A

Control

insertion site	CT1	CT2	CT3	average ratio
intergenic (low)	0.64	0.76	0.69	0.69
intragenic (low)	0.36	0.24	0.31	0.31
intron (low)	0.90	1.00	0.99	0.96
exon (low)	0.10	0.00	0.01	0.04
intergenic (high)	0.65	0.81	0.69	0.72
intragenic (high)	0.35	0.19	0.31	0.28
intron (high)	0.88	1.00	0.99	0.96
exon (high)	0.12	0.00	0.01	0.04

Schizophrenia

insertion site	SZ1	SZ2	SZ3	average ratio
intergenic (low)	0.72	0.63	0.58	0.64
intragenic (low)	0.28	0.37	0.42	0.36
intron (low)	0.99	0.98	0.98	0.98
exon (low)	0.01	0.02	0.02	0.02
intergenic (high)	0.74	0.63	0.57	0.65
intragenic (high)	0.26	0.37	0.43	0.35
intron (high)	0.99	0.98	0.98	0.98
exon (high)	0.01	0.02	0.02	0.02

B

Control

Term	Count	p value
GO:0005856~cytoskeleton	74	5.92E-04
GO:0005509~calcium ion binding	56	0.0031
GO:0005930~axoneme	9	0.0095
GO:0035085~cilium axoneme	7	0.0289
GO:0003779~actin binding	26	0.0322
GO:0044425~membrane part	240	0.0387
GO:0016010~dystrophin-associated glycoprotein complex	6	0.0405

Schizophrenia

Term	Count	p value
GO:0045202~synapse	57	3.09E-09
GO:0030054~cell junction	64	8.57E-06
GO:0044459~plasma membrane part	187	1.49E-05
GO:0004674~protein serine/threonine kinase activity	58	2.27E-05
GO:0044456~synapse part	38	4.44E-05
GO:0004672~protein kinase activity	72	7.45E-05
GO:0030554~adenyl nucleotide binding	147	1.09E-04
GO:0005856~cytoskeleton	126	1.19E-04
GO:0005488~binding	820	1.25E-04
GO:0006468~protein amino acid phosphorylation	76	1.75E-04
GO:0006796~phosphate metabolic process	100	1.98E-04
GO:0006793~phosphorus metabolic process	100	1.98E-04
GO:0016773~phosphotransferase activity, alcohol group as acceptor	80	2.31E-04
GO:0001882~nucleoside binding	148	2.52E-04
GO:0005524~ATP binding	138	2.63E-04
GO:0001883~purine nucleoside binding	147	2.77E-04
GO:0032559~adenyl ribonucleotide binding	139	3.43E-04
GO:0017076~purine nucleotide binding	169	4.19E-04
GO:0000166~nucleotide binding	190	0.0011
GO:0014069~postsynaptic density	17	0.0011
GO:0032553~ribonucleotide binding	161	0.0012
GO:0032555~purine ribonucleotide binding	161	0.0012
GO:0043167~ion binding	323	0.0015
GO:0016043~cellular component organization	204	0.0018
GO:0043169~cation binding	318	0.0021
GO:0016310~phosphorylation	83	0.0021
GO:0046872~metal ion binding	315	0.0025
GO:0016301~kinase activity	85	0.0032
GO:0005737~cytoplasm	489	0.0037
GO:0008092~cytoskeletal protein binding	58	0.0045
GO:0007155~cell adhesion	74	0.0049
GO:0022610~biological adhesion	74	0.0052
GO:0019898~extrinsic to membrane	54	0.0053
GO:0043687~post-translational protein modification	108	0.0169
GO:0030030~cell projection organization	45	0.0219
GO:0005509~calcium ion binding	88	0.0234
GO:0015629~actin cytoskeleton	33	0.0439
GO:0016772~transferase activity, transferring phosphorus-containing groups	90	0.0473
GO:0045211~postsynaptic membrane	21	0.0487

C

Control

Term	Count	p value	FE
height	4	0.0132	7.7
scoliosis	3	0.0316	10.3

Schizophrenia

Term	Count	p value	FE
schizophrenia; schizoaffective disorder; bipolar disorder	5	0.0125	5.2
schizophrenia	29	0.0135	1.6
hypertension	20	0.0194	1.7
bipolar disorder	13	0.0373	1.9

Figure 4. Insertion Site, Gene Ontology, and Disease Association Analyses

(A) L1-insertion site analysis. Proportion of intergenic and intragenic L1 insertion and that of intronic and exonic L1 insertion are given. The low and high mean estimated proportions based on both less and stringent criteria are given. Note that ratios are not significantly different between patients and controls. (B) Gene ontology analysis. p values indicate Bonferroni-corrected modified Fisher's exact test p value. The terms showing $p < 0.05$ are shown for both groups. (C) Disease-association analysis. p values indicate noncorrected modified Fisher's exact test p value. FE, fold enrichment. In both analyses, gene lists generated by the stringent criteria were used. See also Tables S2 and S3 and Figure S4.

to the L1-Hs (Figure S4). Among the detected mobile element insertion sites in each sequenced sample, we first identified brain-specific L1 insertions in each subject (Tables S2 and S3). Although the total number of brain-specific L1 insertion tended to be higher in schizophrenia patients, this was not statistically significant, most likely due to the limited sample size and high interindividual variation. We then compared genomic locations of the insertion sites of brain-specific L1 between patients and controls (Figure 4A). The inter-to-intragenic L1 insertion ratio as well as exonic-to-intronic L1 insertion ratio did not differ between patients and controls. We then compared the affected genes by brain-specific L1 insertion by gene ontology approach. This analysis revealed that the number of enriched terms is higher in schizophrenia than controls, in spite that the number of brain-specific L1 insertions did not significantly differ. We found that neuronal function-related terms such as synapse and protein phosphorylation are clearly overrepresented in schizophrenia compared to controls (Figure 4B). In addition, disease-association analysis revealed that affected genes in patients are specifically enriched in terms related to schizophrenia and bipolar disorder, while those in controls are enriched in nonneuropsychiatric terms such as height and scoliosis (Figure 4C). These results were consistently confirmed when we used less stringent definition of brain-specific L1 insertion (Figure S4). In

addition, enrichment of the L1-inserted genes to the terms related to neuropsychiatric disorders in schizophrenia was also detected by the ingenuity pathway analysis (IPA) (Figure S4).

DISCUSSION

We report that the neuronal genome of schizophrenia contains higher copy number of a retrotransposon, L1. To validate this finding, we utilized iPS cells from patients with schizophrenia carrying the 22q11 deletion and observed an increase in L1 copy number in iPS cell-derived neurons. Moreover, using WGS, we found that L1 preferentially inserted into genes related to synaptic functions and schizophrenia. Animal model studies showed that environmental factors related to infection or inflammation that disturbs early neurodevelopmental processes increase L1 copy number in the brain. Collectively, these results suggest that hyperactive L1 retrotransposition into critical genes during neural development, triggered by genetic and/or environmental factors, contribute to the pathophysiology of schizophrenia. Our results significantly expand the range of neuropsychiatric illnesses linked to aberrant L1 retrotransposition, from Mendelian disease patients with *MECP2* mutations in Rett syndrome (Muotri et al., 2010) and *ATM* mutations in ataxia telangiectasia (Coufal et al., 2011) to schizophrenia, a complex mental disorder.

The observed increase of L1 content in schizophrenia was not due to, or modulated by, biological or experimental artifacts, because changes were measured in two independent patient cohorts and each result was confirmed with two different internal controls. Although the L1 region showing significant increases differed between the two brain sets, this is attributable to cohort differences amplified by the strict threshold we employed. Actually, a significant increase of L1 content was widely observed in all probes in the SATA-normalized data in set II, where neuronal L1 copy number was directly examined (Figure S1). In addition, from the data analysis utilizing lifetime intake of antipsychotics of patients, and from the cell culture and macaque experiments, we conclude that antipsychotics do not affect L1 copy number in the brain. A significant increase was also observed in patients with mood disorders in one internal control in set I (Figure 1C). Future work will clarify whether there are L1 content increases in other mental disorders using larger and/or stratified patient cohorts.

L1 retrotransposition has been detected during adult neurogenesis in the rat hippocampus, indicating that neural progenitor cells retain retrotransposition activity even in adult stages (Muotri et al., 2009). However, we analyzed potential confounding factors, including age, age of onset, and duration of illness, and did not observe any significant correlation with L1 copy number in the brain. The transcript level of L1 in adult brain sample was also increased in patients compared to controls (data not shown). However, elevated expression is unlikely to contribute to increase of L1 copy number in patients, as significant increase of L1 transcripts was detected only in the 5′ region of L1 such as 5′ UTR and ORF1. These results suggest that L1 copy number does not globally increase with aging and that the variation of L1 copy number in patients is probably confined to early neurodevelopmental stages, at least in the prefrontal cortex. This prediction would be consistent with the neurodevelopmental hypothesis of schizophrenia, where abnormalities during critical early periods of brain development may trigger the later appearance of clinical symptoms (Bloom, 1993; Murray et al., 1992; Weinberger, 1987).

In Rett syndrome, increased L1 copy number in human brain was linked to mutations in *MECP2* (Muotri et al., 2010) and *MeCP2* knockout mice also showed increased L1 content (Muotri et al., 2010). It has also been suggested that SOX2 and MECP2 regulate L1 transcription in neurons (Muotri et al., 2005; Yu et al., 2001). However, we did not observe a significant correlation between *MECP2* or *SOX2* expression and brain L1 content, by using the previously performed gene expression analyses on the same sample sets (Iwamoto et al., 2004, 2005) (data not shown). In addition, patients with high levels of L1 copy number (two schizophrenia and one major depression in set I, and two schizophrenia patients in set II) did not show altered *MECP2* or *SOX2* expression levels (data not shown). These findings suggest that the molecular mechanism of increased L1 in schizophrenia is different from Rett syndrome.

In this study, we found that both early environmental and well-defined strong genetic factors for schizophrenia are involved in the increase of L1 copy number in the brain. A recent study using the poly-I:C model indicated that the offspring of this model had exacerbated schizophrenia-like phenotypes, if they were exposed to environmental stress during puberty, suggesting that early environmental factors can lower the threshold for onset of schizophrenia (Giovanoli et al., 2013). Therefore, increased L1 insertions induced by environmental factors may increase the susceptibility to schizophrenia by disrupting synaptic and schizophrenia-related genes in neurons, rather than being a direct cause of the disease. On the other hand, the pathological consequences of increased L1 content in neurons derived from iPS cells of schizophrenia patients with 22q11 deletions remain unclear. We chose patients with 22q11 deletions to examine L1 dynamics where there is a well-defined strong genetic risk for schizophrenia. In *MeCP2*-knockout mice, Rett-like behavioral abnormalities could be rescued by the re-expression of wild-type MeCP2 at both young and adult stages (Cobb et al., 2010; Ehninger et al., 2008), suggesting that L1 content itself may not be directly causal to disease phenotypes but instead modulate phenotypic variability among patients (Muotri et al., 2010). Similarly, we speculate that the L1 increase in schizophrenia patients with 22q11 deletions is likely to modulate phenotypes of schizophrenia rather than a direct cause, because many genes related to schizophrenia, such as *TBX-1*, *SEPT5*, *COMT*, and *PRODH*, are located within the deletion (Hiroi et al., 2013; Karayiorgou et al., 2010). Nevertheless, our findings will facilitate further studies of the mechanism of increased L1 retrotransposition associated with schizophrenia.

