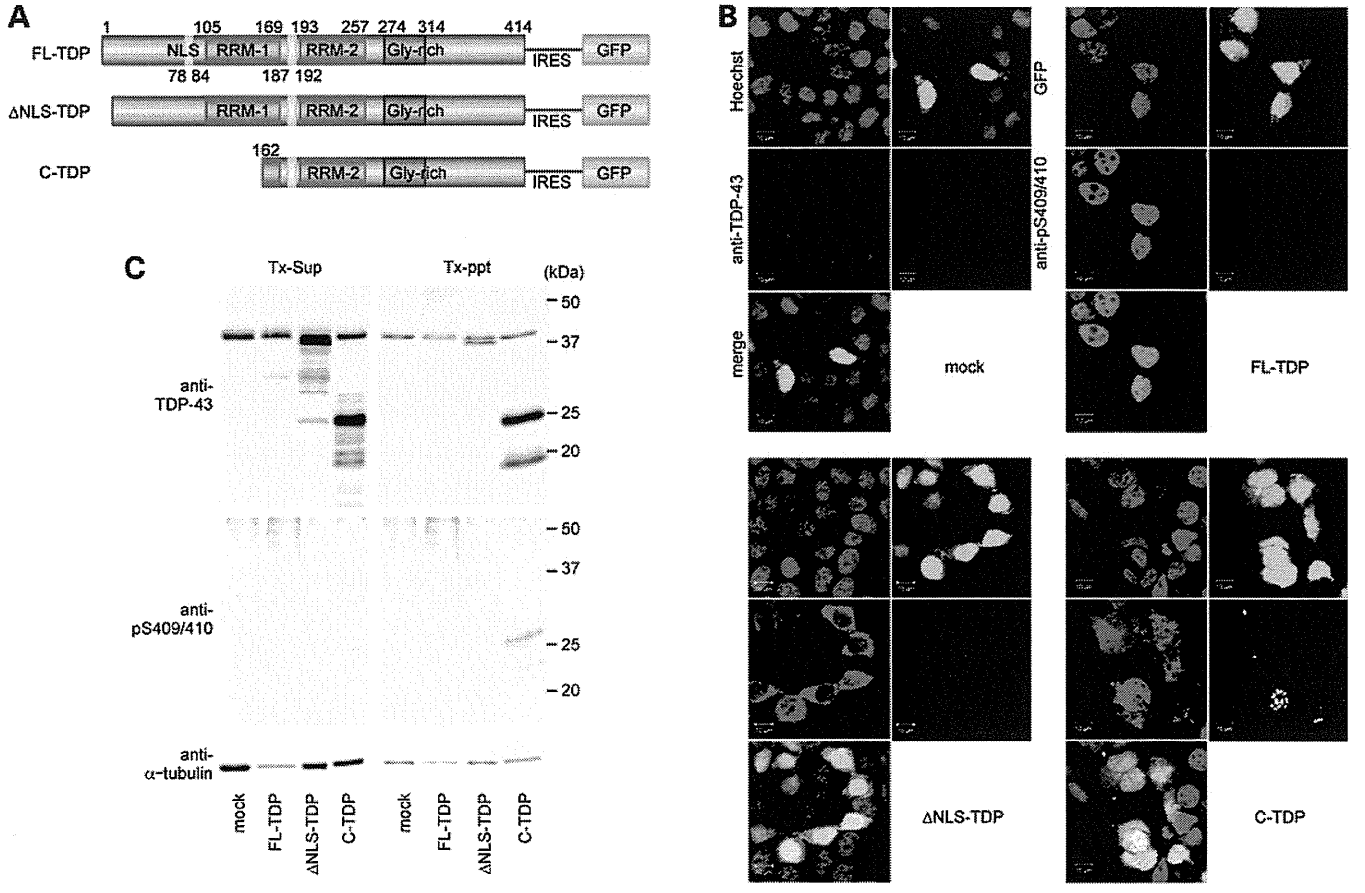

Figure 1. Overexpression of TDP-43 constructs in SH-SY5Y cells. (**A**) Schematic representation of FL-TDP, TDP-43 with deletion of the nuclear localization signal [78–84 residues (23)] (ΔNLS-TDP) and CTF (C-TDP) constructs in lentiviral expression system. RNA recognition motifs (RRM-1 and RRM-2: blue) and glycine-rich domain (Gly-rich: red) are shown. (**B**) SH-SY5Y cells were infected with each TDP-43 virus at 2×10^7 copies/well. At 4 days after infection, the cells were fixed and stained with anti-TDP-43 and anti-phospho-TDP-43 (anti-pS409/410) antibodies. Scale bars, 10 μm. (**C**) Transfected cells were also prepared for western blotting (20, 22). Cell lysates were extracted with 1% Triton X-100 (Tx), and the supernatants (Tx-sup) and insoluble pellets (Tx-ppt) after centrifugation at 100 000*g* for 20 min were analyzed by immunoblotting. The blots were probed with anti-TDP-43, anti-pS409/410 or anti-tubulin antibody.

result obtained in the case of lentiviral expression of FL-TDP (Fig. 2D). On the other hand, the cell cycle distribution of cells expressing GFP-C-TDP was not changed when compared with that of cells transfected with pEGFP empty vector, indicating that apoptosis is not induced in cells with formation of GFP-C-TDP aggregates. Taking these results together, it appears that overexpression of GFP-FL-TDP caused mild suppression of DNA synthesis and striking induction of apoptosis, whereas significant suppression of DNA synthesis and no abnormalities in the cell cycle were seen in cells including GFP-C-TDP aggregates. These results clearly indicate that cell death is induced via distinct molecular pathways upon overexpression of different TDP-43 species.

