【代表分担者：佐谷秀行】

発表者名	論文タイトル名	発表誌	巻・号	ページ	出版年
Takenouchi T, Saya H et al.	Multiple cafe´ au lait spots in familial patients with MAP2K2 mutation.	Am J Med Genet	164	392-396	2014

【研究分担者：小崎健次郎】

発表者名	論文タイトル名	発表誌	巻・号	ページ	出版年
Kosaki R, Takenouchi T, Takeda N, Kagami M, Nakabayashi K, Hata K, Kosaki K.	Somatic CTNNB1 mutation in hepatoblastoma from a patient with Simpson-Golabi-Behmel syndrome and germline GPC3 mutation.	Am J Med Genet	in press	in press	2014

Ⅳ. 研究成果の刊行物・別刷

Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart Association®

Steps Toward Safe Cell Therapy Using Induced Pluripotent Stem Cells

Hideyuki Okano, Masaya Nakamura, Kenji Yoshida, Yohei Okada, Osahiko Tsuji, Satoshi Nori, Eiji Ikeda, Shinya Yamanaka and Kyoko Miura

Circ Res. 2013;112:523-533
doi: 10.1161/CIRCRESAHA.111.256149
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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This article is the first in a new thematic series on **Recent Advances in iPS Cell Research**, which includes the following articles:

Shinya Yamanaka, Guest Editor

Steps Toward Safe Cell Therapy Using Induced Pluripotent Stem Cells

Hideyuki Okano, Masaya Nakamura, Kenji Yoshida, Yohei Okada, Osahiko Tsuji, Satoshi Nori, Eiji Ikeda, Shinya Yamanaka, Kyoko Miura

Abstract: **The enthusiasm for producing patient-specific human embryonic stem cells using somatic nuclear transfer has somewhat abated in recent years because of ethical, technical, and political concerns. However, the interest in generating induced pluripotent stem cells (iPSCs), in which pluripotency can be obtained by transcription factor transduction of various somatic cells, has rapidly increased. Human iPSCs are anticipated to open enormous opportunities in the biomedical sciences in terms of cell therapies for regenerative medicine and stem cell modeling of human disease. On the other hand, recent reports have emphasized the pitfalls of iPSC technology, including the potential for genetic and epigenetic abnormalities, tumorigenicity, and immunogenicity of transplanted cells. These constitute serious safety-related concerns for iPSC-based cell therapy. However, preclinical data supporting the safety and efficacy of iPSCs are also accumulating. In this Review, recent achievements and future tasks for safe iPSC-based cell therapy are summarized, using regenerative medicine for repair strategies in the damaged central nervous system (CNS) as a model. Insights on safety and preclinical use of iPSCs in cardiovascular repair model are also discussed. (*Circ Res*. 2013;112:523-533.)**

Key Words: induced pluripotent stem cell ■ neural stem/progenitor cell ■ transplantation ■ spinal cord injury

Applications for the Use of Induced Pluripotent Stem Cells and Related Concerns: An Overview

Ever since pioneering reports introduced mouse[1] and human-induced[2–4] pluripotent stem cells (iPSCs) to the scientific community and the populace at large, there has been an increasing interest in applications for their use in the fields of biomedical research. These include cell therapy in regenerative medicine and modeling of human disease. By characterizing

Original received September 2, 2011; revision received October 31, 2011; accepted November 1, 2011. In November 2012, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 15.8 days.

From the Departments of Physiology (H.O., K.Y., Y.O., O.T., S.N., K.M.) and Orthopedic Surgery (M.N., O.T., S.N.) and Kanrinmaru Project (K.Y., Y.O.), School of Medicine, Keio University, Tokyo, Japan; Department of Pathology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan (E.I.); Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan (S.Y.); and Genomic Science Laboratory, Dainippon Sumitomo Pharma, Osaka, Japan (K.Y.).

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Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/CIRCRESAHA.111.256149**

Nonstandard Abbreviations and Acronyms	
CNPase	2′,3′-cyclic nucleotide 3′-phosphodiesterase
CNS	central nervous system
EB	embryoid body
ESC	embryonic stem cell
GFAP	glial fibrillary acidic protein
hiPSC	human iPSC
iPSC	induced pluripotent stem cell
PNS	primary neurosphere
NS/PC	neural stem/progenitor cell
SCI	spinal cord injury
SNS	secondary neurosphere

the in vitro phenotype of disease-specific iPSC-derived cells, researchers have gained new insights, not only into the pathophysiology of the particular disorder, but also into strategies for drug screening and the development of novel therapeutic agents.[5] Although disease modeling is emerging as an extremely exciting research field, this issue will not be discussed here because of space limitations. In regard to the application of iPSCs for regenerative medicine, increasing experimental evidence supports their therapeutic benefits.[6] However, some recent reports also indicate risk factors for the use of iPSCs, such as genetic and epigenetic abnormalities that could take place during reprogramming or maintenance in subsequent cell culture.[7–11] Of particular relevance is the potential tumorigenicity[6,12–14] and immunogenicity[15] associated with iPSC-based cell therapy. The purpose of this review article is to summarize previous efforts in the field, as well as the current status of iPSC-based cell therapy. Work from this group that uses iPSC-derived neural stem/progenitor cells (NS/PCs) in preclinical studies for repair of the damaged central nervous system (CNS) is discussed, with a special emphasis on spinal cord injury (SCI). These findings and those from other groups are presented in light of an important task for the future: how should researchers best address the implicit pitfalls of iPSC-based cell therapy?

Early Development of iPSC-Based Technologies

Mouse iPSCs were first established by Takahashi and Yamanaka in 2006,[1] and continuous progress has been made in the methods for their production during recent years. This article does not attempt to fully cover the methodological details of iPSC production; however, the key findings that are related to safety issues of iPSC-based cell therapy are summarized herein.

Initially, iPSCs were generated from mouse fibroblasts by retroviral introduction of the transcription factors *Oct4*, *Sox2*, *c-Myc*, and *Klf4*.[1] The actions of these 4 transcription factors are thought to reprogram somatic cells, including fibroblasts, into embryonic stem cell (ESC)-like pluripotent cells through multiple stochastic epigenetic events (eg, silencing of somatic genes and retroviral genes that putatively activate Dnmt3a and 3b) and activation of various pluripotent genes.[16] The original mouse iPSCs were selected for the expression of *Fbxo15*, a marker of undifferentiated ES cells, and thus are called *Fbxo15*-iPSCs. These cells demonstrated in vitro and in vivo differentiation into various types of cells from all 3 germ layers, but their epigenetic and biological properties differed from ESCs.[1,16] *Fbxo15*-iPSCs are currently understood to be partially reprogrammed iPSCs in which the retroviral transgenes are still expressed and the autoregulatory loops of endogenous *Oct4/Sox2/Nanog* genes are not completely established.[16] In fact, our group could not obtain pups from *Fbxo15*-iPSC-derived chimeric mice, although these cells contributed to embryonic development.[1]

In July 2007, 2 groups independently reported that selection for *Nanog* or *Oct4* expression resulted in germline-competent mouse iPSCs with increased mouse ESC-like gene expression and DNA methylation patterns compared with *Fbxo15*-iPS cells.[17,18] *Nanog* and *Oct4* are known to be crucial for the maintenance of undifferentiated ESCs by forming autoregulatory loops of endogenous *Oct4/Sox2/Nanog* genes.[16] *Nanog*-iPSCs were generated by Okita et al[17] from fibroblasts of transgenic mice containing the *Nanog*-GFP-IRES-Puro^r reporter construct. Fibroblasts from this transgenic mouse do not express the endogenous *Nanog* gene; hence, they are puromycin sensitive. However, by the retroviral transduction of *Oct4*, *Sox2*, *c-Myc*, and *Klf4*, these fibroblasts are reprogrammed into ESC-like cells and start to express the protein products of the endogenous *Nanog* gene, as well the as the *Nanog*-GFP-IRES-Puro^r reporter gene. As such, the cells acquire puromycin resistance and green fluorescent protein expression after transduction. Thus, through selection of puromycin-resistant and green fluorescent protein-positive colonies, iPSC clones with high endogenous *Nanog* expression can be obtained. These reprogrammed *Nanog*-iPSCs, as well as *Oct4*-iPSCs, are likely to be at a more advanced state of development than *Fbxo15*-iPSCs, with a phenotype characterized by activated Dnmt3a and 3b, silenced retroviral transgenes, and established autoregulatory loops of the *Oct4/Sox2/Nanog* genes. However, there is no guarantee that *Nanog*-selected iPSCs or *Oct4*-selected iPSCs are completely equivalent to mouse ESCs.[16] In fact, reactivation of the *c-Myc* retroviral transgene increased tumorigenicity in chimeric mice obtained via blastocyst injection of *Nanog*-selected iPSCs.

In January 2008, Nakagawa et al[19] reported a modified protocol for the generation of mouse iPSCs that did not require the *c-Myc* transgene. Importantly, chimeric mice derived from the *c-Myc*-minus iPSCs did not develop tumors during the study period. Furthermore, the omission of the *c-Myc* transgene resulted in the efficient isolation of iPSCs without drug selection (eg, *Nanog*-puromycin selection). This finding is advantageous for the generation of human iPSCs (hiPSCs), because the human genome does not accommodate the *Nanog*-green fluorescent protein-IRES-Puro^r reporter gene (Figure 1).

From April 2008 to February 2009, 3 articles were published reporting that mouse iPSCs could be generated from cells of various adult somatic origins, including terminally differentiated B lymphocytes,[20] liver and stomach cells,[21] and neural stem cells,[22] although the required combinations of transgenes were different depending on the somatic origin. Thus, the epigenetic state of the somatic cells affected the efficacy of their reprogramming into iPSCs.