Our WGS analysis could not detect increased brain-specific L1 insertions in schizophrenia; however, we found that L1 insertions were more frequent in genes for synaptic function and schizophrenia relative to controls. Evrony et al. cloned one L1 insertion event from 300 single neurons and showed that 2 of 83 cortical neurons from an individual had this insertion, but detection of such a low level mosaic insertion in bulk brain tissue of the same individual was difficult and needed optimization

(Evrony et al., 2012). Thus, rare L1 insertion events could be missed in our WGS analysis. Apart from L1, nonautonomous retrotransposons such as *Alu* and SVA also show an increased copy number in the brain, possibly via the aid of L1 ORF products (Baillie et al., 2011) and their copy number might also be increased in patients. Further studies on the neuronal genome of patients with mental disorders, and supporting mechanistic evidence from animal and cellular models, may establish a broader role for instability of neural genome in the pathophysiology of schizophrenia. We expect that our findings will promote the further study of genomic instability in disease etiology due to L1 retrotransposition in brain development.

EXPERIMENTAL PROCEDURES

Postmortem Samples

Postmortem brain and liver samples were obtained from the Stanley Medical Research Institute. The demographics are summarized in Figure 1B and are described at the web site (http://www.stanleyresearch.org/). Ethics committees of RIKEN and the University of Tokyo Faculty of Medicine approved the study.

Animal Models

Animal experiments were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and guidelines of relevant facilities. For poly I:C model, pregnant mice (C57BL/6) received either a single intraperitoneal injection of poly-I:C (2 mg/ml, Sigma-Aldrich) dissolved in PBS (20 mg/kg) or an equivalent volume of PBS at embryonic day 9.5. At postnatal day 21, tissues were dissected from pups. For macaque models, cynomologus monkeys (*Macaca fascicularis*) (4 years old; all males) were given oral haloperidol (0.25–0.5 mg/kg; Wako Pure Chemical Industries) or vehicle for 2 months (Shibuya et al., 2010). After transiently separating two male monkey neonates (2 weeks old) from dams, neonates received subcutaneous administration of human recombinant EGF (0.3 mg/kg, Funakoshi) for seven times over 11 days and then quickly returned to their dams. Preliminary behavioral assessment of the EGF-treated monkeys was performed at ages of 4 and 6 years and reported (Nawa et al., 2009). These monkeys were sacrificed at the age of 4 and 7 years with the overdose of pentobarbital (26 mg/kg; 65 mg/ml). Experiments were subjected to review by the Ethical Committee of Shinn Nippon Biomedical Lab.

iPS Cells

All procedures for skin biopsy and iPS cell production were approved by the Keio University School of Medicine ethics committee and RIKEN ethics committee. The 201B7 iPS cells were kindly provided by Dr. Yamanaka (Takahashi et al., 2007). For the control WD39, a skin-punch biopsy from a healthy 16-year-old Japanese female obtained after written informed consent was used to generate iPS cells (Imaizumi et al., 2012). 22q11.2 deletion syndrome iPS cells (SA001 and KO001) were generated from a 37-year-old Japanese female patient (Toyosima et al., 2011) and a 30-year-old Japanese female patient, respectively, using the same method used to generate the WD39 (M.T., unpublished data). 22q11 deletion was characterized by the CGH array analysis (Figure S3). Production and maintenance of iPS cells were performed according to the previous studies (Imaizumi et al., 2012; Takahashi et al., 2007). All the iPS cells and differentiated neuronal cell lines were characterized with immunofluorescence staining and their morphologies (Figure S3).

L1 Copy Number Estimation

We performed either Taqman-based quantitative real-time PCR according to Coufal et al. (2009) with minor modifications (100 or 500 pg DNA as starting material and single amplicon analysis) or SYBR-Green-based quantitative real-time PCR according to Muotri et al. (2010). SYBR-Green assay was performed using 500 pg DNA and Power SYBR Green PCR Master Mix (Life Technologies). Primers, probe location, and reaction chemistry are listed in Figure 1A and Table S4. Quantification was performed in triplicate. A nonparametric Mann-Whitney U test was employed for two group comparison and $p < 0.05$ was considered significant.

Whole-Genome Sequencing

WGS of brain and liver samples from controls and schizophrenia patients was performed by Complete Genomics, with the paired-end library preparation and sequencing-by-ligation using self-assembling DNA nanoball (DNB) (Drmanac et al., 2010). Data process, mapping, and detection of variations were performed using the software developed by the Complete Genomics (version 2.2.0.26 and format version 2.2). Among the detected mobile insertion elements, we compared the genomic location of L1 insertion between brain and liver within an individual and identified brain-specific L1 insertions.

Further experimental details are available in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2013.10.053.

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REVIEW

Modeling human neurological disorders with induced pluripotent stem cells

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Abstract

Human induced pluripotent stem (iPS) cells obtained by reprogramming technology are a source of great hope, not only in terms of applications in regenerative medicine, such as cell transplantation therapy, but also for modeling human diseases and new drug development. In particular, the production of iPS cells from the somatic cells of patients with intractable diseases and their subsequent differentiation into cells at affected sites (e.g., neurons, cardiomyocytes, hepatocytes, and myocytes) has permitted the *in vitro* construction of disease models that contain patient-specific genetic information. For example, disease-specific iPS cells have been established from patients with neuropsychiatric disorders, including schizophrenia and autism, as well as from those with neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease. A multi-omics analysis of neural cells originating from patient-derived iPS cells may thus enable investigators to elucidate the pathogenic mechanisms of neurological diseases that have heretofore been unknown. In addition, large-scale screening of chemical libraries with disease-specific iPS cells is currently underway and is expected to lead to new drug discovery. Accordingly, this review outlines the progress made via the use of patient-derived iPS cells toward the modeling of neurological disorders, the testing of existing drugs, and the discovery of new drugs.

Keywords: human disease model, induced pluripotent stem cells, neurological disorders, Parkinson's disease.

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The advent of an aging society is accompanied not only by increases in cancer and heart disease but also by increases in chronic and age-related diseases. Analyses of the pathological mechanisms of various chronic diseases and the development of new therapies for their management are currently underway, based in large part on the impressive research advances of recent years. However, numerous disorders remain with no established means of treating the underlying cause. The fact that complete human disease models are unavailable for these conditions is cited as a major problem in terms of developing new drugs for their control. While animal disease models and human disease-mimetic cell lines have been developed, construction of models that can accurately and thoroughly reproduce human pathology remains difficult. Furthermore, there is ample room for debate as to whether animal and cell line disease models can correctly reflect the phenomena that actually occur in human patients, because of species-specific differences and differences in cell line specificity. Moreover, no disease models exist for many of the rarer conditions.

In recent years, however, Professor Yamanaka of Kyoto University (Kyoto, Japan) launched a method for the preparation of induced pluripotent stem (iPS) cells that have almost the same pluripotency as embryonic stem (ES) cells. This was done by introducing four reprogramming genes, *Oct4, Sox2, Klf4*, and *c-Myc*, into differentiated somatic cells (Takahashi and Yamanaka 2006; Takahashi *et al.* 2007). Yamanaka's method made it possible (with some exceptions) to establish iPS cells from the somatic cells of any individual, regardless of race, genetic background, or state of health (i.e., whether afflicted with a disease). Moreover, the development of *in vitro* differentiation protocols for ES cells toward each

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Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; ERK1/2, extracellular signal-regulated kinase 1/2; ES cell, embryonic stem cell; FD, familial dysautonomia; GSH, reduced glutathione; iPS cell, induced pluripotent stem cell; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; TALEN, transcription activator-like effector nuclease; ZFN, zinc-finger nuclease.

embryonic germ layer paved the way for researchers to apply these techniques to iPS cells, allowing the production of a variety of iPS-derived cells, including hepatocytes, neurons, and cardiomyocytes (Takahashi *et al.* 2007). For example, the use of iPS cells derived from patients with certain neurological diseases permits the preparation of brain cells that contain the actual genetic information of the patients themselves. This is a notable feat, given that such cells have been technologically and ethically difficult to obtain in the past. Moreover, as long as the *in vitro* differentiation system is in place, it may be feasible to produce human disease models for diseases whose causative gene is unknown.

The pathological investigation of disease progression, including disease onset and the time course of disease advance, requires human materials. These materials are difficult to obtain in practice and until recently, researchers had to utilize the tissues of patients in both the early phase and the asymptomatic stage of a particular disease. However, thanks to the present revolution in iPS cell technology, cells differentiated *in vitro* from iPS cells can be used instead of human tissues for these purposes. Furthermore, iPS cell technology can be applied to chemical library screening for drug discovery, as well as to subsequent testing for drug toxicity and efficacy (Fig. 1). As a result, it is expected that the enormous cost and time involved in drug discovery research will be streamlined, and that the ability to discover new drugs will be improved.

In this review article, we outline the current status of neurological disease-specific iPS cell research. In particular, we describe recently obtained knowledge in the form of actual examples from the literature.

Driving iPS cell neural differentiation

Modeling a neurological disease requires developing methods to mimic development to make defined cultures of neurons and/or glia. So far, many studies involving the induction of various types of neurons from ES cells have allowed following the developmental process *in vitro*. Although co-culture with stromal cells, such as PA6, and/or spontaneous aggregation called embryoid bodies were directed to form neural cells in early studies (Kawasaki *et al.* 2000; Okada *et al.* 2004), recent protocols provided us more efficient and specific neural differentiation with a combination of small-molecules in a feeder-free culture system. Dual SMAD signal inhibition by supplementing Noggin and SB431542, inhibiting bone morphogenetic protein (BMP) and transforming growth factor beta, respectively, contributed to rapid and high efficacy of neuroepithelial cells (Chambers *et al.* 2009). These neuroepithelial cells have potential to differentiate into different region-specific central nervous system neurons using appropriate cues, such as Sonic Hedgehog (Shh) and Wnt8 (for midbrain dopaminergic neurons) (Fasano *et al.* 2010; Kriks *et al.* 2011), retinoic acid (RA) and Shh (for spinal cord motor neurons) (Li *et al.* 2005), Shh (for forebrain γ-aminobutyric acid (GABA) interneurons) (Liu *et al.* 2013a). These recent studies provide a promising strategy for controlled production of specific neurons for neurological disorders.

