Simple Derivation of Spinal Motor Neurons from ESCs/iPSCs Using Sendai Virus Vectors

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INTRODUCTION
Amyotrophic lateral sclerosis (ALS) is a progressive and fatal degenerative disorder of motor neurons (MNs). Embryonic stem cells (ESCs)/induced pluripotent stem cells (iPSCs) now help us to understand the pathomechanisms of ALS via disease modeling. Various methods to differentiate ESCs/iPSCs into MNs by the addition of signaling molecules have been reported. However, classical methods require multiple steps, and newer simple methods using the transduction of transcription factors run the risk of genomic integration of the vector genes. Heterogeneity of the expression levels of the transcription factors also remains an issue. Here we describe a novel approach for differentiating human and mouse ESCs/iPSCs into MNs using a single Sendai virus vector encoding three transcription factors, LIM/homeobox protein 3, neurogenin 2, and islet-1, which are integration free. This single-vector method, generating HB9-positive MNs, significantly reduces the efforts required to generate MNs, and it provides a useful tool for disease modeling.

RESULTS
Differentiation of Human iPSCs into MNs with Three Separate SeV Vectors
First, we differentiated human iPSCs into MNs as described in Figure 1A. To detect MNs easily, we used HB9-EGFP knockin human MNs can be obtained from iPSCs, using signaling molecules such as retinoic acid (RA) and Sonic hedgehog (Shh) (Table S1) These methods rely on developmental principles and require changing the combinations of signaling molecules at multiple steps, which is why some methods require more than 4 weeks to produce MNs. In contrast, Hester et al. reported a rapid differentiation method using adenoviral vectors that encode the transcription factors neurogenin 2 (Ngn2), islet-1 (Isl1), and LIM/homeobox protein 3 (Lhx3). These three transcription factors were transduced into neural progenitor cells, and MNs were obtained 11 days after the transduction. Son et al. reported that mouse and human fibroblasts were converted directly into MNs using seven and eight transcription factors, respectively, encoded by retrovirus vectors.


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iPSCs. On day 0, iPSCs were seeded on Matrigel-coated dishes and the medium was changed from ESC medium to neurobasal medium with N2 and B27 supplements. RA, smoothened agonist (SAG), and neurotrophic factors (NTFs) also were added from day 0. For the differentiation to MNs, three separate vectors, SeV-Lhx3/TS7ΔF (SeV-L), SeV18+Ngn2/TS7ΔF (SeV-N), and SeV18+Isl1/TS7ΔF (SeV-I) were transduced into human iPSCs. To test the transduction efficiency of the SeV vectors, we transduced SeV18+EGFP/TS7ΔF (SeV-EGFP) into control iPSCs at the multiplicity of infections (MOIs) of 1, 3, 10, 30, and 100. We observed
Dose-dependent increases of EGFP-positive cells on day 2. However, the ratios of EGFP-positive cells on day 4 compared to day 2 decreased at MOIs of 30–100 (Figure S1). Thus, we chose an MOI of less than 30. On day 14, we observed HB9-positive neurons and Tuj1-positive neurons, and 7.3% ± 1.4% and 16.6% ± 4.8% of total cells were positive for HB9 and Tuj1, respectively (Figures 1B and 1C). The qPCR analysis showed increased expression levels of HB9, ChAT, and MAP2 (Figure 1D). We also confirmed via immunocytochemistry and qPCR analysis that MNs can be generated from human ESCs with these SeV vectors (Figure S2).

To determine which combination of Lhx3, Ngn2, and Isl1 best produces MNs from iPSCs, we transduced one to three of SeV-L-, SeV-N, and SeV-I into human iPSCs, and we evaluated Tuj1 and HB9 expressions by immunocytochemistry. The combination of all three factors and the combination of SeV-L and SeV-N produced both Tuj1- and HB9-positive neurons. The percentage of MNs per neurons was 43.9% ± 6.6% with all three factors and 18.2% ± 1.1% with Lhx3 and Ngn2 (Figure S3). We, therefore, decided to use all three factors for the differentiation of MNs.

