**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<br>2. Decreased SMN protein level (evaluated with WB/IA)<br>3. Rescue phenotype 2 with 1 mmol/l valproic acid |
| Familial dysautonomia[42] (*IKBKAP*) | AR | Infancy | 1. Increased abnormal splicing in differentiated neural crest<br>2. Decreased no. (%) of ASCL1+, Tuj1+ neurons<br>3. Migratory dysfunction (scratch assay)<br>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)<br>2. Differentiate into CD34+/hematopoietic progenitors |
| Dyskeratosis congenita[44] (*XR: DKC1*) | XR (AR/AD) | Adolescence | 1. Elongated telomere in iPSCs (TERT/TERC↑)<br>2. Shortened telomere after differentiation (TERT/TERC↓) |
| LEOPARD syndrome[45] (*PTPN11, RAF1, SHOC2*) | AD | Infancy to adolescence | 1. Enlarged cell size of differentiated cardiomyocyte<br>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)<br>2. Reduced RTT protein level/cell size and rescue by gentamicin (100 µg/ml) at Q244X clone<br>3. Reduced activity-dependent calcium transients<br>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]

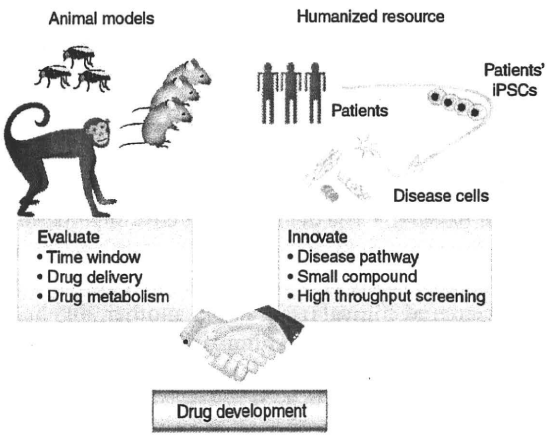


**Figure 2** Combined approach involving animal models and induced pluripotent stem cell (iPSC) technology. The new iPSC technology is complemented by a drug development strategy in preclinical settings that uses animal models and other conventional approaches.

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.

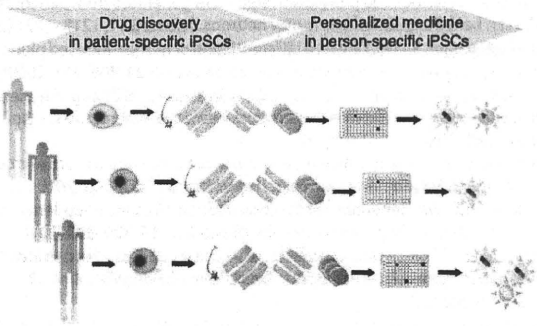


**Figure 3** Personalized medicine based on induced pluripotent stem cell (iPSC) technology. iPSC technology is highly amenable to individualized approaches. Person-specific iPSCs can be derived, differentiated into specific cell types, and used for therapeutic/toxicity response assays.

### 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

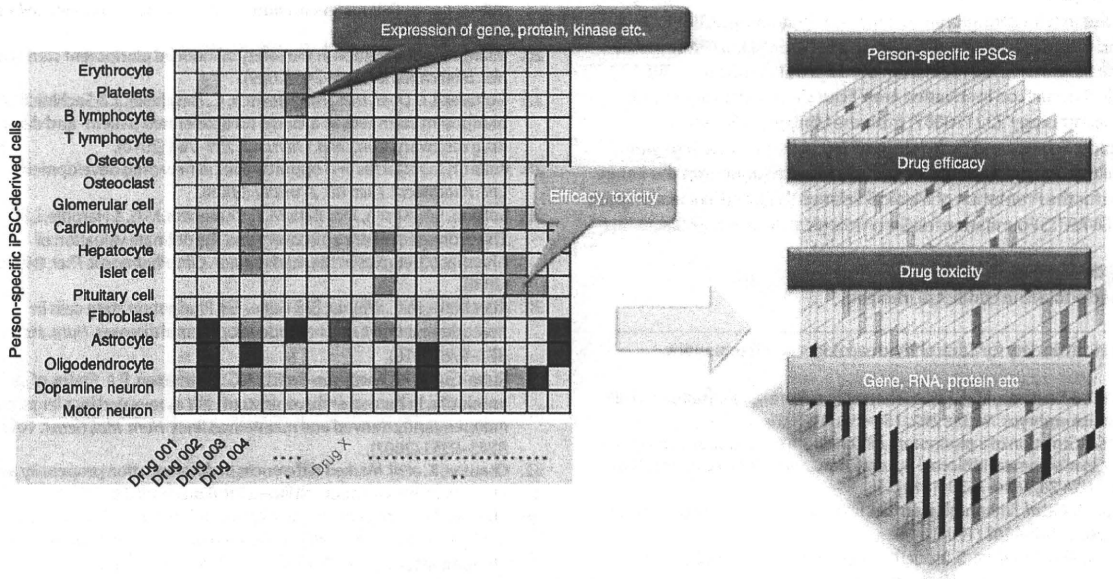


**Figure 4** "Pharmaco-iPSCellomics" by person-specific iPSCs. iPSCs derived from individual subjects/patients can be differentiated into multiple cell types, thereby providing a personalized iPS-cellome platform. This cell-based system can be used for drug discovery and selection of clinical therapeutics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

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### CONFLICT OF INTEREST

The authors declared no conflict of interest.



