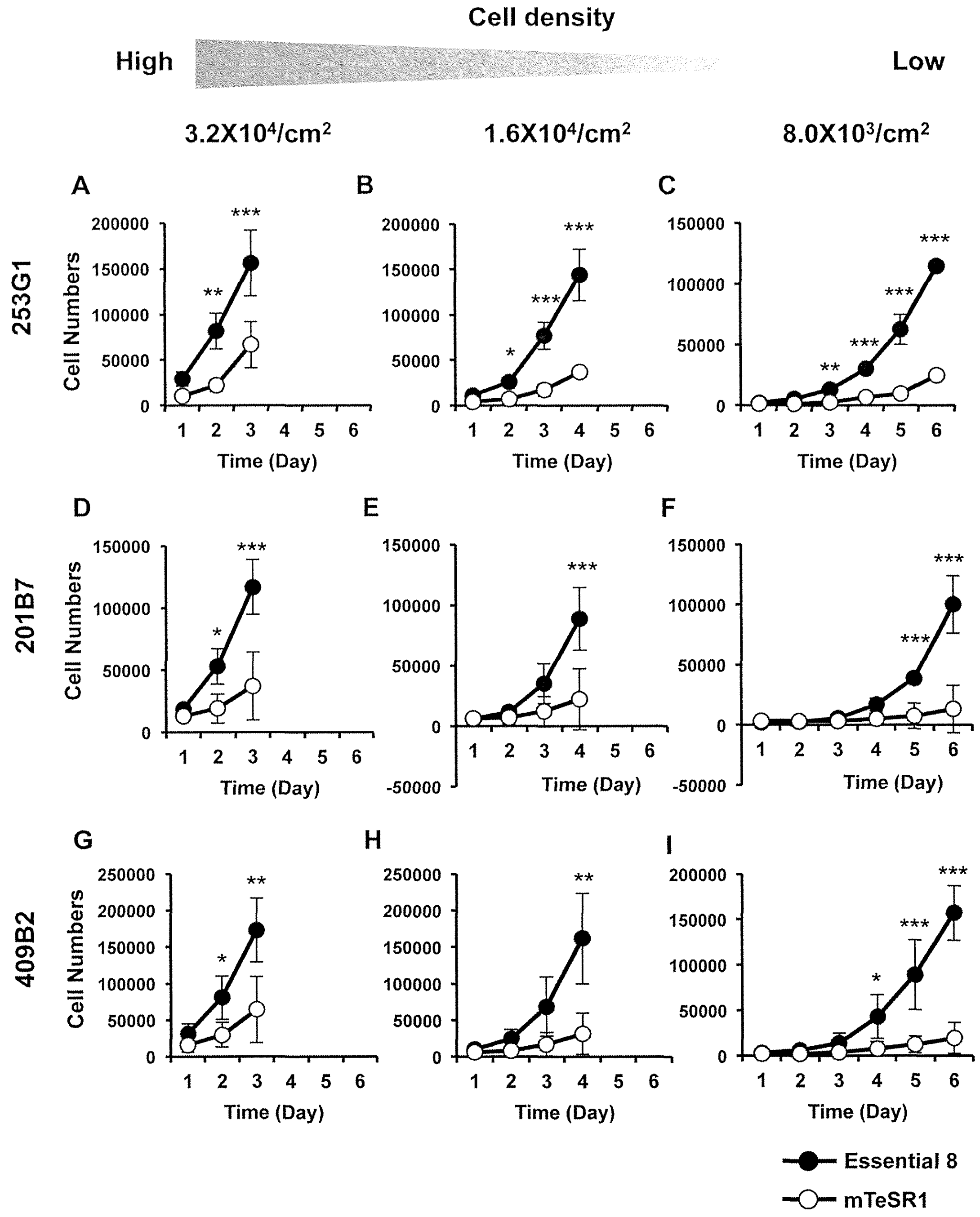
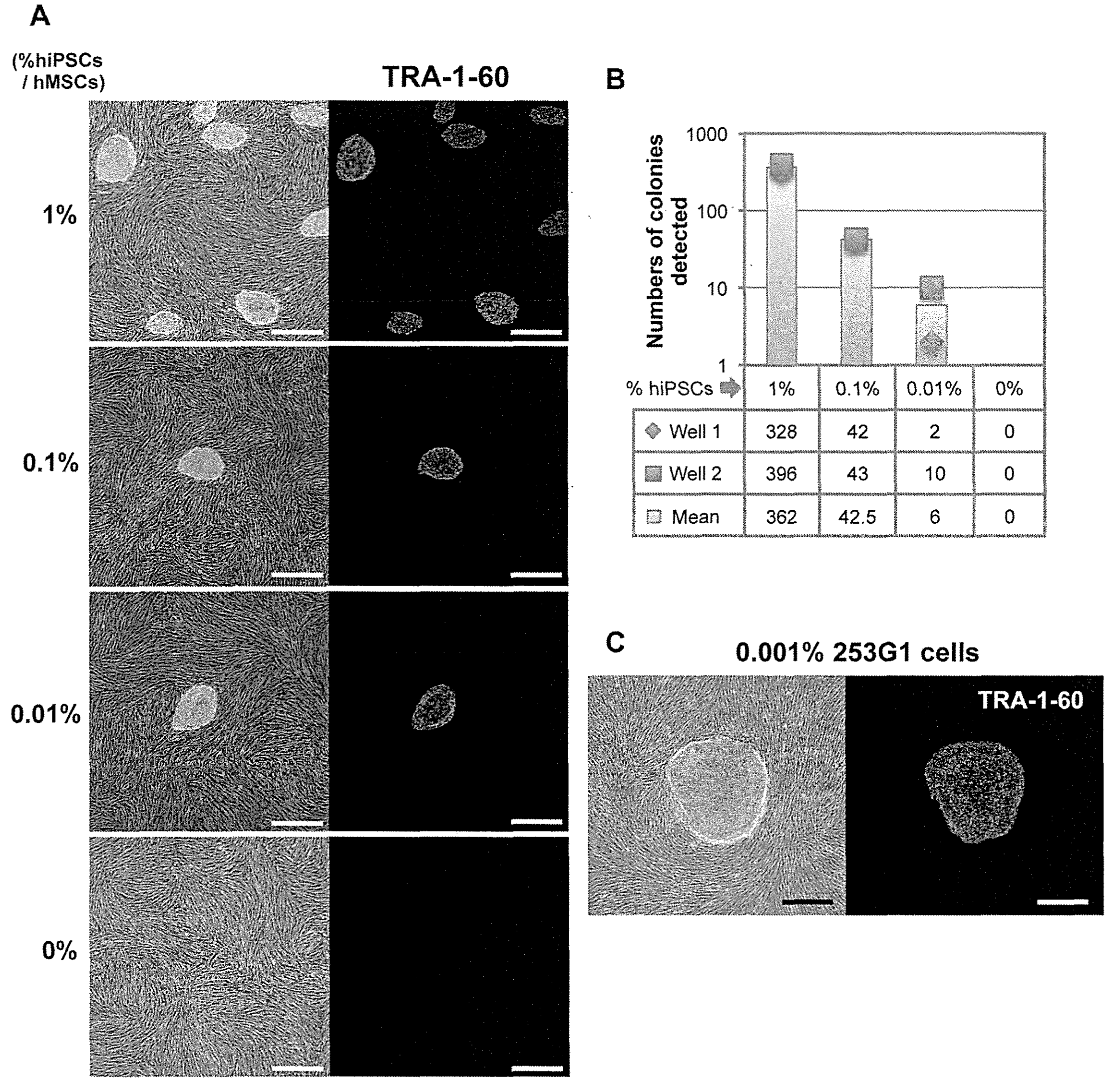


Figure 3. Rapid cell proliferation of hiPSCs plated at low cell density on laminin-521 in Essential 8 medium. (A-I) Quantification of the number of 253G1, 201B7 and 409B2 cells expanded on laminin-521 in Essential 8 or mTeSR1 medium. Cell numbers were counted every 24 h after plating at 3.2×10^4 cells/cm^2 (A, D, G), 1.6×10^4 cells/cm^2 (B, E, H) and 8.0×10^3 cells/cm^2 (C, F, I), respectively. Data are presented as the mean ± standard deviation (SD) of three independent experiments (*$P<0.05$, **$P<0.01$, *** $P<0.001$, two-way repeated-measures ANOVA followed by a Bonferroni post-hoc test).
doi:10.1371/journal.pone.0110496.g003

find any colonies when only hMSCs were cultured on laminin-521 in Essential 8 medium. In addition, immunofluorescence staining with anti-TRA-1-60 antibody showed that these colonies formed in an undifferentiated state (Figure 4A), suggesting that colonies derived from hiPSCs were formed in an hMSC monolayer under conditions with laminin-521 and Essential 8 medium. We also



% hiPSCs ➡	1%	0.1%	0.01%	0%
◆ Well 1	328	42	2	0
▨ Well 2	396	43	10	0
□ Mean	362	42.5	6	0

Figure 4. Detection of hiPSCs spiked into hMSCs on the culture system using laminin-521 and Essential 8 medium. (A) Morphologies of forming colonies derived from 409B2 cells spiked into hMSCs are shown (images in the left). 409B2 cells (1%, 1000 cells; 0.1%, 100 cells; 0.01%, 10 cells; 0%, 0 cells) were spiked into hMSCs (100,000 cells) and co-cultured on laminin-521-coated wells in 6-well plates in Essential 8 medium for 7 days. Expression of the undifferentiated marker, TRA-1-60, in these colonies was assessed using immunofluorescence staining (images in the right). Each experiment was carried out in duplicate. Scale bars, 500 µm. (B) Numbers of the colonies detected in each spiked sample in (A) are shown. Data are present as raw data in each well (shown by plots) or the mean of well 1 and well 2 (shown by bar graphs). (C) Morphology of a forming colony derived from 253G1 cells spiked into hMSCs at the ratio of 0.001% (6 hiPSCs to 600,000 hMSCs) is shown (images in the left). Mixture of those cells was co-cultured on a 100-mm cell culture dish coated with laminin-521 in Essential 8 medium for 9 days. Forming colony was stained with anti TRA-1-60 antibody (images in the right). Experiment was carried out in duplicate. Scale bars, 500 µm.
doi:10.1371/journal.pone.0110496.g004

tested another hiPSC line, 253G1, for undifferentiated cells spiked into hMSCs. We found that 253G1 cells spiked into hMSCs at the ratio of 1% and 0.1% formed approximately 100 and 20 colonies, respectively, on laminin-521 in Essential 8 medium (Figure S4). We detected one colony when 253G1 cells were spiked into hMSCs at a ratio of 0.01% or 0.001% and co-cultured on a laminin-521-coated dish in Essential 8 medium (Figure S4 and Figure 4C). Taken together, our culture system using laminin-521 and Essential 8 medium allows the direct detection of 0.001%–0.01% hiPSCs in hMSCs as a result of efficient cell amplification.

We also confirmed that no colonies were detected when a mixture of hiPSCs and hMSCs were cultured on laminin-521 in MSCGM medium instead of Essential 8 medium. In the absence of laminin-521, several colonies were detected in Essential 8 when hMSCs contained 1% hiPSCs but not when hMSCs contained 0.1% and 0.01% hiPSCs (data not shown). These results suggest that laminin-521 is required to detect trace amounts of hiPSCs in hMSCs (less than 0.1%).