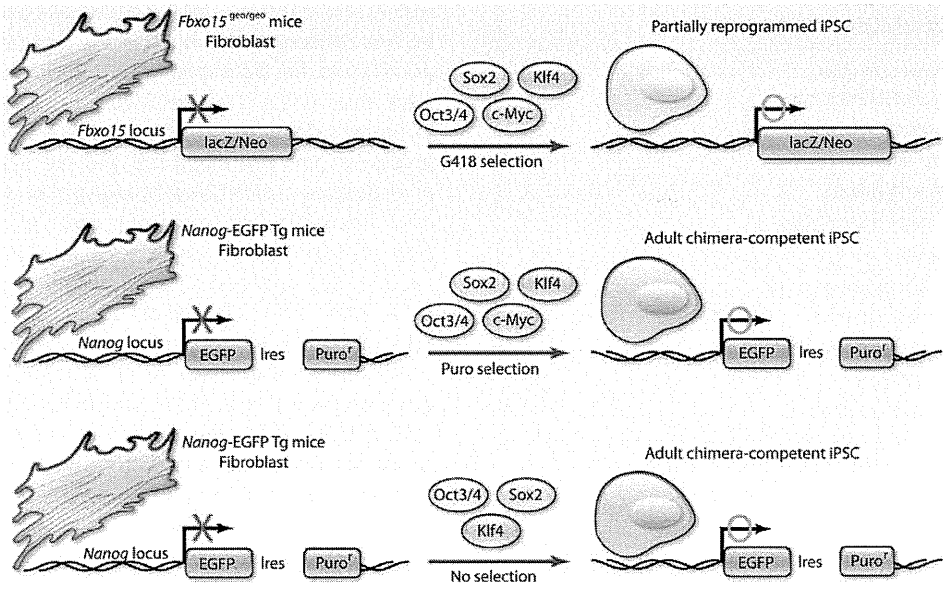

Figure 1. Evolution of methods for the generation of mouse iPSCs. Top, Generation of Fbxo15 induced pluripotent stem cells (iPSCs).[1] Four factors including *c-Myc* were transduced into fibroblasts from *Fbxo15*$^{geo/geo}$ mice. After G418 selection, partially reprogrammed *Fbxo15*-iPSCs were obtained. **Middle,** Generation of *Nanog*-iPSCs.[17] Four transcription factors including *c-Myc* were transduced into fibroblasts derived from transgenic mice carrying the *Nanog*-GFP-IRES-Puro^r reporter gene to generate *Nanog*-enhanced GFP (EGFP) transgenic mice. After puromycin selection, adult chimera-competent *Nanog*-iPSCs were obtained. **Bottom,** Generation of iPSCs with no drug selection.[19] Three transcription factors excluding *c-Myc* were transduced into fibroblasts derived from *Nanog*-EGFP transgenic mice or mice that did not have selection markers. Adult chimera-competent iPSCs were obtained without drug selection. Illustration credit: Ben Smith.

In November 2007, 2 groups independently reported the generation of hiPSCs from adult somatic cells by a retroviral/lentiviral-mediated gene transfer method that used a combination of *Oct4*, *Sox2*, *c-Myc*, and *Klf4*[2] or *Oct4, Sox2, Nanog*, and *Lin28*.[3] These reports stimulated enormous interest in iPSC research for cell therapy applications in human regenerative medicine, as well as human disease modeling, such as creating models of neurological diseases. In particular, there have been continuous efforts toward the establishment of well-characterized iPSCs that are both safe and efficacious for cell therapies. These efforts have involved improving the methods for iPSC generation and iPSC assay systems, as discussed later in this Review. However, a critical question remains, and that is how to actually test for the safety and efficacy of iPSC-based cell therapy. In the following paragraphs, lessons from previous investigations that include studies from this group will be introduced to address this issue.

Partial Reprogramming of Mouse iPSCs Is a High-Risk Factor for iPSC-Based Cell Therapy

Our group has been studying stem cell-based therapy for repair of the damaged CNS and, in particular, repair after SCI.[23–26] This Review now discusses the applicability of iPSC-based cell therapy for regenerating the contused spinal cord. Recent studies have revealed that ESCs have the potential to generate neural cells, including oligodendrocyte precursor cells[27,28] and NS/PCs.[29,30] Notably, clinical trials of human ESC therapies have finally been initiated for SCI patients at the subacute phase of injury after the primary mechanical trauma.[31] However, the use of human ESCs for SCI repair is complicated by both ethical and immunologic concerns, which could be overcome if pluripotent stem cells were derived directly from the patients' own somatic cells.[32–34]

On these grounds, soon after the first publication that introduced mouse iPSCs[1] and the successful establishment of mouse iPSCs, our group began preclinical investigations of iPSC-based cell therapy for SCI.[6,34] As a first step in the preclinical study, NS/PCs were induced from iPSCs and expanded in the form of neurospheres from various types of mouse iPSCs. The neural differentiation capability of the NS/PCs was then examined in vitro. The safety and differentiation potential of neurospheres derived from each iPSC clone were assessed through a series of transplantation experiments. In the initial experiments, 2 *Fbxo15*-iPSCs clones and 3 mouse ESCs (used as controls) were induced to differentiate into NS/PCs according to standard methods. These methods involved the treatment of neutrally biased embryoid bodies (EBs) with either Noggin or a low concentration of retinoic acid, with subsequent neurosphere formation.[35,36] The temporal changes in the differentiation potential of CNS stem cells in vivo were thereby mimicked, including the differentiation of newly generated neurons and gain of gliogenic competency that take place during fetal development.[37]

NS/PCs that were induced during EB formation from mouse ESCs were then expanded to form primary neurospheres in the presence of fibroblast growth factor-2. These primary neurospheres gave rise exclusively to early born neurons. However, after the passage of the primary neurospheres, the resultant secondary neurospheres gave rise to both neurons (mostly interneurons) and glial cells, including astrocytes and oligodendrocytes, because of the epigenetic modifications of genes involved in glial cell development.[36,37] By contrast,

Fbxo15-iPSC-derived secondary neurospheres (SNSs) differentiated into neurons and astrocytes but not into oligodendrocytes, suggesting that the differentiation capability of NS/PCs derived from *Fbxo15*-iPSCs was somehow compromised (Figure 2A and 2B). It is relevant to note that both undifferentiated *Fbxo15*-iPS cells and *Fbxo15*-iPSC-derived SNSs exhibited high expression of all 4 transgenes (*Oct4*, *Sox2*, *c-Myc*, and *Klf4*) that were used to generate the *Fbxo15*-iPSCs (Figure 2C). This suggests that continuous expression of the transgenes restricted the differentiation potential of the *Fbxo15*-iPSC-derived SNSs and rendered them highly tumorigenic. To this point, upregulation of *Oct4* and *c-Myc* are reported in naturally occurring tumors.[38,39] Notably, when *Fbxo15*-iPSC-derived secondary neurospheres were transplanted into the brains (striatal region) of nonobese diabetic (NOD)/severe combined immunodeficient (SCID) mutant mice, they showed robust teratoma formation (Figure 2D). Taken together, these results suggest that partially reprogrammed iPSCs are not suitable for cell therapy, because somatic cells (eg, NS/PCs) induced from these iPSCs still show high teratoma-forming propensities attributed, at least in part, to their incomplete suppression of transgenes encoding *Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*.