Modeling neurological diseases in vitro with disease-specific iPS cells

The iPS cell technology has rapidly expanded worldwide in less than 5 years. Disease-specific iPS cells are now available from patients with a variety of conditions, including nervous system, hematopoietic system, and metabolic system diseases, and investigations of their pathology are progressing at a brisk pace (Dimos *et al.* 2008; Park *et al.* 2008; Bellin *et al.* 2012; Robinton and Daley 2012).

Previous explorations of human neurological and psychiatric disorders were hampered by the difficulty in obtaining patient-derived neural cells or tissues because of the limited accessibility to the brain, except for autopsy samples. On the other hand, researchers have long used patient-derived fibroblasts or immortalized lymphoblasts for study, but these cells do not always recapitulate the pathogenic events of neurological and psychiatric disorders. To overcome these limitations, researchers now take advantage of olfactory tissue, with its enormous capacity for neurogenesis (Makay-Sim, 2013; Sawa and Cascella, 2009; Kano *et al.*, 2013), as well as neural cells induced from disease-specific iPS cells to examine the pathophysiology of these conditions. In fact, a variety of iPS cells derived from patients with the following neurological and psychiatric conditions are currently in wide use: Alzheimer's disease (AD) (Yagi *et al.* 2011; Israel *et al.* 2012; Kondo *et al.* 2013), Parkinson's disease (PD) (Devine *et al.* 2011; Nguyen *et al.* 2011; Seibler *et al.* 2011; Cooper *et al.* 2012; Imaizumi *et al.* 2012; Jiang *et al.* 2012; Liu *et al.* 2012a; Rakovic *et al.* 2012; Sanchez-Danes *et al.* 2012; Reinhardt *et al.* 2013), amyotrophic lateral sclerosis (Dimos *et al.* 2008; Mitne-Neto *et al.* 2011; Bilican *et al.* 2012; Egawa *et al.* 2012), Huntington's disease (Park *et al.* 2008; Zhang *et al.* 2010; An *et al.* 2012; Camnasio *et al.* 2012; HD iPSC Consortium 2012; Jeon *et al.* 2012; Juopperi *et al.* 2012), spinal muscular atrophy (Ebert *et al.* 2009; Chang *et al.* 2011), spinal and bulbar muscular atrophy (Nihei *et al.* 2013), Rett's syndrome (Marchetto *et al.* 2010; Muotri *et al.* 2010; Ananiev *et al.* 2011; Cheung *et al.* 2011; Ricciardi *et al.* 2012), schizophrenia (Brennand *et al.* 2011; Chiang *et al.* 2011; Pedrosa *et al.* 2011; Paulsen Bda *et al.* 2012), Down syndrome (Park *et al.* 2008; Li *et al.* 2012; Weick *et al.* 2013), Dravet syndrome (Higurashi *et al.* 2013; Jiao *et al.* 2013; Liu *et al.* 2013b), familial dysautonomia (FD) (Lee *et al.* 2009, 2012), adrenoleukodystrophy (Jang *et al.* 2011), Cockayne's syndrome (Andrade *et al.* 2012), fragile X-associated tremor/ataxia syndrome (Liu *et al.* 2012b; Crompton *et al.* 2013),

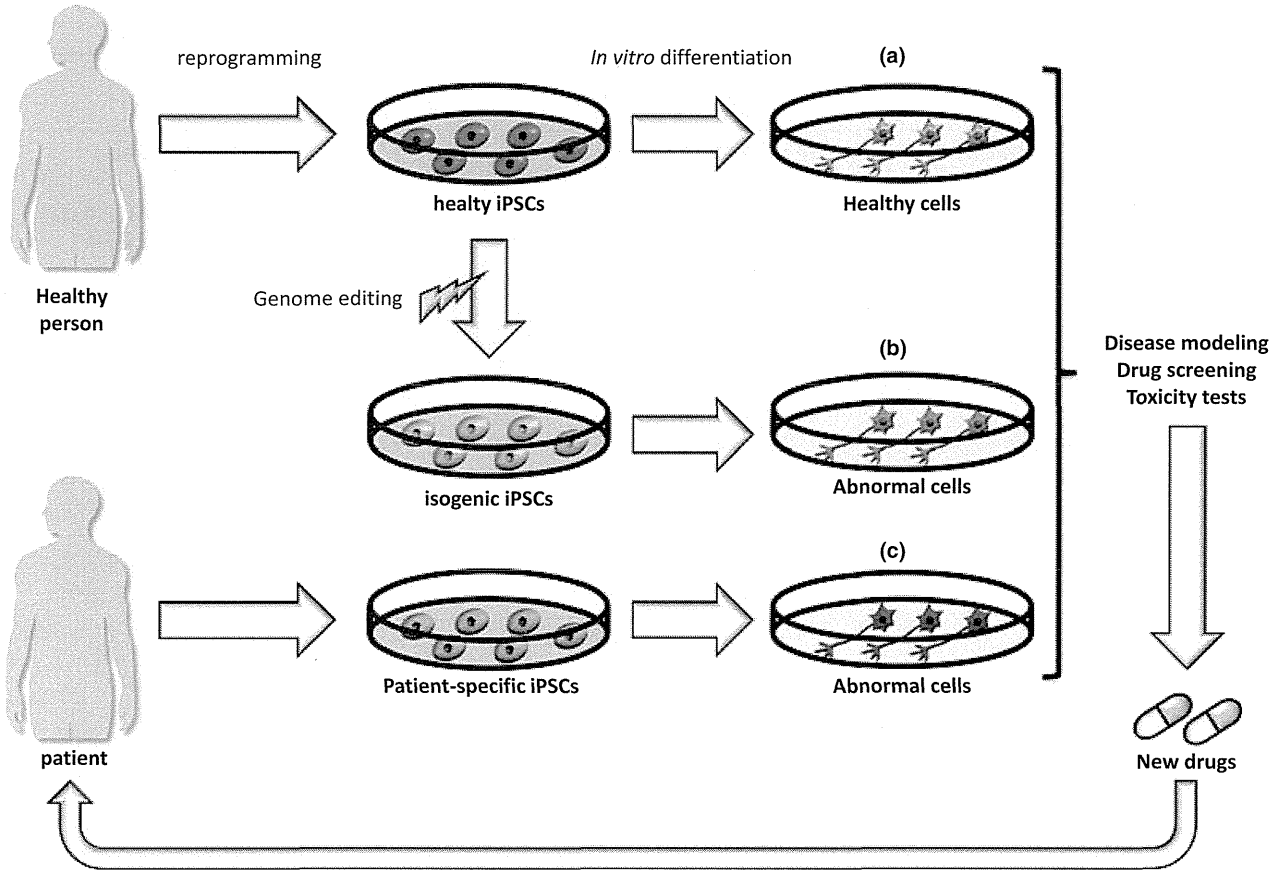

Fig. 1 Application of induced pluripotent stem (iPS) cell technology in disease research. (a, c) iPS cells are established by introducing Yamanaka's four factors (*Oct4, Sox2, Klf4,* and *c-Myc*) into healthy human and patient-derived somatic cells. Later, the iPS cells are induced to differentiate into target cells. The differentiated target cells can then be applied to the analysis of disease pathology, the screening of chemical libraries to identify drug candidates, and toxicity and efficacy testing of the newly identified compounds. Thus, iPS cells are crucially linked to new drug development. (b) Monogenic mutations are induced in iPS cells derived from healthy subjects via genome editing technologies by using helper-dependent adenoviral vectors, the zinc-finger nucleases (ZFNs), the TALENs, and the crisper-Cas9 system, which have all been developed in recent years. The use of isogenic iPS cells makes it possible to precisely analyze pathogenetic mechanisms that are attributable to the effects of a single gene.

and Machado-Joseph disease (Koch *et al.* 2011) (Table 1). We will introduce some examples of the use of these disease-specific iPS cells for the characterization of human neurological disorders in the following sections.

Modeling Familial PDs with disease-specific iPS cells

A number of disease-specific iPS cells were originally obtained from patients with genetic diseases, in which the causative gene was identified mainly because of recent advances in sequencing technology. The incidence rate of these diseases is low, and accordingly, the development of treatment modalities has in general been delayed. These diseases may also be described as conditions in which the cause–effect relationship can readily be identified between the abnormality exhibited by neurons differentiated from the disease-specific iPS cells and the mutation or deletion of the causative gene. The advantages of disease-specific iPS cells have been greatly exploited for rare genetic diseases.

Disease-specific iPS cell research directed toward familial PD is especially active. Parkinson's disease is the second most common neurodegenerative disease after AD. More than 4 million patients are afflicted with PD worldwide, and the prevalence in Japan is about 100–150 cases per population of 0.5 million individuals. There is currently no method of treatment for the underlying cause, and because many patients become symptomatic from the latter half of the 6th decade until the 7th decade of life, the management of PD is a major issue in countries facing an aging population. Although the exact cause–effect relationship of the disorder remains undetermined, PD is thought to stem from a loss of dopaminergic neurons in the substantia nigra of the midbrain. As a result, dopamine content falls below 20% of its normal

