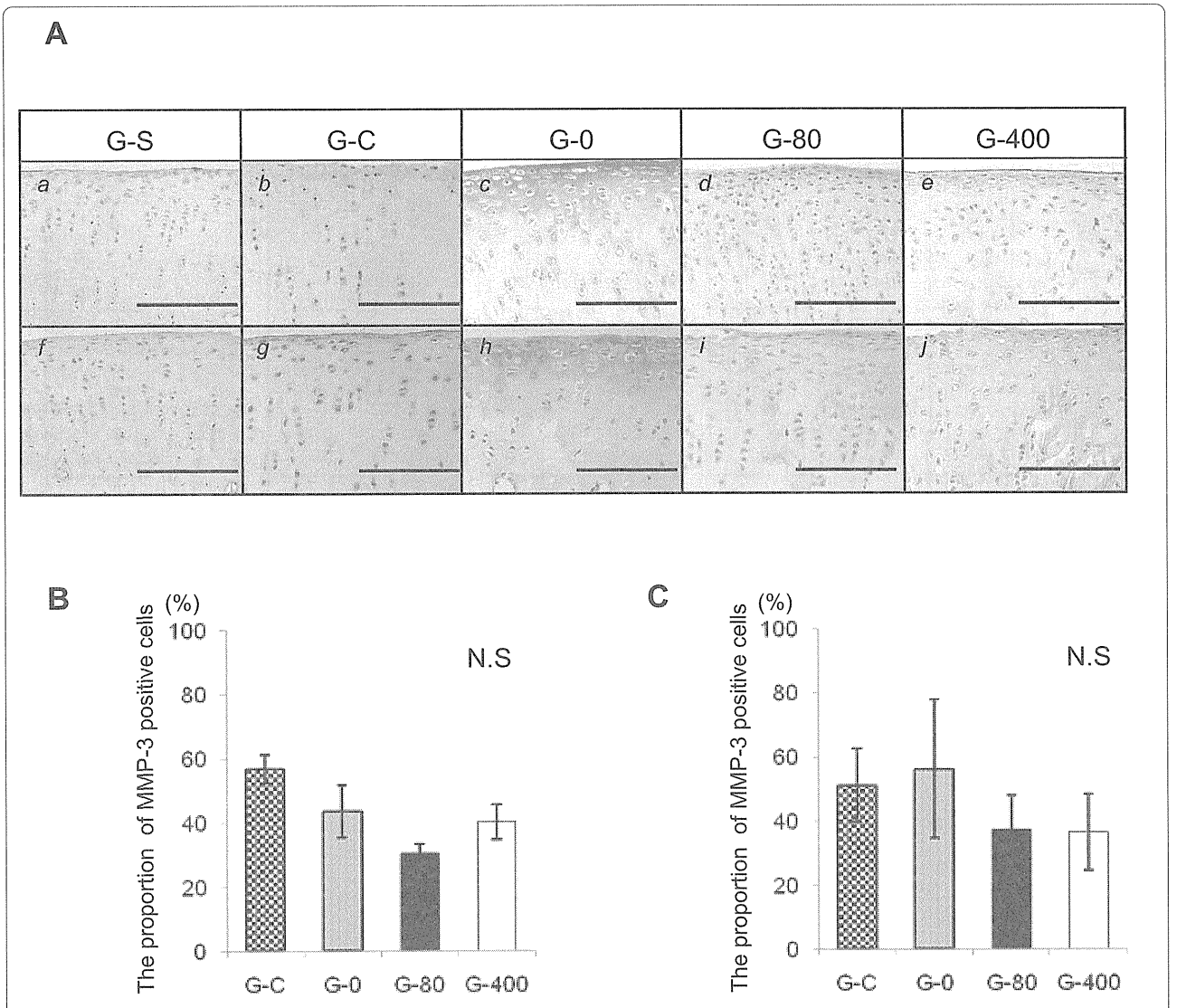

Figure 6 Effect of ONO-8815Ly on the expression of MMP-3 in femoral condyles. (a) Immunostaining for MMP-3 at two weeks after the operation. Sections of (*a* to *e*) medial and (*f* to *j*) lateral femoral condyles were prepared from samples of (*a* and *f*) G-S, (*b* and *g*) G-C, (*c* and *h*) G-0, (*d* and *i*) G-80, and (*e* and *j*) G-400. Magnification, 400×. Scale bar, 200 μm. Proportion of MMP-3-positive cells in **(b)** medial and **(c)** lateral femoral condyles at two weeks after the operation. Values are the mean ± standard deviation. *$P < 0.05$. MMP-3, matrix metalloproteinase-3; N.S, not significant.

Previously, we showed that EP2 signaling enhances the growth of chondrocytes [18,19] and promotes the regeneration of articular cartilage in rabbits with cartilage defects by an EP2-selective agonist [19]. However, in the current study, EP2 signaling failed to promote chondrocyte proliferation (Figure 5). The differences may result from differences in the animal models. In the previous study, the effect of EP2 signaling on articular cartilage was evaluated using the chondral and osteochondral defect models. In that model, cartilage defects are present before initiation of the treatment with an EP2 agonist. Thus, EP2 signaling may promote cartilage regeneration by inducing proliferation of cartilage chondrocytes and, consequently, contributing to ECM reconstruction. On the other hand, in the present study, the articular chondrocytes appeared normal immediately after the ACLMT operation, and EP2 signaling reduced cartilage degeneration caused by traumatic instability of the knee joint. These differences in models might be the cause of difference in the results.

In the present study, the abnormal stress on cartilage tissues induced by joint instability was the main cause of degeneration. The degeneration was more remarkable in the lateral (Figures 2d and 3d) than in the medial components (Figures 2c and 3c), wherein partial meniscectomy was performed. We have no clear explanation for this

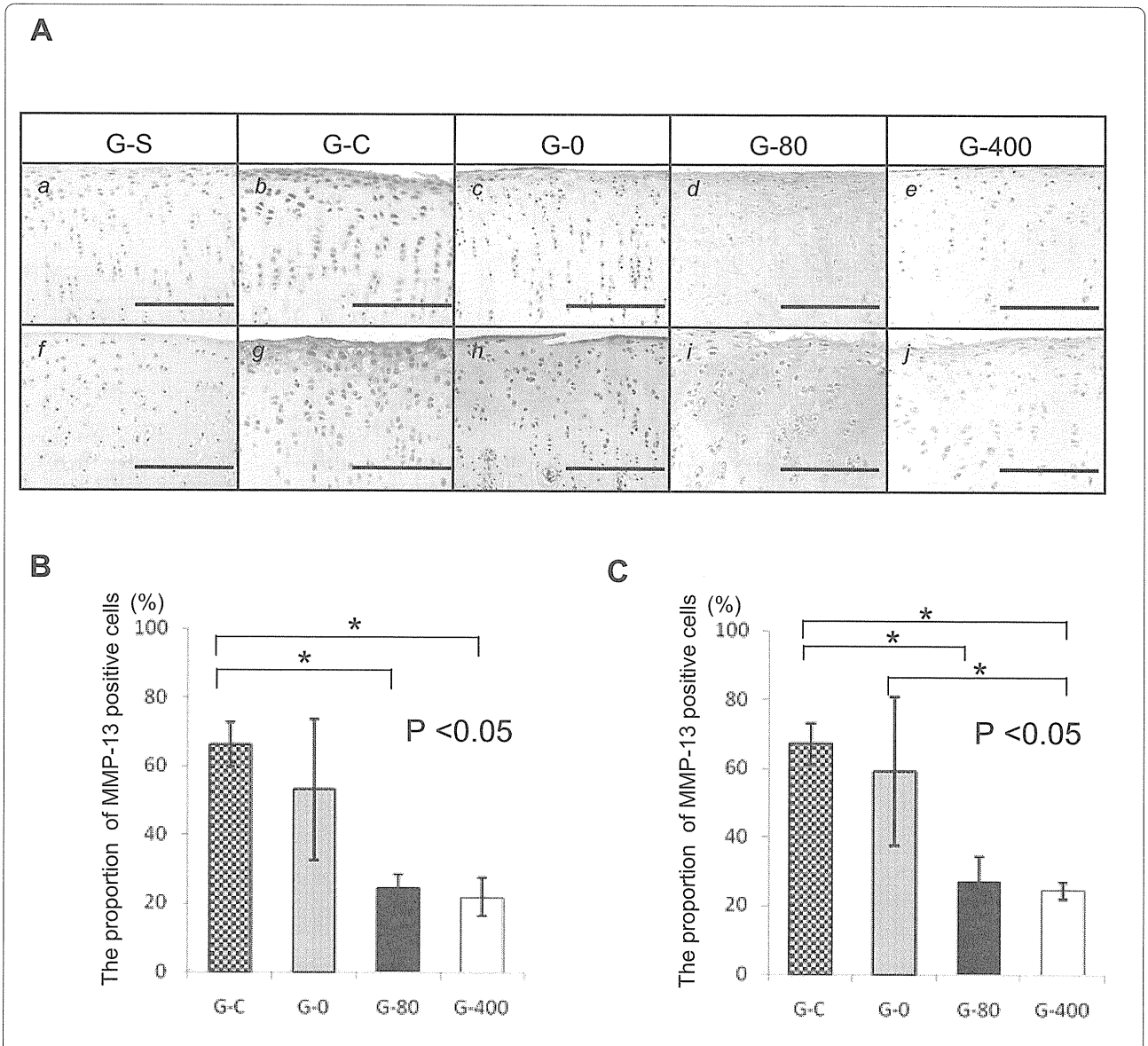

Figure 7 Effect of ONO-8815Ly on the expression of MMP-13 in femoral condyles. (a) Immunostaining for MMP-13 at two weeks after the operation. Sections of (*a* to *e*) medial and (*f* to *j*) lateral femoral condyles were prepared from samples of (*a* and *f*) G-S, (*b* and *g*) G-C, (*c* and *h*) G-0, (*d* and *i*) G-80, and (*e* and *j*) G-400. Magnification, 400×. Scale bar, 200 μm. Proportion of MMP-13-positive cells in **(b)** medial and **(c)** lateral femoral condyles at two weeks after the operation. Values are the mean ± standard deviation. *$P < 0.05$. MMP-13, matrix metalloproteinase-13; N. S, not significant.

result. A study has shown that the lateral components of the rabbit knees were more susceptible to degeneration than the medial components in the ACLMT model [31]. The rabbit knee joints are physiologically in the valgus position, causing excess load on the lateral side, which might explain the susceptibility.

The grade of degeneration at 12 weeks was less prominent than we expected (Figure 4). In this injury model, cartilage degeneration will be induced by abnormal stress due to joint instability. Such abnormal stress takes place during weight-bearing movements of the knee joints. Therefore, to enhance such stress, Park et al. forced the rabbits to move in a confined space (5 m × 5 m) for one hour twice a day, from three days after ACLMT onward [22], which increased the Mankin's score up to 12 points at eight weeks after the operation. Restriction in a small cage in the knee-flexed position, as in our study, may minimize such stresses. In addition, both knees were operated on, which may further decrease the activities of the rabbits. These may cause almost no progression of the disease after two weeks.

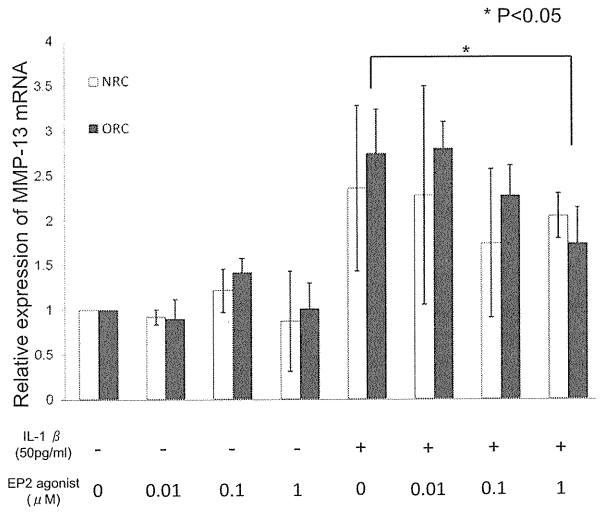

Figure 8 Reduction of IL-1β-induced *MMP-13* mRNA expression by an EP2 agonist on rabbit normal or OA chondrocyte primary culture cells *in vitro*. Expression levels of *MMP-13* mRNA in NRC and ORC at six hours after treatment with IL-1β (50 pg/ml), ONO-AE-259-01, selective EP2 agonist (each at a concentration of 0, 0.01, 0.1, and 1 μM), or a combination of IL-1β and ONO-AE-259-01. GAPDH was used as internal control. Using the ratio obtained for the untreated sample as a standard (1.0), the relative ratio of the treated samples was presented as the relative expression levels of the *MMP-13* gene. Values are the mean ± standard deviation. NRC (n = 4) and ORC (n = 5). *$P < 0.05$. EP2, prostaglandin E2 receptor type 2; IL-1β, interleukin-1β; MMP-13, matrix metalloproteinase-13; NRC, non-treated rabbit chondroctes; ORC, ACLMT-operated rabbit chondrocytes.