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Review

# Neurodegenerative disease-specific induced pluripotent stem cell research

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ABSTRACT

Neurodegenerative disease-specific induced pluripotent stem cell (iPSC) research contributes to the following 3 areas; "Disease modeling", "Disease material" and "Disease therapy".
"Disease modeling", by recapitulating the disease phenotype *in vitro*, will reveal the pathomechanisms. Neurodegenerative disease-specific iPSC-derived non-neuronal cells harboring disease-causative protein(s), which play critical roles in neurodegeneration including motor neuron degeneration in amyotrophic lateral sclerosis, could be "Disease material", the target cell(s) for drug screening. These differentiated cells also could be used for "Disease therapy", an autologous cellular replacement/neuroprotection strategy, for patients with neurodegenerative disease.
Further progress in these areas of research can be made for currently incurable neurodegenerative diseases.



## Contents



Neurodegenerative diseases are caused by the degeneration of selected neurons: cortical neurons in Alzheimers' disease, dementia with Lewy bodies, or frontotemporal lobar degeneration, midbrain dopaminergic neurons in Parkinson's disease, cerebellar neurons in spinocerebellar degeneration, and upper and lower motor neurons in amyotrophic lateral sclerosis (ALS). It is widely believed that neurodegenerative diseases generally arise through the same process; neuronal dysfunction [1], the gradual accumulation of misfolded protein and the acceleration of aggregate formation [2], neuronal death [3], and disease progression caused by non-neuronal cells [4,5].

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Neurodegenerative diseases are still intractable, although studies using molecular biology continue to enhance our understanding of neurodegeneration.

ALS, one of the neurodegenerative diseases, is characterized by the degeneration of upper and lower motor neurons, leading to fatal paralysis. The name "ALS" originated from the pathological observation that a distinct myelin pallor in the lateral part of the spinal cord represents degeneration and loss of the axons of upper motor neurons in the spinal cord [6,7]. This relentless disease is characterized by the degeneration of somatic motor neurons in the spinal cord, brain stem, and cortex. Common symptoms are progressive muscular atrophy, difficulty in swallowing and speech, and respiratory failure. Generally, the disease has a midlife onset; it is found in the 45–60 year age group and the typical disease course is 1 to 5 years. The proportion of affected individuals in the population is 4 to 6 per 100,000, and the lifetime risk is about 1 in 1000 [3,6–8].

Approximately 10% of patients with ALS are inherited (familial ALS, FALS), while the remaining have no family history of ALS (sporadic ALS, SALS) [4–8]. After the missense mutations in the gene that encodes the antioxidant enzyme Cu/Zn superoxide dismutase 1 (SOD1) was found in 10–20% of patients with FALS in 1993, most studies in the field have focused on revealing the mechanism of SOD1-mediated motor neuron degeneration [4–8]. SOD1 changes superoxide radicals into oxygen and hydrogen peroxide. When mutated SOD1 are overexpressed in rodents, this causes phenotypes of an ALS-like motor neuron disease, which is not rescued by overexpression of wild-type SOD1. These observations suggest that a toxic gain of function mechanisms, but neither loss of function nor haploinsufficiency, cause mutant SOD1-mediated FALS. Genetically engineered mutant SOD1 animal models have provided abundant information regarding the possible mechanisms of this disease [4–8].

Several therapies with drugs have shown therapeutic effects in SOD1 transgenic rodents, but clinical trials in humans have not been successful. This suggests that current ALS rodent models can provide only limited or extra insight into the pathogenesis of human ALS. Therefore, for drug screenings, human resources, which possess human molecular signaling pathways different from rodents, are supposed to be required. Human resources could be robustly supplied by differentiating human stem cells, especially neurodegenerative disease-specific induced pluripotent stem cells (iPSCs), which are generated by reprogramming adult fibroblast cells of neurodegenerative disease including ALS [9] by using forced expression of the transcription factors—Klf-4, Sox-2, Oct-4, and c-Myc. The iPSCs have the same advantages as traditional stem cells due to their ability to generate differentiated cells such as neurons and glia from individuals [10–12].

Disease-specific iPSCs [13–21] research is a new field that could contribute to the following 3 areas; "Disease modeling", "Disease material" and "Disease therapy" (Fig. 1).

## Disease modeling

"Disease modeling" by recapitulating the diseases phenotype *in vitro* would make it possible to study how different cell types are involved in the pathobiology of neurodegenerative diseases, and to unravel the cellular mechanisms that may trigger familial, as well as sporadic, forms of the disease. In an ALS study, Dimos et al. successfully directed the differentiation of iPSCs, generated from an elderly patient with FALS and a SOD1 mutation, into motor neurons expressing appropriate motor neuron markers including Hb9 and ISLET [9]. While both spinal muscular atrophy (SMA) [13] and ALS [9] are neurodegenerative diseases of motor neurons, only SMA motor neurons from disease-specific iPSCs show phenotypes. This may be due to the fact that the onset age of ALS is after middle age while most SMA cases begin in childhood. Furthermore, the iPSC-derived neurodegenerative models that have been developed for SMA and familial dysautonomia (FDA) [17] take the autosomal recessive inherited form. In neurodegenerative disease modeling from iPSCs, genetic information, the environment, or senescence all contribute to neurodegeneration, and therefore it would be critical to promote these conditions *in vitro*. These studies have led to new avenues for drug development for neurodegenerative diseases.

