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14. ABSTRACT The limited access to patient neurons greatly impedes the progress of research in dystonia. The major purpose of this project is to develop a novel cellular model system for dystonia research. We have successfully generated human patient-specific neurons through either direct conversion of patients' skin fibroblasts or differentiation of induced pluripotent stem cells (iPSCs). We found that dystonia neurons show developmental delay, thickened nuclear lamina, disrupted nuclear morphology, and impaired nucleocytoplasmic transport. Furthermore, we have uncovered that nuclear lamina protein LMNB1 is upregulated in DYT1 neurons and exhibits abnormal subcellular distribution in a cholinergic motor neuron (MN)-specific manner. Interestingly, downregulation of LMNB1 can largely ameliorate all the cellular defects in DYT1 MNs. These results reveal high value of disease modeling with human patient-specific neurons and indicate that dysregulation of nuclear LMNB1 may constitute a major molecular mechanism underlying DYT1 pathology.					
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1. INTRODUCTION:

Dystonia is the third most common movement disorder and characterized by sustained or intermittent muscle contractions, leading to abnormal movements and postures. Veterans are particularly at risk due to the frequent occurrence after head trauma and potentially irreversible side effects of medications used to treat PTSD (post-traumatic stress disorder). Because the clinical characteristics and underlying causes are very heterogeneous, the pathological mechanisms of dystonia remain largely unknown and the diagnosis and etiological definition of this disorder remain a challenge. However, the limited access to patient neurons greatly impedes the progress of research in dystonia. The major purpose of this project is to develop a novel cellular model system for dystonia research and examine the cellular and molecular pathogenesis of this disease. We will generate human patient-specific neurons through either direct conversion of patients' skin fibroblasts or differentiation of induced pluripotent stem cells (iPSCs). These patient-specific neurons will be extensively examined and compared with healthy control cells at cellular morphology, neurodevelopment, survival, nuclear transport and functions. Expected results emanating from this study will provide novel insights into dystonia pathology and potentially lead to molecular targets for therapeutic interventions.

2. KEYWORDS:

Dystonia, Human Neurons, Motor Neurons, Reprogramming, TOR1A

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major goals:

1. To establish the protocol of directly reprogramming human neurons from adult fibroblasts.

Milestones achieved:

1.1 Obtained all research materials, including fibroblast cell lines are used in this project.
80% completion.

1.2 Established the protocol of reprogramming human neurons from adult fibroblasts.
80% completion. We are able to generate patient-specific motor neurons.

1.3 Verified the neuronal identity, electrophysiology and maturation of reprogrammed neurons.
50% completion. Electrophysiological analysis needs to be done.

1.4 Successfully generated dystonia neurons.
50% completion. Other neuronal subtypes will be generate, including from adulthood-onset patients.

2. To examine the nuclear morphology and nucleocytoplasmic transport in dystonia neurons.

Milestones Achieved:

2.1 Optimized conditions of immune-staining reprogrammed neurons.
100% completion.

2.2 Acquired plenty of confocal micrographs for data analysis.
50% completion. More target proteins will be analyzed.

2.3 Optimized protocols for EM sample preparation.
30% completion. The fixation and embedding condition need to be optimized.

2.4 Acquired plenty of EM data of reprogrammed neurons.
20% completion. More EM samples will be prepared and examined.

3. To examine nucleocytoplasmic transport (NCT) in dystonia neurons.

Milestones Achieved:

3.1 Optimized Fluorescence in situ hybridization (FISH) condition for nuclear mRNA export assay in reprogrammed neurons.
60% completion. The protocol for measuring total mRNA distribution has been established. Next will set up protocols for measuring gene-specific transcripts.

3.2 Optimized condition for dual reporter assay.
100% completion.

3.3 Acquired plenty of confocal micrographs of FISH data and dual reporter data.
70% completion. More samples will be analyzed.

3.4 Quantified the micrographs by ImageJ.
70% completion. More samples will be analyzed.

3.5 Organized data and prepared manuscript (s).
This project has generated four peer-reviewed papers so far, including 3 research papers and 1 invited perspective. (See appendix A-D)

What was accomplished under these goals?

During this reporting period, this project achieved the following accomplishments.

1. We have established the protocol of directly reprogramming human neurons from adult fibroblasts.

First, we chose proper fibroblast lines of dystonia patients and healthy controls. Secondly, we developed the protocol of directly reprogramming motor neurons from adult fibroblasts via lentiviral delivery of transcription factors. Finally, we have successfully generated human neurons from fibroblasts of dystonia patients and healthy controls. This protocol has been published in *Front Mol Neurosci*. (See Appendix A)

2. We have developed two systems to measure the nucleocytoplasmic transport in reprogrammed human neurons.

We have developed two systems to measure the nucleocytoplasmic transport (NCT) of both mRNA and proteins cargos. Using these two systems, we examined NCT activity at the single-cell level by measuring mRNA subcellular distribution and protein transport in directly induced motor neurons (diMNs) from adult human fibroblasts. The results show that mRNA subcellular distribution but not protein transport is affected by neuronal maturation stages, whereas both transport processes are not influenced by the sample sex. This study also provides quantitative methods and optimized conditions for measuring NCTs of mRNAs or protein cargos, establishing a robust way for future functional examinations of NCT activity in directly induced neurons from diseased human patients. (See Appendix A)

3. We have developed another strategy to generate human neurons via human induced pluripotent stem cells (hiPSC).

We developed a protocol to generate functional MNs from human iPSCs via lentiviral delivery of transcription factors. Moreover, we optimized induction conditions by using different combinations of transcription factors and found that a single lentiviral vector expressing three factors [neurogenin-2 (NGN2), insulin gene enhancer 1 (ISL1), and LIM/homeobox 3 (LHX3)] is necessary and sufficient to induce iPSC-derived MNs (iPSC-MNs). These MNs robustly expressed general neuron and MN-specific markers and showed electrical maturation and firing of action potentials within 3 weeks. This approach significantly improved the neuronal survival, yield, and purity, making it feasible to obtain abundant materials for biochemical studies in modeling movement diseases. This method was published in *Am J Physiol Cell Physiol* (See Appendix B). I also published an invited perspective in *Neural Regen Res* and further described this method. (See Appendix C).

4. We have identified the cellular deficits in dystonia neurons.

Using motor neurons (MNs) derived from DYT1 dystonia, we found that dystonia neurons show developmental delay, thickened nuclear lamina, disrupted nuclear morphology, and impaired nucleocytoplasmic transport. Furthermore, we have uncovered that nuclear lamina protein LMNB1 is upregulated in DYT1 neurons and exhibits abnormal subcellular distribution in a cholinergic motor neuron (MN)-specific manner. Such dysregulation of LMNB1 can be recapitulated by either ectopic expression of the mutant *TOR1A* gene or shRNA-mediated downregulation of endogenous *TOR1A* in healthy control MNs. Interestingly, downregulation of LMNB1 can largely ameliorate all the cellular defects in DYT1 MNs. These results reveal high value of disease modeling with human patient-specific neurons and indicate that dysregulation of nuclear LMNB1 may constitute a major molecular mechanism underlying DYT1 pathology. These findings were recently published in *J Neurosci*. (See Appendix D).

What opportunities for training and professional development has the project provided?

During this reporting period, this project provided 3 Graduate Research Assistant (GRA) positions (person semester) and research opportunities for 2 undergraduate researchers. Through participating this project, graduate students learned the basic skills of cell culture, immunostaining, imaging and the advanced techniques of reprogramming human neurons from adult fibroblasts. Undergraduate students worked with graduate students and learned the basic experimental skills and how to analyze the confocal images using ImageJ software.

The PI participated The American Society for Cell Biology (ASCB) Annual Meeting, ASCB/EMBO Virtual Meeting in December 2-16, 2020, and did a poster presentation entitled “*Dysregulated nuclear LMNB1 and impaired nucleocytoplasmic transport in Dystonia patient-derived neurons.*” (See Appendix E)

Graduate students presented their research in our department seminars.

Masuma Akter, (PhD level student) presented “*Generation of human neurons from fibroblast for disease modeling*” in October 15, 2020.

Masuma Akter presented “*Modeling of Neurological movement disorder dystonia by using human specific neurons*” in February 11, 2021.

Haochen Cui (PhD level student) presented “*Nucleocytoplasmic transport and its measurement*” in February 18, 2021.

How were the results disseminated to communities of interest?

The new findings from this project were published in prestigious peer-reviewed journals and the PI also presented the new findings to scientific communities in scientific conference. So far, the findings from this project have generated four papers that have been published in *Journal of Neuroscience*, *Frontiers in Molecular Neuroscience*, *American Journal of Physiology-Cell Physiology*, and *Neural Regeneration Research*. The PI also presented these findings in ASCB/EMBO Virtual Meeting to the scientific communities.
(See appendix A-D)

What do you plan to do during the next reporting period to accomplish the goals?

1. Generate more human neurons from dystonia patients and healthy controls.

Using both direct conversion and iPSC-based approach to generate different neuronal subtypes, including motor neurons, cholinergic interneurons and generic neurons.

2. Examine the cellular defects in DYT1 neurons.

We will examine the nuclear morphology of DYT1 neurons by TEM, and analyze the nuclear lamina and nuclear pore complex.

3. Examine the nuclear mRNA export in DYT1 neurons.

The total mRNA distribution will be examined by FISH with oligo-dT probes and particular gene transcripts will be analyzed by gene-specific probes.

4. Organize the data and prepare more manuscripts.

The new findings will be organized and published in peer-reviewed journals.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We have developed and further optimized the techniques for generation of human neurons from patient skin fibroblasts. These techniques overcome the limited access to human brain cells and provide valuable materials in dystonia research. Using patient-derived neurons, we found that the dystonia neurons show developmental delay, abnormal nuclear morphology, disrupted nuclear transport that is important for cellular functions. We also found that the nuclear protein LMNB1 is higher in patient neurons and show the abnormal distribution. When we used a technique to bring down the LMNB1 protein level and the cellular defects in dystonia neurons were corrected. These findings reveal high value of disease modeling with human patient-specific neurons and indicate that dysregulation of nuclear LMNB1 may constitute a major molecular mechanism underlying DYT1 dystonia pathology. Downregulation of LMNB1 could be a novel target for developing therapeutic interventions in DYT1 dystonia.

What was the impact on other disciplines?

Besides the impact on neuroscience and disease modeling, the findings from this project also provide novel insights into the regulations of nucleocytoplasmic transport, which belongs to the research field of Cell Biology. Abnormal nuclear envelope and dysregulated nuclear lamina will significantly disrupt the nuclear transport.

What was the impact on technology transfer?

The findings from this project reveal high value of disease modeling with human patient-specific neurons and indicate that dysregulation of nuclear LMNB1 may constitute a major molecular mechanism underlying DYT1 dystonia pathology. Downregulation of LMNB1 could be a novel target for developing therapeutic interventions in DYT1 dystonia.

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Nothing to Report

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Because of the pandemic of CovID-19, our university has been closed for about three months (March to June) in 2020. After the university reopen, the laboratory was only partly functional because we have to practice social distancing and follow the maximum occupancy in the lab. For example, the maximum number allowed to simultaneously work in my lab is 2 people. This unprecedented crisis significantly delayed the progress of the research and the expenditure of the fund. Currently, the research activities and collaborations in my lab are fully resumed. I plan to request the extension of the termination date of this project, so that we could fully complete the proposed studies in this project, especially for some time-consuming analysis, such as TEM assay.

Changes that had a significant impact on expenditures

The pandemic of COVID-19 has a significant impact on the expenditures. During university closure, we had to stop or minimize our research activities and collaborations. The expenditure was much less than anticipated. After university reopen, the lab was only partly functional. The overall expenditure during this reporting period was significantly delayed.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**Significant changes in use or care of human subjects**

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

1. **Ding B,*** Akter M, and Zhang C-L. (2020). Differential Influence of Sample Sex and Neuronal Maturation on mRNA and Protein Transport in Induced Human Neurons. *Front Mol Neurosci.* 2020 Apr 3; 13: 46. (See Appendix A)
Published, acknowledgement of federal support (yes).
2. Sepehrimanesh M, and **Ding B*** (2020). Generation and Optimization of Highly Pure Motor Neurons from Human Induced Pluripotent Stem Cells via Lentiviral Delivery of Transcription Factors. *Am J Physiol Cell Physiol.* 319: C771–C780. (See Appendix B) *Published, acknowledgement of federal support (yes).*
3. **Ding B*.** (2021). Generation of patient-specific motor neurons in modeling movement diseases. *Neural Regen Res* 16(9):1799-1800. (See Appendix C)
Published, acknowledgement of federal support (yes).
4. **Ding B***, Tang Y, Ma S, Akter M, Liu ML, Zang T, Zhang CL.(2021). Disease modeling with human neurons reveals LMNB1 dysregulation underlying DYT1 dystonia. *J Neurosci.* 41(9):2024-2038. (See Appendix D)
(Featured Article, Journal of Neuroscience 3 March 2021, 41 (9) 1846)
Published, acknowledgement of federal support (yes).

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Presentation:

Ding B., Yu T. and Zhang C-L. *Dysregulated nuclear LMNB1 and impaired nucleocytoplasmic transport in Dystonia patient-derived neurons*. The American Society for Cell Biology (ASCB) Annual Meeting. December 2-16, 2020. ASCB/EMBO Virtual Meeting. (See Appendix E)

- **Website(s) or other Internet site(s)**

<https://www.jneurosci.org/content/41/9/1846>

This Week in The Journal, *The Journal of Neuroscience*, highlights our new findings in dystonia using in patient-specific neurons. (Publication of Appendix D).

- **Technologies or techniques**

Developed and optimized protocols for generation human neurons from adult fibroblasts. These methods have been shared via publications in peer-reviewed journals (see appendix A-D).

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Baojin Ding	Masuma Akter	Haochen Cui	Jacob Stagray
Project role	PI	Graduate Student	Graduate Student	Graduate Student
ORCID ID	0000-0002-2149-2599	N/A	N/A	N/A
Nearest person month worked	5	3	5	2
Contribution to Project	Supervision for the project, including design experiment, train the graduate students and prepare manuscripts and presentations for scientific meetings.	Performed experiments, including culture cells, reprogramming human neurons, and neuronal survival assay.	Performed experiments, including culture cells, plasmid purification, lentivirus preparation.	Analyzed nuclear morphology by Transmission Electron Microscopy (TEM).
Funding support	Start-up and NIH	University fellowship	RA position from this Award	Department TA position

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

University of Texas Southwestern Medical Center, Dallas, TX.

Dr. Chun-li Zhang's lab in UT Southwestern has generously shared some fibroblast cell lines and plasmids for reprogramming human neurons. We also collaborated with the Zhang Lab for TEM analysis of nuclear morphology of diseased neurons using the services from EM core at UT Southwestern.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to Report

QUAD CHARTS:

Nothing to Report

9. APPENDICES:

Appendix A-Journal article

Ding B,* Akter M, and Zhang C-L. (2020). Differential Influence of Sample Sex and Neuronal Maturation on mRNA and Protein Transport in Induced Human Neurons. *Front Mol Neurosci*. 2020 Apr 3; 13: 46.

Appendix B-Journal article

Sepehrimanesh M, and **Ding B*** (2020). Generation and Optimization of Highly Pure Motor Neurons from Human Induced Pluripotent Stem Cells via Lentiviral Delivery of Transcription Factors. *Am J Physiol Cell Physiol*. 319: C771–C780.

Appendix C-Journal article

Ding B*, Tang Y, Ma S, Akter M, Liu ML, Zang T, Zhang CL.(2021). Disease modeling with human neurons reveals LMNB1 dysregulation underlying DYT1 dystonia. *J Neurosci*. 41(9):2024-2038.

Appendix D-Journal article

Ding B*. (2021). Generation of patient-specific motor neurons in modeling movement diseases. *Neural Regen Res* 16(9):1799-1800.

Appendix E-Poster presentation in ASCB/EMBO virtual meeting

Appendix F-the PI's updated CV



Differential Influence of Sample Sex and Neuronal Maturation on mRNA and Protein Transport in Induced Human Neurons

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Nucleocytoplasmic transport (NCT) across the nuclear envelope (NE) is tightly regulated in eukaryotic cells and is critical for maintaining cellular homeostasis. Its dysregulation leads to aging and neurodegeneration. Because they maintain aging-associated hallmarks, directly reprogrammed neurons from human fibroblasts are invaluable in understanding NCT. However, it is not clear whether NCT activity is influenced by neuronal maturation and sample sex [a key biological variable emphasized by the National Institutes of Health (NIH) policy]. We examined here NCT activity at the single-cell level by measuring mRNA subcellular distribution and protein transport in directly induced motor neurons (diMNs) from adult human fibroblasts. The results show that mRNA subcellular distribution but not protein transport is affected by neuronal maturation stages, whereas both transport processes are not influenced by the sample sex. This study also provides quantitative methods and optimized conditions for measuring NCTs of mRNAs or protein cargoes, establishing a robust way for future functional examinations of NCT activity in directly induced neurons from diseased human patients.

Keywords: reprogrammed human neurons, nucleocytoplasmic transport, nuclear mRNA export, fluorescence *in situ* hybridization (FISH), protein nuclear transport, sex as a biological variable (SABV)

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; BDNF, brain-derived neurotrophic factor; CGN, cerebellar granule neuron; CHAT, choline acetyltransferase; DIG, digoxigenin; DIV, days *in vitro*; DM, dorsomorphin; dpi, days post infection; FBS, fetal bovine serum; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; FISH, fluorescence *in situ* hybridization; FSK, forskolin; GDNF, glial cell-derived neurotrophic factor; GFP, green fluorescent protein; HEK, human embryonic kidney; HST, Hoechst 33342; HD, Huntington's disease; MAP2, microtubule-associated protein 2; MN, motor neuron; diMN, directly induced motor neuron; MOI, multiplicity of infection; NCT, nucleocytoplasmic transport; NE, nuclear envelope; NES, nuclear export sequence; NLS, nuclear localization sequence; NPC, nuclear pore complex; NT3, neurotrophin-3; Nups, nucleoporins; PCN, primary cortical neuron; iPSC, induced pluripotent stem cell; RanBP, Ran binding protein; RanGAP, Ran-GTPase-activating protein; Ran-GEF, Ran-GDP-exchange factor; RFP, red fluorescent protein; RNP, ribonucleoprotein; mRNP, messenger ribonucleoprotein; megaRNP, ultra-large ribonucleoprotein; RVC, ribonucleoside-vanadyl complex; SSC, saline sodium citrate; TUBB3, tubulin beta 3 class III; wpi, weeks post viral infection.

INTRODUCTION

In eukaryotic cells, transcription and translation processes are physically separated by the nuclear envelope (NE). Newly transcribed mRNAs must be exported to the cytoplasm for protein synthesis, while some proteins require to be imported into the nucleus to fulfill their nuclear functions. The nuclear pore complex (NPC) is the principal gateway between the nucleus and cytoplasm. It is one of the largest protein complexes in eukaryotic cells, penetrating and bridging the inner and outer nuclear membrane (Alber et al., 2007; Mohr et al., 2009). In vertebrates, a fully assembled NPC has an estimated molecular mass of 120 MDa, composed of multiple copies of about 30 different proteins that are called nucleoporins (Nups; Beck and Hurt, 2017). Its three-dimensional structure shows an eight-fold rotational symmetry and consists of several major domains, such as cytoplasmic filaments, nuclear basket, central transport channel, and a core scaffold that supports the central channel (Alber et al., 2007; Kabachinski and Schwartz, 2015). The central channel is filled and surrounded with a distinct class of Nups (Grunwald et al., 2011), which contain phenylalanine and glycine (FG) repeats. FG repeats are intrinsically disordered domains (Lemke, 2016), and they directly function in nucleocytoplasmic transport (NCT) through mediating the passage of the soluble transport receptors (Frey et al., 2006; Frey and Gorlich, 2007; Grunwald et al., 2011).

Generally, cargoes of less than 40–60 kDa can passively diffuse through the NPC, but transport of larger macromolecules through the NPC requires the receptor-mediated transport pathways. Although different kinds of cargoes could be mediated by different transport pathways, a general paradigm usually involves different nuclear transport receptors, the small GTPase Ran and its regulatory factors (Grunwald et al., 2011). Many nuclear transport receptors belong to the karyopherin families, including importins and exportins. Importins recognize nuclear localization sequence (NLS) on their cargo proteins and mediate their import into the nucleus. Exportins recognize nuclear export sequence (NES) and mediate their cargo protein export (Lange et al., 2007; Stewart, 2007a). These karyopherins bind NLSs or NESs of their cargoes to the FG Nups and to the GTPase Ran (Moore and Blobel, 1993). The intrinsic GTPase activity of Ran is low, but interactions with Ran binding proteins (RanBPs) and the Ran-GTPase-activating protein (RanGAP) stimulate GTP hydrolysis. RanBPs are large scaffolding proteins that bind Ran and RanGAP. Because RanBPs are anchored in the cytoplasm side of the nuclear membrane, efficient conversion of RanGTP to RanGDP will occur only in the cytoplasm, yielding a nuclear/cytoplasm ratio of RanGTP of approximately 200:1 (Pollard et al., 2017). On the other hand, another Ran regulatory factor, Ran-GDP-exchange factor (Ran-GEF), switches the RanGDP-bound state into a RanGTP-bound state in the nucleus. This exchange further strengthens the differences of the subcellular distribution: a higher RanGDP concentration in the cytoplasm and a higher RanGTP concentration in the nucleus. This RanGTP-RanGDP gradient across the nuclear membrane generates a driving force

for directional NCT processes (Kopito and Elbaum, 2007; Terry and Wente, 2009).

The exportins of karyopherin family and Ran cycle also regulate the export of transfer RNAs (tRNAs), micro RNAs (miRNAs), small nuclear RNAs (snRNAs), and ribosomal RNAs (rRNAs; Rodriguez et al., 2004). However, the export of mRNA is mechanistically different from proteins and other RNAs because it uses a non-karyopherin transport receptor and does not directly depend on the RanGTP-RanGDP gradient. mRNA is exported as a large messenger ribonucleoprotein (mRNP) complex, in which a single mRNA is associated with RNA-binding proteins (RNPs) that have functions in processing, capping, splicing, and polyadenylation (Kabachinski and Schwartz, 2015). In humans, the Nxf1-Nxt1 heterodimer functions as a general mature mRNP export receptor (Stewart, 2007b). Like karyopherins, the Nxf1-Nxt1 complexes can physically interact with FG Nups and mediate RNP cargoes crossing the nuclear membrane through the NPC. However, once part of the mRNP reaches the cytoplasmic face of the NPC, the transport receptor Nxf1-Nxt1 heterodimer will be released in an ATP-dependent manner, rather than by GTP hydrolysis (Montpetit et al., 2011). The protein export receptor Crm1/Xpo1 is also involved in the nuclear export of a number of unspliced and partially spliced viral mRNAs (Forbes et al., 2015). Besides the NPC-dependent pathway for nuclear mRNA export, another NPC-independent mechanism was identified in *Drosophila*. In this mechanism, megarNP (ultra-large ribonucleoprotein) granules containing mRNAs exit the nucleus *via* budding through the nuclear membranes without using a nuclear pore complex (Speese et al., 2012). This NE-budding pathway is likely utilized in cell processes or stages that require high levels of protein synthesis (Jokhi et al., 2013).

Dysregulations of NCT have been shown to be implicated in aging (Mertens et al., 2015) and neurological diseases, such as amyotrophic lateral sclerosis (ALS; Freibaum et al., 2015; Jovicic et al., 2015; Zhang et al., 2015; Kim and Taylor, 2017; Chou et al., 2018; Burk and Pasterkamp, 2019), Huntington's disease (HD; Gasset-Rosa et al., 2017; Grima et al., 2017), and Alzheimer's disease (AD; Eftekharzadeh et al., 2018; Lester and Parker, 2018). Thus, to decipher the pathophysiological mechanisms underlying NCT-defective neurological diseases, the examination of NCT activity in human neurons is extremely critical. Excitingly, direct conversion of neurons from adult fibroblasts overcomes the limited access to human neuronal samples, providing an unprecedented approach in the research of neurological diseases (Mertens et al., 2015; Liu et al., 2016; Victor et al., 2018). How can the NCT activity unbiasedly be examined in these reprogrammed human neurons? Do the reprogramming stages and sample sex affect the NCT activity? The answers to these questions are yet to be elucidated. Particularly, sex as a biological variable (SABV) is a key part of the new National Institutes of Health (NIH) initiative to enhance reproducibility through rigor and transparency (Clayton, 2018). In this study, we attempted to address these questions in motor neurons (MNs) that were directly converted from skin fibroblasts of both male and female healthy human donors.

Nuclear mRNA export is measured by fluorescence *in situ* hybridization (FISH) with digoxigenin (DIG)-labeled oligo-dT probes, and protein NCT is examined by a dual reporter system. These approaches gave rise to strong and reliable results. We also found that developmental stages, but not sample sex, significantly affect the mRNA subcellular distribution. These results provide quantitative methods in the measurement of nuclear transport of both mRNA and protein cargoes, paving the way for further examination of NCT activity in reprogrammed patient-specific neurons.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Human embryonic kidney (HEK) 293T cells [American Type Culture Collection (ATCC)] were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco). Primary skin fibroblasts from apparently healthy human individuals were obtained from the cell repository at the Coriell Institute for Medical Research (Table 1). They were cultured in DMEM supplemented with 15% FBS and 1% penicillin/streptomycin. Two different cell culture media were sequentially employed in reprogramming human neurons. (1) Neuronal induction medium consists of DMEM: F12: neurobasal (1:1:1), 0.8% N2 (Invitrogen), 0.8% B27 (Invitrogen), 1% penicillin/streptomycin, and supplemented with 10 μ M forskolin (FSK; Sigma-Adrich, St. Louis, MO, USA), 1 μ M dorsomorphin (DM; Millipore, Kankakee, IL, USA), and 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ, USA). (2) Neuronal maturation medium consists of DMEM: F12: neurobasal (1:1:1), 0.8% N2, 0.8% B27, 1% penicillin/streptomycin, and supplemented with 5 μ M FSK and 10 ng/ml each of brain-derived neurotrophic factor (BDNF; PeproTech, Rocky Hill, NJ, USA), glial cell-derived neurotrophic factor (GDNF; PeproTech, Rocky Hill, NJ, USA), and neurotrophin-3 (NT3; PeproTech, Rocky Hill, NJ, USA).

Plasmids and Virus Production

As previously described (Liu et al., 2016), a third-generation lentiviral vector (pCSC-SP-PW-IRES-GFP) was used to express NEUROG2-IRES-GFP-T2A-Sox11 and ISL1-T2A-LHX3. The dual reporter 2Gi2R was generously provided by Dr. Fred H. Gage (Salk Institute for Biological Studies; Mertens et al., 2015). Replication-incompetent lentiviruses were produced in

HEK293T cells, and viral supernatants were collected at 48 and 72 h post-transfection. Viral supernatants were filtered through 0.45- μ m syringe filters and titered as previously described (Ding and Kilpatrick, 2013). Lentiviruses were stored at 4°C prior to cell transduction.

Direct Conversion of Human Motor Neurons From Adult Fibroblasts

Lentiviral delivery of four factors (NGN2, Sox11, ISL1, and LHX3) was used as previously described (Liu et al., 2016; Tang et al., 2017). In brief, fibroblasts were plated at a density of 1×10^4 cells/cm² onto Matrigel-coated dishes. Cells were transduced the next day at an approximate multiplicity of infection (MOI) of 10 with lentiviral supernatants supplemented with 6 μ g/ml polybrene (Millipore, Kankakee, IL, USA). Fibroblast medium was refreshed after overnight incubation. One day later, the culture was replaced with neuronal induction medium and half-changed every other day until 14 days post infection (dpi). Induced neurons were then partially enriched with a replating procedure (Liu et al., 2013) and cocultured with primary astrocytes on Matrigel-coated coverslips. The medium was half-changed twice a week until analysis. The neuronal conversion efficiency was calculated with the percentage of GFP (+) cells expressing TUBB3 (TUBB3/GFP), and the MN fraction was calculated with the percentage of TUBB3 (+) cells expressing HB9 (HB9/TUBB3).