Generally, cartilage degeneration in OA is due to the induction of MMP expression. MMP-13 is a product of chondrocytes that reside in cartilage and has a stronger effect that MMP-1 on type II collagen [32]. Some insisted that PGE2 exerts direct inhibitory effects on the expression of MMP-1 [33,34] and MMP-13 [28,33,34] in arthritic chondrocytes, and Sato et al. demonstrated that EP2 signaling was responsible for the down-regulation of MMP-13 *in vitro*, although they used a different agonist [28]. Taken together, EP2 signaling regulates MMP-13 production. In agreement, we showed that production of MMP-13 in articular chondrocytes was reduced when treated with an EP2 agonist *in vivo* (Figure 7) and *in vitro* (Figure 8). Controversially others studies show that PGE2 plays a crucial role in the induction of MMP-13 and MMP-3 in chondrocytes in response to IL-1β in microsomal prostaglandin E synthase-deficient mice [35] or that of PGE2 inhibits chondrocyte maturation [36]. In the current study model, EP2 signaling was shown to inhibit the expression of *MMP-13* mRNA, suggesting that EP2 signaling protects the articular cartilage from degeneration.

MMP-3 is a protease expressed in OA specimens at an early stage [37,38]. MMP-3 cleaves a variety of ECM components such as proteoglycans, collagens, and procollagens [39]. In the current study, ONO-8815Ly had no effect on the production of MMP-3 (Figure 6). Although there is still much to be done, the current study suggested that an EP2 agonist may exert a protective effect on articular cartilage by inhibiting MMP-13.

It is important to clarify whether an EP2 agonist caused inflammation either systemically or locally. PGs are pro-inflammatory lipid mediators whose levels increase in the synovial membrane and synovial fluid of patients with OA. We previously reported that intra-articular administration of an EP2 agonist did not affect the mRNA expression of the *MMP-3*, *TIMP-3*, and *IL-1β* genes in the synovium, or the amounts of TNF-α and C-reactive protein (CRP) in joint fluids. As in our previous study, we found no severe inflammatory changes in the synovium, and no change in the levels of CRP (data not shown), suggesting that this EP2 agonist caused no inflammation either systemically or locally.

The effect of an EP2 agonist did not last long (Figure 4), yet this may be rectified by developing a suitable drug-delivery system. Continuous administration of an EP2 agonist using such a newly developed system could provide a novel therapeutic modality to treat OA.

Conclusions

Stimulation of PGE2 via EP2 prevents degeneration of the articular cartilage during the early stages. The current study suggests that EP2 agonists may exert a protective effect on articular cartilage by inhibiting MMP-13. With a long-term delivery system, the EP2 agonist could be a new therapeutic tool for OA.

Abbreviations

ACLMT: anterior cruciate ligament and menisectomy transaction; COX: cyclo-oxygenase; CRP: C-reactive protein; DMEM: Dulbecco's modified Eagle's medium; ECM: extracellular matrix; EP2: prostaglandin E2 receptor type 2; FBS: fetal bovine serum; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; H&E: hematoxylin & eosin; IGF: insulin-like growth factor; IL: interleukin; MMP: matrix metalloproteinase; OA: osteoarthritis; PBS: phosphate-buffered saline; PCNA: proliferating cell nuclear antigen; PG: prostaglandin; PLGA: polylactic-co-glycolic acid; SD: standard deviation; TNF: tumor necrosis factor.

Acknowledgements

We are grateful to Tomohisa Kato, Akira Nasu, Takashi Kasahara, Kazuo Hayakawa, Kyosuke Kobayashi, Michiko Ueda, Sakura Tamaki, and Yukiko Kobayashi for their assistance. This work was partially supported by Grants-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Education, Culture, Sports, Science, and Technology, and from the Ministry of Health, Labor, and Welfare.

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Authors' contributions

HM performed animal experiments, carried out analysis and interpretation of the data, and drafted the manuscript. TA conceived this study, designed the study, carried out analysis and interpretation of the data, and drafted the manuscript. MF and JY performed animal experiments and carried out analysis of the data. KI performed animal experiments. TM was the chief investigator in the development of materials, and conceived this study. TK designed and performed animal experiments. SF performed animal experiments and obtained samples from animals. HS was responsible for providing materials. NA was responsible for the development of drug delivery system. TO carried out administrative and financial support and helped to draft the manuscript. TN carried out administrative and financial support and helped to draft the manuscript. JT conceived this study, provided financial support, designed experiments, interpreted the data, and drafted the manuscript. All authors have read and approaved the manuscript for publication.

Competing interests

Takayuki Maruyama, Toshiya Kanaji, Shinsei Fujimura, Hikaru Sugihara, and Akio Nishiura are employees of Ono Pharmaceutical Co. Ltd. All other authors have no conflicts of interest.

Received: 19 January 2011 Revised: 28 June 2011
Accepted: 14 September 2011 Published: 14 September 2011

References

1. Peat G, McCarney R, Croft P: **Knee pain and osteoarthritis in older adults: a review of community burden and current use of primary health care.** *Ann Rheum Dis* 2001, **60**:91-97.
2. Buckwalter JA, Saltzman C, Brown T: **The impact of osteoarthritis: implications for research.** *Clin Orthop Relat Res* 2004, **427 Suppl**:S6-15.
3. Goldring SR, Goldring MB: **The role of cytokines in cartilage matrix degeneration in osteoarthritis.** *Clin Orthop Relat Res* 2004, **427 Suppl**: S27-36.
4. Goldring MB, Goldring SR: **Osteoarthritis.** *J Cell Physiol* 2007, **213**:626-634.
5. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chen J, Van Wart H, Poole AR: **Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage.** *J Clin Invest* 1997, **99**:1534-1545.
6. Neuhold LA, Killar L, Zhao W, Sung ML, Warner L, Kulik J, Turner J, Wu W, Billinghurst C, Meijers T, Poole AR, Babij P, DeGennaro LJ: **Postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice.** *J Clin Invest* 2001, **107**:35-44.
7. Hu Y, Xiang JS, DiGrandi MJ, Du X, Ipek M, Laakso LM, Li J, Li W, Rush TS, Schmid J, Skotnicki JS, Tam S, Thomason JR, Wang Q, Levin JI: **Potent, selective, and orally bioavailable matrix metalloproteinase-13 inhibitors for the treatment of osteoarthritis.** *Bioorg Med Chem* 2005, **13**:6629-6644.
8. Narumiya S, Sugimoto Y, Ushikubi F: **Prostanoid receptors: structures, properties, and functions.** *Physiol Rev* 1999, **79**:1193-1226.
9. Laufer S: **Role of eicosanoids in structural degradation in osteoarthritis.** *Curr Opin Rheumatol* 2003, **15**:623-627.
10. Martel-Pelletier J, Pelletier JP, Fahmi H: **Cyclooxygenase-2 and prostaglandins in articular tissues.** *Semin Arthritis Rheum* 2003, **33**:155-167.
11. Bunning RA, Russell RG: **The effect of tumor necrosis factor alpha and gamma-interferon on the resorption of human articular cartilage and on the production of prostaglandin E and of caseinase activity by human articular chondrocytes.** *Arthritis Rheum* 1989, **32**:780-784.
12. Lippiello L, Yamamoto K, Robinson D, Mankin HJ: **Involvement of prostaglandins from rheumatoid synovium in inhibition of articular cartilage metabolism.** *Arthritis Rheum* 1978, **21**:909-917.
13. Li X, Ellman M, Muddasani P, Wang JH, Cs-Szabo G, van Wijnen AJ, Im HJ: **Prostaglandin E2 and its cognate EP receptors control human adult articular cartilage homeostasis and are linked to the pathophysiology of osteoarthritis.** *Arthritis Rheum* 2009, **60**:513-523.
14. Riquet FB, Lai WF, Birkhead JR, Suen LF, Karsenty G, Goldring MB: **Suppression of type I collagen gene expression by prostaglandins in fibroblasts is mediated at the transcriptional level.** *Mol Med* 2000, **6**:705-719.
15. Miyamoto M, Ito H, Mukai S, Kobayashi T, Yamamoto H, Kobayashi M, Maruyama T, Akiyama H, Nakamura T: **Simultaneous stimulation of EP2 and EP4 is essential to the effect of prostaglandin E2 in chondrocyte differentiation.** *Osteoarthritis Cartilage* 2003, **11**:644-652.
16. Di Battista JA, Dore S, Morin N, He Y, Pelletier JP, Martel-Pelletier J: **Prostaglandin E2 stimulates insulin-like growth factor binding protein-4 expression and synthesis in cultured human articular chondrocytes: possible mediation by Ca(++)-calmodulin regulated processes.** *J Cell Biochem* 1997, **65**:408-419.
17. Lowe GN, Fu YH, McDougall S, Polendo R, Williams A, Benya PD, Hahn TJ: **Effects of prostaglandins on deoxyribonucleic acid and aggrecan synthesis in the RCJ 3.1C5.18 chondrocyte cell line: role of second messengers.** *Endocrinology* 1996, **137**:2208-2216.
18. Aoyama T, Liang B, Okamoto T, Matsusaki T, Nishijo K, Ishibe T, Yasura K, Nagayama S, Nakayama T, Nakamura T, Toguchida J: **PGE2 signal through EP2 promotes the growth of articular chondrocytes.** *J Bone Miner Res* 2005, **20**:377-389.
19. Otsuka S, Aoyama T, Furu M, Ito K, Jin Y, Nasu A, Fukiage K, Kohno Y, Maruyama T, Kanaji T, Nishiura A, Sugihara H, Fujimura S, Otsuka T, Nakamura T, Toguchida J: **PGE2 signal via EP2 receptors evoked by a selective agonist enhances regeneration of injured articular cartilage.** *Osteoarthritis Cartilage* 2009, **17**:529-538.
20. Tani K, Naganawa A, Ishida A, Egashira H, Sagawa K, Harada H, Ogawa M, Maruyama T, Ohuchida S, Nakai H, Kondo K, Toda M: **Design and synthesis of a highly selective EP2-receptor agonist.** *Bioorg Med Chem Lett* 2001, **11**:2025-2028.
21. Okada H: **One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate.** *Adv Drug Deliv Rev* 1997, **28**:43-70.
22. Park SR, Park SH, Jang KW, Cho HS, Cui JH, An HJ, Choi MJ, Chung SI, Min BH: **The effect of sonication on simulated osteoarthritis. Part II: alleviation of osteoarthritis pathogenesis by 1 MHz ultrasound with simultaneous hyaluronate injection.** *Ultrasound Med Biol* 2005, **31**:1559-1566.
23. Sakakibara Y, Miura T, Iwata H, Kikuchi T, Yamaguchi T, Yoshimi T, Itoh H: **Effect of high-molecular-weight sodium hyaluronate on immobilized rabbit knee.** *Clin Orthop Relat Res* 1994, 282-292.
24. Mankin HJ, Dorfman H, Lippiello L, Zarins A: **Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data.** *J Bone Joint Surg Am* 1971, **53**:523-537.
25. Noguchi K, Maeda M, Ruwanpura SM, Ishikawa I: **Prostaglandin E2 (PGE2) downregulates interleukin (IL)-1alpha-induced IL-6 production via EP2/EP4 subtypes of PGE2 receptors in human periodontal ligament cells.** *Oral Dis* 2005, **11**:157-162.
26. Noguchi K, Shitashige M, Endo H, Kondo H, Ishikawa I: **Binary regulation of interleukin (IL)-6 production by EP1 and EP2/EP4 subtypes of PGE2 receptors in IL-1beta-stimulated human gingival fibroblasts.** *J Periodontal Res* 2002, **37**:29-36.
27. Tchetina EV, Di Battista JA, Zukor DJ, Antoniou J, Poole AR: **Prostaglandin PGE2 at very low concentrations suppresses collagen cleavage in cultured human osteoarthritic articular cartilage: this involves a decrease in expression of proinflammatory genes, collagenases and COL10A1, a gene linked to chondrocyte hypertrophy.** *Arthritis Res Ther* 2007, **9**:R75.
28. Sato T, Konomi K, Fujii R, Aono H, Aratani S, Yagishita N, Araya N, Yudoh K, Beppu M, Yamano Y, Nishioka K, Nakajima T: **Prostaglandin EP2 receptor signalling inhibits the expression of matrix metalloproteinase 13 in human osteoarthritic chondrocytes.** *Ann Rheum Dis* 2011, **70**:221-226.
29. Rashad S, Low F, Revell P, Hemingway A, Rainsford K, Walker F: **Effect of non-steroidal anti-inflammatory drugs on course of osteoarthritis.** *Lancet* 1989, **2**:1149.
30. Reijman M, Bierma-Zeinstra SM, Pols HA, Koes BW, Stricker BH, Hazes JM: **Is there an association between the use of different types of nonsteroidal antiinflammatory drugs and radiologic progression of osteoarthritis? The Rotterdam Study.** *Arthritis Rheum* 2005, **52**:3137-3142.
31. Shirai T, Kobayashi M, Nishitani K, Satake T, Kuroki H, Nakagawa Y, Nakamura T: **Chondroprotective effect of alendronate in a rabbit model of osteoarthritis.** *J Orthop Res* 2011, **29**:1572-7.
32. Burrage PS, Mix KS, Brinckerhoff CE: **Matrix metalloproteinases: role in arthritis.** *Front Biosci* 2006, **11**:529-543.

