish show widespread adult neurogenesis and new TH^+ cells are added in the olfactory bulb and diencephalon (Grandel et al., 2006). We here show the gradual functional recovery of dopaminergic neurons in the fish transiently exposed to MPTP at the larval stage, as evidenced by increases in spontaneous movement. Such behavioral recovery has been reported in several mammalian species after MPTP treatment (Rose et al., 1989; Elsworth et al., 1990, 2000; Kurlan et al., 1991).

The degree of the recovery is variable and may be dependent on several factors, including the protocol of MPTP treatment, the species, and the method of behavioral evaluation. Interestingly, the functional recovery observed in medaka was not accompanied by the restoration of the number of TH^+ cells or by increase in the amount of dopamine to a normal level in the adult stage. The striatum of MPTP-treated medaka did not show robust denervation of the TH^+ neurons, and this may explain the behavioral recovery observed in our medaka. Such complete behavioral recovery despite the incomplete return of the amount of dopamine may take place as in squirrel monkeys, possibly due to alteration in dopamine metabolism and neuronal sprouting (Petzinger et al., 2006).

In summary we have generated a medaka PD model by treating larval fish with MPTP, and established reliable assays from the larval stage to the adult. Our protocol of inducing PD-like phenotypes and our assay described in this study provides invaluable tools to investigate medaka model of familial PD retrieved from the TILLING library or medaka treated by other toxins or drugs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neures.2009.07.010.

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Human Induced Pluripotent Stem Cells on Autologous Feeders

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Abstract

Background: For therapeutic usage of induced Pluripotent Stem (iPS) cells, to accomplish xeno-free culture is critical. Previous reports have shown that human embryonic stem (ES) cells can be maintained in feeder-free condition. However, absence of feeder cells can be a hostile environment for pluripotent cells and often results in karyotype abnormalities. Instead of animal feeders, human fibroblasts can be used as feeder cells of human ES cells. However, one still has to be concerned about the existence of unidentified pathogens, such as viruses and prions in these non-autologous feeders.

Methodology/Principal Findings: This report demonstrates that human induced Pluripotent Stem (iPS) cells can be established and maintained on isogenic parental feeder cells. We tested four independent human skin fibroblasts for the potential to maintain self-renewal of iPS cells. All the fibroblasts tested, as well as their conditioned medium, were capable of maintaining the undifferentiated state and normal karyotypes of iPS cells. Furthermore, human iPS cells can be generated on isogenic parental fibroblasts as feeders. These iPS cells carried on proliferation over 19 passages with undifferentiated morphologies. They expressed undifferentiated pluripotent cell markers, and could differentiate into all three germ layers via embryoid body and teratoma formation.

Conclusions/Significance: These results suggest that autologous fibroblasts can be not only a source for iPS cells but also be feeder layers. Our results provide a possibility to solve the dilemma by using isogenic fibroblasts as feeder layers of iPS cells. This is an important step toward the establishment of clinical grade iPS cells.

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Introduction

Human pluripotent stem cells, both embryonic stem (ES) cells and induced Pluripotent Stem (iPS) cells, are generally maintained on mouse embryonic fibroblasts (MEF), which are mitotically inactivated by treatment with mitomycin C or γ-ray irradiation [1–3]. However, usage of mouse feeder cells may transfer exogenous antigens, unknown viruses, or zoonotic pathogens to iPS cells. In fact, non-human sialic acid N-glycolylneuraminic acid (Neu5Gc), which is potentially immunogenic, was detected on the surface of human ES cells maintained on MEF feeder [4]. Although feeder-free culture of human ES cells has been reported, it may lead to chromosomal instabilities of human ES cells [5,6]. To avoid these issues, human fibroblasts from neonatal foreskin or ES cell-derived fibroblast-like were used to support self-renewal of human ES cells [7–11]. However, one still have to concern about existence of unidentified pathogens, such as viruses and prions in these non-autologous feeders. Since iPS cells are generated from fibroblasts, it would be ideal if the same fibroblasts can be used for the generation and maintenance of iPS cells.

Results and Discussion

To examine whether human fibroblasts support self-renewal of human iPS cells, we treated four independent human fibroblast lines (1388, 1392, 1503 and NHDF; see Table S1) and SNL cells [12] with mitomycin C, and seeded them on culture plates (Fig. S1). Then, we plated 201B7 iPS cell line [2] derived from 1388 fibroblasts onto these feeder cells with standard density (1:5 dilutions). The passage number of iPS cells was 20 at this point. All the five cell lines of feeder cells were supportive for undifferentiated growth of iPS cells at least 19 additional passages (Fig. 1A). The percentage of TRA-1-60 (a marker for undifferentiated ES cells and iPS cells) positive colonies was similar among different human fibroblasts and SNL cells (Fig. 1B). No significant differences were observed in the plating efficiencies (Fig. 1C). In iPS cells at passage 2 after switching onto various HDF feeders, no significant re-activation of transgenes was observed (Fig. S2). In addition, reverse transcription polymerase chain reaction (RT-PCR) showed that the expression of ES cell marker genes such as *OCT3/4*, *SOX2* and *NANOG* were equally to those of H9 ES cells at passage 19 [1] (Fig. 1D).