To know whether this culture system also works in detecting trace amounts of hiPSCs contaminating other types of cells besides

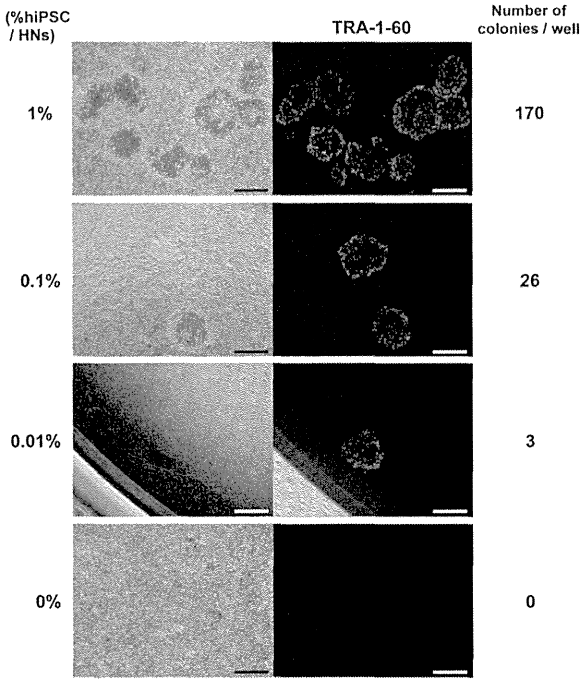


Figure 5. Detection of hiPSCs spiked into human neurons on the culture system using laminin-521 and Essential 8 medium. Morphologies of forming colonies derived from 253G1 cells spiked into human neurons are shown (images in the left). 253G1 cells (1%, 1000 cells; 0.1%, 100 cells; 0.01%, 10 cells; 0%, 0 cells) were spiked into human neurons (100,000 cells) and co-cultured on laminin-521-coated wells in 12-well plates in Essential 8 medium for 6 days. Forming colonies were stained with anti TRA-1-60 antibody (images in the right). HNs, human neurons. Scale bars, 500 µm.
doi:10.1371/journal.pone.0110496.g005

hMSCs, we next tested colony formation of hiPSCs spiked into primary human neurons. Spiked 253G1 cells were co-cultured with human neurons on laminin-521 in Essential 8 medium and clearly formed colonies (Figure 5), which is consistent with the observation using hiPSCs spiked into hMSCs. We detected 170, 26 and 3 colonies that were positive for TRA-1-60 when 253G1 cells were spiked into 1×10^5 human neurons at the ratio of 1, 0.1 and 0.01%, respectively. There was no colony when only human neurons were cultured on our system. These results suggest that this culture system is also useful for detection of trace amounts of hiPSCs not only in hMSCs but also in other types of cells such as human neurons. We also confirmed that no colonies were formed on the well that was not coated with laminin-521 even when human neurons containing 10% hiPSCs were plated (data not shown), indicating that formation of the colonies derived from hiPSCs in human neurons is dependent on laminin-521.

Culture system using laminin-521 and Essential 8 medium has a capacity for direct detection of residual undifferentiated cells contained in differentiating hiPSC cultures

Finally, we examined whether this culture system using laminin-521 and Essential 8 medium is applicable in direct detection of residual hiPSCs contained in differentiated cells derived from hiPSCs. We attempted to differentiate 253G1 cells into MSCs as described in Materials and Methods (Figure 6A). Using this protocol, we observed attached cells with fibroblast-like morphology at the stage of passage 0 MSCs. We confirmed that approximately 20% of these attached cells were positive for staining with anti-CD105 antibody, a MSC marker antibody (Figure S5). During the differentiation process of 253G1 cells into MSCs, we examined the expression levels of residual pluripotency markers in the cell cultures. qRT-PCR analysis revealed that expression of *OCT3/4*, *NANOG* and *LIN28* mRNA were clearly decreased in a time-dependent manner, however, expression levels at the same time point varied markedly among those genes (Figure 6B). In the cells at day 6 of differentiation, mRNA levels of *OCT3/4*, *NANOG* and *LIN28* were 7.3%, 4.8% and 86.4% of the control at day 1, respectively (Figure 6B). At day 14 of differentiation, although *OCT3/4* and *LIN28* were still at detectable levels of 2.6% and 17.2% of control cells, respectively, *NANOG* expression was not detected. These results indicate that the population of residual hiPSCs in differentiating cells, when estimated by the qRT-PCR data, greatly varies and depends on the pluripotency marker gene employed for the estimation. In addition, it is also possible that all the qRT-PCR signals were derived from partially differentiated cells, not from fully undifferentiated cells. To examine colony formation of residual undifferentiated cells in differentiating cell culture, cells at day 6 were dissociated into single cells and replated on laminin-521 in Essential 8 medium. Small cell clusters began to emerge 4 days after plating, rapidly expanded and formed colonies on laminin-521 in Essential 8, while other types of cells gradually decreased their numbers (Figure 6D). After 8 days of culture, 9.5 colonies (the mean of duplicate measurements) were formed from differentiating cells (5×10^4) (Figure 6C) and they were all positive for TRA-1-60 (Figure 6D), indicating that the colonies were derived from residual undifferentiated cells in the differentiating cell cultures. These results suggest that the culture method using a combination of laminin-521 and Essential 8 directly detects residual undifferentiated cells by highly efficient cell amplification. Based on our finding that approximately 0.3 and 6.7 colonies were formed from 1×10^4 MSCs containing 0.01% and 0.1% of 253G1 cells, respectively, in this culture system (Figure S4), and assuming that the sensitivity of the system for hPSCs in EBs are comparable to that in MSCs, the population of the undifferentiated cells in the differentiating cell cultures on day 6 (1.9 colonies/10^4 cells) was estimated to be in between 0.01% and 0.1%. When we tested colony formation using cell cultures on day 14 of differentiation, no colonies were detected on laminin-521 in Essential 8 medium (Figure 6C and data not shown), suggesting that the population of the residual hiPSCs was less than 0.01%.

Discussion

A method to detect residual undifferentiated hPSCs contained in CTPs is required to evaluate product quality during manufacturing processes. In the present study, we propose a novel method to detect a trace amount of undifferentiated hPSCs by highly efficient amplification of those cells *in vitro*. We showed that Essential 8 medium significantly promotes cell growth of hiPSCs dissociated into single cells on laminin-521 compared with the conventional medium, mTeSR1. In addition, Essential 8 medium allowed robust proliferation of hiPSCs even at low cell density on laminin-521. We also demonstrated that 0.001%–0.01% hiPSCs spiked into primary hMSCs were clearly detected and formed colonies on laminin-521 in Essential 8. Similarly, we confirmed that 0.01% hiPSCs spiked into primary human neurons were also detectable on this system. Moreover, we showed that residual undifferentiated hiPSCs contained in differentiating cells were

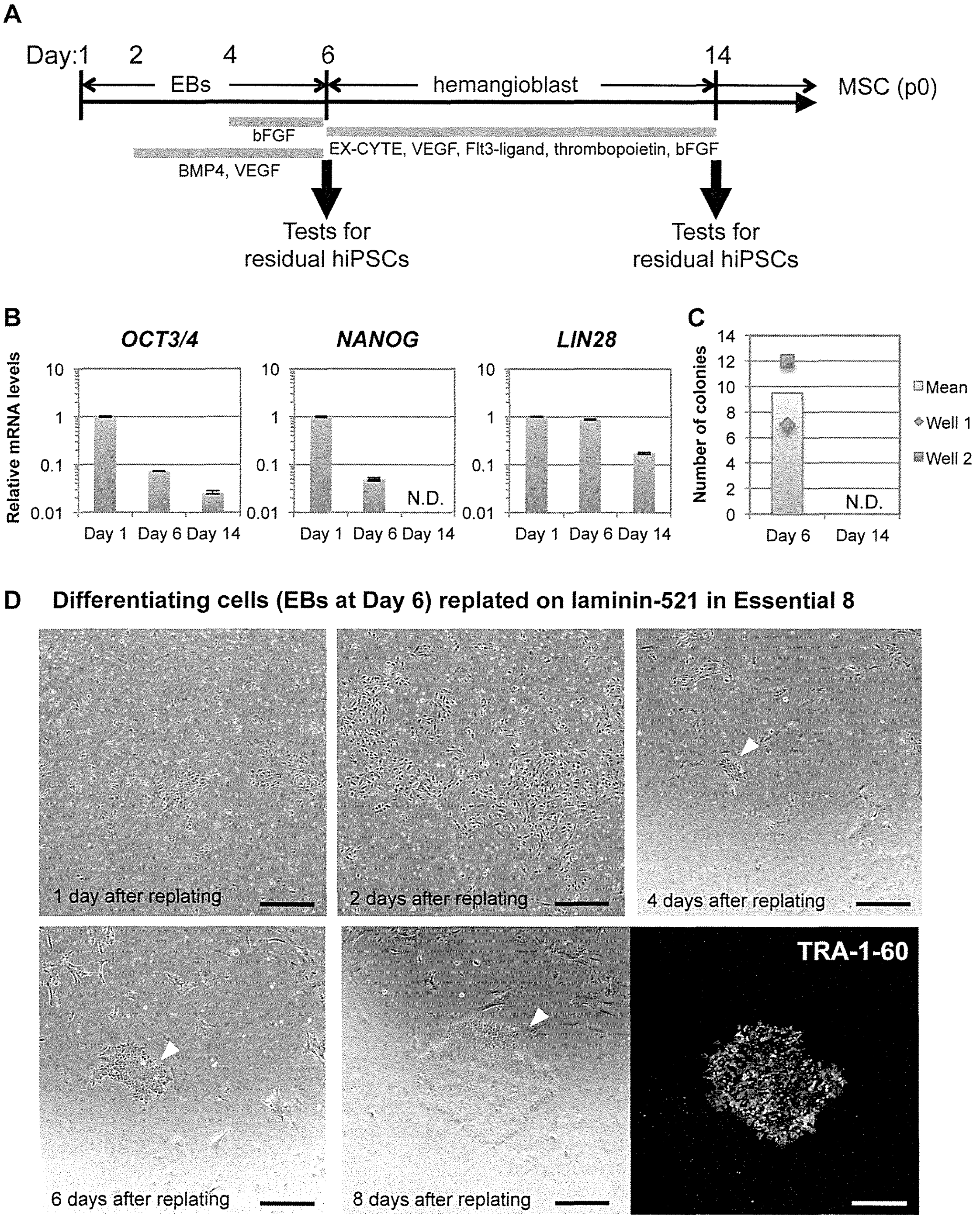


Figure 6. Detection of residual undifferentiated cells contained in differentiating cell cultures. (A) Differentiation scheme of 253G1 cells into MSCs is shown. (B) Expression levels of undifferentiated cell markers (*OCT3/4, NANOG* and *LIN28*) in each cell culture were determined using qRT-PCR. Relative mRNA expression levels are presented as ratios to the level of that in 253G1 cells at Day 1. Results are the mean ± SD (n = 3). (C) Numbers of the forming colonies derived from residual undifferentiated cells in differentiating cell culture at Day 6 or Day 14 are shown. Experiments were carried out in duplicate. Data are present as raw data in each well (shown by plots) or the mean of well 1 and well 2 (shown by bar graphs). (D) Phase contrast images of forming colonies derived from residual undifferentiated cells are shown. Cells at Day 6 of differentiation (EBs) were dissociated into single cells by Accutase and cultured on laminin-521-coated wells in Essential 8 medium (5×10^4/well). After 4 days of culture, small clusters emerged and then started to grow rapidly. Finally, they formed colonies that were positive for TRA-1-60 (shown by immunofluorescence staining, green). Arrowheads indicate a colony derived from same origin. Scale bars, 500 μm.
doi:10.1371/journal.pone.0110496.g006

detectable by forming colonies on laminin-521 in Essential 8 in the process of hMSC differentiation. These results indicate that a culture system utilizing a combination of laminin-521 and Essential 8 medium provides a direct and highly sensitive method for detecting undifferentiated hPSCs. To our knowledge, this is the first report to show a direct and highly-sensitive *in vitro* method for detecting undifferentiated hPSCs as impurities in CTPs.