33. Nishitani K, Ito H, Hiramitsu T, Tsutsumi R, Tanida S, Kitaori T, Yoshitomi H, Kobayashi M, Nakamura T: **PGE2 inhibits MMP expression by suppressing MKK4-JNK MAP kinase-c-JUN pathway via EP4 in human articular chondrocytes.** *J Cell Biochem* 2010, **109**:425-433.
34. Pillinger MH, Rosenthal PB, Tolani SN, Apsel B, Dinsell V, Greenberg J, Chan ES, Gomez PF, Abramson SB: **Cyclooxygenase-2-derived E prostaglandins down-regulate matrix metalloproteinase-1 expression in fibroblast-like synoviocytes via inhibition of extracellular signal-regulated kinase activation.** *J Immunol* 2003, **171**:6080-6089.
35. Gosset M, Pigenet A, Salvat C, Berenbaum F, Jacques C: **Inhibition of matrix metalloproteinase-3 and -13 synthesis induced by IL-1beta in chondrocytes from mice lacking microsomal prostaglandin E synthase-1.** *J Immunol* 2010, **185**:6244-6252.
36. Clark CA, Li TF, Kim KO, Drissi H, Zuscik MJ, Zhang X, O'Keefe RJ: **Prostaglandin E2 inhibits BMP signaling and delays chondrocyte maturation.** *J Orthop Res* 2009, **27**:785-792.
37. Bau B, Gebhard PM, Haag J, Knorr T, Bartnik E, Aigner T: **Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro.** *Arthritis Rheum* 2002, **46**:2648-2657.
38. Kevorkian L, Young DA, Darrah C, Donell ST, Shepstone L, Porter S, Brockbank SM, Edwards DR, Parker AE, Clark IM: **Expression profiling of metalloproteinases and their inhibitors in cartilage.** *Arthritis Rheum* 2004, **50**:131-141.
39. Klatt AR, Paul-Klausch B, Klinger G, Kuhn G, Renno JH, Banerjee M, Malchau G, Wielckens K: **A critical role for collagen II in cartilage matrix degradation: collagen II induces pro-inflammatory cytokines and MMPs in primary human chondrocytes.** *J Orthop Res* 2009, **27**:65-70.

doi:10.1186/ar3460
Cite this article as: Mitsui *et al.*: Prostaglandin E2 receptor type 2-selective agonist prevents the degeneration of articular cartilage in rabbit knees with traumatic instability. *Arthritis Research & Therapy* 2011 **13**:R146.

Acta Neuropathol (2011) 122:223–229
DOI 10.1007/s00401-011-0842-y

ORIGINAL PAPER

Clinicopathologic study on an ALS family with a heterozygous E478G *optineurin* mutation

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Received: 10 April 2011 / Accepted: 20 May 2011 / Published online: 5 June 2011

Abstract We investigated a family manifesting amyotrophic lateral sclerosis (ALS) with a heterozygous E478G mutation in the *optineurin* (*OPTN*) gene. Clinically, slow deterioration of motor function, mood and personality changes, temporal lobe atrophy on neuroimaging, and bizarre finger deformity were noted. Neuropathologically, TAR DNA-binding protein 43 (TDP-43)-positive neuronal intracytoplasmic inclusions were observed in the spinal and medullary motor neurons. In these cells, the immunoreactivity of nuclear TDP-43 was reduced. Consecutive sections revealed that the inclusions were also reactive with anti-ubiquitin and anti-p62 antibodies, but noticeably negative for OPTN. In addition, TDP-43/p62-positive glial cytoplasmic inclusions (GCIs) were scattered throughout the spinal cord and the medullary motor nuclei. Furthermore, Golgi fragmentation was identified in 70% of the anterior horn cells (AHCs). The presence of AHCs with preserved nuclear TDP-43 and a fragmented Golgi apparatus, which are unrecognizable in sporadic ALS, indicates that patients with the E4787G *OPTN* mutation would manifest Golgi fragmentation before loss of nuclear TDP-43. In the neocortex, GCIs were sparsely scattered among the primary motor and temporal cortices, but no neuronal TDP-43-positive inclusions were detected. In the amygdala and the ambient gyrus, argyrophilic grains and ballooned neurons were seen. The thorough neuropathologic investigations performed in this work demonstrated that OPTN-positive inclusion bodies, if any, were not prominent. We postulate that optineurinopathy is closely linked with TDP-proteinopathy and speculate that this heterozygous E478G mutation would cause ALS by acting through a dominant-negative mechanism.

Keywords Amyotrophic lateral sclerosis · Optineurin · TDP-43 · Golgi fragmentation

Electronic supplementary material The online version of this article (doi:10.1007/s00401-011-0842-y) contains supplementary material, which is available to authorized users.

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Introduction

We recently reported that mutations in the gene encoding optineurin (*OPTN*) cause amyotrophic lateral sclerosis (ALS) [15]. *OPTN* had previously been identified as a causative gene of primary open-angle glaucoma (POAG) [18]. However, the sites of mutation in the *OPTN* gene found in ALS patients were distinct from those in cases with POAG. In addition, we demonstrated that OPTN is co-localized with TAR DNA-binding protein of 43 kDa (TDP-43) or Cu/Zn superoxide dismutase (SOD1) in the pathognomonic inclusion bodies of sporadic ALS (SALS) or familial ALS associated with *SOD1* mutation (SOD1-FALS), respectively [15]. The presence of OPTN immunoreactivity in TDP-43-positive inclusions of SALS patients was subsequently confirmed by other investigators [9, 17]. In addition, we recently demonstrated that OPTN is also co-localized with *fused* in *sarcoma* (FUS) within basophilic inclusions of ALS with the *FUS* mutation and in basophilic inclusion body disease [10]. Our findings thus indicate that OPTN associates with each of three major ALS-related proteins, i.e., TDP-43, SOD1, and FUS, suggesting that the underlying pathomechanism in ALS might be attributable to dysfunctional OPTN.

We identified eight ALS cases associated with three distinct types of *OPTN* mutation (OPTN-ALS) [15]: two siblings with a homozygous deletion of exon 5, two cases with a homozygous Q398X nonsense mutation, and four patients with a heterozygous E478G missense mutation within its ubiquitin-binding domain. Detailed clinicopathological features of patients with each mutation remain unknown. Moreover, whereas the pathomechanism causing the disease by the homozygous mutations is speculated to be a loss of function resulting from nonsense-mediated mRNA decay of the transcript, that of the heterozygous E478G mutation remains uncertain.

Here, we provide further clinicopathologic information about Family 4 [15] with the E478G mutation. Although their clinical features and our neuropathologic findings have previously been reported in brief [15], we obtained some new and novel information by examining the living patient and interviewing her daughters, and by investigating the autopsied material thoroughly.

Subjects and methods

Clinical features

Three siblings were affected in this family (Fig. 1a). Their mother died at age 32 from heart disease. Their father then married the mother's younger sister and had four more children (denoted by the diamond symbol). The father was over 80 years old at death, and all of his other four children are now over 60 years of age with no signs of ALS.

The demographic and clinical features of the three OPTN-ALS patients are summarized in Table 1.

Patient III-1 had noted right-hand weakness at age 58. Muscle weakness of all four limbs, dysarthria, and dysphagia followed. Her nieces noticed that she had become irritable and touchy. She was diagnosed as having ALS and died of pneumonia after artificial ventilation for several months at age 63.

Patient III-2 suffered from right-hand weakness at age 56. Flexion deformity of her fingers gradually developed four years later. Examinations at age 61 disclosed dysarthria, atrophy, fasciculation in the tongue, and exaggerated deep tendon reflexes and bilateral extensor plantar responses in all four extremities. She was depressed but not demented. A cranial MRI demonstrated mild atrophy of the medial temporal region (Fig. 1b). She died of CO_2 narcosis without respiratory support at age 66.

Patient III-3 suffered from right-hand weakness at age 64. Leg weakness, dysarthria, and dysphagia followed slowly afterward. She could communicate well with others until age 75, when she became taciturn and depressive. A cranial CT scan at age 76 showed pronounced temporal lobe atrophy (Fig. 1c). Examinations at age 78 revealed generalized atrophy and fasciculation of skeletal muscles, reduced deep tendon reflexes, and bilateral extensor plantar responses. Atrophy of the tongue was mild. Conspicuously, her fingers were bizarrely deformed, resulting in difficulty in passive movement of any finger joints (Fig. 1d). We observed 4-Hz rhythmic tremor of the fingers of her left hand. She was awake, and eye contact was preserved, but appeared expressionless and mute. She is alive after 14 years from the onset without respiratory support.

No patients developed decubitus, ophthalmoplegia, glaucoma, or cardiac or muscular abnormalities. Blood tests, including those on alkaline phosphatase and creatine phosphokinase, were normal. Chest and spine X-rays did not show any evidence of Paget's disease.

We had previously identified a heterozygous missense mutation (c.1743A>G, E478G, exon14) in the *OPTN* gene of Patients III-2 and III-3 [15]. Genetic analysis and cognitive testing were not performed on the other family members because of the lack of informed consent.

Neuropathological examinations

Formalin-fixed, paraffin-embedded 6-μm-thick sections were deparaffinized and stained with hematoxylin and eosin (H&E) or subjected to Gallyas–Braak silver impregnation. For immunohistochemistry, after antigen retrieval by heat/autoclaving (10 min at 121°C in 10 mM sodium citrate buffer, pH 6.0), the sections were incubated with a given

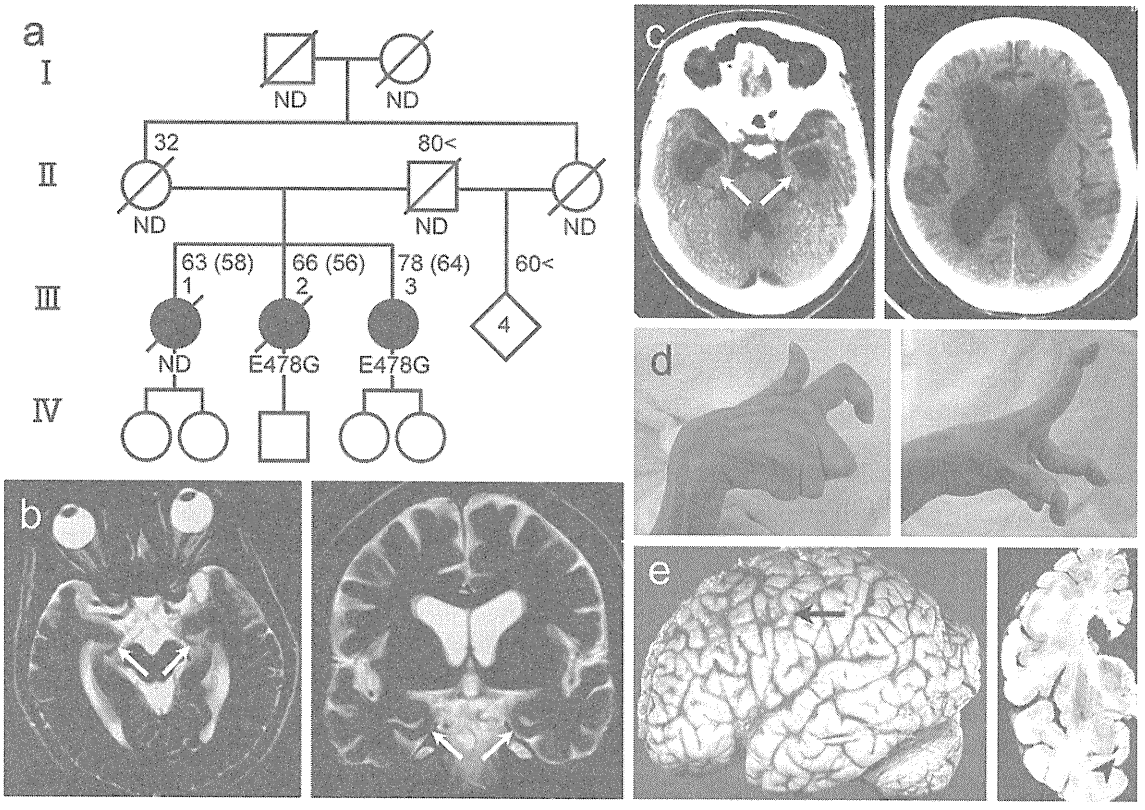
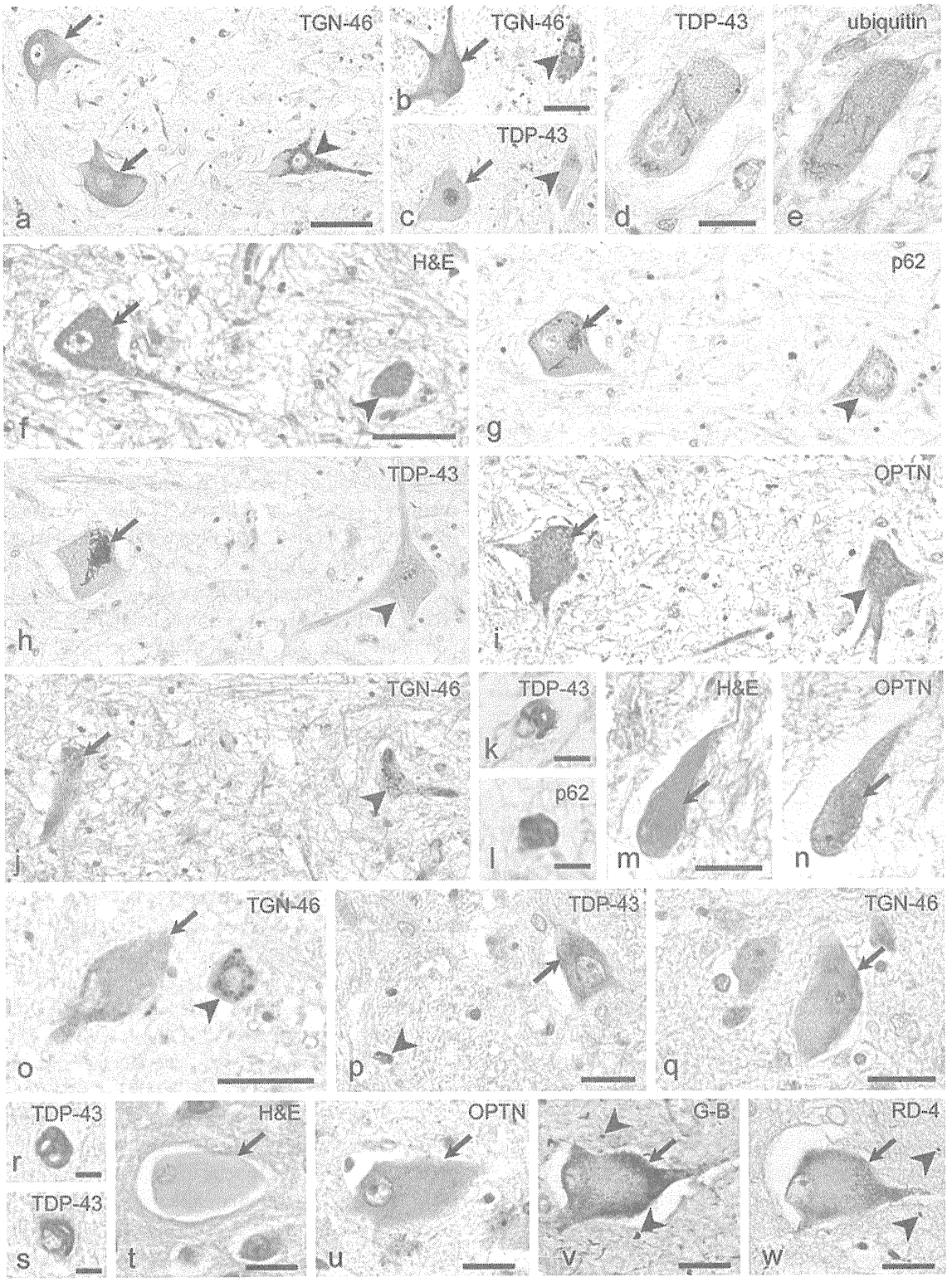