**Differentiation to MNs by Lhx3, Ngn2, and Isl1 in a Single SeV Vector and Time-Lapse Imaging**

To increase the percentage of MNs per neurons, we designed a single SeV vector encoding Lhx3, Ngn2, and Isl1 (SeV-L-N-I). Each transgene was connected with the transcription termination (E), trinucleotide intergenic (I), and transcription restart sequence (S) of the Sendai virus (EIS sequence). We examined the differentiation of MNs using this vector (Figure 2A). On day 14, we observed both HB9-positive neurons and ChAT-positive neurons, and 6.2% ± 1.6% of the total cells were neurons and 5.3% ± 1.5% were MNs. The percentage of MNs per neurons was 85.6% ± 1.7% (Figures 2B and 2C). We confirmed no MNs were obtained without SeV-L-N-I or with SeV-EGFP vector only using the current protocol. Without RA and SAG, HB9-positive cells were 48.5% ± 1.5% of neurons (Figures S4A–S4C). We also analyzed populations other than neurons (Figure S4D). The qPCR analysis showed increased expression levels of HB9, ChAT, and MAP2 (Figure S4E). When MNs were co-cultured with human myocytes differentiated from a human myogenic cell line, Hu5/E18, the formation of neuromuscular junctions was confirmed by co-localization of HB9-EGFP-positive neurites with α-bungarotoxin-stained acetylcholine receptors (Figure S4F).

To capture when HB9-positive cells emerge, we conducted time-lapse imaging analysis using HB9-EGFP knockin iPSCs. Time-lapse imaging of EGFP was started on day 1, and EGFP-positive cells were observed on day 2. The number of EGFP-positive cells gradually increased, but some of them disappeared as time passed. On day 3, neuron-like morphology was observed (Movie S1).

**Differentiation to MNs by a Single SeV Vector Encoding Lhx3, Ngn2, Isl1, and EGFP**

Since the efficiency for the differentiation of neural lineages appeared low based on the total number of cells, to investigate the MN and neuron differentiation efficiency in SeV-infected cells, we designed an SeV-L-N-I-EGFP vector, which could label SeV-infected cells, and we transduced the three factors into iPSCs (Figure 3A). We found that >90% of SeV-L-N-I-EGFP-infected cells had differentiated into MNs and neurons (Figures 3B and 3C). The qPCR analysis showed increased expression levels of HB9, ChAT, and MAP2 (Figure 1D). We also confirmed via immunocytochemistry and qPCR analysis that MNs can be generated from human ESCs with these SeV vectors (Figure S2).

To confirm that our method is applicable to the research of MNDs, we generated human iPSCs from the fibroblasts of a familial ALS patient with mutant superoxide dismutase 1 (SOD1 ALS) by transducing the four transcription factors Oct3/4, Sox2, Klf4, and c-Myc, as previously reported37 (Table S4). The iPSCs were examined immunocytochemically for the ESC markers SSEA4 and NANOG (Figure S5A), and they were confirmed to retain the SOD1 gene mutation (Figure S5B). The generation of another familial ALS patients (mutant TAR DNA-binding protein, 43 kDa [TDP-43]-mediated ALS [TDP-43 ALS]), as well as control-derived iPSC lines, was reported previously.7 When we differentiated human ALS iPSCs into MNs using SeV-L-N-I (Figures S5C and S5D), SOD1-ALS iPSC-derived neurons presented an accumulation of misfolded SOD1 (Figures 6A and 6B), and TDP-43-ALS iPSC-derived neurons exhibited cytosolic TDP-43 aggregation (Figures 6C and 6D). These cellular phenotypes were not specific to MNs (Figures S5E and S5F).

Next, we generated iPSCs from embryonic fibroblasts of ALS model mice carrying mutant SOD17 or mutant TDP-4348 or from littermate controls by transducing Oct3/4, Sox2, Klf4, and c-Myc, as previously reported39,40 (Figure S6A; Table S5). We differentiated the iPSCs into MNs to examine their phenotypes via immunocytochemistry (Figures S6B and S6C). MNs derived from mouse SOD1-ALS iPSCs were positive for misfolded SOD1, while those derived from mouse control iPSCs were negative (Figure S6D). MNs derived from mouse TDP-43-ALS iPSCs did not display the cytosolic aggregates of TDP-43.
Figure S6E), which is consistent with a report on TDP-43-transgenic mice.41

DISCUSSION
Along with the development of stem cell technology, stem cell-derived MNs have been utilized for modeling MNDs in vitro. However, the heterogeneity of these MN populations presents a potential issue for disease modeling and analysis. To obtain more homogeneous MNs, we used a single SeV vector that encodes three transcription factors. SeV, known as murine parainfluenza virus type 1, is a negative sense, single-stranded RNA virus of the family Paramyxoviridae. SeV vectors are cytoplasmic RNA vectors that do not integrate
into host genomes. They can be transduced into both dividing and non-dividing cells, and short-term exposure is enough for efficient transduction. SeV vectors can accommodate up to 5 kb of insertion.