Primary Cultures

Primary cortical neurons (PCNs) and cerebellar granule neurons (CGNs) were isolated from CD1 embryonic day-18 mice (E18) and 6-day-old pups (P6) of either sex, respectively, as previously described (Ding et al., 2013, 2016, 2018). All animal work was carried out with permission from the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Southwestern Medical Center. Individual culture experiments were performed using cells prepared from the same litter. Cells were plated at a density of 5×10^4 cells/cm² onto coverslips (Thermo Fisher Scientific, Waltham, MA, USA) in cell culture plates coated with poly-D-lysine/laminin (Invitrogen) in Neurobasal medium (Invitrogen) containing B-27 serum-free supplement (50 $\times$; Invitrogen). The medium was half-changed twice a week until analysis.

Immunocytochemistry

Cells were cultured to the desired time points. They were fixed with 4% paraformaldehyde (PFA; Sigma-Adrich, St. Louis, MO, USA) in phosphate buffered saline (PBS) for 15 min at room

TABLE 1 | Fibroblast cell lines used in this study.

	Coriell Cat#	RRID	Sex	Age at sampling	Tissue type	Product
F1	GM07522	CVCL_F162	Female	19 years	Skin	Fibroblast
F2	GM04506	CVCL_7413	Female	20 years	Skin	Fibroblast
F3	GM08400	CVCL_7483	Female	37 years	Skin	Fibroblast
M1	GM03652	CVCL_7397	Male	24 years	Skin	Fibroblast
M2	GM00495	CVCL_7289	Male	29 years	Skin	Fibroblast
M3	GM00024	CVCL_7269	Male	31 years	Skin	Fibroblast

RRID, Research Resource Identifier.

temperature (RT) and permeabilized with blocking buffer [PBS containing 3% bovine serum albumin (BSA) and 0.2% Triton X-100] for 1 h. Cells were then incubated with primary antibodies in blocking buffer overnight at 4°C, followed by washing and incubation with fluorophore-conjugated corresponding secondary antibodies. Primary antibodies used in this study are goat anti-CHAT (Millipore, Kankakee, IL, USA; 1:200), mouse anti-HB9 (DSHB; 1:500), chicken anti-MAP2 (Abcam; 1:10,000), mouse or rabbit anti-TUBB3 (Covance; 1:2,000). Nuclei were stained with Hoechst 33342 (HST; Thermo Fisher Scientific, Waltham, MA, USA).

Fluorescent *in situ* Hybridization

FISH was performed as described previously (Li et al., 2016) with modifications. Briefly, cultured neurons were washed once with DPBS (Gibco) and fixed with 4% PFA in PBS for 30 min at RT. Cells were further permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at RT and twice washed with ice-cold PBS. For RNase A-treated controls, samples were incubated with 0.5 mg/ml of RNase A (New England BioLabs) in 10 mM Tris-HCl buffer (pH 7.4) for 1 h at 37°C. The samples were equilibrated with hybridization buffer composed of 2× saline sodium citrate (SSC), 10% dextran sulfate (average molecular weight 500,000; Sigma-Aldrich, St. Louis, MO, USA), 10 mM ribonucleoside-vanadyl complex (RVC; New England Biolabs), and 20% formamide (Thermo Fisher Scientific, Waltham, MA, USA). A mixture of DIG-labeled oligo-dT or dA probes (0.2 ng/μl) containing yeast tRNA (0.2 μg/μl) were heated to 95°C for 5 min and chilled on ice immediately. Probes were then combined with equal volumes of 2× hybridization buffer and added to samples for overnight incubation at 37°C. Anti-DIG antibody (sheep anti-DIG; Sigma-Aldrich, St. Louis, MO, USA) and fluorophore-conjugated corresponding secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) were used to detect oligo-dT signals. Anti-MAP2 antibody (Abcam) and HST were used to determine neuronal soma and nucleus, respectively. All reagents and solutions were prepared in nuclease-free water.

Protein Nuclear Transport Assay

Protein nuclear transport was analyzed by using a dual reporter 2Gi2R (Mertens et al., 2015). Cultured cells were transduced with a lentivirus, which expressed a fused double-GFP with an NES (2GFP-NES) and a double RFP containing an NLS (2RFP-NLS). To validate this reporter system, transduced HEK cells were treated with 50 nM leptomycin B (Alfa Aesar) and then analyzed by confocal microscopy during a time course. For directly reprogrammed neurons, the reporter lentivirus was cotransduced with reprogramming factors, and the signal densities and distributions of GFP and RFP were analyzed at the indicated time points.

Confocal Microscopy and Quantification

Confocal images were obtained with a Zeiss-LSM700 or Leica SP5 confocal microscopes. The ImageJ software (NIH) was used to quantify fluorescence intensity from images of confocal slices. For each neuron, as large an area as possible was measured within the nucleus or cytoplasm. An unbiased approach for data

collection was employed. The person who analyzed the images was completely blinded to the sample information. The mean values were used to quantify an average signal density. For FISH assays, the average of oligo-dT signals in soma (both nucleus and cytoplasm) was used to evaluate the overall mRNA expression levels. mRNA subcellular distribution was measured by the ratio of nuclear to cytoplasmic oligo-dT signals (dT_{nuc}/dT_{cyt}). For the dual reporter assay, GFP and RFP signals were separately measured in the nucleus and cytoplasm. The ratio of nuclear to cytoplasmic GFP (GFP_{nuc}/GFP_{cyt}) was used to evaluate protein export, while the ratio of cytoplasmic to nuclear RFP (RFP_{cyt}/RFP_{nuc}) was used to measure protein nuclear import.

Statistics

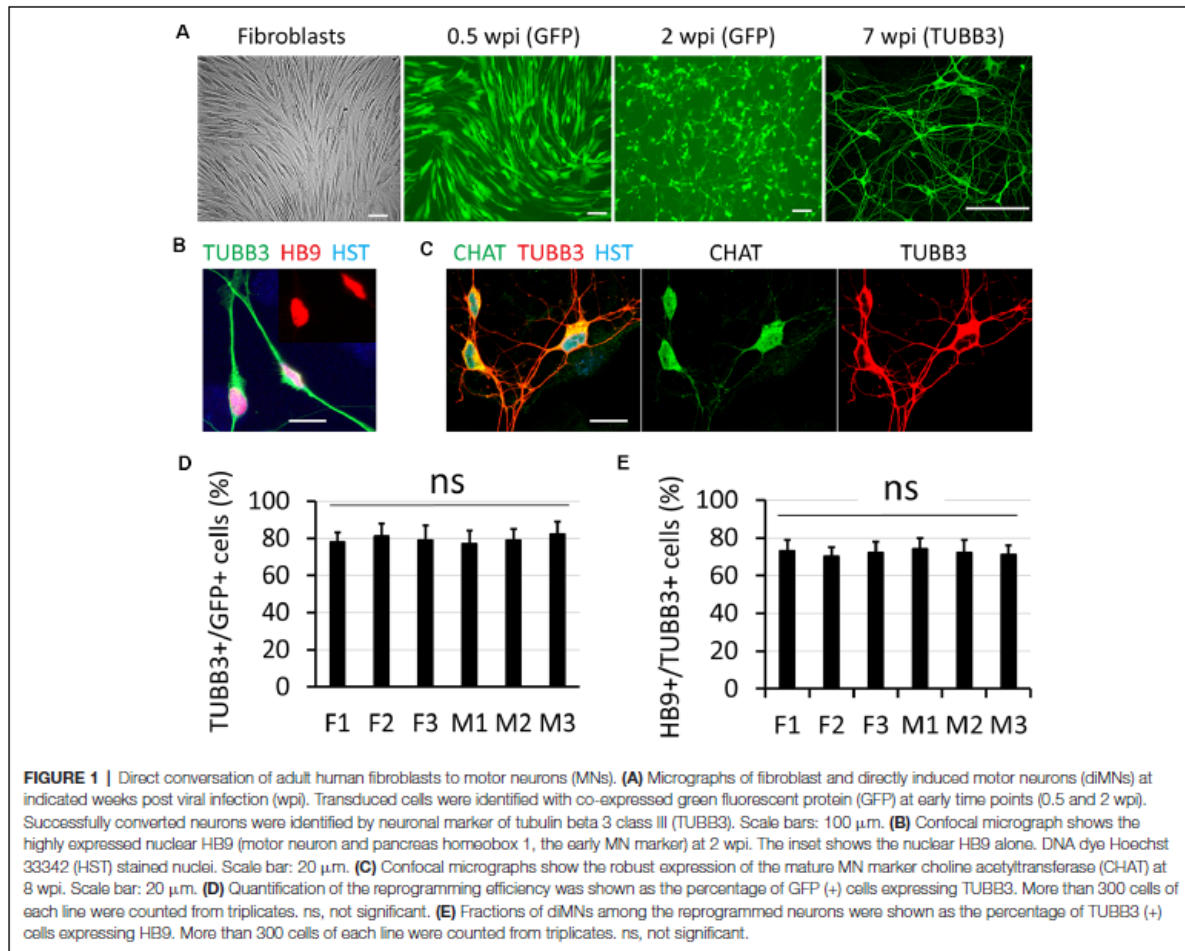
An unpaired two-tailed Student's *t*-test was used for experiments consisting of two groups. For experiments consisting of more than two groups, one-way analysis of variance (ANOVA) was used with either Tukey (when comparing samples with each other) or Dunnett (when comparing samples to a control) *post hoc* tests. The results are expressed as mean ± SD of three biological replicates, and *P* < 0.05 was treated as significant.

RESULTS

Direct Conversion of Adult Human Fibroblasts to Motor Neurons

Disruption of NCT is particularly involved in movement disorders (Da Cruz and Cleveland, 2016; Li and Lagier-Tourenne, 2018), such as ALS which is also characterized with progressive loss of MNs (Freibaum et al., 2015; Jovicic et al., 2015; Zhang et al., 2015; Boeynaems et al., 2016; Chou et al., 2018; Burk and Pasterkamp, 2019). To understand how defective NCT contributes to diseases, measuring NCT activity directly in human MNs became extremely important. In this study, we generated human MNs from adult fibroblasts through direct reprogramming (Liu et al., 2016). Transduced fibroblasts can be identified with co-expressed GFP, and they gradually became neuron-like and grow complex neurites over time (Figure 1A). Immunocytochemistry confirmed their expression of the neuronal marker tubulin beta 3 class III (TUBB3) and early MN marker HB9 (Figure 1B; Arber et al., 1999). At later stages, such as 6–8 weeks post viral infection (wpi), reprogrammed neurons expressed high levels of choline acetyltransferase (CHAT), the mature MN marker (Figure 1C). Thus, adult fibroblasts could be efficiently and directly converted into MNs, which were hereafter referred as directly induced MNs (diMNs). We examined skin fibroblasts from six apparently healthy donors, three males and three females (Table 1). No significant difference was observed between male and female donors at the neuronal conversion efficiency and the MN fraction (Figures 1D,E). About 80% transduced GFP+ cells expressed TUBB3 (Figure 1D). Among these TUBB3+ neurons, approximately 75% of them highly expressed nuclear HB9 (Figures 1B,E).

Based on this study and previous reports (Mertens et al., 2015; Liu et al., 2016; Smith et al., 2016), we summarized the major features associated with the reprogramming process in Table 2.

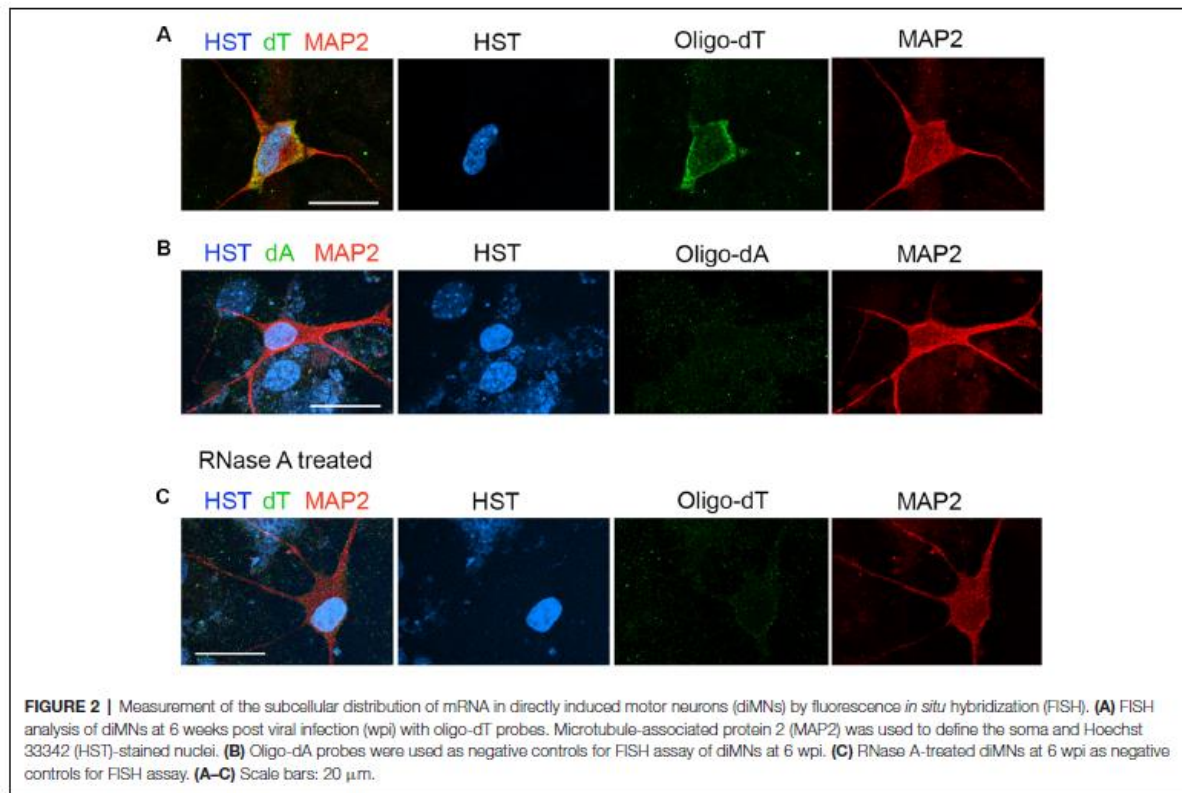
**TABLE 2 |** Biological features associated with the direct reprogramming process.

Stages (wpi)	Major biological features	References
1	High expression of the reprogramming factors and reporter; chromatin remodeling; changes of global gene expression	This study and Smith et al. (2016)
2	Morphological changes with extensive neurite outgrowths; expression of neuronal markers such as TUBB3 and nuclear HB9 in diMNs	This study and Liu et al. (2016)
3	Expression of genes essential for neuronal function: voltage-gated channels, neurotransmitters, transporters, pre- and post-synaptic proteins, etc.; high levels of mRNAs in the nucleus	This study and Mertens et al. (2015)
4	Complex multipolar morphology; robust expression of markers for mature neurons including MAP2 and CHAT; high nuclear mRNA levels	This study and Liu et al. (2016)
6	Typical neuronal morphology; high levels of markers for mature neurons such as CHAT, VACHT, and synaptic proteins; reduced nuclear mRNA levels	This study and Liu et al. (2016)
8	Fully functional neurons with synaptogenesis and mature electrophysiology; formation of neuromuscular junctions between diMNs and myotubes	Liu et al. (2016)

TUBB3, tubulin beta 3 class III; MAP2, microtubule-associated protein 2; CHAT, choline acetyltransferase; VACHT, vesicular acetylcholine transporter.

During the first week, the transduced cells highly express the reprogramming factors, which remodel the chromatin structure and induce global changes on gene expression (Smith et al., 2016). The cells rapidly change morphology with outgrowing neurites and high expression of markers for general neurons

at 2–4 wpi (Mertens et al., 2015; Liu et al., 2016). By 6–8 wpi, the reprogrammed neurons become functionally mature, characterized by synaptogenesis, firing of repetitive action potentials, and formation of neuromuscular junctions (NMJs; Liu et al., 2016).



Measurement of mRNA Distribution in Directly Induced Motor Neurons by Fluorescence *in situ* Hybridization

The distribution of total poly(A) RNAs in single diMNs was measured by FISH with oligo-dT probes as previously described (Packard et al., 2015; Li et al., 2016). Following hybridization, immunostaining of microtubule-associated protein 2 (MAP2) was used to identify the soma, and the DNA dye HST was used to define the nucleus. At the late development stage of 6 wpi diMNs, strong oligo-dT signals could be detected in both the nucleus and cytoplasm, but the majority of signals localize in the cytoplasm (Figure 2A). No obvious signals were detected with the oligo-dA probe (Figure 2B) or in diMNs that were treated with RNase A (Figure 2C), suggesting that the oligo-dT signals in FISH assays are specific.

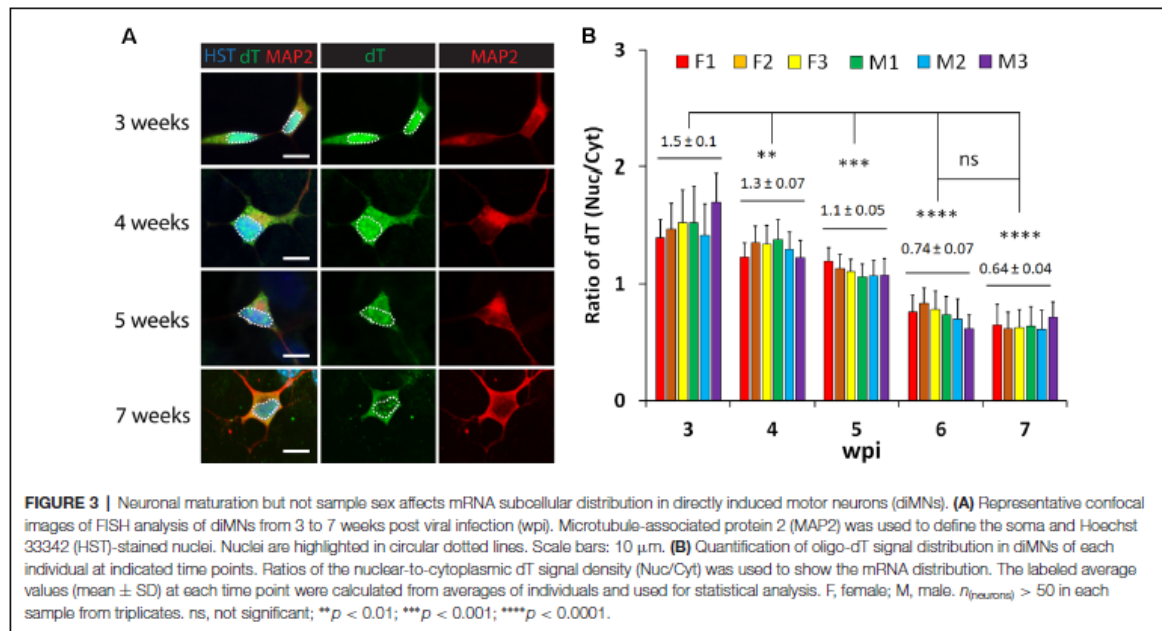
Neuronal Maturation But Not Sample Sex Affects mRNA Subcellular Distribution in Directly Induced Motor Neurons

To examine whether neuronal maturation affects mRNA subcellular distribution, we conducted a time-course analysis of diMNs by FISH. At 3 wpi, oligo-dT signals were highly enriched in the nucleus (Figure 3A). Although diMNs at this stage outgrow neurites and express markers for general neurons

(MAP2 and TUBB3) and MNs (HB9; Figures 1, 3), their somas are still under transformation from an oval morphology of fibroblasts to the multipolar morphology of typical MNs. With diMN maturation, subcellular distribution of mRNAs gradually changed, with decreased signals in the nucleus and conversely increased signals in the cytoplasm. By 7 wpi, the signal density was obviously higher in the cytoplasm than that in the nucleus (Figure 3A). To quantify the subcellular distribution of poly (A) RNAs, the average signal density was separately measured in the nucleus and cytoplasm. The ratio of nuclear to cytoplasmic oligo-dT signal density (Nuc/Cyt) was significantly decreased within the first 6 wpi but reached a steady state thereafter (Figure 3B). These findings indicate that mRNA subcellular distribution is significantly affected by neuronal maturation stages and that it can be steadily measured at 6 wpi or later. A thorough analysis, however, failed to show any effect of sample sexes on mRNA subcellular localization in diMNs (Figure 3B).

mRNA Distribution Is Influenced by Neuronal Maturation in Primary Mouse Neurons

To determine whether the influence of neuronal maturation on mRNA subcellular distribution is a common feature or unique to diMNs, we examined primary mouse neurons at different



maturation stages. Primary mouse cortical neurons (PCNs) were isolated from E18 embryos. After culturing for 2 days *in vitro* (DIV), they showed strong oligo-dT signals in both the nucleus and the cytoplasm (Figure 4A). The specificity of FISH assays was verified by using the oligo-dA probe that showed no any signals (Figure 4B). The overall oligo-dT signals and the ratio of nuclear to cytoplasmic signal density (Nuc/Cyt) significantly decreased when examined at 14 DIV (Figures 4C–E). We also analyzed mRNA distribution in primary mouse CGNs, which were isolated from P6 pups (Figures 5A–C). These postnatal neurons mature faster than those prenatal PCNs in culture, and they are considered as mature with robust marker expression at 6 DIV (Ding et al., 2013; Leto et al., 2016). Consistent with the results in PCNs, total mRNA levels and the ratio of nuclear to cytoplasmic signal density (Nuc/Cyt) gradually decreased with the CGN maturation process (Figures 5D,E). These results clearly indicate that the influence of neuronal maturation on mRNA subcellular distribution is not unique to diMNs but also occurs in primary mouse neurons.

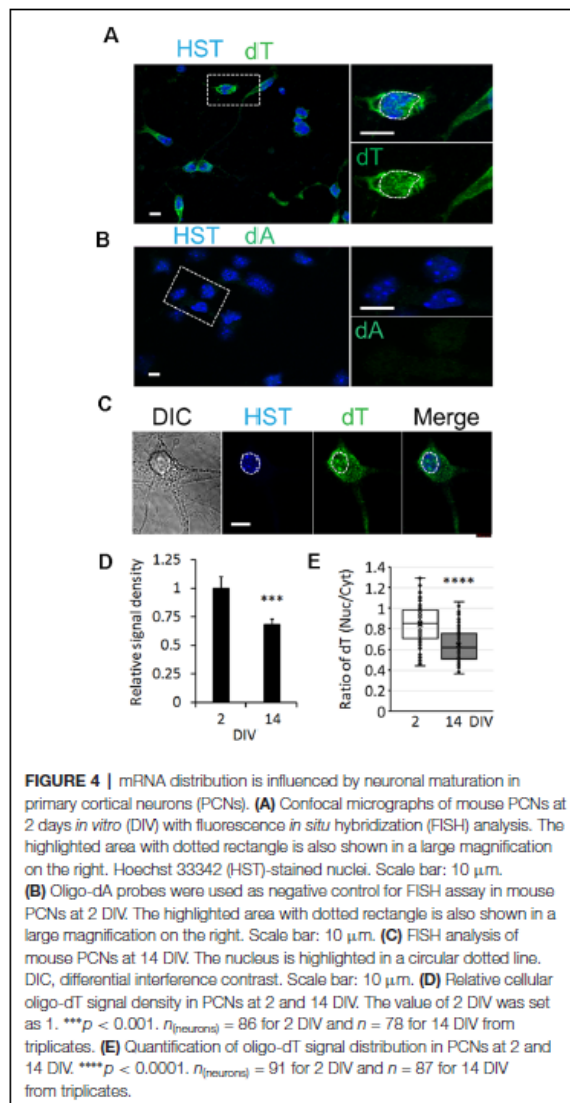
Protein Nucleocytoplasmic Transport Measurement

We employed a dual reporter system (2Gi2R) to measure protein NCT activity in single diMNs. This reporter system consists of a fused double-GFP with an NES (2GFP-NES) and a double RFP containing an NLS (2RFP-NLS; Mertens et al., 2015). NES and NLS are conserved signal sequences that are recognized by nuclear transport receptors of exportins and importins, respectively (Christie et al., 2016; Cagatay and Chook, 2018). Thus, both protein export and import can be analyzed with this dual reporter in single cells. In cells with normal NCT

activity, GFP and RFP are respectively localized in the cytoplasm and nucleus, whereas such distribution will be disrupted when NCT activity is impaired (Figure 6A). An increased ratio of nuclear to cytoplasmic GFP ($\text{GFP}_{\text{nuc}}/\text{GFP}_{\text{cyt}}$) represents disrupted protein export, while a higher ratio of cytoplasmic to nuclear RFP ($\text{RFP}_{\text{cyt}}/\text{RFP}_{\text{nuc}}$) indicates a compromised protein nuclear import.

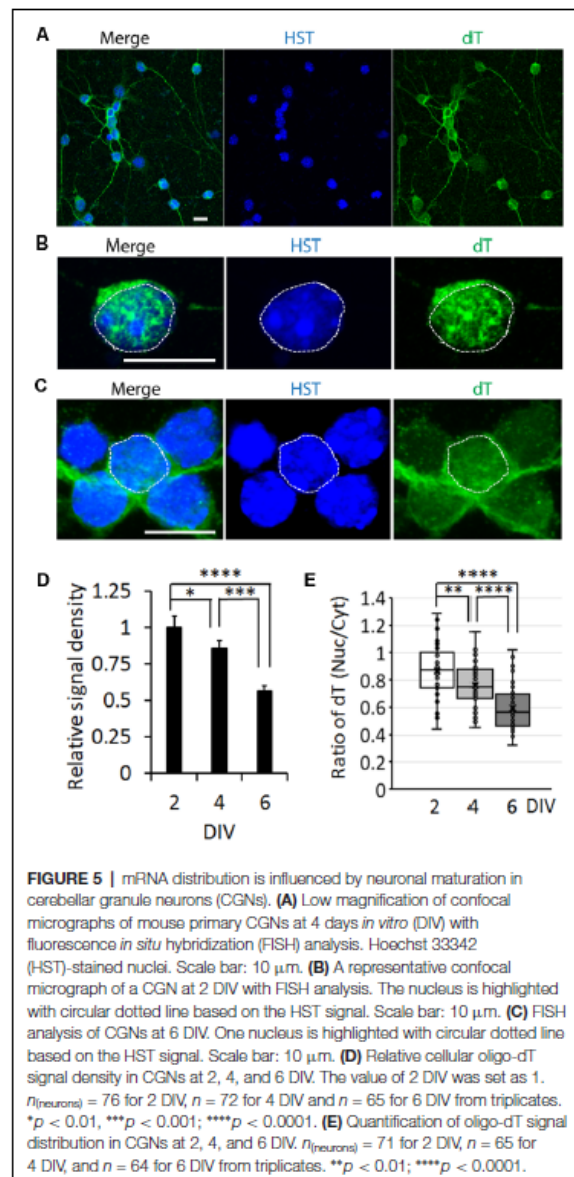
We first validated the 2Gi2R reporter in HEK cells with the treatment of leptomycin B, a nuclear export inhibitor (Jang et al., 2003). Both GFP and RFP were exclusively localized in the cytoplasm and nucleus of transduced cells, respectively (Figure 6B). In the presence of leptomycin B, GFP gradually accumulated inside the nucleus. A significant increase of the ratio of nuclear to cytoplasmic GFP ($\text{GFP}_{\text{nuc}}/\text{GFP}_{\text{cyt}}$) was observed at 10 min and later following exposure to leptomycin B (Figures 6B,C). On the other hand, the distribution of RFP was not significantly affected (Figures 6B,C). These results agree well with previous reports that leptomycin B is a highly specific inhibitor of nuclear export receptor CRM1 (Jang et al., 2003; Mutka et al., 2009), suggesting that this reporter system is reliable in protein NCT measurement.