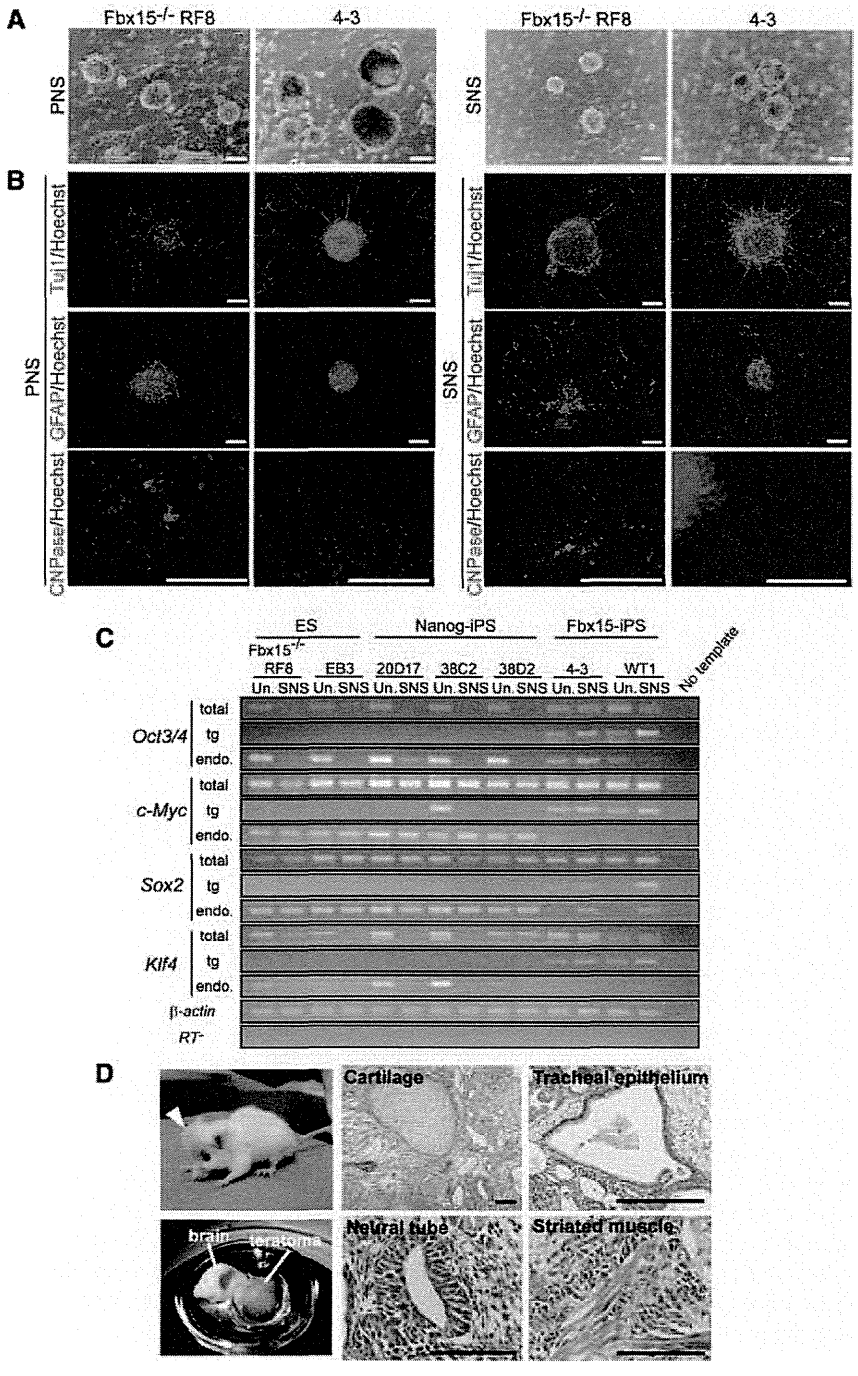

Figure 2. Characterization of neural stem/progenitor cells (NS/PCs) derived from *Fbxo15* induced pluripotent stem cells (iPSCs) in vitro. A, Neurosphere-like cell aggregates derived from *Fbxo15*-iPSCs (line 4-3). Scale bar, 200 μm. **B**, Immunocytochemical analyses of Tuj1 (class III β-tubulin), glial fibrillary acidic protein (GFAP), and 2′,3′-cyclic nucleotide 3[prime]-phosphodiesterase (CNPase) in differentiated primary neurospheres (PNSs) or secondary neurospheres (SNSs) derived from *Fbxo15*-iPSCs (line 4-3). Scale bar, 100 μm. **C**, Total RNA was isolated from undifferentiated cells (Un.) or SNSs of each cell clone and processed for reverse transcription polymerase chain reaction (RT-PCR) analysis with primers amplifying the coding regions of the 4 transgenes (total), endogenous transcripts only (endo.), or transgene transcripts only (tg). **D**, Immature teratomas derived from *Fbxo15*-iPSC (line 4-3)-derived SNSs. Large tumors were observed 4 weeks after transplantation of *Fbxo15*-iPSC (line 4-3)-derived SNSs (2 left images). These tumors were examined by histological methods using hematoxylin-eosin staining (right). Scale bar, 100 μm.

Influence of the Somatic Origin of iPSCs for Safety Issues of Cell Therapy Using iPSC-Derived NS/PCs.

As a next step, mouse iPSCs generated with more advanced reprogramming (as reported by Okita et al in 2007,[17] Nakagawa et al in 2008, [19] and Aoi et al in 2008[21]) were examined instead of *Fbxo15*-iPSCs for their neural differentiation abilities and tumor-forming propensities. As described above, adult chimera-competent mouse iPSCs have been isolated by drug selection for the expression of pluripotency-associated genes such as *Nanog* and *Oct4*; and more recent approaches have allowed their generation in the absence of drug selection.[19] In these reports, tumor formation but not teratoma formation was observed to varying degrees and was ascribed to transgene (especially *c-Myc*) activation. However, this tumor-forming propensity in adult chimera mice might not necessarily correlate with tumorigenic risks of iPSC-based cell therapy in humans because of the different species-specific mechanisms underlying tumor formation. However, considering the variations in reprogramming methods reported to date, the safety and therapeutic implications of these variations must be thoroughly evaluated before iPSCs are used in cell therapies for human patients.

The teratoma-forming propensity of SNSs derived from advanced reprogramming mouse iPSC lines was next evaluated.[12] Mouse iPSC lines differ in terms of their somatic origin, as well as the method originally used for iPSC generation, that is, drug selection and/or use of *c-Myc* transgenes. However, the mouse iPSC lines examined in this study were all established with retroviral transgenes. Surprisingly, the presence or absence of the *c-Myc* transgenes used in the generation of the iPSCs did not affect the tendency of the SNSs to form teratomas. This differs from the tumorigenic inclinations of the adult chimeric mice, which are attributable to the reactivation of the *c-Myc* retrovirus.[16] However, in the case of iPSC-derived SNSs, reactivation of *c-Myc* or other transgenes was not observed, nor did the SNSs go on to form teratomas on transplantation. Furthermore, the use of drug selection did not affect the teratoma-forming propensity of the SNSs.

On the other hand, tumorigenic tendencies varied significantly depending on the somatic tissue of origin of the parent iPSC, showing good correlation with the persistence of undifferentiated cells within the SNS. For example, SNSs derived from iPSCs that were generated from adult tail-tip fibroblast iPSCs showed the highest teratoma-forming propensity. Those derived from iPSCs generated from mouse embryonic fibroblasts and stomach tissues showed the lowest propensity and were comparable to SNSs derived from ESCs. In fact, only 1 of 11 tail-tip fibroblast-iPSC lines (line 335D1) was free from teratomas after transplantation into the striatum. Our current hypothesis is that undifferentiated cells that are continually present within the SNS could act as a source of differentiation-resistant and teratoma-initiating cells. It will be of great interest to examine whether the presence of undifferentiated cells also correlates with the teratoma-forming propensity of iPSCs generated from other somatic cells, such as cardiomyocytes. Moreover, transplantation applications for human patients will necessitate the examination of the teratoma-forming properties of NS/PCs derived from hiPSCs established from various somatic origins.

The mechanisms underlying the different teratoma-forming propensities of SNSs derived from various mouse iPSC lines remain to be determined. However, the fact that the somatic origin of the iPSCs significantly influences this propensity might suggest the involvement of epigenetic mechanisms, although the involvement of genetic changes cannot be excluded. Genes that are differentially expressed between ESCs and iPSCs are termed reprogramming-resistant genes. These genes resist the induction of a transcriptional state in iPSCs that is identical to that seen in ESCs.[40] Resistance of reprogramming might result from any of the following 3 mechanisms: (1) insufficient induction of ESC-specific genes; (2) insufficient suppression of somatic cell-specific genes; and (3) excess induction of iPSC-specific genes. In the case of teratoma formation by SNSs, it seems likely that the second mechanism could be involved, because the tissue of origin of the parent iPSC is tightly associated with the tendency of the SNS to generate teratomas. It is therefore crucially important to identify the reprogramming-resistant genes that are associated with tumor formation, from the viewpoint of safety concerns for iPSC-based cell therapy.

Transplantation of Safe Mouse iPSC Clone-Derived NS/PCs for the Repair of SCI

As described above, many types of iPSCs have thus far been established, and each type exhibits different biological properties (eg, the capacity to form teratomas after neural differentiation and transplantation, discussed above). Thus, detailed evaluation of each iPSC line, including differentiation potential and tumorigenic activity in different contexts, should be investigated to establish the safety of that line and its effectiveness for cell transplantation therapies. On these grounds, this group examined the therapeutic potential of NS/PCs derived from mouse iPSCs in an SCI model.[6]

Neurospheres from safe iPSCs (38C2 mouse embryonic fibroblasts- and 335D1 tail-tip fibroblasts-iPSC lines), which had been pre-evaluated as nontumorigenic after their transplantation into the NOD/SCID mouse brain,[12] were first considered. These neurospheres were transplanted into the spinal cord 9 days after a contusion injury. The neurospheres differentiated into all 3 neural lineages (neurons, astrocytes, and oligodendrocytes) without forming teratomas or other tumors. The graft-derived oligodendrocytes participated in remyelination and induced axonal regrowth of host 5HT(+) serotonergic fibers, which are associated with the locomotor functions of the hindlimbs.[41] The therapeutic effects of the mouse embryonic fibroblasts-iPSC line (38C2)-derived NS/PCs were very similar in their regenerative capabilities to NS/PCs derived from either mouse fetal striatal regions[42] or mouse ESCs.[30] The 3 types of NS/PCs all stimulated recovery of locomotor function in the same mouse SCI contusion model (Figure 3). By contrast, iPSC-derived neurospheres pre-evaluated as unsafe showed robust teratoma formation on transplantation and sudden locomotor functional loss after preliminary functional recovery, putatively because of a tumor mass effect in the SCI model.

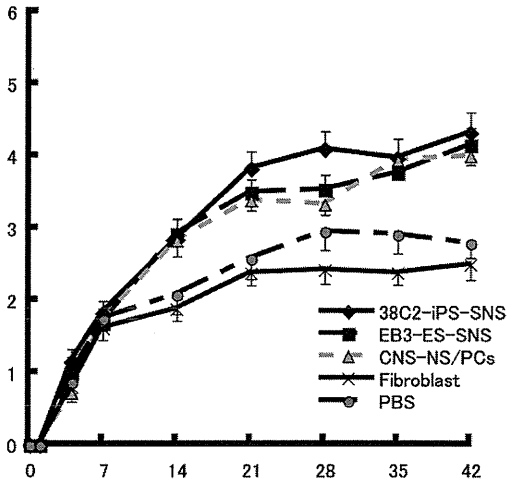

Figure 3. Therapeutic effects of secondary neurospheres (SNSs) derived from 3 independent sources (fetal mouse brains, mouse embryonic stem cells [ESCs], and mouse induced pluripotent stem cells [iPSCs]) in a mouse spinal cord injury (SCI) model. Summary of previous studies showing the time course of functional recovery of hindlimbs evaluated by the locomotor rating of the Basso Mouse Scale (BMS)[87] in SCI mice (adult female C57BL/6J mice) with a contusion injury at thoracic level 10 induced using an Infinite Horizon impactor (60 kdyn; Precision Systems, Lexington, KY). These mice were transplanted with 5×10^5 SNSs derived from mouse iPSCs (38C2 line; n=19)[6]; mouse ESCs (EB3 line; n=15)[6]; or dissociated striatal regions derived from the brains of fetal mice (embryonic day 14 C57BL/6J mice; n=8).[42] Other mice were injected with adult fibroblasts instead of SNSs (n=13).[6] Negative control mice were injected with phosphate buffered saline (n=12).[6] Cells were injected into the lesion epicenter at 9 days after the injury. As shown here, the therapeutic effects of neural stem/progenitor cells (NS/PCs) derived from 3 independent sources were very similar to one another. $^*P<0.05$, $^{**}P<0.01$.

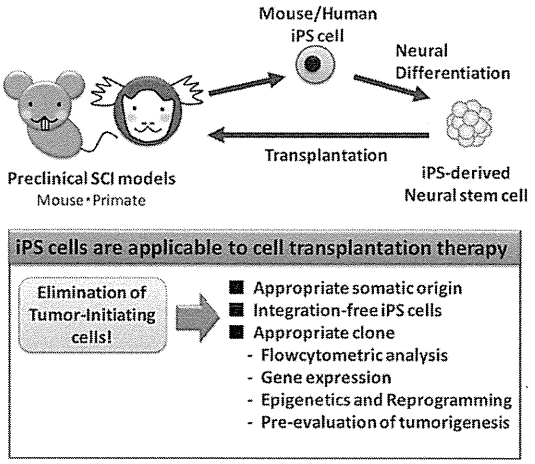

Figure 4. Preclinical studies for spinal cord injury (SCI) repair using induced pluripotent stem cell (iPSC)-derived neural stem/progenitor cells (NS/PCs). NS/PCs were generated from mouse or human iPSCs. The NS/PCs were subsequently transplanted into SCI models that used mice or nonhuman primates.[44] Appropriate selection of the iPSCs resulted in significant functional recovery without tumor formation. For future clinical applications, intensive validation of safety issues will be necessary, as indicated in the Figure.