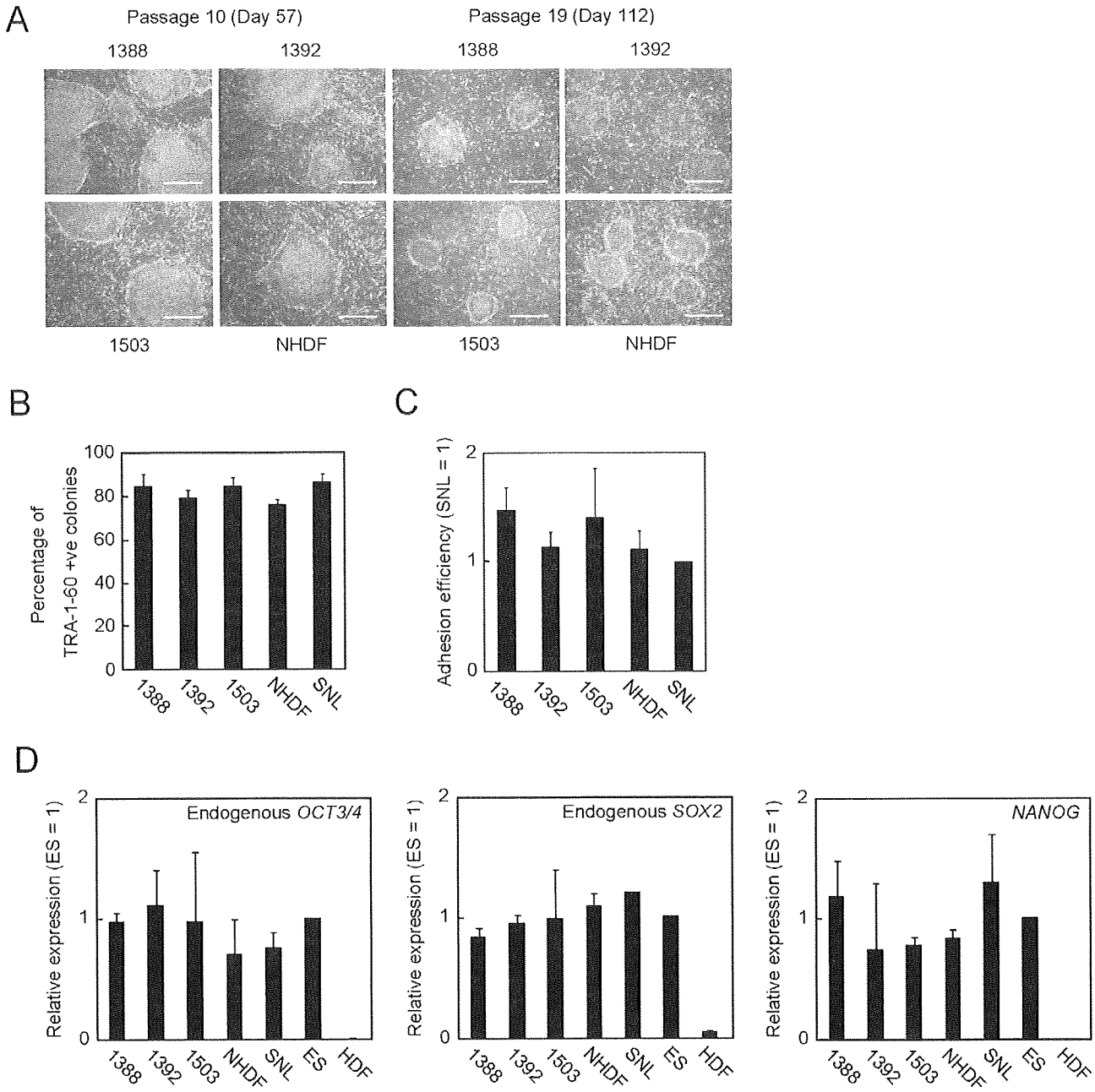

Figure 1. HDF can maintain self-renewal of established human iPS cells. A. Images of iPS cells maintained on each fibroblast at passage number 10 and 19. Bars indicate 200 μm. B. 201B7 iPS cells at passage number 19 were plated on each feeder, and incubated for 6 days. The graph shows the percentage of TRA-1-60 positive colonies. Three individual assays were performed. Error bars indicate standard deviation. C. The number of colonies was counted and compared with the results of SNL feeder. This graph showed the average of three independent experiments. Error bars mean standard deviation. D. RT-PCR of undifferentiated ES cell markers. 201B7 iPS cells maintained on each HDF over 100 days were lysed, and their total RNAs were purified. One microgram of RNA sample was used for cDNA synthesis. qPCR was performed with the primers for endogenous *OCT3/4*, endogenous *SOX2*, *NANOG* and *G3PDH*. Data were normalized with the value of *G3PDH*. The graphs showed the average of three experiments. Error bars indicate standard deviation.
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Conditioned medium (CM) of MEF or SNL allows feeder-free culture of iPS cells. To test whether CM of fibroblasts could maintain self-renewal of iPS cells without feeder cells, we seeded 201B7 iPS cells onto Matrigel-coated plates in CM from each human fibroblast line or SNL. As a control, we used non-conditioned medium supplemented with bFGF. Cells in non-conditioned medium failed to form tightly packed colonies, whereas those in each CM grew healthily with typical undifferentiated ES-like morphologies (Fig. S3A). RT-PCR revealed that iPS cells maintained in each CM expressed undifferentiated ES cell marker genes such as *OCT3/4*, *SOX2*, *NANOG* and *TERT* at similar levels to those in iPS cells or human ES cells cultured on SNL feeder layers (Fig. S3B). Quantitative PCR (qPCR) confirmed that no significant alternations in the expression levels

of *OCT3/4*, *SOX2* and *NANOG* transcripts among CM from different feeders (Fig. S3C). These data demonstrated that human neonatal and adult fibroblasts could be utilized as feeder cells of human iPS cells.

Next, we examined whether human iPS cells could be established without non-autologous feeder cells. We introduced the four reprogramming factors into the four human fibroblast lines by retroviral transduction. Six days after infection, we plated the transduced cells at 5×10^5 cells on 100-mm dishes either with SNL feeders, with isogenic human fibroblast feeder, or without feeder cells. Next day, we started cultivation using human ES cell culture medium. In the plates without feeders, the plated cells became confluent within a several days and showed an appearance resembling feeder cells. Around three weeks after transduction, ES-like colonies began to emerge on the feeder cell-like layer (Fig. 2A). We observed no significant differences in the numbers of ES-like colonies among on SNL feeders, on isogenic fibroblast feeders or feeder-free condition (Table S2). On day 25 after transduction, we picked up ES-like colonies from the plates without feeders and transferred them onto new plates with mitomycin C-treated each parental fibroblasts as feeders. Human iPS cells derived from each of the four fibroblast lines used in this study grew normally and maintained the undifferentiated morphologies on corresponding autologous feeders for at least 18 passages (Fig. 2B).

RT-PCR showed that established clones at passage number 5 expressed endogenous *OCT3/4*, *SOX2*, *NANOG* and *TERT* transcripts at similar levels to those in 201B7 iPS cells, which were established on SNL feeder cells, and H9 ES cells (Fig. S4A). The retroviruses of the four factors were effectively silenced, which is a hallmark of complete reprogramming (Fig. S4B). Even after additional 15 passages, the expression of *OCT3/4*, *SOX2* and *NANOG* in these iPS cells were comparable to those of ES cells and iPS cells maintained on SNL feeder cells (Fig. 2C). Immunoprecipitation assay with anti-methylated cytosine antibody revealed that the promoter regions of pluripotent-associated genes such as *OCT3/4* and *NANOG* locus were almost completely unmethylated in iPS cells established and maintained on the autologous feeders at passage number 5, like in H9 ES cells (Fig. S5). In addition, iPS cells generated with the autologous feeders showed normal karyotypes at least after 26 times passages (Fig. S6).

To evaluate pluripotency of iPS cells generated and maintained on autologous feeders, we performed *in vitro* differentiation assay. These iPS clones formed embryoid bodies using the floating culture condition. After 16-day differentiation, we detected SOX17 (endoderm), α-smooth muscle actin (α-SMA, mesoderm) and NESTIN (ectoderm) positive cells in the culture (Fig. 2D, Fig. S7). We also confirmed that undifferentiated markers such as *OCT3/4*, *SOX2* and *NANOG* decreased and other differentiated markers such as *AFP*, *PDGFRα* and *PAX6* increased (Fig. S8). In addition, we injected iPS cells at passage number 9 into testes of immune-deficient mice for teratoma formation. After 8 to 12 weeks, all clones we tested developed teratoma containing various tissues including gut-like epitheliums (endoderm), cartilages (mesoderm) and neural rosettes (ectoderm) (Fig. 2E). These data confirmed pluripotency of iPS cells, which were established and maintained on the autologous feeder cells.

To examine the compatibility of iPS cells and feeder cells, we plated iPS cells derived from the four HDF onto mitomycin C-treated parental fibroblasts or SNL cells with all the possible combinations. After six days, we stained the cells with TRA-1-60 antibody, and counted the number of positive colonies. More than 80% of colonies in all the combinations showed morphology of undifferentiated ES-like cells and were positive for TRA-1-60 (Fig. S9).

Our results demonstrated that human iPS cells can be generated and maintained on autologous fibroblasts as feeder layers. Furthermore, human iPS cell can be generated even without any additional feeders, since non-reprogrammed fibroblasts can serve as feeders. The maintenance of iPS cells can also be achieved without feeders, by using conditioned medium of human fibroblasts. All of the tests performed in this study revealed that iPS cells derived from four independent HDF maintained autologous feeders kept pluripotency during at least 100 day culture (Table S3). The most of reprogrammed cell colonies on isogenic feeders are uniformly undifferentiated (Fig. S9).

In general, we can obtain more than ten millions of fibroblasts from 5-mm square skin biopsy at the passage number 3 with our standard protocol [13]. For iPS cell generation with retroviruses, we need less than 1×10^5 fibroblasts. An alternative method using an episomal vector system requires one million of fibroblasts [14]. Thus, we have enough amounts of surplus fibroblasts to be used as autologous feeder cells. We did notice that iPS cells cultured on SNL feeders are easier to passage than those on MEF and human fibroblast feeders.

Our data that all four HDF lines tested in this study could support both generation and maintenance of iPS cells, does not guarantee that every fibroblasts can be used as feeders cells for human pluripotent cells [15]. We also tested that whether 14 HDF lines were supportive for maintenance of ES cells and iPS cells. Both KhES3 ES cell line and 201B7 could grow normally on eleven out of 14 HDF lines, two MEF lines (ICR and C57BL6) and SNL [16] (Table S1, Fig. S1). In co-culture with three lines (1554, 1616 and TIG107), at least either KhES3 or 201B7 could not stay at undifferentiated state even at passage number 2. At least, among on 11 supportive fibroblasts and MEF and SNL, no significant differences were observed in growth rate of ES cells and iPS cells. Unsupportive lines are indistinguishable from HDF lines by their morphologies or growth speed. Probably, support activity for self-renewal of ES cells and iPS cells do not depend on at least passage number and donor's sex or race. Unsupportive lines tested in this study are derived from donors at 68, 77 and 81 years old. On the other hand, we found that HDF derived from donors at 69 and 73 years old could support maintenance of undifferentiated state of both ES cells and iPS cells. Further detailed analyses will be required for decision whether donor's ages of feeder cells are important or not.

Recent study by Unger and colleagues demonstrated that iPS cells derived from human fetal fibroblasts could be established and maintained on isogenic feeder cells [17]. On the other hand, we assessed that neonate and adult fibroblast-derived iPS cells can be generated and maintained on autologous feeders. Inspection of established iPS cells is always necessary before clinical trials even in autologous cell transplantation therapy. However, the culture system established in this study demonstrated that fibroblasts from an individual could play dual roles as source of iPS cells and feeder cells, probably contributing to efficient processing for clinical grade-pluripotent stem cells. Actually, the system still includes animal components such as albumin, insulin and trypsin. Xeno-free culture is basically required for therapeutic usage of iPS cells. However, even if it is for removing the xenogenic components, the culture condition should not be oppressive for pluripotent cells. Isogenic culture from the start is one of true worth of iPS cells because autologous fibroblasts can not normally inhabit when ES cells are established from blastocysts. Our result is an important step toward the generation of clinical-grade human iPS cells suitable for future medical applications.