In this study, highly efficient amplification of undifferentiated hPSCs has been uniquely applied to quality control of CTPs. Amplified hPSC colonies were visible using phase-contrast microscopy and also immunofluorescence staining using pluripotency antibodies, which enabled direct detection of hPSCs contaminating CTPs. Our method distinguished between undifferentiated cells and other cells *in vitro*, and overcame the disadvantage of other *in vitro* methods such as flow cytometry and qRT-PCR. The flow cytometry analysis detects known marker molecules expressed in undifferentiated hPSCs using antibodies and proteins. Signals originating from non-specific detection commonly affect sensitivity of the assay as background. Our *in vitro* method can lower the background arising from non-specific detection and is expected to specifically detect residual undifferentiated hPSCs in CTPs. The qRT-PCR method is highly sensitive and can rapidly quantify undifferentiated cell contamination in CTPs. However, in the present study, gene expression levels of pluripotency markers during the differentiation process of hiPSCs into MSCs varied markedly among those marker genes (Figure 6B). Moreover, there remains a possibility that expression signals of marker genes were not derived from totally undifferentiated hPSCs, but from partially differentiated cells. Indeed, the expression level of *LIN28* did not decrease so much during the differentiation as those of the other genes, which was not obviously associated with the differentiation status of the cells in EBs on Day 6 (Figure 6B), although we have previously reported that *LIN28* was a useful marker for monitoring the level of residual hiPSCs in RPE cells derived from hiPSCs [3]. Thus, it is difficult to determine the presence of residual hiPSCs simply by qRT-PCRs. In contrast, direct detection method using the highly efficient amplification system can clearly detect the presence of intact undifferentiated cells. Based on the result from direct detection of residual hiPSCs when tested the cells on Day 6 of differentiation (approximately 0.01%–0.1%) (Figure 6C-D), it is conceivable that the qRT-PCR signals for the pluripotency marker genes (Figure 6B) are partly derived from residual hiPSCs but mainly derived from partially differentiated cells. Similarly, in the case of the cells at Day 14 of differentiation, the majority of the qRT-PCR signals of *OCT3/4* and *LIN28* (Figure 6B) are considered to be attributable to partially differentiating cells but not to intact hiPSCs. Combination of the *in vitro* methods including our cell culture method would mutually support useful quality assessment of CTPs to detect undifferentiated hPSCs.

In addition to the detection of undifferentiated cells, this culture system using laminin-521 and Essential 8 medium allows further characterization of the undifferentiated cells if they are maintained *in vitro* or inoculated into immunodeficient animals. Analyses for the properties of the residual undifferentiated cells would be necessary not only for the quality assessment of CTPs, but also for improvement of quality specifications of hPSCs as a raw/intermediate material for production of CTPs.

Here, we showed that our culture system is able to detect 0.01% of 409B2 hiPSCs and 0.001% of 253G1 hiPSCs, both of which were spiked into hMSCs (Figure 4). The detection sensitivity for hiPSCs spiked into hMSCs was different between the two hiPSC lines, although such a difference in cell growth on laminin-521 was not found between these two cell lines (Figure 3). This difference may be attributable to the difference in the growth potential of hPSCs in the specific environment provided by CTPs. Kanemura *et al.* have recently demonstrated that hiPSCs co-cultured with iPSC-derived RPE undergo apoptosis by pigment epithelium-derived factor (PEDF) secreted from hiPSC-derived RPE [15], showing that CTPs themselves have the potential to affect cell growth of hPSCs. In the present study, the influence of the co-culture system with hMSCs to the proliferation of hiPSCs might have been different between the two cell lines.

The mechanism by which laminin-521 and Essential 8 medium enhance hiPSCs cell proliferation remains unclear. Rodin *et al.* have recently shown that addition of E-cadherin to laminin-521 permitted the efficient clonal expansion of hESCs [7]. E-cadherin is known to be the primary cell-cell adhesion molecule and essential for hESC survival [16]. We observed that anti-E-cadherin antibody decreased growth potential of hiPSCs under our experimental conditions (data not shown). Therefore, E-cadherin signaling may play some important roles in the rapid cell growth on laminin-521 in Essential 8 medium.

Tumorigenicity is one of the major safety concerns for CTPs derived from hPSCs that are transplanted into patients. However, testing strategies for the tumorigenicity of hPSC-derived CTPs have not yet been established. Here, we introduced a novel testing method for directly detecting a trace amount of undifferentiated hPSCs *in vitro*. The ability of each tumorigenicity-associated test should be taken into consideration to evaluate tumorigenicity of residual undifferentiated hPSCs as impurities in products. *In vivo* tumorigenicity tests using immunodeficient animals can detect tumorigenic cells including undifferentiated hPSCs, but this method is costly and time-consuming. The flow cytometry analysis and qRT-PCR are rapid, but these methods indirectly detect tumorigenic cells depending on marker molecules. Risk of tumorigenicity in hPSCs-derived CTPs should be assessed, based on the results from an appropriate combination of these tumorigenicity-associated tests. Our novel method will contribute to establishment of the testing strategies for tumorigenicity in products, following evaluation of the quality of CTPs derived from hPSCs for the future regenerative medicine/cell therapy.

Supporting Information

Figure S1 (A) Quantification of the number of dissociated 201B7 cells expanded on laminin-521 or Matrigel in Essential 8 or mTeSR1 medium. Data are presented as the mean ± standard deviation (SD) of three independent experiments (**$P<0.01$, two-way ANOVA followed by a Bonferroni post-hoc test). LN521, laminin-521. MG, Matrigel. (B) Quantification of the number of dissociated 201B7 cells expanded on laminin-521 or LM511-E8 in Essential 8 or mTeSR1 medium. Results are presented as the mean ±SD (n = 3) (***$P<0.001$, two-way ANOVA followed by a Bonferroni post-hoc test).
(TIF)

Figure S2 (A-B) Expression levels of undifferentiated markers (*OCT3/4, NANOG, SOX2* and *LIN28*) in 201B7 cells (A) and 409B2 cells (B) subcultured on laminin-521 in Essential 8 were determined using qRT-PCR. Relative mRNA expression levels are presented as ratios to the level of that in control cells subcultured on Matrigel in mTeSR1 medium by colony passage. Results are presented as the mean ± SD (n = 3). (C-D) Expression levels of markers for the differentiation of embryoid bodies (EBs) derived from 201B7 cells (C) and 409B2 cells (D): endoderm (*GATA6, SOX17*), mesoderm (*CDH5, FOXF1*), and ectoderm (*SOX1, PAX6*). Relative mRNA expression levels are presented as

ratios to the level of that in control cells (EBs at day 10). Results are presented as the mean ± SD (n = 3).
(TIF)

Figure S3 Quantification of the number of 253G1 cells expanded on laminin-521 in Essential 8 or mTeSR1 medium. Cell numbers were counted at day 6, 9, and 12 after plating at 8.0×10^3 cells/cm^2 or 8.0×10^2 cells/cm^2.
(TIF)

Figure S4 Morphologies of forming colonies derived from 253G1 cells spiked into hMSCs are shown (images in the left). 253G1 cells (1%, 300 cells; 0.1%, 30 cells; 0.01%, 3 cells; 0%, 0 cells) were spiked into hMSCs (30,000 cells) and co-cultured on 12-well plates coated with laminin-521 in Essential 8 medium for 9 days. Expression of the undifferentiated cell marker, TRA-1-60, in these colonies was assessed using immunofluorescence staining (images to the right). Each experiment was carried out in duplicate.
(TIF)

Figure S5 Phase contrast images of the cells at day 18 of differentiation (at the stage of passage 0 MSCs) are shown. Expression of MSC marker, CD105, in these cells was examined using immunofluorescence staining (images to the right). Arrowheads indicate the cells that were positive for CD105.
(TIF)

Table S1 Sequences of the primers and probes for qRT-PCR.
(DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: KT SY YS. Performed the experiments: KT. Analyzed the data: KT SY TK HS AU YS. Contributed reagents/materials/analysis tools: KT SY TK HS AU YS. Wrote the paper: KT SY YS. Acquired the funding: HS AU YS.

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PATHOBIOLOGY IN FOCUS

A practical guide to induced pluripotent stem cell research using patient samples

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Approximately 3 years ago, we assessed how patient induced pluripotent stem cell (iPSC) research could potentially impact human pathobiology studies in the future. Since then, the field has grown considerably with numerous technical developments, and the idea of modeling diseases 'in a dish' is becoming increasingly popular in biomedical research. Likely, it is even acceptable to include patient iPSCs as one of the standard research tools for disease mechanism studies, just like knockout mice. However, as the field matures, we acknowledge there remain many practical limitations and obstacles for their genuine application to understand diseases, and accept that it has not been as straightforward to model disorders as initially proposed. A major practical challenge has been efficient direction of iPSC differentiation into desired lineages and preparation of the large numbers of specific cell types required for study. Another even larger obstacle is the limited value of *in vitro* outcomes, which often do not closely represent disease conditions. To overcome the latter issue, many new approaches are underway, including three-dimensional organoid cultures from iPSCs, xeno-transplantation of human cells to animal models and *in vitro* interaction of multiple cell types derived from isogenic iPSCs. Here we summarize the areas where patient iPSC studies have provided truly valuable information beyond existing skepticism, discuss the desired technologies to overcome current limitations and include practical guidance for how to utilize the resources. Undoubtedly, these human patient cells are an asset for experimental pathology studies. The future rests on how wisely we use them.

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The potential influence of induced pluripotent stem cell (iPSC) technology for pathobiology studies is revolutionary.[1] Once established from any given patient, iPSCs serve as enduring resources to provide various functional cell types, essentially forever, which retain genomic information from the original patient. For this reason, as well as based upon expectations of their applications for cellular transplantation therapy, iPSC research has been growing exponentially within the short number of years since the original method was published by Takahashi and Yamanaka in 2006.[2] Technical feasibility and high reproducibility are two additional reasons why the method has prevailed worldwide so quickly. Fundamentally, iPSC generation does not require sophisticated equipment or technical expertise, and all the materials required for generation are commercially available. Owing to more recent technological advances, one can now routinely generate iPSCs from patient peripheral blood cells without concern of exogenous gene integration. Accordingly, we can say iPSC technology has become a standard research tool in experimental medicine, like polymerase chain reaction, small interfering RNA, knockout mice and others.