In summary, pre-evaluated safe iPSC-derived NS/PCs and ESC-derived NS/PCs[30] showed similar therapeutic effects in an in vivo SCI model, suggesting that the availability of the human equivalent of these cells would provide a promising cell source for transplantation therapy after CNS damage.[34]

Transplantation of hiPSC-Derived NS/PCs Promoted Functional Recovery After SCI in NOD/SCID Mice

As discussed in the preceding section, once the safety issue is overcome, hiPSCs could be a potential cell source for regenerative medicine in human patients. Therefore, the therapeutic potential of transplantation of hiPSC-derived neurospheres for SCI in NOD/SCID mice was next investigated. The hiPSC clone (201B7 line)[2] used in this work was established using 4 reprogramming factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) through retroviral transduction. The grafted hiPSC-derived neurospheres survived, migrated, and differentiated into all 3 neural lineages within the injured spinal cord. The transplanted hiPSC-derived neurospheres resulted in significant functional recovery through cell-autonomous, as well as nonautonomous, mechanisms. No tumor formation was observed in the hiPSC-derived neurosphere-grafted mice during an observation period of ≤112 days. Furthermore, continuous functional recovery was observed during this time period. Hence, NS/PCs derived from hiPSCs were an effective cell source for transplantation therapy in a murine model of SCI.[34] Recently, hiPSC (201B7 line) neurospheres were also successfully transplanted into a nonhuman primate SCI injury model, resulting in a functional recovery with no sign of tumor formation.[43,44] The neurospheres promoted significant functional recovery with no tumor formation for ≤3 months after transplantation.[43,44]

In summary, NS/PCs were induced from mouse and hiPSCs and transplanted into mouse and/or nonhuman primate SCI models. The NS/PCs stimulated functional locomotor recovery without forming detectable tumors. These results were accomplished via selection of the appropriate safe iPSC lines (Figure 4).

Potential Immunogenicity in iPSC-Based Cell Therapy

A recent study indicated that iPSCs are potentially immunogenic.[15] This conclusion was made after transplantation of undifferentiated mouse iPSCs and assays for teratoma formation. However, in the case of the work from this group, transplantation of predifferentiated NS/PCs from appropriately pre-evaluated mouse iPSCs into the damaged mouse spinal cord showed no evidence of tumorigenesis or immunogenicity. Furthermore, the NC/PCs differentiated into normal trilineage neural cells in the injured spinal cord[6] in a similar way to ESC-derived NS/PCs[30] and fetal CNS-derived NS/PCs.[42]

To further address the concern of immunogenicity, global gene expression profiles were compared among undifferentiated mouse ESCs (EB3 line), mouse iPSCs (38C2 line), and predifferentiated ESC/iPSC-derived NS/PCs. Principal component analysis and hierarchical clustering analysis revealed that predifferentiated NS/PCs were clustered closely with

mouse fetal NS/PCs and separated completely from undifferentiated ESCs/iPSCs (Figure 5A and 5B). Moreover, expression levels of the representative pluripotent markers *Nanog* and *Oct4* were drastically downregulated and neuronal markers were strongly upregulated during neural differentiation (Figure 5C). Zhao et al[15] suggested that the potential immunogens *Hormad1* (a tumor antigen) and *Spt1* (a tissue-specific antigen) were abnormally upregulated in undifferentiated iPSCs and/or iPSC-derived teratomas. However, in this work, the expression levels of *Hormad1* and *Spt1* were undetectable in iPSC-derived NS/PCs, and the expression levels were very low even in undifferentiated mouse iPSCs (Figure 5D). These results strongly indicate that predifferentiated NS/PCs possess entirely different properties than undifferentiated ESCs/iPSCs and that selection of appropriate iPSC lines and proper differentiation of iPSCs will greatly reduce the potential risk of immunogenicity.

Recent Progress for the Generation of Safer hiPSCs

The above-mentioned results indicate that the safety of iPSC-based cell therapy depends on appropriate selection of iPSC lines. On the other hand, retroviral transgene activation and/or retroviral insertion mutagenesis are admittedly risk factors for the tumorigenesis of iPSC-derived cells. For this reason, increasing efforts have been directed recently toward the generation of insertion-less or insertion-free iPSCs using chemical compounds,[45–47] adenovirus vectors,[48] transposons,[49,50] plasmids,[51] recombinant proteins,[52,53] episomal vectors,[54,55] Sendai virus vectors,[56–59] and modified RNA.[60] Efforts are also underway to modify the transgenes and/or chemical compounds used in an attempt to both improve the quality of iPSCs and the efficiency of iPSC generation. These strategies include the development of inhibitors for histone deacetylase[61] and protein kinases (mitogen activated protein kinase and glycogen synthase kinase-3)[62] and the replacement of *c-Myc* with *L-Myc*.[14] Reprogramming

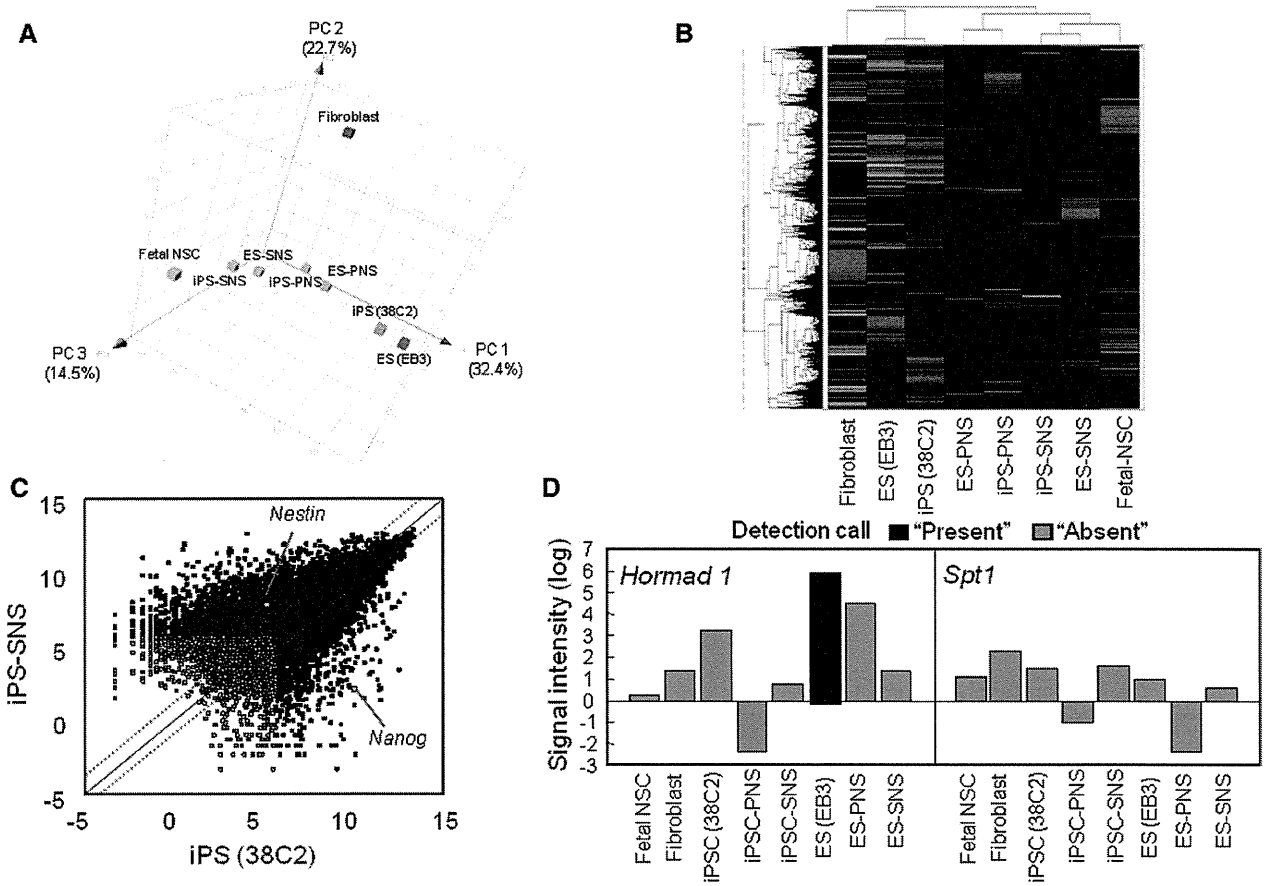

Figure 5. Global gene expression analysis of induced pluripotent stem cell (iPSC)-derived neural stem/progenitor cells (NS/PCs) and expression levels of genes encoding potential immunogens. Global gene expression analysis was carried out using Affymetrix GeneChip technology with standard protocols (accession No. GSE31725). Signal detection and quantification were performed using the MAS5 algorithm, and global normalization was performed so that the average signal intensity of all probe sets was equal to 100. **A**, Principal component analysis of gene expression data (33314 probe sets). Color key: blue cube, fibroblast; green cubes, neurospheres; red cube, embryonic stem cells (ESCs; EB3 line); pink cube, iPSCs (38C2 line). **B**, Hierarchical clustering analysis of gene expression data (33314 probe sets). The signal intensity of each gene was normalized and calculated for visualization. **C**, Scatter plot using microarray data of iPSCs and iPSC-derived secondary neurospheres (SNSs). Values represent the logarithmical signal intensity. Red dotted lines indicate a 2-fold increase or decrease between 2 samples. A signal intensity of 50 was set as the cutoff line for gene expression. Black squares, Expression in at least one sample; gray squares, no expression in either sample. **D**, Expression of genes encoding potential immunogens in undifferentiated ESCs/iPSCs and ESC/iPSC-derived neurospheres. Values represent the logarithmical signal intensity.