Table 1 Neurological diseases modeled with iPS cells

Disease	Gene	Cell type differentiated from iPSCs	Drug tests	References
Adrenoleukodystrophy	ABCD1	Oligodendrocytes and neurons	Lovastatin, 4-phenylbutyrate	(Jang *et al.* 2011)
Alzheimer's disease	PS1, PS2, APP, sporadic	Cortical neurons	γ-、β-secretase inhibitor, docosahexaenoic acid	(Yagi *et al.* 2011; Israel *et al.* 2012; Kondo *et al.* 2013)
Amyotrophic lateral sclerosis	SOD1, VAPB, TDP43	Motor neurons and glial cells	Anacardic acid, trichostatin A, spliceostatin A, garcinol	(Dimos *et al.* 2008; Mitne-Neto *et al.* 2011; Bilican *et al.* 2012; Egawa *et al.* 2012)
Cockayne's syndrome	ERCC6	iPSCs and neurons	N/A	(Andrade *et al.* 2012)
Down syndrome	Trisomy 21	Neurons and neural progenitors	N/A	(Park *et al.* 2008; Li *et al.* 2012; Weick *et al.* 2013)
Dravet syndrome	SCNA1A	GABAergic neurons	N/A	(Higurashi *et al.* 2013; Jiao *et al.* 2013; Liu *et al.* 2013b)
Familial dysautonomia	IKBKAP	Neural crest progenitor cells	Kinetin, SKF-86466	(Lee *et al.* 2009, 2012)
Fragile X-associated tremor/ataxia syndrome	FMR1	Neurons	N/A	(Urbach *et al.* 2010; Liu *et al.* 2012b)
Huntington's disease	HTT	Glutamatergic neurons and GABAergic neurons	N/A	(Park *et al.* 2008; Zhang *et al.* 2010; An *et al.* 2012; Camnasio *et al.* 2012; HD iPSC Consortium 2012; Jeon *et al.* 2012; Juopperi *et al.* 2012)
Machado-Joseph disease	ATXN3	Glutamaterigic neurons	Calpain	(Koch *et al.* 2011)
Parkinson's disease	LRRK2, PINK1, SNCA, PARKIN, sporadic	Dopaminergic neurons	Coenzyme Q10, rapamycin, GW5074, LRRK2-IN1, PD0325901	(Devine *et al.* 2011; Nguyen *et al.* 2011; Seibler *et al.* 2011; Cooper *et al.* 2012; Imaizumi *et al.* 2012; Jiang *et al.* 2012; Liu *et al.* 2012a; Rakovic *et al.* 2012; Sanchez-Danes *et al.* 2012; Reinhardt *et al.* 2013)
Rett's syndrome	MECP2, CDKL5	Neurons and neural progenitor cells	IGF1, gentamicin	(Marchetto *et al.* 2010; Muotri *et al.* 2010; Ananiev *et al.* 2011; Cheung *et al.* 2011; Ricciardi *et al.* 2012)
Schizophrenia	DISC1, sporadic	Neurons	Loxapine, valproic acid	(Brennand *et al.* 2011; Chiang *et al.* 2011; Pedrosa *et al.* 2011; Paulsen Bda *et al.* 2012)
Spinal muscular atrophy	SMN1	Motor neruons	Valproic acid, tobramycin	(Ebert *et al.* 2009; Chang *et al.* 2011)
Spinal and bulbar muscular atrophy	CAG repeat in the androgen receptor gene	Motor neurons	17-allylaminogeldanamycin	(Nihei *et al.* 2013)

level, defects develop in the circuits linking the cerebrum and the basal ganglia to which dopaminergic neurons project, and patients exhibit stereotypical motor symptoms, including bradykinesia, rigidity, tremors, and postural instability.

Previous research indicates that approximately 10% of PD patients have a form of disease that is caused by a mutation in a specific gene, and whose occurrence is termed 'familial'. For example, familial PD develops because of mutations in

the *LRRK2*, *PINK1*, *SNCA*, *PARKIN*, *DJ-1*, *ATP1A3*, *UCHL1*, and *GBA* genes. These genes are also thought to play an important role in the pathogenetic mechanism of sporadic Parkinson's disease. Because the motor symptoms do not develop until close to 70% of the dopaminergic neurons in the substantia nigra are lost, the molecular mechanism that predominates during the initial stage of PD remains unknown. However, iPS cells derived from PD patients can theoretically serve as a useful disease model by providing a tool to investigate the time course of changes from the onset of PD until the pathology has become apparent. Especially, the iPS-derived dopaminergic neuron is reasonable as PD patients have substantial loss of these neurons during the disease. We therefore focused our recent research efforts toward establishing iPS cells from early-onset familial PD (PARK2) patients with a mutation in the *PARKIN* gene to investigate the efficacy of disease-specific iPS cells for modeling purposes (Imaizumi *et al.* 2012).

We prepared PARK2 iPS cells by using recombinant retrovirus vectors to introduce the four aforementioned reprogramming genes, that is, *Oct4*, *Klf4*, *Sox2*, and *c-Myc*, into the skin cells of two PARK2 patients, patient PA and patient PB (Imaizumi *et al.* 2012). First, based on previous research on animal models of PD, we assumed that these patients would have higher levels of oxidative stress than normal individuals. Hence, we analyzed the amounts of reactive oxygen species (ROS) and reduced glutathione (GSH) in the neurons induced from PARK2 iPS cells. The results showed an inverse relationship between low GSH content and high ROS levels. We also observed activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (Zhang *et al.* 2011), which serves as a defense mechanism against intensified oxidative stress. These observations are consistent with previous data showing a predominant decrease in GSH and a concomitant increase in Nrf2 signaling in the substantia nigra of the postmortem brains of PD patients (Venkateshappa *et al.* 2012; Bassik *et al.* 2013). Thus, oxidative stress is apparently already high in the brain before the onset of Parkinson's disease and during the initial stages after its onset.

In support of the above-described work, the dopamine D1/D2 receptor antagonist apomorphine (Jang *et al.* 2013), the selective monoamine oxidase B inhibitor deprenyl (Benraiss *et al.* 2013), and the dopamine D2 receptor antagonist bromocriptine (Merkle and Eggan 2013), all of which are used clinically as drugs for the treatment of PD, up-regulate the Nrf2 pathway together with their main pharmacological effects. Moreover, the anti-convulsant drug zonisamide has a protective effect on dopaminergic neurons that is mediated by its dopamine synthesis-stimulating and mild monoamine oxidase-inhibiting actions; however, the drug also activates the synthesis of GSH, which is governed by the Nrf2 pathway (Liu *et al.* 2012b). Therefore, the therapeutic indications for zonisamide have been expanded to include its use as an anti-parkinsonian agent. Thus, if zonisamide can directly activate the central Nrf2 pathway in a more efficient manner, it is expected to find utility as a highly effective drug for the prevention of the onset and/or progression of PD.

Mitochondria are essential intracellular organelles that function in energy production. Mitochondria release significant amounts of ROS as byproducts of energy metabolism. For this reason, mitochondrial energy production is considered to be the main cause of oxidative stress. Up until now, it has been a technical challenge to make detailed structural observations of the mitochondria obtained from the postmortem brain tissue of PD patients, because of the need to minimize chemical changes in the tissues caused by cell death. As an alternative approach, we conducted detailed electron microscopic observations of the mitochondria in neurons induced from the iPS cells of PARK2 patients. Interestingly, abnormal mitochondria were observed in the iPS-derived neurons originating from both of the PARK2 patients investigated in this study, patient PA and patient PB (Fig. 2). Because no abnormal mitochondria were observed in PARK2-derived fibroblasts or in the PARK 2 iPS cells themselves, these abnormal organelles would appear to be a neuron-specific phenomenon.

In addition, the PARKIN protein is reportedly involved in mitochondrial autophagy (mitophagy) (Narendra *et al.* 2008; Matsuda *et al.* 2010). Therefore, we employed the mitochondria uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone to reduce mitochondrial membrane potential. carbonyl cyanide *m*-chlorophenyl hydrazone caused the inner membrane of the mitochondria of neurons derived from healthy subjects to disappear, whereas no disappearance of the inner membrane was observed in the neurons derived from the iPS cells of PARK2 patients. These findings suggest that abnormal mitochondria may have accumulated in the cells, because the mechanism that controls mitochondrial quality does not function properly in PARK2 iPS cell-derived neurons (Fig. 3).

Accumulation of α-synuclein-rich Lewy bodies has been reported in the neurons of patients with sporadic Parkinson's disease, based on analyses of postmortem brain tissue (Shults 2006). However, it appears that Lewy bodies usually do not accumulate in the postmortem brain tissue of PARK2 patients (Farrer *et al.* 2001). We therefore analyzed the postmortem brain tissue of PARK2 patient PA. Surprisingly, we observed an abundance of α-synuclein in the brain tissue of this patient. On the other hand, an analysis of the postmortem brain tissue of the father of patient PB, who had an identical *PARKIN* gene mutation and a similar genetic background as patient PB, revealed no α-synuclein buildup. Next, we analyzed the expression of α-synuclein in the iPS cell-induced neurons of patient PA and patient PB. A significant accumulation of α-synuclein was found only in the iPS cell-induced neurons of patient PA, thus mimicking the situation in the patient's postmortem brain tissue. No such

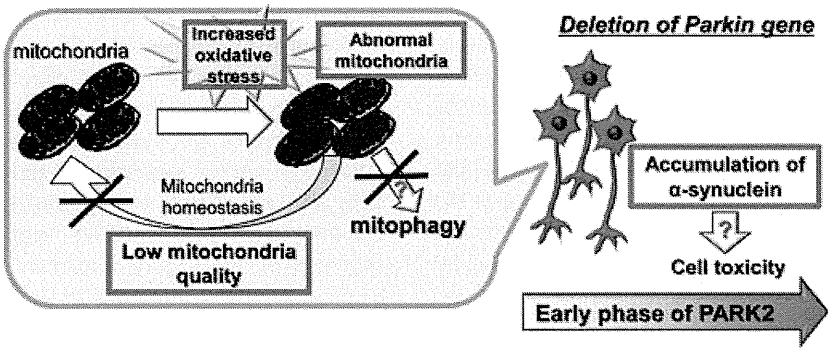

Fig. 2 Summary of the results of our study with PARK2 induced pluripotent stem (iPS) cell-derived neurons. An analysis of familial Parkinson's disease (PD) (PARK2) patient-derived iPS cells in which the *PARKIN* gene is defective revealed an increase in oxidative stress, abnormal mitochondrial morphology, and low mitochondrial quality. In addition, α-synuclein accumulation was observed in the neurons originating from PARK2 patient (patient PA)-derived iPS cells. The same patient showed Lewy body accumulation in the postmortem brain. Based on these results, further analyses using disease-specific human iPS cell-derived neurons should make it possible to reproduce the pathology and degree of disease progression in patients' brains, and to analyze pathological conditions that are close to those observed *in situ*.