Human fibroblasts from healthy donors were then transduced with lentiviruses expressing the 2Gi2R reporter and the reprogramming factors. In the resulting diMNs, both GFP and RFP were mainly localized in the cytoplasm and nucleus, respectively (Figure 6D). We also examined but failed to observe an effect of neuronal maturation on protein NCT activity, indicated by unaltered subcellular distribution of the GFP and RFP reporters at 4, 6, or 8 wpi (Figures 6E,F). Similarly, protein NCT activity is not affected by sample sexes during this time-course analysis (Figures 6E,F).

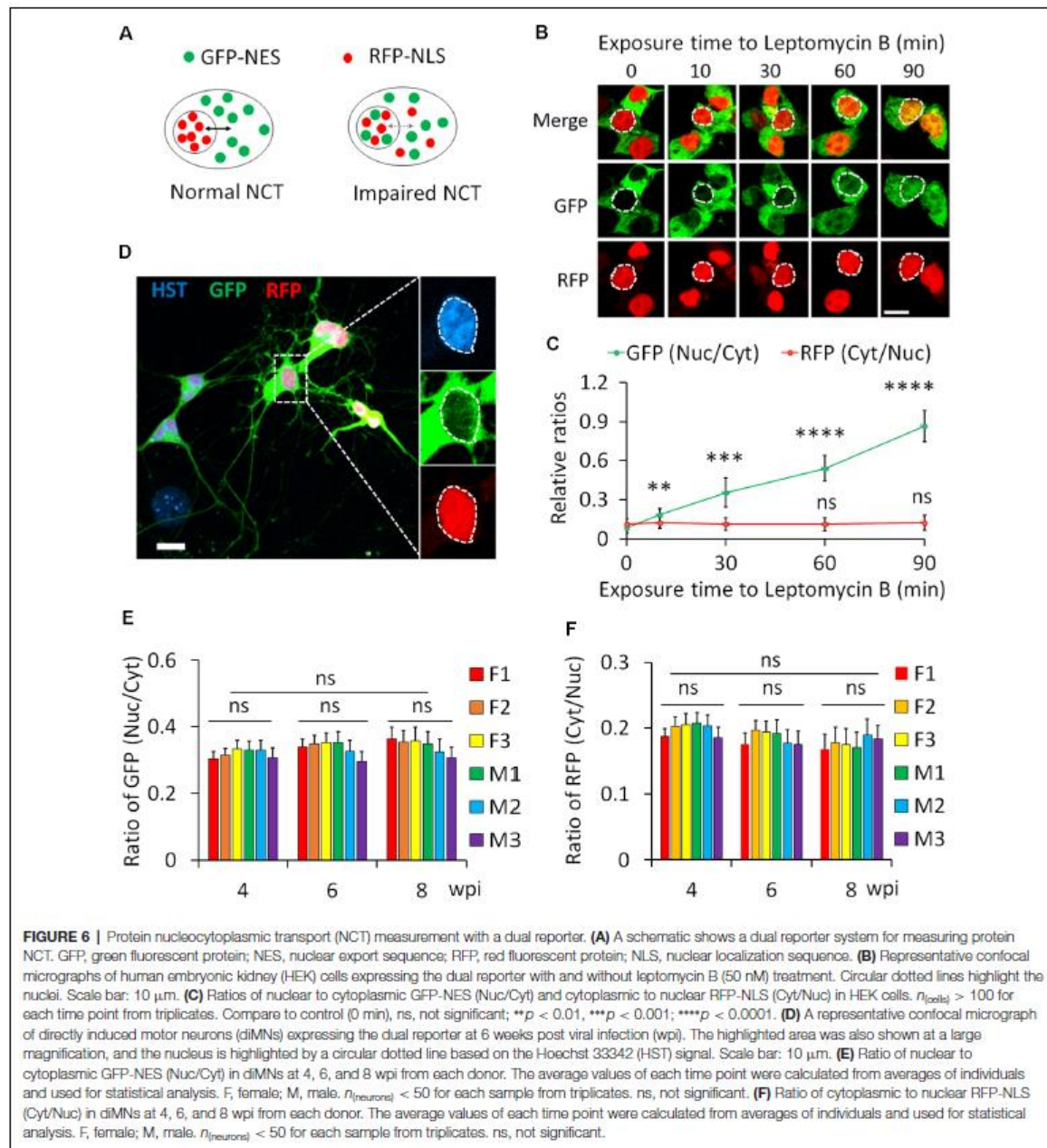


DISCUSSION

In this study, we examined nuclear transport of both mRNAs and proteins in diMNs from adult human fibroblasts. Combining confocal microscopy with quantitative single-cell analysis, the results of this study demonstrate a robust way to measure NCT of both mRNA and protein cargoes in cultured neurons. Importantly, a time-course analysis revealed that neuronal maturation but not sample sex significantly affects mRNA subcellular distribution, whereas both of these biological variables have no effect on protein transport. These results pave a way for future detailed investigations of NCT activity under pathological conditions.



Defective NCT has emerged as a common pathogenic factor of many neurodegenerative diseases, including the movement disorder ALS (Freibaum et al., 2015; Jovicic et al., 2015; Zhang et al., 2015; Chou et al., 2018; Burk and Pasterkamp, 2019). To understand the contributions of disrupted NCT to the pathogenesis of diseases, examinations of NCT activity directly in reprogrammed human neurons became invaluable approaches. Human neurons could be generated by direct conversion from patient fibroblasts or induced pluripotent stem cells (iPSCs)-based reprogramming and differentiation (Barker et al., 2018; Mertens et al., 2018). As iPSCs are epigenetically reset to an



embryonic state during the reprogramming process, certain age-associated features are erased in iPSC-neurons (Lapasset et al., 2011; Soria-Valles and López-Otin, 2016). Directly reprogrammed neurons from donors can maintain aging-associated hallmarks, providing an excellent model system in the research of age-related neurodegenerative diseases (Mertens et al., 2015, 2018; Tang et al., 2017; Victor et al., 2018).

In this study, using diMNs, we have systematically examined the nuclear mRNA export by FISH with digoxigenin-labeled oligo-dT probes. Compared to directly fluorophore-conjugated probes, the oligo-dT signals in this method could be further amplified by anti-digoxigenin antibody and fluorophore-conjugated secondary antibodies, providing much stronger signals for the quantification assay. Most importantly, the

specificity of oligo-dT signals was confirmed by a lack of signal when digoxigenin-labeled oligo-dA was used as probes, or when samples were treated with RNase A. Combined with immunostaining of neuron markers and nuclear dye, the soma and the nucleus of neurons can be clearly defined. Thus, the nuclear and cytoplasmic mRNAs can be measured, and their ratio will reveal the subcellular distribution of mRNAs. The increased ratio of nuclear to cytoplasmic oligo-dT signals (dT_{nuc}/dT_{cyt}) will indicate the impaired nuclear mRNA export. Time-course analysis indicated that neuronal maturation significantly affected the mRNA subcellular distribution in reprogrammed MNs. Interestingly, similar effects were observed when examining developmental stages on the mRNA subcellular distribution in mouse primary neurons. This could be due to the higher nuclear transcriptional levels at early developmental stages when neurons undergo rapid neurite outgrowth. Thus, to obtain reliable results of NCT activity, using reprogrammed human neurons at late mature stages is vitally critical. In our condition, the best developmental stage to measure the mRNA subcellular distribution of diMNs is 6 wpi.

Compared to nuclear mRNA export, different signaling pathways are employed for protein nuclear transport (Stewart, 2007a; Kabachinski and Schwartz, 2015). In this study, we also simultaneously analyzed protein nuclear import and export using a dual reporter, in which GFP-NES and RFP-NLS were co-expressed in transduced neurons. By 4 wpi, both GFP and RFP signals are strong enough for quantification analysis. The increased ratio of nuclear to cytoplasmic GFP (GFP_{nuc}/GFP_{cyt}) will suggest the impaired protein export, while the higher ratio of cytoplasmic to nuclear RFP (RFP_{cyt}/RFP_{nuc}) will indicate the compromised protein nuclear import. Consistent with the results of mRNA subcellular distribution in diMNs, the sample sex did not significantly affect protein nuclear transport. Different from mRNA subcellular distribution, protein NCT did not show any significant difference in diMNs within 4–8 wpi, suggesting that protein NCT activity does not significantly change within this time frame. The differential influence of reprogramming stages on mRNA and protein NCT could be because they are mediated by different signaling pathways. Our results also support the notion that the higher transcriptional levels in early-stage neurons, rather than the NCT machinery itself, significantly influence the poly (A) RNA subcellular distribution.

However, we did not analyze the protein NCT at time points earlier than 4 wpi because of the relatively low expression levels of both GFP-NES and RFP-NLS. Therefore, the differences of protein NCT activity at very early reprogramming stages cannot be excluded as a possibility. Another limitation in this study is that only overall poly (A) RNAs have been examined, rather than the specific transcripts. If digoxigenin-

labeled gene-specific probes are employed, we are confident that the subcellular distribution of a particular mRNA can be measured in cultured neurons using our approach. The measurement of the subcellular distribution of specific transcripts would be more important in identification of mislocalized mRNAs in patient-derived neurons. Our study indicates that the sample sex does not significantly influence the mRNA subcellular distribution and protein NCT in diMNs. However, given the facts that sample sex is a biological variable in many biological processes, sex-matched controls are still recommended in using reprogrammed human neurons to model human diseases.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

All animal work was carried out with approvals from the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center. Primary human skin fibroblasts were all obtained from the cell repository at the Coriell Institute for Medical Research. No approval was required from the institutional review board (IRB) at University of Texas Southwestern Medical Center.

AUTHOR CONTRIBUTIONS

BD conceived and performed most experiments and acquired and analyzed data. MA performed leptomycin B experiment. BD and C-LZ prepared the manuscript. All authors reviewed and approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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METHODS IN CELL PHYSIOLOGY | *Making Cell Culture More Physiological*

Generation and optimization of highly pure motor neurons from human induced pluripotent stem cells via lentiviral delivery of transcription factors

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Sepehrimanesh M, Ding B. Generation and optimization of highly pure motor neurons from human induced pluripotent stem cells via lentiviral delivery of transcription factors. *Am J Physiol Cell Physiol* 319: C771–C780, 2020. First published August 12, 2020; doi: 10.1152/ajpcell.00279.2020.—Generation of neurons from human induced pluripotent stem cells (hiPSCs) overcomes the limited access to human brain samples and greatly facilitates the progress of research in neurological diseases. However, it is still a challenge to generate a particular neuronal subtype with high purity and yield for determining the pathogenesis of diseased neurons using biochemical approaches. Motor neurons (MNs) are a specialized neuronal subtype responsible for governing both autonomic and volitional movement. Dysfunctions in MNs are implicated in a variety of movement diseases, such as amyotrophic lateral sclerosis (ALS). In this study, we generated functional MNs from human iPSCs via lentiviral delivery of transcription factors. Moreover, we optimized induction conditions by using different combinations of transcription factors and found that a single lentiviral vector expressing three factors [neurogenin-2 (NGN2), insulin gene enhancer 1 (ISL1), and LIM/homeobox 3 (LHX3)] is necessary and sufficient to induce iPSC-derived MNs (iPSC-MNs). These MNs robustly expressed general neuron markers [microtubule-associated protein 2 (MAP2), neurofilament protein (SMI-32), and tubulin β -3 class III (TUBB3)] and MN-specific markers [HB9 and choline acetyltransferase (ChAT)] and showed electrical maturation and firing of action potentials within 3 wk. This approach significantly improved the neuronal survival, yield, and purity, making it feasible to obtain abundant materials for biochemical studies in modeling movement diseases.

human induced pluripotent stem cells (hiPSCs); lentivirus; motor neurons; transcription factors

INTRODUCTION

Motor neurons (MNs) are a specialized neuronal subtype responsible for innervating musculature in the periphery and governing both autonomic and volitional movements (6). The process of MN specification *in vivo* consists of several sequential developmental stages, including neural induction in embryonic ectoderm, positioning along rostrocaudal and dorsoventral axes, and differentiation of specified neuronal precursors into postmitotic neuronal subtypes. In this developmental sequence, the interactions between intrinsic genes and extrinsic environmental clues ensure that numerous groups of genes should be turned on and off in response to extracellular signals in an elegant spatiotemporal manner (8, 23, 40). The combined actions of transcription factors, chemicals, and small molecules together with the bone morphogenic protein (BMP), Wntless

and Int-1 (WNT), and fibroblast growth factor (FGF) signaling pathways have unique influences on neuronal identity and cell fate (2, 6, 23). Malfunctions in MNs caused by either degeneration or dysregulation are involved in a variety of movement diseases, such as amyotrophic lateral sclerosis (ALS), progressive muscular atrophy (PMA), spinal and bulbar muscular atrophy (SBMA), and dystonia (13, 16, 30, 42, 52). So far, there are no specific treatments available to cure these diseases because of unclear pathophysiological mechanisms. Generation of patient-specific MNs provides an unprecedented *in vitro* model system and greatly facilitates studies in deciphering the pathogenesis of these diseases (29, 33, 45).

MNs could be generated via direct conversion from adult fibroblasts by delivery of four transcription factors—neurogenin-2 (NEUROG2), insulin gene enhancer 1 (ISL1), LIM/homeobox 3 (LHX3), and sex-determining region Y (SRY)-box transcription factor 11 (Sox11)—with two lentiviruses (9, 33). However, the relative low purity and poor yield of directly converted MNs severely limit the applications that require a highly pure and large amount of cells, such as gene expression analysis by next-generation sequencing (NGS), and protein coimmunoprecipitation (Co-IP) and mass spectrometry (MS) analysis. As human induced pluripotent stem cells (hiPSCs) possess almost unlimited expansion capability, the approach of iPSC-based reprogramming and differentiation is feasible to generate a large number of human neurons for biochemical studies. Importantly, iPSCs could be prepared from a variety of readily available tissues such as skin and blood (49, 57). For instance, patient peripheral blood mononuclear cells (PBMCs) or skin fibroblasts could be used to generate hiPSCs by Yamanaka factors and then further induced into specific neuronal subtypes (55). These induced neurons have the same genetic background as the donors and could survive for a long time in cultured vessels to achieve fully functional maturation. These advantages make the iPSC-based approach a powerful tool in modeling neurological diseases (15, 18, 24).

Currently, there are several approaches to generate MNs from hiPSCs (Table 1). Small molecules such as growth factors and chemicals plus transcription factors are routinely used for neuronal induction. These factors either directly regulate genes or target signaling pathways that play critical roles in neurogenesis and differentiation. For example, GSK3 β inhibitors such as CHIR99021 combine with the dual Sma and Mad proteins (SMAD) inhibitors, including SB 431542, a TGF- β receptor I kinase inhibitor, and LDN, a BMP type I receptor inhibitor, to induce neural progenitor cells (NPCs; 4, 17, 21, 31). In this way, it is crucial to use BMP inhibitors (Noggin and/or dorsomorphin), γ -secretase inhibitors (DAPT, tert-butyl

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Table 1. Approaches for generation of human iPSC-MNs

Induction Factors*	Positive Markers	Characteristics†	Ref.
SB 431542, CHIR99021, dorsomorphin, and compound E	ChAT, HB9, SOX11, PAX6, nestin, OLIG2, TUJ1, neurofilament, and MAP2	MNs that form synaptic connections with bursting behavior, defined ion currents, and induced APs as well as calcium spikes (maturation time: 21 days)	(4)
SB 431542 and FGF2 or Noggin	HB9, ISL1, PAX6, SOX11, synapsin, OLIG2, and TUJ1	Rostrocaudal MNs that form synapses and give spontaneous postsynaptic currents (maturation time: 15 days)	(22)
SB 431542, Noggin, FGF2, and ROCK inhibitor	HB9, ISL1, PAX6, rosette markers, Nestin, PLZF, ZO1, OTX2, BF1, FOXG1, AP2, and PAX7	Spinal MNs and midbrain dopamine neurons (maturation time: 11 days)	(7)
SB 431542, dorsomorphin, BDNF, retinoic acid, and Smoothed agonist	ISL1/2, FOXP1, HOXA5, MAP2, TUJ1	Lateral motor column identity with a rostral phenotype that shows electrical spiking and activity (maturation time: 24 days)	(25)
RA and purmorphamine	ChAT, HB9, SMI-32, OLIG2, TUJ1	Caudovernalized spinal MNs with spontaneous and depolarization-induced action potentials (maturation time: 8 days)	(46)
SB 431542, CHIR99021, dorsomorphin, and RA	ChAT, HB9, SMI-32	Spinal cord MNs (maturation time: 24 days)	(17)
RA, Smoothed agonist, BDNF, GDNF, and DAPT	ChAT, HB9, ISL1, SMI-32, TUJ1	MNs that form physical and functional synapses with spontaneously occurring action potentials (maturation time: 32–38 days)	(20)
SB 431542, dorsomorphin, RA, smoothed agonist, GDNF, BDNF, and CNTF	ChAT, HB9, TUJ1	MNs with normal total neurite length, normal maximum neurite length, and normal number of nodes per MN (maturation time: 26–29 days)	(53)
SB 431542, dorsomorphin, RA, purmorphamine, cAMP, BDNF, GDNF, IGF-1	ChAT, HB9, ISL1	MNs that form end plate-like structures and induce clustering of acetylcholine receptors (maturation time: 42 days)	(48)
SB 431542, dorsomorphin, β -mercaptoethanol, insulin, CHIR99021, RA, purmorphamine, Compound E	ChAT, OLIG2, HOXB4, and SMI-32	Spinal cord neuron with spontaneous firing, containing glutamate receptors and voltage-dependent calcium channels (maturation time: 18 days)	(21)
SB 431542, LDN, CHIR99021, RA, Smoothed agonist, DAPT, BDNF, GDNF, and cAMP	ChAT, HB9, ISL1, MAP2, TUJ1, and synaptophysin	Spinal MNs with radial projection of neurites, defined electrophysiological characteristics, fast-activating, fast-inactivating inward Na^+ currents and large outward K^+ currents (maturation time: 12 days)	(31)
Purmorphamine, RA, dibutyl-cAMP, BDNF, GDNF, and IGF-1	ChAT, HB9, ISL1, NGN2, OLIG2, LIM3, MNR2, FLAG, and DYKDDDDK tag	Spinal interneurons (maturation time: 28 days)	(3)
SB 431542, dorsomorphin, B18R, syn-TFs mRNAs of neurogenin and NeuroD families, forskolin, BDNF, GDNF, and NT-3	ChAT, HB9, and ISL1	Canonical MNs with stimulus-induced channel activities and electrical properties that exhibit action potential; this method decreases the differentiation time (maturation time: 10 days)	(19)
pLV-TRET-Ngn2-2A-Ngn1	ChAT, GLUT1, MAP2, and synapsin	Cortical neurons with functional synaptic activity and physiological action potential; this method has fewer medium changes (maturation time: 14 days)	(5)
NEUROG2, Sox11, ISL1, and LHX3	HB9, ChAT, TUBB3, MAP2, and synapsin	Low MNs with typical morphology and markers and high induction efficiency and purity (maturation time: 14 days)	(13, 51)

Chemicals include SB 431542 (a TGF- β receptor I kinase inhibitor), CHIR99021 [a glycogen synthase kinase-3 (GSK-3) inhibitor], dorsomorphin [an adenosine 5'-monophosphate-activated protein kinase (AMPK) inhibitor], compound E and DAPT (γ -secretase inhibitors), Y-27632 [a Rho-associated protein kinase (ROCK) inhibitor], retinoic acid (RA; a regulator of cell growth and differentiation), Smoothed agonist and purmorphamine [Hedgehog (Hh) signaling pathway activators], and forskolin (adenylyl cyclase activator that increases intracellular cAMP concentration as well as affects calcium currents and inhibits MAP kinase). Moreover, growth factors and small peptides comprise fibroblast growth factor 2 [FGF2, also referred to as basic fibroblast growth factor (bFGF), which stimulates neural precursor cell proliferation], Noggin [an inhibitor of bone morphogenic protein (BMP) signaling], brain-derived neurotrophic factor (BDNF; a canonical nerve growth factor), glial cell-derived neurotrophic factor [GDNF; supports the survival of dopaminergic and motor neurons (MNs)], ciliary neurotrophic factor (CNTF; promotes neurotransmitter synthesis and neurite outgrowth), insulin-like growth factor 1 (IGF-1), and neurotrophin-3 (NT-3; stimulates growth and differentiation of neurons and synapses). Finally, transcription factors enumerate as neurogenin-2 (NEUROG2 or NGN2), sex-determining region Y (SRY)-box transcription factor 11 (Sox11), insulin gene enhancer 1 (ISL1, or ISL LIM/homeobox 1), and LIM/homeobox 3 (LHX3; detailed functions are presented in Fig. 2B). ChAT, choline acetyltransferase; MAP2, microtubule-associated protein 2; SMI-32, neurofilament protein; TUJ1 or TUBB3, tubulin β -3 class III. HB9, homeobox gene 9; PAX6, paired box protein; PLZF, promyelocytic leukemia zinc finger; ZO-1, zona occludens protein; OTX2, orthodenticle homeobox 2; BF1, brain factor 1; FOXG1, forkhead box G1; PAX7, paired box protein 7; HOXA5, homeobox A5; Syn-TFs, synthetic transcription factors; DAPT, tert-butyl (2S)-2-[[[(2S)-2-[[[2-(3,5-difluorophenyl)acetyl]amino]propanoyl]amino]-2-phenylacetate. *Generation of human induced pluripotent stem cell (iPSC)-derived MNs (iPSC-MNs) can be done via chemicals, growth factors, small peptides, and transcription factors.

†Maturation time is defined by different criteria in different studies.

(2S)-2-[[[(2S)-2-[[2-(3,5-difluorophenyl)acetyl]amino]propanoyl]amino]-2-phenylacetate or compound E), antioxidants [cAMP or β -mercaptoethanol (β -ME)], and insulin-like growth factor 1 (IGF-1) or insulin to boost NPC proliferation. Then, retinoic acid (RA), purmorphamine, or Smoothened agonist can activate sonic hedgehog (SHH) signaling and induce choline acetyltransferase (ChAT)-positive MNs (3, 21, 31). The presence of brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), or neurotrophin-3 (NT-3) in the culture medium is necessary to maintain the neuronal survival and promote neuronal maturation (20, 48, 51). Regardless of the extensive use of these small molecules, these methods have poor efficiency, produce clear heterogeneity in neuronal subtypes, and are time and cost consuming. Therefore, new methods of creating iPSC-derived MNs (iPSC-MNs) are being developed (Table 1). Lentiviral delivery of transcription factors is an alternative approach (51).

In this study, we generated functional mature MNs via hiPSC-based induction and differentiation. Human iPSCs were induced into NPCs and then transduced with a set of transcription factors as used in the direct conversion approach (13, 51). Moreover, we closely examined and compared the induction efficiency with different combinations of factors. We found that the transduction of NPCs with a single lentivirus coexpressing three factors (NEUROG2, ISL1, and LHX3) is necessary and sufficient to generate iPSC-MNs. Most importantly, by using a single lentiviral vector, the transduction efficiency was maximized and simultaneously the cell death was minimized. Our study provides an optimized approach to generate highly pure iPSC-MNs with very high yield, making it feasible to obtain abundant materials for biochemical studies in modeling movement diseases.

METHODS

Cell lines and culture conditions. Human embryonic kidney (HEK) 293T cells [American Type Culture Collection (ATCC) cat. no. CRL-11268, RRID:CVCL_1926] and human wild-type iPSCs (Coriell cat. no. GM23476, RRID:CVCL_T841) were purchased from ATCC and Coriell Institute for Medical Research, respectively. Human iPSCs were maintained in complete mTeSR1 medium (STEMCELL Technologies cat. no. 85850) on Matrigel (Corning cat. no. 356234)-coated dishes at 37°C and 5% CO₂, and the medium was replaced daily. As in previous reports (9, 51), the detailed medium recipes were as follows:

- 1) HEK medium (lentivirus preparation medium) consisted of DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS; Corning) and 1% penicillin-streptomycin (P/S; GIBCO).
- 2) iPSC medium (complete mTeSR1 medium) consisted of mTeSR1 basal medium supplemented with mTeSR1 supplement (STEMCELL Technologies) and 1% P/S.
- 3) KnockOut Serum Replacement (KOSR) medium consisted of KOSR (Thermo Fisher Scientific), 1% GlutaMax (GIBCO), 1% nonessential amino acid (NEAA; GIBCO), 50 μ M β -mercaptoethanol (β -ME; GIBCO), and 1% P/S.
- 4) Neurosphere (NSP) medium consisted of DMEM/F-12 medium containing 1% N2 (Invitrogen), 1% GlutaMax, 1% NEAA, 1% P/S, 50 μ M β -ME, 8 μ g/mL heparin (STEMCELL Technologies), 20 ng/mL basic fibroblast growth factor (bFGF), and 20 ng/mL epidermal growth factor (EGF; PeproTech).
- 5) NPC medium consisted of DMEM/F-12 and neurobasal medium (1:1) containing 0.5% N2 (Invitrogen), 1% B27 (Invitrogen), 1% GlutaMax, 1% NEAA, 50 μ M β -ME, 1% P/S, 10 ng/mL EGF, 10 ng/mL bFGF, and 1% P/S.

- 6) Neuronal maturation medium consisted of DMEM-F-12-neurobasal medium (2:2:1), 0.8% N2, 0.8% B27, and 1% P/S, supplemented with 5 mM forskolin (FSK; Sigma-Aldrich) and 10 ng/mL each of BDNF (PeproTech), GDNF (PeproTech), and neurotrophin-3 (NT-3; PeproTech).

Note that it is critical to freshly prepare a complete culture medium and use it up within 1 wk. A very old complete culture medium (>2 wk) may affect cell growth and even cause contamination.

Plasmids and virus production. As previously described (13, 33, 51), a third-generation lentiviral vector (pCSC-SP-PW-IRES-GFP) was used to express NEUROG2-IRES-GFP (plasmid 1), NEUROG2-IRES-GFP-T2A-Sox11 (plasmid 2, Addgene no. 90214), and ISL1-T2A-LHX3 (plasmid 3, Addgene no. 90215). The DNA sequence of NEUROG2-IRES in plasmid 2 was subcloned into plasmid 3 and constructed NEUROG2-IRES-ISL1-T2A-LHX3 (plasmid 4), in which three transcription factors are coexpressed in a single lentiviral vector (Fig. 2A). Replication-incompetent lentiviruses were produced in HEK293T cells, and viral supernatants were collected at 48 h and 72 h posttransfection. Viral supernatants were filtered through 0.45- μ m syringe filters and titered as previously described (9, 12). Lentiviruses were stored at 4°C before cell transduction.

Note that high-quality supercoiled plasmid is critical to prepare lentivirus with good titer. The purity and the supercoiled conformation of plasmids could be examined by NanoDrop and running agarose gels as in a previous report (12).

NPC generation. NPCs were generated as previously reported (13, 56). Briefly, hiPSCs were cultured in mTeSR1 medium with 10 μ M all-trans-retinoic acid (RA; Sigma) and 0.5 mM valproic acid (VPA; Sigma) on Matrigel-coated six-well plates for 7 days. iPSCs were then digested with Versene (Thermo Fisher Scientific) and gently pipetted into small clumps supplemented with 10 μ M Y-27632 Rho-associated protein kinase (ROCK) inhibitor (STEMCELL Technologies). Cell clumps were aggregated in the KOSR medium for 4 days, followed by culturing in the NSP medium for another 1 wk. Neurospheres were then formed and dissociated into single cells by Accutase (Innovative Cell Technologies). NPCs were maintained and expanded on Matrigel-coated six-well plates in NPC medium. When cells reached ~90% confluence, NPCs were frozen in freezing medium (NPC medium supplemented with 10% DMSO) and kept in liquid nitrogen for long-term storage.