Fig. 1 Clinical and neuropathologic findings of the familial amyotrophic lateral sclerosis (FALS) patients with an *optineurin* (*OPTN*) mutation. The three patients in the family pedigree are indicated by the *solid circles* (**a**). A heterozygous E478G mutation in the *OPTN* gene was detected in Patients III-2 and III-3. *ND* not determined. Age at death or current age and age at disease onset are indicated *n (m)*. Deceased individuals are indicated by the *oblique line*. A cranial MRI of Patient III-2 at age 65 (**b**) reveals mild atrophy of the ambient gyri (*arrows*). A cranial CT scan of Patient III-3 at age 76 (**c**) reveals conspicuous atrophy of the medial temporal lobes (*arrows*) and mild atrophy of the frontal lobe. Gradually progressive bizarre deformity of the hands of Patient III-3 is striking (**d**). Photographs of the brain from Patient III-2 (**e**) reveal slight atrophy of the motor cortex (*arrow*) and of the ambient gyrus (*arrowhead*)

Table 1 Demographic and clinical features of patients with a heterozygous E478G *OPTN* mutation

Patient	III-1	III-2	III-3
Age at onset (years)	58	56	64
Gender	Female	Female	Female
Symptom at onset	Right-hand weakness	Right-hand weakness	Right-hand weakness
Upper motor neuron signs	Unknown	+	+
Lower motor neuron signs	+	+	+
Cognitive symptoms	Personality change	Depression	Depression
Other clinical features	–	Finger deformity	Finger deformity, Parkinsonian tremor
Neuroimaging	Unknown	Mild temporal lobe atrophy	Marked temporal lobe atrophy
Disease duration (years)	5	10	>14
Artificial ventilation	For several months	–	–
Cause of death	Pneumonia	CO_2 narcosis	Alive
Genetic analysis	Unavailable	E478G in *OPTN* gene	E478G in *OPTN* gene

primary antibody (listed in Online Resource 1) overnight at 4°C. Bound antibodies were detected with the appropriate Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA), with 3,3′-diaminobenzidine tetrahydrochloride used as the chromogen. All sections were counterstained with hematoxylin after immunohistochemistry. Some sections were stained with H&E, photographed, decolorized with 70% ethanol, and then immunostained for OPTN. The tissues from three age-matched neurologically normal subjects served as controls.



We assessed staining specificity by replacing the primary antibodies with an appropriate amount of non-immune rabbit serum or phosphate-buffered saline solution containing 3% bovine serum albumin. No deposits of reaction products were seen in the sections thus treated (data not shown).

Procedures involving use of human material were performed in accordance with ethical guidelines set by Shiga University of Medical Science and the Helsinki Declaration of 1983. No frozen tissue was available.

◀ **Fig. 2** Representative photomicrographs of the lumbar anterior horn (**a–n**), the facial nucleus (**o**, **p**), and the cerebral cortices (**q–w**) from Patient III-2. Immunostaining with anti-trans-Golgi-network 46 (*TGN-46*) antibody demonstrates evident fragmentation of the Golgi apparatus (GA) in some of the anterior horn cells (AHCs, *arrows*), in comparison with the preserved GA (*arrowhead*) in others (**a**). Consecutive sections stained with anti-TGN-46 (**b**) and anti-TDP-43 (**c**) antibodies indicate a neuron with normal nuclear TDP-43 immunoreactivity and obviously fragmented GA (*arrow*). The other neuron in these sections has a normal GA with preserved TDP-43 nuclear staining (*arrowhead*). A noticeable skein-like cytoplasmic inclusion immunoreactive for TDP-43 (**d**) and ubiquitin (**e**) is identifiable in consecutive sections. The physiological nuclear TDP-43 immunoreactivity is absent (**d**). Five consecutive sections stained with H&E (**f**) and immunostained for p62 (**g**), TDP-43 (**h**), optineurin (**i**), and GA (**j**) in this order reveal that a TDP-43-positive skein-like inclusion (**h**, *arrow*) is also reactive with anti-p62 antibody (**g**, *arrow*), which inclusion is indiscernible on the H&E-stained section (**f**, *arrow*). Note that the inclusion is devoid of optineurin (OPTN-I)-labeling (**i**, *arrow*). The GA is fragmented in this neuron (**j**, *arrow*) compared with the spared AHC with preserved TDP-43 nuclear staining (**f–j**, *arrowheads*). Glial cytoplasmic inclusions (GCIs) immunoreactive with anti-TDP-43 (**k**) and anti-p62 (**l**) antibodies are scattered throughout the spinal cord. The eosinophilic cytoplasmic hyaline region of this AHC (**m**) was decolorized and re-stained with the OPTN-C antibody (**n**), resulting in positive staining; however, prominent OPTN-positive inclusion bodies were not evident. GA fragmentation is apparent in this motor neuron of the facial nucleus immunostained with TGN-46 antibody (**o**, *arrow*), whereas another neuron has a preserved GA (*arrowhead*). By staining with anti-TDP-43 antibody, a skein-like inclusion (**p**, *arrow*) and a GCI (*arrowhead*) are clearly identifiable in the facial nucleus. A Betz cell within the primary motor cortex (**q**) shows reduced immunoreactivity with TGN-46 antibody (*arrow*). Only sparsely scattered TDP-43-positive GCIs are detectable in the frontal (**r**) and the temporal (**s**) cortices. Ballooned neurons in the ambient gyrus (**t–w**, *arrows*) are immuno-positive in their entire cytoplasm for OPTN (**u**), stained at their periphery by Gallyas–Braak (*G–B*) silver staining (**v**, *arrow*), and are reactive with anti-4-repeat tau (*RD-4*) antibody (**w**, *arrow*). Argyrophilic grains (**v**, *arrowheads*), immuno-positive for 4-repeat tau (**w**, *arrowheads*), are also observed. *Scale bars* 50 μm (**a**, **b**, **f**, **o**), 20 μm (**d**, **m**, **p**, **q**, **t–w**), and 10 μm (**k**, **l**, **r**, **s**)

Results

The brain of Patient III-2 weighed 1,250 g. Macroscopically, the primary motor and medial temporal cortices appeared slightly atrophic (Fig. 1e).

Throughout the spinal cord, the anterior horns and the corticospinal tracts had degenerated. Additional immunohistochemical investigation revealed characteristic fragmentation of the Golgi apparatus (GA) in the anterior horn cells (AHCs; Fig. 2a). Quantitative analysis using a method described elsewhere [8] revealed that 72.8% (75/103) of the AHCs from eight distinct spinal cord segments had fragmented GAs. Analysis of consecutive sections immunostained for GA and TDP-43 revealed GA fragmentation not only in all the AHCs with reduced nuclear TDP-43 immunoreactivity but also in a substantial number of those with preserved nuclear TDP-43 (Fig. 2b, c). In contrast, a normal staining pattern for GAs was observed for non-motor neurons.

More importantly, we identified TDP-43/ubiquitin-positive skein-like inclusions in AHCs (Fig. 2d, e). The nucleus of these inclusion-bearing neurons was invariably immunonegative for TDP-43. Consecutive sections revealed that the TDP-43-positive inclusions were also reactive with anti-p62 antibody; they were difficult to recognize on H&E-stained sections and noticeably negative for OPTN on use of either the OPTN-C or OTPN-I antibodies (Fig. 2f–i). This finding was confirmed by double immunofluorescence investigation (Online Resource 2). The GA in AHCs with such inclusions was fragmented (Fig. 2j). We identified inclusions in 12.5% (19/152) of AHCs on 20 cervical and lumbar cord sections immunostained for TDP-43. In addition, TDP-43/p62-positive glial cytoplasmic inclusions (GCIs) were scattered throughout the spinal cord (Fig. 2k, l).

Careful examination of 265 AHCs on 30 H&E-stained sections revealed no Bunina bodies or round hyaline inclusions in these cells. Cystatin C immunohistochemistry failed to detect Bunina bodies in 182 AHCs examined. Eosinophilic intracytoplasmic regions were noted in several AHCs, which showed immunoreactivity when decolorized and then re-stained with each of the anti-OPTN antibodies (Fig. 2m, n). Occasionally, these eosinophilic retentions appeared to have formed inclusion-like structures; however, OPTN-positive prominent inclusion bodies delineated by a distinct margin were completely unrecognizable.

In the hypoglossal and facial nuclei, motoneurons were depleted in number, the GA was fragmented, and TDP-43-positive inclusions were identified (Fig. 2o, p). Betz cells were mildly depleted in number, and the remaining cells had reduced immunoreactivity for GA (Fig. 2q). TDP-43-immunoreactive GCIs were sparsely scattered among the medullary motor nuclei (Fig. 2p), primary motor and temporal cortices (Fig. 2r, s), putamen, and thalamus, but no neuronal intracytoplasmic inclusions were found other than in the spinal and medullary motor neurons. No intranuclear inclusions were identifiable throughout the central nervous system.

In the amygdala and the ambient gyrus, numerous argyrophilic, 4-repeat tau-positive grains, and several ballooned neurons were seen (Fig. 2t–w). The cytoplasm of these neurons was eosinophilic, and diffusely immunopositive for OPTN and phosphorylated neurofilaments; the cells were stained at their periphery by Gallyas–Braak silver impregnation and with anti-4-repeat tau antibody. There was faint, if any, immunoreactivity indicating ubiquitin, and the cells were negative for p62, α-synuclein, 3-repeat tau, TDP-43, FUS, SOD1, and ApoE. This III-2 case corresponded to argyrophilic grain disease (AGD), stage II [5, 20].

By amyloid β and AT8-immunohistochemistry this case was graded as amyloid stage A and NF stage II,

respectively [3]. Immunostaining for α-synuclein, FUS, and SOD1 revealed no pathologies. Additional genetic analysis of Patients III-2 and III-3 revealed no mutations in their *TARDBP*, *GRN* or *VCP* genes.

Discussion

Motor symptoms of our patients were indistinguishable from those of SALS. However, the rate of deterioration was noticeably slow in both of the genetically proven patients, suggesting that slow progression might be a feature of patients with a heterozygous E478G *OPTN* mutation. Progression was faster for their elder sister, whose DNA was unavailable, showed, implying intrafamilial variability. All the patients developed personality and mood changes, and neuroimaging showed medial temporal lobe atrophy. These features are consistent with those of AGD [5], which was confirmed neuropathologically for Patient III-2. In SALS, concomitant AGD is reported in 7.7–22% of cases [14, 20]. Whether FALS with mutated *OPTN* would be prone to coincide with AGD awaits clarification. Furthermore, finger deformity was observed in our patients. This feature might be a consequence either of dystonia or of chronic arthritis induced by disinhibited NF-κB because of the *OPTN* mutation [15]. The parkinsonian tremor observed in Patient III-3 could be a manifestation of the E478G mutation or simply coincidental.