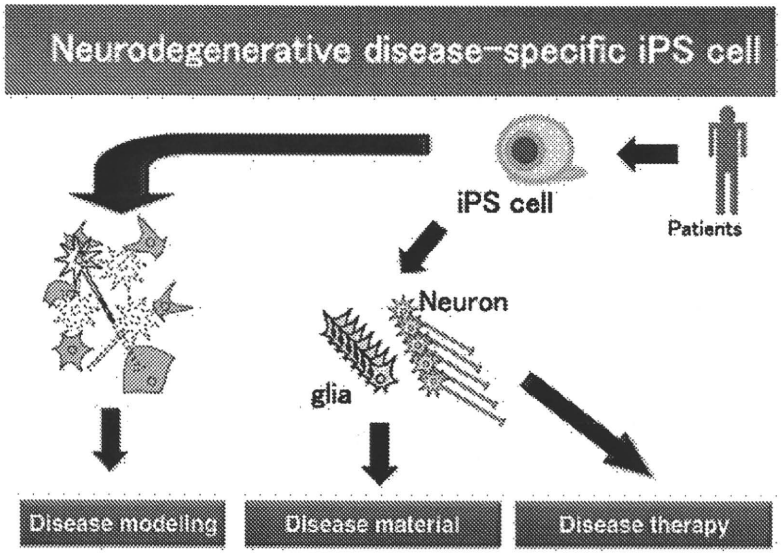


**Fig. 1 – Neurodegenerative disease-specific iPSC research contributes to the following 3 areas; "Disease modeling", "Disease material" and "Disease therapy".**

## Disease material

Affected cells in neurodegenerative disease(s) as "Disease material", which cannot be collected from patients, can be generated from disease-specific iPSCs. These cells possess the genetic information of the patient. Non-neuronal cells, including glial cells [4,5], can be a target of neurodegenerative disease-specific iPSC research based on non-cell autonomous neurodegeneration hypothesis demonstrated by following studies.

The absence of motor neuron degeneration with the synthesis of mutant SOD1, under the control of neuron-specific promoter, provided the initial supportive evidence that the disease probably does not arise from damages caused within motor neurons through cell-autonomous mechanisms [22]. The onset of the disease was delayed and the survival period was extended when part of mutant SOD1 in motor neurons was removed using the Cre–loxP system [23], although, after the disease onset, the period of disease progression was almost unaffected. Similarly, virus-mediated small interfering RNA knock-down, within which the CNS selectively suppressed mutant SOD1 in motor neurons, showed a robust delay in onset [24–26]. On the other hand, based on the results of further studies on such transgenic animals, disease progression is determined by the amount of mutant SOD1 in astrocyte/microglia [23,27]. This suggests that the amount of mutant SOD1 in motor neurons (cell autonomous) is relevant to disease initiation, while progression is dependent on non-neuronal cells, including astrocytes and microglia (non-cell-autonomous).

A chimeric mice study that included a combination of wild-type cells and cells expressing mutant SOD1 directly showed an evidence for a non-cell-autonomous disease mechanism [28]. Motor neurons surrounded by wild-type neighbors survived longer with no degeneration despite mutant SOD1 expression, while motor neurons that were genetically normal were damaged by neighboring mutant SOD1-expressing cells. These results support the idea that non-cell autonomous mechanisms contribute to neurodegeneration in mutant SOD1-mediated ALS.

Embryonic stem cell (ESC) research [30–33] also contributes to our understanding of non-cell-autonomous mechanisms in neurodegenerative disease. It has been demonstrated that mutant SOD1-expressing astrocytes diminish the survival of motor neurons over a 2-week period compared to normal glial cells by the co-culture of primary, mutant SOD1-expressing astrocytes with primary motor neurons purified from embryos, or with motor neurons generated by the differentiation of mouse embryonic stem cells. Astrocyte-derived toxicity, which is transferred by astrocyte conditioned media, acts on wild-type as well as mutant-SOD1 motor neurons, and is specific to motor neurons with no effect on sensory neurons or interneurons [29,30]. Studies also showed that human ESC-derived motor neurons are also sensitive to the toxic effect of glial cells carrying an ALS-causing mutation, which supports previous suggestions that non-neuronal cells contribute to the pathogenesis of ALS [31,32]. Astrocytes expressing ALS-linked mutated SOD1 release factors that are toxic to motor neurons. Further studies may show the relevance to these factors with the reduced expression of GLT-1 that is commonly seen in human ALS [33]. These experiments have provided an *in vitro* paradigm for the use of stem cell-derived co-culture experiments in exploring cell–cell interactions in ALS or in other neurodegenerative diseases.