Basic approaches to utilize patient iPSCs for disease mechanism studies are well demonstrated in the literature. Essentially, when patient iPSCs are differentiated into disease-relevant cell types, they can recapitulate, at least in part, molecular and phenotypic changes seen in patients. Using this system, we can further investigate how disease-related phenotypes develop 'in a dish', or even test whether novel therapeutic approaches can reverse these changes. Pioneering

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studies proved that these concepts are indeed valid for certain clinical disorders of both monogenic and polygenic origins. Thus, the future looks quite promising in general. However, when the concept is applied to model a wide range of diseases, we often encounter practical limitations and obstacles for their genuine application to understand diseases, and realize their application has not been as straightforward as initially proposed. First, despite numerous published protocols, *in vitro* differentiation of iPSCs is challenging, often requiring tremendous effort for optimization until the system becomes useful in other laboratories. Second, even after differentiation is successfully achieved, a major obstacle frequently resides in limited value of the *in vitro* outcomes, which may not closely represent disease conditions.

As we have witnessed triumphal examples and experienced many practical obstacles at the same time, we are gradually recognizing ways to utilize patient iPSCs more wisely. Three years have passed since we wrote the previous review in *Laboratory Investigation*,[1] and during this time, we have had the opportunity to manage a core facility for patient iPSC research at the University of Florida. Thus, we feel this is a good time to revisit the issue of 'modeling diseases in a dish' using patient iPSCs, and try to elucidate where we are now with the technology. We target general experimental pathologists as primary readers of the present review, particularly those who are interested in starting patient iPSC research to study a disease of their interest, but not yet sure whether the direction will justify the effort. As there are many outstanding review articles available for recent technological advances in iPSCs,[3–5] here we will focus more on introducing practical issues and solutions for pathobiology applications, leaving extensive details to the references.

EXEMPLARY CASES

To understand how patient iPSC research is generally conducted, it is useful to introduce a few exemplary cases briefly, in which patient iPSCs have been wisely and beneficially utilized. As iPSCs retain genomic information from the original patient, theoretically we can analyze phenotypic and functional characteristics manifested from changes in the individual genome. Initially, early-onset monogenic disorders, where a single genetic aberration is considered to cause severe deleterious effect on cellular function, have been studied preferentially using iPSCs.

Early-Onset Monogenic Disease

An exemplary work proving the concept, 'modeling diseases in a dish' was first published in January 2009 by Ebert *et al*.[6] The authors successfully established iPSCs from patients with spinal muscular atrophy, differentiated them into motor neurons, and demonstrated the premature death of neurons *in vitro*, a phenotype reflecting the disorder. Importantly, the study further proposed that disease iPSCs could be utilized to screen novel drugs that could de-repress the *SMN2* gene, a close homolog of the mutated *SMN1* gene. SMN2 is normally not expressed in neurons but could mitigate the disease phenotype when induced. It should be noted that the *SMN2* gene only exists in humans but not in rodents, thus this type of drug screening would only be possible using human neurons.

Late-Onset Monogenic Disease

Modeling late-onset disease in a dish is a more difficult task because some environmental factors, for example, oxidative stressors, may be involved in disease progression. Nevertheless, Nguyen *et al*[7] demonstrated, for instance, that a phenotype of a familial Parkinson's disease (PD) can be evaluated *in vitro*. The authors generated iPSCs from a patient with a mutation in the leucine-rich repeat kinase 2 (*LRRK2*) gene and differentiated the iPSCs into dopaminergic neurons. The resultant dopaminergic neurons were more susceptible to oxidative stressors (hydrogen peroxide, MG-132 and 6-hydroxydopamine), compared with those from control iPSCs. The study also demonstrated that the patient iPSC-derived dopaminergic neurons had an increase in α-synuclein, which is one of the major components of Lewy bodies, a hallmark of PD pathology.

Proving the Causal Mutation and Elucidating a Novel Mechanism

LRRK2-G2019S is the most commonly identified mutation, but it is only found in a few percent of the sporadic PD patients. Genome-wide association studies suggested that many other polymorphisms in other genomic loci are linked to the disease phenotypes and clinical courses. To that end, the exact pathological mechanism caused by the *LRRK2-G2019S* mutation needed to be elucidated using isogenic controls. Reinhardt *et al*[8] applied genomic engineering technology to correct the *G2019S* mutation in patient iPSCs. They confirmed *LRRK2-G2019S* indeed induced pathological changes of dopaminergic neurons such as deficit in neurite outgrowth, defect in autophagy, increase in α-synuclein, and higher susceptibility to oxidative stress. Furthermore, the study demonstrated the *LRRK2-G2019S* mutation is associated with activation of extracellular signal-regulated kinases (ERKs), which leads to transcriptional dysregulation of CPNE8, MAP7, UHRF2, ANXA1 and CADPS2, resulting in neural degeneration. By demonstrating an ERK inhibitor-mediated amelioration of the neurodegeneration, the study indeed indicated a novel therapeutic approach for patients with PD.

Polygenic Disorder or Disease of Unknown Causes

In the case of polygenic disorders or sporadic diseases with unknown causes, it is more challenging to obtain useful outcomes using patient-derived iPSCs. Israel *et al*[9] successfully investigated neural phenotypes derived from both familial and sporadic Alzheimer's disease. One of the

	Helpful	Harmful
	Strengths	**Weaknesses**
Internal Origin	☐ Clear merits to use patient iPSCs ☐ Strong research history for the disease ☐ Accessibility to number of patients (or *iPSC clones*) ☐ Preexisting collaborative strengths to develop the study	☐ May take time to establish differentiation protocols in your lab ☐ Differentiated cells may not be pure or mature enough for your study
	Opportunities	**Threats**
External Origin	☐ High expectations for developing novel model systems ☐ High expectations from societies of particular diseases ☐ External and internal grant opportunities	☐ Competitors working on similar directions ☐ Competing animal models

Figure 1 SWOT analysis before start patient iPSC research. It is critical to analyze all the strengths and potential problems you have before you initiate patient iPSC research. A local iPSC core facility may also assist you to analyze individual projects and create a research design.

two sporadic patient's iPSCs showed higher levels of the pathological markers amyloid-β (1-40), phosphor-tau(Thr231) and active glycogen synthase kinase-3β (aGSK-3β), as those derived from familial Alzheimer's disease, while the other case did not. These observations offered new opportunities to investigate the mechanisms underlying heterogeneity among sporadic cases. For such studies, however, a larger number of patients and controls would ideally be required.

Imprinting Disorders

In addition to genetic diseases, the iPSC models facilitate investigation of epigenetic-related diseases such as Beckwith–Wiedemann syndrome, Silver–Russell syndrome, Angelman syndrome and Prader–Willi syndrome. Unlike genetics based on the DNA sequence, epigenetic processes involve DNA methylation and histone modulation. One of the most important epigenetic phenomena is genomic imprinting by which genes are expressed in a parent-of-origin-specific manner. Abnormality of the imprinting mechanism during development causes epigenetic diseases. The methylation status of imprinting genes is maintained during iPSC generation and subsequent cultivation, implying that imprinting disease iPSCs are worth investigating to elucidate mechanism of imprinting abnormality.[10] Patient iPSCs from Angelman and Prader–Willi syndrome have been established and utilized for examination of epigenetic and transcriptomic abnormalities, and for testing compounds aimed at correcting the epigenetic aberrations.[11,12] One must use caution when analyzing epigenetic aberrations in imprinting disease iPSCs because the process of iPSC generation is associated with epigenetic dynamics that may bias interpretation.[13] However, iPSCs with *in vitro* multipotency have been an invaluable tool to clarify molecular mechanisms as a simulator of developmental defects.[14,15]

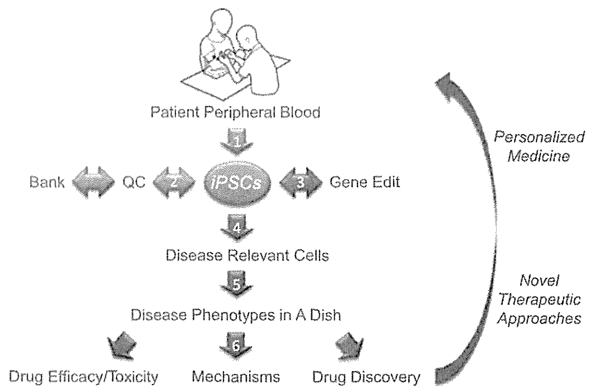

Figure 2 A typical work flow of patient iPSC research and tips for individual steps. (**1**) iPSC generation (~3 weeks)—multiple clones from multiple patients using non-integrating reprogramming vectors; (**2**) Quality control (QC) and storage (1–4 weeks)—first by morphology and pluripotency markers, then ideally by gene expression profiling, teratoma formation, karyotyping, exome analysis, and mycoplasma testing; (**3**) Isogenic controls made by gene editing serve as ideal controls; (**4**) Differentiation (2–10 weeks)—consult a local iPSC core or colleagues to identify the best available protocols; (**5**) Disease recapitulation—set realistic goals to demonstrate unique pathological changes *in vitro*; (**6**) Study further disease mechanisms—molecular 'omic' analyses are often used here. 'Green' highlighted parts are usually taken care by a local iPSC core facility (if desired), whereas 'blue' highlighted parts will typically be performed by individual investigators.

PRACTICAL ADVICE BEFORE YOU BEGIN

These exemplary cases certainly make us feel hopeful that we can apply patient iPSCs to various diseases. Taking all the progress and current issues into consideration, which we discuss more in detail in the following section, we have compiled practical tips you may find useful when starting patient iPSC research. First, it is essential to analyze whether the project is worth pursuing, as with any other new research projects. A SWOT analysis, for an example as shown in Figure 1, will guide you to identify the potential internal and external strengths and weaknesses of your direction. Unfortunately, the field is highly competitive, and the funding is scarce; thus it is critical to fully analyze the status of your project before beginning. In the end, the most important factor in the analysis is whether you have unique and significant question(s) that are likely answered using patient iPSCs.