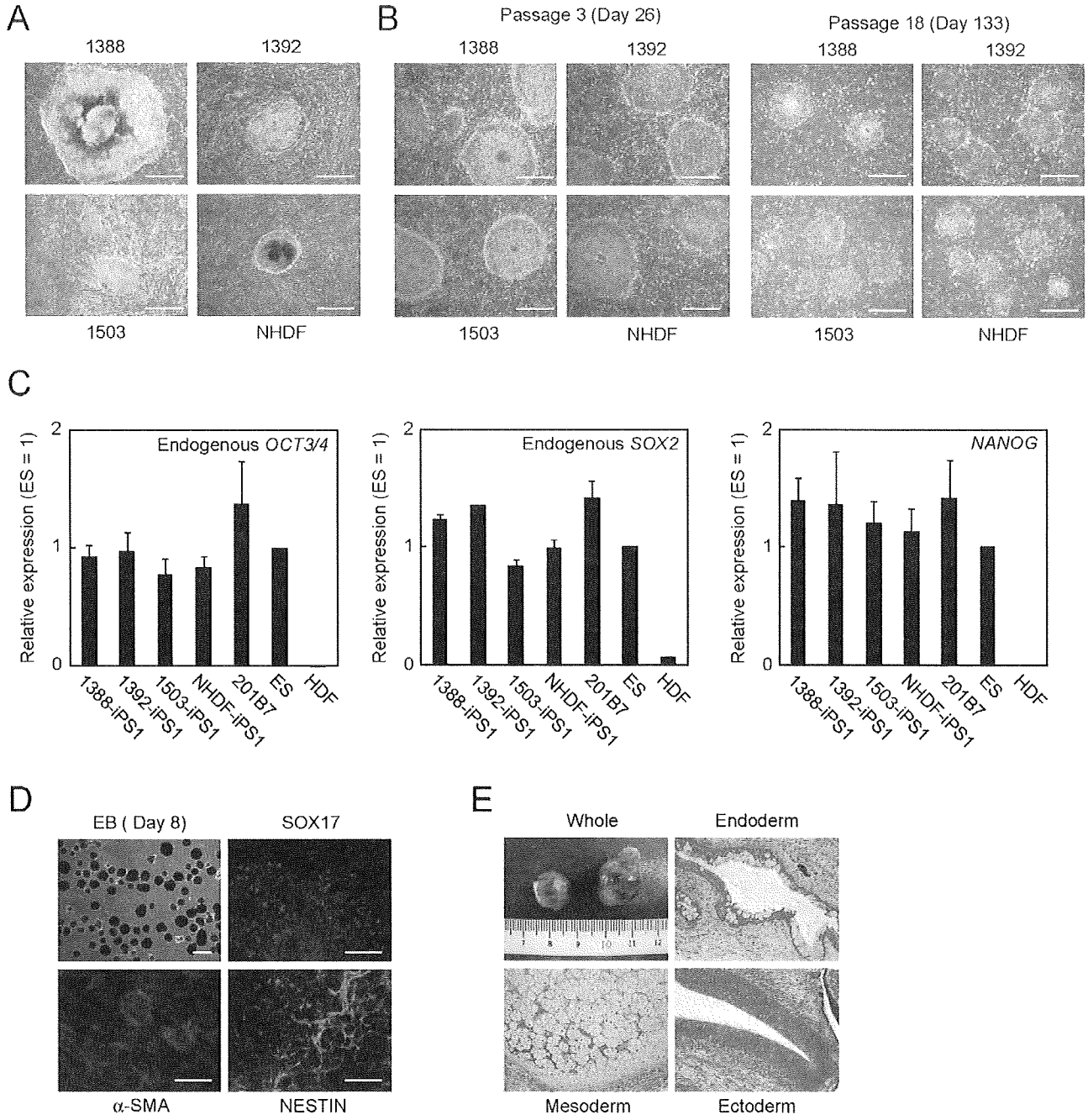

Figure 2. Generation and maintenance of human iPS cells on autologous feeders. A. Images of primary iPS cell colonies. We introduced 4 reprogramming factors into 1388, 1392, 1503 or NHDF. The colonies were photographed 25 days after transduction. Bars indicate 200 μm. B. Images of established iPS clones. We isolated iPS clones and transferred onto each isogenic fibroblast line. The cells at passage 3 and 18 were photographed. Bars indicate 200 μm. C. Quantification of the expression of pluripotent stem cell markers. Total RNA of iPS cells established from four independent fibroblast lines maintained on isogenic feeders, H9 ES cells and HDF was purified, and used for reverse transcription. The graphs show the average of three independent experiments. Error bars indicate standard deviation. Data were normalized with the results of *G3PDH*. D. In vitro differentiation of iPS cells. iPS cells were transferred to suspension culture to form embryoid bodies for 8 days. Embryoid bodies were transferred to gelatin-coated plated, and incubated another 8 days. The cells were stained with anti-SOX17 (red), anti-α-SMA (red) or anti-NESTIN (green) antibodies. Nucleuses were stained with Hoechst 33342 (blue). Bars indicate 100 μm. E. Teratoma formation of iPS cells. Paraffin-embedded sections were stained with hematoxylin and eosin.
doi:10.1371/journal.pone.0008067.g002

Materials and Methods

Cell Culture

Human dermal fibroblasts (HDF) were purchased from Cell applications Inc or obtained from National Institute of Biomedical Innovation. HDF, 293FT and PLAT-E [18] were maintained Dulbecco's modified eagle medium (DMEM, Nacalai tesque) contained 10% fetal bovine serum (FBS, Invitrogen) and 0.5% penicillin and streptomycin (Invitrogen). The medium for human iPS cells (hES medium) consisted of DMEM/F12 (Invitrogen),

20% Knockout serum replacement (KSR, Invitrogen), 2 mM L-glutamine (Invitrogen), 1×10^{-4} M non essential amino acids (Invitrogen), 1×10^{-4} M 2-mercaptoethanol (Invitrogen) and 0.5% penicillin and streptomycin supplemented with 4 ng/ml recombinant human basic fibroblast growth factor (bFGF, WAKO).

Generation of iPS Cells

iPS cells were established from HDF as described previously with some slight modifications [2]. In brief, we firstly introduced mouse solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 (*Slc7a1*) gene which encodes ecotropic retrovirus receptor by lentiviral transduction. Transfectants were plated at 2×10^5 cells per 60 mm dish and incubated overnight. The next day, into the cells *OCT3/4*, *SOX2*, *KLF4* and *c-MYC* were introduced by retroviral infection. Six days later, the cells were harvested by trypsinization, and plated at 5×10^5 cells per 100 mm dish. The medium was replaced on the next day with hES cell medium, and cultured for another 20 days. At day 25 post-induction, ES-like colonies were mechanically dissociated and transferred on to 24-well plate on each isogenic feeder. We designated this point as passage 1.

Feeder Cells

We added phosphate buffered saline (PBS, Nacalai tesque) containing 12 μg/ml mitomycin C directly into fibroblast culture in subconfluent, and incubated at 37°C for 3 hours. After treatment, the cells were washed twice with PBS and harvested by trypsinization. The cells were plated at 1×10^6 cells per 24-well plate, 6-well plate, 3 of 60 mm dishes or 100 mm dish.

Conditioned Medium

We plated fibroblasts at 3×10^5 cells per 60 mm dish, and incubated overnight. Next day, the medium was replaced with 3 ml of hES medium, and incubated for 24 hours. After incubation, the supernatant of fibroblast culture was collected and filtered. We added 4 ng/ml bFGF before use.

Differentiation

iPS cells were harvested by treatment with CTK solution consisting of 0.1 mg/ml collagenase IV (Invitrogen), 0.25% trypsin (Invitrogen), 0.1 mM $CaCl_2$ (Nacalai tesque) and 20% KSR, and then suspended cell clumps in hES medium plus 10 μM Y-27632 without bFGF [19]. The cells were transferred to ultra low binding plate (Corning). After 8-day floating culture, embryoid bodies were transferred on to gelatin-coated plate, and incubated another 8 days. After incubation, the cells were fixed with PBS containing 4% paraformaldehyde and then incubated in PBS containing 5% normal goat or donkey serum (Chemicon), 1% bovine serum albumin (BSA, Nacalai tesque), and 0.2% TritonX-100. The primary antibodies were as follows; anti-SOX17 (1:300, R & D systems), anti-α-smooth muscle actin (α-SMA, 1:500, DAKO) and anti-NESTIN (1:1000, Abcam). The secondary antibodies were as follows; Cyanine 3-labeled anti-goat IgG (1:500, Zymed), Alexa 546-labeled anti-mouse IgG (1:500, Invitrogen) and Alexa 488-labeled anti-rabbit IgG (1:1000, Invitrogen). Nucleuses were stained with 1 μg/ml Hoechst 33342 (Invitrogen).