In addition to the SOD1-mediated ALS studies, there are supporting evidence showing non-cell autonomous neurodegeneration in spinocerebellar ataxias (SCA), which is one of neurodegenerative diseases with characteristics of cerebellar neurodegeneration that lead to progressive motor incoordination [5]. The most affected cells are cerebellar Purkinje neurons. Non-neuronal neighbors to these neurons are Bergmann glia, cerebellar-specific astrocytes, with long finger-like processes to enwrap the dendritic trees of Purkinje cells [34]. SCA7 is caused by polyQ expansion in the gene encoding ataxin-7. Transgenic mice that showed mutant polyQ expression only in Bergmann glia and other astrocytes by using the GFAP promoter would be sufficient to cause Purkinje cell degeneration. Moreover, the Purkinje cell degeneration was remarkably similar to that induced by polyQ expansion under the control of the Prion promoter, both neuronal and glial promoters, demonstrating non-cell-autonomous Purkinje cell degeneration [5,35].

These findings suggest that disease-causative protein in glial cells could be target molecule(s) in targets cell(s) in drug screening by using disease-specific iPSCs. Although it is still unclear whether glial cells in patients elicit the same toxicity as ALS model astrocytes, as the latter commonly harbor multiple copies of mutant SOD1 [36]. An analysis of iPSC-derived glial cells from patients with mutant SOD1-induced ALS could possibly reveal whether a single gene copy renders human glial cells as toxic as those harboring multiple mutant SOD1 copies [36]. As a consequence, it is critical to analyze the mechanisms for disease pathways and to perform drug screening by using human resources derived from neurodegenerative disease-specific iPSCs followed by evaluating drug delivery, optimal safe dose or time window in animal model studies.

## Disease therapy

There is more than one method for generating iPSCs (i.e., retrovirus, lentivirus, adenovirus, plasmid vector, small compounds, protein transduction etc.) [37] from multiple origins. The iPSCs also could provide an autologous cellular replacement/neuroprotection strategy for patients with neurodegenerative diseases [36], "Disease therapy". In addition to transplantation of specific neurons from iPSCs for replacement therapy, transplantation of glial cells from iPSCs can be used for neuroprotection [36]. Before the transplantation approach can be applied clinically, numerous hurdles must be overcome. For these putative stem cell-based therapies, we must first identify the optimal cell dose and source, and/or the route of delivering the cells [36]. Transplantation therapies would consist of the delivery of a combination of subtypes of neuronal cells to provide both cellular replacement and neuroprotection.

Further progress in these areas of research can be developed for currently incurable neurodegenerative diseases.

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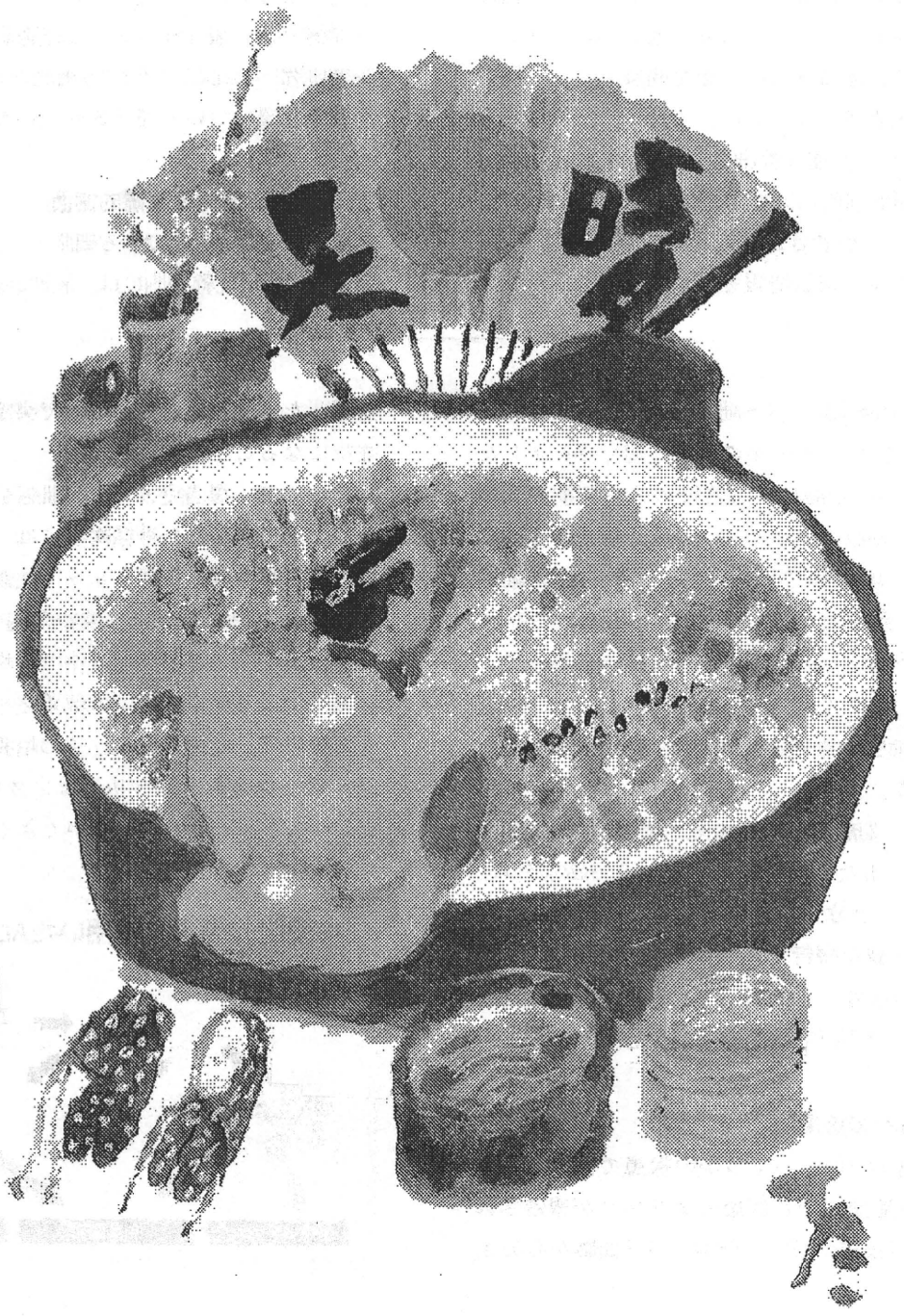