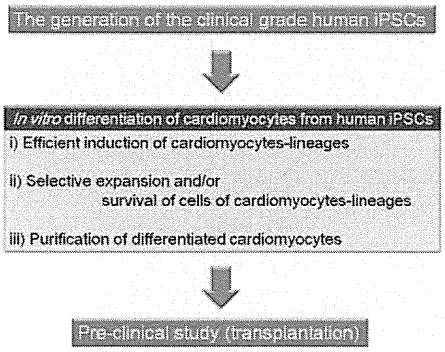

Figure 6. Safety and preclinical use of human induced pluripotent stem cells (iPSCs) in cardiovascular repair model. The steps toward safe human iPSC-based cell therapy for severe heart diseases, such as myocardial infarction, would include efforts on the generation of clinical-grade human iPSCs, in vitro differentiation of cardiomyocytes from human iPSCs, and preclinical study (transplantation) for cardiac diseases models to assess the safety and effectiveness.

of somatic cells is also being explored using mature microRNAs[63,64] and maternal transcription factor *Glis1*.[65] Another method to enhance reprogramming is by reducing p53-activity via induction of the dominant-negative form of *p53* or its short hairpin RNA.[55] The rationale behind this approach is that p53 suppresses the generation of iPSCs through the p53-p21 pathway.[66] Despite this rapid evolution of iPSC technology, fail-safe approaches for the generation of clinical-grade iPSCs have not yet been established and are part of an ongoing process.[67]

Safety and Preclinical Use of iPSCs in a Cardiovascular Repair Model

The rapid progress of stem cell technologies described above has triggered an increasing interest in the use of pluripotent stem cells including ESCs and iPSCs in cardiovascular repair. On the generation of clinical-grade hiPSCs, how can these cells be applied for regenerative medicine of cardiovascular diseases? One of the strongest advantages of using pluripotent stem cells for cardiovascular repair will be the highly expandable and self-renewal nature of these cells, which could provide an unlimited source of particular types of cardiovascular cells, including cardiomyocytes for cell therapy of severe heart diseases, such as myocardial infarction. On the other hand, as is the case in cell therapy for CNS disorders,[6,33] the potential pitfall of pluripotent stem cell-based therapy for the treatment of severe heart diseases is teratoma-forming propensity,[68] which is associated with the contamination of undifferentiated pluripotent stem cells and/or differentiation-resistant cells. Furthermore, a significantly larger number of cells would be required for cell therapy of severe heart diseases compared with that of SCI. Considering these issues, the large-scale preparation of clinical-grade cardiomyocytes would require addressing the following issues after the generation of clinical-grade human ESCs/iPSCs: (1) efficient induction of cardiomyocyte lineages from pluripotent stem cells; (2) selective expansion and/or survival of cells of cardiomyocyte lineages derived from the pluripotent stem cells; and (3) purification of differentiated cardiomyocytes derived from pluripotent stem cells and elimination of residual undifferentiated pluripotent stem cells (Figure 6). These steps are likely to be common for hESCs and hiPSCs and should be followed by performing preclinical testing for safety and effectiveness. Here, we introduce each strategy one by one.

Efficient Induction of Cardiomyocyte Lineages

As in neural differentiation,[35–37] the efficient induction of cardiomyocyte lineages from pluripotent stem cells in vitro requires the recapitulation of the microenvironmental factors that play a role during mesodermal and cardiac development. In fact, multiple steps are involved in cardiomyocyte development, including initial mesodermal differentiation, emergence of the cardiac myoblast, cardiac myoblast proliferation, and cardiomyocyte maturation.[69] In mouse embryonic development, the bone morphogenetic protein antagonist Noggin is transiently but strongly expressed in the heart-forming region during gastrulation and induces the mesendoderm for cardiogenic development. Yuasa et al[70] took advantage of this fact to develop an effective protocol for obtaining cardiomyocytes from mouse ESCs by inhibition of bone morphogenetic protein signaling. In contrast to the in vitro neural differentiation protocol of mouse ES cells, in which Noggin is administered for a prolonged period during EB formation,[35] transient Noggin treatment (from 3 days before to 1 day after EB formation) is important for the induction of cardiomyocytes. In fact, the transient Noggin treatment protocol is one of the most efficient protocols for cardiomyocyte differentiation, yielding an ≈100-fold increase in the number of cardiomyocytes compared with the control. In addition to the Noggin protocol, various techniques have been developed for the efficient induction of cardiomyocytes from pluripotent stem cells including iPS cells and human cells.[71–75]

Selective Expansion and/or Survival of Cells of Cardiomyocyte Lineage

Precise regulation of the bone morphogenetic protein,[70,76] Wnt,[77–79] hedgehog,[80] and Notch pathways[71,81] has been reported to play an important role in particular steps of cardiac development.[69] On the other hand, granulocyte colony-stimulating factor was identified as a humoral factor that uniquely promotes the proliferation of cardiomyocytes derived from mouse ESCs,[82] consistent with the expression profile of granulocyte colony-stimulating factor and its receptor in embryonic cardiac development. Administration of extrinsic granulocyte colony-stimulating factor was also found to promote the proliferation of hiPSC-derived cardiomyocytes, indicating that granulocyte colony-stimulating factor can be used to obtain high yields of cardiomyocytes from hESCs/hiPSCs for their potential application in regenerative medicine of heart diseases.

Purification of Differentiated Cardiomyocytes

The formation of teratomas in response to transplantation of undifferentiated pluripotent stem cells[68] implies that the purification of pluripotent stem cell-derived cardiomyocytes before transplantation is essential. For this purpose, various combinations of cardiomyocyte-specific reporters have been used to obtain highly pure cardiomyocytes from pluripotent stem cells,[83–87] although this method requires genetic modification of the cells. Recently, Hattori et al[13] developed a nongenetic

cardiomyocyte purification method (>99% purity) based on the fact that differentiated cardiomyocytes are extremely enriched in mitochondria. In this method, a mitochondria-selective fluorescent dye and a flow cytometer (a mitochondrial method) are used for the purification of hiPSC-derived cardiomyocytes. Notably, hESC-derived cardiomyocytes purified by this method did not induce teratoma formation after transplantation into NOD/SCID mice. Thus, this mitochondrial method could potentially contribute to the safety of hiPSC-based cell therapy for severe heart diseases, although high-speed flow cytometry of clinical grade is required for the application of this method to the treatment of human patients. Thus, further technological improvements would be required to purify large amounts of clinical-grade cardiomyocytes.

Although the above-mentioned steps are essential for the large-scale preparation of clinical-grade purified cardiomyocytes derived from human ESCs/iPSCs, their safety and effectiveness should be assessed, and methods developed for the administration of therapeutic cells into damaged hearts should be optimized using large animals as preclinical models (Figure 6).

Conclusions: Perspectives for Safe iPSC-Based Cell Therapy

Despite some precautionary data and critical attitudes, accumulating preclinical evidence supports the effectiveness of iPSC-based cell therapy on the selection of appropriate iPSC clones. Continuous development of safer iPSCs has resulted from insertion-free systems and the use of new transgenes. Nevertheless, before clinical application of iPSC-based cell therapies is achieved, these safety concerns must be assuaged through a thorough examination of the quality of both iPSCs and iPSC-derived cells, in terms of genetic and epigenetic status, differentiation capability both in vitro and in vivo, and tumorigenicity. Initial studies will require transplantation of these cells into immune-deficient animals, with subsequent long-term observation.

Acknowledgments

We thank Dr S. Shibata, H. Naka, Dr Francois Renault-Mihara, S. Ishii, Dr M. Nakamura, Dr T. Shimazaki, Dr W. Akamatsu, and Prof K. Fukuda for technical assistance and scientific discussion and all the members of Dr Okano's laboratory and Dr Yamanaka's laboratory for encouragement and kind support.

Sources of Funding

This work was supported by Grants-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science and the Ministry of Education, Culture, Sports, Science and Technology of Japan (to H.O. and S.Y.); the Project for Realization of Regenerative Medicine with support for the core institutes for iPS cell research from Ministry of Education, Culture, Sports, Science and Technology of Japan (to S.Y., M.N., and H.O.); the Ministry of Health, Labor, and Welfare (to H.O. and M.N.); the Leading Project of Ministry of Education, Culture, Sports, Science and Technology of Japan and the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program) of the Japanese Society for the Promotion of Science (to S.Y. and H.O.); Research Fellowships for Young Scientists from the Japanese Society for the Promotion of Science (to K.M.); and by a Grant-in-Aid for the Global Center of Excellence program from Ministry of Education, Culture, Sports, Science and Technology of Japan to Keio University.

Disclosures

H. Okano is the scientific consultant of San Bio, Inc; Eisai Co Ltd; and Daiichi Sankyo Co Ltd. K. Yoshida is employed by Dainippon Sumitomo Pharma Co Ltd; he worked in Dr Okano's laboratory in Keio University for 1 year as a collaborative research fellow. S. Yamanaka is a member of scientific advisory boards of iPearian Inc and iPS Academia Japan. The remaining authors report no conflicts.

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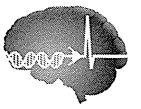

Molecular Brain

RESEARCH Open Access

The long non-coding RNA nuclear-enriched abundant transcript 1_2 induces paraspeckle formation in the motor neuron during the early phase of amyotrophic lateral sclerosis

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Abstract

Background: A long non-coding RNA (lncRNA), nuclear-enriched abundant transcript 1_2 (NEAT1_2), constitutes nuclear bodies known as "paraspeckles". Mutations of RNA binding proteins, including TAR DNA-binding protein-43 (TDP-43) and fused in sarcoma/translocated in liposarcoma (FUS/TLS), have been described in amyotrophic lateral sclerosis (ALS). ALS is a devastating motor neuron disease, which progresses rapidly to a total loss of upper and lower motor neurons, with consciousness sustained. The aim of this study was to clarify the interaction of paraspeckles with ALS-associated RNA-binding proteins, and to identify increased occurrence of paraspeckles in the nucleus of ALS spinal motor neurons.