When the analysis is positive, Figure 2 illustrates an actual workflow of the study with estimated time lines. Unless you have extensive experience in human pluripotent stem cell culture, it is easiest to consult with an iPSC core facility or colleagues to generate patient iPSCs. In a typical study of a monogenic disorder, generation of three iPSC clones from three individual patients is minimally required, along with an equivalent number of controls; however, such number can vary considerably depending on your questions. The quality of iPSC clones should also be controlled by the core facility to

Table 1 List of iPSC banks and registries by disease

Diseases	Institute	Website
General	Corriel Institute/NIGMS	http://ccr.coriell.org/Sections/Collections/NIGMS/ipsc_list.aspx?PgId=696
	American Type Cell Collection	http://www.atcc.org/Products/Cells_and_Microorganisms/Stem_Cells/Human_IPS_Pluripotent.aspx
	RIKEN Bioresource Center Cell Bank	http://www.brc.riken.jp/lab/cell/english/index_hps.shtml
	Wi-Cell	http://www.wicell.org/home/stem-cell-lines/order-stem-cell-lines/obtain-stem-cell-lines.cmsx
	Boston University, Center for Regenerative Medicine	http://www.bu.edu/dbin/stemcells/ips_cell_bank.php
	U MASS International Stem Cell Registry	http://www.umassmed.edu/iscr/Genetic-Disorders-Lines/
	U Connecticut Stem Cell Core	http://stemcellcore.uchc.edu/services/distribution.html
	Harvard Stem Cell Institute	http://stemcelldistribution.harvard.edu/shoppingCart/index
Neural	Corriel Institute/NINDS	http://ccr.coriell.org/Sections/Collections/NINDS/ipsc_list.aspx?PgId=711&coll=ND
Mental	NIMH Stem Cell Center	http://nimhstemcells.org/catalog.html

Currently available information of iPSC bank and registry (June 2014). Please note that the list here may not cover all the available sites.

meet the current standard, as discussed later. If patient iPSC clones or fibroblasts already exist in publicly available libraries, you can save substantial amount of time and cost. Table 1 shows a list of disease iPSC bank and registry, in which you may be able to find the lines of your interest. Additional information is available in a recent review article specifically discussing this topic.[16]

As we discuss later, iPSC differentiation should ideally be performed in collaboration between your lab and the iPSC core or a person who has iPSC expertise. In the steps of disease recapitulation and further mechanism studies, it is particularly important to set practical goals for patient iPSC research. First, you should accurately estimate purity, quantity and maturation status of the resultant iPSC-derived differentiated cells. Depending on those factors, you can identify what types of assays can be performed with the prepared cells. In general, patient iPSCs will hold the most value in identifying molecular changes caused by pathogenic mutations in certain human cell types. ‘Omic’ level screening will be particularly useful there; and isogenic iPSC clones with the mutation corrected through gene editing would serve as ideal controls in such tests, as discussed later.

TECHNICAL IMPROVEMENT AND REMAINING CHALLENGES

iPSC Generation

Viral methods

Methods for achieving reprogramming have progressed significantly from the groundbreaking work completed by Yamanaka and colleagues. The variety of reprogramming approaches stems from an interest to develop methods that do not integrate DNA into the host genome, making them feasible for eventual use in clinical applications. Virus-mediated reprogramming is commonly used for its capacity to efficiently transduce cells of interest. Original methods using retrovirus[2,17] and lentivirus[18] remain widely used. The disadvantage is that these viruses integrate transgenes randomly into the host genome upon infection. This has the potential to cause unpredictable changes in the genome and result in aberrant transgene expression, which can potentially impact data interpretation and differentiation potential. Although scientists have devised ways to remove the transgenes after reprogramming is complete (using loxP sites and Cre recombinase),[19] it is still necessary to thoroughly screen clones for confirmation of excision and loxP site retention that may alter endogenous gene expression. For these reasons, methods to reprogram cells have since focused on naturally non-integrating approaches.

Improvements using viruses that do not integrate into the genome, including adenovirus and Sendai virus, are becoming increasingly popular. The use of adenovirus was first applied to iPSC reprogramming shortly after the initial reprogramming reports.[20,21] Adenovirus was chosen for its inability to integrate into the genome and ability to provide high transgene expression for a limited amount of time as the virus is reduced with each cell division. Although successful, the incidence of tetraploid cells following reprogramming has limited its usefulness.[20]

Sendai virus has recently been developed for reprogramming because it is non-cytopathic and remains in the cytoplasm of host cells.[22] In addition, it has the ability to reprogram peripheral blood mononuclear cells (PBMCs) in addition to other somatic cells (including fibroblasts). In addition to the non-integrating nature of this virus, it is cleared by culturing cells at an elevated temperature, or treatment with siRNA against the large protein (L-gene) of the virus. Recently, a modification has also been introduced that enables clearance by microRNA 302L, naturally produced by pluripotent cells, which recognizes an inserted microRNA targeting sequence that was incorporated into the vector (Nakanishi, personal communication).

Non-viral methods

Non-viral methods include minicircle and episomal plasmids, piggyBac transposon, RNA transfection, protein transduction, and microRNA transfection. Traditional transfection was successfully used to reprogram mouse cells using polycistronic plasmids.[23,24] However, extensive screening was necessary to find clones without integration. In addition, repeated transfections were necessary to maintain high transgene expression. Minicircle DNA was first applied to reprogram adipose stem cells.[25] Polycistronic minicircle is advantageous because transfection efficiency is improved and it is diluted out more slowly during cell division, thus reducing the number of transfections required. Unfortunately, both conventional and minicircle DNA reprogram at much lower efficiency and also require more hands on time due to multiple transfections. Episomal plasmids can be stably introduced into cells using drug selection, and can be removed after drug selection is discontinued. Yu *et al*[26] first showed feasibility of this method in 2009 by reprogramming human foreskin fibroblasts, although unfortunately this method also yielded low efficiency. The piggyBac transposon system enables the removal of all exogenous elements, cleaner than the Cre-loxP system. In 2009, multiple groups demonstrated high efficiency reprogramming using tetracycline-inducible or polycistronic expression of the reprogramming factors.[27–29] Although removal of the transgenes was demonstrated by sequencing, transposase-mediated excision of transgenes was shown to also induce microdeletion of genomic DNA, which could pose a problem for future use.

Methods described thus far carry the risk of unexpected persistence or genetic modification. To circumvent this, scientists have been devising methods, which do not introduce DNA into the host cell. mRNA synthesized *in vitro* from cDNA of the reprogramming factors was demonstrated to be successful in 2010.[30] This method utilized host cells translation machinery, although it requires five consecutive transfections to be successful. Protein delivery is an alternative to nucleic acid introduction. Harnessing the ability of reprogramming factors tagged with C-terminus polyarginine domains to transduce through the cell membrane, two groups showed feasibility.[31,32] Protein delivery method eliminates the need to screen for integration of transgenes. However, efficiency was lower, and multiple rounds of transduction were necessary. In 2011, mature double-stranded microRNA including mir-200c, mir-302s and mir369s family of microRNAs were shown to reprogram somatic cells by direct transfection.[33] Although this method resulted in lower efficiency, it provides a viable method compatible with clinical use.

Ultimately, these methods and modifications have laid the groundwork for improving methodology. Combination of these methods with small molecules has been shown to improve reprogramming. In 2013, Deng's group used a cocktail of seven compounds to reprogram mouse somatic cells into iPSCs at efficiency comparable to standard reprogramming techniques.[34] The ability to apply this technique to human cells would be an exciting advance in the field. Although many methods focus on efficiency, it is important to note that efficiency alone is not the most important aspect of the reprogramming process. In the end, it is more important to obtain a number of high quality iPSCs clones. Generally, fewer than 10 clones per individual are needed, especially if using a non-integrating method where exhaustive transgene screening is not necessary.

Practical considerations

Starting cell type before reprogramming is an important consideration. Dermal fibroblasts and PBMCs are the most common starting cells, and while most methods nearly always reprogram dermal fibroblasts successfully, using a method that also works for PBMCs increases flexibility. Benefits include reduced processing time (biopsy outgrowth can require up to 1 month *vs* isolation of PBMCs from a blood draw can be completed within an hour). In addition, a blood draw is less invasive and particularly useful for obtaining cells from pediatric patients. Ultimately, starting cell type may vary depending on the questions to be asked. If initial assays using fibroblasts can be useful for disease understanding, it may be advantageous to reprogram those cells. Regardless of delivery method (virus, plasmid and so on), utilizing polycistronic plasmids to introduce all reprogramming factors at once is easier and increases the likelihood of successfully reprogramming.

Commercial availability of multiple reprogramming methods is also increasing. Although cost may be an issue, it is possible to send samples to be reprogrammed using various non-integrating methods or to purchase ready to use reagents to complete the procedure in the lab. In addition, it is important to realize the reprogramming process itself is not the only barrier to overcome. It is imperative to learn proper culture techniques. To this end, many commercially available cell culture media are available (Life Technologies, ReproCell, Stem Cell Technologies and so on) that can ease the transition for researchers who are new to the culture techniques required to propagate these cells. Even for seasoned scientists, commercial protocols and products enable quick improvements and it is advantageous to keep up to date to reduce labor and improve quality of iPSC culture.

In addition to various culture media, there are also a number of different substrate iPSCs can be cultured on (mouse embryonic fibroblasts, Matrigel, Vitronectin, Geltrex and so on). In addition, iPSCs themselves are generally an intermediate resource before differentiation to various lineages. As such, the vast variety of differentiation protocols generally has different starting cell culture conditions. Usually, these are referred to as feeder dependent or feeder free. For this reason, it may be advantageous to generate frozen stocks cultured by feeder-dependent and feeder-free techniques to reduce the labor involved if testing out a number of protocols.

Quality Control

Variability within iPSC clones (either genetic, epigenetic or phenotypic) has been a concern in patient iPSC research. Unless each iPSC clone is carefully evaluated, researchers could potentially run into issues with data misinterpretation when using this approach.

Quest for genome stability

To investigate characteristics of iPSCs derived from monogenic disorders, one of the important issues is to validate retention of the gene mutation in iPSCs and to identify additional mutations introduced during iPSC generation. By comparing genomes of parental cells and iPSCs, exome analysis may be a prerequisite for subsequent medical research of pathogenesis and drug discovery. Whole-exome analysis covering protein-coding sequences is sufficient to investigate pre-existing and additional mutations, although the recent platform of exome analysis has expanded to include not only coding but also untranslated, non-coding RNA and their adjacent regions. The number of single-nucleotide mutations per cell genome was estimated from 22 human iPSCs by extensive exome analysis on protein-coding sequences.[35] Generally, iPSCs are considered to have a comparable nucleotide substitution rate independent of donor cells, except for cells from patients with a genome instability syndrome, a DNA repair disorder or a DNA damage response syndrome. However, acquisition of novel mutations during passages is indeed unavoidable, and banking of early passage iPSC clones is therefore essential once suitable disease iPSCs are established and characterized.

Quest for quality control

In addition to genomic analysis, general characteristics of disease iPSCs such as morphological analysis, *in vitro* differentiation by embryoid body formation, teratoma formation by injection of iPSCs into immunodeficient animals, karyotypic analysis, short tandem repeat analysis, pluripotency markers such as Oct4/3, Sox2, Nanog, SSEA4, Tra-1-60 and Tra-1-81, and gene expression of exogenous and endogenous pluripotency-associated genes are usually performed. Before banking, contamination of mycoplasma, bacteria, virus and endotoxins should ideally be tested. Generally, morphology of iPSCs provides us enormous information including purity, quality, transformation, undifferentiated state and other cell contamination. In addition to these standard quality controls, profiling of RNA expression, DNA methylation and glycans can be added for monitoring when necessary.[10,13,36,37] These comprehensive analyses would also elucidate pathogenic states such as aberrant genomic methylation and gene expression of patient iPSCs.