RNA pol II and some transcription factors are co-localized with TDP-43 inclusions and their activities are suppressed

It has been reported that transcriptional dysregulation is one of the central pathogenic mechanisms in Alzheimer's disease, Parkinson's disease and Huntington's disease (24). A number of transcriptional regulators, such as Sp1 and CREB, interact with protein aggregates, resulting in functional disruption of these transcriptional factors in brain, followed by neurodegeneration (25–27). Therefore, to check whether these transcriptional factors are also associated with aggregates of TDP-43 CTF, we examined the localization of these factors as well as RNA pol II, which is a major enzyme responsible for transcription of protein-encoding genes. SH-SY5Y cells were transfected with GFP-C-TDP and incubated for 3 days, followed by immunohistochemical analyses. As shown in Figure 5A, inclusions composed of GFP-C-TDP were found to be positive for anti-RNA pol II antibody in cells expressing GFP-C-TDP. Furthermore, endogenous Sp1 and CREB were also co-localized with these inclusions. This result clearly shows that not only endogenous RNA pol II but also Sp1 and CREB are recruited to aggregated GFP-C-TDP. Next, we tried to confirm that endogenous RNA pol II interacts with TDP-43 biochemically. Cells expressing GFP, GFP-FL-TDP, GFP-N-TDP, GFP-C-TDP or GFP-ΔNLS-TDP were lyzed in RIPA buffer and the lysates were subjected to immunoprecipitation with anti-GFP antibody-tagged Dynabeads, followed by immunoblotting using several antibodies. We found that endogenous RNA pol II bound with phosphorylated form of GFP-C-TDP to a greater extent than did GFP-FL-TDP, GFP-N-TDP and

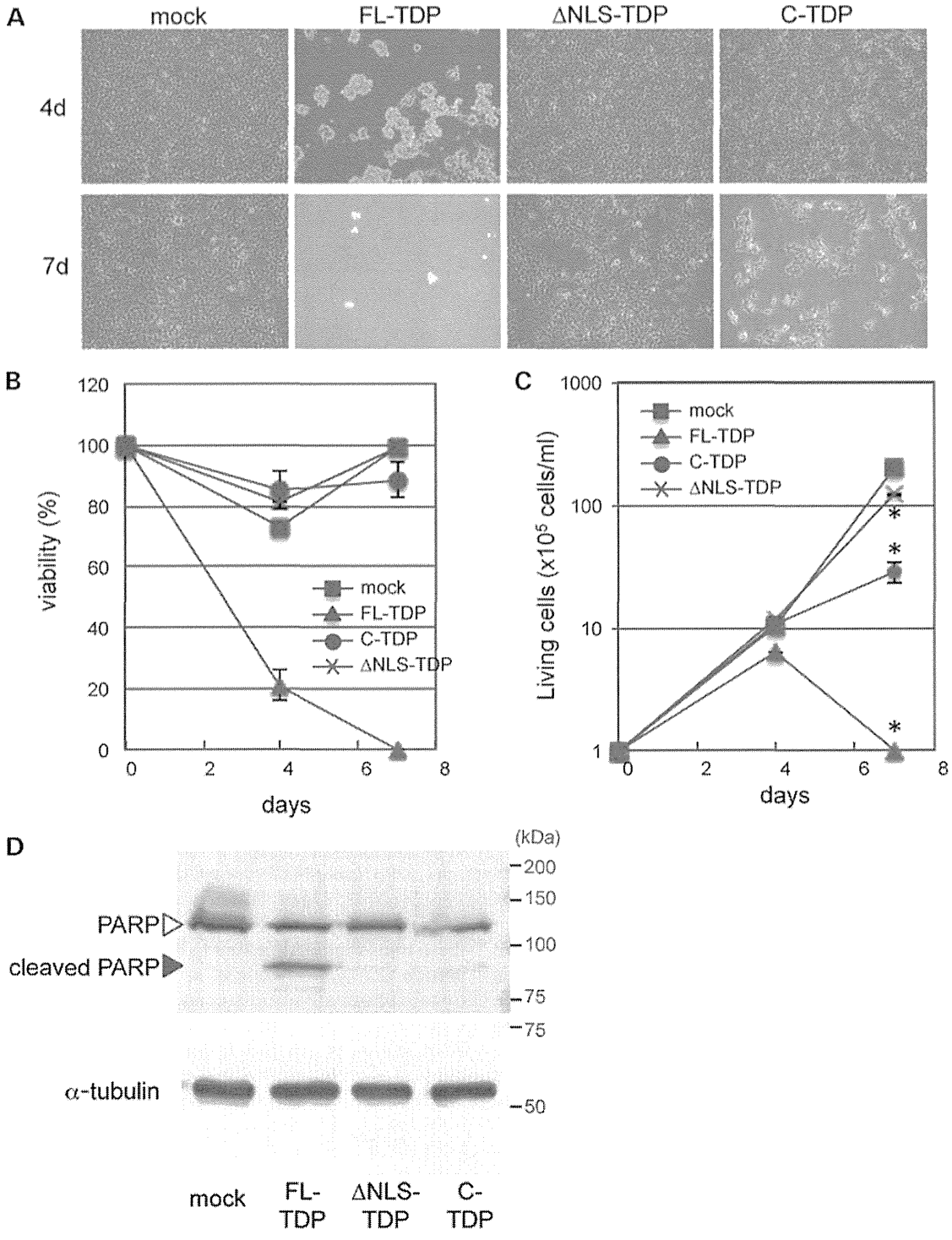

Figure 2. Cytotoxic effects of overexpressed TDP-43 in SH-SY5Y cells. SH-SY5Y cells were infected with each TDP-43 virus at 2×10^7 copies/well. At 4–7 days after infection, transfected cells were subjected to microscopic analyses, followed by cell death assay. They were assessed with light microscopy (× 10 objective) at 4 and 7 days after transfection (**A**), and viability was examined by the trypan blue dye exclusion method (**B**) and living cells were counted using an automated cell counter TC10 (Bio-Rad) (**C**). The experiments were repeated three times; the illustrated results are typical. Data are means ± SEM. $^*P < 0.01$ versus 'mock' by Student's *t*-test. (**D**) Transfected cells were also subjected to immunoblot analyses using anti-PARP and anti-tubulin antibodies. Note that PARP was cleaved in cells expressing FL-TDP, indicating that apoptosis is induced in these cells.