Expression Analyses

We performed RT-PCR as described previously [2]. In brief, the cells were lysed with Trizol reagent (Invitrogen), and then total RNA was purified. RNA samples were treated with Turbo DNA free (Ambion) to remove genomic DNA contamination. One microgram of DNase treated RNA was used for first-strand complementary DNA (cDNA) synthesis with Rever tra ace -α- (Toyobo) and oligo dT_{20} primer. qPCR was performed using SYBR Premix ExTaq II (Takara). Primer sequences were listed in Table S4 [2,20,21,22].

Methylation Assay

Four microgram of genomic DNA was mechanically shared by sonication, and boiled at 95°C for 10 minutes. Then shared genomic DNA was incubated with pan-mouse IgG magnetic beads (Invitrogen) -conjugated anti-5-methyl cytosine antibody (Eurogentec) supplemented with 5 μg/ml BSA and 25 μg/ml yeast tRNA (Ambion) overnight at 4°C. Beads were washed three times with PBS containing 0.05% TritonX-100. Beads were suspended in 0.15 ml of TE containing 1% SDS, and incubated at 65°C for 5 minutes. The elution was repeated with an additional 0.15 ml of 1% SDS/TE. The eluates were treated with Protease K at 50°C for 2 hours, and then extracted with phenol: chloroform: isoamyl alcohol, and purified by ethanol precipitation. Primer sequences are provided in Table S4.

Supporting Information

Figure S1 Images of mitomycin C-treated HDF, MEF and SNL. Bars indicate 200 μm.
Found at: doi:10.1371/journal.pone.0008067.s001 (2.91 MB TIF)

Figure S2 The expression of four reprogramming factors in iPS cells on various HDF feeders at passage 2. RT-PCR was performed with the primers for endogenous and total (common in endogenous and transgene) OCT3/4, SOX2, KLF4 and c-MYC. Data were normalized with the value of NAT1. The graphs showed the average of triplicate. Error bars indicate standard deviation.
Found at: doi:10.1371/journal.pone.0008067.s002 (0.31 MB TIF)

Figure S3 A. Image of 201B7 iPS cells maintained in CM of each HDF. Bars indicate 200 μm. B. RT-PCR of undifferentiated ES cell markers. iPS cells maintained on feeders (F) or in feeder-free culture with conditioned medium (CM) were lysed, and their total RNAs were purified. One microgram of RNA sample was used for cDNA synthesis. PCR was performed with the primers for endogenous OCT3/4, endogenous SOX2, NANOG, TERT and NAT1. C. qPCR of the expression of OCT3/4, SOX2 and NANOG in 201B7 iPS cells maintained on various feeder cells or in their conditioned medium. Data were normalized with the value of G3PDH. The graphs showed the average of three experiments. Error bars indicate standard deviation.
Found at: doi:10.1371/journal.pone.0008067.s003 (1.67 MB TIF)

Figure S4 A. RT-PCR of undifferentiated ES cell markers. Total RNAs of iPS cells established from four independent fibroblast lines and maintained on each parental fibroblast were isolated and used for reverse transcription. PCR was performed with the primers for endogenous OCT3/4, endogenous SOX2, NANOG, TERT and NAT1. B. The expression of OCT3/4 (total and endogenous), SOX2 (total and endogenous) and NANOG were quantified by qPCR. Data were normalized with the value of G3PDH. The graphs showed the average of triplicate. Error bars indicate standard deviation.
Found at: doi:10.1371/journal.pone.0008067.s004 (0.75 MB TIF)

Figure S5 CpG methylation statuses at promoter regions of ES cell marker genes in iPS cells maintained on autologous feeders. Immunoprecipitants by anti-5-methyl cytosine (mDIP) antibody or normal mouse IgG, or pre-immunoprecipitated DNA (Input) were used for qPCR as a template. The data was calculated as (mDIP −

normal IgG)/Input. Each data was normalized by the result of H9 ES cells. The data indicate the results of qPCR in triplicate of two independent experiments. Error bars indicate standard deviation. Found at: doi:10.1371/journal.pone.0008067.s005 (0.25 MB TIF)

Figure S6 Images of G-band staining of iPS cells.
Found at: doi:10.1371/journal.pone.0008067.s006 (0.68 MB TIF)

Figure S7 Images of differentiated iPS cells in vitro. iPS cells differentiated via embryoid body formation. Red or green signals indicate SOX17-, α-SMA- or NESTIN-positive cells. Nucleuses were stained with Hoechst 33342 (blue). Bars indicate 100 μm.
Found at: doi:10.1371/journal.pone.0008067.s007 (4.63 MB TIF)

Figure S8 iPS cells maintained on isogenic feeders (U) or differentiated by embryoid body formation (D) were lysed with Trizol reagent. Total RNA was purified and treated with DNase to remove genomic DNA contamination. One microgram of DNase-treated RNA sample was used for first-strand cDNA synthesis with oligo dT20 primer. PCR was performed with the primers listed in Supplemental Table 2.
Found at: doi:10.1371/journal.pone.0008067.s008 (0.39 MB TIF)

Figure S9 Images of iPS cells from four HDF on various HDF feeders or SNL. Red signals indicate TRA-1-60 positive cells. Nucleuses were visualized by Hoechst 33342 staining. Bars indicate 200 μm.
Found at: doi:10.1371/journal.pone.0008067.s009 (3.96 MB TIF)

Table S1 The list of HDF lines used in this study.
Found at: doi:10.1371/journal.pone.0008067.s010 (0.04 MB DOC)

Table S2 The number of ES-like and total colonies from four HDF on SNL, on isogenic fibroblasts, or in feeder-free condition.
Found at: doi:10.1371/journal.pone.0008067.s011 (0.03 MB DOC)

Table S3 Experiments performed in this study.
Found at: doi:10.1371/journal.pone.0008067.s012 (0.04 MB DOC)

Table S4 Primer sequences.
Found at: doi:10.1371/journal.pone.0008067.s013 (0.06 MB DOC)

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Author Contributions

Conceived and designed the experiments: KT SY. Performed the experiments: KT MN MY TI. Analyzed the data: KT. Contributed reagents/materials/analysis tools: KT. Wrote the paper: KT SY.

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2. iPS細胞作製の最先端と作製されたiPS細胞株間の特性差異についての最新の知見

長船健二

最初の樹立の報告以降，わずか数年の間で，iPS細胞研究は生物学および医学における先駆的な一大研究領域となった．マウス，ラット，ブタ，そして，霊長類など複数の動物種，生体内の多くの細胞種からのiPS細胞樹立が報告された．体細胞が未分化幹細胞に初期化される機構は，ほとんど未解明のままであるが，その機構の解明に基づく真に安全で効率のよいiPS細胞の樹立方法が，近い将来に確立されることが期待される．幹細胞株間の分化能や安全性の差異をはじめとする特性を理解したうえで，より適切な維持培養法と分化誘導法を開発することが，再生医療の実現化を加速させる．

はじめに

簡便な遺伝子操作にて体細胞から作製可能な多能性幹細胞であるiPS細胞（induced pluripotent stem cell：人工多能性幹細胞）が，マウス[1]に続いてヒトにおいても開発され[2)3)]，それ以降，世界中で激しい研究競争が繰り広げられている．そのなかにおいても，最近のiPS細胞研究で最も進展した分野は，ゲノムへの導入遺伝子の組み込みを必要としない，あるいは，それを残さない新しいiPS細胞樹立方法の開発と，疾患特異的iPS細胞を用いた疾患モデル作製研究ではないかと考える．疾患モデル研究における最新の知見は他稿に譲る（**第6章-5**参照）として，本稿においては，iPS細胞作製についての最新の知見，および，われわれの報告を含めた幹細胞株間の特性差異についての知見を要約し，それらに基づく今後のiPS細胞研究の課題と展望について述べてみたい．

[キーワード&略語]
iPS細胞，初期化機構，樹立法，分化能，株間の特性差異
GSK-3：glycogen synthase kinase-3（グリコーゲン合成酵素キナーゼ3）
MAPK：mitogen-activated protein kinase（分裂促進因子活性化タンパク質キナーゼ）
shRNA：small hairpin RNA（低分子ヘアピン型RNA）
siRNA：small interfering RNA（低分子干渉RNA）

Recent advances in iPS cell generation and characterization of inter-line variability among iPS cell lines