Quest for suitable controls of disease iPSCs

In addition to disease-derived iPSCs, preparation of suitable control iPSCs are required for elucidation of disease mechanisms and drug discovery. One of the ideal controls is genetically corrected iPSCs. To correct gene mutation in disease iPSCs, ZFN, TALEN and CRISPR/Cas-based methods for genome editing can be used. Alternatively, introduction of exogenous genes that are mutated in disease iPSCs may be used, but the expression level of the exogenous gene may bias phenotypes. Another control is iPSCs obtained from the same age, gender and ethnic group. Usually, iPSCs from more than three independent patients and from more than three independent healthy donors need to be analyzed to conclude that observed pathogenic phenotypes are due to endogenous genotypes of the disease iPSCs. However, genetic correction and preparation of age-, gender- and ethnic-matched controls is labor intensive. To circumvent this, commonly available iPSCs from healthy donors may be used for comparison. MRC5-derived (fetal lung fibroblast) iPSCs have been utilized as a control in several previous reports,[13,37–41] and can be obtained from the public bank. If MRC5-iPSCs do not demonstrate pathogenic phenotypes that disease iPSCs do under the same experimental condition, MRC5-iPSCs would serve as a practical control.

Differentiation

Lack of practical differentiation protocols

Depending on the desired disease or field of study, there may be ample protocols for investigators to turn to (as in the case of neurodegenerative disease modeling).[3,5] However, unless the particular lab is well versed in the biology of both pluripotent stem cells and differentiated cell types, the likelihood of reproducing a protocol in a reasonable time is uncertain. In general, differentiation protocols take advantage of particular cytokines, culture media and extracellular matrices, thus making these protocols quite expensive. Often, after differentiation, cell populations of interest need to be separated using specific surface markers to achieve sufficient purity. In addition to the expense, most protocols are time consuming and slow in data collection. In general, common obstacles in published differentiation protocols include low reproducibility, low yield, high cost and multiple steps, which often utilize complicated procedures. Thus, except for a few relatively straightforward lineages such as neural progenitors, we are still lacking very practical protocols to prepare a large number of disease-relevant cell types. Developing simple, easy and affordable methods, where the process can be applied to robust large-scale cell differentiation from patient iPSCs, is truly desired in the field.

Uncertain quality of differentiated cells
Depending on the cell type, iPSC differentiated cells may not proliferate well in the long term. As with human primary cells, doubling times while maintaining proper phenotype will most likely be limited, making it more difficult to carry out desired experiments. Furthermore, the possibility of freezing a batch of cells for later use may be unrealistic, giving investigators a 'one shot' per differentiation scenario to obtain meaningful data. This can become taxing if a differentiation protocol takes months from start to finish as in the case of vascular cell differentiation with a 2-month long protocol.[42] Also, unless the differentiation protocol is well established in an investigator's own hands, a portion of the obtained cells will need to be used to assess the proper phenotype. Despite a successful differentiation protocol, investigators may run into issues if these cells are to be used in functional assays. iPSC-derived cells may have the proper phenotype but may be too immature to also possess the normal function of the cells. In that case, investigators will have to optimize such conditions for their specific interests keeping in mind the physiological relevance of their *in vitro* assays.

Practical considerations
Although there remain many issues to be improved, some iPSC differentiation protocols are relatively straightforward, and have been successfully used by multiple groups to obtain mesoderm,[42–44] endoderm[45] and ectodermal[46–48] lineages. These protocols utilize available materials, the procedures are uncomplicated, the methods include simple cell purification steps such as sorting, and their reproducibility and usefulness have been demonstrated by other investigators. There are many additional protocols available in the literatures (many that share commonalities, while others are distinct). As the field is constantly changing, updated information is best obtained through an iPSC core facility or colleague scientists. We emphasize here again that one should try to reproduce the protocol(s) in a side-by-side collaboration with a scientist who has expertise in iPSCs and another scientist with experience of the targeted differentiating lineage. Knowing the biology of both ends, the cells you start with and those you end up with, is critical to reproducing protocols in a reasonable time.

Disease Modeling

How to fill the discrepancies from real disease

Although generation of disease-relevant cell types from patient-derived iPSC is a standard strategy for studying a 'disease in a dish' as described above, many human diseases arise from multicellular interactions in the context of tissue architecture, organ or whole-body homeostasis. Therefore, it is essential to further advance model systems to represent a more complex physiological environment similar to the body. When your hypothesis requires the interactions of different cell types for pathogenesis, multiple cell types in a co-culture setting will certainly provide further functional insights for the disease. As an exemplary work, the co-culture of glial cells from ALS iPSCs with neurons from normal iPSCs demonstrated the non-cell autonomous effect of diseased glial cells for aberrant survival of neurons.[49] Similarly, aberrant controls in vasculature tone would be better understood when co-culturing endothelial and vascular smooth muscle cells together rather than using a single cell type.

Admirably, iPSCs possess pluripotency comparable to embryonic stem cells (ESCs), which are originated from the embryonic blastocyst stage embryo. Both iPSCs and ESCs are competent to early developmental cues. Once proper cues are given, initial specification occurs to induce differentiation. The multiple types of differentiated cells are autonomously organized and interact with each other leading to subsequent fate specification like the cascade of embryonic development. To take maximum advantage of this self-organizing ability of pluripotent stem cells, several groups have developed sophisticated 3D culture protocols for making organoid structures *in vitro*. One example is the so-called 'mini-brain' consisting of tissue layers that mimic the brain cortex. Using this culture technique, Knoblich's group demonstrated that iPSCs derived from a microcephalic patient indeed formed a smaller brain than iPSCs from a healthy control.[50] Similarly, several organoid culture techniques for iPSCs have evolved to generate other tissue types and organs (optic cup, pituitary gland).[51,52] Lack of vascular supply is the major limiting factor to grow more functional units in organoid culture. Remarkably, Taniguchi's group was able to generate a transplantable small liver unit from human iPSCs. They co-cultured hepatic endoderm cells differentiated from iPSC with human mesenchymal stem cells and human umbilical vein endothelial cells in a loosely solidified extracellular matrix. These cells autonomously formed the functional units of the liver *in vitro* with the support of microvasculature. Upon transplantation of the unit into immunodeficient mice, the liver bud quickly connected to the host vascular networks and further functional maturation occurred.[53]

Advances in differentiation protocols heavily rely on our knowledge of the molecular mechanisms of embryonic development. Our knowledge is not sufficient to provide the optimal environment for desired morphogenesis from iPSC *in vitro* culture. Nevertheless, simple inoculation of iPSCs into immunodeficient animals is able to form teratomas, which comprise cells from all three germ layers (endoderm, mesoderm and ectoderm). As mature tissue organization (gut epithelial, cartridge and so on) can be observed in the tumor, it will be feasible to assess the histopathological phenotype of patient-derived iPSC using this methodology. For instance, iPSCs from dominant genetic disorders with oncogenesis may develop cancer in teratomas over time. Patients with familial adenomatous polyposis develop adenoma and adenocarcinoma in colon. Similarly, iPSCs from familial adenomatous polyposis may generate adenoma and adenocarcinoma in colon-like mucosa in teratomas. iPSCs from degenerative disorders may exhibit degeneration or apoptosis of cells in corresponding tissues of teratomas. It is also noteworthy that histopathological analysis of implanted cells into immunodeficient animals may support *in vitro* phenotypes of iPSCs during the differentiation process.

To model systemic disease, it is compelling to reconstitute the human pathological process in experimental animals. For example, type I diabetes is recognized as a type of autoimmune disease, in which three major cell lineages (hematopoietic cells, pancreatic β cells and thymus epithelial cells) have important roles. Melton's group has reconstituted the human version of these three lineages into animals by transplantation into immunodeficient mice.[54] A more rigorous approach is led by Nakauchi's group, where they successfully generated a whole kidney or pancreas derived from iPSCs in the pig by blastocyst complementation. They transferred donor pig iPSC into pancreatogenesis- or nephrogenesis-disabled blastocyst stage pig embryos, and demonstrated the embryos were born as chimeras having pancreas or kidney exclusively derived from the donor pig iPSCs.[55] Any blastocyst complementation using human iPSC into animals has not been performed yet because of ethical issues, but theoretically it is feasible to generate whole functional human organs in animals using the same strategy. This humanized animal or hybrid animal approach using patient-derived iPSC would be a next-generation disease model for studying human pathology.

Gene Editing

Rapidly evolving gene-editing technology has been shown valuable in patient iPSC research as well, as described above with an exemplary case.

TALEN

Transcription activator-like effector nucleases (TALENs) are composed of a DNA-binding domain that is capable of directing the *Fok*I nuclease to a specific target site. Two TALENs, recognizing left and right arms of the target site, respectively, can bring two *Fok*I monomers close together for the formation of a functional dimer, which generates a DNA double-strand break (DSB) on the target site.[56,57] The TALEN-induced DSBs activate the DNA repair system within cells, which stimulates non-homologous end joining (NHEJ) in the absence of a homologous DNA template. The error-prone nature of this repair mechanism results in the introduction of nucleotide mismatches, insertions or deletions. However, in the presence of a homologous template DNA, the DSB triggers homologous recombination, introducing desired DNA sequence alterations. The TALENs have rapidly gained prominence as a novel genome-editing tool, which were successfully applied to create site-specific gene modifications in model organisms such as yeast, plants, zebra fish, mouse, rat and human cells, including human pluripotent cells.[58–62] TALEN has also been used to generate single base-pair mutations, linking single-nucleotide polymorphisms to specific human disease.[63] Furthermore, TALENs have even been utilized to eliminate the mutant form of mitochondrial DNA from patient-derived cells.[64] Currently, TALEN plasmids targeting 18 740 protein-coding human genes have been assembled using a high-throughput Golden-Gate cloning system.[65] Delivery of these TALENs can be achieved by injection of DNA or mRNA encoding TALENs or even the TALEN proteins directly.[62,66,67]

CRISPR

The CRISPR system is another effective genome-editing tool, which utilizes Cas9 nuclease to cleave DNA and chimeric guide RNA (gRNA) to target Cas9 to a specific region in the genome.[68,69] The Cas9-gRNA-mediated genome editing has been shown to have improved efficiencies over TALENs and it is also easier to implement.[68–72] Moreover, it allows simultaneous editing of more than one site through expression of multiple gRNAs.[68,69] This approach was used to create mice carrying five different mutant genes in a single step,[73] and also was shown to generate large deletions of genomic regions by directing Cas9 cleavages at the two sites flanking the desired deletion.[68] Wu *et al*[74] have even shown in mice that a dominant mutation in *Crygc* gene that causes cataracts could be rescued by a Cas9-mediated DSB on the mutant allele, which triggered homology-directed repair based on the endogenous WT allele. More recently, a clone library encoding short gRNAs targeting all open reading frames in the human genome has been generated. Combined use of this library with Cas9 enabled the generation of random gene knockouts in the human genome, which can be screened for desired phenotypes to link genes to their functions.[75,76] The CRISPR technology has been used to cure a mouse model of a human fatal liver disorder (type I tyrosinemia) caused by a single genetic mutation in the fumarylacetoacetate hydrolase gene.[77] This defect in tyrosine catabolism causes toxic accumulation of the amino acid, leading to liver failure. CRISPR-mediated genome editing could one day help treat many diseases caused by single mutations, such as hemophilia and Huntington's disease.