Results: *In situ* hybridization (ISH) and ultraviolet cross-linking and immunoprecipitation were carried out to investigate interactions of NEAT1_2 lncRNA with ALS-associated RNA-binding proteins, and to test if paraspeckles form in ALS spinal motor neurons. As the results, TDP-43 and FUS/TLS were enriched in paraspeckles and bound to NEAT1_2 lncRNA directly. The paraspeckles were localized apart from the Cajal bodies, which were also known to be related to RNA metabolism. Analyses of 633 human spinal motor neurons in six ALS cases showed NEAT1_2 lncRNA was upregulated during the early stage of ALS pathogenesis. In addition, localization of NEAT1_2 lncRNA was identified in detail by electron microscopic analysis combined with ISH for NEAT1_2 lncRNA. The observation indicating specific assembly of NEAT1_2 lncRNA around the interchromatin granule-associated zone in the nucleus of ALS spinal motor neurons verified characteristic paraspeckle formation.

Conclusions: NEAT1_2 lncRNA may act as a scaffold of RNAs and RNA binding proteins in the nuclei of ALS motor neurons, thereby modulating the functions of ALS-associated RNA-binding proteins during the early phase of ALS. These findings provide the first evidence of a direct association between paraspeckle formation and a neurodegenerative disease, and may shed light on the development of novel therapeutic targets for the treatment of ALS.

Keywords: Long non-coding RNA, Paraspeckle, NEAT1_2, TDP-43, FUS/TLS, Amyotrophic lateral sclerosis, Electron microscopy

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Background

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by loss of upper and lower motor neurons. The clinical symptoms of ALS typically develop at between 50 to 70 years of age, leading to skeletal muscle weakness including respiratory failure. The overall median tracheostomy free survival is 2.5 years [1]. Among the genes associated with familial ALS, mutations in TAR DNA-binding protein-43 (TDP-43), fused in sarcoma/translocated in liposarcoma (FUS/TLS), optineurin and SQSTM1, and hexanucleotide repeat expansion in C9ORF72 were also identified in sporadic ALS cases [2-7]. Wild-type (WT) TDP-43 and FUS/TLS are predominantly observed in the nucleus by immunostaining [8,9]. Besides full-length WT TDP-43, 35-kDa and 18–26-kDa C-terminal fragments are produced via caspase-dependent and -independent pathways [8,10]. The 26-kDa C-terminal TDP-43 fragment aggregated insolubly in the cytoplasm of ALS motor neurons with ubiquitination and phosphorylation [11,12]. We previously demonstrated that the 35-kDa C-terminal fragment functions in the formation of stress granules in the cytoplasm, which induces mRNA stabilization and translational arrest against stresses [8]. Similarly, FUS/TLS mutants linked with ALS, which lacked the nuclear import activity, demonstrated mislocalization to the cytoplasm and formed a stress granule-like structure [9]. TDP-43 and FUS/TLS play critical roles in RNA processing [13]; however, the association of these RNA-binding proteins with ALS pathogenesis remains mostly unknown.

As another specific finding to sporadic ALS, the A-to-I RNA editing efficiency of mRNA encoding the GluA2 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor varied greatly, from 0% to 100% among the motor neurons of sporadic ALS cases. This observation was in marked contrast to control motor neurons, all of which demonstrated 100% editing efficiency [14].

Paraspeckle is known as one of factors which have influence on edited RNAs [15,16]. Among nuclear bodies which are important for RNA processing, paraspeckle is in close proximity to nuclear speckles [17-20]. Based on bioinformatics analyses, the nuclear-enriched abundant transcript1 (NEAT1) locus generates two types of non-coding RNAs (ncRNAs) from the same promoter in the human genome: 3.7 kb NEAT1_1 (MENε) and 23 kb NEAT1_2 (MENβ) [21,22]. Notably, NEAT1_2 long non-coding RNA (lncRNA) is essential for paraspeckle formation [17-20]. Recent reports, using individual nucleotide-resolution ultraviolet (UV) cross-linking and immunoprecipitation (iCLIP), CLIP-seq and photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) procedures, displayed that NEAT1_2 lncRNA was one of RNAs bound by both TDP-43 and FUS/TLS [23-27].

Previous electron microscopic observations indicated that the paraspeckle corresponds to a specific structure of the interchromatin granule-associated zones (IGAZ) [17,28-30]. In the current model, paraspeckles consist of NEAT1 ncRNA and more than 40 paraspeckle proteins including paraspeckle protein-1 (PSP1)/paraspeckle component1, $p54^{nrb}$/non-POU domain-containing octamer-binding protein (NONO), polypyrimidine tract binding protein-associated splicing factor (PSF), RNA polymerase II and other proteins [16,31-34]. Among these proteins, $p54^{nrb}$ and PSF are core paraspeckle proteins that trigger the formation of paraspeckles through an interaction with NEAT1_2 lncRNA.

Building upon these previous findings, we investigated the association of paraspeckles with TDP-43 and FUS/TLS in the nucleus and the alteration in paraspeckle formation in spinal motor neurons of ALS patients.

Results

TDP-43 and FUS/TLS are enriched in nuclear paraspeckles in cultured cells

Characteristics of NEAT1_2 lncRNA and paraspeckle formation have been elucidated mainly using cultured cells including HeLa cells [16-20,28-34]. To investigate the cellular basis for the pathogenesis of ALS, we initially examined the subcellular localization of WT, mutant and/or truncated forms of TDP-43 and FUS/TLS by exogenously expressing tagged proteins in HeLa cells. Both WT and the 35-kDa C-terminal fragment of TDP-43 protein were widely distributed throughout the nucleus; however, their characteristic aggregates in the nucleus coincided with 93.8 ± 10.6% and 89.7 ± 13.9% of the NEAT1_2 foci, respectively (Figure 1A, B). These findings mean that almost all NEAT1_2 foci overlap with parts of TDP-43-forming nuclear aggregates. Similar enrichment to NEAT1_2 foci was also observed with tagged WT FUS/TLS (96.9 ± 10.2%; Figure 1A) as well as endogenous TDP-43 and FUS/TLS (Additional file 1: Figure S1A). By contrast, the 26-kDa C-terminal fragment of TDP-43 formed few aggregates in the nucleus, and was distributed throughout both the nucleus and the cytosol, as shown in a previous article [8], demonstrating a marked lack of affinity for NEAT1_2 foci (15.8 ± 17.9%; Figure 1A). Additionally, ALS-associated TDP-43 mutants and FUS/TLS mutants were colocalized with NEAT1_2 lncRNA as frequently as WT TDP-43 and WT FUS/TLS (TDP-43^{A315T}: 93.3 ± 12.7%, TDP-43^{A382T}: 98.0 ± 8.2%, FUS/TLS^{R514S}: 95.8 ± 9.3%, and FUS/TLS^{P525L}: 98.4 ± 5.8%; Additional file 1: Figure S1B, C).

The short form of NEAT1 ncRNA, NEAT1_1, is produced from the 5′-end of NEAT1. Although an *in situ* hybridization probe targeting NEAT 1_1 ncRNA, that is