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1 iPS細胞樹立可能な細胞種および動物種

これまでの多くの報告では，iPS細胞は，成体あるいは胎児の線維芽細胞から作製されているが，成体由来の神経幹細胞[4]，皮膚の角化細胞[5]，血液細胞[6]～[8]をはじめとするさまざまな体細胞[9][10]，そして，出生前診断時に採取される羊水中および絨毛の細胞[8]や臍帯血[11][12]からも樹立が報告された．よって，初期化（再プログラム化，リプログラミング）は，樹立効率が細胞種で異なる可能性があるが，生体内のほとんどの細胞種で可能であることが予想される（**図1A**）．

マウス以外のより大型で寿命の長い動物種からのiPS細胞の樹立は，疾患に対する新しい動物モデルを作製することやiPS細胞から作製された臓器細胞の移植後の安全性を検証する優れた系の開発に繋がる．現在までのところ，マウスとヒトのiPS細胞とくらべて培養条件の確立が十分ではない可能性もあるが，サル（rhesus monkey：アカゲサル）[13]，ラット[14][15]，ブタ[16][17]，そして最近，京都大学の中村らよりイヌからのiPS細胞樹立も報告されている（**図1B**）[18]．

2 初期化機構の解明

分化細胞が未分化状態に初期化されるメカニズムは，iPS細胞研究における生物学的な面で最も興味深い謎の1つであるが，その機構はほぼ未解明のままである．

初期化誘導因子に関しては，オリジナルである山中4因子の組合わせ（Oct4/Sox2/Klf4/c-Myc）[1][2]が最も広範に使用されているが，ウィスコンシン大学（アメリカ）のトムソンらの組合わせ（Oct4/Sox2/Nanog/Lin28）を用いてもiPS細胞が樹立されている[3]．最近，Sox2を内因性に高発現する神経幹細胞が，Oct4単独でiPS細胞に初期化されることが報告されたが[4]，現在までのところ，Oct4を用いないで初期化された報告が存在しないため，前述の因子のなかでは，Oct4が中心的な分子であると考えられる．また，最近，核内オーファン受容体であるEsrrb遺伝子が，Oct4/Sox2との組合わせでiPS細胞を樹立するとの興味深い報告がなされた[19]．さらに，ヒストン脱アセチル化酵素阻害剤であるバルプロ酸[20]やBIX-01294/BayK8644の2剤[21]が，それぞれ，Oct4/Sox2，そして，Oct4/Klf4との組合わせでiPS細胞を誘導できるなど，化合物により初期化因子が置き換えられることが示された（**図1C**）．

初期化機構の解明に向けて，iPS細胞とその元となった体細胞などで超高速シークエンサーを用いた遺伝子発現解析やエピゲノム解析などの試みも開始され，分化細胞系譜に特異的な転写因子の抑制とDNA脱メチル化が初期化にとって重要なステップであることが報告されているが[22]，この研究領域のますますの進展が期待される．

3 新規のiPS細胞樹立法

iPS細胞樹立の最初の報告では，レトロウイルスやレンチウイルスベクターを用いた遺伝子導入が行われた[1]～[3]．かつて，免疫不全症の患者に対して，レトロウイルスベクターを用いた遺伝子治療が施行されたが，ゲノムへのベクターの挿入による癌原遺伝子の過剰発現に起因する白血病発症という深刻な合併症が生じ，死亡例も報告された[23][24]．同様に，iPS細胞においてもゲノムに組み込まれたベクターの影響により，それから作製された臓器細胞を移植に用いた場合，将来的にその細胞が悪性化するなどのさまざまな危険性が懸念される．

この問題の解決を目指して，ゲノムへの導入遺伝子の組み込みを必要としない，あるいは，それを残さない新しいiPS細胞樹立方法が次々と報告されている（**図1D**）．まず，ゲノムへの組み込みの危険性の少ないアデノウイルスベクターを用いたiPS細胞樹立がマウス[25]，そして，ヒトにおいても報告された[26]．また，ウイルスベクターを用いず2つのプラスミド（Oct4/Klf4/Sox2を繋いだプラスミドとc-Mycのみのもの）を用いた方法や[27]，4因子を繋いだ1つのプラスミドによる方法にて，マウスiPS細胞が樹立された[28]．

その他にも，piggyBacトランスポゾン[29]～[31]やCre-loxPシステム[32]によりiPS細胞樹立後に導入遺伝子を切り出す方法も報告された．また，エピゾーマルベクターoriP/EBNA1を用いてベクターを含まないiPS細胞の単離を可能とする方法や[33]，初期化因子のリコンビナントタンパク質を導入することによりマウス[34]およびヒト[35]でiPS細胞が樹立された．さらに最近，核に取り込まれないセンダイウイルスベクターを用いたiPS細胞樹立の報告がディナベック社の房木らのグルー

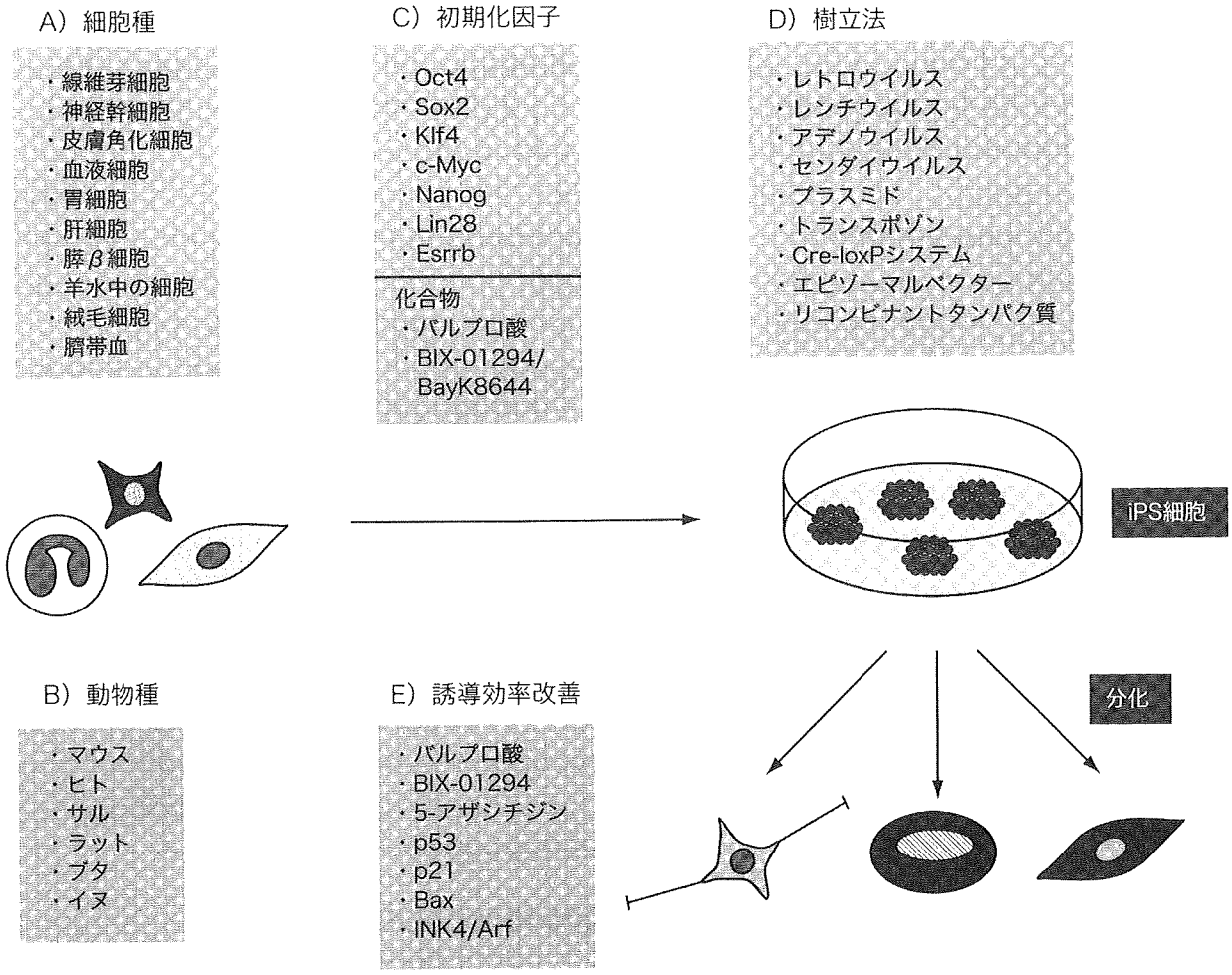

図1　iPS細胞作製に関する知見の要約
さまざまな細胞種，動物種，初期化因子の組合わせ，樹立法を用いてiPS細胞が作製され，誘導効率を改善する方法も報告されている