# iPS細胞作製技術を用いたALS治療法開発

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准教授　井上治久

## 1. はじめに

筋萎縮性側策硬化症(ALS)におきまして、病変の首座は、運動ニューロンといわれる、体の随意運動をコントロールする神経細胞にあります。運動ニューロンは、頭蓋骨や脊椎骨で囲まれた中枢神経系に位置するため、直接に病態を解析するには限界がありました。そのため、遺伝学的解析、病理組織・遺伝子改変動物/細胞モデルなどの生化学的・組織学的解析を中心にこれまで研究が進められてきました。すなわち、患者さんの病態を間接的にしか解析することが出来ませんでした。

2006年に人工多能性幹細胞(induced Pluripotent Stem cells: iPS細胞)の作製技術が開発されました。そのことにより患者さんご自身の皮膚の細胞から、患者さんご自身の遺伝情報を有する運動ニューロンを含めた中枢神経系の細胞を入手することが可能になりました。そのことによって、これまで知られていなかったALSという疾患の新たな病因が明らかになる可能性が生まれました。また、患者さんご自身の細胞を生きたまま入手することができることから、培養皿の中でALSという疾患の病態が再現できる可能性が生まれ、病態解明・創薬開発がすすむことが期待されています(図)。

本稿では、現在までのiPS細胞研究の基礎的側面、疾患研究への応用、iPS/ES細胞を用いたALS研究、今後の展望について述べさせていただきます。

## 2. iPS細胞研究の基礎的側面

様々な細胞に分化できる細胞を、多能性幹細胞といいます。多能性幹細胞には、胚性幹細胞(Embryonic Stem cells: ES細胞)やiPS細胞などがあります。

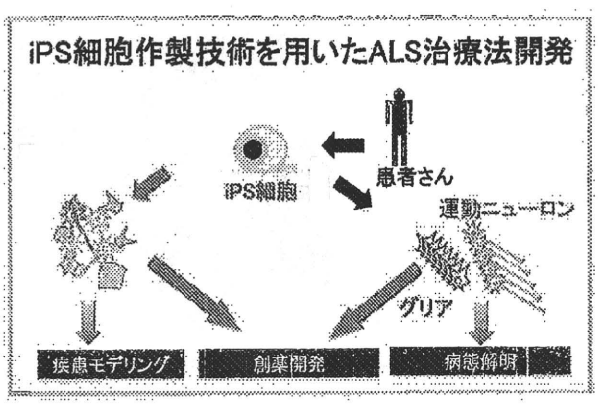
ヒトES細胞は、1998年に初めて樹立されました。しかし、ヒトES細胞は、受精後の発生初期の胚の一部を用いて樹立されるため、多くの疾患から樹立することは、現時点では困難です。そしてES細胞に特徴的に発現しているOct3/4・Sox2・Klf4・c-Mycという遺伝子の皮膚線維芽細胞への導入により、2006年にマウスで、2007年にはヒトでES細胞類似の多能性幹細胞樹立がなされ、iPS細胞と命名されました。

現在では、皮膚以外に、血液、胃等、体内の様々な部位から、また、ウイルスを用いる方法以外に、DNA、RNA、小分子化合物を用いる方法等、様々な方法での作製が報告されています。今後は、侵襲ができるだけ少なく、効率よく、作製にかかる期間が短い方法での樹立方法開発が進むと考えられます。

## 3. 疾患研究への応用

iPS細胞誕生以降、いくつかの疾患で、患者さん由来のiPS細胞を用いた疾患モデリングが報告されています。疾患モデリングとは、iPS細胞から分化誘導した細胞が、培養皿の中で実際の病気と同様の症状になることです。