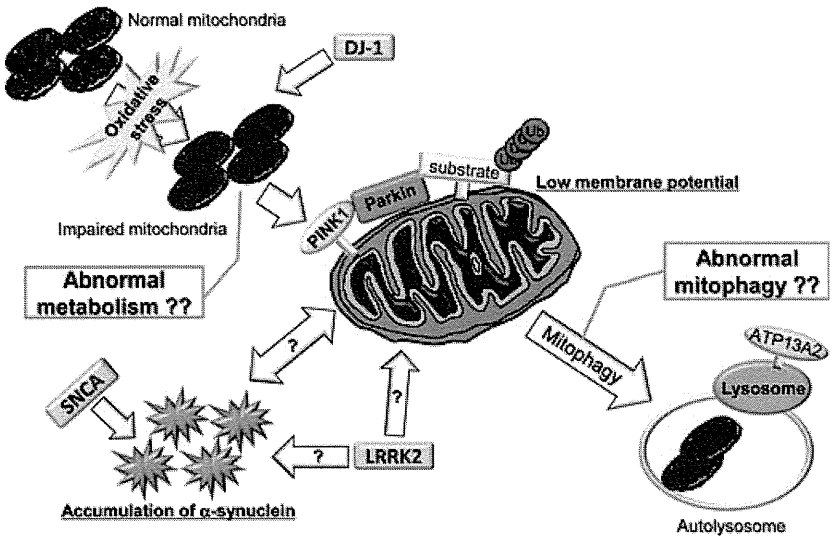

Fig. 3 Working model for familial Parkinson's disease (PD) risk factors: mediated mitochondrial quality control. Impaired mitochondria that have a low membrane potential develop as a result of increased oxidative stress and other factors. Mitochondrial quality is maintained by the familial PD factors PINK1 (PARK6) and PARKIN (PARK2). These factors pass into impaired mitochondria and guide the mitochondria toward degradation. DJ-1 (PARK7), another familial Parkinson's disease factor, is involved in a thioredoxin-mediated mechanism that protects mitochondria against oxidative stress, and a low mitochondrial membrane potential has been reported in LRRK2 mutants (PARK8). In addition, mutation of ATPase type 13A2 (ATP13A2, or PARK9), an H^+-ATPase involved in lysosomal acidification, causes abnormal lysosome function and abnormal protein degradation. On the other hand, α-synuclein oligomers cause mitochondrial impairment in familial PD (PARK1, 4) and are associated with a missense abnormality of the gene (SNCA) that encodes α-synuclein. In this case, a negative feedback loop is formed in which the mitochondrial impairment acts as a trigger for the formation of α-synuclein oligomers. These results strongly suggest that a breakdown of quality control in the impaired mitochondria plays a critical role in the onset of PD.

accumulation was observed in the iPS cell-induced neurons of patient PB, which was consistent with the analysis of the patient's father's postmortem brain tissue.

This series of findings demonstrated that is possible to model many aspects of the cellular changes seem in patients. We still have a long way to go in modeling the

disease-especially the 3D organization and different cell types including glia, etc.

Many additional studies of iPS cells originating from familial PD patients (involving *LRRK2*, *PINK1*, *SNCA*, and *PARKIN* gene mutations) have been reported by other groups (see references in Table 2). Thus, by utilizing these disease-specific iPS cells, an investigator can actually reproduce the pathology of an individual patient's disease in a test tube. Furthermore, disease-specific iPS cells are proving extraordinarily useful in analyzing disease pathology in greater detail than that afforded by other methods (Table 2).

In addition, replication experiments with isogenic iPS cells, and rescue experiments via genetic repair, are likely to allow the precise analysis of pathogenetic mechanisms resulting from changes in single genes. A variety of innovations have made the performance of these experiments tenable, including the development of the helper-dependent adenoviral vector (Suzuki *et al.* 2008), zinc-finger nucleases (Zhou *et al.* 2009), transcription activator-like effector nucleases (TALENs) (Miller *et al.* 2011), and the crisper-Cas9 system (Cong *et al.* 2013; Mali *et al.* 2013) (Fig. 2). For example, Reinhardt *et al.* (2013) demonstrated neurite outgrowth abnormalities, induction of dopaminergic neuron death by the addition of 6-hydroxydopamine, tau and α-synuclein deposition, and gene expression changes in dopaminergic neurons induced from iPS cells prepared from a patient with a *LRRK2* gene mutation (G2019S mutation). The investigators also established patient-specific iPS cells in which the G2019S mutation was repaired with zinc-finger nucleases, as well as isogenic leucine-rich repeat kinase 2 (LRRK2) iPS cells in which the G2019S mutation was introduced into normal cells. These iPS cells were used to show that the dopaminergic neuron abnormalities stemmed from a gain-of-toxic-function mutation of LRRK2 and an enhanced extracellular signal-regulated kinase 1/2 phosphorylation. However, it is essential to conduct further isogenic control and rescue experiments to confirm the phenotype obtained using patient/disease-specific iPS cells for the analyses of disease-specific iPS cells derived from many monogenic mutation diseases, and not just PD.

Challenges in modeling sporadic PD with patient-derived iPS cells

Unlike diseases caused by deletions or mutations within a specific gene (i.e., the familial Parksonism-related genes *LRRK2*, *PINK1*, *SNCA*, or *PARKIN*), it is not easy to construct human disease models for sporadic diseases in a test tube, even by using disease-specific iPS cells. The difficulty is because of the fact that these diseases are impacted by environmental as well as genetic factors. Nonetheless, a recent study successfully modeled certain aspects of the sporadic PD and LRRK2 PD by using neurons derived from a variety of patient-specific iPS cells (Sanchez-Danes *et al.* 2012). The results showed that either sporadic PD- or LRRK2-PD-iPS-derived dopaminergic neurons showed morphological alterations as well as alterations in autophagic clearance. This study demonstrates that phenotypes in neurons representing LRRK2 mutation were also observed in those from sporadic PD patients. These phenotypes associated with sporadic and LRRK2 PD might be helpful to understand pathogenesis of sporadic PD.

iPS cells based human disease models for genetic and sporadic AD

Recent work using neurons derived from the iPS cells of sporadic AD patients revealed an accumulation of amyloid β, as was observed previously in neurons derived from the iPS cells of familial AD patients with mutations in *PRESENILIN1*, *PRESENILIN2*, or amyloid precursor protein (APP) (Yagi *et al.* 2011; Israel *et al.* 2012). Presenilin 1 and 2 function as components of the γ-secretase intra-membrane protease complex. Although a γ-secretase inhibitor effectively inhibited the amyloid β buildup in neurons derived from the iPS cells of familial AD patients with *PRESENILIN1* and *PRESENILIN2* mutations, a β-secretase inhibitor, but not a γ-secretase inhibitor, engendered the same effect in neurons derived from the iPS cells of sporadic AD patients and *APP*-mutant familial AD patients (Yagi *et al.* 2011; Israel *et al.* 2012). Moreover, neurons derived from the iPS cells of *APP*-mutant patients and one of two sporadic AD patients (patient 1) showed an accumulation of Aβ oligomers, as well as increased endoplasmic reticulum and oxidative stress. Interestingly, docosahexaenoic acid treatment ameliorated the stress response in the sporadic AD iPS cell-derived neurons of patient 1, but had no effect on the iPS cell-derived neurons originating from the other sporadic AD patient (Kondo *et al.* 2013).

Thus, while there continues to be room for further experimentation, these observations reveal the impressive capacity of iPS cell research to elucidate pathological phenomena and to judge the clinical effectiveness of drugs in sporadic as well as genetic diseases. Furthermore, the likelihood that iPS cell technology will lead to the development of new strategies and methods of disease treatment seems high.

Potential applications of disease-specific iPS cells for drug screening

In recent years, several analyses have utilized disease-specific iPS cells to explore the pathogenetic mechanisms of disease. In addition, a number of attempts to utilize these cells for drug screening have also been reported. For instance, Lee and colleagues established iPS cells from patients with FD, a disorder caused by a point mutation in

Table 2 Familial PD modeled with iPS cells

Gene	Mutations	iPSC-derived cells	Phenotype in human iPSC-derived cells	Use of isogenic & gene corrected iPSC	Drug testing	References
SNCA	Triplication of SNCA	Dopaminegic neurons	Double amount of alpha-synuclein protein	N/A	N/A	Devine *et al.* 2011
Parkin	Heterozygous deletions of exon 3 and exon 5/Homozygous deletion of exon 3	Dopaminegic neurons	Increased spontaneous dopamine release; Decreased dopamine uptake and dopamine transporter-binding sites; Elevated ROS by increasing MAO transcripts	Lentiviral expression of WT-parkin	N/A	Jiang *et al.* 2012
	Homozygous deletion of exon 2-4/Homozygous deletion of exon 6,7	Neural cells including dopaminergic neurons	Increased oxidative stress; Abnormal mitochondrial morphology and impaired mitochondrial homeostasis; Accumulation of alpha-synuclein protein	N/A	N/A	Imaizumi *et al.* 2012
PINK1	Q456X nonsense/V170G missense	Dopaminegic neurons	Impaired recruitment of PARKIN to mitochondria; Increased mitochondrial copy number; Up-regulation of PGC-1alpha	Lentiviral expression of wild-type PINK1	N/A	Seibler *et al.* 2011
	V170G missense	Dopaminegic neurons	Lack of Valinomycin-induced mitophagy	N/A	N/A	Rakovic *et al.* 2012
PINK1/LRRK2	Q456X nonsense (PINK1)/Heterozygous R1441C and homozygous G2019S mutation (LRRK2)	Neural cells including dopaminergic neurons	Production of reactive oxygen species (PINK1); Mitochondrial respiration (PINK1 and LRRK2); Proton leakage (PINK1); Intraneuronal movement of mitochondria (LRRK2)	N/A	Coenzyme Q10, rapamycin, or GW5074	Cooper *et al.* 2012
LRRK2	Homozygous G2019S mutation	Dopaminegic neurons	Increased expression of oxidative stress-response genes (HSPB1, NOX1, and MAOB) and alpha-synuclein protein; Sensitive to caspase-3 activation and cell death caused by hydrogen peroxide, MG-132, and 6-hydroxydopamine	N/A	N/A	Nguyen *et al.* 2011
	Homozygous G2019S mutation	Neural stem cells	Susceptibility to proteasomal stress; Passage-dependent deficiencies in nuclear-envelope organization; Clonal expansion and neuronal differentiation	Isogenic corrected LRRK2 iPSC/mtLRRK2 KI ESC	LRRK2-In-1	Liu *et al.* 2012a

Table 2 (continued)

Gene	Mutations	iPSC-derived cells	Phenotype in human iPSC-derived cells	Use of isogenic & gene corrected iPSC	Drug testing	References
	Heterozygous G2019S mutation	Dopaminegic neurons	Dysregulation of CPNE8, MAP7, UHRF2, ANXA1, and CADPS2; Increased extracellularsignal-regulated kinase 1/2 (ERK) phosphorylation; Increased expression of MAPT mRNA and TAU protein as well as Increased alpha-synuclei protein; Neurite outgrowth phenotype; Increased sensitivity to 6-Hydroxydopamine, rotenone and oxiditative stress	Isogenic corrected LRRK2 iPSC/mtLRRK2 KI iPSCs	LRRK2-IN1, PD0325901	Reinhardt *et al.* 2013
LRRK2/sporadic	G2019S mutation/Sporadic	Dopaminegic neurons	Increased accumulation of alpha-synuclein: Fewer and shorter neurites; Increased apototic cells; Abnormal autophagic clearance	N/A	N/A	Sanchez-Danes *et al.* 2012

the *IKBKAP* (inhibitor of nuclear factor-κB kinase complex-associated protein) gene (Lee *et al.* 2009). Neural crest progenitor cells induced from the iPS cells of FD patients exhibited decreases in *IKBKAP* expression levels, reduced differentiation efficiency into neurons, and attenuated migratory ability (Lee *et al.* 2009). The same investigators also screened 6912 compounds in an effort to uncover drugs capable of increasing the expression of the *IKBKAP* gene in the induced FD patient-derived neural crest precursor cells. One of the hit drugs was termed 'SKF-86466', which has since been confirmed to augment the expression of nervous system marker genes in the induced neural crest precursor cells (Lee *et al.* 2012). These results indicate that large-scale screening of chemical libraries with patient-derived cells, an impossible goal in the past, is rapidly becoming a reality through the utilization of disease-specific iPS cells.