Neuropathologically, neuronal intracytoplasmic inclusions immuno-positive for TDP-43, ubiquitin, and p62 were unequivocally identified in the spinal and medullary motoneurons. They were morphologically indistinguishable from those observed in SALS. However, OPTN was noticeably not co-localized within the inclusions, in contrast with those of SALS [15]. Although negative immunohistochemical results inherently warrant further investigation, this finding suggests that not only the mutated but also the wild-type OPTN would be impaired in its association with TDP-43. The molecular link between OPTN and TDP-43 is unknown. OPTN might function in TDP-43 transportation for degradation, and hence, dysfunctional OPTN could cause TDP-43 mislocalization, resulting in neurodegeneration.

TDP-43 pathology associated with FALS (ALS-TDP) and/or frontotemporal lobar degeneration (FTLD-TDP) has been reported [13] in patients with mutations in genes encoding TDP-43 (*TARDBP*), progranulin (*GRN*), and valosin-containing protein (*VCP*), and in one case with a mutation in *ANG* encoding angiogenin, who manifested atypical clinicopathological features [21]. TDP-43 pathology indistinguishable from that of SALS and/or FTLD was observed for mutations in *TARDBP* [13], through both gains and losses of function [22]. Patients with *GRN* mutations manifest FTLD, and TDP-pathology develops principally in the neocortex [12], through a haploinsufficiency mechanism [2, 4]. *VCP* mutations were originally identified in patients with inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD) [23]. Identification of TDP-43, but not VCP, within ubiquitinated inclusions in these cases implies that *VCP* mutations lead to a dominant-negative loss of VCP function, with degradation of TDP-43 [16]. More recently, *VCP* mutations were also shown to cause autosomal dominantly inherited FALS [11]. One autopsy case revealed motor neuron degeneration with intracytoplasmic TDP-43-positive inclusions and Bunina bodies in the remaining cells. Our patients showed no mutation of their *TARDBP*, *GRN*, or *VCP* gene. However, the association of *OPTN* with Paget's disease, found by a recent genome-wide association study [1], and the similar biological function of OPTN and VCP warrant further investigation.

The presence of TDP-43 pathology has been reported in 60% of AGD cases [6]. In those cases, TDP-43-positive structures were mainly observed in the limbic regions and lateral occipitotemporal cortex [6]. The difference between the distribution of TDP-43 pathology of our patient and that of AGD cases implies that the pathomechanism of TDP-43 pathology in optineurinopathy would be distinct from that in AGD.

GA fragmentation in our Patient III-2 is plausible, because OPTN plays an important role in maintaining the GA [19]. The number of AHCs with GA fragmentation for our case (72.8%) was notably higher than that reported for SALS (8.3–52.6%, mean 29.6%) [8]. However, because this percentage varies markedly in SALS patients, it remains to be elucidated whether the ratio of GA fragmentation in AHCs of OPTN-FALS patients would be generally higher than that of SALS patients. Moreover, the presence of AHCs with preserved nuclear TDP-43 and showing fragmented GA, unrecognizable in SALS [7], indicates that patients with the E478G *OPTN* mutation would manifest GA fragmentation before loss of nuclear TDP-43. The relevance of GA fragmentation and TDP-43 nuclear staining to ALS awaits further clarification. In contrast, consistently preserved GA of non-motor cells implies that unrecognized GA-maintaining systems other than the OPTN system are operating in those neurons, affording them less vulnerability to dysfunctional OPTN.

The mutations of the *OPTN* gene causing FALS are unique in that recessive and dominant traits have similar symptoms. The mechanisms of neurodegeneration in the homozygous deletion of exon 5 and the homozygous Q398X nonsense mutation are conceivably speculated to be a loss function resulting from nonsense-mediated mRNA decay of the transcript. In contrast, the pathomechanisms operating in the case of the heterozygous E478G mutation remain unknown. The mechanism of dominant mutations causing a disease is assumed to be toxic gain-of-function, loss of function because of haploinsufficiency, or a dominant-

negative loss of OPTN function. Among these, a gain-of-function mechanism would be implausible because diseases caused by such a mechanism are usually associated with the presence of distinct inclusion bodies consisting of mutant proteins. However, the thorough neuropathologic investigations performed in this work demonstrated that OPTN-positive inclusion bodies, if any, were not prominent in our patient. A haploinsufficiency mechanism would be also unlikely, because individuals with the heterozygous exon 5 deletion or Q398X mutation, in whom half of the amount of OPTN is abolished by nonsense-mediated mRNA decay, manifest no motor neuron signs although the number of such subjects examined thus far is small. In contrast, a dominant-negative loss-of-function mechanism would be a possibility; being similar to that for patients with *VCP* mutations who manifest FTLD [16]; ubiquitinated inclusions identified in the AHCs of our patient demonstrated immunoreactivity for TDP-43, but not for OPTN. OPTN is reported to form homohexamers [24] and, thus, mutant OPTN could conceivably impair the formation of properly functioning hexamers, thus having a dominant-negative effect. This notion is consistent with the fact that the four patients with proved heterozygous E478G *OPTN* mutation [15] had later onset and longer disease duration (55.0 ± 6.7 years, longer than 7.6 ± 5.5 years (1 patient is still alive), respectively) than those with homozygous *OPTN* null mutations (41.3 ± 8.5 years and 4.0 ± 3.6 years, respectively).

For determination of the clinicopathologic features and pathomechanism of FALS with mutated *OPTN*, further studies with additional cases are needed.

Acknowledgments This work was supported in part by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (no. 21500336) and from the Japan Science and Technology Agency (AS2211173G). We thank Professor Yoshifumi Yokota (Department of Biochemistry and Bioinformative Sciences, University of Fukui) for his helpful advice, Drs Makio Takahashi, Kengo Uemura, and Hirofumi Yamashita (Department of Neurology, Kyoto University Graduate School of Medicine) for valuable discussions, Dr Masaya Hashimoto (Takao Hospital) for providing clinical information on Patient III-3, and Miss Hitomi Nakabayashi for her excellent technical assistance.

Conflict of interest The authors declare they have no conflict of interest.

References

1. Albagha OM, Visconti MR, Alonso N et al (2010) Genome-wide association study identifies variants at CSF1, OPTN and TNFRSF11A as genetic risk factors for Paget's disease of bone. Nat Genet 42:520–524
2. Baker M, Mackenzie IR, Pickering-Brown SM et al (2006) Mutations in progranulin cause tau-negative frontotemporal dementia linked to chromosome 17. Nature 442:916–919
3. Braak H, Braak E (1991) Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 82:239–259
4. Cruts M, Gijselinck I, van der Zee J et al (2006) Null mutations in progranulin cause ubiquitin-positive frontotemporal dementia linked to chromosome 17q21. Nature 442:920–924
5. Ferrer I, Santpere G, van Leeuwen FW (2008) Argyrophilic grain disease. Brain 131:1416–1432
6. Fujishiro H, Uchikado H, Arai T et al (2009) Accumulation of phosphorylated TDP-43 in brains of patients with argyrophilic grain disease. Acta Neuropathol 117:151–158
7. Fujita Y, Mizuno Y, Takatama M, Okamoto K (2008) Anterior horn cells with abnormal TDP-43 immunoreactivities show fragmentation of the Golgi apparatus in ALS. J Neurol Sci 269:30–34
8. Gonatas NK, Stieber A, Mourelatos Z et al (1992) Fragmentation of the Golgi apparatus of motor neurons in amyotrophic lateral sclerosis. Am J Pathol 140:731–737
9. Hortobágyi T, Troakes C, Nishimura AL et al (2011) Optineurin inclusions occur in a minority of TDP-43 positive ALS and FTLD-TDP cases and are rarely observed in other neurodegenerative disorders. Acta Neuropathol 121:519–527
10. Ito H, Fujita K, Nakamura M et al (2011) Optineurin is co-localized with FUS in basophilic inclusions of ALS with FUS mutation and in basophilic inclusion body disease. Acta Neuropathol 121:555–557
11. Johnson JO, Mandrioli J, Benatar M et al (2010) Exome sequencing reveals VCP mutations as a cause of familial ALS. Neuron 68:857–864
12. Josephs KA, Ahmed Z, Katsuse O et al (2007) Neuropathologic features of frontotemporal lobar degeneration with ubiquitin-positive inclusions with progranulin gene (PGRN) mutations. J Neuropathol Exp Neurol 66:142–151
13. Mackenzie IR, Rademakers R, Neumann M (2010) TDP-43 and FUS in amyotrophic lateral sclerosis and frontotemporal dementia. Lancet Neurol 9:995–1007
14. Martinez-Lage P, Munoz DG (1997) Prevalence and disease associations of argyrophilic grains of Braak. J Neuropathol Exp Neurol 56:157–164
15. Maruyama H, Morino H, Ito H et al (2010) Mutations of optineurin in amyotrophic lateral sclerosis. Nature 465:223–226
16. Neumann M, Mackenzie IR, Cairns NJ et al (2007) TDP-43 in the ubiquitin pathology of frontotemporal dementia with VCP gene mutations. J Neuropathol Exp Neurol 66:152–157
17. Osawa T, Mizuno Y, Fujita Y, Takatama M, Nakazato Y, Okamoto K (2011) Optineurin in neurodegenerative diseases. Neuropathology. doi:10.1111/j.1440-1789.2011.01199.x
18. Rezaie T, Child A, Hitchings R et al (2002) Adult-onset primary open-angle glaucoma caused by mutations in optineurin. Science 295:1077–1079
19. Sahlender DA, Roberts RC, Arden SD et al (2005) Optineurin links myosin VI to the Golgi complex and is involved in Golgi organization and exocytosis. J Cell Biol 169:285–295
20. Saito Y, Ruberu NN, Sawabe M et al (2004) Staging of argyrophilic grains: an age-associated tauopathy. J Neuropathol Exp Neurol 63:911–918
21. Seilhean D, Cazeneuve C, Thuriès V et al (2009) Accumulation of TDP-43 and alpha-actin in an amyotrophic lateral sclerosis patient with the K17I ANG mutation. Acta Neuropathol 118:561–573
22. Van Deerlin VM, Leverenz JB, Bekris LM et al (2008) TARDBP mutations in amyotrophic lateral sclerosis with TDP-43 neuropathology: a genetic and histopathological analysis. Lancet Neurol 7:409–416
23. Watts GD, Wymer J, Kovach MJ et al (2004) Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. Nat Genet 36:377–381
24. Ying H, Shen X, Park B, Yue BY (2010) Posttranslational modifications, localization, and protein interactions of optineurin, the product of a glaucoma gene. PLoS One 5:e9168

The Use of Induced Pluripotent Stem Cells in Drug Development

H Inoue[1,2,3] and S Yamanaka[1,2,4]

Induced pluripotent stem cell (iPSC) technology is revolutionizing medical science, allowing the exploration of disease mechanisms and novel therapeutic molecular targets, and offering opportunities for drug discovery and proof-of-concept studies in drug development. This review focuses on the recent advancements in iPSC technology including disease modeling and control setting in its analytical paradigm. We describe how iPSC technology is integrated into existing paradigms of drug development and discuss the potential of iPSC technology in personalized medicine.

The ability of cells to differentiate into various cell types—known as "pluripotency"—is a hallmark of embryonic stem cells (ESCs). Stem cells belong to one of two major categories according to their potency of differentiation: organ-specific stem cells and pluripotent stem cells. Organ-specific stem cells generally have limited potential for growth and differentiation. In contrast, pluripotent stem cells, such as ESCs[1–3] and induced pluripotent stem cells (iPSCs),[4–6] replicate in culture dishes and are theoretically capable of giving rise to any of the cell types found in the body (Figure 1).

The development of cellular reprogramming techniques leading to iPSCs has dramatically changed the landscape of stem cell research and application by providing a modality that circumvents the two major issues hampering fulfillment of the great potential of human ESCs.[4–6] One is the ethical issue associated with the derivation of human ESCs from human fertilized eggs, and the other is the immunological incompatibility between ESC-derived donor organs or cells and the recipients because of histocompatibility–antigenic factors.[4–6] As iPSCs are transforming the field of regenerative medicine, the reprogramming approach is also becoming a platform for drug discovery research.

DISCOVERY OF iPSCs

Reprogramming inducers

Transduction of four genes encoding transcription factors highly functional in ESCs (i.e., Oct3/4, Sox2, Klf4, and c-Myc) was discovered to be sufficient to trigger reprogramming of both mouse and human somatic cells and to generate cells closely resembling the respective ESCs.[4–6] The term coined for these reprogrammed ESC-like cells was "iPSCs."[4] Subsequent research from our laboratory as well as from others has revealed several alternative methods for generating iPSCs.[7–9]

Among the quartet of transcription factors involved in reprogramming,[9] Oct3/4 is expressed specifically in ESCs and germ cells but not in somatic cells.[9] The forced expression of Oct3/4 in mouse or human Sox2-expressing neural stem cells can give rise to iPSCs, albeit with low reprogramming efficiency.[9] There are reports of iPSC generation even in the absence of the Oct3/4 transgene, but the efficiency of generation is very low.