これまで、患者さんのiPS細胞を用いて、疾患モデリングに成功した神経疾患には、脊髄性筋萎縮症、家族性自律神経失調症、レット症候群等があります。それぞれ疾患に罹患する神経細胞の細胞数減少等、疾患表現型などを培養皿の中で再現し、さらには既存薬剤による疾患表現型の改善を示すこと等に成功しています。このことは、その培養皿の中のモデルが、新たな薬剤をスクリーニング(選別していくこと)することに用いることができる可能性を示して



います。

## 4. iPS/ES細胞を用いたALS研究

1993年、家族性ALSの一部の家系で、SOD1という遺伝子に変異があることが明らかになりました。1994年、ヒトの変異SOD1遺伝子を有するマウス（変異SOD1マウス）が作製され、ヒトALSと同様の症状を呈することが明らかになりました。その際に、治療法開発が一気に加速することが期待されましたが、実際にはマウスで有効だったほとんどの薬剤が、患者さんでは有効ではありませんでした。その一つの原因としては、ヒトとマウスの細胞が、異なっていること（細胞内部の信号伝達等の違いによる可能性等があります）が考えられました。よって、ヒト、しかも患者さんご自身の細胞を用いた創薬開発が求められていました。そのような状況の中、ヒトiPS細胞が誕生しました。

しかしながら、変異SOD1マウスを用いた研究により、多くのALSの病因も明らかになっています。その中でも重要な病因として、脊髄運動ニューロン自体だけではなく、その周辺の変異SOD1を有するグリア細胞が、脊髄運動ニューロン細胞死を増強、加速していることが明らかになりました。2007年ハーバード大学医学部のケビン・エッガン博士らのグループや、コロンビア大学のグループが、変異SOD1マウスから作製したグリア細胞が、マウス脊髄運動ニューロンの細胞死を生じることを報告しました。2008年には、エッガン博士のグループや他のグループが、ヒトES細胞から分化誘導した脊髄運動ニューロンを変異SOD1を有するグリア細胞と一緒に培養すると、それが正常のヒトES細胞由来脊髄運動ニューロンであっても、細胞死に至ることを報告しました。そのことは、変異SOD1を有するグリア細胞が脊髄運動ニューロンに対する毒性を有していることを示しています。その報告の中で、プロスタグランジンといわれる生理活性脂質がグリアの毒性に関連しており、創薬の標的となりうることが示されています。本年度、国際幹細胞学会において、エッガン博士は、ヒトES細胞から分化誘導した脊髄運動ニューロンをGFPというクラゲ由来の蛍光タンパク質で光らせ、培養皿の中で変異SOD1を有するグリア細胞と一緒に培養し、タイムラプスイメージングという動画撮影で運動ニューロンの細胞死が起こる瞬間の映像を、世界で初めて報告しました（日本のTV番組でも放映されました）。

2008年、エッガン博士らのグループは、高齢のALS患者さんの皮膚線維芽細胞からiPS細胞を作製し、さらにiPS細胞から脊髄運動ニューロンまで分化誘導が可能であることを報告しました。しかし、その後、世界において、未だ、ALS患者さんのiPS細胞を用いた疾患モデリングに関する報告はありません。iPS細胞を用いて疾患モデリングに成功した先述の疾患とALSとの違いには、①ALSが比較的高齢になってから発症する疾患であること（加齢変化）、②孤発性ALSの場合には遺伝的背景以外の環境の影響が発病に関連している可能性があること（環境要因）、③ALSではグリア細胞等の脊髄運動ニューロン以外の細胞が脊髄運動ニューロン細胞死への強い影響を有する可能性があること（周辺細胞の毒性）、の3点が挙げられます。私達の研究室も含め、これらの三つの要素を巧みに取り入れた培養皿の中での疾患モデリングの試みを、世界中の研究室が行っています。

これまで、私達の研究室では、疾患モデリングの試みに加えて、多くの大学、製薬会社等と共同で、脊髄運動ニューロンやグリア細胞と他の細胞ができるだけ混じらないように純化し、大量に入手する方法を開発しています。現在、患者さん由来の脊髄運動ニューロンやグリア細胞の詳細な解析を行い、新たな創薬の標的を同定しつつあります。その中でも、変異SOD1によるALSに対しては複数の治療薬候補を選定すると同時に、動物モデルを用いた検証に

も取り組み始めています。

### 5. おわりに

iPS細胞作製技術というこれまでの歴史上に存在しなかった革命的技術を用いることによって、培養皿の中でのALSの疾患モデリング、病態解明、創薬開発が加速しています。あらゆる可能性、考えられうるすべての手法、分野を超えて協力しうるすべての研究者との共同研究によって、世界中の研究者も私達も、全速力で、ALSという疾患の制圧を目指しています。

最後になりましたが、本研究にご協力いただいております患者さん、ご家族の皆様、そして協会の方々に、心より御礼申し上げます。どうぞ今後とも何卒よろしくお願い申し上げます。

## iPS細胞研究所　山中伸弥所長より JALSA会員への メッセージをいただきました。

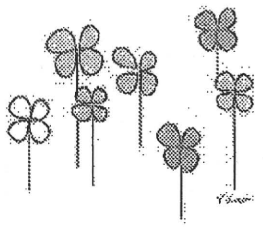
京都大学iPS細胞研究所（CiRA）では、井上治久准教授を中心にALSの患者さんから提供していただいた細胞からiPS細胞を作製し、それをさらに分化させた運動神経細胞等を用いて、治療薬探索の準備を進めています。他の研究者や製薬企業とも協力しながら研究を強力に推進し、新しい治療法の開発に貢献したいと思います。