GFP-ΔNLS-TDP (Fig. 5B). We also examined the transcriptional activities of Sp1 and CREB in cells containing inclusions of GFP-C-TDP by means of luciferase assay. SH-SY5Y cells were transfected with mCherry (mC)-tagged full-length (mC-FL), ΔNLS (mC-ΔNLS) or CTF of TDP-43 (162–414 residues, mC-C), followed by co-transfection of pFR-Luc together

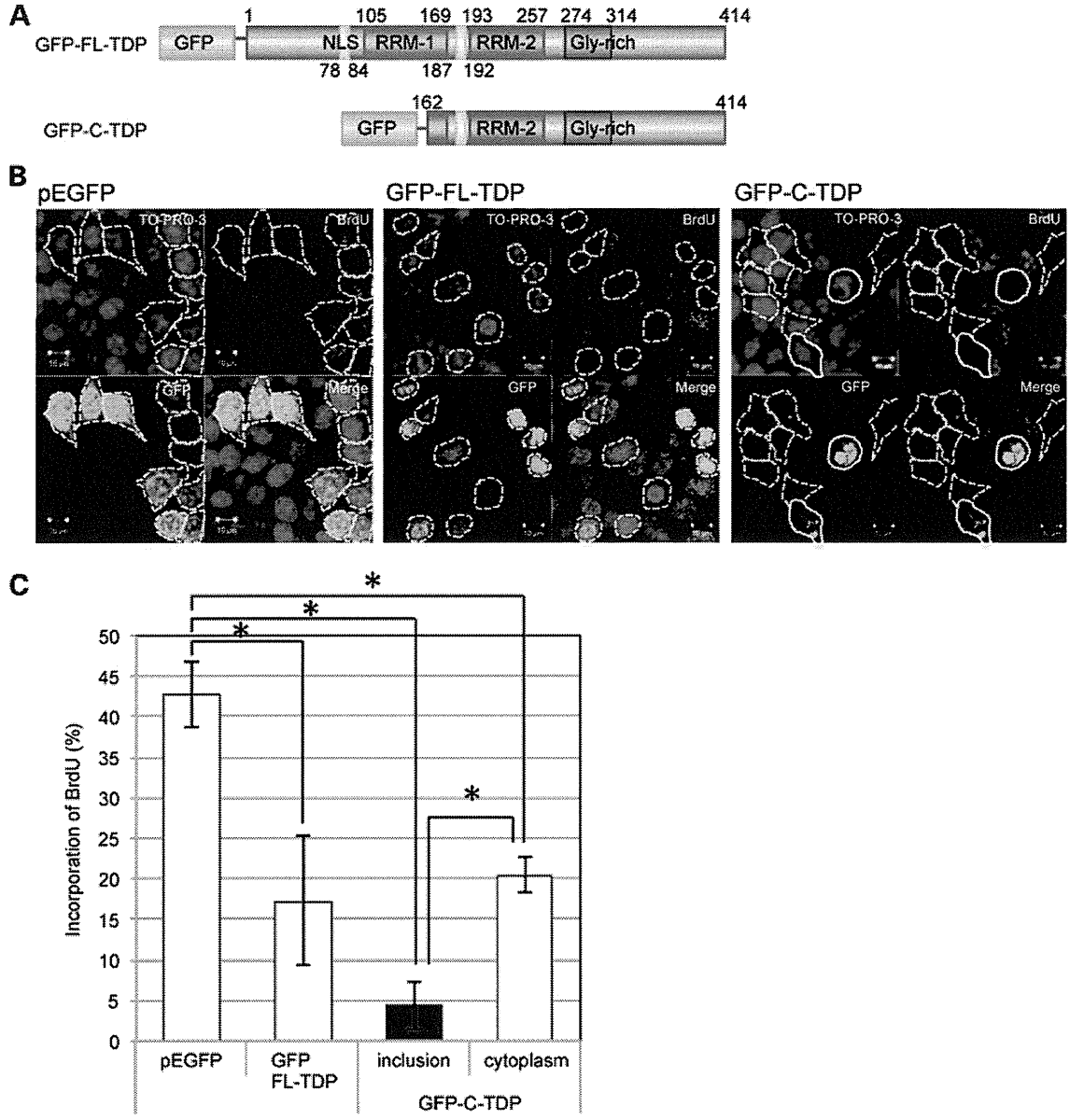

Figure 3. Comparison of cytotoxic effects of FL-TDP and its CTF. (**A**) Schematic representation of GFP-FL-TDP and GFP-C-TDP constructs in transient expression systems. (**B** and **C**) SH-SY5Y cells were transfected with the empty (pEGFP), GFP-FL-TDP or GFP-C-TDP vector for 3 days. After incubation, DNA synthesis was measured by BrdU uptake assay, using a confocal laser microscope (B). Scale bars, 10 μm. The positions of cells are indicated with broken white lines. Cells with GFP-C-TDP inclusions are indicated with white lines. The ratio (%) of the numbers of BrdU-positive cells to the numbers of GFP-positive cells was calculated as the incorporation ratio of BrdU (C). At least eight areas per sample were analyzed ($n = 8–16$), and the experiments were repeated three times; the illustrated results are typical. Data are means ± SEM. $^*P < 0.01$ by Student's *t*-test.

with pGAL4-Sp1 or pGAL4-CREB. At 48 h after transfection, cells were harvested and the luciferase activity was measured. Figure 5C shows that the transcriptional activities of Sp1 and CREB were significantly suppressed not only in cells transfected with mC-C but also in cells expressing mC-FL. We also observed co-localization of GFP-FL-TDP with Sp1, as well as co-localization of GFP-FL-TDP with CREB (Supplementary Material, Fig. S2). However, transcriptional dysregulation caused by the expression of GFP-FL-TDP appears to be due to its high toxicity (Fig. 2B), rather than the co-localization. Alternatively, it is possible that cellular damage resulting from overexpression of GFP-FL-TDP may cause transcriptional suppression of Sp1 and CREB.