プよりなされた[36]．

以上，さまざまな樹立法が報告されているが，多くの方法で共通していることは，オリジナルのレトロウイルスベクターとくらべて樹立効率が低い点である．また，アデノウイルスやプラスミドも低い確率でゲノムに組み込まれる可能性があることや，初期化因子として依然として癌関連の遺伝子であるc-Myc/Klf4を用いていることが今後の改善点としてあげられる．

4 iPS細胞誘導効率の改善

体細胞を初期化してiPS細胞を誘導する効率は，依然として高くはない．これは，初期化因子発現のタイミング，バランス，発現量などが正確に調節されることが必要である可能性，あるいは，稀少なゲノム・エピゲノム状態の変化を起こした細胞集団のみが選択される可能性などが考えられるが，機序は不明のままである．前述のバルプロ酸[20]，BIX01294[21]，そして，5－アザシチジン（5-azacytidine）[22]などの化合物がiPS細胞誘導効率を高めることが報告されている（**図1E**）．

近年，iPS細胞誘導効率に関して，癌抑制遺伝子であるp53について注目が集まっている．まず2008年に，山中4因子にp53のsiRNA（small interfering RNA）を加えることでiPS細胞クローンの出現率が100倍に上昇するが，多くは部分的に初期化されたものであり，iPS細胞の特徴の1つである*in vivo*での奇形腫形成を示さないことが報告されていた[37]．しかし最近，山中らを含む複数のグループより，iPS細胞形成にお

けるp53の役割について，より解明の進んだ報告が同時になされた[38)~42)]．まず山中らによると，p53遺伝子のノックアウトマウス由来の線維芽細胞では，驚くべきことに3因子（Oct4/Sox2/Klf4）導入にて10%もの細胞がiPS細胞に初期化され，同マウス由来の終末分化したT細胞からiPS細胞を作製することさえ可能であった[38)]．同様の所見が，p53のshRNA（small hairpin RNA）によるノックダウンを用いて別のグループからも報告され，さらに，p53の下流にあるp21やBax遺伝子の発現を低下させることでもiPS細胞誘導効率が上がることも示された[39)]．また，別の2グループが，p53の発現を制御するInk4/Arf遺伝子の発現低下が初期化効率を高めることを報告し[40) 41)]，さらに，別のグループによって，p53がテロメア長の短い細胞のiPS細胞への初期化を阻害することが示された[42)]．以上より，p53を取り囲む分子ネットワークが初期化を阻害し，このネットワークの破綻がiPS細胞誘導効率を高めることが示唆される（**図1E**）．

5 iPS細胞株間の特性および差異

われわれは，異なる17の個体由来のヒトES細胞（embryonic stem cell：胚性幹細胞）株を比較解析し，株間で分化能が大きく異なっており，神経外胚葉，中内胚葉組織など分化しやすい細胞系譜も株間で顕著な差があることを示した（**図2**）[43)]．このようなヒトES細胞株間の分化能の差を生じている原因として，異なる人種・家系に由来する余剰胚から作製されていることによる遺伝的素因の違いや，各ES細胞株間での異なるエピゲノム状態の関与などが予想されるが，現時点では全く不明である．

このようなヒトES細胞株間における分化能の定量的な差は，体細胞核移植法で作製されたES細胞やiPS細胞の株間においても，同様に存在する可能性が示唆される．特にiPS細胞に関しては，未分化状態のES細胞と同じマーカー遺伝子を発現し，三胚葉への分化能を示すことのみによって定義されており，現時点では，iPS細胞を分子的に定義するより明確で客観的な基準がない．また，レトロウイルスやレンチウイルスベクターを用いて樹立されたiPS細胞のゲノムにはベクターが複数の染色体にランダムに組み込まれており[9)]，同一個体由来のiPS細胞株間でも遺伝的背景が異なる．さらに，iPS細胞には，前述のようにさまざまな樹立法や細胞種を用いて作製された細胞株があり，異なる方法により作製されたiPS細胞が，全く均一なものであるか否かは不明である．

最近，テトラプロイド凝集胚形成法※1にてマウスiPS細胞が完全な個体を形成する分化能を有することが示されたが[44)~46)]，その生存率や成功率はiPS細胞株間で異なり，iPS細胞樹立に要した日数と関連があることが認められている[44)]．また最近，異なる組織由来のマウスiPS細胞から分化誘導された神経細胞を移植した際に奇形腫形成を合併する率が，由来組織によって異なり，株間で安全性が異なることが示された（**第4章-3参照**）[47)]．

現在までのところ，多くのiPS細胞株間で分化能を詳細に比較解析した報告はほとんど存在しないが，分化能や安全性など株間の差異は，細胞療法などの移植医療に使用する細胞を効率よく作製するために，また，移植後の重篤な合併症を防ぐために大変重要な点であり，今後，異なるiPS細胞株間で詳細に比較検討する必要があると考える．

6 iPS細胞研究の課題と展望

iPS細胞は，再生医療の開発など医学面での注目が高い一方で，生物学的にも大変興味深い疑問を提示している．特に初期化機構の解明は始まったばかりであり，この機構の解明が，ひいてはiPS細胞の樹立効率の低さを克服すると期待される．また，初期化機構の解明に基づいて，ゲノムへの導入遺伝子の組み込みがないため安全で，かつ，レトロウイルスベクターを用いたものと同等かそれ以上の誘導効率を有するiPS細胞樹立方法の開発および普及が期待される．また今後，iPS細胞を分子的に定義するより明確で客観的な基準の決定，すなわち「iPS細胞の標準化」にも貢献する

※1 テトラプロイド凝集胚形成法

野生型の2つの胚を電気的に融合させたテトラプロイド（四倍体）胚とディプロイド（二倍体）であるES細胞を凝集させ，ES細胞からクローン（個体）を作製する方法．このキメラ胚では，テトラプロイド細胞は胚体自体には寄与できないが，胎盤などの胚体外組織には寄与できるという性質があり，100%ES細胞由来の個体が作製できる．ES細胞の分化能を判定する最も厳しい評価基準と言われている．