Note that all-trans-RA is light and temperature sensitive. Keep it from light during storage and performance. To maintain an effective concentration of chemicals, it is important to change the induction medium daily.

Neural differentiation. Neural differentiation was conducted as in previous reports (13, 51) with further modifications. For spontaneous differentiation, NPCs were plated onto Matrigel-coated plates at a density of 5×10^4 cells/cm² and cultured with neuronal maturation medium containing 5 mM FSK and 10 ng/mL each of BDNF, GDNF, and NT-3. The medium was half-changed every other day until analysis. For transcription factor-induced differentiation, NPCs were seeded onto Matrigel-coated plates at a density of 3×10^4 cells/cm² overnight and infected with lentiviruses expressing desired transcription factors. In this study, three different combinations of transcription factors were employed to generate iPSC neurons: 1) NEUROG2 alone (N, *lentivirus 1*); 2) NEUROG2, ISL1, LHX3, and Sox11 (N-S-I-L, *lentiviruses 2* and 3); and 3) NEUROG2, ISL1, and LHX3 (N-I-L, *lentivirus 4*; Fig. 2A). The multiplicity of infection (MOI) was about 2 for a single virus and 4 for the cocktail of two lentiviruses (1:1). The next day, the medium was replaced with a neuronal maturation medium containing 5 mM FSK and 10 ng/mL each of BDNF, GDNF, and NT-3. Neurons were then dissociated by Accutase at 5 days postinfection (dpi) and replated onto astrocyte-coated coverslips at a density of 1×10^3 cells/cm². Mouse astrocytes were isolated from *postnatal day 1* (P1) pups and cultured in DMEM with 15% FBS as reported (47). The medium was changed twice a week until analysis.

Note that at transduction, the proper NPC density (50–60% confluence) is critical to obtain high-yield iPSC-MNs. Too high and too low cell density at transduction will cause more cell death.

Immunostaining. Cultured cells at indicated time points were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 min at room temperature and then incubated in a blocking buffer [PBS containing 3% bovine serum albumin (BSA) and 0.2% Triton X-100] for 1 h for permeabilization and blocking. Cells were then incubated with primary antibodies in a blocking buffer at 4°C overnight, followed by washing and incubation with fluorophore-conjugated corresponding secondary antibodies. The primary antibodies used in this study were

microtubule-associated protein 2 (MAP2, Abcam cat. no. ab5392, RRID: AB_2138153, 1:10,000; and Sigma-Aldrich cat. no. M4403, RRID: AB_477193, 1:500), tubulin β -3 class III (TUBB3, 1:2,000; Covance Research Products Inc. cat. no. MMS-435P, RRID:AB_2313773, and cat. no. PRB-435P-100, RRID:AB_2313773), HB9 (1:500; DSHB, cat. no. 81.5C10-c), ChAT (1:200, Millipore cat. no. AB144P, RRID: AB_2079751), vesicular glutamate transporter 1 [VGLUT1, 1:100; University of California, Davis/National Institutes of Health (NIH) NeuroMab Facility cat. no. 75-066, RRID:AB_2187693], VGLUT3 (1:100; University of California, Davis/NIH NeuroMab Facility cat. no. 75-073, RRID:AB_2187711), γ -aminobutyric acid (GABA, 1:1,000; Sigma-AI-

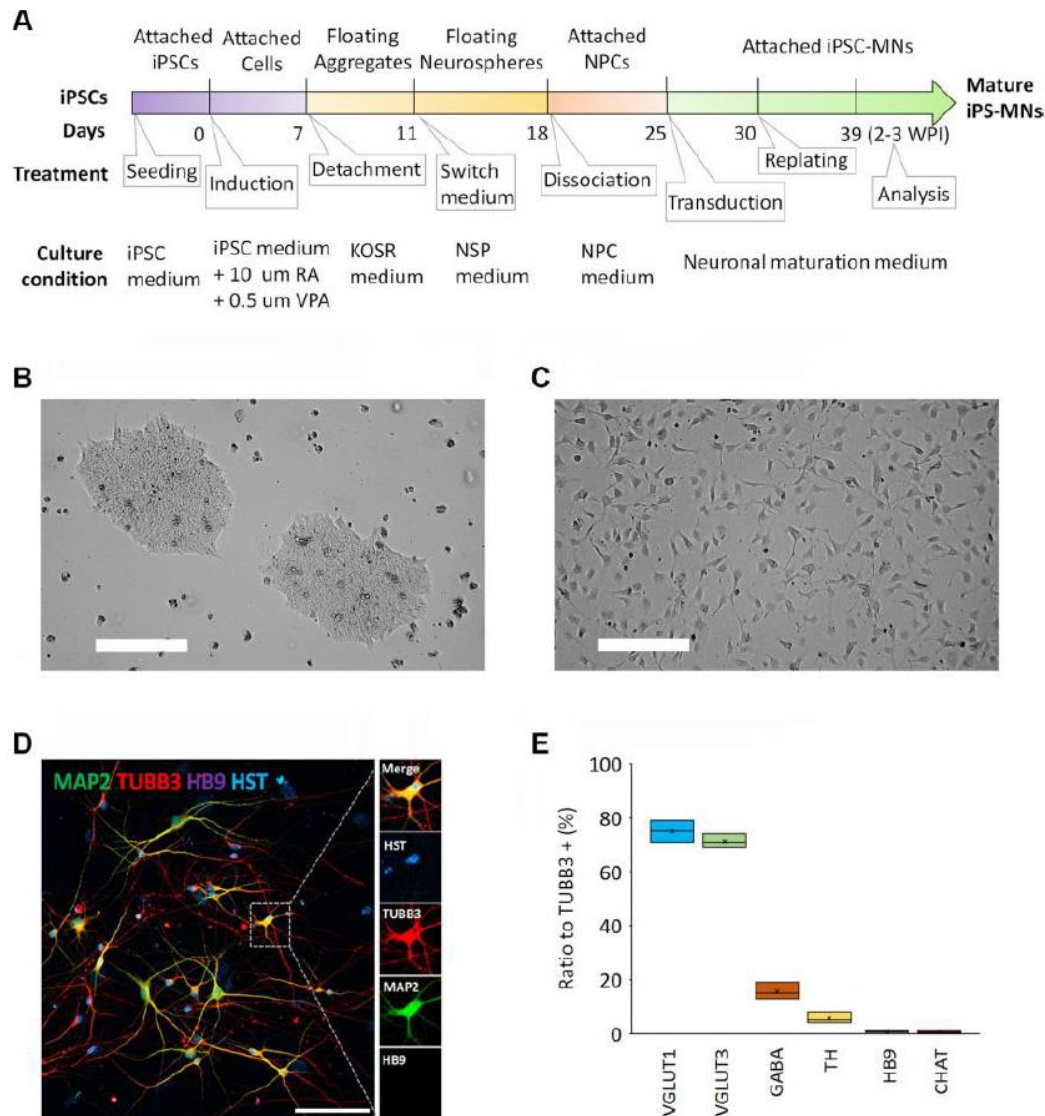


Fig. 1. Generation of human neurons from induced pluripotent stem cells (iPSCs). **A:** a schematic shows the process of the generation of human motor neurons (MNs) from iPSCs. The starting induction time was set as day 0. The age of iPSC-derived MNs (iPSC-MNs) was counted from lentiviral transduction. KOSR, KnockOut Serum Replacement; NPCs, neuronal progenitor cells; NSP, neurosphere; RA, retinoic acid; VPA, valproic acid; WPI, weeks post-viral infection. **B:** a representative phase contrast micrograph of human iPSCs. Scale bar, 200 μ m. **C:** a representative phase contrast micrograph of NPCs. Scale bar, 200 μ m. **D:** a confocal micrograph of spontaneously differentiated neurons from NPCs at 3 wk. Hoechst 33342 (HST)-stained nuclei. Scale bar, 100 μ m. **E:** quantification of neuron identity shown as the ratios of specific markers to tubulin β -3 class III (TUBB3)+ cells. Number of neurons $n > 300$ from 3 biological replicates. ChAT, choline acetyltransferase; GABA, γ -aminobutyric acid; MAP2, microtubule-associated protein 2; TH, tyrosine hydroxylase; VGLUT1, vesicular glutamate transporter 1; VGLUT3, vesicular glutamate transporter 3.

drich cat. no. A2052, RRID:AB_477652), tyrosine hydroxylase (TH, 1:1,000; LifeSpan cat. no. LS-C149142-200, RRID:AB_11145263), and SMI-32 (1:5,000; BioLegend cat. no. 801701, RRID:AB_2564642). The secondary antibodies used in this study were purchased from Jackson ImmunoResearch Laboratories. They were Alexa Fluor (AF)488 anti-mouse (cat. no. 715-545-150, RRID:AB_2340846), AF594 anti-mouse (cat. no. 715-585-151, RRID:AB_2340855), AF647 anti-mouse (cat. no. 715-605-150, RRID:AB_2340862), AF488 anti-rabbit (cat. no. 711-545-152, RRID:AB_2313584), AF594 anti-rabbit (cat. no. 711-585-152, RRID:AB_2340621), AF647 anti-rabbit (cat. no. 711-605-152, RRID:AB_2492288), AF488 anti-chicken (cat. no. 703-545-155, RRID:AB_2340375), and AF594 anti-chicken (cat. no. 703-585-155, RRID:AB_2340377; 10, 11, 14, 33, 51). The nuclei were stained with Hoechst 33342 (HST, 1 μ g/mL; Thermo Fisher Scientific).

Imaging and quantification. Living cells were visualized in culture plates with a CKX53 inverted microscope (Olympus). Immunostaining images were obtained with a Leica SP5 confocal microscope in the Microscopy Center at the University of Louisiana at Lafayette. ImageJ software (NIH) was used to quantify the neurons based on specific markers. An iPSC-induced neuron was defined by highly expressed neuron-specific markers TUBB3 and/or MAP2. An MN was defined by the robust expression of nuclear HB9 [also named motor neuron and pancreas homeobox 1 (MN1)] at early developmental stages and/or high levels of ChAT in the soma at late mature stages. The purity of iPSC-differentiated MNs was calculated by the ratio of HB9+ cells to TUBB3+ cells at the early developmental stages and the ratio of ChAT+ cells to TUBB3+ cells at the late mature stages. The yield and the surviving neurons were counted on the basis of TUBB3 signals and normalized to the number of starting materials.

Neuronal survival assay. Induced neurons were replated onto astrocyte-coated 96-well plates at a cell density of 1×10^3 cells/cm² and cultured in neuronal maturation medium. The medium was changed twice a week until analysis with immunostaining at desired time points. The surviving neurons were counted on the basis of having typical neuronal morphology after immunostaining of TUBB3 and showing clear nuclei with HST staining. The number of neurons at the first week post-viral infection (WPI) was set as 100% and used for normalization of surviving neurons at 2 and 3 WPI. Three wells of neurons were examined under each condition at each time point.

Electrophysiology. Whole cell patch-clamp recordings were used to verify the neuronal electrophysiological maturation as in previous reports (13, 33) with minor modifications. Briefly, induced MNs were cultured on astrocyte-coated glass coverslips for 3 WPI and used for analysis. Cells were maintained at 30°C in a submersion chamber with Tyrode solution containing 150 mM NaCl, 4 mM KCl, 2 mM MgCl₂, 3 mM CaCl₂, 10 mM glucose, and 10 mM HEPES at pH 7.4 (adjusted with KOH) and 300 mOsm. The recording pipettes (~6–9 M Ω) were filled with an intracellular solution containing 0.2 mM EGTA, 130 mM K-gluconate, 6 mM KCl, 3 mM NaCl, 10 mM HEPES, 4 mM ATP-Mg, 0.4 mM GTP-Na, and 14 mM phosphocreatine-di (Tris) at pH 7.2 and 285 mOsm. Action potentials (APs) were recorded in current-clamp and elicited by a series of current injections ranging from –20 to 200 pA at 20-pA increments and 800 ms in duration. All current-clamp recordings were made at resting membrane potential or without any current injection. Data analysis was performed with Clampfit 10.3 software (Molecular Devices). The AP trace immediately above the threshold was used to determine the delay of the first spike as the length of time and the start of current steps to the peak of AP. The same AP trace was used to measure the AP threshold as the corresponding voltage when there was the sharpest change of the trace slope. The above-indicated AP trace was also measured to determine the maximum velocity of the rise and decay. AP frequency was obtained by dividing the maximum number of spikes during the current step protocol by the step time duration (800 ms).

Statistical analysis. Data are presented as means $\pm$ SE from at least three biological replicates. Statistical analysis was conducted by one-way analysis of variance and statistical significance was set at a *P* value of <0.05.

RESULTS

Human MNs cannot be obtained via spontaneous differentiation of iPSC-NPCs. To generate a large number of MNs with high purity, we tried the iPSC-based approach. As previously reported (13, 51), human iPSCs were first induced into NPCs by exposure to all-trans-RA and VPA in the culture medium (Fig. 1, A–C). NPCs were rapidly expanded with a population doubling time (PDT) of ~35 h. Human iPSC-derived NPCs could spontaneously differentiate into neurons if they are cultured in neuron maturation medium. We have systematically analyzed the neuronal identity with immunostaining of different markers in the third week, a time point at which most types of NPC-derived neurons reach the maturation stage (13, 51). Generic neuron markers including MAP2 and TUBB3 revealed that these spontaneously differentiated neurons are a mixture of different neuronal populations. They vary greatly in cellular morphology and soma size (Fig. 1D). MNs can be identified by robustly expressed nuclear HB9 at early stages and high levels of ChAT in the soma at late mature stages (1,

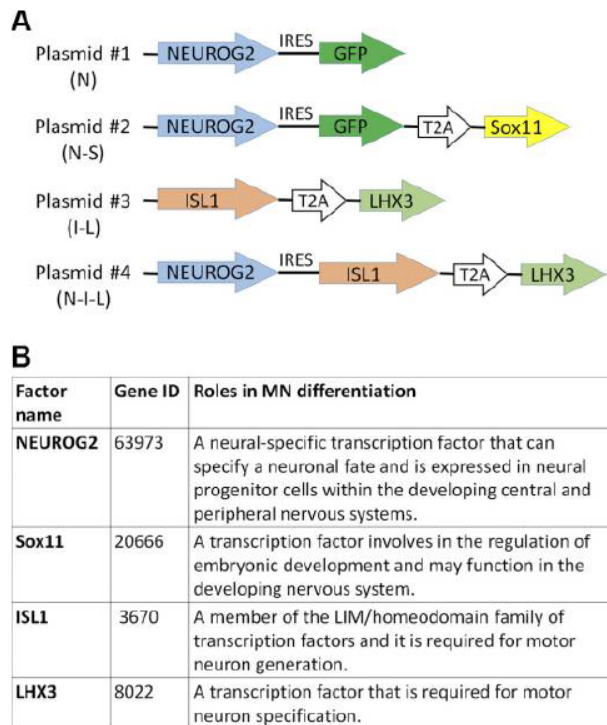


Fig. 2. Strategies to generate human induced pluripotent stem cell (iPSC)-derived motor neurons (MNs; iPSC-MNs) via lentiviral delivery of transcription factors. **A:** schematic diagrams show various transcription factors expressed in different lentiviral vectors (9, 33, 51). GFP, green fluorescent protein; IRES, internal ribosome entry site; ISL1 (I), insulin gene enhancer 1 or ISL LIM/homeobox 1; LHX3 (L), LIM/homeobox 3; NEUROG2 (N), neurogenin-2; Sox11 (S), mouse sex-determining region Y (SRY)-box transcription factor 11. **B:** roles of transcription factors involved in MN differentiation (28, 34, 37, 43).

41). However, most of these spontaneously differentiated neurons are glutamatergic neurons (~75%); ~15% are GABAergic, and ~5% are dopaminergic (TH+). There are very rare (<1%) MNs (HB9+) and cholinergic neurons (ChAT+; Fig. 1E). Therefore, it is not feasible to obtain human MNs via spontaneous differentiation of iPSC-NPCs.

Strategies to generate highly pure human MNs via lentiviral delivery of transcription factors. Lentiviral delivery of transcription factors is an efficient way to generate human MNs from iPSC-NPCs (13, 51). As NPCs are very sensitive to lentivirus dosage, to obtain high-quality MNs through this method, preparation of high-quality lentiviruses and addition of the proper amount at transduction are critical. Too high virus dosage will cause cell death and lead to low yield. On the other hand, if the dosage is too low, the purity of MN will be sacrificed because NPCs may not be efficiently transduced with lentiviruses and will generate more non-MNs via spontaneous differentiation (Fig. 1, D and E).

In our previous study, we successfully generated iPSC-MNs via lentiviral delivery of transcription factors (13, 51). In this

method, NPCs are infected with two lentiviruses expressing four factors (N-S-I-L, the cocktail of *lentiviruses* 2 and 3; Fig. 2A). These factors play critical roles in either neuron differentiation or MN generation and specification (Fig. 2B). However, we noted some variations in MN purity, survival, and yield among different sets of experiments. Besides the impact of lentivirus quality, another factor that affects the MN outcomes could be the cotransduction of two lentiviruses. Some NPCs may not be simultaneously infected with both of these viruses, and thus they cannot be successfully induced into the MN subtype, leading to variations in MN purity in different preparations. A higher dosage of viruses will increase the transduction efficiency, but it will also cause more cell death and result in a low yield of mature MNs. Therefore, to generate highly pure MNs with high yield, we need to minimize the virus dosage to infect NPCs and meanwhile need to ensure that most NPCs are infected with virus and transcription factors are sufficiently expressed.

To this end, we have optimized the induction strategies with different combinations of transcription factors. Among our four previously used factors (N-S-I-L; 13, 51), Sox11 plays a general role in the development of the nervous system (Fig. 2B), and it is critical for direct conversion of MNs from fibroblasts (9, 33, 51). Given that NPCs are already of neuronal lineage, Sox11 may not be required for the generation of MNs from NPCs. If this is the case, a single virus expressing three factors (N-I-L) could be sufficient to induce iPSC-MNs and may significantly improve the MN purity and yield. Thus, we constructed a lentiviral vector in which three factors were simultaneously expressed (N-I-L, *plasmid* 4; Fig. 2A). As controls, other combinations of lentiviral vectors were also included in this study (Fig. 2A). We systematically examined and compared the induction of iPSC-MNs using three different combinations of factors: 1) NEUROG2 alone (N, *lentivirus* 1);

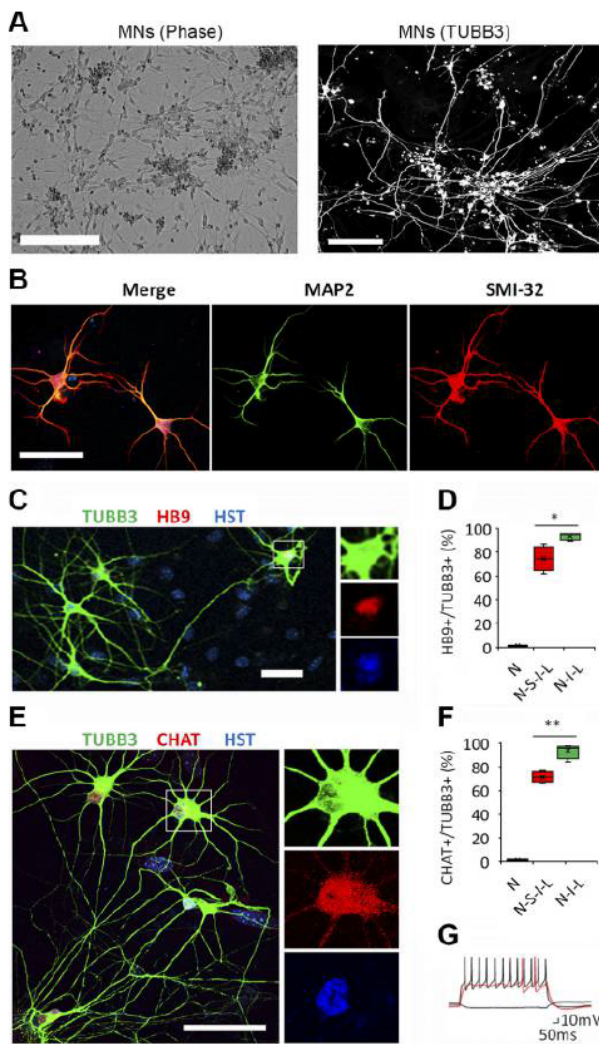


Fig. 3. Verification of motor neuron (MN) identity, purity, and maturation under different induction conditions. **A:** representative micrographs of induced pluripotent stem cell (iPSC)-derived MNs (iPSC-MNs) at 1 wk post-viral infection (WPI). *Left:* phase contrast. Scale bar, 200 μ m. *Right:* immunostaining of tubulin β -3 class III (TUBB3). Scale bar, 100 μ m. **B:** confocal micrographs of MNs at 1 WPI. Microtubule-associated protein 2 (MAP2) antibody recognizes dendrites, and neurofilament protein (SMI-32) antibody recognizes neurofilaments. Scale bar, 50 μ m. **C:** confocal micrograph of MNs at 1.5 WPI. Neuronal marker TUBB3 shows the soma and neuron processes, Hoechst 33342 (HST) stained nuclei, and nuclear HB9 was used as the early MN marker. The MN highlighted with a rectangle is also shown at a high magnification with separated channels. Scale bar, 50 μ m. **D:** quantification of MN fractions (HB9+/TUBB3+) under the indicated induction conditions. N, insulin gene enhancer 1 or ISL LIM/homeobox 1 (ISL1); L, LIM/homeobox 3 (LHX3); N, neurogenin-2 (NGN2); S, mouse sex-determining region Y (SRY)-box transcription factor 11 (Sox11). Number of neurons $n = 493$ for N, 407 for N-S-I-L, and 462 for N-I-L from 4 biological replicates. Student's t test was used for statistical analysis (N-S-I-L vs. N-I-L). $*P < 0.05$. **E:** confocal micrograph of MNs at 2 WPI. Neuronal marker TUBB3 shows the soma and neuron processes, HST stained nuclei, and choline acetyltransferase (CHAT) was used as a mature MN marker. The MN highlighted with a rectangle is also shown at a high magnification with separated channels. Scale bar, 50 μ m. **F:** quantification of MN fractions (CHAT+/TUBB3+) under the indicated induction conditions. Number of neurons $n = 465$ for N, 412 for N-S-I-L, and 436 for N-I-L from 4 biological replicates. Student's t test was used for statistical analysis (N-S-I-L vs. N-I-L). $**P < 0.01$. **G:** repetitive action potential (AP) waveforms recorded under current-clamp mode of iPSC-MNs at 3 WPI. The precondition sweep and the sweep immediately above threshold (in red) are also shown.

2) *NEUROG2*, *ISL1*, *LHX3*, and *Sox11* (N-S-I-L, the cocktail of *lentiviruses* 2 and 3); and 3) *NEUROG2*, *ISL1*, and *LHX3* (N-I-L, *lentivirus* 4; Fig. 2A).

Verification of MN identity and maturation. NPCs were cultured on Matrigel-coated six-well plates. When the cellular confluence was ~50% (Fig. 1C), proper amounts of lentiviruses were added at MOI of ~2 to infect NPCs. Within 1 WPI, neurites rapidly grew out from the soma, and cells became MN-like with very long axons (Fig. 3A). Immunostaining indicated that these induced neurons highly expressed generic neuron markers including *TUBB3* (Fig. 3A), *MAP2*, and neurofilament protein (SMI-32; Fig. 3B). The highly expressed *HB9* in nuclei (Fig. 3C) and the *ChAT* in the soma (Fig. 3E) verified that these induced neurons were of MN identity. We quantified the MN numbers among neuronal populations under different induction conditions. As expected, very few *HB9*+ neurons and *ChAT*+ neurons can be identified in the group of *NEUROG2* alone (N). Excitingly, induction with a single virus expressing three factors (N-I-L) showed significantly higher MN fractions than the combination of four factors (N-S-I-L; Fig. 3, D and F). These results indicate that *Sox11* is dispensable in the generation of MNs from NPCs. A single virus expressing three factors is sufficient to induce MNs and even gives rise to a higher purity.

To verify MN electrophysiological maturation, action potentials (APs) were recorded using a whole cell patch-clamp. Repetitive AP waveforms can be easily recorded under current-clamp of iPSC-MNs at 3 WPI (Fig. 3G). These results strongly support the idea that mature and functional human MNs can be obtained from iPSC-derived NPCs via lentiviral delivery of three transcription factors (N-I-L).

Optimized condition significantly improved the survival and yield of iPSC-MNs. Next, we examined and compared the survival and yield of iPSC-MNs that were induced under two different conditions. A surviving neuron was defined by two

criteria: 1) *TUBB3* immunostaining shows typical neuronal morphology without obvious degeneration, and 2) the soma contains a clear nucleus with HST staining (Fig. 3, C and E). Excitingly, the ratios of surviving neurons under the optimized condition (N-I-L) were higher than with the previous method (N-S-I-L), increased about 2-fold and 2.3-fold at 2 and 3 WPI, respectively (Fig. 4, A and B). Similarly, we quantified surviving neurons at 3 WPI and calculated the final yield with normalization to the starting materials. Strikingly, the yield of iPSC-MNs under the optimized condition was ~3 times that of the previous method (Fig. 4, C and D). These results demonstrate that the optimized condition using a single lentivirus expressing three factors dramatically improves the survival and the yield of iPSC-MNs.

DISCUSSION

Generation of neurons from hiPSCs greatly facilitates studies in understanding the regulatory mechanisms of neuronal development, determining the pathogenesis of neurological diseases, identifying molecular targets for therapeutic interventions, and screening drugs in a more human neuron-specific manner (15, 24, 29, 35, 36, 54). To achieve more reliable and disease-relevant findings, at least the following criteria should be considered to evaluate the cellular system in modeling human neurological diseases. First, the specific neuronal subtype that is particularly affected in the disease should be used in the research. For example, patient-derived MNs are the best neuronal subtype in modeling neurological diseases that target MNs. Second, neurons are able to achieve functional maturation. Some disease-related phenotypes may not be noticed until the diseased neurons reach the late mature stages, especially for modeling aging-dependent neurodegenerative diseases. Synaptic proteins, neurotransmitter-related enzymes, and electrophysiological activities are good measures to examine the

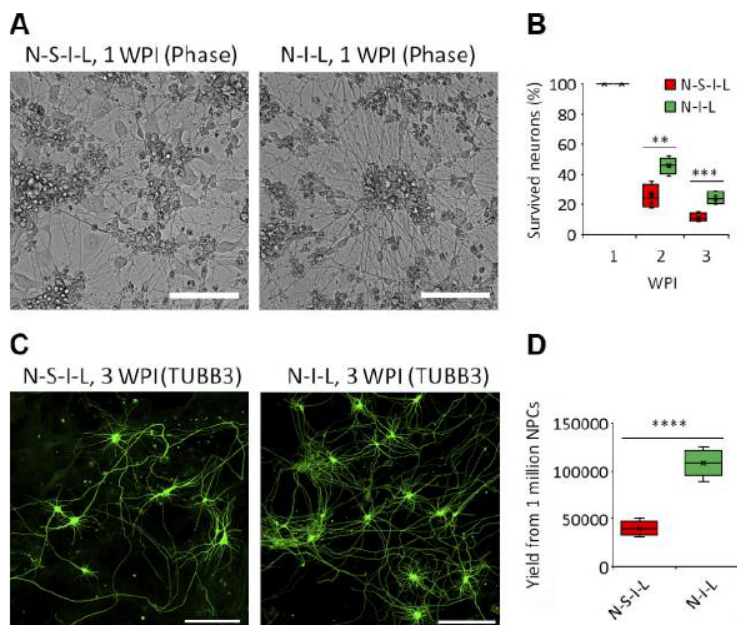


Fig. 4. Optimized induction condition dramatically improved induced pluripotent stem cell (iPSC)-derived motor neuron (MN; iPSC-MN) survival and yield. **A**: phase contrast micrographs of iPSC-MNs induced via the indicated combinations of transcription factors at 1 wk post-viral infection (WPI). Scale bars, 100 μ m. **B**: surviving neurons under the indicated conditions. The number of neurons at 1 WPI was set as 100%. I, insulin gene enhancer 1 or ISL LIM/homeobox 1 (*ISL1*); L, LIM/homeobox 3 (*LHX3*); N, neurogenin-2 (*NGN2*); S, mouse sex-determining region Y (*SRY*)-box transcription factor 11 (*Sox11*). Number of neurons $n > 3,000$ from 4 biological replicates. Student's *t* test was used for statistical analysis (N-S-I-L vs. N-I-L). ** $P < 0.01$; *** $P < 0.001$. **C**: representative micrographs of iPSC-MNs induced via the indicated transcription factors at 3 WPI with immunostaining of tubulin β -3 class III (*TUBB3*). Scale bars, 100 μ m. **D**: yield of iPSC-MNs at 3 WPI from 1 million neuronal progenitor cells (NPCs) at transduction. The results represent 4 biological replicates. Student's *t* test was used for statistical analysis. **** $P < 0.0001$.

maturation and functions. Third, the purity and the yield should be good enough for analysis. Generally, immunostaining of specific markers could be able to distinguish the target neurons from other types of cocultured cells. However, to biochemically identify dysregulated factors in diseased neurons, large amounts of specific neurons with very high purity are required. Finally, culture the neurons more physiologically and maximize the relevance to in vivo conditions. Using extracellular matrix (ECM) protein coated culture vessels and culture medium supplemented with neurotrophic factors, and coculture of neurons and glial cells could be necessary to make the neuronal culture more physiological and improve the survival.