RNA pol II co-localizes with TDP-43 inclusions in FTLD-TDP brain

To study the association of RNA pol II and transcriptional factors with TDP-43 aggregates in diseased brains, we carried out immunostaining of sporadic FTLD-TDP brain with several antibodies. As shown in Figure 6A and B, dystrophic neurites (DNs) were immunopositive for both anti-RNA pol II and

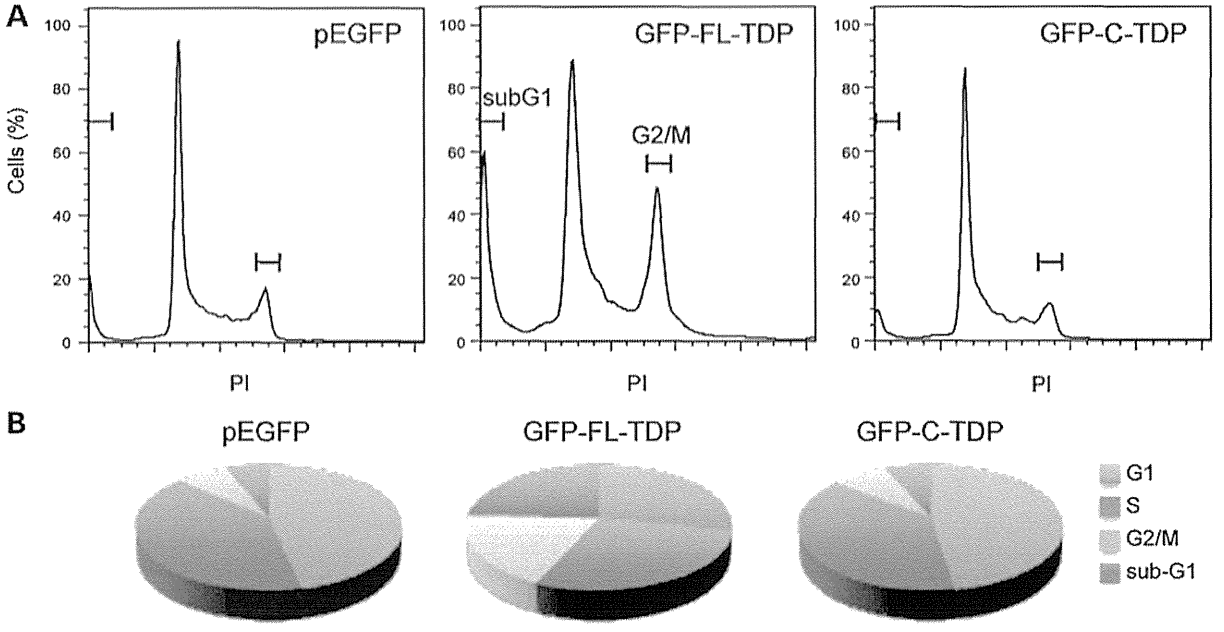

Figure 4. Analyses of the cell cycle distribution of cells transfected with GFP-tagged TDP-43. At 48 h after transfection of SH-SY5Y cells with GFP-tagged TDP-43 constructs or pEGFP empty vector, cells were stained with PI and analyzed with a flow cytometer. The results of flow-cytometric analyses are shown (**A**). The proportions of cells in G1, S, G2/M and subG1 phases were calculated using the Watson Pragmatic model (**B**). Note that growth arrest at G2/M phase was observed in GFP-FL-TDP-transfected cells, but not in GFP-C-TDP-transfected cells.

anti-pS409/410. In fluorescence-microscopic analyses, RNA pol II was co-localized with phosphorylated TDP-43 in DNs (Fig. 6D–F), clearly indicating that RNA pol II is indeed recruited to TDP-43 inclusions in FTLD-TDP brain as well as in cultured cells expressing GFP-C-TDP (Fig. 5A). On the other hand, unfortunately, antibodies to transcriptional factors Sp1, CREB and TAF II p130 failed to stain phosphorylated TDP-43 inclusions. These antibodies also failed to stain the nuclei of normal neurons in FTLD brains (data not shown). These results suggest the possibility that transcriptional activity is dysregulated *in vivo* as well as in cultured cells.

Apoptosis is not induced in FTLD-TDP brains

To test whether apoptosis is induced in FTLD-TDP brains, we performed immunoblot analyses of human brain lysates using anti-PARP and pS409/410 antibodies. As controls, we analyzed two control brains without any protein deposition and unaffected cerebellum of FTLD-TDP, in which no accumulation of TDP-43 and no atrophy has been reported. As shown in Figure 7, caspase-3-cleaved PARP was not detected in these control brains and the cerebellum of the FTLD-TDP case as well as in the temporal cortex of the FTLD-TDP case showing accumulation of TDP-43. This result indicates that apoptosis is not induced in affected neurons containing phosphorylated and aggregated TDP-43 *in vivo*.

DISCUSSION

Aberrant protein aggregates in affected neurons are well-known hallmarks of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, but the mechanisms by which these aggregates elicit neuronal degeneration remain unclear. In TDP-43 proteinopathy, inclusion bodies composed of phosphorylated, ubiquitinated and fragmented TDP-43 were found in neuronal cells of brains or spinal cords of patients. Recently, it was reported that prion-like propagation of aggregated TDP-43 is associated with the onset and progression of TDP-43 proteinopathy (22,28). So, in order to clarify the significance of TDP-43 inclusions in disease pathogenesis, we tried to establish cellular models with stable or transient TDP-43 expression in SH-SY5Y cells.

When we overexpressed FL-TDP in SH-SY5Y cells, significant cell death, suppression of cell growth, cleavage of PARP and increased cell populations at the G2/M and sub G1 phase were detected. However, intracellular inclusions of TDP-43 were not observed in these cells. These results suggest that an abnormally increased level of TDP-43, but not its aggregates, may be necessary for induction of cellular damage leading to apoptotic cell death. Indeed, several studies reporting that endogenous TDP-43 expression is tightly regulated and is critical for survival are consistent with this idea. For example, overexpression of wild-type TDP-43 caused motor neuron degeneration in yeast and rodents (15,17), knockout of TARDBP in mice led to embryonic lethality (29–32), heterozygous knockout mice develop motor impairments with age (30), and conditional knockout mice exhibit rapid postnatal lethality (29). TDP-43 is also regulated at the mRNA level through a negative feedback loop (3). These studies indicate that cellular TDP-43 levels are under tight control and perturbation of normal TDP-43 function is detrimental. Furthermore, it was reported that wild-type human TDP-43 expression causes mitochondrial aggregation in transgenic mice (33). This result suggests the possibility that apoptotic cell death found in cells expressing FL-TDP in this study may be caused by TDP-43-induced mitochondrial dysfunction.