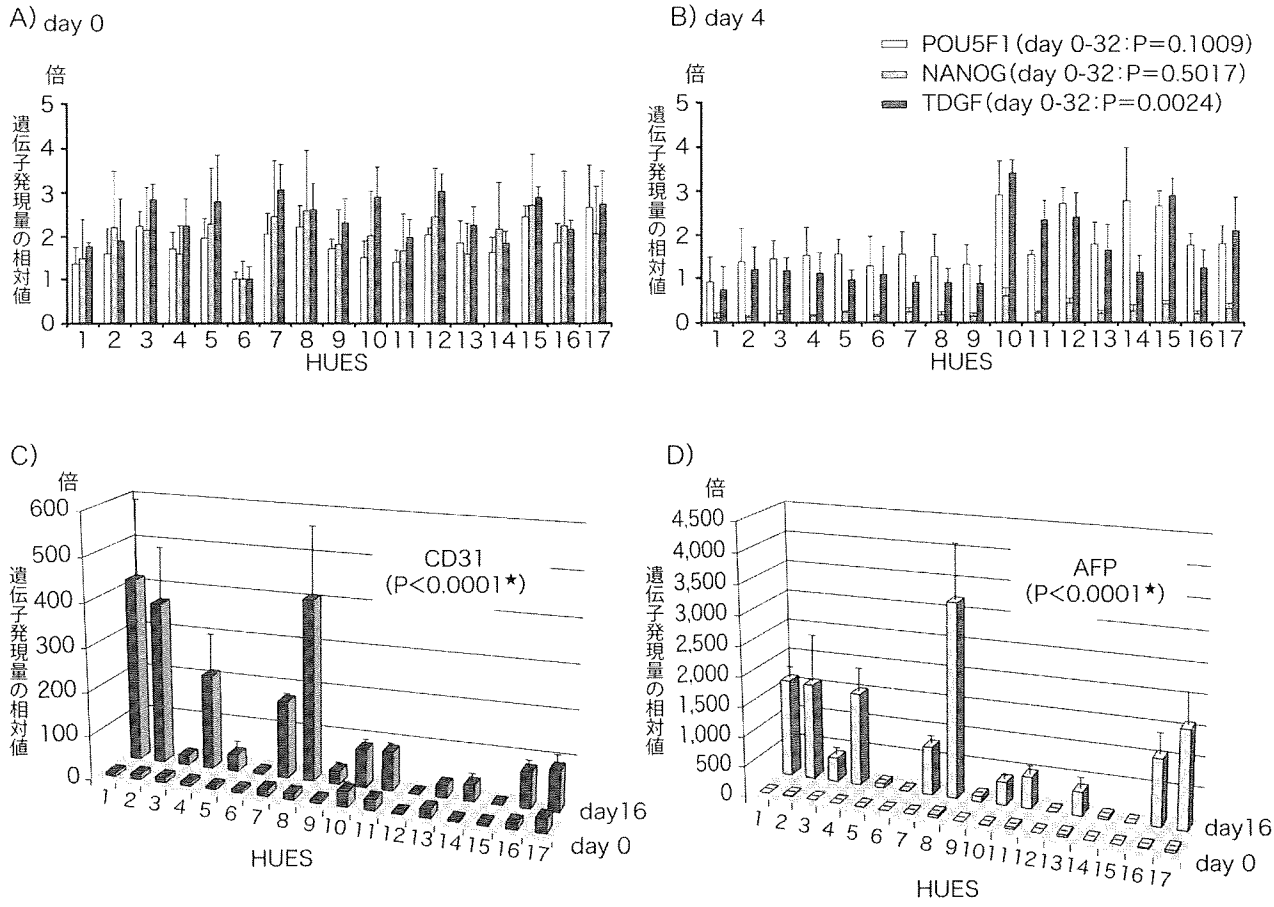

図2　ヒトES細胞株間の分化能の顕著な差異

17種類のヒトES細胞株（HUES1-17）由来の胚様体における自発性分化時の遺伝子発現の多様性．mRNA発現量の定量的PCRによる解析結果．day 0（A）およびday 4（B）における未分化状態マーカー遺伝子（*POU5F1*, *NANOG*, *TDGF*）．C）day 0および16における血管内皮（中胚葉）マーカー遺伝子*CD31*．D）day 0および16における内胚葉マーカー遺伝子*AFP*．それぞれのグラフは，17細胞株間で最も発現量の小さい株の値を1として，それに対する倍率を示している．未分化マーカー遺伝子の発現量は，17細胞株間で差が小さく統計学的有意差はないが，*CD31*および*AFP*の発現量は，顕著な違いがあり，株間で有意差を認める（P<0.0001, ANOVA for repeated measurements，文献43より）

ものと期待される．

細胞株間の分化能や安全性の差異/不均一性については，その正確な評価を可能とする分化プロトコールの開発を行い，目的に応じた細胞株を選択して使用できるシステムの構築が必要である．あるいは，逆のアプローチとして，近年，MAPK（mitogen-activated protein kinase）およびGSK-3（glycogen synthase kinase-3）の2つの酵素の阻害剤を使用する培養法で，異なる系統に由来するマウスES細胞株がより均一になることが示されているが[48]，同様の培養法が特にヒトES/iPS細胞に対しても開発され，細胞株間の差異を最小限とし，株間をより均一にする技術の開発が望まれる（**図3**）．

おわりに

iPS細胞研究はすさまじいスピードで進展を続けている．また，世界中の大学や研究機関のみならず，産業界，官公庁，マスメディアをも巻き込んで，1つの社会現象を引き起こしたと言っても過言ではないと思われる．毎日のように新しい論文の発表や報道が行われるため，すべての知見を網羅することは困難であり，本誌が出版されるころにはiPS細胞に対する認識も多

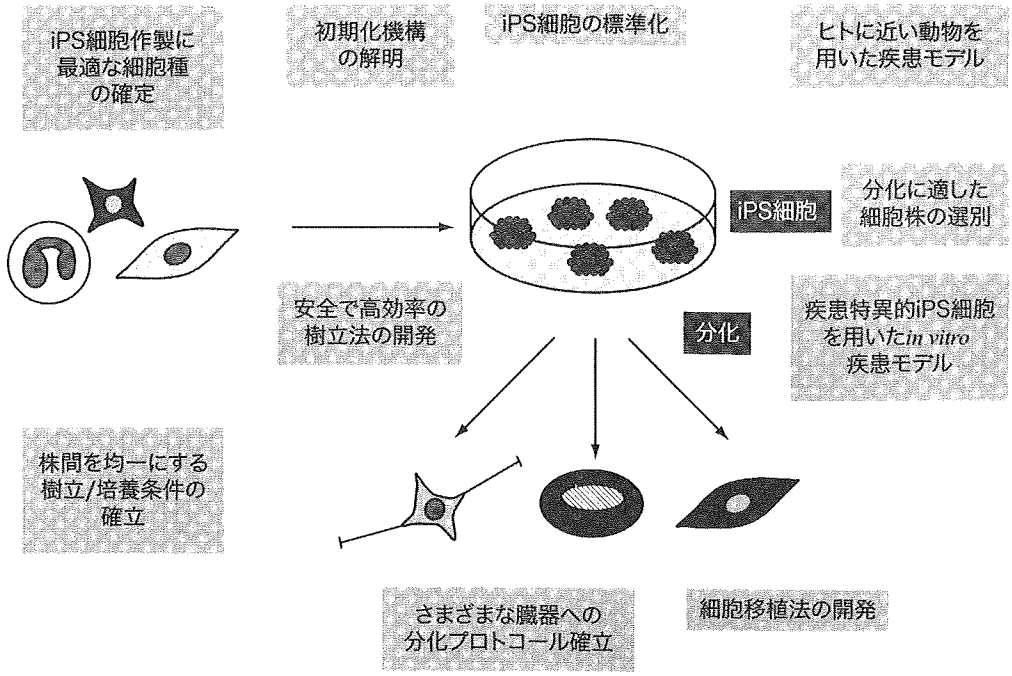

図3　iPS細胞研究の課題と今後の展望
初期化機構の解明に基づく安全で高効率なiPS細胞樹立法の開発と再生医療の実現化に向けた主要臓器細胞への分化誘導 / 移植法の開発，そして，難治性疾患に対するよりヒトに近い動物モデルおよび疾患特異的iPS細胞を用いた*in vitro*モデル作製による病態解析と治療薬開発への貢献が期待される

少変わっているのかもしれない．しかしながら，本稿がiPS細胞研究の知識の整理に少しでも役立ち，1人でも多くの若者や他領域の研究者が，iPS細胞研究に参入し，この本邦から誕生した技術を起点として新たなブレークスルーを次々と生み出すことを願ってやまない．

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iPS細胞を用いた創薬研究
—薬効・副作用評価系への活用—

Drug discovery using induced pluripotent stem cells
- Application to drug screening and toxicology assay -

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Key Words:　induced pluripotent stem cells, drug discovery, screening

Abstract

新薬を創生することが年々難しくなっている。2008年の米国食品医薬品局（FDA）の新薬承認数は24とやや持ち直したが，ここ数年は20前後で推移しており1990年代に比較して明らかに減少している。これは，審査基準の厳格化，とくに副作用試験に対する要求基準のアップに加え，創薬に適したターゲット分子の減少や難易度の高い疾患への挑戦などが要因と考えられる。このような状況下，製薬企業は低分子化合物を中心とした創薬に加えて，抗体医薬や核酸医薬など新たな医薬品の形態に取組み，新技術も積極的に活用しつつ厳しい競争に打ち勝つ努力をしている。ES細胞や体性幹細胞など幹細胞関連技術は従来から創薬への応用の可能性が言及されてきたが，iPS細胞は患者組織から作製できること，使用に関して倫理的な問題がほとんどないことから創薬への期待が非常に大きい。創薬研究のプロセスは多くのステップが必要であるが，その中でも薬効スクリーニング，毒性評価へのiPS細胞の活用について述べる。

■iPS細胞を用いた薬効スクリーニング

医薬品開発の初期の重要なステップであるスクリーニングは数十万あるいは100万種以上の化合物から活性のある候補化合物を見つけ出す作業である。短期間で数多くの化合物を評価するためには，再現性に優れたスループットの高いアッセイ系（スクリーニング系）を構築しなければならない。近年のイメージング技術の進歩により，細胞を用いたセルベースアッセイでもスループットの高いアッセイが可能になってきた。創薬スクリーニング用の細胞としては，培養方法や遺伝子操作が比較的簡単であることから，がん細胞株や不死化細胞株が広く用いられている。

しかし，これらの細胞はヒト組織の生理的な性質あるいは病態の異常を忠実に反映している保障はない。動物やヒト組織由来の初代培養細胞も使用されるが，増殖能に限界があり均質な材料が常に入手できないことから，スクリーニングのハイスループット化と安定的な供給の面で問題がある。ヒト細胞の性状を反映し供給面での問題を解決したソースとしてヒトiPS細胞から分化成熟させた細胞の利用が考えられる。iPS細胞の樹立にはヒト胚を利用しないので倫理的な障壁がなく，企業がスクリーニング等の創薬開発に積極的に使用することについても抵抗はない。

iPS細胞を用いた分化誘導研究はまだ緒についたばかりであるが，すでに，神経細胞（1）（2），心筋細胞（3），網膜色素上皮細胞（4）など一部の成熟組織細胞への分化が報告され，生体の組織細胞の機能を反映したヒト分化細胞の取得が可能とな