Future directions using disease-iPS cells

Recent advances in the iPS cell technology enable us to study not only monogenetic diseases but also sporadic disease. Furthermore, isogenic iPS cell controlled with the same genetic background would be more adequate to analyze the subtle changes in disease models. However, there are still some limitations in iPS cell technology. For example, current iPS cell-derived neurons showed poor maturational state. Patani *et al.*, showed that transcriptome profile of ES cell-derived neurons was more similar to fetal neurons rather than adult ones (Patani *et al.* 2012). Therefore, current iPS cell-derived neurons were not fully suitable for late-onset neurological disease, such as AD and PD, and disorders of neuronal circuits, such as autism and epilepsy. Moreover, the iPS cell-derived neurons following well-established neural differentiation protocols were still showed mixed population. For example, purity of ES cell-derived midbrain dopaminergic neurons was 60–80% and their properties *in vitro* were different from those in the A9 area and the striatum. However, recent direct reprogramming technology using neural factors on fibroblasts or iPS cells showed rapid production, nearly 100% purity, and functional maturation of neurons (induced neurons; iNs) (Vierbuchen *et al.* 2010; Caiazzo *et al.* 2011; Zhang *et al.* 2013). Therefore, inducing neural transcription factors on fibroblasts or iPS cells has a potential to achieve neuronal potential like adult neurons.

Furthermore, major challenges in modeling neurological disease focused on genetic disorders. However, most neurological disorders are mainly polygenic and dependent of environmental factors. To address those common diseases using iPS technology, it is because of make epigenetic modulation and/or common variant on iPS cell-derived neurons. Improving culture conditions to recapitulate the *in vivo* environment using tissue engineering

techniques, such as three dimensional culture system or microfluidics technology, is a need for future approaches.

Conclusions

In this review, we turned our attention toward neurological disease-specific iPS cells and described the current status of research in the field. While there continue to be many issues that must be resolved from the standpoint of conducting iPS cell research, including generating homogenous populations of iPS cells and formulating methods of efficiently inducing and authenticating target cell types, research in this area has been expanding worldwide since the first report of the establishment of disease-specific iPS cells in 2008 (Dimos *et al.* 2008; Park *et al.* 2008; Bellin *et al.* 2012; Robinton and Daley 2012). Current research indicates that disease-specific iPS cell technology can accurately reflect conditions before the onset of clinical disease or, in many cases, during the initial stages of the disease. By combining disease-specific iPS cell technology with whole-genome analysis and non-invasive imaging technology, dramatic progress in the elucidation of pathogenetic mechanisms is expected. Moreover, applications in new drug development, as well as in sensitivity testing, safety testing, and toxicity testing of existing drugs, are likely to proceed at an accelerated pace through the use of disease-specific iPS cell technology. Our fervent hope is that the achievements in the field of disease-specific iPS cell research will be made available to all patients for whom no treatment of the underlying disease cause currently exists.

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The miR-17/106–p38 axis is a key regulator of the neurogenic-to-gliogenic transition in developing neural stem/progenitor cells

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Neural stem/progenitor cell (NSPC) multipotency is highly regulated so that specific neural networks form during development. NSPCs cannot respond to gliogenic signals without acquiring gliogenic competence and decreasing their neurogenic competence as development proceeds. Coup-tfI and Coup-tfII are triggers of these temporal NSPC competence changes. However, the downstream effectors of Coup-tfs that mediate the neurogenic-to-gliogenic competence transition remain unknown. Here, we identified the microRNA-17/106 (miR-17/106)–p38 axis as a critical regulator of this transition. Overexpression of miR-17 inhibited the acquisition of gliogenic competence and forced stage-progressed NSPCs to regain neurogenic competence without altering the methylation status of a glial gene promoter. We also identified *Mapk14* (also known as p38) as a target of miR-17/106 and found that *Mapk14* inhibition restored neurogenic competence after the neurogenic phase. These results demonstrate that the miR-17/106–p38 axis is a key regulator of the neurogenic-to-gliogenic NSPC competence transition and that manipulation of this axis permits bidirectional control of NSPC multipotency.

neural development | glia | fate determination | differentiation | neurogenesis

Treatments of central nervous system (CNS) injury and diseases have become more promising with advances in modern medicine. Recent progress in stem cell biology has drawn attention to stem cells as innovative resources for transplantation therapies and individualized drug screenings (1, 2). Multipotent neural stem/progenitor cells (NSPCs) that give rise to all types of neural cells can now be readily obtained from induced pluripotent stem cells. However, specific and efficient induction of homogeneous target cell populations from NSPCs remains difficult because of the complex mechanisms that regulate NSPC development and differentiation. Therefore, further elucidation of how specific cell types can be generated from NSPCs is required to facilitate therapeutic applications.

We recently used a newly developed embryonic stem cell (ESC)-derived neurosphere culture system to investigate the molecular mechanisms that govern NSPC differentiation (3). Although NSPCs are multipotent, and are thus able to differentiate into neurons and glial cells, neurogenesis largely precedes gliogenesis during CNS development in vertebrates. The neurogenesis-to-gliogenesis switch requires temporal identity transitions of NSPCs (4). Importantly, our neurosphere culture system recapitulates neural development in vivo. Using this system, we found that Coup-tfI and Coup-tfII (also known as Nr2f1 and Nr2f2, respectively) are critical molecular switches in the temporal identity transition of NSPCs (3). Remarkably, Coup-tfs do not repress neurogenesis or promote gliogenesis but, instead, change the competence of NSPCs. Although Coup-tfs permit alterations by changing the responsiveness of NSPCs to extrinsic gliogenic signals, the critical regulators and/or drivers of this process remain largely unknown. The aim of this study was to determine the molecular machinery underlying the neurogenic-to-gliogenic competence transition of NSPCs.

Results

Identification of miR-17/106 as Downstream Effectors of *Coup-tfs*. We first attempted to identify candidate genes that are downstream of *Coup-tfs*. We searched for genes that are responsible for inhibition of gliogenesis and/or sustained generation of early-born neurons in *Coup-tf*-knockdown (KD) cultures. To this end, we investigated the direct DNA binding sites of Coup-tfs by performing chromatin immunoprecipitation sequencing. We then compared global gene and microRNA (miRNA) expression profiles between control and *Coup-tf*-KD neurospheres from mouse ESCs, using microarray analyses. Candidate genes were defined as genes with expression levels that were down-regulated as development proceeded in control neurospheres but that remained in a steady state in *Coup-tf*-KD neurospheres. We cloned and constructed lentiviral libraries for overexpression (OE) and KD of the candidate genes: 150 transcription factors

Significance

Neural stem/progenitor cells (NSPCs) restrict their differentiation potential by developmental stage-dependent temporal specification. Thereby, specific and efficient induction of homogeneous target cell populations remains a challenge in stem cell biology. Here, we provided a potential solution by identifying the molecular machinery responsible for neurogenic-to-gliogenic transition of NSPCs, a process we termed "competence change." We identified the microRNA-17/106–p38 axis as a critical regulator of the competence change, although epigenetic regulation seemed to be the regulatory program behind it, by our previous Coup-tf study. NSPCs sustained and restored neurogenic potential by competence regulation. Moreover, at least a part of neuron-subtype specification was regulated independent of it. Control of these multilayered regulatory programs seems promising for rigorous manipulation of cytogenesis from NSPCs.

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and 83 miRNAs, which also included miRNA clusters. We then used the ESC-derived neurosphere culture system to functionally screen the candidate genes, as previously described (*SI Appendix*, Tables S1 and S2 and *SI Appendix*, *Materials and Methods*) (3). After screening the candidate genes, we identified the miR-17-92 miRNA cluster as a functional downstream effector of Coup-tfs that regulated NSPC competence, although not as their direct target.

The miR-17-92 cluster was originally identified as a cluster of oncogenic miRNAs and is associated with the development of various organs (5, 6). The microarray results showed that the expression levels of all of the miRNAs that compose this cluster were higher in *Coup-tf*-KD neurospheres that were passaged twice (p2) than in control p2 neurospheres. Normally, the majority of cells in p2 neurospheres differentiate into astrocytes, and fewer than 20% become neurons. However, OE of the miR-17-92 cluster caused these cells to differentiate almost exclusively into neurons under the differentiation conditions without mitogen (Fig. 1*A*).

The miR-17-92 cluster encodes six distinct miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a), as well as their star (*) strands. To determine which of these miRNAs are responsible for the neurogenic phenotype, we individually overexpressed each miRNA by infecting NSPCs with lentiviruses. The lentiviruses permitted simultaneous OE of the miRNA of interest and green fluorescent protein (GFP). Only miR-17(-5p) OE replicated the phenotype induced by the intact miR-17-92 cluster (Fig. 1*A*).

MiR-17 has two additional paralogs with high similarity and identical 7mer seed sequences (nucleotides 2–8; Fig. 1*B*) that are critical for binding to target mRNAs (Fig. 1*B*), miR-106b and miR-106a, which are encoded by the miR-106b-25 and miR-106a-363 clusters, respectively (7). The target mRNAs of these two miRNAs should have highly overlapping sequences. These paralogs were individually overexpressed in NSPCs to examine whether they also affect the competence transition. OE of either miRNA led to a neurogenic phenotype, although the effects of miR-106b were stronger than those of miR-106a (Fig. 1*A*). MiR-20a and miR-20b are also extremely similar to miR-17, but we did not observe any phenotype when it was overexpressed (Fig. 1 *A* and *B*). We hypothesized that the total amounts of mature miR-17 and miR-106 are critical for the regulation of neurogenic competence in NSPCs at early developmental stages. Therefore, we assessed the expression levels of miR-17/106 in neurospheres by quantitative PCR (qPCR). All three miRNAs were down-regulated during development, and the expression levels at the p2 stage were less than half of the levels observed at p0 (Fig. 1*C*). Comparable declines in miR-17/106 were observed in NSPCs sorted from the cerebral cortex of *Nestin*-EGFP transgenic mice (Fig. 1*D*) (8). However, forced OE of miR-17 resulted in a 7.5-fold increase in the expression level of miR-17 in p2 neurospheres compared with that observed in control miR-LacZ-OE neurospheres (Fig. 1*E*).