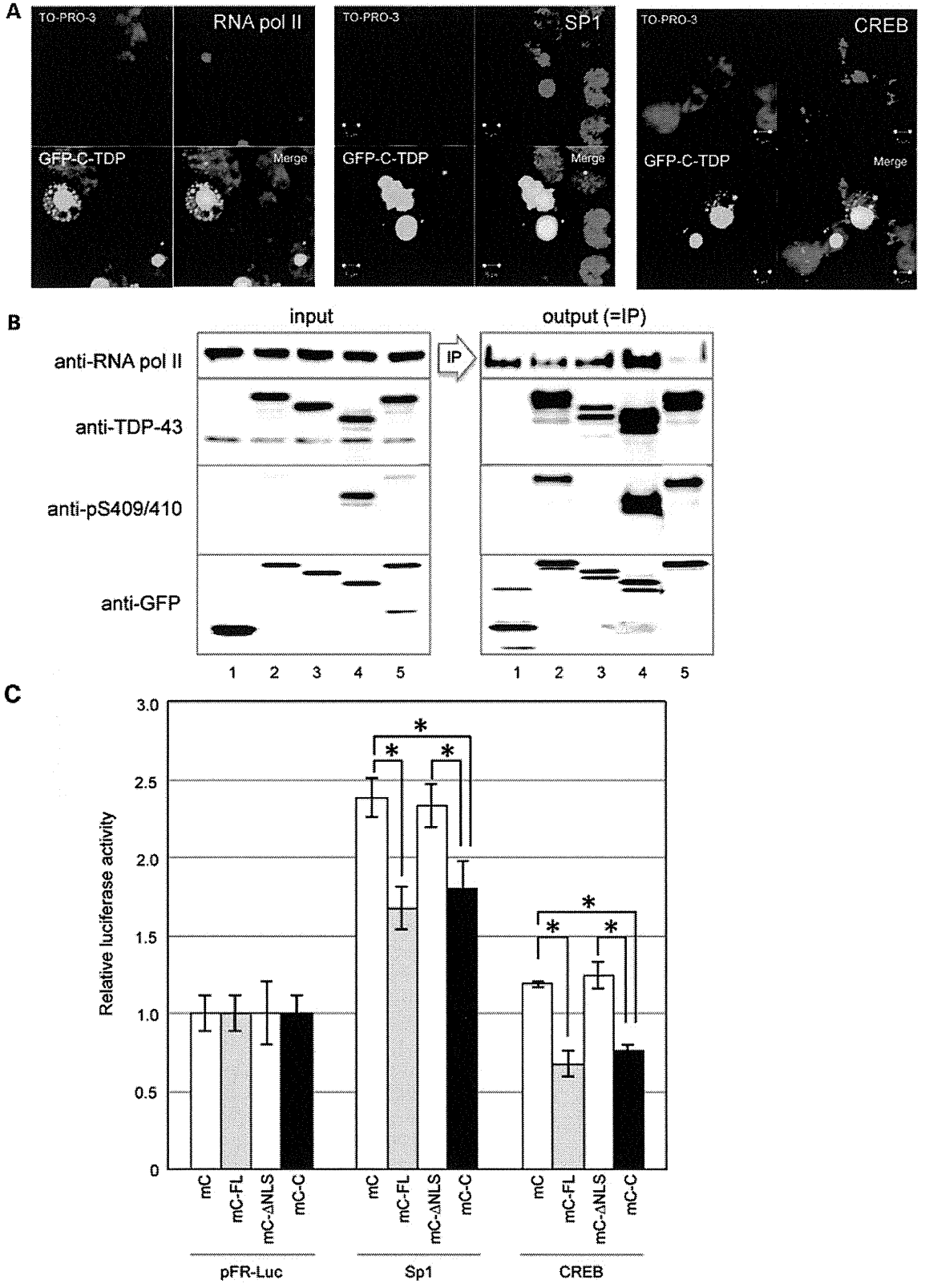

Figure 5. Co-localization of RNA pol II, Sp1 and CREB with intracellular inclusions of TDP-43 CTF. (**A**) At 72 h post-transfection with GFP-C-TDP, SH-SY5Y cells were stained with antibodies for RNA pol II, Sp1 and CREB, and observed with a confocal laser microscope. Scale bars, 5 μm. (**B**) Immunoprecipitation of cells transfected with GFP-tagged TDP-43 was performed with anti-GFP, and each sample was subjected to immunoblotting with anti-RNA pol II, TDP-43, pS409/410 and GFP antibodies. 1: pEGFP; 2: GFP-FL-TDP; 3: GFP-N-TDP (TDP-43 N-terminal fragment of 1–161 residues); 4: GFP-C-TDP and 5: GFP-ΔNLS-TDP. (**C**) SH-SY5Y cells were transfected with mCherry (mC)-tagged ΔNLS-TDP-43 (mC-ΔNLS) or CTF (mC-C). On the next day, cells were co-transfected with pFR-Luc together with pGAL4-Sp1 or pGAL4-CREB. At 48 h after the second transfection, cells were collected and luciferase activity was measured. At least three points were measured for each sample ($n = 3–6$), and the experiment was repeated three times; the illustrated results are typical. Data are means ± SEM. $^*P < 0.01$ by Student's *t*-test.