Sox2, which is a key partner of Oct3/4, is expressed almost exclusively in ESCs, germ cells, and nerve cells. The deletion of Sox2 causes the death of the embryo, suggesting its crucial role in embryogenesis.[9] Sox family proteins, including Sox2, show functional overlap with each other. Although the conventional reprogramming method requires Sox2 transgene, inhibition of the transforming growth factor beta (TGF-β) was shown to be capable of replacing Sox2 in reprogramming mouse embryonic fibroblasts.[9] Moreover, in some cell types, such as neural stem cells, melanocytes, and melanoma cells, the Sox 2 transgene is not necessarily a requirement for iPSC generation.[9] These findings indicate the opportunistic nature of Sox transgene requirement in iPSC reprogramming.

Kruppel-like transcription factor 4 (Klf4) is a downstream target gene of the signaling pathway of the cytokine leukemia inhibitory factor—Stat3. Klf4 has overlapping functions with other Klf transcriptional factors (Klf2 and Klf5).[10] During the reprogramming process, Klf4 binds to the Oct3/4-Sox2 complex[11] and, together with homeobox protein PBX1, it underpins iPSC identity by regulating expression of Nanog, one of

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Received 29 November 2010; accepted 4 February 2011; advance online publication 23 March 2011. doi:10.1038/clpt.2011.38

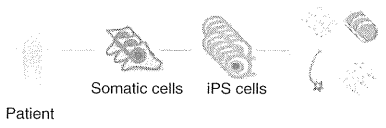

Figure 1 Generation of induced pluripotent stem cells (iPSCs) and their differentiation potential. iPSCs are derived from easily accessible somatic cells. In contrast to organ-specific stem cells, pluripotent stem cells such as embryonic stem cells and iPSCs show the ability to differentiate into many different cell types in culture. This allows *in vitro* generation of specific tissue cell types with the characteristics of the disease phenotype, from patient-derived iPSCs.

the pluripotency-defining proteins.[12] The Klf4 transgene is not necessary for reprogramming under certain conditions such as histone deacetylase inhibition[13,14] and in the absence of the tumor suppressor gene *Trp53*.[15]

The reprogramming process is highly enhanced by c-Myc,[16] although its inclusion in the reprogramming process should be discouraged, given its clear oncogenic potential. c-Myc expression is ubiquitous, in contrast to the other Myc family members, N- and L-Myc.[9] L-Myc and c-Myc mutants, all of which have little transformation activity, were shown to promote the generation of human iPSCs with more efficiency and specificity as compared with wild-type c-Myc.[7]

For these reasons, the original quartet of reprogramming factors (Oct3/4, Sox2, Klf4, and c-Myc) are not necessary under certain conditions and could be modified in accordance with the experimental context. Clearly, it is necessary to obtain a better understanding of the mechanisms underlying somatic cell reprogramming in order to fully validate the iPSC technology.

iPSC/ESC differentiation repertoire and tumorigenicity

In vitro culture and the differentiation of stem cells provide us with opportunities for disease modeling, drug discovery, and cell replacement therapy. The generation of specific functional cell types from ESCs/iPSCs has been demonstrated, including neural cells, vascular endothelia, smooth muscle cells, cardiomyocytes, hematopoietic cells, pancreatic insulin-producing cells, and hepatocyte-like cells.[17–23] The current differentiation repertoire includes more than 200 types of somatic cells.[24] These cells may be applied in regenerative medicine, and work is ongoing to overcome the remaining hurdles. Significant challenges in iPSC-based regenerative medicine include (i) the tumorigenic potential inherent to the reprogramming methods, (ii) the difficulty in achieving highly targeted differentiation, and (iii) the complexity of cellular transplantation techniques.[25]

Eradicating the tumorigenic potential of iPSC-derived cells is of fundamental importance to further enhance clinical transfer of the technology. Interestingly, the teratoma-forming propensities of secondary neurospheres, after transplantation into the brains of nonobese/severe combined immunodeficient mice, vary significantly depending on the origin of the tissue from which the iPSCs were derived.[26] For example, secondary neurospheres from iPSCs generated from adult tail-tip fibroblasts of mice showed the highest propensity for tumorigenicity, whereas those from iPSCs originating from mouse embryonic fibroblasts and gastric epithelial cells showed the lowest such propensity, the latter being comparable, in this regard, to those obtained from ESCs. Secondary neurospheres from hepatocyte iPS cells showed an intermediate teratoma-forming propensity. The use of iPSCs in regenerative medicine clearly requires further improvement of differentiation protocols in order to minimize tumorigenicity.

iPSC-BASED DISEASE MODELING

There are many potential causes for the failed translation of drug discovery from levels of molecular and animal models to human therapeutics. In particular, the success of preclinical phases of drug development is based on animal models.[27] Furthermore, <10% of the compounds that enter the clinical phase of testing reach the stage of market approval; the estimated cost of the entire drug development process is US$1.2–1.7 billion per drug.[27–29] Drug discovery/development platforms using iPSC-based disease models could be useful in filling the gap between animal models and clinical trials.

iPSC technology is expected to provide innovative tools for drug development via high-throughput therapeutic/toxicity screening, using differentiated cells from patient-derived iPSCs. This disease-modeling approach to drug discovery will also increase our understanding of disease progression and biology in specific cell types, which could possibly lead to redefining known aspects of diseases.[30] Patient-specific iPSCs provide not only genetic information but also potential phenotype attributes. In addition, iPSCs can be generated from patients irrespective of whether the disease is in the familial or the sporadic form. Drug screening platforms can be developed to test compounds (including biologics such as small hairpin RNAs) that are able to make the disease-related phenotype revert to that of the non-disease control.[30]

The available lines of human ESCs are variable with regard to epigenetic information, expression profile, and differentiation propensity.[31,32] Significant intrinsic variability also remains in iPSC lines, and abnormal expression of imprinted genes has been detected in a significant number of them.[33] These inter-iPSC differences were attributed to the introduction of reprogramming factors using randomly integrating viral vectors, and/or to persistent donor cell gene expression.[34] However, even if iPSCs are generated in the absence of integrating factors, intrinsic variability remains,[35–37] including in the matter of neuronal differentiation competence.[38] Moreover, expression profile analysis of integration-free human iPSCs has shown an expression signature in iPSCs that is distinct from those of both the original population and standard human ESCs.[35] It is also reported that there is a strong correlation between gene expression signatures and specific laboratories, in both ESC and iPSC lines, because of differences in the *in vitro* microenvironment.[39] These observations suggest that further dissecting the intrinsic variability of iPSCs may provide clues regarding the wild-type iPSCs that would be most suitable as experimental controls and the number of control lines that should be obtained for each experiment.[35] Despite these variations, however, many

Table 1 Disease modeling using disease-specific iPSCs

Disease (responsible gene)	Inheritance pattern	Age of onset	Recapitulated phenotype/proof of drug efficacy
Spinal muscular atrophy[41] (*SMN1, SMN2*)	AR	Infancy to adolescence	1. Decreased no. (%) of ChAT$^+$/Tuj1$^+$ neurons 2. Decreased SMN protein level (evaluated with WB/IA) 3. Rescue phenotype 2 with 1 mmol/l valproic acid
Familial dysautonomia[42] (*IKBKAP*)	AR	Infancy	1. Increased abnormal splicing in differentiated neural crest 2. Decreased no. (%) of ASCL1+, Tuj1+ neurons 3. Migratory dysfunction (scratch assay) 4. Partial rescue phenotype 1, 2 with 100 μmol/l kinetin
Fanconi anemia[43] (*FANC A~N*)	AR/XR	First decade ~4/5th decade	1. Unsuccessful at obtaining iPSCs from patient's fibroblast → after "*in vitro* genetic correction" of patient's fibroblast Successful in obtaining iPSCs (chromosomal instability) 2. Differentiate into CD34+/hematopoietic progenitors
Dyskeratosis congenita[44] (*XR: DKC1*)	XR (AR/AD)	Adolescence	1. Elongated telomere in iPSCs (TERT/TERC↑) 2. Shortened telomere after differentiation (TERT/TERC↓)
LEOPARD syndrome[45] (*PTPN11, RAF1, SHOC2*)	AD	Infancy to adolescence	1. Enlarged cell size of differentiated cardiomyocyte 2. Inactivated RAS-MAPK pathway (bFGF induction)
Rett syndrome[46] (*MeCP2*)	XR	6–18 Months	1. Reduced no. of glutamatergic synapses and morphological alterations (synapsin puncta at dendrites), rescued by IGF-1 (ng/ml) 2. Reduced RTT protein level/cell size and rescue by gentamicin (100 μg/ml) at Q244X clone 3. Reduced activity-dependent calcium transients 4. Reduced spontaneous postsynaptic currents

AD, autosomal dominant; AR, autosomal recessive; IA, immunological analysis; IGF-1, insulin-like growth factor 1; iPSC, induced pluripotent stem cell; TERC, telomerase RNA component; TERT, telomerase reverse transcriptase gene; WB, western blot analysis; XR, X-linked recessive.

lines of disease-specific iPSCs are being generated,[40] given that several studies have actually recapitulated the phenotypes of diseases in the iPSC-derived targeted cell population and that this approach now finds a place on the drug development platform as a useful tool to complement *in vivo* experiments (Table 1).[41–46]

To avoid both inter- and intrapatient clonal variations of iPSCs, it is necessary to purify targeted cells by fluorescence-activated cell sorting or magnetic sorting using fluorescent or magnet-labeled antibodies[27] or by high-content analysis.[47,48] The control of the prominent heterogeneity of iPS-derived differentiated cells presents a technological challenge; this continues to be the major limitation of standardized high-throughput screening, although further modifications in differentiation protocols are under way in our laboratory.

iPSC-BASED TOXICITY SCREENING

The progressive attrition of medicinal products in the long pipeline between "hit" identification and the market has become one of the concerns of the pharmaceutical industry in the past decade.[48] The development cost of a marketable product is continuing to grow.[27–29,49] In 2001, development was abandoned because of lack of efficacy in 30% of the medicines that entered clinical trials and in another 30% because of safety concerns[49] such as cardiotoxicity and hepatotoxicity. The effective development of new drugs therefore requires predictive toxicity assays of adequate accuracy during preclinical testing. The use of human iPSCs and robust protocols to differentiate them into cardiomyocytes and hepatocytes should be able to provide straightforward assays for analyzing certain aspects of drug metabolism and for assessing probable side effects. However, technological hurdles still exist with respect to achieving the desired maturity of differentiated cells[50] and minimizing the substantial heterogeneity of iPS-derived differentiated cells for the assay. Despite these limitations, significant progress has been made.

The drug-induced blockade of the ether-a-go-go related gene 1 (*hERG1*) channel is reportedly associated with an increased duration of ventricular repolarization, causing prolongation of the QT interval (i.e., long-QT syndrome).[51–54] Data related to the electrophysiological capacity and responsiveness of human iPSC-derived cardiomyocytes in response to several cardiac and noncardiac drugs have been reported.[51–54] Cardiac toxicity screening tools based on these approaches will soon become available.

The efficient generation of functional hepatocyte-like cells from iPSCs has been also reported.[20,21] The use of three-dimensional culture as well as co-culture systems (e.g., associating Kupffer and/or endothelial cells with hepatocytes in order to mimic the *in vivo* hepatic context) are among the strategies now recognized to enhance the generation of even more mature cells.[49]

To establish toxicity screening tools using iPSC technology, validation is essential. In particular, it is crucial to show high fidelity of the iPSC-based toxicity screening tools in reproducing, *in vitro,* the toxicity profiles of "hit" drugs that had been eliminated from the development pipeline because of safety concerns.

CHALLENGES IN iPSC-BASED APPROACHES

Aging process and environmental effects

Several diseases that are characterized by onset in early life have been successfully modeled using iPSC technology.[41–46] On the other hand, in some diseases (including neurodegenerative

diseases) that are age dependent, patient-specific iPSC-derived neural cells may not immediately manifest the disease phenotype as compared with normal control cells, under basal cell culture conditions.[30,55,56] This may also apply to drug toxicity that shows age-dependent susceptibility. Identification of disease/toxicity-related phenotypes in short-term settings *in vitro* appears particularly challenging, but it may be possible to achieve by mimicking the aging process with stressors such as oxygen reactive species, proinflammatory factors, or toxins.[30,55,56] Identification of new and more effective and relevant stressors that can accelerate the process of eliciting phenotypes in models of late-onset diseases will therefore be an important goal for future disease modeling.[30,55,56]

Even patients with monogenetic diseases manifest large genotype–phenotype variability. Therefore, it would be more difficult to establish disease modeling from sporadic-disease iPSCs, given the complexity of the different genetic backgrounds and environmental cues involved.[27,30] It will be both challenging and exciting to examine whether the same phenotype as seen in monogenic-disease modeling could be recapitulated in sporadic-disease-iPSC-derived modeling by reproducing environmental effects *in vitro*.[27,30,55,56]

Definition of "control"

Whether in selecting a therapeutic or in toxicity assays using patient-specific iPSC-derived cells, the use of well-defined, non-disease control cells is crucial. Recent genome-wide association studies[57] have demonstrated that every person has disease-relevant single-nucleotide polymorphisms, and it is therefore impossible to categorically define iPSCs that represent perfect non-disease control.