In this study, using the lentiviral delivery of transcription factors, we have successfully generated functional human MNs from iPSCs and further optimized the induction conditions. This optimized approach minimized the virus dosage by switching two viruses expressing four factors to a single virus expressing three factors and thus significantly improved the neuronal survival and gave rise to high yield and purity. This approach makes it feasible to obtain abundant materials for biochemical studies in determining the pathogenesis of MN-related diseases.

As noninfected cells will spontaneously differentiate into non-MNs, generation of higher-purity MNs requires higher transduction efficiency. Thus, the preparation of high-quality lentivirus is very important to achieve good infection efficiency (>95%). On the other hand, a higher dosage of virus will cause more cell death and lead to low yield. In our practice, we routinely test the titer of active virus in each preparation (12) and use a proper virus dosage to infect NPCs. Thereby the transduction efficiency and neuron survival could be well balanced based on active virus titer. In this study, a single virus was used to coexpress essential and sufficient factors to generate MNs, and thus we can maximize the transduction efficiency and minimize cell death, obtaining highly pure iPSC-MNs with high yield.

Compared with other approaches, iPSC-MNs induced by lentiviral delivery of transcription factors possess unique features, such as high purity (>90%) and fully functional maturity. These neurons robustly express MN marker HB9, ISL1, and ChAT and mature neuron markers MAP2 and presynaptic proteins and show electrical maturation and firing of action potentials within 3 wk (13, 51). These unique features make such iPSC-MNs an excellent research system in modeling movement disorders in vitro. Most importantly, in this study we further optimized the induction conditions and significantly improved survival and yield, making it feasible to obtain a large amount of functional mature iPSC-MNs.

Generation of abundant and highly pure MNs from patient-specific iPSCs is the prerequisite for biochemically determining the pathogenesis of movement disorders and identifying molecular targets for therapeutic interventions. For example, genome-wide analysis of gene expression using RNA-sequencing (RNAseq) techniques and determining the transcriptional and epigenetic regulations of gene expression using chromatin immunoprecipitation (ChIP; 10, 11, 14, 39, 44). Additionally, using patient-derived neurons, studies in RNA-protein interactions could reveal the regulation and dysregulation of RNA metabolism, and Co-IP-MS analysis could identify dysregulated factors in diseased neurons (32, 45). All these biochemical approaches require large amounts of pure materials.

Another big challenge in modeling neurological diseases using iPSC-derived neurons is how to culture the neurons more physiologically, especially for long-term culture. Chemical or physical modifications of the cell culture plates, such as coating with ECM proteins, have been demonstrated to be an efficient method to better mimic in vivo cell behavior (26). In some studies, neuroglial coculture is required for modeling certain diseases, for exploring neuroglial interactions, and for a long-term survival (27, 38, 50). In this study, we cultured iPSCs, NPCs, and MNs on Matrigel-coated plates. For long-term cultures (>2 wk), iPSC-MNs were seeded onto culture plates coated with a monolayer of astrocytes. These measures make the culture more physiological and maximize the relevance to in vivo conditions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

B.D. conceived and designed research; M.S. and B.D. performed experiments; M.S. and B.D. analyzed data; M.S. and B.D. interpreted results of experiments; B.D. prepared figures; M.S. and B.D. drafted manuscript; M.S. and B.D. edited and revised manuscript; M.S. and B.D. approved final version of manuscript.

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Perspective

Generation of patient-specific motor neurons in modeling movement diseases

Baojin Ding*

Generating neurons from human induced pluripotent stem cells (hiPSCs) overcomes the limited access to human brain tissues and greatly facilitates the research in neuroscience (Karagiannis et al., 2019). However, it is still a big challenge to obtain a particular neuronal subtype with high purity and yield to conduct certain studies, such as determining the pathogenesis of diseased neurons using biochemical approaches. Motor neurons (MNs) are a specialized neuronal subtype responsible for innervating musculature in the periphery and governing both autonomic and volitional movements. Dysfunctions in MNs are implicated in a variety of movement diseases, such as amyotrophic lateral sclerosis (ALS), progressive muscular atrophy, and dystonia (Sances et al., 2016; Ding et al., 2020b). ALS belongs to MN diseases, which are caused by gradual degeneration and death of MNs in the brain (upper MNs) and/or in the spinal cord (lower MNs). Several pathogenic mechanisms are involved in ALS, including glutamate excitotoxicity, dysregulated interactions between neurons and glial cells, intracytoplasmic and intranuclear aggregation of certain proteins and RNAs, impaired nucleocytoplasmic transport, and changes in the axon terminals and neuromuscular junctions (NMJs) (Sances et al., 2016). So far, there are no specific treatments available to cure these diseases due to unclear pathophysiological mechanisms. Generation of patient-specific MNs will provide valuable *in vitro* model systems in deciphering the pathogenesis of these diseases. Recently, we have developed an approach by which functional MNs could be generated from hiPSCs via lentiviral delivery of three transcription factors (Figure 1). These MNs robustly expressed generic neuronal markers, MN-specific markers, and showed electrical maturation and firing of action potentials within 3 weeks (Sepehrmanesh and Ding, 2020). Compared to previous methods (Tang et al., 2017), this approach significantly improved the neuronal survival, purity, and yield, making it feasible to obtain abundant patient-specific MNs for biochemical studies in modeling movement diseases.

Patient-derived MNs could be generated by two kinds of approaches. One is the direct conversion from adult fibroblasts (Ding et al., 2020a). The other approach is hiPSC-based induction and differentiation. However, the relatively low purity and poor yield of directly converted MNs severely limit the studies that require highly pure and large amount of cells, such as gene expression analysis by next-generation sequencing, and protein co-immunoprecipitation and mass spectrometry analysis. An iPSC-based approach will overcome these limitations. iPSCs could be prepared from a variety of readily available tissues such as skin fibroblasts with Yamanaka factors (Takahashi and Yamanaka, 2006) or obtained from international stem cell banks (Kim et al., 2017). These induced neurons retain

the specific mutations that are derived from patient donors or engineered by CRISPR makes it possible to directly evaluate the impact of disease-relevant mutations. Most importantly, iPSCs possess almost unlimited expansion capability and are suitable for preparing large amount of materials. These unique features make iPSC-based method a very powerful tool in neuroscience research. iPSC-derived human neurons have been extensively used in understanding of regulatory mechanisms in neuronal development, determining the pathogenesis of neurological diseases, identifying molecular targets for therapeutic interventions, and screening drugs in a more human neuron-specific manner (Karagiannis et al., 2019).

In order to acquire more reproducible and disease-relevant findings in modeling diseases, at least the following four criteria should be considered in evaluation of the cellular system (Sepehrmanesh and Ding, 2020). First, generation of specific neuronal subtype that is particularly affected in the disease. For example, patient-derived MNs are the best neuronal subtype in modeling neurological diseases that target MNs, such as ALS. Second, neurons are able to achieve functional maturation. Some disease-related phenotypes may not be noticed until the diseased neurons reach the late mature stages, especially for modeling aging-dependent neurodegenerative diseases. Examinations of synaptic proteins, neurotransmitter-related enzymes, neurotransmitter release, and electrophysiological activities are good measures to evaluate the maturation and functions. Third, the purity and the yield should be good enough for performing the analysis. Generally, immunostaining of specific markers could be able to distinguish the target neurons from other types of co-cultured cells. However, to biochemically identify dysregulated factors in diseased neurons, large amounts of specific neurons with high purity and yield are required. Lastly, we need to culture the neurons more physiologically and maximize the relevance to *in vivo* conditions. Using extracellular matrix proteins coated culture vessels, supplemented with neurotrophic factors in culture medium, and co-culture with glia cells could be necessary to make the culture more physiological and improve the neuronal survival. Even though these criteria will be strictly followed, researchers still need to be cautious to interpret the significance of the results in understanding the pathogenesis of diseases.

Currently, several approaches are being used to generate MNs from hiPSCs. Small molecules such as growth factors and chemicals together with transcription factors are widely used for neuronal induction. These factors either directly regulate genes or target signaling pathways that play critical roles in neurogenesis and differentiation. For example, GSK3 β inhibitors (such as CHIR99021) combined with

the dual Sma and Mad proteins inhibitors (such as SB 431542, and LDN) induce iPSCs into neural progenitor cells (NPCs) (Fujimori et al., 2018). Additionally, other small molecules such as bone morphogenetic protein inhibitors, γ -secretase inhibitors, all-trans-retinoic acid (RA), antioxidants and insulin-like growth factor 1 or insulin have important effects on NPC proliferation and changing into choline acetyltransferase-positive MNs. The presence of brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor, or neurotrophin-3 (NT-3) in the culture medium is necessary to maintain the neuronal survival and promote neuronal maturation (Ding et al., 2018; Karagiannis et al., 2019). Regardless of the extensive use of these small molecules, these methods have poor efficiency, and produce clear heterogeneity in neuronal subtypes. Therefore, new methods of creating iPSC-MNs are being developed.

In our protocol (Sepehrmanesh and Ding, 2020), hiMNs were first induced into NPCs by addition of RA and valproic acid in mTeSR1 culture medium (Figure 1A–D). Large amount of NPCs could be prepared and frozen for a long-term storage. To efficiently induce NPCs into MNs, transduction of proper amount of lentivirus expressing transcription factors is critical. Because non-infected NPCs could spontaneously differentiate into non-MNs in neuronal maturation medium, generation of higher purity of MNs requires higher transduction efficiency (Sepehrmanesh and Ding, 2020). This could be achieved by preparation of high quality lentivirus. On the other hand, a higher dosage of lentivirus would cause more cell death and lead to low yield. In our practice, we routinely test the titer of active virus in each preparation and use a proper virus dosage to infect NPCs (Ding and Kilpatrick, 2013). Thereby the transduction efficiency and neuronal survival could be balanced well based on titers of active virus. Compare to the previous approach in which NPCs were infected by two lentiviruses expressing four transcription factors, neurogenin-2 (NEUROG2), insulin gene enhancer 1 (ISL1), LIM/homeobox 3 (LHX3), and SRY-Box Transcription Factor 11 (Sox11; Tang et al., 2017), and the optimized protocol used a single virus co-expressing three essential and sufficient factors (NEUROG2, ISL1 and LHX3). This improvement further maximizes the transduction efficiency and simultaneously minimizes the virus dosage at transduction, resulting in less cell death and a higher yield. These iPSC-MNs robustly expressed generic neuronal markers such as microtubule-associated protein 2 and tubulin beta 3 class III, and early MN marker of nuclear HB9 (also known as MNX1 for motor neuron and pancreas homeobox 1) and the late marker of choline acetyltransferase (Figure 1E–G). Within 3 weeks post-viral infection, action potentials could be detected by whole-cell patch-clamp recordings (Figure 1H), suggesting that these iPSC-MNs achieved electrical maturation and functions (Sepehrmanesh and Ding, 2020).

Besides the purity and yield of iPSC-derived neurons, another big challenge in modeling neurological diseases is how to culture neurons more physiologically, especially for long-term cultures. Chemical or physical modifications of the cell culture plates, such as coating with extracellular matrix proteins, have demonstrated to be an efficient method to better mimic *in vivo* cell behavior. Neuroglia co-culture is required for modeling certain

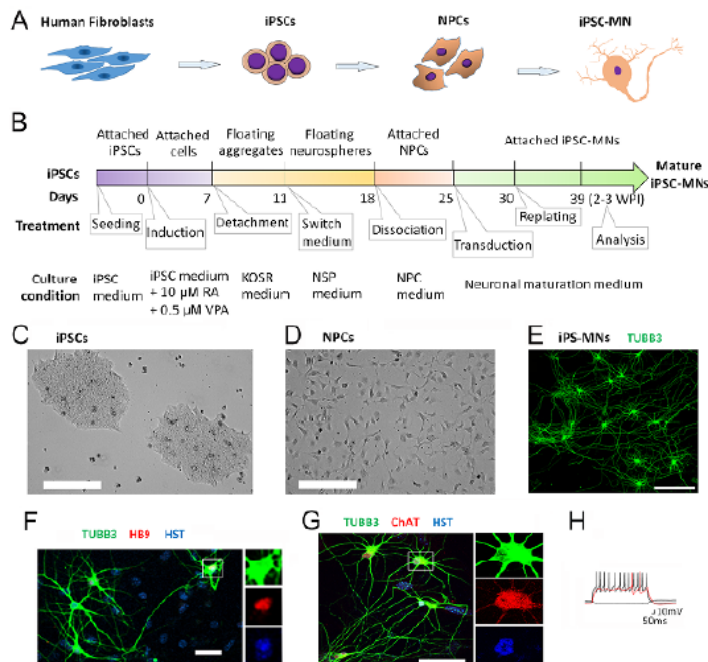


Figure 1 | Generation of motor neurons (MNs) from human induced pluripotent stem cells (hiPSCs). (A) A schematic shows the process of the generation of human MNs from iPSCs. (B) The detailed process of generating iPSC-MNs. The starting induction time was set as day 0. The age of iPSC-MNs was counted from lentiviral transduction. (C) A representative phase contrast micrograph of human iPSCs. Scale bar: 200 μ m. (D) A representative phase contrast micrograph of NPCs at lentiviral transduction. Scale bar: 200 μ m. (E) A representative fluorescence micrograph of iPSC-MNs induced by three transcription factors (NEUROG2, ISL1 and LHX3) at 3 WPI with immunostaining of TUBB3. Scale bar: 100 μ m. (F) A confocal micrograph of iPSC-MNs at 1.5 WPI. Neuronal marker TUBB3 shows the soma and neuron processes, Hoechst 33342 (HST) stained nuclei, and nuclear HB9 was used as the early MN marker. The rectangle highlighted MN was also shown at a large magnification with separated channels. Scale bar: 50 μ m. (G) A confocal micrograph of MNs at 2 WPI. Choline acetyltransferase (ChAT) was used as a mature MN marker. The rectangle highlighted MN was also shown at a large magnification with separated channels. Scale bar: 50 μ m. (H) Repetitive action potential (AP) waveforms recorded under current-clamp mode of iPSC-MNs at 3 WPI. HB9: MNX1 for motor neuron and pancreas homeobox 1; iPSC-MN: iPSC-derived MN; KOSR: knockout serum replacement; NPCs: neuronal progenitor cells; NSP: neurosphere; RA: retinoic acid; TUBB3: tubulin beta 3 class III; VPA: valproic acid; WPI: weeks post-viral infection. Adapted from Sepehrmanesh and Ding (2020).

diseases, exploring neuroglia interactions, and for a long-term survival (Nadadthur et al., 2019). In our studies, we cultured iPSCs, NPCs, and MNs on Matrigel-coated plates. For long-term cultures (more than 2 weeks), iPSC-MNs were seeded onto culture plates coated with a monolayer of astrocytes. Meanwhile, neurotrophic factors (BDNF, GDNF and NT-3) were routinely added in neuronal maturation medium. These measures make the culture more physiological, promote neuronal maturation and survival, and maximize the relevance to *in vivo* conditions.

Compare to other chemical-based approaches, iPSC-MNs induced by lentiviral delivery of transcription factors possess unique features, such as high purity (> 90%) and fully functional maturity (Ding et al., 2020b; Sepehrmanesh and Ding, 2020). These unique features make iPSC-derived MNs become excellent research materials in determining the pathogenesis of MN-related diseases. However, lentiviral vectors are derived from the human immunodeficiency virus. They have the potential risks for generation of replication-competent lentivirus and could randomly integrate viral DNA into host genome. Thus, the lentiviral vector-based gene delivery limits the clinical applications for intervention therapy. Because of the superior

biosafety and the mild immune response in humans, Adeno-associated virus (AAV) is widely used as a vector for human gene therapy. Therefore, another research emphasis in neural regeneration field would be generating highly pure MNs for clinical applications using recombinant AAV-based approaches. The major drawback of AAV vector is its limited cloning capacity. In the future, using CRISPR engineering to generate paired patient-specific iPSC lines and isogenic controls, or tagged with fluorescent or reporters will enable researchers to examine the specific subcellular compartments and the activation of specific genes or pathways under physiological and pathological conditions. The growing of "mini brains" (organoids) from hiPSCs will provide a novel research system to model neurological diseases and test therapies.

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Disease Modeling with Human Neurons Reveals LMNB1 Dysregulation Underlying DYT1 Dystonia

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DYT1 dystonia is a hereditary neurologic movement disorder characterized by uncontrollable muscle contractions. It is caused by a heterozygous mutation in *Torsin A* (*TOR1A*), a gene encoding a membrane-embedded ATPase. While animal models provide insights into disease mechanisms, significant species-dependent differences exist since animals with the identical heterozygous mutation fail to show pathology. Here, we model DYT1 by using human patient-specific cholinergic motor neurons (MNs) that are generated through either direct conversion of patients' skin fibroblasts or differentiation of induced pluripotent stem cells (iPSCs). These human MNs with the heterozygous *TOR1A* mutation show reduced neurite length and branches, markedly thickened nuclear lamina, disrupted nuclear morphology, and impaired nucleocytoplasmic transport (NCT) of mRNAs and proteins, whereas they lack the perinuclear "blebs" that are often observed in animal models. Furthermore, we uncover that the nuclear lamina protein LMNB1 is upregulated in DYT1 cells and exhibits abnormal subcellular distribution in a cholinergic MNs-specific manner. Such dysregulation of LMNB1 can be recapitulated by either ectopic expression of the mutant *TOR1A* gene or shRNA-mediated downregulation of endogenous *TOR1A* in healthy control MNs. Interestingly, downregulation of LMNB1 can largely ameliorate all the cellular defects in DYT1 MNs. These results reveal the value of disease modeling with human patient-specific neurons and indicate that dysregulation of LMNB1, a crucial component of the nuclear lamina, may constitute a major molecular mechanism underlying DYT1 pathology.

Key words: cholinergic motor neurons; dystonia; human neurons; nuclear LMNB1; nucleocytoplasmic transport; *TOR1A*

Significance Statement

Inaccessibility to patient neurons greatly impedes our understanding of the pathologic mechanisms for dystonia. In this study, we employ reprogrammed human patient-specific motor neurons (MNs) to model DYT1, the most severe hereditary form of dystonia. Our results reveal disease-dependent deficits in nuclear morphology and nucleocytoplasmic transport (NCT). Most importantly, we further identify LMNB1 dysregulation as a major contributor to these deficits, uncovering a new pathologic mechanism for DYT1 dystonia.

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*B.D. and Y.T. contributed equally to this work. The author order was determined by workload and leadership in the project.

The authors declare no competing financial interests.

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Introduction

Dystonia is a common movement disorder characterized by sustained or intermittent muscle contractions causing abnormal movements and/or postures (Albanese et al., 2013). The clinical characteristics and underlying causes of dystonia are very heterogeneous, so the cellular mechanism for dystonia remains elusive (Lohmann and Klein, 2013). Childhood-onset Torsin dystonia, also called DYT1 dystonia, represents the most frequent and severe form of hereditary isolated dystonia (Albanese et al., 2013; Dauer, 2014). Most cases of typical DYT1 dystonia are caused by a heterozygous 3-bp deletion (Δ GAG) in exon 5 of the *TOR1A* gene, resulting in the loss of one of the two adjacent glutamate residues (E302 or E303) in the carboxy-terminal region of Torsin A protein (TOR1A Δ E; Ozeliuss et al., 1997; Charlesworth et al., 2013).

Protein TOR1A belongs to the superfamily of AAA+ (ATPases associated with diverse cellular activities) proteins (Ozeliuss et al., 1997). It is widely distributed throughout the

human CNS (Rostasy et al., 2003). Most TOR1A proteins are embedded in the lumen of the endoplasmic reticulum and the endomembrane space of the nuclear envelope (NE; Vander Heyden et al., 2011). Accumulating evidence indicates that TOR1A plays an important role in membrane-cytoskeleton interactions (Laudermilch and Schlieker, 2016). At the NE, TOR1A is associated with the linkage between NE and cytoskeleton via binding the KASH domain of nesprins (Nery et al., 2008). It is also involved in the regulation of NE membrane morphology (Naismith et al., 2004; Goodchild et al., 2005; Laudermilch et al., 2016), the localization and function of nuclear pore subunits (VanGompel et al., 2015), and the nuclear egress of large ribonucleoprotein granules (Jokhi et al., 2013). Furthermore, TOR1A regulates neurite extension (Hewett et al., 2006) and synaptic vesicle recycling (Granata et al., 2008), underscoring its critical functions in neuronal development and function.

Although TOR1AΔE has been shown to be a loss-of-function mutation (Torres et al., 2004; Goodchild et al., 2005; Zhao et al., 2013), the cellular mechanisms underlying DYT1 dystonia remain largely unknown. Traditionally, dystonia is considered as a disorder of the basal ganglia (Tewari et al., 2017), supported by the findings in *Tor1a*-mutant mice including the early structural and functional plasticity alterations in the corticostriatal synapses (Maltese et al., 2018) and the dysregulation of the striatal dopaminergic neurons (Ip et al., 2016). The cerebellum via direct modulation of the basal ganglia is also shown as a major site of dysfunction in *Tor1a* knock-out mice (Fremont et al., 2017; Tewari et al., 2017). One cellular hallmark in *Tor1a* knock-out mice is the abnormal NE morphology in multiple areas of the CNS including the large ventral horn of the spinal cord (Goodchild et al., 2005), indicating that the lower motor neurons (MNs) might be particularly affected. Unlike the majority of human DYT1 patients who are affected by the heterozygous TOR1A mutation, mice with the identical mutation as a heterozygote nonetheless failed to exhibit any pathologic phenotypes (Goodchild et al., 2005), clearly indicating there might be species-dependent differences on disease mechanisms. However, DYT1 has never been previously modeled in human neurons.

Through cell fate reprogramming, we successfully generated human cholinergic MNs from fibroblasts of DYT1 patients and healthy controls via two strategies: direct conversion (diMNs) and differentiation through induced pluripotent stem cells (iPSCs; iMNs). We found that DYT1 neurons generated by both methods show impairments in neuron development, NE morphology, mRNA nuclear export, and protein transport. We further discovered that nuclear lamin B1 (LMNB1) is dysregulated in both expression and subcellular localization in patient MNs. Importantly, LMNB1 downregulation can largely ameliorate defects of NE morphology and nuclear transport in patient neurons. These findings indicate that LMNB1 dysregulation may contribute to DYT1 dystonia and provide a novel molecular target for intervention.

Materials and Methods

Cell lines and culture condition

HEK 293T cells (CRL-11268) and SH-SY5Y (CRL-11266) cells were purchased from ATCC. DYT1 fibroblast and healthy controls, GM03211 (DYT1-1, male, 30 years old), NDS00305 (DYT1-2, male, 22 years old), GM02304 (DYT1-3, female, 17 years old), NDS00301 (DYT1-4, female, 53 years old), GM00024 (Control-1, male, 31 years old), GM03652 (Control-2, male, 24 years old), GM04506 (Control-3, female, 20 years old), and AG07473 (Control-4, female, 50 years old) were obtained from

the Coriell Institute for Medical Research or NINDS Human Cell and Data Repository (NHCDR). Human age-matched wild-type (WT) iPSC (GM23476) was obtained from Coriell and H9 embryonic stem cell (ESC) line (WA09) was purchased from WiCell Research Institute. Human iPSCs were maintained in complete mTeSR1 medium (STEMCELL Technologies) on Matrigel (Corning) coated dishes at 37°C and 5% CO₂, and the medium was daily replaced.

The medium recipes were as following:

- (1) HEK medium: DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S).
- (2) Fibroblast medium: DMEM supplemented with 15% FBS and 1% P/S.
- (3) Neuronal induction medium: DMEM:F12:neurobasal (2:2:1), 0.8% N2 (Invitrogen), 0.8% B27 (Invitrogen), 1% P/S, and supplemented with 10 mM Forskolin (FSK, Sigma-Aldrich), 1 mM dorsomorphin (DM; Millipore), and 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech).
- (4) Neuronal maturation medium: DMEM:F12:neurobasal (2:2:1), 0.8% N2 (Invitrogen), 0.8% B27 (Invitrogen), 1% P/S, and supplemented with 5 mM FSK and 10 ng/ml each of BDNF, GDNF, and NT3 (PeproTech).
- (5) ESC medium: DMEM/F12 medium with 20% KnockOut Serum Replacement (KOSR; Thermo Fisher Scientific), 1% GlutaMax, 1% non-essential amino acid (NEAA), 50 μM β-mercaptoethanol (β-ME), 1% P/S, and 10 ng/ml bFGF (PeproTech).
- (6) Neurosphere medium (NSP medium): DMEM/F12 medium containing 1% N2, 1% GlutaMax, 1% NEAA, 50 μM β-ME, 1% P/S, 8 μg/ml Heparin, 20 ng/ml bFGF, and 20 ng/ml epidermal growth factor (EGF; PeproTech).
- (7) Neural progenitor cell medium: DMEM/F12 and neurobasal medium (1:1) containing 0.5% N2 (Invitrogen), 1% B27 (Invitrogen), 1% GlutaMax, 1% NEAA, 50 μM β-ME, 1% P/S, 10 ng/ml EGF and 10 ng/ml bFGF.
- (8) SH-SY5Y medium: DMEM supplemented with 15% FBS and 1% P/S.
- (9) SH-SY5Y differentiation medium: DMEM supplemented with 3% FBS, 10 μM all-trans-retinoic acid (RA; Sigma) and 1% P/S.