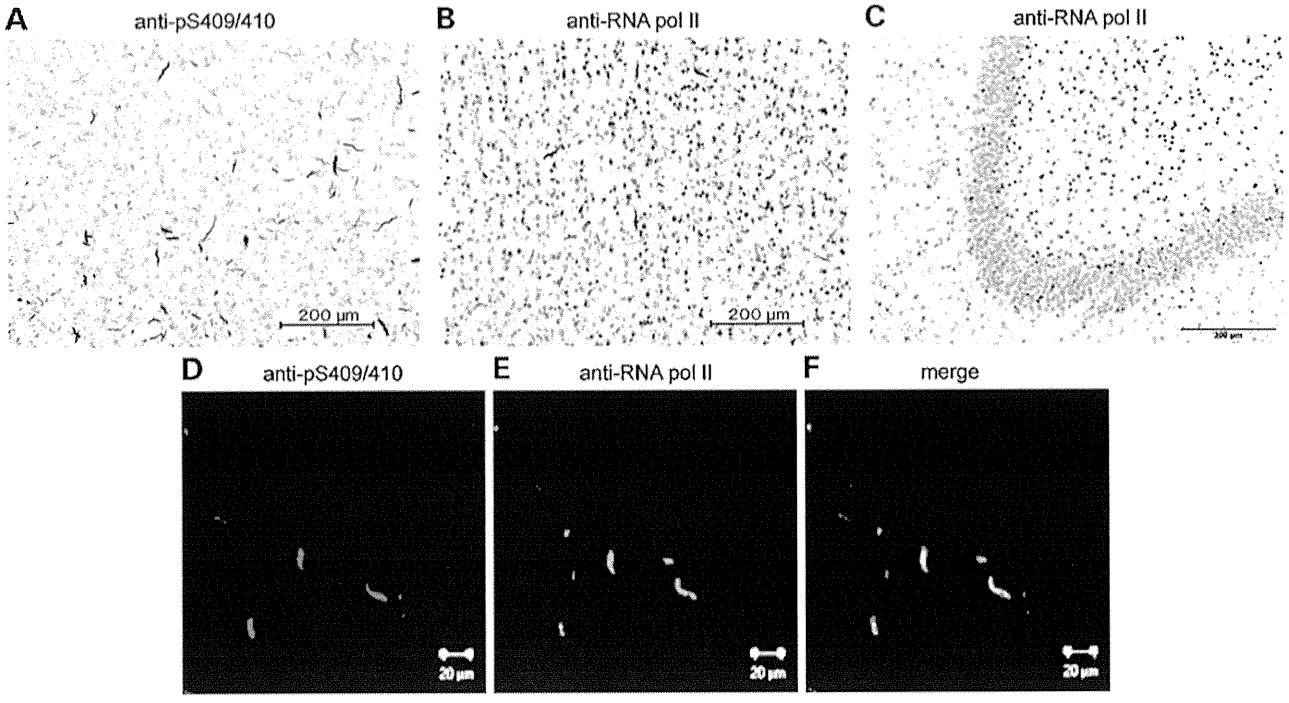

Figure 6. RNA pol II co-localizes with DNs in FTLD-TDP brain. Immunohistochemical stainings of sporadic FTLD-TDP (**A** and **B**) and control brain (**C**) were performed with anti-pS409/410 and anti-RNA pol II antibodies. (A–C) Bright-field images. Scale bars, 200 μm. (**D**–**F**) fluorescence images of sporadic FTLD-TDP. (A and D), anti-pS409/410; (B, C and E), anti-RNA pol II; (F), merge of (D) and (E). Scale bars, 20 μm.

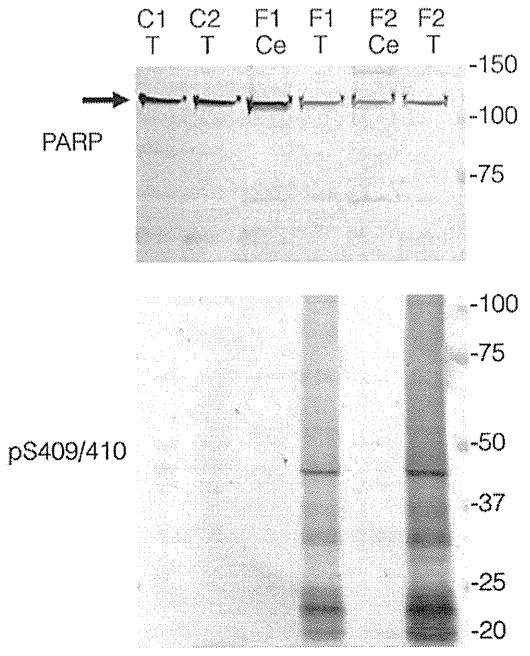

Figure 7. Apoptosis is not induced in affected neurons of FTLD-TDP brains. Immunoblot analyses of Tris-soluble (upper) and Sarkosyl-insoluble fraction (lower) prepared from human brain tissues (C1 and C2: control brains, F1 and F2: FTLD-TDP brains) were performed using anti-PARP (upper) and anti-pS409/410 (lower) antibodies. Ce, cerebellum; T, temporal cortex. Bands of endogenous whole PARP (~120 kDa: arrow) were detected in controls and patients, but no caspase-cleaved band (~95 kDa) was detected in affected or non-affected brains.

It remains unclear, however, how TDP-43 induces neuronal apoptotic cell death.

TDP-43 is a heterogeneous nuclear ribonucleoprotein and functions in RNA transcription and pre-mRNA splicing (34–38). In several genes, TDP-43 has been shown to bind directly to pre-mRNAs and regulate their splicing (36–42). In fact, widespread dysregulation of pre-mRNA splicing has been found in TDP-43-depleted cultured cells, TDP-43-depleted mouse brain and affected tissues from ALS patients (36–38, 41–44). These results suggest that dysregulation of pre-mRNA splicing is associated with ALS pathogenesis (38). Pre-mRNA splicing is mainly regulated by the spliceosome, which is a complex of small nuclear ribonucleoproteins (snRNPs) (38). The biogenesis of spliceosomes is regulated in Gemini of coiled bodies (GEMs) (38, 45–47). TDP-43 is likely to associate with GEMs in cultured cells (7), suggesting that TDP-43 contributes to GEM formation or function (38). Recently, it has been reported that the number of GEMs and the level of uridine-rich snRNA were decreased in spinal motor neurons of ALS patients (38, 48), suggesting that abnormal splicing caused by spliceosome disruption results in motor neuron death in ALS. Furthermore, several RNA processing genes have been shown to be mutated or genetically associated with ALS, including not only TDP-43, but also FUS/TLS, again suggesting that disordered RNA processing may be a key pathogenic mechanism in development of ALS (49). To our knowledge, the evidence presented here is the first to show that the formation of inclusions composed of TDP-43 CTF is cytotoxic: in cells with these inclusions, we observed a significant decrease of BrdU uptake, sequestration of RNA pol II, Sp1 and CREB into cytoplasmic aggregates of TDP-43 CTF, and

decreased transcriptional activities of Sp1 and CREB. Furthermore, RNA pol II was co-localized with these inclusions both in cultured cells and FTLD-TDP brain. These results also support the idea that transcriptional deregulation plays a critical role in the degenerative cascade in TDP-43 proteinopathy.