Nonetheless, we think that the following two approaches are valid for deriving iPSC-positive (disease) and negative (non-disease) controls: (i) deductive and (ii) inductive. Deductive controls would include non-disease iPSC/ESC lines with modification (e.g., disease gene transgenic and disease gene knock-in), disease gene–corrected iPSC/ESC lines generated from disease iPSC/ESCs, and iPSCs with non-disease alleles from an individual patient in somatic mosaicism (Table 2). Deductive approaches define negative and positive controls in similar genetic backgrounds, providing benchmarks of disease modeling to specify differences between disease and non-disease control, whereas contributors other than the targeted gene(s) are not considered. On the other hand, inductive controls may be non-disease iPS cell lines or iPSCs from healthy individuals or from other patients (positive control). This approach could be less complicated than the deductive method, especially if noise from iPSC variations can be further reduced.

For the deductive control setting of disease modeling, the tools for achieving expression or knockout of disease genes in hiPSCs/ESCs by random integration of vectors (including viruses, bacterial artificial chromosomes, synthetic gene delivery reagents, and a transposon/transposase system) are useful.[58–60] Also, the current development of engineered nucleases makes targeted genome modification an attractive tool with therapeutic potential that may go beyond the development of drug screening tools.[58]

Table 2 Proposed definition of "control" in induced pluripotent stem cell research

Deductive approach
Embryonic stem cell line with and without disease-introducing genetic modification
Non-disease induced pluripotent stem (iPS) cell line with and without disease-introducing genetic modification
Disease iPS cell with and without disease-correcting genetic modification
iPS cell from somatic mosaic with and without disease allele
Inductive approach
iPS cell from a patient and a disease-free family member
Disease genetic risk-ascertained iPS cell lines (preferably as a risk-absent non-disease control)
iPS cell lines from disease-phenotyped individuals (healthy or disease control)

iPSC-BASED NOVEL DRUG DEVELOPMENT PLATFORM

iPSC-based *in vitro* phase III

Diseases can be divided into rare, monocausal genetic diseases and a large group of sporadic, multifactorial diseases. No large-scale disease modeling is currently available for the latter group. Technological advances in rapid and easy iPSC generation on a large scale will realize the possibility of both *in vitro* phase III and case–control studies by using non-disease and disease controls derived from age/gender-matched donors or from family members regardless of age/gender.[30] One of the factors facilitating the process could be to obtain a blood sample from each patient in order to generate iPSCs. iPSC generation from peripheral blood drops from each patient would allow case–control studies to be carried out, although several issues must still be resolved prior to the use of iPSCs from peripheral blood cells.[61–64] First, the differentiation potency of these iPSCs must be analyzed further.[61] Peripheral blood–derived iPSCs may preserve epigenetic memories of having been blood cells and may therefore exhibit preferred differentiation into hematopoietic lineages rather than into other cell types.[61,65]

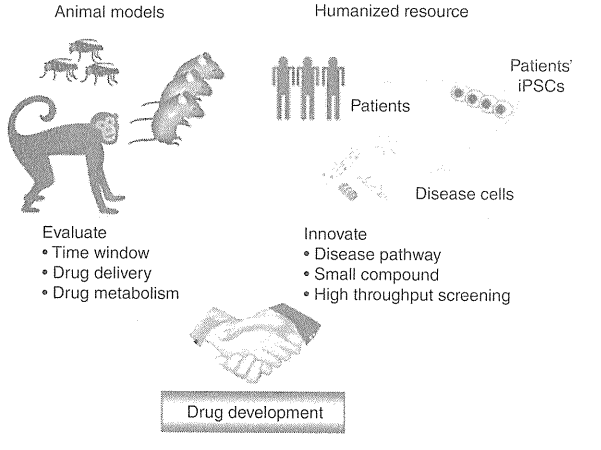

Figure 2 Combined approach involving animal models and induced pluripotent stem cell (iPSC) technology. The new iPSC technology is complemented by a drug development strategy in preclinical settings that uses animal models and other conventional approaches.

Another issue is how long and to what extent iPSC clones from terminally differentiated cells can be expanded.[61–64] Finally, the effect of the presence of pre-existing T-cell receptor rearrangements on the properties of iPSC or differentiated cells needs to be determined.[61,66] Besides minimizing the invasive biopsy procedures, reducing the time required for iPSC differentiation, resulting in lower costs, would be essential for large cohort studies, potentially leading to the discovery of novel drug targets.

iPSCs and animal models

Cell lines and animal models contribute to the exploration of disease mechanisms and drug development for various diseases. However, the animal models do not always demonstrate the same phenotypes as those seen in humans.[55] For instance, in mice the type and/or distribution of cardiac ion channels are different from those in humans, demonstrating a relatively shorter duration of action potential and higher heart rate (600 bpm).[67] An *in vitro* analysis of human cardiomyocytes is therefore critical to an understanding of the mechanism of genetics-related arrhythmias in humans.[67] Also, compounds that demonstrate significant benefit in animal models may fail to show effectiveness in clinical trials in humans.[55,68,69] The use of transgenic mice of mutant superoxide dismutase (*SOD1*), a gene found to be associated with amyotrophic lateral sclerosis,[70] enabled the identification of several compounds that relieve the disease phenotype, including vitamin E and creatine.[71–73] However, when these compounds were tested in humans, no clinical improvements were observed.[71–73] The toxicity of compounds is sometimes missed in cell lines and animal models because specific interactions with human biological processes cannot be recapitulated in these systems.[27] Also, the use of animal models for toxicity assays may be ethically problematic, the animals may be expensive to purchase and maintain, and the process may be difficult to automate.[27] Clearly, we require different drug screening models that complement these systems and represent the human condition with high fidelity.[74] iPSCs are expected to fulfill these requirement and are amenable to the demands of drug development. There are nonetheless great advantages associated with cell line–based models (which could be used for homologous culture, yielding reproducible results) and for animal models (which provide information regarding optimal time window, drug delivery, metabolism, etc.) (Figure 2). Integrated drug screening systems, consisting of disease-specific iPSC-based models as well as cell lines and animal models, would greatly enhance the efficiency of translational drug research.

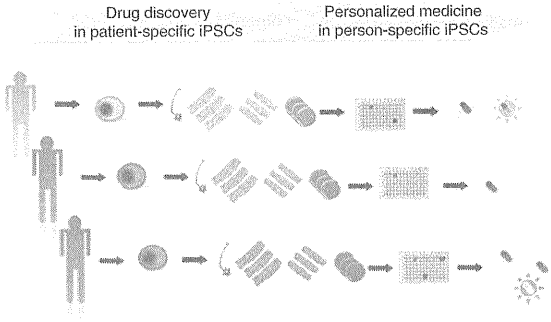

Figure 3 Personalized medicine based on induced pluripotent stem cell (iPSC) technology. iPSC technology is highly amenable to individualized approaches. Person-specific iPSCs can be derived, differentiated into specific cell types, and used for therapeutic/toxicity response assays.

Personalized medicine

The striking advantage of using iPSCs rather than ESC-based approaches is that iPSCs can be derived from any individual with relative ease, thereby allowing development of a personalized study platform on individual genomic information. iPSCs and

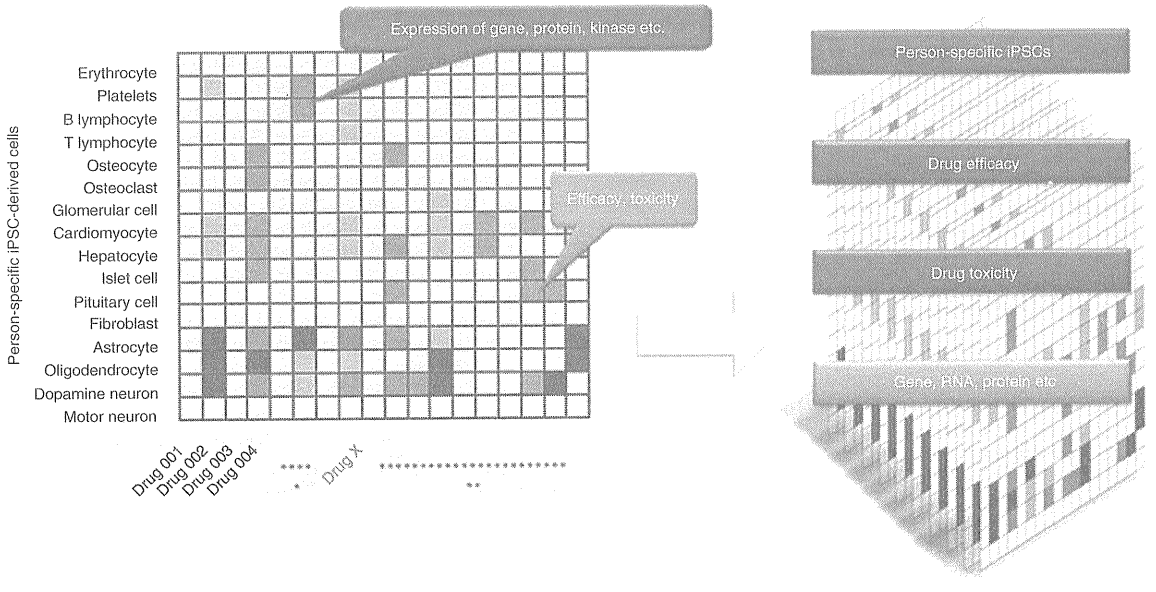

Figure 4 "Pharmaco-iPSCellomics" by person-specific iPSCs. iPSCs derived from individual subjects/patients can be differentiated into multiple cell types, thereby providing a personalized iPS-cellome platform. This cell-based system can be used for drug discovery and selection of clinical therapeutics with various biomarker end points.

differentiated cells from the iPSCs retain their personal identity, like an alter ego, suggesting that iPSC technology can be applied to disease-, patient-, and finally person-specific approaches to examine the individual differences in pharmacokinetic/pharmacodynamic features (Figure 3). Given that everyone will almost certainly become a patient at least once in his or her lifetime, individual iPSC-based predictive therapeutic and toxicity profiling of all drugs available in multiple cell types will be a logical and attractive approach. This "pharmaco-iPSCellomic" analysis (Figure 4) could eventually be available in an array-based format for high-throughput assay before specific drug therapy is prescribed for a particular disease condition.

CONCLUSION

The potential of iPS cell technology in drug discovery is enormous.[75] At the same time, the technology is still in its infancy with numerous challenges to overcome before its clinical translation is complete. The long journey has just begun. It may take years to reach the eventual goals, but the iPSC technology itself, combined with existing methods and models, will begin to contribute to the development of new cures.

ACKNOWLEDGMENTS

We express our sincere gratitude to all our coworkers and collaborators, to Shinya Ito for critical reading of the manuscript, and to Takayuki Kondo for drawing the figures. We are also grateful to Rie Kato, Eri Nishikawa, Sayaka Takeshima, Yuko Otsu, Haruka Hasaba, and Kazumi Murai for their valuable administrative support. We apologize to the researchers whose contributions to iPSC-based advancements in drug discovery we could not cite because of space limitations.

This study was supported in part by a grant from the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST) of the Japan Society for the Promotion of Science (JSPS) (S.Y.), Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and Ministry of Education, Culture, Sports, Science and Technology (MEXT) (S.Y.), a grant from the Leading Project of MEXT (S.Y., H.I.), a grant from Yamanaka iPS cell special project (S.Y., H.I.), the Japan Science and Technology Agency and Core Research for Evolutional Science and Technology (CREST) (H.I.), Grant-in-Aid from the Ministry of Health and Labour (H.I.), a Grant-in-Aid for Scientific Research (21591079) from the JSPS (H.I.), a Grant-in-Aid for Scientific Research on Innovative Area "Foundation of Synapse and Neurocircuit Pathology" (22110007) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (H.I.), a research grant from the Takeda Science Foundation (H.I.), a research grant from the Kanae Foundation for the Promotion of Medical Science (H.I.), and a research grant from the NOVARTIS Foundation for Gerontological Research (H.I.).

CONFLICT OF INTEREST

The authors declared no conflict of interest.