Plasmid construction and virus production

A third-generation lentiviral vector (*pCSC-SP-PW-IRES-GFP*) was used to express *NEUROG2-IRES-GFP-T2A-Sox11*, *NEUROG2-IRES-Sox11*, or *ISL1-T2A-LHX3*. cDNAs for TOR1A and TOR1AΔE were provided by Gonzalo E. Torres (Torres et al., 2004) and were individually subcloned into *pCSC-SP-PW-IRES-GFP* vector at the BamHI and XhoI restriction sites. Another third-generation lentiviral vector (*LV-CAG-mCherry-miRE-Luc*) was used to express TOR1A-shRNAs or LMNB1-shRNAs as described in previously report (Pelossof et al., 2017). Briefly, shRNA oligos targeting the gene coding regions were synthesized, PCR amplified with flanking miR-30 sequences, and ligated into the above lentiviral vector at the XhoI and EcoRI restriction sites. The targeting sequences are AACGGTGTTCACCAAGTTAGAT (TOR1A-shRNA-1), CAGCAAGATCATCGCAGAGAATA (TOR1A-shRNA-2), AGCTTCTGTGTAAGTTA (LMNB1-shRNA-1), or CAGACTGT CATCAGAGATGAA (LMNB1-shRNA-2). All plasmids were verified by restriction enzyme digestions and DNA sequencing. The dual reporter 2Gi2R was generously provided by Fred H. Gage (Mertens et al., 2015). The Moloney murine leukemia virus-based retroviral vectors (pMXs) carrying OCT4, SOX2, KLF4, or c-MYC were obtained from Addgene (Takahashi et al., 2007). Each of these vectors was co-transfected with packaging plasmids (*pCMV-Gag-Pol* and *pCMV-VSVG*) into HEK293T cells for retrovirus production. Replication-incompetent lentiviruses were produced and viral supernatants were collected at 48 and 72 h posttransfection (Ding and Kilpatrick, 2013). The viral supernatants were filtered through 0.45-μm syringe filters and stored at 4°C before cell transduction.

Direct conversion of adult fibroblasts into MNs

Lentiviral delivery of four factors (*NEUROG2*, *Sox11*, *ISL1*, and *LHX3*) was used as previously described (Liu et al., 2016; Tang et al., 2017; Ding et al., 2020). In brief, fibroblasts were plated at a density of 1×10^4 cells/

cm² onto Matrigel-coated dishes. Cells were transduced the next day with lentiviral supernatants supplemented with 6 mg/ml polybrene. Fibroblast medium was refreshed after overnight incubation. One day later, the culture was replaced with neuronal induction medium and half-changed every other day until 14 d postinfection (dpi). A replating procedure (Liu et al., 2013) was used to purify induced neurons. The purified cells were cultured in neuronal maturation medium onto Matrigel-coated coverslips with or without the presence of astrocytes depending on desired experiments. The medium was half changed twice a week until analysis.

DYT1 iPSC generation

DYT1 fibroblasts were plated into gelatin-coated 10 cm dishes (8×10^5 cells/dish) and transduced with a cocktail of four retroviruses (1:1:1:1) expressing *OCT4*, *SOX2*, *KLF4*, and *c-MYC*. Two days later, fibroblasts received a second round of virus infection and was marked as day 0. On day 5, cells were replated with fibroblast medium at 2×10^5 cells per well in Matrigel-coated six-well plates. Culture medium was replaced with human ESC medium on day 6, and then changed to mTeSR1 medium in the following days. Cells were treated with 0.5 mM VPA (Sigma) for 10 d starting on day 5. Based on ESC-like colony morphology, iPSC colonies were picked around three to four weeks. The picked colonies were then expanded and maintained in Matrigel-coated plates with mTeSR1 medium.

Neural progenitor cells and neuronal differentiation

Neural progenitor cells were generated as previously reported with minor modifications (Yang et al., 2014). Briefly, iPSCs or ESCs were cultured in mTeSR1 medium with 10 μ M RA and 0.5 mM VPA in Matrigel-coated 6-cm plates for 7 d. Cells were then digested with Versene and gently pipetted into small clumps supplemented with 10 μ M Y-27632. Cell clumps were aggregated in KOSR medium for 4 d, followed by culturing in NSP medium for another week. The resulting neurospheres were then dissociated into single cells with Accutase (Innovative Cell Technologies), and maintained in neural progenitor cell medium. For MN differentiation (Tang et al., 2017; Sepehrmanesh and Ding, 2020), neural progenitor cells were plated into Matrigel-coated plates at a density of 3×10^5 cells/cm² and transduced with a cocktail of two lentiviruses (1:1) expressing *NEUROG2-IRES-GFP-T2A-Sox11* and *ISL1-T2A-LHX3*. Culture medium was replaced the next day with neuronal maturation medium. Neurons were dissociated with Accutase on day 6 and replated onto astrocyte-coated coverslips for long-term culture.

Immunostaining, confocal microscopy, and image analysis

Cultured cells at the indicated time points were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature (RT) and then permeabilized and blocked for 1 h in blocking buffer (PBS containing 0.2% Triton X-100 and 3% BSA). They were subsequently incubated overnight with primary antibodies in blocking buffer at 4°C, followed by washing and incubation with corresponding fluorophore-conjugated secondary antibodies. Cell nuclei were counterstained with Hoechst 33342 (HST; Thermo Fisher Scientific). Primary antibodies used in this study were choline acetyltransferase (CHAT; Millipore, AB144P, 1:200), GFP (Aves, GFP-1020, 1:1000), HB9 (DSHB, 81.5C10, 1:500), lamin A (LMNA)/LMNC (Abcam, ab40567, 1:300), LMNB1 (Proteintech, 12987-1-AP, 1:500), MAB414 (BioLegend, 902901, 1:500), MAP2 (Sigma, M4403, 1:500), MAP2 (Abcam, ab5392, 1:10,000), NUP98 (Santa Cruz, sc-74578, 1:200), NUP359 (Millipore, ABN1385, 1:100), SMI32 (BioLegend, 801701, 1:5000), SYN1 (Cell Signaling, 5297, 1:200), TUBB3 (Covance, MMS-435P, 1:2000), and TUBB3 (Covance, PRB-435P-100, 1:2000).

Confocal images were obtained with a Nikon AIR or Zeiss LSM700 (63 $\times$ /1.4NA) confocal microscope. Neurite length was measured with the ImageJ software and shown as relative length per neurite (Ding et al., 2013, 2016, 2018). Neurites with direct connections to the soma were counted and shown as primary branches per cell. For nuclear morphology assay, we zoomed in on individual nucleus by using the ImageJ software and defined the abnormal nucleus based on two criteria: (1) at least one obvious sharp angle or a deep invagination; and (2) at least half area

of the nucleus losing the smooth outline. Signals of nuclei (HST) or nuclear lamins were used to distinguish the nucleus and cytoplasm.

Fluorescent in situ hybridization (FISH)

FISH was performed as described previously (Ding et al., 2020). Briefly, cultured cells were washed once with PBS, and fixed with 4% PFA for 30 min at RT. Cells were permeabilized with 0.2% Triton X-100 for 10 min, followed by two washes with PBS. Samples were equilibrated with hybridization buffer composed of 2 $\times$ SSC, 10% dextran sulfate, 10 mM ribonucleoside-vanadyl complex (RVC; New England Biolabs) and 20% formamide. A mixture of digoxigenin (DIG)-labeled oligo-dT or dA probes (0.2 ng/ μ l) and yeast tRNA (0.2 μ g/ μ l) were heated to 95°C for 5 min and immediately chilled on ice. Probes were then combined with equal volumes of 2 $\times$ hybridization buffer and incubated with samples overnight at 37°C. Anti-DIG antibody (Sigma, 11333089001, 1:100) and corresponding fluorophore-conjugated secondary antibodies (Thermo Fisher Scientific) were used to detect oligo-dT signals. Anti-MAP2 (Abcam, ab5392, 1:10,000) antibody and HST were used to determine neuronal soma and nuclei, respectively. Fluorescence density of confocal images were analyzed with the ImageJ software. The “mean” values of the nucleus and the cytoplasm in each neuron were used to calculate the ratio of dT signal in nucleus to cytoplasm (Ding et al., 2020). We measured as large an area as possible for each neuron and employed an unbiased approach for data collection. The person who analyzed the images was completely blinded to the sample information.

Western blot analysis

Cells were lysed in lysis buffer composed of 50 mM Tris-HCl buffer (pH 8.0), 150 mM NaCl, 1% NP40, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitor cocktail (Roche). Equal amounts of cell lysates (20 μ g per lane) were used for SDS-PAGE and western blot analysis as previously described (Ding et al., 2018). Primary antibodies and dilutions were Actin (Sigma, A5441, 1:60,000), TOR1A (Cell Signaling, 2150, 1:1000), TUBB3 (Covance, MMS-435P, 1:10,000), LMNB1 (Proteintech, 12987-1-AP, 1:2000), LMNB2 (Abcam, ab97513 1:1000), GLE1 (Abcam, ab96007, 1:2000), LAP2A (Abcam, ab5162, 1:2000), LAP2B (Thermo Fisher Scientific, A304-840A, 1:2000), and RANGAP1 (Cell Signaling, 36067, 1:1000).

Quantitative real-time PCR analysis

Total RNA was extracted from cultured cells using TRIzol (Life Technologies), and genomic contamination was removed using TURBO DNase (Life Technologies). cDNA synthesis reactions were performed using 0.5 μ g of RNA from each sample with the SuperScriptIII First-Strand kit (Life Technologies) and random hexamer primers. Real-time PCR was performed in triplicate using primers, SYBR GreenER SuperMix (Invitrogen), and the ABI 7900HT thermocycler (Applied Biosystems). Target mRNA levels were normalized to the reference gene *HPRT* by 2^{−ΔΔC_t} method as described previously (Ding et al., 2013, 2016, 2018). PCR primer sequences (from 5' to 3') were CTGGTGGATGGGGTCTCCTA (qTOR1A-F), GACACAGACAACGCGTGTTC (qTOR1A-R), AGGAAAGCGGAAGAGGGTTG (qLMNB1-F), and GCCTCCCATTTGGTTGATCCT (qLMNB1-R).

Transmission electron microscopy (TEM)

Cells on Matrigel-coated glass coverslips were fixed overnight at 4°C with a solution composed of 4% PFA, 1% glutaraldehyde, 1.5 μ M MgCl₂ and 5 μ M CaCl₂ in 0.1 M cacodylate buffer (pH 7.4). Samples were then twice washed with cacodylate buffer, treated for 1.5 h at RT with 1% osmium tetroxide (OsO₄; EMS) and 0.8% K₃Fe(CN)₆ in cacodylate buffer, and prestained with 2% uranyl acetate in water for 2 h in the dark at RT. The subsequent steps of dehydration, infiltration, and embedding were the same as previously described (Li et al., 2016). The resin blocks were polymerized overnight at 60°C and sectioned with a diamond knife (Diatome) on a Leica Ultracut UCT 6 ultramicrotome (Leica Microsystems). Sections were poststained with 2% uranyl acetate in water and lead citrate. Images were acquired on a JEOL JEM 1200EX TEM equipped with a tungsten source at 120 kV using a Morada SIS camera (Olympus). The electron-dense areas immediately

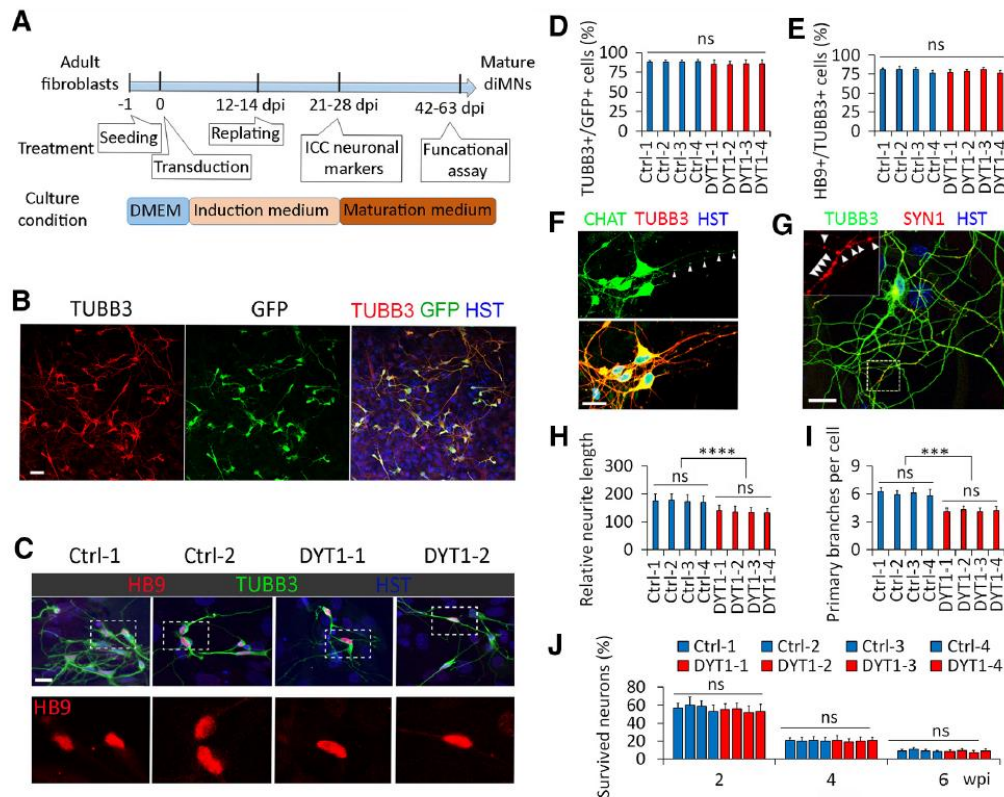


Figure 1. Direct conversion of DYT1 patient fibroblasts to MNs. **A**, A schematic of the direct conversion procedure. **B**, A lower magnification view of the reprogrammed neurons at 19 d postviral infection (dpi). Virus-transduced cells are indicated by the co-expressed GFP. Scale bar: 50 μ m. **C**, Confocal images of the reprogrammed neurons at 19 dpi. Higher magnification views of nuclear HB9 are shown in the bottom panels. Scale bar: 20 μ m. **D**, Quantification of the reprogramming efficiency ($n = 259$ for Ctrl-1, $n = 249$ for Ctrl-2, $n = 237$ for Ctrl-3, $n = 243$ for Ctrl-4, $n = 257$ for DYT1-1, $n = 254$ for DYT1-2, $n = 302$ for DYT1-3, and $n = 257$ for DYT1-4 from triplicates; ns, not significant). **E**, Fractions of diMNs among the reprogrammed neurons ($n = 257$ for Ctrl-1, $n = 273$ for Ctrl-2, $n = 252$ for Ctrl-3, $n = 281$ for Ctrl-4, $n = 229$ for DYT1-1, $n = 219$ for DYT1-2, $n = 289$ for DYT1-3, and $n = 233$ for DYT1-4 from triplicates; ns, not significant). **F**, Robust expression of the MN marker ChAT at 7 wpi. Arrowheads indicate ChAT+ puncta. Scale bar: 20 μ m. **G**, Expression of the presynaptic marker SYN1 at 9 wpi. The inset shows discrete SYN1+ puncta (indicated by arrow heads). Scale bar: 50 μ m. **H**, Quantitative analysis of neurite length at 2 wpi ($n = 123$ for Ctrl-1, $n = 126$ for Ctrl-2, $n = 137$ for Ctrl-3, $n = 125$ for Ctrl-4, $n = 114$ for DYT1-1, $n = 127$ for DYT1-2, $n = 118$ for DYT1-3, $n = 113$ for DYT1-4 from triplicates; ns, not significant; **** $p = 2.56E-05$, t test). **I**, Neurite number of the primary branches examined at 4 wpi ($n = 163$ for Ctrl-1, $n = 153$ for Ctrl-2, $n = 157$ for Ctrl-3, $n = 145$ for Ctrl-4, $n = 152$ for DYT1-1, and $n = 143$ for DYT1-2, $n = 125$ for DYT1-3, and $n = 131$ for DYT1-4 from triplicates; ns, not significant; *** $p = 0.00032$, t test). **J**, A time course analysis of diMN survival [each line n (neurons) > 2000 from triplicates; ns, not significant].

beneath the nuclear membrane were measured with ImageJ (NIH) and the ratio of electron-dense area to the nuclear perimeter was used to evaluate the thickness of nuclear lamina. By using the ImageJ software, we zoomed in on the nuclear member and quantified the number of nuclear pore complexes (NPCs) and the pore sizes.

Electrophysiology

Neurons cultured on astrocyte-coated glass coverslips for 35 d or longer were used for analysis. Whole-cell patch-clamp recordings were performed under visual guidance using GFP-fluorescence to identify GFP+ cells when cells were maintained at 30°C in a submersion chamber with tyrode solution containing 150 mM NaCl, 4 mM KCl, 2 mM MgCl₂, 3 mM CaCl₂, 10 mM glucose, and 10 mM HEPES at pH 7.4 (adjusted with KOH) and 300 mOsm. The recording pipettes (~6–9 M Ω) were filled with an intracellular solution containing 0.2 mM EGTA, 130 mM K-glucuronate, 6 mM KCl, 3 mM NaCl, 10 mM HEPES, 4 mM ATP-Mg, 0.4 mM GTP-Na, 14 mM phosphocreatine-di(Tris) at pH 7.2 and 285 mOsm. Series and input resistance were measured in voltage-clamp with a 400 ms, 10-mV step from a -60-mV holding potential (filtered at 30 kHz and sampled at 50 kHz). Cells were used for analysis only if the series resistance was <30 M Ω and was stable throughout the experiment. The input resistance ranged from 0.2 to 2 G Ω . Currents were

filtered at 3 kHz, acquired and digitized at 10 kHz using Clampex10.3 software (Molecular Devices). Action potentials (APs) were recorded in current-clamp and elicited by a series of current injections ranging from -20 to 200 pA at 20-pA increments and 800 ms in duration. All current-clamp recordings were made at resting membrane potential or without any current injection. Data analysis was performed with Clampfit10.3 software (Molecular Devices). The AP trace immediately above threshold was used to determine the delay of first spike as the length of time and the start of current steps to the peak of AP. The same AP trace was used to measure AP threshold as the corresponding voltage when there was the sharpest change of the trace slope. The above indicated AP trace was also measured to determine maximum velocity of the rise and decay. AP frequency was obtained by dividing the maximum number of spikes during the current steps protocol with the step time duration (800 ms).

Statistical analysis

Unpaired two-tailed Student's t tests were used to compare one experimental sample with its control. One-way analysis of variance was used when comparing multiple samples, with either Tukey's (when comparing samples with each other) or Dunnett's (when comparing samples to a control) *post hoc* tests. Results are expressed as mean $\pm$ SD of at least three biological replicates, and $p < 0.05$ is treated as significant.

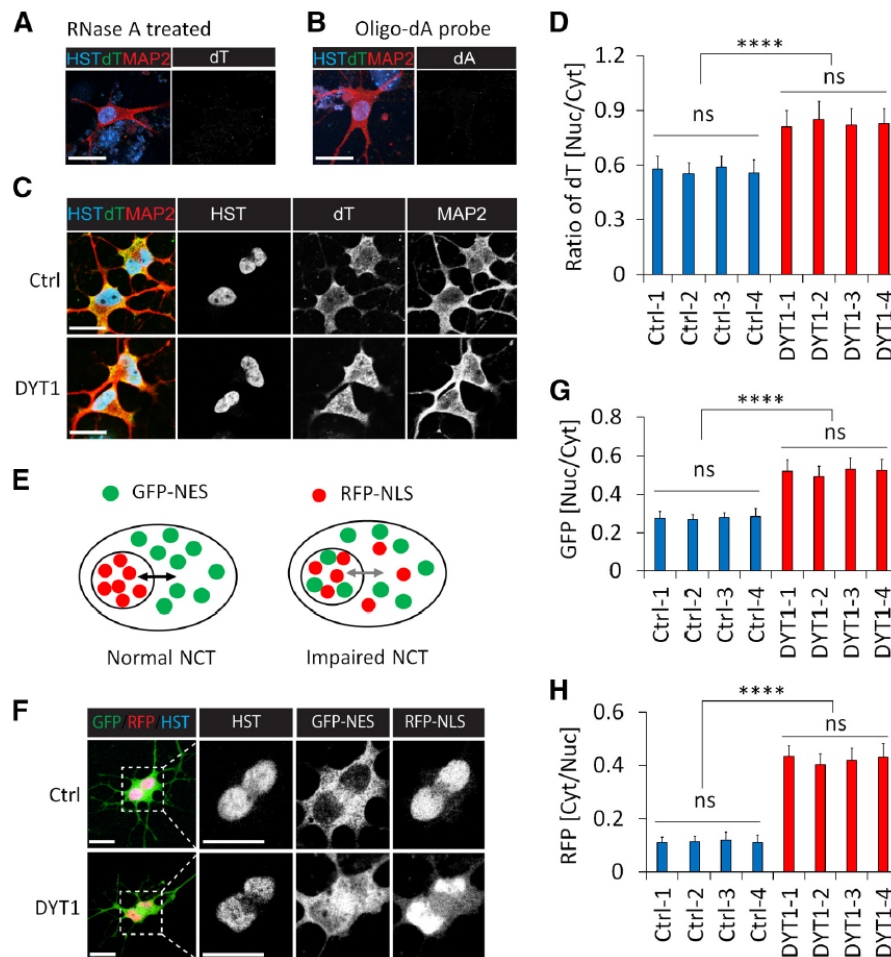


Figure 2. Impaired NCT in DYT1-diMNs from fibroblasts. **A**, RNase A-treated diMNs as negative controls for FISH. Cells were analyzed at 6 wpi. Scale bar: 20 μ m. **B**, Oligo-dA probes as negative controls for FISH assays at 6 wpi. Scale bar: 20 μ m. **C**, FISH analysis of diMNs with oligo-dT probes at 6 wpi. Scale bar: 20 μ m. **D**, Quantification of oligo-dT signal distribution ($n = 65$ for Ctrl-1, $n = 67$ for Ctrl-2, $n = 72$ for Ctrl-3, $n = 70$ for Ctrl-4, $n = 64$ for DYT1-1, $n = 63$ for DYT1-2, $n = 74$ for DYT1-3, and $n = 68$ for DYT1-4 from four replicates; ns, not significant; **** $p = 3.83E-08$, t test). **E**, A dual reporter system for measuring protein NCT. NES, nuclear export signal; NLS, nuclear localization signal. **F**, Representative confocal images of reporter distribution in diMNs at 6 wpi. Scale bar: 20 μ m. **G**, Subcellular distribution of GFP-NES in diMNs ($n = 62$ for Ctrl-1, $n = 64$ for Ctrl-2, $n = 76$ for Ctrl-3, $n = 74$ for Ctrl-4, $n = 67$ for DYT1-1, and $n = 68$ for DYT1-2, $n = 75$ for DYT1-3, and $n = 73$ for DYT1-4 from four replicates; ns, not significant; **** $p = 5.15E-10$, t test). **H**, Subcellular distribution of RFP-NLS in diMNs ($n = 62$ for Ctrl-1, $n = 64$ for Ctrl-2, $n = 76$ for Ctrl-3, $n = 74$ for Ctrl-4, $n = 67$ for DYT1-1, and $n = 68$ for DYT1-2, $n = 75$ for DYT1-3, and $n = 73$ for DYT1-4 from four replicates; ns, not significant; **** $p = 4.15E-12$, t test).

Results

Directly induced cholinergic MNs from fibroblasts of DYT1 patients

Because dystonia is a movement disorder and the ventral horn neurons in the spinal cord are most severely affected in *Tor1a*-mutant mice (Goodchild et al., 2005), we first generated MNs from human fibroblasts using a direct reprogramming method as we have previously described (Liu et al., 2016; Fig. 1A). The fibroblasts of four DYT1 patients and four age-matched healthy controls were used for this study. Although sex does not affect the reprogramming efficiency (Ding et al., 2020), all healthy controls were selected to be sex-matched to patient cell lines. Transduction with reprogramming factor-expressing lentivirus enabled adult human fibroblasts to gradually become neuron-like and grow complex neurites with time. Immunocytochemistry confirmed their expression of the neuronal marker TUBB3 (Fig. 1B) and early MN marker HB9

(Fig. 1C; Arber et al., 1999). Neuronal conversion efficiency was similar among all these fibroblasts, with $\sim 85\%$ of virus-transduced GFP+ cells as TUBB3+ neurons (Fig. 1D). Approximately 80% of these neurons showed strong HB9 expression and there was no difference between different cell lines (Fig. 1E). At later stages, such as seven or nine weeks postviral infection (wpi), reprogrammed neurons expressed high levels of ChAT and exhibited discrete puncta of the presynaptic marker synapsin 1 (SYN1; Fig. 1F, G). Thus, fibroblasts from DYT1 patients as well as healthy controls could be efficiently and directly converted into mature cholinergic MNs, which were hereafter referred as diMNs (directly induced MNs).

DYT1-diMNs show impaired development but normal survival

DYT1 dystonia can also be classified as a neurodevelopmental disorder (Dauer, 2014). To track the development of human

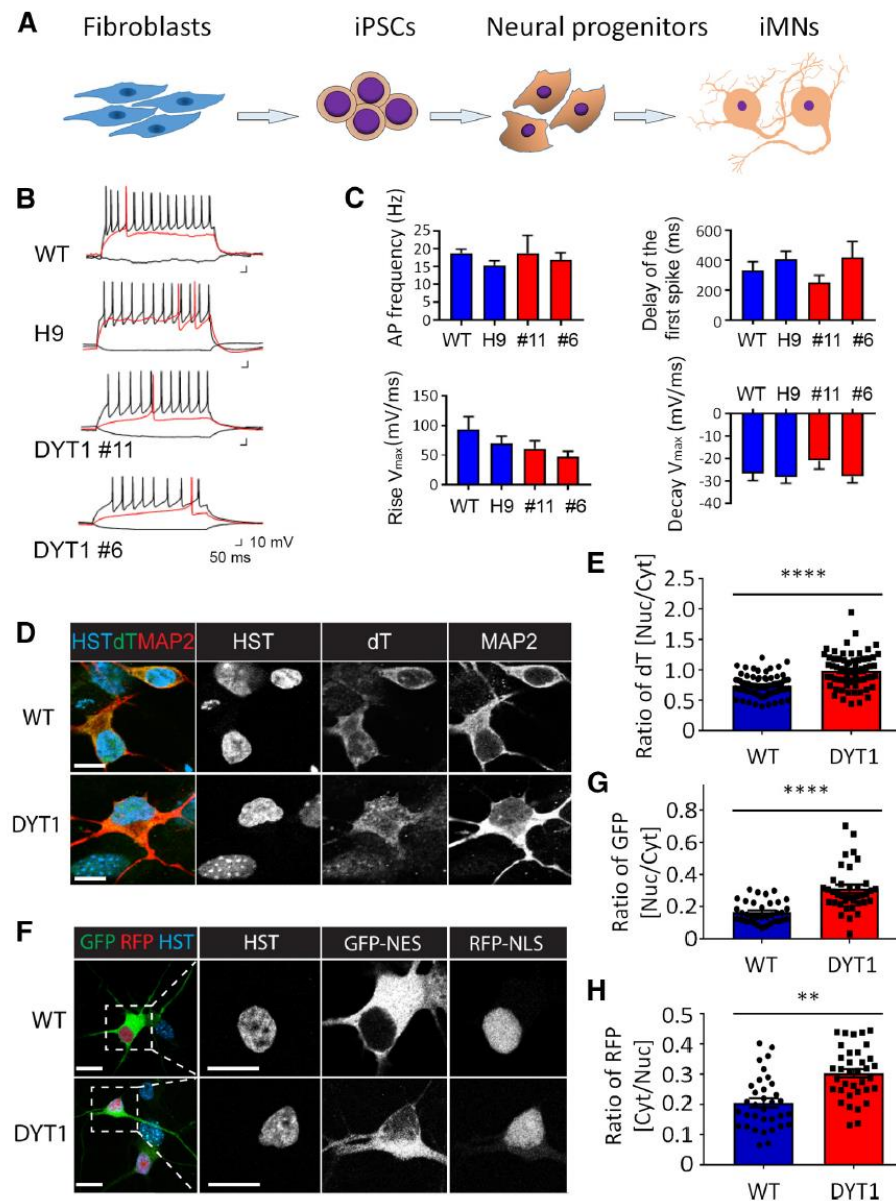


Figure 3. NCT impairments in DYT1-iMNs from iPSCs. **A**, A schematic of the iPSC-based procedure to generate iMNs. **B**, Repetitive AP waveforms recorded under current-clamp mode for the control (WT and H9) and two clones of DYT1 iMNs (#11 and #6) at 5 wpi. The precondition sweep and the sweep immediately above threshold (in red) are also shown. **C**, Quantifications of AP frequency, delay of the first spike, rise and decay V_{max} in the indicated iMNs, respectively ($n = 30$ for each line from 4 biological replicates). **D**, Representative confocal images of FISH assay in iMNs at 3 wpi. Scale bar: 10 μ m. **E**, Subcellular distribution of mRNAs in iMNs by FISH assay ($n = 85$ for WT and 73 for DYT1 from triplicates; **** $p = 7.13 \times 10^{-5}$, t test). **F**, Representative confocal images of iMNs with the dual reporter at 3 wpi. Scale bar: 20 μ m. **G**, Subcellular distribution of GFP-NES in iMNs ($n = 34$ for WT and $n = 36$ for DYT1 from triplicates; **** $p = 3.42 \times 10^{-6}$, t test). **H**, Subcellular distribution of RFP-NLS in iMNs ($n = 34$ for WT and $n = 36$ for DYT1 from triplicates; ** $p = 0.0043$, t test).