The present study demonstrated that the ratio of MNs to neurons was higher when using a single SeV vector in comparison with three different SeV vectors for the transduction of Lhx3, Ngn2, and Isl1. The differentiation of neural lineage cells to MNs increases the percentage of HB9-positive cells per total cells compared to that of iPSCs. However, this method requires the dissociation and passage of cells and the change of compounds. On the other hand, the direct addition of a single vector to iPSCs is a very simple method, and the rapid differentiation of

Figure 3. MN Differentiation Using a Single SeV Vector Encoding Lhx3, Ngn2, Isl1, and EGFP

(A) Outline shows the experimental procedure to generate MNs using SeV-L-N-I-EGFP from iPSCs. (B) Immunostaining for HB9, Tuj1, MAP2, and ChAT on day 14 is shown. Scale bars, 20 μm. (C) Differentiation efficiency of MNs in SeV-infected cells is shown. The percentages of HB9-positive and Tuj1-positive cells per EGFP-positive cells were 92.8% ± 1.2% and 97.7% ± 1.2% on day 14, respectively. Error bars are SEM (n = 3). (D) The qPCR analysis for HB9, MAP2, and ChAT is shown. Student’s t test was used for statistical comparison (*p < 0.05). Error bars are SEM (n = 3).
MNs is beneficial for research application. Moreover, immunocytochemistry of MNs derived from control and ALS patient iPSCs showed that MNs produced by this method are useful for research on MNDs.

We also showed via time-lapse imaging that HB9-EGFP-positive cells emerged within 2 days after the transduction of SeV-L-N-I and that these cells extended neurites on day 3. Some of the cells gradually disappeared, perhaps because the SeV vectors may have had some cytotoxic effects or because we could not change the medium during time-lapse imaging.

There are still some challenges to be resolved. First, the number of infecting vectors may vary between individual cells. Second, the SeV vectors should be easily removable from the transduced cells after differentiation. Removable SeV vectors are now being developed. In addition, the homogeneity of the MNs needs to be further improved. Although further studies are required to determine whether this method is applicable to other types of neurons, we expect it will provide a new approach for research on neurodegenerative diseases.
In conclusion, we established a simple and useful method for differentiating human iPSCs into MNs with a single SeV vector encoding multiple transcription factors. This method will help to facilitate stem cell-based research on MNDs.

MATERIALS AND METHODS

The generation and use of human iPSCs was approved by the Ethics Committees of the respective departments, including Kyoto University. The procedures for generation of mouse iPSCs were performed in accordance with the Kyoto University Animal Institutional Guidelines, and all experiments were approved by the Center for iPS Cell Research and Application (CiRA) Animal Experiment Committee.

Derivation of Human Fibroblasts and Generation of iPSCs

Human fibroblasts were obtained with written consent. The iPSCs were generated according to a method previously described. After selecting iPSC colonies, iPSCs were cultured and passaged on an SNL feeder layer. The medium was primate embryonic stem cell medium (ReproCELL) with 4 ng/mL basic fibroblast growth factor (Wako Chemicals) and 50 mg/mL penicillin and streptomycin. The medium was changed every day and iPSCs were passaged about once a week.

Transduction Ratio by SeV Vectors into ESCs/iPSCs

To decide the transduction ratio by SeV vectors, SeV-EGFP (ID Pharma) was transduced into control iPSCs. The iPSCs were treated with collagenase type IV, trypsin, and knockout serum replacement (CTK) dissociation solution (ReproCELL) for 2 min, dissociated to single cells with Accumax (Innovative Cell Technologies), and transferred onto a 96-well plate coated with Matrigel (Becton Dickinson). Cells were fixed on day 2 and day 4. Images were captured by In Cell Analyzer 6000 (GE Healthcare).