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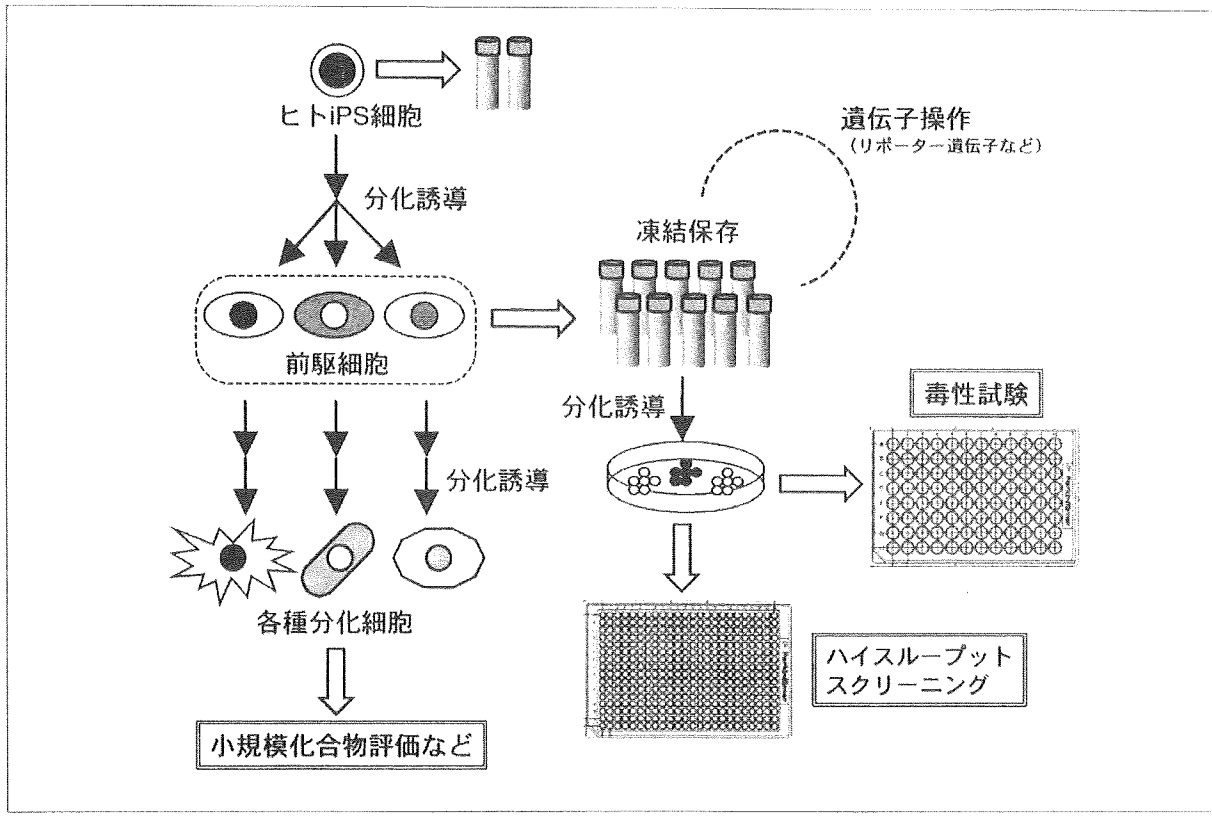

図　iPS細胞を用いた創薬研究の流れ
iPS細胞から分化誘導したヒト成熟組織細胞は小規模な化合物評価に利用できる。一方，大規模な薬効スクリーニングに用いるためには，分化誘導の効率化や前駆細胞の凍結保存など利便性および品質の安定性に繋がる細胞供給システムの確立が必要である。

ってきた。これらの細胞を用いた中枢神経系疾患，心疾患などの薬剤スクリーニングへの応用が期待される。例えば，ヒトiPS細胞由来のヒト神経細胞を用いて，神経細胞死，神経突起伸張，電気生理などに関した機能的なアッセイが構築可能であり，さらに，iPS細胞からの各種成熟神経への選択的な分化培養法で得られたドーパミン神経，アセチルコリン神経などの機能的なヒト成熟ニューロンを用いて神経伝達物質に関したアッセイも構築可能であろう。今後，上記組織以外にもヒトiPS細胞から様々な組織の細胞への分化誘導系が研究され，ヒト組織を反映したヒト成熟組織が入手可能になると予想される。

このように期待の大きいiPS細胞であるが，実際に使用するためには解決すべき課題も少なくない。現状では，iPS細胞から成熟組織細胞への分化誘導には比較的長期間の培養期間と複数のステップが必要であり，成熟細胞の純度も高くない。大規模スクリーニングのためには大量の細胞の供給が必要であり，また，再現性があり信頼できる実験結果を得るためには，分化細胞の性状の安定化が必須である。使用目的にもよるが，成熟細胞の純度を上げるために分化誘導効率の向上や目的細胞の濃縮・精製方法の確立が必要である。

さらに，利便性の面から，分化培養の途中段階，例えば特定の組織への分化がコミットされた前駆細胞の段階で凍結保存が可能となれば，供給面のみでなく再現性の面からもメリットは大きい。大量に保存された前駆細胞から少数のステップで必要な組織への分化が実現できれば，創薬スクリー

表　iPS細胞の創薬利用に焦点を当てたベンチャー企業

	Key Scientists（創業者，アドバイザー）	iPS細胞関連のプロダクト・サービスおよび技術	主なiPS細胞関連特許（ライセンスを含む）	備考
ReproCELL Inc.（日本）	中辻憲夫 中内 啓光	・iPS細胞培地 ・iPS細胞由来心筋細胞の提供 ・iPS由来心筋細胞を用いた毒性評価	マウス及びヒトiPS細胞（Yanamaka，2006）	
iPierian（米）	George Daley Douglas Melton Lee Rubin Deepak Srivastava Corey Goodman	・疾患特異的iPS細胞の作製と分化誘導 ・疾患特異的iPS細胞を用いたDrug Discovery	ヒトiPS細胞（Sakurada，2007）	Gladstone Instituteおよび京都大学と共同研究
Cellular Dynamic International（米）	James A. Thomson Craig T. January Timothy J. Kamp Igor Slukvin	・iPS由来分化細胞の提供 ・iPS由来心筋細胞を用いた毒性評価 ・血球細胞からiPS作製 ・プラスミドによるiPS作製技術	ヒトiPS細胞（Thompson，2007）	Rocheと提携
Fate Therapeutics（米）	Philip Beachy Sheng Ding Rudolf Jaenisch David Scadden Leonard Zon	・iPS細胞の作製 ・iPS由来細胞を用いた再生医療と創薬応用 ・タンパク質にあるいは化合物によるiPS作製	転写因子によるリプログラミング（Jaenisch，2004	創薬応用を目目指したiPS細胞研究ネットワークCATALYSTを構築

ニングへの応用は飛躍的に広がると思われる（図）。

■iPS細胞を用いた副作用評価

ヒトの成熟組織細胞を安定的に供給できるという点で，iPS細胞は副作用（毒性）評価にも活用が期待されている。心毒性，肝毒性，神経毒性などへの応用がまず考えられ，創薬の初期の段階から品質の安定したヒト組織を用いた毒性評価が可能になれば，ヒトへの外挿性が上がり臨床試験の成功確率の上昇に繋がると期待できる。毒性試験は製薬企業各社が開発した医薬品そのものの競争力に直接関係しないので，各社が協力して標準的な評価法の開発を行なうことが可能である。また，iPS細胞を用いた毒性評価が可能になれば，各社連携してデータを蓄積し将来的に申請資料に使えるように当局と早期から協議をしていくことが望まれる。日本においても，新エネルギー・産業開発機構（NEDO）のプロジェクト「iPS細胞等幹細胞産業応用促進基盤技術開発」，医薬基盤研究所が主体となったスーパー特区「ヒトiPS細胞を用いた新規in vitro毒性評価系の構築」などが立ち上がり，iPS細胞を用いた心毒性，肝毒性評価系構築に向けて研究がスタートしている。前者はユーザーフォーラムという形で複数の製薬企業が参加しており，後者は将来製薬協との連携を視野に入れている。これらのプロジェクトを中心に毒性評価系の標準化に向けて産官学が協力し，iPS細胞を用いた毒性評価の早期実用化が望まれる。

iPS細胞の毒性研究への応用として利用価値が高いと思われるのは，前臨床試験や臨床試験において毒性が原因で開発中止となった薬剤の毒性発現メカニズムの解析である。iPS細胞を用いると，毒性発現の対象となるヒト成熟組織細胞を用いたメカニズム解析が可能になり，先行化合物の副作用を軽減したバックアップ化合物の研究開発に繋がる。また，実際に副作用が発現した患者組織からiPS細胞を作製し，その分化細胞を用いることが出来れば，毒性発現のメカニズムの研究はより効率