DYT1-diMNs, we examined neurite outgrowth and primary branches at the early reprogramming stages of 2 and 4 wpi as described in previous studies (Ding et al., 2013, 2016, 2018; Ding, 2015). DYT1-diMNs had significantly shorter neurites (Fig. 1H) and fewer primary branches (Fig. 1I) than controls. On the other hand, no significant differences were identified on neuronal survival within six weeks (Fig. 1J). These results support the notion that DYT1 dystonia is caused by alterations in neuronal function rather than by neurodegeneration (Granata et al., 2009).

DYT1-diMNs exhibit dysfunctional nucleocytoplasmic transport (NCT)

Torsin has been shown to participate in nuclear export of mRNAs via a non-conventional mechanism called NE budding in *Drosophila* (Speese et al., 2012). In *Torsin* mutants, disruption of mRNA nuclear export led to slowed growth and impaired development and plasticity of neuromuscular junctions (Jokhi et al., 2013). To examine whether mRNA export was similarly impaired in DYT1-diMNs, we performed FISH, as previously described (Packard et al., 2015; Li et al., 2016; Ding et al., 2020).

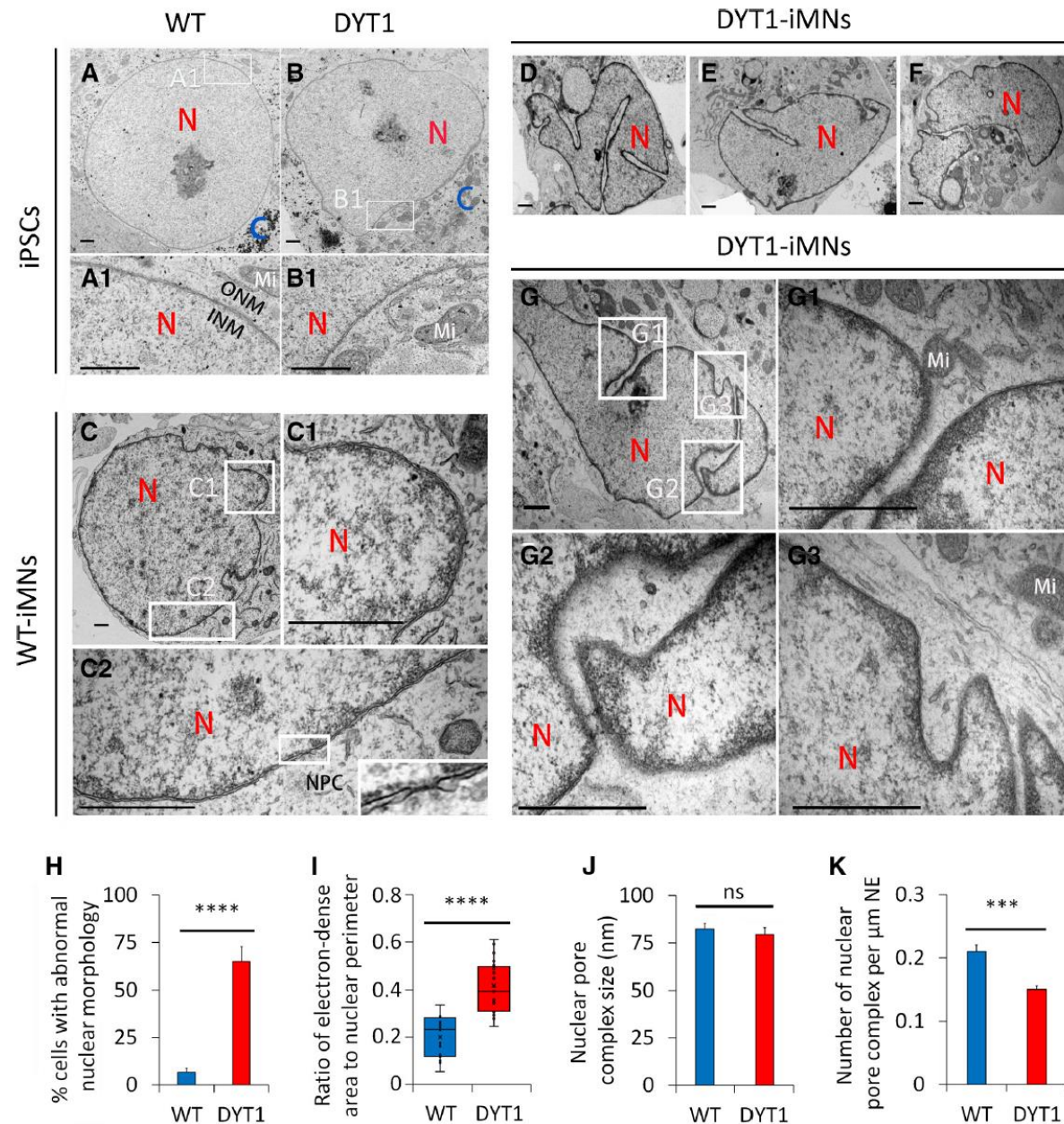


Figure 4. NE deformation in DYT1-iMNs. **A**, Representative electron micrograph of WT-iPSC. A higher magnification view of the boxed region is shown in **A1** of the bottom panel. N, nucleus; C, cytoplasm; INM, inner nuclear membrane; ONM, outer nuclear membrane; Mi, mitochondria. Scale bars: 1 μm . **B**, Representative electron micrograph of DYT1-iPSC. A higher magnification view of the boxed region is shown in **B1** of the bottom panel. Scale bars: 1 μm . **C**, Representative electron micrographs of WT-iMN at 3 wpi. Higher magnification views of the boxed regions are shown in **C1**, **C2**, respectively. NPC, nuclear pore complex. Scale bars: 1 μm . **D–G**, Representative electron micrographs of DYT1-iMNs at 3 wpi. Higher magnification views of the boxed regions are shown in **G1**, **G2**, **G3**, respectively. Scale bars: 1 μm . **H**, iMNs with abnormal nuclear morphology at 3 wpi [n (nuclei) = 47 for WT and 42 for DYT1 from triplicates; **** p = 4.31E-05, t test]. **I**, Ratio of electron dense area immediately beneath the nuclear membrane to the nuclear perimeter in iMNs at 3 wpi [n (nuclei) = 24 for WT and 22 for DYT1 from triplicates; **** p = 2.15E-05, t test]. **J**, Nuclear pore size in iMNs at 3 wpi [n (nuclei/nuclear pore) = 17/56 for WT and 21/34 for DYT1 from triplicates; ns, not significant, t test]. **K**, Number of nuclear pore structure in iMNs at 3 wpi [n (nuclei) = 35 for WT and 32 for DYT1 from triplicates; *** p = 0.000263, t test].

We used DIG-labeled oligo-dT as probes to detect the subcellular distribution of Poly (A) mRNAs. FISH specificity was confirmed by a lack of signals when samples were treated with RNase A or when DIG-labeled oligo-dA was used as a probe (Fig. 2A,B). mRNA nuclear export was measured by the ratio of nuclear to

cytoplasmic oligo-dT signals ($dT_{\text{nuc}}/dT_{\text{cyt}}$). In healthy control diMNs, most mRNAs were localized in the cytoplasm when examined at 6 wpi (Fig. 2C,D). In contrast, substantial amounts of mRNAs were accumulated inside the nucleus of DYT1-diMNs (Fig. 2C,D).

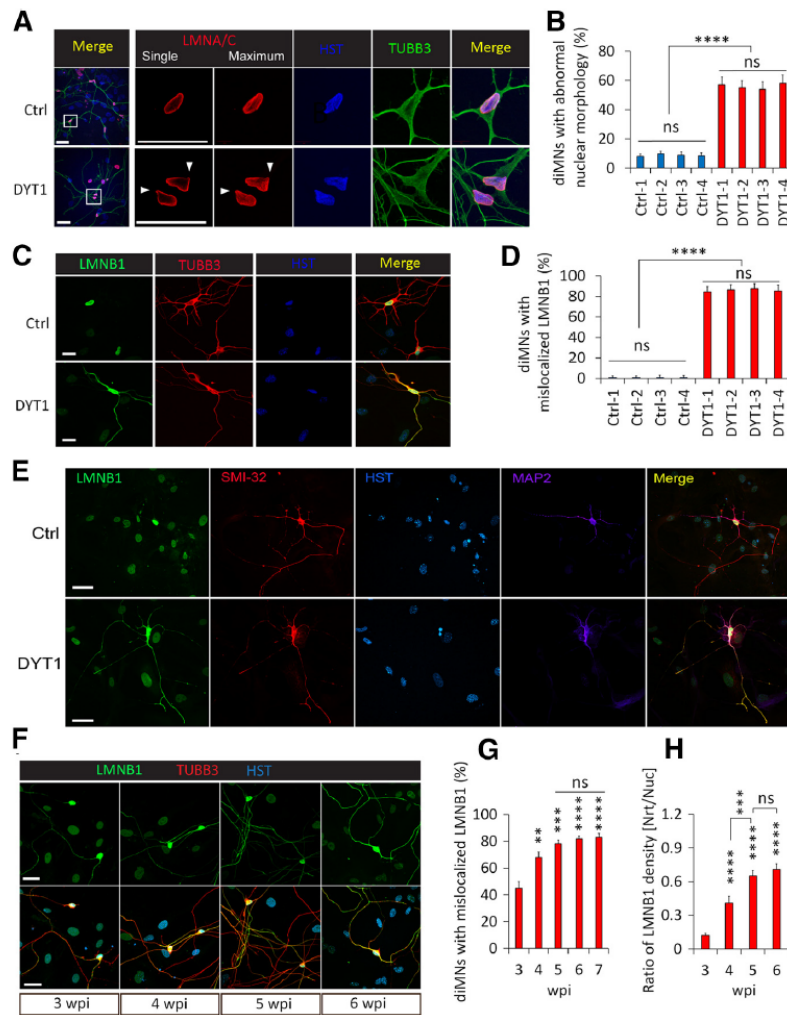


Figure 5. Abnormal nuclear morphology and mislocalized LMNB1 in DYT1-diMNs. **A**, Representative confocal images of diMNs at 4 wpi. Arrowheads indicate the sharp and rigid angles in nuclear membrane. Scale bar: 50 μ m. **B**, Quantification of diMNs with abnormal nuclear morphology at 4 wpi ($n > 100$ for each line from four replicates; ns, not significant; **** $p = 2.38E-09$, t test). **C**, Representative confocal images of diMNs at 6 wpi. Scale bar: 20 μ m. **D**, Percentages of diMNs with mislocalized LMNB1 at 6 wpi ($n = 410$ for Ctrl-1, $n = 418$ for Ctrl-2, $n = 490$ for Ctrl-3, $n = 378$ for Ctrl-4, $n = 358$ for DYT1-1, $n = 349$ for DYT1-2, $n = 378$ for DYT1-3, and $n = 376$ for DYT1-4 from four replicates; ns, not significant; **** $p = 6.43E-11$, t test). **E**, Representative confocal images of diMNs at 6 wpi. Scale bar: 50 μ m. **F**, A time course analysis of LMNB1 mislocalization in DYT1-diMNs. Scale bar: 50 μ m. **G**, Frequency of LMNB1 mislocalization in DYT1-diMNs at the indicated time points ($n = 458$ for 3 wpi, $n = 425$ for 4 wpi, $n = 327$ for 5 wpi, $n = 314$ for 6 wpi, and $n = 213$ for 7 wpi from triplicates; ns, not significant; compare to 3 wpi, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ANOVA). **H**, Subcellular distribution of LMNB1 (neurite vs nucleolus) in DYT1-diMNs at the indicated time points ($n = 126$ for 3 wpi, $n = 114$ for 4 wpi, $n = 128$ for 5 wpi, and $n = 107$ for 6 wpi from triplicates; ns, not significant; *** $p < 0.001$, **** $p < 0.0001$, ANOVA or t test).

Interestingly, we noticed obvious MAP2 signals in the nucleus of DYT1-diMNs (Fig. 2C), suggesting that protein NCT might also be compromised. We used a dual reporter system (2Gi2R) to further examine NCT (Ding et al., 2020). This reporter system consists of a fused double-GFP with a nuclear export signal (2GFP-NES) and a double RFP containing a nuclear localization signal (2RFP-NLS; Mertens et al., 2015). In cells with normal NCT activity, GFP and RFP are localized in the cytoplasm and nucleus, respectively; in cells with compromised NCT, this subcellular distribution will be disrupted (Fig. 2E). An increased ratio of nuclear to cytoplasmic GFP (GFP_{nuc}/GFP_{cyt}) represents

impaired protein export, while a higher ratio of cytoplasmic to nuclear RFP (RFP_{cyt}/RFP_{nuc}) indicates compromised protein nuclear import. Both GFP and RFP were mainly localized in the cytoplasm and nucleus, respectively, in control diMNs when examined at 6 wpi (Fig. 2F–H). In contrast, DYT1-diMNs had a substantial amount of GFP accumulated inside the nucleus and obvious RFP detected in the cytoplasm (Fig. 2F). As such, the ratios of GFP_{nuc}/GFP_{cyt} and RFP_{cyt}/RFP_{nuc} were significantly higher for DYT1-diMNs than control neurons (Fig. 2G,H). Together, FISH analysis and dual reporter assays clearly indicate that both mRNA nuclear export and protein NCT are impaired in DYT1-diMNs.

NCT impairments in MNs from DYT1 iPSCs

To verify our findings, we examined iPSC-derived MNs (Fig. 3A). One iPSC clone from a healthy control (WT) and two clones from a DYT1 patient (#6 and #11) were successfully generated. The well-characterized human ESC line H9 was included as an additional control. We differentiated these pluripotent stem cells into neural progenitors (Yang et al., 2014), which were further induced to become MNs (Tang et al., 2017; Sepehrimanesh and Ding, 2020). These iPSCs-derived or ESCs-derived MNs were hereafter referred as iMNs. Electrophysiological properties of iMNs were examined by whole-cell patch-clamp recordings at 4 wpi. All patched iMNs fired repetitive APs on current injections (Fig. 3B). No apparent differences were observed between DYT1 and control iMNs on AP frequency, delay of the first spike, and rise and decay of V_{max} (Fig. 3C). Interestingly, nuclear mRNA export and protein NCT were significantly compromised in DYT1-iMNs (Fig. 3D–H), though these defects were less severe than in DYT1-diMNs (Fig. 2). For example, the cytoplasmic localization of RFP-NLS was less obvious in DYT1-iMNs than DYT1-diMNs (compare Figs. 3F,H and 2F,H). Such a difference may reflect an aging effect on disease phenotypes, since diMNs retain aging-associated features, whereas iMNs are rejuvenated during the reprogramming process (Mertens et al., 2015; Tang et al., 2017).

Abnormal NE but no perinuclear blebs in DYT1 MNs

One cellular hallmark in *Tor1a*-mutant mice (*Tor1a* knock-out or *Tor1a*^{ΔE/ΔE}) is the abnormal NE morphology in multiple areas of the CNS (Goodchild et al., 2005; Tanabe et al., 2016). It is characterized by an accumulation of blebs in the perinuclear

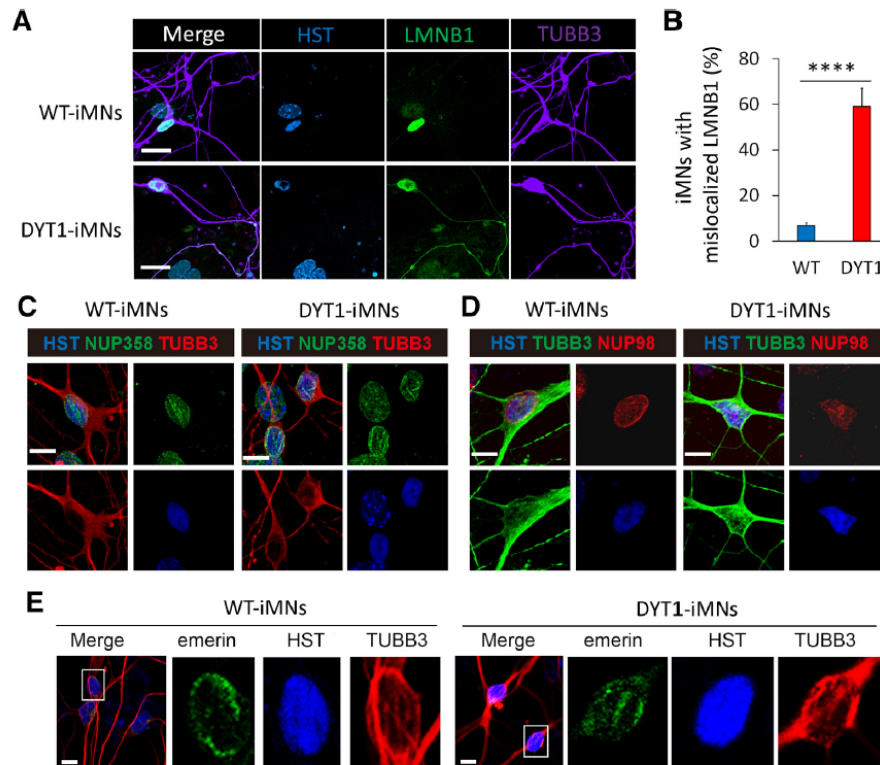


Figure 6. LMNB1-specific mislocalization in DYT1-iMNs. **A**, Representative confocal images of the indicated markers in iMNs at 3 wpi. Scale bar: 20 μ m. **B**, Subcellular localization of LMNB1 in iMNs at 3 wpi ($n = 397$ for WT and $n = 423$ for DYT1 from triplicates; **** $p = 2.42E-06$, t test). **C–E**, Representative confocal images of the indicated markers in iMNs at 3 wpi. Scale bars: 10 μ m.

space, morphologically similar to the budding granules in the body-wall muscle in *torsin*-mutant flies (Speese et al., 2012; Jokhi et al., 2013). We first examined whether such abnormalities also occur and contribute to impaired NCT in human DYT1 neurons. Nuclear morphology was studied by TEM. At the iPSC stage, both WT and DYT1 cells had smooth and round nuclei with clear double nuclear membranes, except for a few small wrinkles in some DYT1-iPSCs (Fig. 4A,B). After differentiation into mature iMNs, most WT cells retained the regular, smooth nuclear shapes with occasional wrinkles and light invaginations (Fig. 4C). In contrast, the nuclear morphology of DYT1-iMNs showed many sharp angles and deep invaginations (Fig. 4D–H). However, we failed to observe any bleb structures at the perinuclear space in DYT1-iMNs from three batches of samples. If bleb structures exist, the frequency must be very low. Such a result indicates that the mechanisms underlying the abnormal NCT might differ between human DYT1 patients and other animal models such as *Drosophila* (Jokhi et al., 2013) and mouse (Goodchild et al., 2005; Tanabe et al., 2016). Interestingly, we found that DYT1-iMNs exhibited thickened nuclear lamina with high electron-dense areas immediately beneath the nuclear membranes, especially in regions with abnormal angles and invaginations (Fig. 4G1–G3). Quantification showed a much higher ratio of the electron-dense area to the nuclear perimeter in DYT1-iMNs than controls (Fig. 4J). Interestingly, although the NPC size was comparable between WT-iMNs and DYT1-iMNs (Fig. 4J), the number of NPCs at TEM was significantly lower in DYT1 neurons (Fig. 4K). These results agree with a recent report showing that Torsin functions in nuclear pore biogenesis

(Rampello et al., 2020) and suggest that the impaired NCT in DYT1 neurons might be because of a reduction of NPC number.

LMNB1 mislocalization in DYT1 MNs

Since deformed NE and thickened nuclear lamina are salient features of DYT1-iMNs (Fig. 4G,I), they were further examined by immunocytochemistry and confocal microscopy. Nuclear lamina consists of A-type and B-type lamins, which are intermediate filament proteins and form the meshwork beneath the inner nuclear membrane to support the nuclear morphology and structural integrity of the NE (Capell and Collins, 2006; Burke and Stewart, 2013; Shah et al., 2017; Tatli and Medalia, 2018). LMNA and LMNC are the major A-type lamins encoded by a single *LMNA* gene in humans (Burke and Stewart, 2013). Staining for LMNA/C showed their exclusive localization in the nuclear periphery in both control and DYT1-diMNs (Fig. 5A). However, unlike the regular ovoid nucleus with a smooth outline in control cells, ~60% of DYT1-diMNs showed abnormal nuclear morphology with sharper and rigid angles (Fig. 5A,B), consistent with what was observed under TEM (Fig. 4D–H).

Similar to LMNA/C, staining of the major B-type nuclear lamins LMNB1 revealed its normal nuclear localization in control diMNs (Fig. 5C). Unexpectedly, a substantial amount of LMNB1 were detected in the cytoplasm and neurites of DYT1-diMNs when examined at 6 wpi (Fig. 5C). Quantification showed ~80% of DYT1-diMNs exhibited mislocalized LMNB1, whereas such mislocalization was rarely detected in healthy controls (Fig. 5D). Co-staining with SMI-32 and MAP2 indicated that the mislocalized LMNB1 was distributed in both axons and dendrites

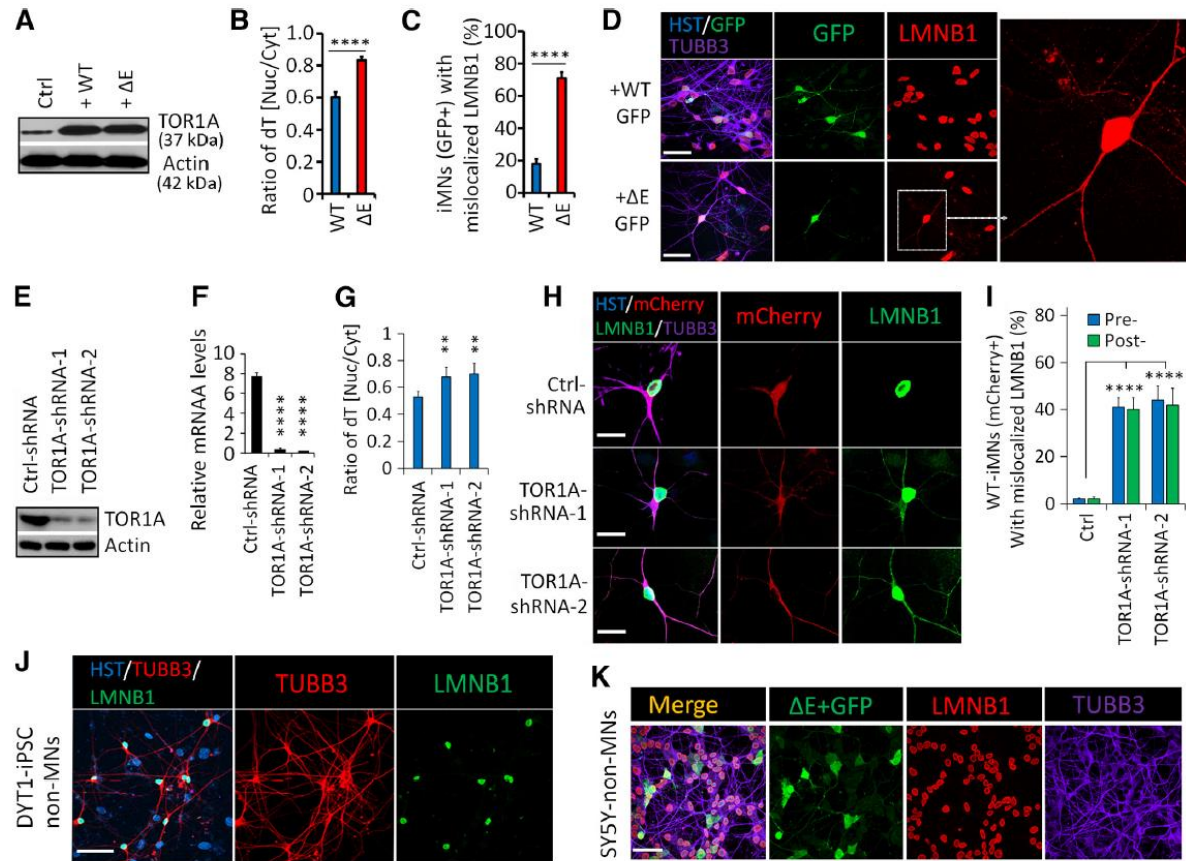


Figure 7. TOR1A hypoactivity disrupts NCT and LMNB1 localization in a MN-specific manner. **A**, Protein expression of the ectopically expressed TOR1A WT or ΔE in iMNs; Actin serves as a loading control. **B**, The effect of TOR1A expression on mRNA distribution assayed by FISH in iMNs at 3 wpi ($n = 99$ for WT and $n = 78$ for DYT1 from triplicates; $****p = 5.44E-05$, t test). **C**, Quantification of LMNB1 mislocalization in iMNs at 3 wpi ($n = 82$ for WT and $n = 75$ for DYT1 from triplicates; $****p = 2.48E-06$, t test). **D**, Representative confocal images of iMNs with ectopic (GFP+) TOR1A WT or ΔE at 3 wpi. Scale bar: 50 μm . **E**, Western blots showing shRNA knock-down of TOR1A protein expression in iMNs. **F**, qRT-PCR showing downregulation of TOR1A mRNA by shRNAs ($n = 3$; $****p = 6.58E-05$, ANOVA). **G**, The effect of TOR1A downregulation on mRNA subcellular distribution assayed by FISH in iMNs at 3 wpi ($n = 95$ for Ctrl, $n = 87$ for TOR1A-shRNA-1, and $n = 78$ for TOR1A-shRNA-2 from triplicates; $**p = 0.00327$, ANOVA). **H**, Representative confocal images of iMNs with the indicated shRNAs (mCherry+) at 3 wpi. Scale bar: 25 μm . **I**, The effect of TOR1A downregulation on LMNB1 subcellular distribution in iMNs. ShRNA lentiviruses were added at either predifferentiation (neural progenitors) or postdifferentiation (MNs at 5 dpi) stages; n (pre and post) = 126 and 113 for Ctrl, $n = 118$ and 97 for TOR1A-shRNA-1, and $n = 103$ and 94 for TOR1A-shRNA-2 from triplicates; $****p = 4.08E-10$ (Ctrl vs shRNA-1) and $5.78E-9$ (Ctrl vs shRNA-2), t test. **J**, Representative confocal images of DYT1-iPSC spontaneously differentiated non-MNs at 3 wpi. Scale bar: 100 μm . **K**, Representative confocal images of SY5Y-derived non-MNs with ectopic expression of TOR1A ΔE (GFP+) at 4 wpi. Scale bar: 50 μm .