A mutant version of the Cas9 was further reported which cleaves only one strand of the target DNA, generating single-strand nicking, thus favors HR DNA repair over NHEJ (error prone), increasing desired DNA changes over random mutations.[70] Recently, a nuclease-defective Cas9 enzyme has been utilized to label genomic loci, allowing for visualization of *in vivo* of their partitioning in live cells.[78] Most interestingly, the catalytically inactive Cas9 nuclease, in complex with a gRNA, can bind to a specific site, which physically blocks the RNA polymerase, thus silencing the target gene.[79] Similarly, the catalytically inactive Cas9 was fused to known transcriptional activator domains and targeted to specific promoter regions by corresponding gRNAs, upregulating the target gene expression.[80] The ability to artificially control the expression of specific target genes not only enables us to better understand gene functions but also to manipulate cell fate through controlled expression of desired sets of pathway genes.

CONCLUDING REMARKS

Undoubtedly, patient iPSCs are an enduring asset for experimental pathology studies, with some exemplary applications introduced above and many more in published literature. Additional technical improvement, particularly in iPSC differentiation methods and three dimensional cultures, as well as expansion of patient iPSC banking, will further accelerate the field. From a pathologist perspective, patient iPSC banking will serve as a powerful addendum to existing tissue banks. Their value is unlimited, as once established, they serve as an enduring and expandable resource for live patient cells. For instance, it is almost impossible to obtain hepatocytes from a rare metabolic disease through liver biopsy of a large number of patients at one given time and place. However, through iPSC banking, such resources will be available to any researcher, any place in the world, and at any time. Banking iPSCs of large patient cohorts with a clinical and GWAS database would be particularly useful in order to identify molecular mechanisms underlying certain genetic links to the disease or individual patients' drug efficacy and toxicity. The future rests on how properly we prepare the resource and how wisely we use it.

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DISCLOSURE/CONFLICT OF INTEREST

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Ataxia telangiectasia derived iPS cells show preserved x-ray sensitivity and decreased chromosomal instability

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Ataxia telangiectasia is a neurodegenerative inherited disease with chromosomal instability and hypersensitivity to ionizing radiation. iPS cells lacking ATM (AT-iPS cells) exhibited hypersensitivity to X-ray irradiation, one of the characteristics of the disease. While parental ataxia telangiectasia cells exhibited significant chromosomal abnormalities, AT-iPS cells did not show any chromosomal instability *in vitro* for at least 80 passages (560 days). Whole exome analysis also showed a comparable nucleotide substitution rate in AT-iPS cells. Taken together, these data show that ATM is involved in protection from irradiation-induced cell death.

The technology to generate human induced pluripotent stem cells (iPS cells) has impacted various medical fields, such as clinical applications and drug discovery, as well as basic biological science on reprogramming of differentiated cells[1,2]. The most recent attention has been placed on their potential use in cell-based transplantation. Using *in vitro* differentiation, iPS cells, like embryonic stem cells (ES cells), can provide an unlimited source of useful cell types for transplantation. The use of iPS cells in clinical application and research has been largely welcomed by society because use of these cells avoids the substantial ethical concern of cellular origin that plagues ES cells. The fact that the cells are autologous for patients could be another advantage in transplantation. Soon after human iPS cell technology was introduced, researchers also began to realize an additional and possibly greater value for the technology as a system to model human diseases[3]. Since iPS cells can be generated from skin biopsies or blood samples, retain all the genomic information from the original patients, and can be differentiated *in vitro* into cell types which are not easily accessible in patients, iPS cells can be utilized to study how genetic aberrancies in the patient manifest in target cells *in vitro*.

Ataxia telangiectasia (AT) is a rare neurodegenerative inherited disease characterized by early-onset progressive cerebellar ataxia, telangiectasias of the eyes and skin, immunodeficiency, chromosomal instability, hypersensitivity to ionizing radiation, and increased risk of cancer[4]. AT is caused by a defect in the *ATM* gene, which is responsible for recognizing and correcting DNA damage, and for destroying the cells when the errors cannot be corrected. One feature of ATM protein is its rapid increase in kinase activity immediately after double-strand DNA break formation[5]. The phenotypic manifestation of AT is due to the broad range of phosphorylation of substrates for the ATM kinase, involving DNA repair, apoptosis, G_1/S, intra-S checkpoint and G_2/M checkpoints, gene regulation, translation initiation, and telomere maintenance[6]. Therefore, a defect in *ATM* has severe consequences, and may lead to tumor formation. For example, the increased risk for breast cancer in AT patients implicates the involvement of ATM in the interaction and phosphorylation of BRCA1 and its associated proteins following DNA damage[7].

Though the molecular basis of AT, such as a defect in the *ATM* gene and the effect that has on the broad range of substrates for the ATM kinase has been well established, the linkage between the loss of ATM function and various clinical outcomes remain still unclear. *Atm*-deficient mice have been created to recapitulate the human disease and then characterized to understand the relationship between the AT phenotype and the pleiotropic function of *Atm*[8]. Mice homozygous for *Atm* disruption show growth retardation, neurologic dysfunction, immunologic abnormalities, lymphoreticular malignancies, chromosomal instability, and extreme sensitivity to ionizing radiation. However, oculocutaneous telangiectasias and remarkable histological evidence of neuronal degeneration, which are characteristics of human AT patients, have not been seen in these mice. The mouse model for AT is, therefore, very useful, but limited for understanding the human disease.

Mouse iPS cells from tail-tip fibroblasts of *Atm*-deficient mice have been reported[9,10]. Reprogramming efficiency is greatly reduced in the fibroblasts of *Atm*-deficient mice. Likewise, *ATM*-deficient human pluripotent stem cells, i.e. ES cells and iPS cells, have successfully been established by disrupting *ATM* gene[11] and from patients with ataxia telangiectasia[12,13], respectively. These pluripotent stem cells exhibit disease-specific characteristics such as radiosensitivity and cell cycle checkpoint defects, and therefore serve disease model cells for clarification of pathogenic mechanism and screening novel compounds to treat the disease. The AT-iPS cell platform was indeed used to screen low-molecular compounds[12].

In the present study, we attempted to generate iPS cells from fibroblasts of ataxia telangiectasia (AT-iPS cells), and successfully established the cells from the fibroblasts of AT patients. The reprogramming efficiency was very low as previously reported in the establishment of murine ATM-KO iPS cells. Human AT-iPS cells exhibited hypersensitivity to X-ray irradiation. Unexpectedly, the human AT-iPS cells did not show any chromosomal instability *in vitro*, i.e. maintenance of intact chromosomes lasted for at least 80 passages (560 days). These results indicate that the established human AT-iPS cells may be useful for the exploration of the mechanism of reprogramming, for clarifying the pathogenesis of AT, and for facilitating novel therapeutic interventions of the human disease. The possible mechanisms for the low reprogramming efficiency and for stable maintenance of their chromosome in the AT-iPS cells will also be discussed.

Results

Generation of iPS cells from human AT cells. It has been reported that fibroblasts from *Atm*-deficient mice show remarkably low reprogramming efficiency compared to normal fibroblasts. Thus, we examined whether iPS cells could be efficiently generated from human cells having a mutation in the *ATM* gene (AT1OS cells, Figure 1A) by using the vesicular stomatitis virus G glycoprotein (VSV-G) retroviral transduction system (Figure 1B). By using this system, the transduction efficiency was 53.8% ± 11.9% (mean ± standard deviation) as estimated by enhanced green fluorescent protein (EGFP) expression (Figure 1C). Southern blot analysis with cDNA probes for each of four transgenes (*OCT-3/4, SOX2, KLF-4*, and *c-MYC*) confirmed that each clone had chromosomal integration of the exogenously infected genes (Supplemental Figure S1). When the reprogramming factors OCT3/4, SOX2, KLF4 and c-MYC were introduced in 2.0×10^5 AT1OS cells, only 10 iPS colonies were successfully generated. We compared the reprogramming efficiency of AT-iPS cells with that of MRC5-iPS cells that were generated by the same VSV-G retrovirus construct and protocol. The efficiency of AT-iPS cell colony generation (0.005%) was approximately 1/100, compared with that of MRC5-iPS cell generation (0.5%). Morphological characteristics of AT-iPS cells were similar to those of other intact iPS cells and ES cells (Figure 1D). Immunohistochemical analyses demonstrated that expression of the pluripotent cell-specific nuclear proteins, OCT3/4, SOX2 and NANOG, and the keratan sulfate proteoglycan TRA-1-60 (Figure 1E, Supplemental Figure S2) was consistent with the profile observed in hES cells. Hierarchical clustering analysis and principle component analysis of gene chip analysis data revealed that AT-iPS cells were grouped into the same category as MRC5-iPS cells, but not grouped into the ES cell category and parental cell category, regardless of gene set: all genes, neural genes, DNA-damage genes, and cell cycle-related genes (Supplemental Figure S3).

Teratoma formation of AT-iPS cells. To address whether the AT-iPS cells have competence to differentiate into specific tissues, teratoma formation was performed by implantation of AT-iPS cells at the subcutaneous tissue (1.0×10^7 cells/site) of immunodeficient NOD/SCID mice. Four independent AT-iPS cell clones induced teratomas within 6–10 weeks after implantation. Histological analysis of paraffin-embedded sections demonstrated that the three primary germ layers were generated as shown by the presence of ectodermal glia and neuroepithelium, mesodermal muscle and cartilage, endodermal ciliated epithelium morphologically in the teratoma (Figure 1F). Thus, all AT-iPS cell clones examined had potential for multi-lineage differentiation *in vivo*.

Characterization of AT-iPS cells. We examined the expression of the mutated *ATM* gene in AT-iPS cells by RT-PCR for amplifying the sequence including exon 31 of the *ATM* gene to confirm that the established cells were AT-derived (Figure 2A, Supplemental Table S1). AT-iPS cells clearly retained expression of the mutated *ATM* gene that had a deletion of 165 bp corresponding to the deletion of exon 31, showing that these cell clones were actually AT1OS derived. We also performed protein blot analysis on AT-iPS and MRC5-iPS cells (Supplemental Figure S4). ATM was detected at the protein level in MRC5-iPS cells, but not in AT-iPS cells (Supplemental Figure S4A, B). p53 was expressed at a similar level in AT-iPS and MRC5-iPS cells, and phosphorylation of p53 on serine-15 was similar in AT-iPS and MRC5-iPS cells (Supplemental Figure S4C, D).

The proliferative capacity of four AT-iPS cell clones was measured and compared with that of three MRC5-iPS cell clones (Figure 2B). No significant differences in proliferation rates were detected between the AT-iPS cell clones and the MRC5-iPS cell clones. Continuous observation through 18 passages revealed that AT-iPS cells continued to expand at a rate similar to MRC5-iPS cells, and could be cultured for more than 20 passages. Neither cessation of cell proliferation like senescence nor apoptosis/cell death was detected during cultivation through 20 passages.

Stem cell-associated gene expression in AT-iPS cells. The expression profiles of stem cell-associated genes were examined with qualitative RT-PCR to confirm the iPS cell characteristics of the established cell clones. The expression of the endogenous reprogramming factor genes (*KLF4, SOX2, OCT3/4*, and *c-MYC*) were undetectable or very low in the parental AT1OS cells, but were all activated in AT-iPS cells (Figure 2C to F). While the transgenes were fully silenced in AT-iPS cells (Figure 2G), expression of pluripotent cell-specific genes, such as *DNMT3B, NANOG*, and *TERT*, were activated in all AT-iPS cell clones to a similar extent of those in control hES cells and MRC5-iPS cells (Figure 2H to J, Supplemental Figure S5).