1. Evans, M.J. & Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156 (1981).
2. Martin, G.R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78**, 7634–7638 (1981).
3. Thomson, J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
4. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676 (2006).
5. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872 (2007).
6. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–1920 (2007).
7. Nakagawa, M., Takizawa, N., Narita, M., Ichisaka, T. & Yamanaka, S. Promotion of direct reprogramming by transformation-deficient Myc. *Proc. Natl. Acad. Sci. USA* **107**, 14152–14157 (2010).
8. Warren, L. *et al.* Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* **7**, 618–630 (2010).
9. Takahashi, K. Direct reprogramming 101. *Dev. Growth Differ.* **52**, 319–333 (2010).
10. Jiang, J. *et al.* A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nat. Cell Biol.* **10**, 353–360 (2008).
11. Wei, Z. *et al.* Klf4 interacts directly with Oct4 and Sox2 to promote reprogramming. *Stem Cells* **27**, 2969–2978 (2009).
12. Chan, K.K. *et al.* KLF4 and PBX1 directly regulate NANOG expression in human embryonic stem cells. *Stem Cells* **27**, 2114–2125 (2009).
13. Huangfu, D. *et al.* Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat. Biotechnol.* **26**, 795–797 (2008).
14. Huangfu, D. *et al.* Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nat. Biotechnol.* **26**, 1269–1275 (2008).
15. Kawamura, T. *et al.* Linking the p53 tumour suppressor pathway to somatic cell reprogramming. *Nature* **460**, 1140–1144 (2009).
16. Singh, A.M. & Dalton, S. The cell cycle and Myc intersect with mechanisms that regulate pluripotency and reprogramming. *Cell Stem Cell* **5**, 141–149 (2009).
17. Dimos, J.T. *et al.* Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* **321**, 1218–1221 (2008).
18. Karumbayaram, S. *et al.* Directed differentiation of human-induced pluripotent stem cells generates active motor neurons. *Stem Cells* **27**, 806–811 (2009).
19. Tateishi, K., He, J., Taranova, O., Liang, G., D'Alessio, A.C. & Zhang, Y. Generation of insulin-secreting islet-like clusters from human skin fibroblasts. *J. Biol. Chem.* **283**, 31601–31607 (2008).
20. Si-Tayeb, K. *et al.* Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology* **51**, 297–305 (2010).
21. Sullivan, G.J. *et al.* Generation of functional human hepatic endoderm from human induced pluripotent stem cells. *Hepatology* **51**, 329–335 (2010).
22. Taura, D. *et al.* Induction and isolation of vascular cells from human induced pluripotent stem cells–brief report. *Arterioscler. Thromb. Vasc. Biol.* **29**, 1100–1103 (2009).
23. Narazaki, G. *et al.* Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. *Circulation* **118**, 498–506 (2008).
24. Boheler, K.R. Stem cell pluripotency: a cellular trait that depends on transcription factors, chromatin state and a checkpoint deficient cell cycle. *J. Cell. Physiol.* **221**, 10–17 (2009).
25. Murry, C.E. & Keller, G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell* **132**, 661–680 (2008).
26. Miura, K. *et al.* Variation in the safety of induced pluripotent stem cell lines. *Nat. Biotechnol.* **27**, 743–745 (2009).
27. Gunaseeli, I., Doss, M.X., Antzelevitch, C., Hescheler, J. & Sachinidis, A. Induced pluripotent stem cells as a model for accelerated patient- and disease-specific drug discovery. *Curr. Med. Chem.* **17**, 759–766 (2010).
28. Kaitin, K.I. Obstacles and opportunities in new drug development. *Clin. Pharmacol. Ther.* **83**, 210–212 (2008).
29. Sollano, J.A., Kirsch, J.M., Bala, M.V., Chambers, M.G. & Harpole, L.H. The economics of drug discovery and the ultimate valuation of pharmacotherapies in the marketplace. *Clin. Pharmacol. Ther.* **84**, 263–266 (2008).
30. Marchetto, M.C., Winner, B. & Gage, F.H. Pluripotent stem cells in neurodegenerative and neurodevelopmental diseases. *Hum. Mol. Genet.* **19**, R71–R76 (2010).
31. Rugg-Gunn, P.J., Ferguson-Smith, A.C. & Pedersen, R.A. Status of genomic imprinting in human embryonic stem cells as revealed by a large cohort of independently derived and maintained lines. *Hum. Mol. Genet.* **16**(spec. no. 2), R243–R251 (2007).
32. Osafune, K. *et al.* Marked differences in differentiation propensity among human embryonic stem cell lines. *Nat. Biotechnol.* **26**, 313–315 (2008).
33. Pick, M., Stelzer, Y., Bar-Nur, O., Mayshar, Y., Eden, A. & Benvenisty, N. Clone- and gene-specific aberrations of parental imprinting in human induced pluripotent stem cells. *Stem Cells* **27**, 2686–2690 (2009).
34. Ghosh, Z., Wilson, K.D., Wu, Y., Hu, S., Quertermous, T. & Wu, J.C. Persistent donor cell gene expression among human induced pluripotent stem cells contributes to differences with human embryonic stem cells. *PLoS ONE* **5**, e8975 (2010).

35. Marchetto, M.C., Yeo, G.W., Kainohana, O., Marsala, M., Gage, F.H. & Muotri, A.R. Transcriptional signature and memory retention of human-induced pluripotent stem cells. *PLoS ONE* **4**, e7076 (2009).
36. Ramos-Mejia, V., Muñoz-Lopez, M., Garcia-Perez, J.L. & Menendez, P. iPSC lines that do not silence the expression of the ectopic reprogramming factors may display enhanced propensity to genomic instability. *Cell Res.* **20**, 1092–1095 (2010).
37. Zhou, H. *et al.* Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* **4**, 381–384 (2009).
38. Hu, B.Y. *et al.* Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. *Proc. Natl. Acad. Sci. USA* **107**, 4335–4340 (2010).
39. Newman, A.M. & Cooper, J.B. Lab-specific gene expression signatures in pluripotent stem cells. *Cell Stem Cell* **7**, 258–262 (2010).
40. Park, I.H. *et al.* Disease-specific induced pluripotent stem cells. *Cell* **134**, 877–886 (2008).
41. Ebert, A.D. *et al.* Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature* **457**, 277–280 (2009).
42. Lee, G. *et al.* Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature* **461**, 402–406 (2009).
43. Raya, A. *et al.* Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature* **460**, 53–59 (2009).
44. Agarwal, S. *et al.* Telomere elongation in induced pluripotent stem cells from dyskeratosis congenita patients. *Nature* **464**, 292–296 (2010).
45. Carvajal-Vergara, X. *et al.* Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome. *Nature* **465**, 808–812 (2010).
46. Marchetto, M.C. *et al.* A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. *Cell* **143**, 527–539 (2010).
47. Barbaric, I., Gokhale, P.J. & Andrews, P.W. High-content screening of small compounds on human embryonic stem cells. *Biochem. Soc. Trans.* **38**, 1046–1050 (2010).
48. Zanella, F., Lorens, J.B. & Link, W. High content screening: seeing is believing. *Trends Biotechnol.* **28**, 237–245 (2010).
49. Laustriat, D., Gide, J. & Peschanski, M. Human pluripotent stem cells in drug discovery and predictive toxicology. *Biochem. Soc. Trans.* **38**, 1051–1057 (2010).
50. Rubin, L.L. Stem cells and drug discovery: the beginning of a new era? *Cell* **132**, 549–552 (2008).
51. Tanaka, T. *et al. In vitro* pharmacologic testing using human induced pluripotent stem cell-derived cardiomyocytes. *Biochem. Biophys. Res. Commun.* **385**, 497–502 (2009).
52. Yoshida, Y. & Yamanaka, S. Recent stem cell advances: induced pluripotent stem cells for disease modeling and stem cell-based regeneration. *Circulation* **122**, 80–87 (2010).
53. Yokoo, N. *et al.* The effects of cardioactive drugs on cardiomyocytes derived from human induced pluripotent stem cells. *Biochem. Biophys. Res. Commun.* **387**, 482–488 (2009).
54. Asai, Y., Tada, M., Otsuji, T.G. & Nakatsuji, N. Combination of functional cardiomyocytes derived from human stem cells and a highly-efficient microelectrode array system: an ideal hybrid model assay for drug development. *Curr. Stem Cell Res. Ther.* **5**, 227–232 (2010).
55. Wichterle, H. & Przedborski, S. What can pluripotent stem cells teach us about neurodegenerative diseases? *Nat. Neurosci.* **13**, 800–804 (2010).
56. Lee, G. & Studer, L. Induced pluripotent stem cell technology for the study of human disease. *Nat. Methods* **7**, 25–27 (2010).
57. Satake, W. *et al.* Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease. *Nat. Genet.* **41**, 1303–1307 (2009).
58. Cabaniols, J.P., Mathis, L. & Delenda, C. Targeted gene modifications in drug discovery and development. *Curr. Opin. Pharmacol.* **9**, 657–663 (2009).
59. Giudice, A. & Trounson, A. Genetic modification of human embryonic stem cells for derivation of target cells. *Cell Stem Cell* **2**, 422–433 (2008).
60. Saha, K. & Jaenisch, R. Technical challenges in using human induced pluripotent stem cells to model disease. *Cell Stem Cell* **5**, 584–595 (2009).
61. Yamanaka, S. Patient-specific pluripotent stem cells become even more accessible. *Cell Stem Cell* **7**, 1–2 (2010).
62. Staerk, J. *et al.* Reprogramming of human peripheral blood cells to induced pluripotent stem cells. *Cell Stem Cell* **7**, 20–24 (2010).
63. Seki, T. *et al.* Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell Stem Cell* **7**, 11–14 (2010).
64. Loh, Y.H. *et al.* Reprogramming of T cells from human peripheral blood. *Cell Stem Cell* **7**, 15–19 (2010).
65. Kim, K. *et al.* Epigenetic memory in induced pluripotent stem cells. *Nature* **467**, 285–290 (2010).
66. Serwold, T., Hochedlinger, K., Inlay, M.A., Jaenisch, R. & Weissman, I.L. Early TCR expression and aberrant T cell development in mice with endogenous prerearranged T cell receptor genes. *J. Immunol.* **179**, 928–938 (2007).
67. Sabir, I.N., Killeen, M.J., Grace, A.A. & Huang, C.L. Ventricular arrhythmogenesis: insights from murine models. *Prog. Biophys. Mol. Biol.* **98**, 208–218 (2008).
68. DiBernardo, A.B. & Cudkowicz, M.E. Translating preclinical insights into effective human trials in ALS. *Biochim. Biophys. Acta* **1762**, 1139–1149 (2006).
69. Scott, S. *et al.* Design, power, and interpretation of studies in the standard murine model of ALS. *Amyotroph. Lateral Scler.* **9**, 4–15 (2008).
70. Rosen, D.R. *et al.* Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **362**, 59–62 (1993).
71. Desnuelle, C., Dib, M., Garrel, C. & Favier, A. A double-blind, placebo-controlled randomized clinical trial of alpha-tocopherol (vitamin E) in the treatment of amyotrophic lateral sclerosis. ALS riluzole-tocopherol Study Group. *Amyotroph. Lateral Scler. Other Motor Neuron Disord.* **2**, 9–18 (2001).
72. Groeneveld, G.J. *et al.* A randomized sequential trial of creatine in amyotrophic lateral sclerosis. *Ann. Neurol.* **53**, 437–445 (2003).
73. Shefner, J.M. *et al.*; NEALS Consortium. A clinical trial of creatine in ALS. *Neurology* **63**, 1656–1661 (2004).
74. Ebert, A.D. & Svendsen, C.N. Human stem cells and drug screening: opportunities and challenges. *Nat. Rev. Drug Discov.* **9**, 367–372 (2010).
75. Nishikawa, S., Goldstein, R.A. & Nierras, C.R. The promise of human induced pluripotent stem cells for research and therapy. *Nat. Rev. Mol. Cell Biol.* **9**, 725–729 (2008).

特集1

神経系におけるiPS細胞
iPS細胞の活用も含めた神経機能修復の現状と将来

iPS細胞技術の神経疾患研究での有用性および今後の課題

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SUMMARY

iPS細胞作製技術の開発により，これまで生体から入手困難であった患者由来組織の細胞を研究材料として使用できるようになった．その結果，神経疾患特異的iPS細胞から疾患の標的細胞である神経細胞あるいはグリア細胞に分化誘導し，*in vitro*での疾患モデルの構築や，構築された疾患モデルを利用した創薬プラットフォームの創出が進められている．また，iPS細胞の*in vivo*での活用法としては，ヒト血球抗原（human leukocyte antigen: HLA）ハプロタイプが一致するヒトiPS細胞から分化誘導した細胞を移植する他家移植，または，患者由来の細胞を*in vitro*で治療した細胞を用いた自家移植に関する研究などが進められている．本稿では，iPS細胞を用いた神経疾患研究の現況と今後の課題について述べる．

KEY WORDS
創薬
移植
神経細胞
グリア細胞
モデリング

はじめに

神経変性疾患や精神疾患に関する現在の知見は死後病理組織の解析に基づくものが多い．しかしながら，死後病理組織は疾患の末期での病態を反映し，必ずしも疾患の発症前もしくは進行期での病態を反映するものではない．さらに，死後病理組織は疾患の影響以外に，死後変化などを含め，二次的な影響を受けている可能性がある．一方，原因遺伝子が明らかな疾患では動物モデル・細胞モデルでの解析が進められているが，これらのモデルを用いた治療実験で効果を有した薬剤が臨床試験で必ずしも効果を示すとは限らない[1,2]．

2007年にヒトiPS細胞技術が開発され[3,4]，患者自身の体細胞からiPS細胞を作製し，疾患の標的細胞へ分化させることが可能になった．このことはさらに，患者由来の標的細胞を用いた*in vitro*での疾患モデリング・創薬プラットフォームの開発を可能にしつつある．

また，これまでES細胞や胎児由来細胞が移植細胞のリソースとして使用されてきたが，倫理的・量的制限および拒絶反応が問題であった．しかしながら，iPS細胞技術は移植の新たなツールを提供した．本稿では，神経疾患特異的iPS細胞を用いた疾患モデルの構築・創薬プラットフォームの創出・移植に関する現

1344-0128/11/ ¥100/JCOPY