Differentiation of MNs from Human ESCs/iPSCs Using SeV Vectors

ESCs/iPSCs were treated with CTK dissociation solution for 2 min and feeder cells were removed with PBS. Then ESCs/iPSCs were dissociated to single cells with Accumax, and they were transferred onto Matrigel-coated plates with MN medium containing a 1:1 mixture of Neurobasal medium (Thermo Fisher Scientific) and DMEM/F12 (Thermo Fisher Scientific), supplemented with 0.5% N2 (Thermo Fisher Scientific), 1% B27 (Thermo Fisher Scientific), 0.5 mM L-glutamine, 1% non-essential amino acids (NEAA), 1% penicillin/streptomycin, 50 ng/mL bFGF (PeproTech), and 0.1 ng/mL EGF (PeproTech).
1 μM retinoic acid (Sigma-Aldrich), 1 μM smoothened agonist (Enzo Life Sciences), 10 ng/mL brain-derived neurotrophic factor (BDNF; R&D Systems), 10 ng/mL glial cell-derived neurotrophic factor (GDNF; R&D Systems), 10 ng/mL neurotrophin-3 (NT-3; R&D Systems), and 10 μM Y-27632 (Nacalai Tesque). At the same time, the ESCs/iPSCs were infected with SeV-L-N-I, SeV-L-N-I-EGFP, or combinations of SeV-L, SeV-N, and SeV-I (ID Pharma) on day 0. MOIs were 5 or 10. The transduction of SeV vectors to human ESCs/iPSCs was conducted just once. The number of cells per well was 5.0 × 10^4 in 96-well plates and 1.0 × 10^6 in 12-well plates. The medium was changed to MN medium without Y-27632 on day 1 and then changed every 3 days.

For phenotype assays, cells were treated with Accumax plus 10 μM Y-27632 and transferred onto poly-L-lysine- and Matrigel-coated glass dishes on day 7. For immunocytochemistry and qPCR analyses, cells were assessed on day 14.

### Differentiation of MNs from Mouse iPSCs Using an SeV Vector

The iPSCs were trypsinized into single cells and plated on Matrigel-coated plates with MN medium. At the same time, the iPSCs were infected with SeV-L-N-I (ID Pharma) on day 0. The MOI was 5 because mouse iPSCs were damaged at an MOI of 10. The medium was changed to MN medium without Y-27632 on day 27632 on day 1 and day 4. Cells were assessed by immunocytochemistry on day 6.

### RNA Extraction, cDNA Synthesis, and qPCR

RNA was isolated using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. The cDNA was synthesized using the ReverTra Ace-α Kit (Toyobo). The qPCR was performed with SYBR Premix Ex TaqⅡ (Takara) by the StepOne Plus instrument (Applied Biosystems). Primer sequences are described in Table S6.

### Co-culture of Human MNs with Human Myogenic Cells

The Hu5/E18 cell line was purchased from RIKEN BioResource Center. Hu5/E18 cells were maintained and differentiated as previously reported. Cells were maintained in DMEM with high glucose (Nacalai Tesque) containing 20% fetal bovine serum (Gibco). Cells were differentiated into human myocytes in DMEM containing 5 μg/mL holo-transferrin bovine (Sigma-Aldrich), 10 μg/mL insulin (bovine, Sigma-Aldrich), 10 nM sodium selenite (Sigma-Aldrich), and 2% horse serum (Gibco) 7 days before SeV-L-N-I transduction into iPSCs. The iPSCs were transduced with SeV-L-N-I on day 0, dissociated with Accumax plus 10 μM Y-27632, and then transferred onto Hu5/E18-cultured plates on day 7. The medium was changed to MN medium. Cells were fixed with 4% paraformaldehyde (pH 7.4) for 30 min on day 14 and assessed by immunocytochemistry.

### Electrophysiological Recordings

Human iPSCs were transduced with SeV-L-N-I vector on day 0 and plated onto astrocytes on day 7. Electrophysiological recording and analysis were performed under microscopy in combination with differential interference contrast (DIC) imaging on day 21, as previously described. During the electrophysiological recording, cells were maintained at 30°C and continuously superfused with oxygenated Krebs-Ringer solution consisting of 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, and 20 mM glucose. To examine whether iPSC-derived MNs were functionally active, action potentials were measured in current-clamp mode with a potassium chloride-based electrode solution composed of 140 mM KCl, 2 mM MgCl₂, 10 mM HEPES, and 1 mM EGTA, adjusted to pH 7.4 with NaOH. For the recording, an EPC 9 amplifier (HEKA) was used, and the data were analyzed with Patchmaster software (HEKA). Primary astrocytes were cultured from post-natal day (P1) mouse in DMEM containing 10% FBS.

### Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (pH 7.4) for 30 min. The cells were then permeabilized with 0.2% Triton X-100, and non-specific binding sites were blocked with Block Ace (Yukijirushi). Cells were incubated with primary antibodies at 4°C overnight and with secondary antibodies at room temperature for 1 hr. Fluorescent images were captured using IN Cell Analyzer 6000, and the percentage of MNs or neurons was calculated using IN Cell Developer Toolbox v1.9 (GE Healthcare). For phenotype assays, images were acquired by Delta Vision (GE Healthcare). The primary antibodies were as follows: HB9 (Developmental Studies Hybridoma Bank [DSHB], 1:200), TuJ1 (Covance, 1:200), TuJ1 (Chemicon, 1:500) for Figures 6A and 6C, ChAT (Chemicon, 1:100), HOXB4 (DSHB, 1:50), HOXC6 (Abcam, 1:200), HOXC9 (Abcam, 1:200), HOXC10 (Abcam, 1:200), misfolded SOD1 (MEDIMABS, B8H10, 1:200), misfolded SOD1 (MEDI MABS, A5C3, 1:200), TDP-43 (Proteintech, 1:200), human Nanog (ReproCELL, 1:500), SSEA4 (Millipore, 1:200), SSEA1 (Chemicon, 1:1,000), Nestin (Millipore, 1:200), GFAP (Dako, 1:2,000), Iba1 (Wako Pure Chemicals Industries, 1:500), CNPase (Cell Signaling Technology, 1:100), SOX17 (R&D Systems, 1:200), and zSMA (Dako, 1:500). TuJ1 (Chemicon) was used to co-immunostain with TDP-43.

### Time-Lapse Imaging

For time-lapse imaging, 35-mm glass-bottom dishes (MatTek) were coated with poly-L-lysine (Sigma-Aldrich) and Matrigel. Human iPSCs transduced by SeV-L-N-I were plated on the dishes on day 0. The medium was changed to FluoroBrite DMEM (Thermo Fisher Scientific) supplemented with 0.5% N2, 1% B27, 1 μM retinoic acid, 1 μM smoothened agonist, 10 ng/mL BDNF, 10 ng/mL GDNF, and 10 ng/mL NT-3 on day 1. Time-lapse imaging was started 24 hr after plating using BioStation IM-Q (Nikon). Images were captured every 30 min.

### Statistical Analysis

All data are shown as mean ± SEM. Data were analyzed by Student’s t test or one-way ANOVA followed by Dunnett’s post-hoc test; p values < 0.05 were considered significant. Statistical analyses were performed with SPSS version 21 (IBM).
SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures, six tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.omtm.2016.12.007.

AUTHOR CONTRIBUTIONS
H.I. conceived the study. K.G., K.I., and K.K. designed, conducted, and analyzed the experiments and prepared the figures. H.I. and M.I. discussed the vector design. K.G., K.I., K.K., and H.I. wrote the manuscript. K.M., K.A., N.N., M.I., and A.K. provided the materials. H.Y. and R.T. provided advice regarding the data and the manuscript. All authors reviewed the manuscript.

CONFLICTS OF INTEREST
M.I. is a board member of ID Pharma Co., Ltd.

ACKNOWLEDGMENTS
We would like to express our sincere gratitude to all our coworkers and collaborators, including Noriko Endo, Mayumi Yamada, and Ruri Taniguchi for their valuable administrative support, and Takumi Kanaya, Takeo Yamamoto, Kaoru Takizawa, and Takashi Hironaka for their valuable technical support. We acknowledge Peter Karagianis for providing critical reading. Funding for this project was received in part from the Program for Intractable Diseases Research utilizing disease-specific iPSCs from Japan Agency for Medical Research and Development (AMED) to H.I., from the Research Project for Practical Applications of Regenerative Medicine from AMED to H.I., from the grant for Core Center for iPS Cell Research of Research Center Network for Realization of Regenerative Medicine from AMED to H.I., and from the Daiichi Sankyo Foundation of Life Science to H.I.

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