トピックス

# メコバラミンの治験　中間解析報告

ALS治療薬として治験を行っているメコバラミンについて、エーザイ株式会社より以下の中間解析報告を受領しましたので紹介致します。

メコバラミンのALSに関する治験は，2006年より開始し、既に患者様の登録は2010年8月末に締め切らせて頂いております。その後、2010年10月末に本剤の有効性及び安全性に関する中間解析が、エーザイとは独立した効果安全性評価委員会によって実施されました。その結果に基づき、同委員会において当該治験の継続可否について検討が行なわれ、有効性及び安全性に関する中止基準に該当しなかったため、弊社は同委員会より「継続」の旨の勧告書を受領いたしました。当該勧告書に基づき、本治験を引き続き継続させて頂くことを決定致しましたので、ご報告申し上げます。なお、当該中間解析は弊社とは完全に独立した形で実施されておりますため、弊社は解析結果の詳細について一切知らされておりません。この点につきましてご了承下さいますよう何卒宜しくお願い申し上げます。



第1回

# ALSフォーラム

～ALS最前線～

# 記録集

2010年8月28日
東京ドームホテル

サノフィ・アベンティス株式会社

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# ❶-4

# iPS細胞作製技術を用いた神経変性疾患の研究

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ヒトiPS細胞は2007年に誕生した。ヒトの皮膚細胞にES細胞（胚性幹細胞）に特徴的な転写因子を3～4個導入することによって、ヒトの皮膚細胞がES細胞様の細胞に初期化されることが発見された。

この技術を応用して神経変性疾患においては、患者の細胞からiPS細胞を樹立し、神経細胞やグリア細胞に分化誘導することによって、創薬もしくは移植医療への応用、あるいは病態を再現することができれば、病態解明につながることが期待されている。今回は、iPS細胞を用いた神経変性疾患に関して、現在我々が進めている研究も含めて紹介する。

## 1. 疾患の再現、薬物スクリーニング

ハーバード大学の研究者らは2008年に、SOD1変異のある高齢ALS患者から採取した皮膚線維芽細胞からiPS細胞を樹立し、さらに運動神経細胞への分化誘導に成功した[1]。また同年、ウィスコンシン大学のグループはSMN1遺伝子の変異によりスプライシングの異常をきたし、運動神経が特異的に減少する脊髄性筋萎縮症患者の線維芽細胞からiPS細胞を作製し、分化誘導した運動神経において神経の減少を再現し、有効性が証明されている薬剤を作用させると、蛋白質SMN1量を増加することを報告した[2]。また、I-κ-B kinase complex associated protein（IκB AP）遺伝子変異によって発症する家族性自律神経失調症から作製された自律神経の前駆細胞（神経提細胞）では、遊走能異常や異常遺伝子発現が再現されたほか、既知の薬剤の有効性が確認された[3]。

そこで我々は、SOD1変異ALS患者の病態解明のためのiPS細胞を樹立する前に、ヒトiPS細胞を運動ニューロンに分化誘導しうるかを確認した。SOD1変異ALS患者の細胞からiPS細胞クローンを10～30個樹立し、ES細胞のマーカーを発現していること、さらに導入遺伝子が10%以下になるようにサイレンシングされていることを確認し、確認できたクローンについて核型を検討した。同時にALS患者が有していたSOD1遺伝子変異が引き継がれていることを確認した後、免疫不全マウスの中で3胚葉に分化するES細胞の表現型を有していること、運動神経にも確実に分化誘導できることを確認してから実験に用いた。

## 2. Cloning variationとcloning selection

iPS細胞は分化誘導効率や、増殖速度が異なることから、clonal varietyがあるといわれる。このclonal varietyの原因としては、様々な要因が考えられている。我々は、導入遺伝子が確実にキャンセルされているか（サイレンシング）を指標にアンバイアスでクローンを選択した。

## 3. 神経細胞死を標的とした薬物スクリーニング

変異SOD1を有する星状膠細胞が毒性を有していることはヒトES細胞から分化誘導された運動ニューロンですでに証明されている。我々は、変異SOD1を有する星状膠細胞上で、SOD1変異ALS患者の運動神経、もしくは変異を有さない運動神経を標識し共培養を行った。確実に神経変性が生じるかをできるだけバイアスのない状況下で、1つひとつをできるだけ高速で、どれくらいの細胞が減少しているのかを現在解析している。

また、前述の非自律性神経変性で問題となる星状膠細胞の毒性については、SOD1変異ALS患者、変異を持たない患者（対照）数名ずつから星状膠細胞を誘導し、同定法の1つであるマイクロアレイを用いて遺伝子変異の違いがある遺伝子を同定した。