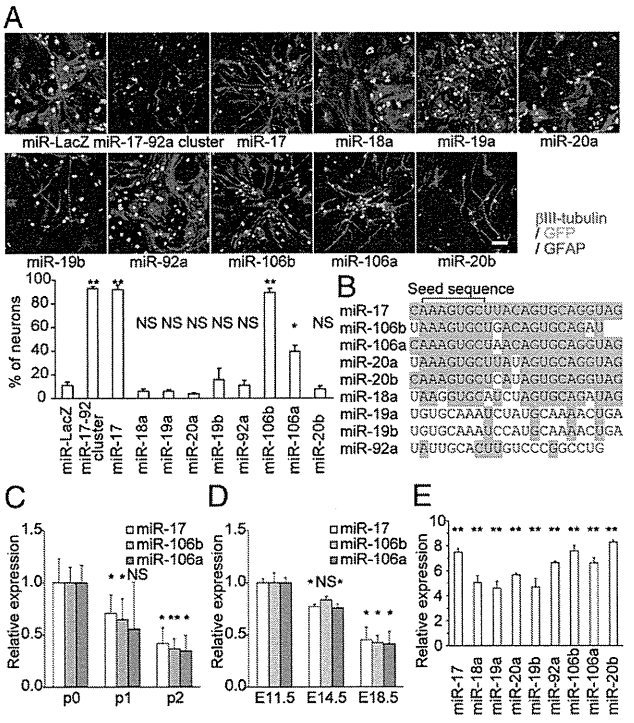

Fig. 1. OE of miR-17/106 inhibits the neurogenic-to-gliogenic transition of NSPCs in vitro. (*A*) Representative immunocytochemical images of differentiated p2 neurospheres infected with lentiviruses of the indicated miRNAs and GFP at p0 stage. MiR-LacZ was used as a control. (Scale bar, 50 μm.) The table indicates the percentage of βIII-tubulin + neurons among the total number of virus-infected cells ($n = 3$). (*B*) Sequence comparison of miRNAs encoded by the miR-17-92, miR-106b-25, and miR-106a-363 clusters. Nucleotides that are identical to the corresponding nucleotides in miR-17 are shown in green. (*C*) qPCR of miR-17/106 in neurospheres at the p0, p1, and p2 stages ($n = 3$). (*D*) qPCR of miR-17/106 in developing NSPCs sorted from the cortex of *Nestin*-EGFP transgenic mouse embryos at E11.5, E14.5, and E18.5 ($n = 3$). (*E*) qPCR of each miRNA in miRNA-OE cells (compared with miR-LacZ-OE cells; $n = 3$). Results are shown as mean ± SEM in *A* and *E* and mean ± SD in *C* and *D*. NS (not significant), $P > 0.05$; *$P < 0.05$; **$P < 0.01$.

MiR-17 Regulates the Neurogenic-to-Gliogenic Transition, but Not the Neuron-Subtype Specification. *Coup-tf*-KD causes sustained generation of Islet 1 (Isl-1)-expressing neurons, a subpopulation of early-born neurons that cannot be observed at the normal p2 neurosphere stage (3). Notably, in contrast to *Coup-tf*-KD, the miR-17 OE did not result in cytogenesis of Isl-1-positive neurons (*SI Appendix*, Fig. S1). However, during the candidate gene screening, the transcription factors Onecut1/2 were identified as positive regulators of Isl-1-positive cell production. OE of *Onecut2* increased production of Isl-1-positive neurons, and KD of *Onecut1/2* caused early termination of the production of Isl-1-positive neurons (Fig. 2 *A* and *B*). In support of these findings, a recent study demonstrated that Onecut factors directly regulate Isl-1 expression and maintain Isl-1 production during motor neuron diversification (9). These results suggest that the neurogenic-to-gliogenic transition in NSPCs, and at least a part of neuron-subtype specification, are independently regulated downstream of *Coup-tfs*.

MiR-17 Regulates NSPC Competence Without Altering the Methylation Status of the *Gfap* Promoter. The transition from early developmental neurogenic competence to late developmental gliogenic competence can be identified by changes in NSPC responsiveness to gliogenic cytokines (3). Leukemia inhibitory factor (LIF) and bone morphogenetic protein 2 (BMP2) are well-studied extrinsic gliogenic factors that promote gliogenesis in the later stages of NSPC development by activating the JAK-STAT pathway and BMP signaling, respectively (10–12). LIF does not act as a gliogenic factor in early developmental NSPCs, as the STAT3-binding sites in the promoters of glial-associated genes are epigenetically silenced by DNA methylation (13). Furthermore, BMP signaling promotes neuronal, but not glial, differentiation in the early stages of NSPC development (12, 14). Therefore, to examine whether miR-17 alters the responsiveness of NSPCs to gliogenic cytokines, we exposed miR-17-OE NSPCs to LIF and BMP2. The miR-17-OE neurospheres were strongly resistant to cytokines and exclusively differentiated into neurons at the p2 stage (Fig. 3*A*).

We next investigated the temporally regulated DNA methylation status of the STAT3-binding site and its surrounding CpG sites in the *Gfap* promoter. Glial fibrillary acidic protein (GFAP) is commonly used as a marker of astrocytes. Coup-tfs are transiently up-regulated in developing NSPCs during midgestation. NSPCs then lose their plasticity and only produce late-born neurons and glial cells. *Coup-tf*-KD in ESC-derived neurospheres

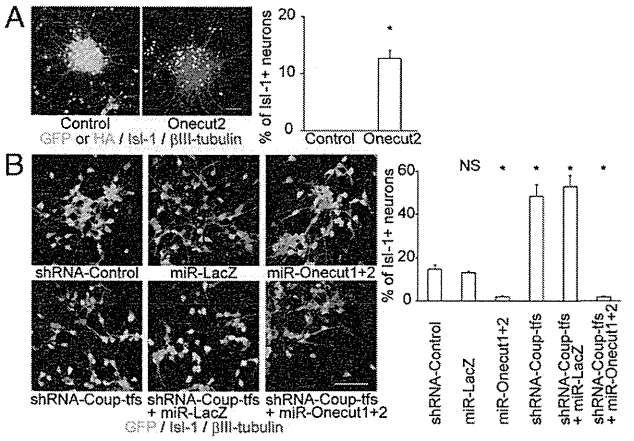

Fig. 2. Onecut1/2 regulate production of Isl-1-positive neurons downstream of Coup-tfs. (*A*) OE of HA-tagged Onecut2 in p2 neurospheres resulted in prolonged production of Isl-1 + neurons ($n = 3$). The control with nuclear-localized EGFP is also shown. (Scale bar, 50 μm.) (*B*) Repression of *Onecut1* and *Onecut2* by specific artificial miRNAs reduced the production of Isl-1 + neurons and eliminated the *Coup-tf*-KD phenotype in p1 neurospheres ($n = 3$). GFP fluorescence stemmed from the lentiviral construct containing the indicated miRNA/shRNA. (Scale bar, 50 μm.) Results are shown as mean ± SEM. NS, $P > 0.05$; $^*P < 0.05$.

maintains the early epigenetic status of the *Gfap* promoter (3). Therefore, we initially expected that the molecular effectors/drivers responsible for changes in NSPC competence would regulate the epigenetic status of neuronal or glial specification-associated genes. However, surprisingly, no significant changes in CpG methylation were observed in miR-17-OE p2 neurospheres (Fig. 3*B*). These results suggest that the changes in methylation status of glial genes are not enough for the abolition of neurogenic potential of NSPCs and that neurogenic competence can be maintained independent of the acquisition of gliogenic competence by the activation of miR-17/106 pathway.

MiR-17 OE Forces Restoration of Neurogenic Competence in Gliogenic NSPCs. We next sought to determine whether miR-17/106 OE could restore neurogenic competence in stage-progressed NSPCs. To address this question, we used lentiviruses to overexpress miR-17 in neurospheres at the onset of the p3 stage. Normally, neurospheres exclusively differentiate into glial cells at this time. We previously found that *Coup-tf*-KD results in restoration of neuropotency in limited populations of p3 NSPCs, which have not yet lost their plasticity (3). Surprisingly, miR-17 OE led to a marked restoration of neuropotency in p3 neurospheres within 1 wk. In contrast, the vast majority of cells in the control p3 neurospheres differentiated into astrocytes (Fig. 3*C*). A neurogenic phenotype similar to that induced by miR-17 OE was observed in primary cultured neurospheres derived from the subventricular zone (SVZ) of the mouse forebrain at postnatal day 30 (P30) (*SI Appendix*, Fig. S2). Furthermore, similar to our observations in neurospheres that overexpressed miR-17 from stage p0 to p2 (Fig. 3*B*), the DNA methylation status of the *Gfap* promoter did not significantly change (Fig. 3*D*). These results further support the idea that competence changes are essential prerequisites for gliogenesis.

MiR-17/106 Determine the Neurogenic Competence of NSPCs. We next used loss-of-function experiments to confirm whether miR-17/106 are downstream effectors of Coup-tfs. We used RNA decoys, referred to as "tough decoy" RNAs (TuDs), to efficiently and stably suppress the activity of specific miRNA family members (15). Lentivirus vectors were constructed that expressed a miR-17 family-specific TuD (TuD-miR-17/106), a miR-17*-specific TuD (TuD-miR-17*), or a control TuD (TuD-miR-LacZ), and we confirmed that these TuDs induced degradation of each target miRNA (Fig. 4*A*). In these experiments, *Coup-tfs* were silenced in neurospheres with a *Coup-tf*-specific short hairpin RNA (shRNA) from the p0 stage onward. Similar to miR-17-OE neurospheres, the neurogenesis-to-gliogenesis transition was inhibited in these *Coup-tf*-silenced neurospheres (3). TuD-miR-17/106 expression largely abolished the neurogenic phenotype induced by *Coup-tf*-KD at the p2 stage, whereas the TuD-miR-17* and TuD-miR-LacZ controls had no significant effect (Fig. 4*B*). Normally, p0 neurospheres barely differentiate into glia even in the presence of gliogenic cytokines. However, OE of TuD-miR-17/106 (but not TuD-miR-17*) inhibited endogenous miR-17/106 (Fig. 4*A*) and resulted in the induction of precocious gliogenesis, but only in the presence of LIF and BMP2 (Fig. 4*C*). These results demonstrate that miR-17/106 are responsible for the early developmental neurogenic competence of NSPCs downstream of Coup-tfs. Furthermore, these data show that down-regulation of miR-17/106 is an essential prerequisite for initiation of gliogenesis.