Our results have shown that perturbation of the expression of FL-TDP elicits apoptotic cell death, and intracellular TDP-43 aggregation causes aberrations in RNA metabolism. The overproduction of TDP-43 might also lead to formation of intracellular TDP-43 aggregates, while decreased levels of TDP-43 protein could also influence TDP-43 expression, because TDP-43 itself auto-regulates its mRNA levels through a negative feedback loop (3). Intracellular TDP-43 aggregate formation may cause aberrant TDP-43 mRNA levels due to decreased levels of normal TDP-43 in nuclei, and this is known to be one of pathological characteristics found in brains of TDP-43 proteinopathy patients. On the other hand, lacking of apoptosis in FTLD-TDP brains containing phosphorylated and accumulated TDP-43 observed in this study suggests that non-apoptotic cytotoxicity induced by TDP-43 aggregates rather than soluble TDP-43 may be closely related to the neurodegenerative mechanisms of TDP-43 proteinopathy. Therefore, it is likely that the loss of function and the gain of toxic function of TDP-43 are mutually associated with the onset of TDP-43 proteinopathy.

We conclude that dysregulation of FL-TDP expression causes neuronal apoptosis, while formation of intracellular aggregates of TDP-43 CTF induces defects in RNA metabolism. Our results suggest that plural pathways lead to TDP-43-induced cellular dysfunction, contributing to the degeneration cascades associated with onset of TDP-43 proteinopathy.

MATERIALS AND METHODS

Antibodies

A monoclonal antibody specific for TDP-43 (anti-TDP-43) was purchased from ProteinTech. An antibody specific for phosphorylated TDP-43 at both Ser409 and Ser410 antibodies (anti-pS409/410) were prepared as described (12,50). Anti-PARP antibody (#9542) was purchased from Cell Signaling. A monoclonal anti-RNA poly II antibody, which recognizes both the phosphorylated and non-phosphorylated forms of the C-terminal heptapeptide repeat region of RNA pol II, was purchased from Active Motif. A polyclonal anti-Sp1 antibody was purchased from Bethyl Laboratories and a monoclonal anti-CREB antibody (M01) was purchased from Abnova. Anti-GFP antibody was obtained from MBL (Nagoya, Japan). Anti-tubulin α antibody was purchased from Sigma-Aldrich. Anti-mouse IgM conjugated with Alexa-568 (A-21043) and anti-rabbit IgG conjugated with Alexa-568 (A-11011) were obtained from Molecular Probes.

Viral transduction of TDP-43 constructs

FL-TDP, NTF (1–161 residues: N-TDP) and CTF [162–414 residues: C-TDP (20)] of TDP-43 were subcloned into the pCL36-C1L-CMp-IRES-GFP lentivirus expression vector (51). HEK 293T cells were transfected with vector containing the insert or the empty vector along with Packing Mix (pCAG-kGP4.1R, pCAG4-RTR2 and pCAGGS-VSV-G vectors) for 40 h (with medium replacement after 6 h). Virus particles were pelleted by ultra-centrifugation (5800*g*, Beckman SE28 rotor, 16 h, 4°C). Viruses were then suspended in Hanks Balanced Salt Solution and stored at −80°C until use. For transfection, virus (1×10^7 copies/ml) was added to 2×10^5 SH-SY5Y cells/ml.

Cell culture and transfection

SH-SY5Y cells were cultured in DMEM/F12 medium (Sigma) supplemented with 10% fetal calf serum, penicillin–streptomycin–glutamine (Invitrogen), and MEM non-essential amino acid solution (Invitrogen). The cells were maintained at 37°C under a humidified atmosphere of 5% (v/v) CO_2 in air. They were grown to 50% confluence in six-well culture dishes for transient expression, and transfected with expression plasmids using FuGENE6 (Roche) according to the manufacturer's instructions. TDP-43 expression plasmids for FL-TDP and C-TDP were constructed as previously described (20,23).

Cell proliferation assay

Cell proliferation was determined with a 5-Bromo-2′-deoxyuridine Labeling and Detection Kit II (Roche). Transfected SH-SY5Y cells were grown on coverslips for 3 days, and then incubated for 10 h at 37°C in culture medium containing 10 μM BrdU. After incubation, cells were washed briefly, fixed and processed for immunostaining according to the manufacturer's instructions.

Cell cycle analysis

Cells were harvested at 72 h after transfection, fixed in 70% ethanol, treated with RNase A (1 mg/ml) for 30 min, and then stained with PI (50 μg/ml). DNA content was analyzed using an EPICS XL flow cytometer (Beckman Coulter).

Immunohistochemical analysis

SH-SY5Y cells were grown on coverslips and transfected as described above. After incubation for the indicated times, cells were fixed with 4% paraformaldehyde and stained with primary antibody (anti-TDP-43, anti-pS409/410, anti-RNA pol II, anti-Sp1 and/or anti-CREB antibody) at 1:1000 dilution. The cells were washed and further incubated with anti-rabbit IgG-conjugated Alexa-568 (1:1000), and then with TO-PRO-3 (1:3000, Invitrogen) or Hoechst 33342 (1:2000, Lonza) to counterstain nuclear DNA. Finally, they were analyzed using a LSM5 Pascal confocal laser microscope (Carl Zeiss).

Human brain tissues were obtained from Tokyo Metropolitan Institute of Medical Science (Tokyo, Japan). This study was approved by the local research ethics committee of Tokyo Metropolitan Institute of Medical Science (approval no. 12-3). Small blocks of human brain were dissected at autopsy or from fresh-frozen brain samples and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 days. Following cryoprotection with 15% sucrose in 0.01 M phosphate-buffered saline (pH 7.4), blocks were cut on a freezing microtome at 30 μm thickness. The free-floating sections were incubated with anti-pS409/410 or anti-RNA pol II antibody for 72 h.