Karyotypic analysis of AT-iPS cells during cultivation. AT is a chromosome instability syndrome. The patients' cells frequently show chromosomal aberrations such as spontaneous chromatid/chromosome breaks, triradials, quadriradials and telomeric associations as well as numerical anomalies. In general, fibroblastic cell lines derived from AT patients accumulate chromosomal aberrations with an increase in passage number. Therefore, we performed karyotypic analyses of the AT1OS parental cells and AT-iPS cell clones at various passages (until 41 passages for more than 10 months). Parental AT1OS cells frequently exhibited chromosomal abnormalities, such as deletion, addition and translocation (Figure 3A).

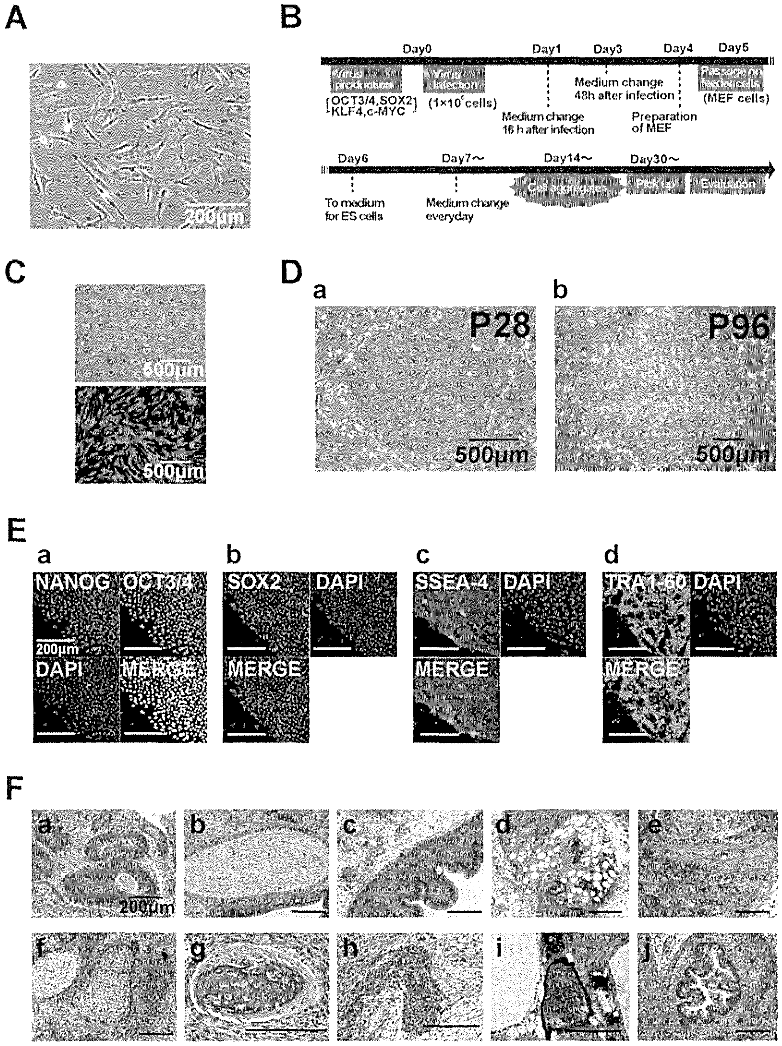

Figure 1 | Generation of iPS cells from ataxia telangiectasia-derived cells. (A). Phase-contrast image of AT1OS ataxia telangiectasia-derived cells. (B). Protocol for iPS cell generation. (C). Infection efficiency as assessed by retrovirus carrying the EGFP gene. (D). Phase-contrast microphotographs of AT-iPS cell clones at passages 28 and 96. (E). Immunocytochemical analysis of AT-iPS cells using antibodies to NANOG (a), OCT3/4 (a), SOX2 (b), SSEA-4 (c), and TRA-1-60 (d). (F). Histology of teratoma generated by AT-iPS cells. (a): ectodermal glia and neuroepithelium, (b): cartilage, (c): intestinal epithelium, (d): adipose tissue, (e): smooth muscle, (f): epidermis, (g): bone, (h): hepatocytes, (i): retina, (j): intestine.

In contrast, most cells of the four AT-iPS cell clones had an intact karyotype at passage 13 to 16 (Figure 3B–D). Even after a long cultivation period (passage 41), karyotypes of all cells of the four AT-iPS cell clones remained intact. Morphological characteristics of AT-iPS cell colonies, i.e. the growth of flat and aggregated colonies, did not significantly change even at passage 96 (Figure 1D). Also, AT-iPS cells retained high alkaline phosphatase activity and teratoma formation after a long-term cultivation (Supplemental Figure S6).

Elongated telomere length in AT-iPS cells. Telomere lengths in AT1OS cells and AT-iPS cells were measured (Table 1). TIG-1 at 34 population doublings served as a telomere length standard (6.91 kbp). The established ATiPS-262, -264, and -024 cells had 13.14, 15.64, and 16.54 kbp in telomere length, while the parental AT1OS cells were 4.13 kbp. The results clearly show that AT-iPS cells gain elongated telomeres after iPS cell generation.

Genomic alteration during AT-iPS cell cultivation. Because AT1OS cells exhibited considerable chromosomal abnormalities *in vitro*, we performed a structural alteration analysis using a SNP genotyping array for AT-iPS cells in ATiPS-262 cells at passage 17, ATiPS-263 cells at passage 27, ATiPS-264 cells at passage 25, and ATiPS-024 cells at passage 25. Compared to parental AT1OS cells, we identified 12 unique structural alterations (Figure 4A, B). Among these genomic alterations, no common chromosomal region was detected in the AT-iPS cells. We also performed exome analysis on the AT-iPS cells to clarify the number of genetic alterations that occur when cells are induced to become pluripotent. The number of bases that our sequencer produced were 18.0, 17.2, 17.4, 17.8 and 18.1 Gb, and mean mapped depths of coverage were 91.7, 89.7, 88.0, 83.3, and 90.0 reads for ATiPS-262, ATiPS-263, ATiPS-264, and ATiPS-024 cells, respectively. In total, 212 SNVs were called. A 23,314-kb copy-neutral loss of heterozygosity (CNLOH) in ATiPS-262 cells, a 3,586-kb deletion in ATiPS-262 cells, and a 234-kb

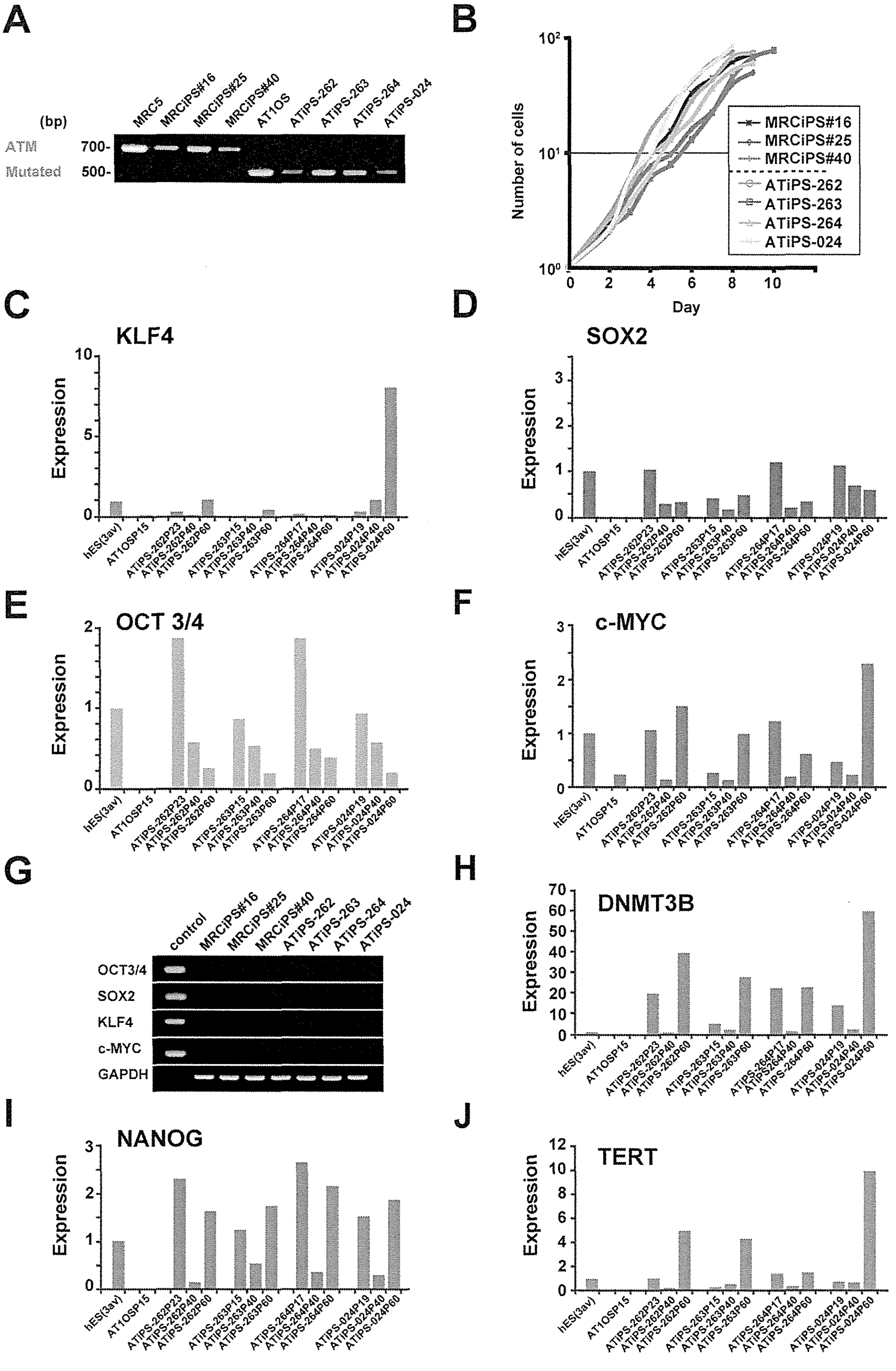

Figure 2 | Expression of the endogenous genes and the transgenes. (A). Expression of intact and mutated *ATM* gene in MRC5-iPS and AT-iPS cells. (B). Growth curves of AT-iPS and MRC5-iPS cells. Cell number was counted on the indicated day after cells (10^5 cells/dish) were seeded on matrigel-coated 6-well plates. (C). Expression of the endogenous *KLF4* gene. (D). Expression of the endogenous *SOX2* gene. (E). Expression of the endogenous *OCT-3/4* gene. (F). Expression of the endogenous *c-MYC* gene. (G). Expression of the *OCT-3/4, SOX2, KLF4,* and *c-MYC* transgenes in each iPS cell at passage 10 (more than 30 population doublings). (H). Expression of the *DNMT-3B* gene. (I). Expression of the *NANOG* gene. (J). Expression of the *TERT* gene.