## 4. 封入体を標的とした薬物スクリーニング

一方、SOD1と同様、重要な遺伝子であるTDP-43遺伝子変異については、TDP-43変異ALS患者から誘導したヒトの運動神経の解析を行っている。

ヒトのリソースとしてiPS細胞を利用することにより、今後はさらに新しい情報が見つかることが考えられる。

### 参考文献

1) Dimos JT, et al. Science 321：1218-1221, 2008
2) Ebert AD, et al. Nature 457：277-280, 2009
3) Lee G, et al. Nature 461：402-406, 2009

## Session ❶ 討論

### 1) TDP-43

**座長** ヒトTDP-43を発現するトランスジェニックマウスモデルの表現型に関連して、マウス脳組織にも細胞培養と同じような変化がありましたか？

**野中** 運動機能異常のような表現型が出ていたので、6ヵ月ぐらいのときに一度、脳を観察したのです。患者脳でみられるようなリン酸化TDP-43抗体に陽性な凝集体は脳でも脊髄でもほとんど存在していませんでした。海外のいくつかのグループも同様の結果でした。今後は、マイクロアレイなどで解析してみてはどうかと考えているところです。

**会場** 蛋白質というのは凝集を形成すると思うのですが、それらが野生型のTDP-43を巻き込むようなことが起こり得るのかどうかについてご教示いただけますか。

**野中** TDP-43以外の、タウ、シヌクレインの凝集体形成モデルも研究しているのですが、TDP-43は断片化すれば、発現しただけで凝集体ができますが、タウやシヌクレインは発現させただけでは凝集体はできません。シヌクレインを発現したところにタウの線維を入れても凝集しないことから、シヌクレイン同士、タウ同士という組み合わせがあるようで、TDP-43に関しても同様にクロスシードしなかったというデータが出ています。

### 2) FUS/TLS

**会場** 塩基性封入体は、従来いわれている塩基性封入体とはちょっと違うように思いますが、ユビキチンがよく染まるといった特性はありますか。

**会場** ユビキチンに関しては変化しやすく、遺伝性のものがよく染まるように思いますが、それ以外ではなかなか染色の差が出ていません。また塩基性封入体を染色する際、遺伝性がないほうが染色されにくいという感触はあるものの、形態は多彩だと思われます。

**青木** おそらく、FUS/TLS遺伝子異常がある家系での塩基性封入体とない家系での封入体が見分けられるようになれば、染色の差も明らかにできるとは思いますが、現時点ではまだはっきりしていません。

### 3) Optineurin

**会場** Optineurinは緑内障も遺伝性に起こすファクターとのことですが、緑内障を起こすメカニズムとALSを起こすメカニズムとの間に、何か共通したファクターがあるのでしょうか。臨床医としては、神経系にoptineurinが沈着するようであれば目の症状に注意する必要があるのでしょうか。

**川上** 緑内障に関しては、最初は分泌された異常蛋白が前房で詰まって眼圧が上がるという説が唱えられていました。しかし、前房にはoptineurinが分泌されないことが明らかになり、最近、岐阜大学でトランスジェニックマウスが作成されました。メカニズムの説明に関しては、Rab8との相互作用が悪くなって、運送されなくなって緑内障が発症するといわれています。ALSにおける変異に関しては、Rab8の相互作用はみていませんが、NF-κBに対する作用が異なるので、機序的には違うものと考えています。

**会場** Optineurinで染めてみると、SOD1や孤発性ALSも染まりますし、非常に広い範囲のものが染色されるということで、共通の経路にoptineurinがあるとのことですが、経路のかなり下流でoptineurinが関係しているというエビデンスはあるのでしょうか。

**川上** 推測ですが、まず1つはSOD1の場合、主な原因はSOD1変異であることが明らかなため、共通なメカニズムではあるものの、より末梢のところでエフェクターとなっている可能性が考えられます。もう1つは、細胞内の物質輸送に絡んでいるため、封入体処理や封入体形成のときに関係している可能性はあると思われます。

### 4) iPS細胞

**会場** ALSなど疾患発症の家系の場合に、iPS細胞を作製して神経分化させた時点で、すでに表現型の違いはあるのでしょうか。もしないとすれば、そのあとどう培養すれば疾患モデルが作製できるか教えてください。

**青木** 疾患モデルです。これまで疾患モデル開発に成功しているものでは孟どが劣性遺伝形式で、

表現型が現れやすいかもしれません。ただし、運動ニューロンがいくつあるのか、または最初に分化誘導から何個あったかをモニタリングするのは非常に困難です。しかし、分化誘導の効率も加味しながら、それらの細胞をモニタリングすると、神経変性、神経細胞死ができるのではと考えています。

**会場** iPS細胞での共培養と、SOD1のトランスジェニックマウスの共培養とでは、コピー数が決定的に異なると思いますが、コピー数をover expressionすることで表現型を呈する時間が短くなると考えれば、逆にsingle copyで変異が出ているiPS細胞で、同様の表現型を再現できるかどうかについて、先生のお考えをお聞かせください。

**井上** 星状膠細胞として選択できるものは疾患遺伝子を有するマウス、有さないマウス、疾患遺伝子を有するヒト、有さないヒト由来の4通りの実験を組むことが可能です。すなわち、コピー数の影響を含め、様々な角度から調べることが可能です。共培養（コカルチャー）というのは、そういう組み合わせができる点が利点になります。