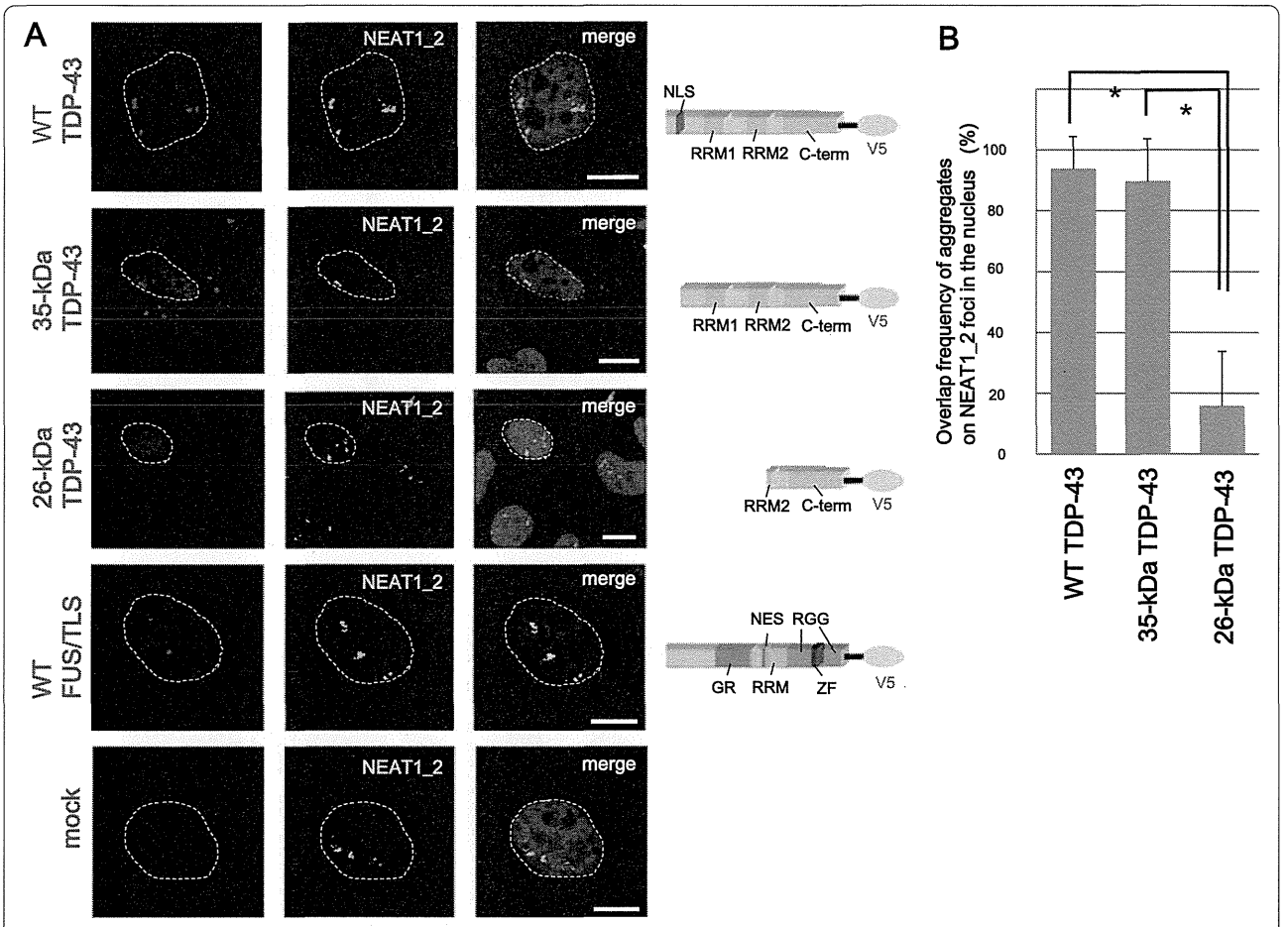

Figure 1 TDP-43 and FUS/TLS aggregate at nuclear NEAT1_2 foci. A. At 48 hours after transfection with wild-type (WT) and 35-kDa TDP-43 fragment and WT FUS/TLS with the V5 tag at the C terminus, HeLa cells were fixed with 4% paraformaldehyde, hybridized with fluorescein isothiocyanate (FITC)-labeled RNA probe against NEAT1_2 long non-coding RNA (lncRNA), and double-immunostained with polyclonal anti-FITC and monoclonal anti-V5 antibodies. Schematic diagram (right) represents the TDP-43 isoforms and FUS/TLS. NLS, Nuclear localization signal; RRM, RNA recognition motif; C-term, C-terminal domain; GR, glycine-rich motif; NES, nuclear export signal; RGG, arginine-glycine-glycine motif ; ZF, zinc-finger motif. Dotted lines represent the outline of the nucleus. Scale bars, 10μm. **B.** The Y-axis shows what percent NEAT1_2 foci is overlapped by TDP-43 nuclear aggregates in (A). The 26-kDa TDP-43 fragment hardly accumulates in nuclei and lacks affinity for NEAT1_2 foci. Data represent mean ± s.d. (n = 50 for each transfection of TDP-43 isoforms). *$P < 0.0001$ (unpaired Student's *t*-test).

shown as NEAT1_1/1_2 probe in Additional file 1: Figure S1D (upper), could not precisely distinguish NEAT1_1 foci from NEAT1_2 foci, most NEAT1_1 foci were also colocalized frequently with nuclear aggregates formed by WT TDP-43 and WT FUS/TLS (Additional file 1: Figure S1D, lower).

Another nuclear body, the Cajal body, is related to RNA metabolism; however, NEAT1_2 foci demonstrated a complete different distribution from Cajal bodies labeled with the marker coilin (Figure 2A, upper). Consistent with a previous report that 40% of TDP-43 nuclear bodies overlapped with Cajal bodies [35], endogenous TDP-43 that did not overlap with NEAT1_2 foci overlapped with the Cajal bodies separately (Figure 2A, lower). In light of TDP-43 and FUS/TLS protein colocalization to NEAT1_2 lncRNA, we tested whether endogenous TDP-43 and FUS/TLS bound directly to NEAT1_2 lncRNA. The RNA-protein complex was immunoprecipitated from UV cross-linked HeLa cells using polyclonal anti-TDP-43 and anti-FUS/TLS antibodies with stringent washes in high-salt buffer to spoil protein-protein interactions. The immunoblotting assay verified specific precipitations by using monoclonal anti-TDP-43 and anti-FUS/TLS antibodies (Figure 2B). After bound RNA was isolated, NEAT1_2 lncRNA levels were quantified by reverse transcription (RT) and polymerase chain reaction (PCR). NEAT1_2 lncRNA was enriched in anti-TDP-43 and anti-FUS/TLS immunoprecipitants compared with control IgG immunoprecipitants (Figure 2C). Paraspeckle formation requires NEAT1_2 lncRNA and core paraspeckle proteins, which subsequently recruit other paraspeckle-associated factors and NEAT1_1

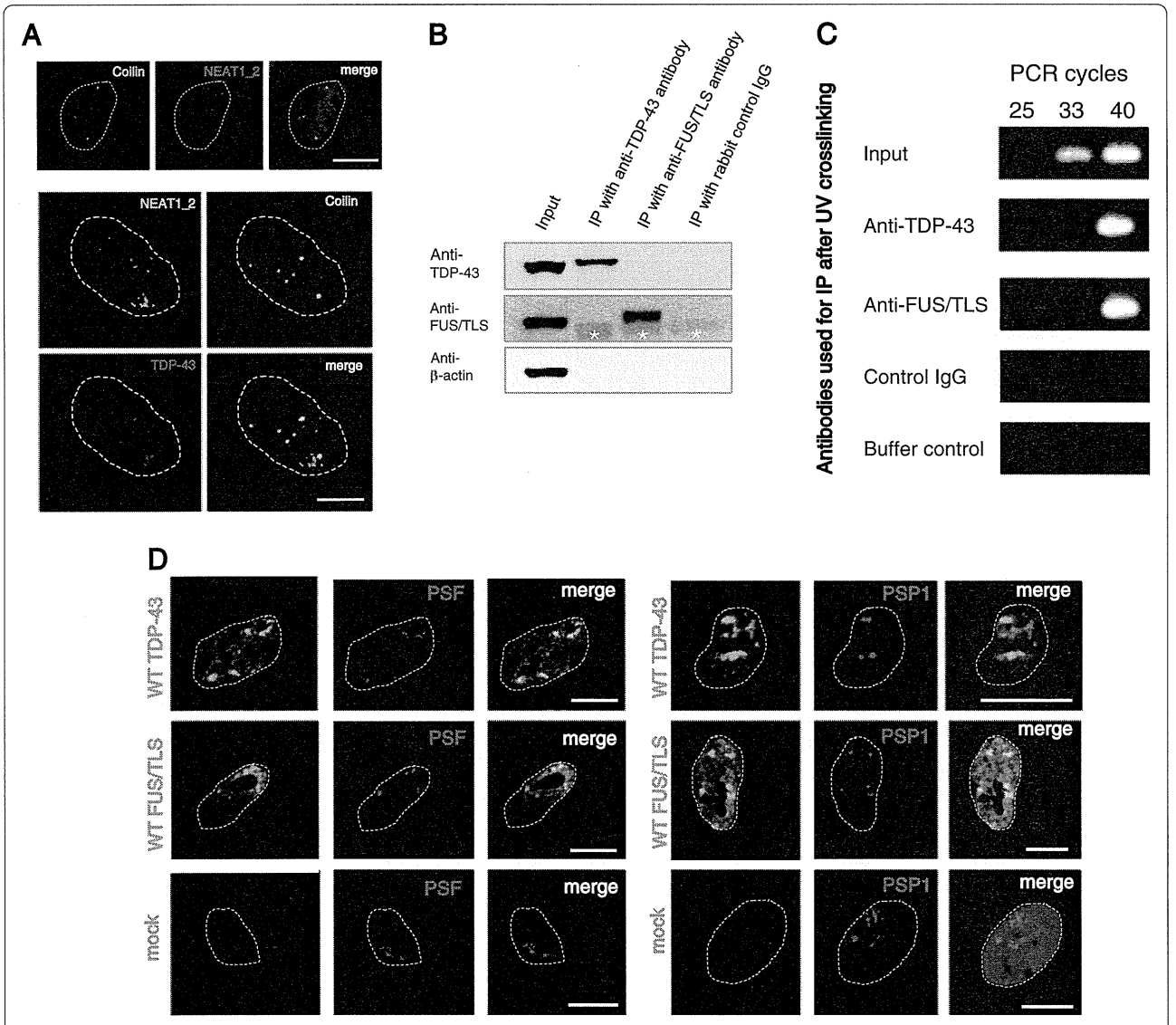

Figure 2 Both TDP-43 and FUS/TLS bind to NEAT1_2 lncRNA and are colocalized with paraspeckle proteins. A. Characterization of NEAT1_2 foci and Cajal bodies (marker: coilin). After *in situ* hybridization using DIG-labeled NEAT1_2 probe, untransfected HeLa cells were triple-labeled with monoclonal IgG1 anti-DIG, monoclonal IgG2b anti-coilin and polyclonal anti-TDP-43 antibodies. Upper: NEAT1_2 lncRNA demonstrates a different localization pattern from Cajal bodies. Lower: endogenous TDP-43 overlaps with both NEAT1_2 foci and Cajal bodies. Dotted lines represent the outline of the nucleus. **B**. Using monoclonal antibodies, full-length TDP-43, FUS/TLS and β-actin bands are shown by immunoblotting. Immunoprecipitaion (IP) followed by solid washes in high-salt buffer purified the specific protein against each antibody. Protein-protein interactions were abolished. By combined RNAs, the complexes treated with 254 nm ultraviolet (UV) crosslinking and IP were shifted to the higher molecule compared to the bands of input without UV treatment. *Non-specific detections of the rabbit IgG heavy chains. **C**. NEAT1_2 lncRNA directly binds to TDP-43 and FUS/TLS. Following UV crosslinking in HeLa cells, NEAT1_2 RT-PCR bands are detected with the indicated number of PCR cycles after IP using each antibody. **D**. Immunofluorescence of HeLa cells, which exogenously expressed WT TDP-43 or FUS/TLS with the V5 tag, was carried out at 48 hours after transfection. Monoclonal or polyclonal anti-V5 along with anti-PSF or anti-PSP1 antibodies were used. Dotted lines represent the outline of the nucleus. Scale bars, 10 μm.

ncRNA [19,29]. Therefore, to determine whether TDP-43 and FUS/TLS formed paraspeckles in cultured cells, immunocytochemistry was performed to examine the intra-nuclear localization of the paraspeckle proteins, PSF and PSP1. Nuclear aggregates of TDP-43 and FUS/TLS colocalized with PSF and PSP1 (Figure 2D). Taking these findings together, NEAT1_2 foci were considered to form paraspeckles with TDP-43 and FUS/TLS.