Following treatment with the appropriate secondary antibody, labeling was detected using the avidin-biotinylated horseradish peroxidase (HRP) complex system coupled with diaminobenzidine (DAB) reaction to yield a brown precipitate. In some sections, the DAB reaction was intensified with nickel ammonium sulfate to yield a dark purple precipitate. Moreover, the sections were incubated with secondary antibodies, labeled with FITC for anti-pS409/410 or with Rhodamine for RNA pol II and then observed under a fluorescence microscopy.

Immunoprecipitation and western blotting

SH-SY5Y cells grown in a six-well plate were transfected with GFP-tagged TDP-43 expression vectors (20). After incubation for 3 days, cells were harvested and lyzed in TX buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 5 mM ethylene glycol tetraacetic acid (EGTA), 1% TX and protease inhibitor cocktail (Roche)] by brief sonication on ice. The lysates were incubated with anti-GFP antibody-linked Dynabeads (Invitrogen) for 4 h at 4°C. The immunoprecipitated Dynabeads complexes were washed five times with TX buffer. Proteins were eluted by boiling in SDS sample buffer and then processed for western blot analysis. Each sample was separated by 12% (v/v) SDS-PAGE using Tris–glycine buffer system, and proteins were transferred onto polyvinylidene difluoride membrane (Millipore). The blots were incubated overnight with each primary antibody at room temperature, followed by incubation with HRP-conjugated secondary antibody. Signals were detected using the ECL plus Western Blotting Detection System (GE Healthcare).

Luciferase assay

In the GAL4-Sp1 expression vectors, the 147 N-terminal codons of the yeast transcription factor GAL4 containing its DNA-binding domain were fused to fragments coding for the N-terminal regions of Sp1 and CREB. The expression vectors of pGAL4-Sp1 and pGAL4-CREB, and luciferase reporter plasmid pFR-Luc were prepared as described previously (52). SH-SY5Y cells were transfected with mCherry-tagged TDP-43 constructs. Next day, these cells were co-transfected with pFR-Luc together with pGAL4-Sp1 or pGAL4-CREB. At 48 h after the second transfection, cells were collected and luciferase activity was measured with a Luciferase Assay kit (Stratagene) according to the manufacturer's instructions. At least three points of each sample were measured ($n = 3–6$), and the experiment was repeated three times; the illustrated results are typical.

Preparation of human brain homogenates

Brain samples for immunoblot analyses were prepared as previously described (12,22). Briefly, frozen brain tissue from two controls or two patients with FTLD-TDP (type C) was homogenized in 10 volumes (w/v) of homogenization buffer (HB: 10 mM Tris–HCl, pH 7.4, 0.8 M NaCl, 1 mM EGTA and 10% sucrose). Aliquots of the homogenates were ultracentrifuged at 100 000*g* for 20 min at 4°C, and the supernatant was recovered as Tris-soluble fraction for immunoblotting analyses. Remaining lysate homogenates were incubated at 37°C for 30 min in HB buffer containing 2% Sarkosyl, and centrifuged at 20 000*g* for 10 min. The supernatants were ultracentrifuged at 100 000*g* for 20 min and the resulting pellets were used as Sarkosyl-insoluble fraction for immunoblotting analyses.

Statistical analysis

All values in the figures are shown as mean ± SEM. Statistical analysis was performed using the unpaired, two-tailed Student's *t*-test. A P value of 0.01 or less was considered to be statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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PATHOBIOLOGY IN FOCUS

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

Katherine E Santostefano[1], Takashi Hamazaki[2], Nikolett M Biel[1], Shouguang Jin[3], Akihiro Umezawa[4] and Naohiro Terada[1]

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

[1]Department of Pathology, Immunology and Laboratory Medicine, Center for Cellular Reprogramming, University of Florida College of Medicine, Gainesville, FL, USA; [2]Department of Molecular Genetics and Microbiology, Center for Cellular Reprogramming, University of Florida College of Medicine, Gainesville, FL, USA; [3]Department of Pediatrics, Osaka City University, Osaka, Japan and [4]Department of Reproductive Biology and Pathology, National Research Institute for Child Health and Development, Tokyo, Japan
Correspondence: Dr N Terada, MD, PhD, Department of Pathology, Immunology and Laboratory Medicine, Center for Cellular Reprogramming, University of Florida College of Medicine, PO Box 100275, Gainesville, FL 32610, USA.
E-mail: terada@pathology.ufl.edu

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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
Internal Origin	**Strengths** ❑ 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	**Weaknesses** ❑ May take time to establish differentiation protocols in your lab ❑ Differentiated cells may not be pure or mature enough for your study
External Origin	**Opportunities** ❑ High expectations for developing novel model systems ❑ High expectations from societies of particular diseases ❑ External and internal grant opportunities	**Threats** ❑ 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.

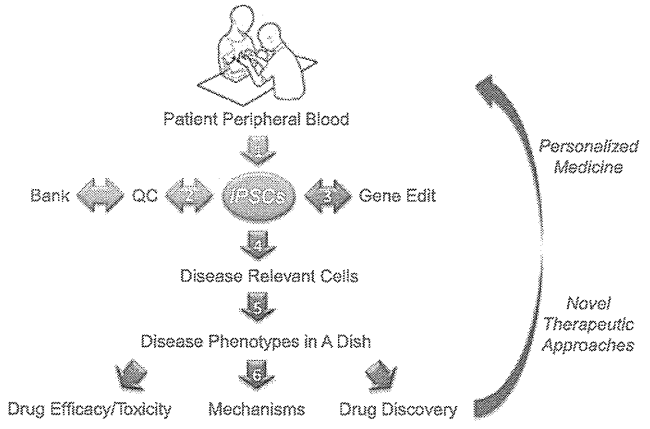

Figure 2 A typical work flow of patient iPSC research and tips for individual steps. (**1**) iPSC generation (~3 weeks)—multiple clones from multiple patients using non-integrating reprogramming vectors; (**2**) Quality control (QC) and storage (1–4 weeks)—first by morphology and pluripotency markers, 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.

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]

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	Coriell 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	Coriell 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 poly-arginine 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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