Functional Roles of miR-17/106 in Developing NSPCs. To better understand the functional roles of miR-17/106 in vivo, we first investigated their expression patterns in the developing CNS. The in vivo expression patterns of miR-17 and miR-106b were similar at embryonic day 11.5 (E11.5) and P0. The levels of both miRNAs in the brain [including the ventricular zone (VZ)] declined as

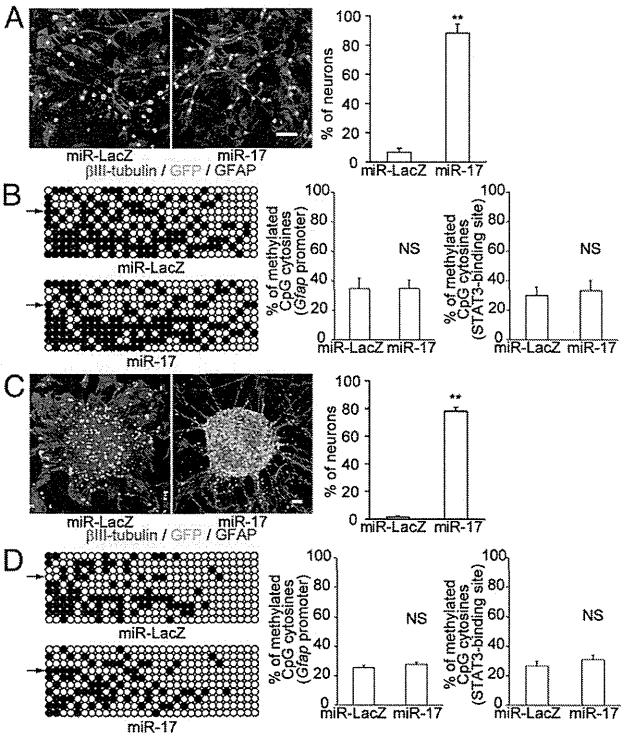

Fig. 3. MiR-17 regulates NSPC competence without altering the epigenetic status of the *Gfap* promoter. (*A*) MiR-17-OE p2 neurospheres did not undergo gliogenesis in response to LIF (10 ng/mL) and BMP2 (100 ng/mL) ($n = 3$). (Scale bar, 50 μm.) (*B*) The CpG methylation status of the *Gfap* promoter was analyzed by bisulfite sequencing. A total of 10 possible CpG cytosines in the *Gfap* promoter, including the CpG site in the STAT3-binding sequence (indicated by the arrows), were available for methylation. The percentages of methylated CpG cytosines in the *Gfap* promoter (*Left*) and the STAT3-binding region (*Right*) are shown ($n = 5$). (*C*) MiR-17 OE restored neuropotency in p3 neurospheres ($n = 3$). (Scale bar, 50 μm.) (*D*) The CpG methylation status of the *Gfap* promoter was analyzed by bisulfite sequencing, as described for *B* ($n = 5$). Results are shown as mean ± SEM. NS, $P > 0.05$; $^{**}P < 0.01$.

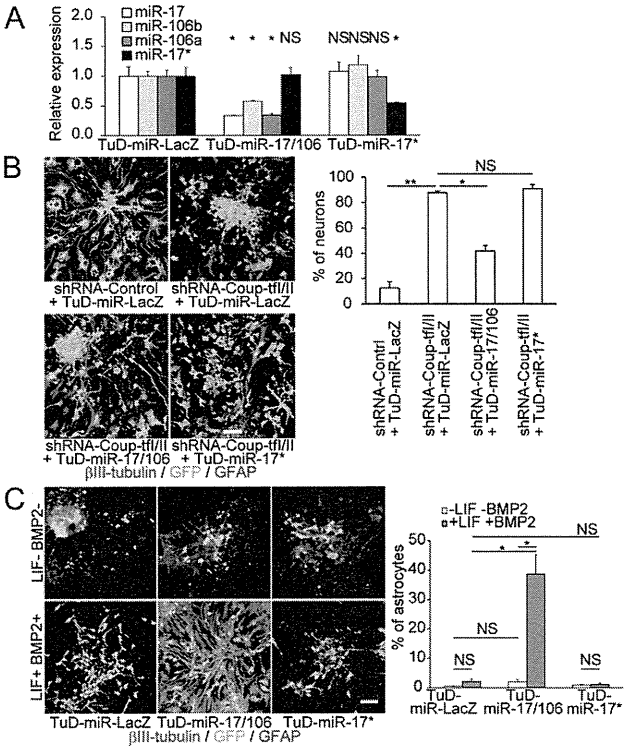

Fig. 4. Down-regulation of miR-17 eliminates the *Coup-tf*-KD phenotype in p2 neurospheres and causes precocious gliogenesis from p0 neurospheres. (*A*) qPCR of endogenous miR-17, 106b, 106a, and 17* in TuD-expressing p0 neurospheres ($n = 3$). (*B*) TuD-miR-17/106 OE eliminated the neuronal phenotype in *Coup-tf*-KD p2 neurospheres and restored the astrocytic phenotype, as evidenced by the reduced number of neurons and increased GFAP immunostaining ($n = 3$). (Scale bar, 50 μm.) (*C*) TuD-miR-17/106 OE caused abnormal early gliogenesis from p0 neurospheres only in the presence of LIF and BMP2 ($n = 3$). (Scale bar, 50 μm.) Results are shown as mean ± SD in *A* and mean ± SEM in *B* and *C*. NS, $P > 0.05$; $^*P < 0.05$; $^{**}P < 0.01$.

development progressed (*SI Appendix*, Fig. S3). Next, we analyzed the in vivo roles of miR-17/106 by overexpressing miR-17 in the developing mouse brain via in utero lentiviral microinjections. Consistent with the in vitro results, most cells (97.6 ± 0.6%, mean ± SEM) infected with the miR-17-OE lentivirus at E10.5 were fated to become neurons in the cerebral cortex by P30. In contrast, only 62.2 ± 2.8% of the cells infected with the control miR-LacZ lentivirus were fated to become neurons (Fig. 5). Thus, miR-17 OE inhibited gliogenesis in vivo in a cell-autonomous fashion. In contrast, we observed no precocious gliogenesis by TuD-miR-17/106 OE in vivo. TuD-miR-17/106 promoted precocious gliogenesis only when we supplied gliogenic cytokines in vitro. Therefore, the effects of TuD-miR-17/106 may have been suppressed by an insufficient amount of gliogenic factors (16) and the presence of antigliogenic factors (17–19) during the neurogenic period when miR-17/106 are highly expressed.

Identification of p38 as a Target of miR-17/106. Normally, miRNAs regulate the translation and/or degradation of multiple target mRNAs (20). To further identify the molecular mechanisms that underlie competence changes of NSPCs, we next attempted to identify the target mRNAs of miR-17/106. We first carried out proteomics analyses using the Isobaric Tags for Relative and Absolute Quantitation method (21) to identify proteins that were down-regulated by miR-17 OE in p2 neurospheres. Candidate genes were then scanned using our original computer program. This program searched for mRNA sequences that were complementary to miRNA seed sequences of particular miRNAs. An initial list of 40 genes was obtained (*SI Appendix*, Table S3). We then shortened the list by bioinformatic evaluation with Ingenuity Pathway Analysis and focused on 10 genes that were associated with the TGF-β signaling pathway. Finally, we functionally screened 10 candidate genes with the neurosphere culture system. Through these procedures, we identified the mRNA encoding p38 (also known as mitogen-activated protein kinase 14 or *Mapk14*) as a direct target of miR-17/106 during the regulation of the NSPC competence transitions (*SI Appendix*, *Materials and Methods*).

p38 protein expression increased during neurosphere passaging and was 2.78-fold and 3.16-fold higher in p2 neurospheres than in p0 neurospheres by Western blot and proteomics analyses, respectively (*SI Appendix*, Fig. S4*A* and Table S3). We also confirmed the increase in p38 expression in developing NSPCs by analyzing Nestin$^+$ cell populations of the SVZ of wild-type mouse forebrains at E11.5, E14.5, and E18.5, using fluorescence-activated cell sorting and immunohistochemical analysis (*SI Appendix*, Fig. S4 *B* and *C*). Furthermore, p2 neurospheres exhibited higher neuropotency than controls on exposure to SB203580, a specific p38 inhibitor, during their growth (Fig. 6*A*). p2 neurospheres subjected to KD of p38 using a p38-specific shRNA also exhibited higher neuropotency than control neurospheres (Fig. 6*B*). In addition, a reporter assay conducted using the miR-17/106-binding site in the 3′ untranslated region of p38 mRNA revealed a direct interaction between p38 mRNA and miR-17 (*SI Appendix*, Fig. S5). Finally, OE of miR-17/106-resistant p38 mRNA that lacked the entire 3′ untranslated region reversed the neurogenic phenotype induced by miR-17 OE to a gliogenic phenotype (Fig. 6*C*) and induced precocious gliogenesis in p0 neurospheres (Fig. 6*D*).

In Vivo Functional Roles of p38 in Developing NSPCs. To determine the in vivo roles of p38, we performed gain- and loss-of-function studies via in utero lentiviral microinjection. Consistent with the in vitro results, precocious GFAP expression was observed in the cerebral cortex at E17.5 when p38 was overexpressed. In utero injections of the p38-OE lentivirus resulted in cell clusters with low GFAP expression in the SVZ. In contrast, control cells that overexpressed enhanced GFP did not form cell clusters or express GFAP (Fig. 7*A*). p38 KD experiments using a lentivirus that expressed p38-specific shRNA revealed a decrease in the number of VZ and SVZ cells that expressed acyl-CoA synthetase bubblegum family member 1 (Acsbg1) at E17.5 (Fig. 7*B*). Acsbg1 is a gray matter astrocyte marker that is useful for the detection of GFAP-positive mature astrocytes and GFAP-negative immature astrocytes in the cerebral cortex (22). These observations suggest that p38 is critically involved in the initiation of gliogenesis.

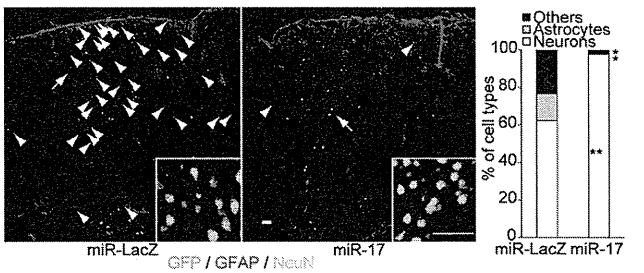

Fig. 5. In vivo roles of miR-17/106 in developing mouse forebrains. Lentiviruses that permitted OE of miR-LacZ or miR-17 were microinjected in utero into the cerebral ventricle of mouse embryos at E10.5, and the fates of the infected cells were examined in the cerebral cortex at P30 by immunohistochemistry ($n = 3$). Neuronal nuclei were stained with an antibody against NeuN. Arrowheads indicate nonneuronal cells, which include GFAP$^+$ astrocytes. Most GFAP-negative nonneuronal cells (defined as "Others" in the graph) appeared to be immature astrocytes. Higher magnification images of the cells indicated with arrows are shown as insets. (Scale bars, 50 μm.) $^*P < 0.05$; $^{**}P < 0.01$.