NEAT1_2 lncRNA is not expressed in motor neurons in control mouse spinal cord

Next, we examined NEAT1_2 distribution in the nervous system of WT control mice *in vivo*. Some reports

have demonstrated that NEAT1_2 expression is abundant in restricted cells including the epithelial cells of the esophagus, forestomach, and surface epithelium of zymogenic region of the stomach in adult mice but not in embryonic stem cells [36]. No previous reports have described NEAT1_2 expression in the spinal cord, aged tissues, or any human tissues. To test the distribution of NEAT1_1 ncRNA and NEAT1_2 lncRNA in each tissue including the nervous system in mice, quantitative RT-PCR was performed using 8-week-old mouse tissue extracts. NEAT1 ncRNA was highly expressed in lung, heart, and kidney (Figure 3A) and markedly dominated by NEAT1_1 expression, consistent with a previous report [36]. Notably, expression levels of NEAT1_1 ncRNA were modest, and NEAT1_2 lncRNA was hardly detectable in the central nervous system. Therefore, we further investigated NEAT1_2 distribution specifically in single spinal motor neurons. *In situ* hybridization followed by fluorescent immunohistochemistry (RNA-FISH) revealed no NEAT1_2 expression in the nuclei of spinal motor neurons from 8-week-old and 2-y-old mice (Figure 3B). NEAT1_1 ncRNA was expressed in the spinal glial cells of both 8-week-old and 2-y-old mice, and was also expressed at low levels in the spinal motor neurons of both young and aged mice (Additional file 2: Figure S2).

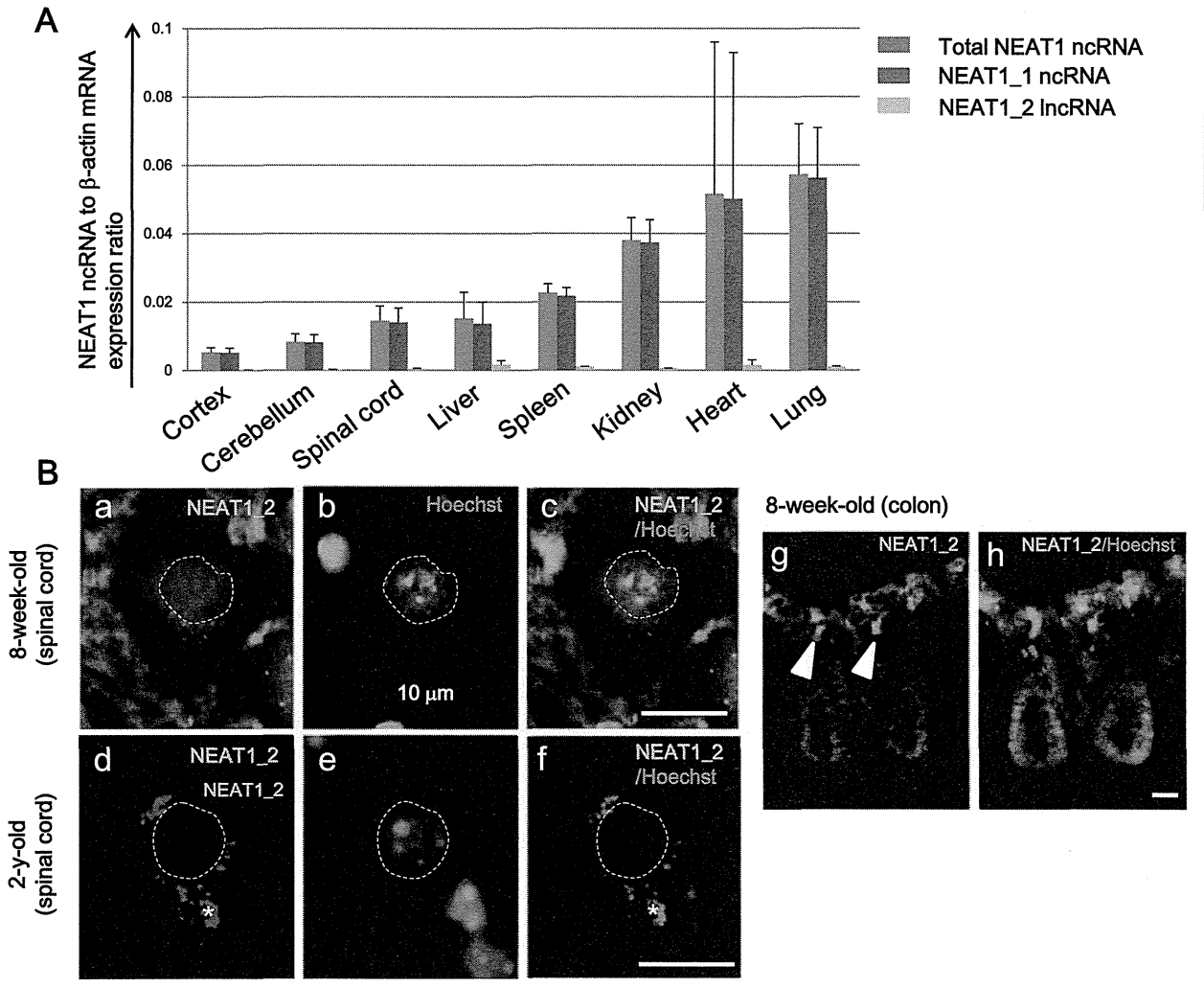

Figure 3 NEAT1_2 lncRNA is not expressed in the nuclei of WT mouse spinal motor neurons. A. The expression levels of total NEAT1, NEAT1_1 and NEAT1_2 transcripts in tissues of 8-week-old mice are displayed using quantitative RT-PCR. Data represent mean ± s.d. (n = 3). **B.** NEAT1_2 nuclear lncRNA does not appear in the control mouse spinal cord. (a-c) a motor neuron in 8-week-old mouse spinal cord. (d-f) a motor neuron in 2-y-old mouse spinal cord. The motor neurons in the ventral horn were distinguished from glial cells by their morphological features. Dotted lines represent the outline of the nucleus. Arrowheads: colon epithelial cells served as a positive control for NEAT1_2 detected by *in situ* hybridization (g, h). Lipofuscin in the cytoplasm, which is easily formed in aged motor neurons and has autofluorescence, is denoted as an asterisk (d, f). A long-path filter was used to distinguish Hoechst from autofluorescence. Scale bars, 10 μm.

Paraspeckle formation occurs in motor neurons in the spinal cords of human ALS patients

While NEAT1_2 lncRNA interacted with ALS-associated proteins in human cell lines (Figure 1A and B, Figure 2C and Additional file 1: Figure S1A), no NEAT1_2 lncRNA was observed in spinal motor neurons in mice *in vivo* (Figure 3B). According to these findings, we hypothesized that NEAT1_2 lncRNA has a particular role in motor neurons in sporadic ALS patients. To test this hypothesis, the fresh frozen spinal cords of six sporadic ALS cases (age: 73.2 ± 8.9 y) and six control cases (age: 83.2 ± 4.4 y) were prepared (Table 1). Contrary to the results in mice (Figure 3B), RNA-FISH demonstrated that more than 80% of human spinal motor neurons in ALS cases displayed NEAT1_2 foci in the nuclei (Figure 4A, 5C). Using sense probe as a negative control, the possibility that antisense probe against NEAT1_2 lncRNA recognized other non-specific intranuclear RNAs was excluded in the human spinal cord (Additional file 3 Figure S3). Parts of endogenous TDP-43 aggregates in the nucleus coincided with nearly all NEAT1_2 foci in motor neurons in ALS cases (Figure 4A), consistent with findings in cultured cells (Figure 1A and B, Figure 2C and Additional file 1 Figure S1A).

Next, to test whether NEAT1_2 lncRNA in human motor neurons formed paraspeckle structure, the nuclear distribution pattern of PSP1 was examined with immunohistochemistry and visualized with DAB (Figure 4C). PSP1 was often observed as an aggregated form in the nuclei of motor neurons as well as surrounding glial cells. In addition, RNA-FISH demonstrated that PSF, PSP1, and $p54^{nrb}$ were colocalized with NEAT1_2 foci in the nuclei of ALS motor neurons (Figure 4D and E, and Additional file 4: Figure S4). In control cases, more than 60% of motor neurons demonstrated no NEAT1_2 foci (Figure 4B, 5C); however, the remaining motor neurons contained NEAT1_2 foci with paraspeckle proteins in the nuclei (Additional file 4: Figure S4). These results suggest that paraspeckle proteins have affinity for NEAT1_2 foci in motor neurons in both ALS and control cases.

Paraspeckles appear predominantly in spinal motor neurons in the early phase of the pathological process

To test whether paraspeckles in the motor neuron were formed significantly in sporadic ALS, occurrence rates of NEAT1_2 lncRNA in human motor neurons were quantified. All motor neurons were regarded as the subjects of the number count in the ventral horns of several spinal cord slices at a constant thickness of 14 μm. The motor neurons were definitively-distinguishable from surrounding glia cells according to the morphology, specific structures including lipofuscin, or the size that is more than approximately 35 μm. To evaluate occurrence rates of NEAT1_2 lncRNA at the same stage of TDP-43 distribution, we subdivided the pathological stages of ALS spinal motor neurons into four classes (Table 2, Figure 5A).

Table 1 Profiles of individual ALS and control cases in this study

Patient	Sex/Age (y)	Disease	Duration of illness (months)	Duration on respirator (months)	TDP-43 cytoplasmic aggregation in spinal motor neurons	Cause of death	Postmortem delay until resection (min)	Number of spinal motor neurons examined in this study
A	Female / 66	sporadic ALS	38	26	+	Pneumonitis	82	74
B	Male / 59	sporadic ALS	45	26	+	empyema	113	104
C	Female / 76	sporadic ALS	39	18	+	pneumonitis	127	93
D	Male / 76	sporadic ALS	12	-	+	pneumonitis	112	92
E	Male / 83	sporadic ALS	27	-	+	pneumonitis	123	143
F	Female / 79	sporadic ALS	31	-	+	pneumonitis	240	127
C1	Female / 79	acute myocardial infarction	-	-	-		97	64
C2	Male / 87	Alzheimer's disease, lung cancer, hypertension	-	-	-		240	47
C3	Female / 86	dementia, acute myocardial infarction, hypertension	-	-	-		240	43
C4	Female / 81	colon cancer, post-cerebral infarction	-	-	-		128	36
C5	Male / 78	metastatic brain tumor	-	-	-		103	100
C6	Male / 88	dementia	-	-	-		250	49

The average ages in ALS and control cases were 73.2 ± 8.9 y and 83.2 ± 4.4 y, respectively. All ALS cases in this study showed TDP-43 cytoplasmic aggregation in the spinal cord. Age, age of the patient at death; Patient A-F, individuals with ALS; C1-C5, normal controls with no neurological disorder affecting the spinal cord.