(Fig. 5E). A time course analysis revealed that cytoplasmic LMNB1 in DYT1-diMNs became progressively more frequent, with 45% at 3 wpi and 80% at 6–7 wpi (Fig. 5F,G). The cytoplasmic fraction of LMNB1 also greatly increased with time and reached a plateau at 6 wpi (Fig. 5H). Importantly, LMNB1 mislocalization was also observed in DYT1-iMNs from iPSCs (Fig. 6A). Such mislocalization could be detected as early as 1.5 wpi and in ~60% of DYT1-iMNs when examined from 2 to 8 wpi (Fig. 6B). In contrast, we failed to detect any obvious mislocalization of several other nuclear proteins, including LMNA/C (Fig. 5A), the nuclear pore subunits NUP98 (Fig. 6C) and NUP358 (Fig. 6D), and the inner nuclear membrane protein emerin (Fig. 6E) in DYT1-iMNs.

TOR1A hypoactivity disrupts NCT and LMNB1 localization
To determine whether these above defects were caused by dysregulation of TOR1A or largely influenced by the genetic background of human patients, we employed two approaches. One was to ectopically express either TOR1A or TOR1A ΔE mutant

in WT-iMNs. These MNs could be identified by the co-expressed GFP reporter. Western blot analysis showed that both TOR1A and TOR1A ΔE were expressed at a similar level (Fig. 7A). Interestingly, ectopic TOR1A ΔE mutant but not the WT TOR1A disrupted mRNA nuclear export (Fig. 7B) and LMNB1 subcellular distribution (Fig. 7C,D). In a second approach, we downregulated TOR1A expression through shRNAs in WT-iMNs. shRNA-expressing cells were marked by the co-expressed mCherry reporter. Each of the two separate shRNAs against TOR1A could efficiently down-regulate its expression at both protein and mRNA levels (Fig. 7E,F). Consistently, downregulation of endogenous TOR1A led to defective export of nuclear mRNAs (Fig. 7G) and altered subcellular localization of LMNB1 (Fig. 7H). Such LMNB1 mislocalization was independent of the neuronal differentiation process because MNs infected with the TOR1A-shRNA virus at either predifferentiation (neural progenitors) or postdifferentiation (MNs at 5 dpi) stages exhibited similar results (Fig. 7I). Together, these results strongly support that NCT impairment and LMNB1 mislocalization are caused by TOR1A hypoactivity.

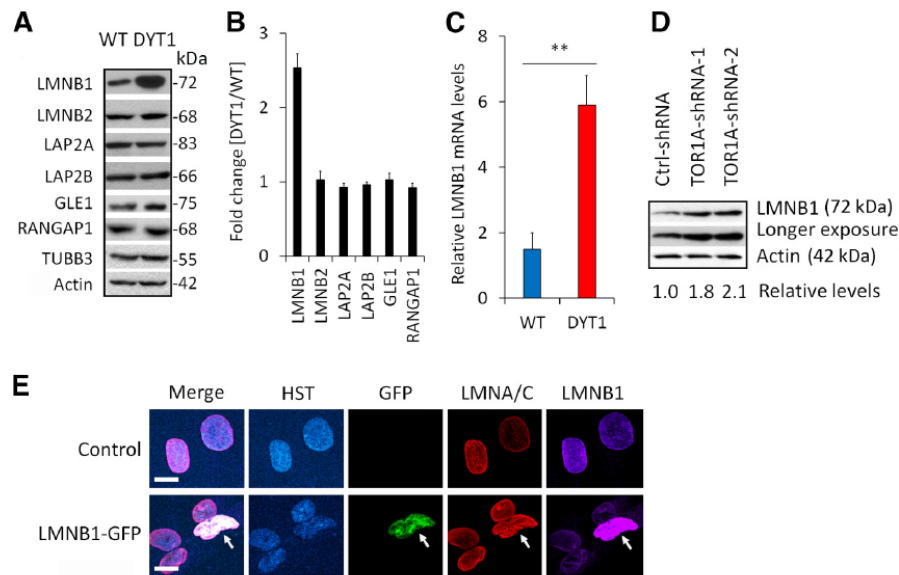


Figure 8. Specific upregulation of LMNB1 in DYT1 MNs. **A**, Western blottings for the indicated proteins in WT-iMNs and DYT1-iMNs at 11 dpi. TUBB3 and Actin are loading controls. **B**, Fold changes (DYT1 to WT) of the indicated proteins in **A** after normalization to the loading control ($n = 3$). **C**, Relative *LMNB1* mRNA levels analyzed by qRT-PCR in iMNs at 11 dpi ($n = 3$; $**p = 0.0092$, t test). **D**, Western blottings for LMNB1 expression (shorter and longer exposure) in iMNs with the indicated shRNAs at 11 wpi. Actin serves as a loading control. Values at the bottom indicate the relative LMNB1 protein levels. **E**, Confocal micrographs of fibroblasts with or without overexpression of LMNB1 (fused with GFP). Arrow indicates a cell with LMNB1 overexpression. Scale bars: 10 μ m.

To examine whether hypoactivity of TOR1A also leads to LMNB1 mislocalization in other neuronal subtypes, we generated non-MNs via two approaches. One was spontaneous differentiation of DYT1 neural progenitors into generic neurons (Fig. 7J), which mainly consisted of glutamatergic and GABAergic neurons (Sepehrimanesh and Ding, 2020). The second approach was to ectopically express *TOR1A* ΔE in generic neurons that were differentiated from human SH-SY5Y neuroblasts (Fig. 7K). Interestingly, we did not notice any mislocalized LMNB1 in either of these generic neurons (Fig. 7J,K). These results suggest that TOR1A hypoactivity may cause LMNB1 mislocalization in a MN-specific manner.

Specific upregulation of LMNB1 in DYT1 MNs

LMNB1 expression in iPSCs-derived iMNs was then examined by western blot analysis. Beta-actin and the neuron-specific microtubule TUBB3 were used as protein loading controls. Strikingly, the LMNB1 protein level was dramatically higher (2.5-fold) in DYT1-iMNs than in WT controls (Fig. 8A,B). Such a result was consistent with immunocytochemistry showing brighter LMNB1 signals and cytoplasmic accumulation in DYT1-diMNs (Fig. 5C,E). Notably, qRT-PCR analysis showed that *LMNB1* transcripts in DYT1-iMNs were 3- to 5-fold higher than those in WT controls (Fig. 8C), indicating that increased LMNB1 protein was mainly contributed by upregulated gene expression. In sharp contrast, no significant differences were observed for lamin-associated proteins, LAP2A and LAP2B (Fig. 8A,B), which are known to interact with LMNA/C and LMNB1 (Wilson and Foisner, 2010). We also failed to detect any significant changes on LMNB2 (another B-type nuclear lamina), GLE1 (a factor involved in mRNA export), and RANGAP1 (a nuclear pore-associated protein involving in nuclear transport; Fig. 8A, B). LMNB1 protein levels were also increased in WT-iMNs when *TOR1A* was downregulated by shRNAs (Fig. 8D). These

results indicate that LMNB1 is uniquely dysregulated on both protein expression and subcellular localization in DYT1 MNs or MNs with hypoactive TOR1A. To directly examine the effect of LMNB1 upregulation, we tried several batches of experiments to overexpress LMNB1 in MNs. Nonetheless, all MNs with ectopic LMNB1 (indicated by the co-expressed GFP) died soon after virus infection. As an alternative, we examined LMNB1 overexpression in fibroblasts of a healthy human patient. Interestingly, cells with ectopic LMNB1 (GFP+) showed a severely deformed nuclear morphology (Fig. 8E), supporting the hypothesis that LMNB1 upregulation underlies the abnormal nuclear morphology in DYT1-MNs.

Phenotypic amelioration of DYT1 MNs via LMNB1 downregulation

To determine whether LMNB1 dysregulation contributes to the pathologic phenotypes in DYT1 MNs, we knocked down its expression through a shRNA approach. The shRNA-expressing cells were marked with the co-expressed mCherry reporter. A shRNA against luciferase was used as a control. Two shRNAs were identified to be efficient in reducing the expression of endogenous LMNB1 determined by both western blots and immunocytochemistry (Fig. 9A,B). Interestingly, downregulation of LMNB1 resulted in a much-reduced frequency of LMNB1 mislocalization in DYT1-iMNs (Fig. 9C). It also significantly improved neurite outgrowth (Fig. 9D), increased the number of primary branches (Fig. 9E), and markedly reduced the percentage of cells with abnormal nuclear morphology (Fig. 9F). When compared with the control shRNA, FISH analysis with DIG-labeled oligo-dT probes showed that *LMNB1*-shRNAs significantly reduced nuclear accumulation of mRNAs in DYT1-iMNs (Fig. 9G,H). Together, these results clearly indicate that LMNB1 dysregulation is a salient pathologic feature contributing to the delayed neuronal development, abnormal nuclear morphology, and impaired NCT.

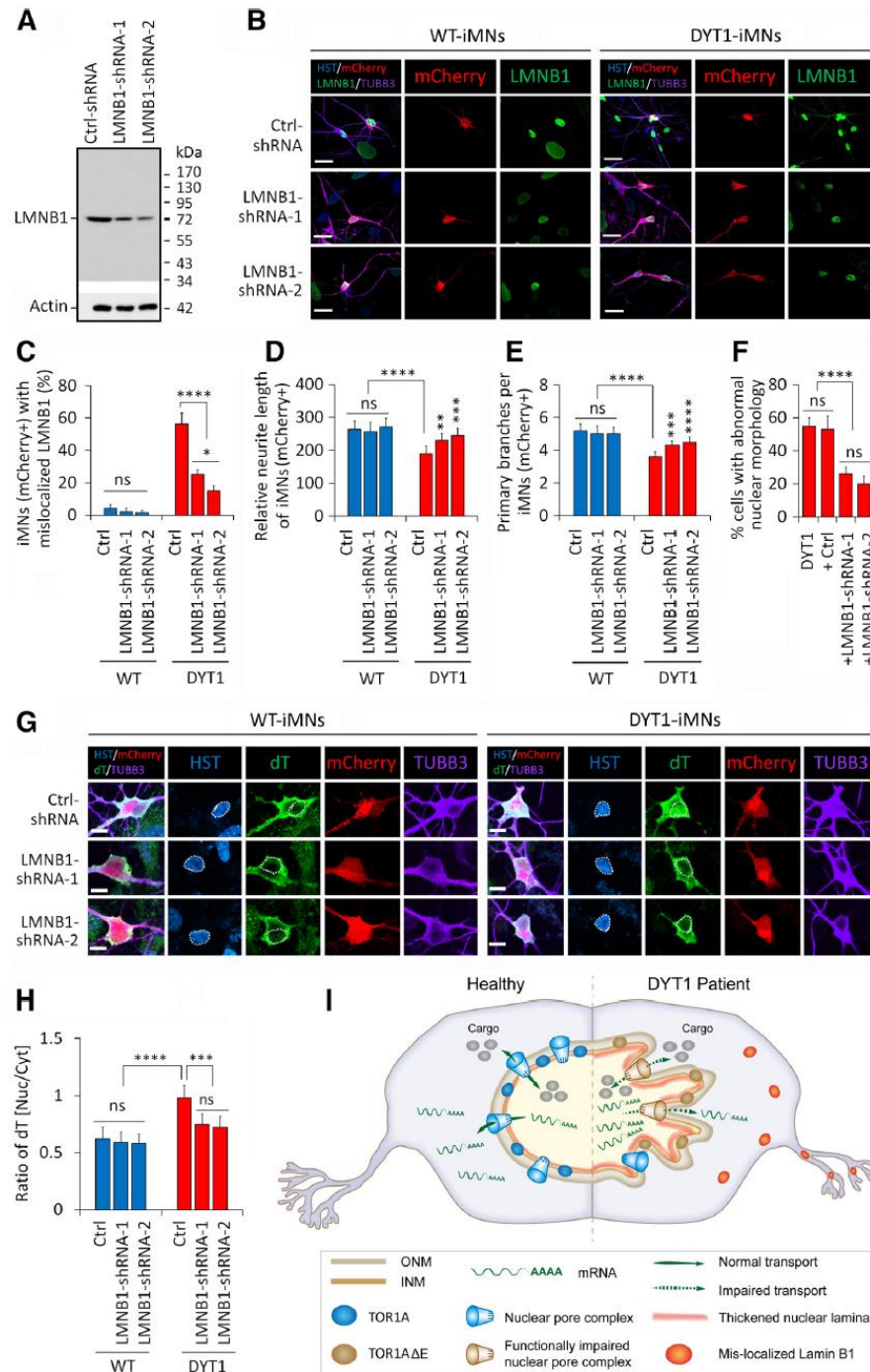


Figure 9. Phenotypic amelioration of DYT1 MNs via LMNB1 downregulation. **A**, Western blottings showing shRNA-mediated downregulation of LMNB1. The single band of LMNB1 on the whole blotting membrane indicates the specificity of LMNB1 antibody. Actin serves as a loading control. **B**, Representative confocal images of iMNs with the indicated shRNAs (mCherry+) at 3 wpi. Scale bar: 25 μ m. **C**, Percentage of iMNs (mCherry+) with mislocalized LMNB1 in **B** [n (neurons) > 200 for each condition from triplicates; ns, not significant; * p = 0.032, **** p < 0.0001, ANOVA]. **D**, Quantitative analysis of neurite length of iMNs (mCherry+) at 2 wpi under the indicated conditions [n (neurons) > 100 for each sample from triplicates; ns, not significant; ** p = 0.0074, *** p = 0.00017 (vs DYT1-Ctrl), ANOVA; **** p = 1.26E-05 (WT-Ctrl vs DYT1-Ctrl), t test]. **E**, Neurite number of the primary branches in iMNs (mCherry+) at 4 wpi [n (neurons) > 100 each sample from triplicates; ns, not significant; **** p < 0.00043, **** p = 7.42E-05 (vs DYT1-Ctrl), ANOVA; **** p = 2.833E-15 (WT-Ctrl vs DYT1-Ctrl), t test]. **F**, Percentage of DYT1-iMNs with abnormal nuclear morphology at 3 wpi (n = 104 for DYT1, n = 98 for Ctrl-shRNA, n = 72 for LMNB1-shRNA1 and n = 69 for LMNB1-shRNA2 from triplicates; ns, not significant; **** p = 5.34E-08, ANOVA). **G**, Representative confocal images of FISH assay of iMNs at 3 wpi. shRNA-expressing cells are identified by the co-expressed mCherry. Dotted lines highlight nuclei. Scale bar: 10 μ m. **H**, Quantification of oligo-dT signal distribution in **G** [n (neurons) > 100 for each sample from triplicates; ns, not significant; **** p = 0.00063, ANOVA; **** p = 5.34E-09 (WT-Ctrl vs DYT1-Ctrl), t test]. **I**, A working model for DYT1 pathogenesis. The healthy MNs have normal nuclear morphology and NCT activity. In DYT1 MNs, LMNB1

Discussion

Induced human neurons as novel models for DYT1 dystonia
Most cases of DYT1 dystonia are caused by a single amino acid deletion in TOR1A under a heterozygous background. Interestingly, mice with the identical *Tor1a* mutation as a heterozygote failed to show any pathologic phenotypes (Goodchild et al., 2005), suggesting that species-specific differences may play a role in DYT1 pathology. Nonetheless, the limited access to patient neurons has greatly impeded the progress of DYT1 dystonia research. In this study, we generated DYT1 MNs via two strategies: direct conversion from patient fibroblasts and iPSC-based reprogramming and differentiation. These neurons are in the same genetic background as human patients and maintain endogenous levels of TOR1A expression. As such, they provide an unprecedented *in vitro* cellular model for dystonia research.

DYT1 MNs from both strategies show similar phenotypes including delayed neuronal development, abnormal nuclear morphology, and disrupted mRNA nuclear export and protein nuclear transport. Unlike iPSCs, which are epigenetically reset to an embryonic state (Lapasset et al., 2011; Soria-Valles and López-Otín, 2016), directly reprogrammed neurons from donors retain features associated with age and disease (Mertens et al., 2015; Tang et al., 2017). We consistently find that DYT1-diMNs from fibroblasts exhibit more severe defects than DYT1-iMNs from iPSCs in neurite outgrowth, nuclear morphology, and NCT. However, the iPSC-based strategy has several unique advantages: both the yield and the purity of iMNs are much higher than diMNs, and the culture time to obtain mature neurons is also shorter. Therefore, iMNs are suitable for experiments requiring a large amount of samples, whereas diMNs can provide aging-relevant neurons for single cell analysis. Both strategies should be valuable in DYT1 dystonia research.

Our study further provides evidence that lower MNs are implicated in the pathogenesis of dystonia. We reveal for the first time that LMNB1 is upregulated and mislocalized in DYT1 MNs. Most interestingly, downregulation of LMNB1 can significantly improve development, NE morphology, and mRNA nuclear export of DYT1 MNs. Our results support a model that upregulated LMNB1 causes thickening of the nuclear lamina, thereby leading to abnormal nuclear morphology and impaired NCT in DYT1 MNs (Fig. 9I). Progressive LMNB1 accumulation and subcellular mislocalization are key pathologic features of DYT1 MNs and may constitute a novel molecular target for intervention.

Increased nuclear LMNB1 causes abnormal NE morphology

Nuclear LMNB1 is an essential scaffolding component of the lamina meshwork beneath the NE. It plays important roles in determining nuclear morphology, DNA regulation, and gene expression (Broers et al., 2006; Turgay et al., 2017). Overexpression of LMNB1 is shown to increase nuclear rigidity (Ferrera et al., 2014). This is consistent with our result that LMNB1 overexpression can disrupt nuclear morphology in fibroblasts. An abnormal extra copy of *LMNB1* gene causes autosomal dominant leukodystrophy (ADLD) characterized by loss of myelin in the brain and the spinal cord, leading to movement problems (Lin et al., 2014; Padiath, 2016; Köhler et al., 2018). Consistently, we find that LMNB1 expression is

obviously increased in DYT1 MNs, and the NE morphology is abnormal at both light-microscope and electron-microscope levels. Downregulation of LMNB1 in DYT1 MNs can alleviate the defects in neuron development, NE morphology, and mRNA nuclear export, suggesting that increased LMNB1 contributes to these abnormalities in DYT1 neurons.

Despite intensive efforts examining the NE structure by TEM, we failed to detect bleb-like structures in DYT1 MNs. Such a result is in sharp contrast to what was observed in *Drosophila* with *torsin* mutant or in mice with *Tor1a* knock-out or *Tor1a*^{ΔE/ΔE} (Goodchild et al., 2005; Jokhi et al., 2013; Tanabe et al., 2016). This discrepancy may be caused by species differences and/or functional redundancy between different human *Torsin* genes in NE morphology maintenance (Laudermilch et al., 2016; Li et al., 2020). Only one *torsin* gene exists in *Drosophila* whereas there are four *Torsin* genes in humans. Additionally, all human patients are heterozygous with one remaining WT copy of *TOR1A* gene that may also diminish such severe abnormalities as nuclear blebs. On the other hand, the formation of these bleb-like structures is cell cycle dependent (Rampello et al., 2020) and developmentally regulated (Tanabe et al., 2016). The bleb-like structures could occur transiently before neuronal differentiation but be quickly resolved at later stages, at which the samples we have examined. As such, it would be minor, if any, for a potential contribution of a defective NE-budding mechanism (Speese et al., 2012) to the impaired mRNA export in differentiated DYT1 MNs. In contrast, our results rather suggest that an impaired NPC-mediated transport pathway is the major causing factor for the defects observed in human DYT1 MNs.

Defective nuclear pore biogenesis and impaired NCT

The NE and embedded NPCs play critical roles in regulating NCT of both transcripts and proteins (Köhler and Hurt, 2007; Grünwald et al., 2011; Katahira, 2015; Beck and Hurt, 2017). Accumulating evidence indicates that *Torsin* and its cofactor system could be involved in the nuclear pore biogenesis or homeostasis (Laudermilch et al., 2016; Chase et al., 2017; Rampello et al., 2020). In *Torsin*-deficient cells, one distinguished cellular feature is the “blebbing” structures at perinuclear space as discussed above. This phenotype has been observed in neural tissues of DYT1 mouse models (*Tor1a* knock-out or *Tor1a*^{ΔE/ΔE} overexpression; Goodchild et al., 2005; Tanabe et al., 2016) and mammalian cells with combined knock-outs of multiple *Torsins* (Laudermilch et al., 2016; Rampello et al., 2020). Interestingly, a subset of FG-Nups have been identified at the electron-dense base of NE blebs and the size of this density is similar to mature NPCs (Laudermilch et al., 2016). By using *Torsin* deletion HeLa cell line (4TorKO with deletion of all four *Torsin* genes *TOR1A*, *TOR1B*, *TOR2A*, and *TOR3A*), Rampello et al. (2020) recently demonstrated that *Torsin* ATPase deficiency leads to defects in nuclear pore biogenesis. Supporting such a conclusion, we find significantly fewer NPC structures in DYT1 MNs than controls.

Although the precise mechanism of NPC assembly is not fully elucidated, two different assembly pathways have been proposed. One pathway occurs postmitotically while NE reforms and the other occurs during interphase (Doucet et al., 2010). The model of an inside-out extrusion of the NE reveals a multistep process of the nuclear pore assembly during interphase (Otsuka, 2016). This assembly starts from the evaginations of inner nuclear membrane as intermediates, which initially contain the nuclear and cytoplasmic ring component of the NPC. While protruding toward the outer nuclear membrane, several subcomplexes of the NPC will be assembled, and then the inner and outer nuclear

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upregulation leads to thickened nuclear lamina, cytoplasmic localization, abnormal nuclear morphology, and impaired NCT, which may subsequently lead to mislocalization of mRNAs and proteins and dysfunction of neurons.

membranes are fused together. Some components, such as the cytoplasmic filament NUP358, are added to the NPC after this fusion event, completing the assembly of mature NPCs from intermediates (Otsuka, 2016). Recent reports suggest that Torsin plays an important role in adding NUP358 to form mature NPCs in the interphase insertion pathway. During postnatal maturation, *Tor1a*-null mouse neurons develop mislocalized and dysfunctional NPCs that lack NUP358 (Pappas et al., 2018). In *Torsin*-deficient cells, NUP358 is underrepresented relative to FG-nucleoporins in nuclear envelopes (Rampello et al., 2020). In current study, our measurement of the nuclear pore size under TEM fails to find significant differences between DYT1 MNs and controls. A caveat is that the resolution of TEM may not detect the diameter differences between immature (without NUP358) and mature NPCs (with NUP358). Given that ~50% of all NPCs are installed via a postmitotic insertion pathway (Doucet et al., 2010), it may not be surprising that the nuclear pore size is relatively normal in mature DYT1 neurons, a developmental stage at which Torsin has less of an effect on NPC assembly. Nonetheless, the abnormally assembled NPCs at early developmental stages may cause persistent defects on neuronal nuclear pores and NCT (Pappas et al., 2018).

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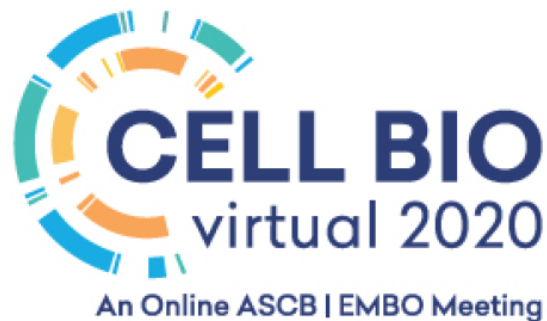
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Appendix E-Poster presentation

9/29/2020

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Current Date/Time: 7/30/2020 11:52:00 AM

Dysregulated nuclear LMNB1 and impaired nucleocytoplasmic transport in Dystonia patient-derived neurons

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³Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX.

Abstract:

Dystonia is the third most common movement disorder characterized by sustained or intermittent muscle contractions. There is no specific treatment to cure this disease due to its unclear pathological mechanisms. The childhood onset DYT1 dystonia represents the most frequent and severe form of hereditary dystonia, providing an excellent example to understand this disease. DYT1 dystonia is caused by a heterozygous mutation in *torsin A (TOR1A)*, a membrane-embedded ATPase. While animal models provide insights into disease mechanisms, significant species-dependent differences exist since mice with the identical heterozygous mutation fail to show pathology. Here, we model DYT1 by using human patient-derived motor neurons. These neurons with the heterozygous *TOR1A* mutation show markedly thickened nuclear lamina, disrupted nuclear morphology, and impaired nucleocytoplasmic transport, whereas they lack the perinuclear "blebs" that are often observed in animal models. Importantly, we further uncover that the nuclear lamina protein LMNB1 is specifically dysregulated in expression and subcellular localization. LMNB1 downregulation can largely ameliorate all the cellular defects in DYT1 motor neurons. These results reveal the value of disease modeling with human neurons and provide novel molecular mechanisms underlying DYT1 dystonia and potentially other neurological diseases with impaired nucleocytoplasmic transport.

This work was supported by NIH grants (NS092616, NS099073, and NS088095 to C.L.Z.; R21NS112910 to B.D.), and Friends of the ADC and NIH ADC grant (NIH/NIA P30-12300-21 to B.D.) and DoD PRMRP grant (W81XWH2010186 to B.D.).

:

Appendix F-The PI'S updated CV

CURRICULUM VITAE Baojin Ding M.D., PH.D.

TITLE AND CONTACT INFORMATION

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EDUCATION

2010 Ph.D. in Biochemistry and Molecular Biology of Veterinary Medical Sciences,
Louisiana State University, School of Veterinary Medicine, Baton Rouge, LA

2004 M.S. of Medicine in Clinical Laboratory
Wenzhou Medical College, Wenzhou, Zhejiang, P. R. China

2001 Bachelor Degree of Medicine (M.D. equivalent)
Medical College of Qingdao University, Qingdao, Shandong, P. R. China

RESEARCH SUPPORT

Current

NIH/NINDS R21 (Grant Number: NS112910) (PI) 04/01/2020-09/30/2021

Title: *Determining the Pathogenesis of DYT1 Dystonia in Reprogrammed Human Neurons*

Source: NINDS Exploratory Neuroscience Research Grant [PA18-358]

Overall goal: To determine the pathogenesis of childhood onset DYT1 dystonia in patient-specific neurons that are generated by direct conversion and iPSC-based reprogramming and differentiation.

DoD (W81XWH2010186) Peer Reviewed Medical Research Program (PRMRP) Discovery Award (PI)
; 03/15/2020-3/14/2022

Title: *Determining the Pathogenesis of Dystonia in Reprogrammed Human Neurons*

Source: Department of Defense, Congressionally Directed Medical Research Programs (CDMRP)

Overall goal: To determine the pathogenesis of adulthood onset dystonia via directly reprogramming human neurons from patient fibroblasts.

Faculty Start-up Package (PI) 2018-2022

Title: New laboratory set up.

Source: University of Louisiana at Lafayette

Enhancement of Biology Research and Teaching through Personal Flow Cytometry (Co-PI) 06/01/2020-06/30/2021, _____

Source: Louisiana Board of Regents Support Fund, Departmental Enhancement

Goals: To improve the educational and research infrastructure in the department of Biology in UL Lafayette

Completed

Lahaye Faculty Development Grant (PI) 2019

This fund is intended to support undergraduate researchers working on biomedical studies.

NIH/NIA P30-12300-21 (PI) 2017 -2018

Title: *Nucleocytoplasmic Transport Defect in Alzheimer's Disease*

Source: The Friends of the Alzheimer's Disease Center and NIH Alzheimer's Disease Center

Major goal: to understand how nucleocytoplasmic transport defect contributes to Alzheimer's disease (AD) by using mouse primary neurons, mammalian cell lines and directly reprogrammed neurons (diNs) from fibroblasts of AD patients.

PROFESSIONAL EXPERIENCE

2018- Present Assistant Professor

Department of Biology, University of Louisiana at Lafayette, Lafayette, LA 70503

Research area: Cell Biology, Neuroscience and Neurological Diseases

2016- 2018 Assistant instructor

Department of Molecular Biology, Center for Regenerative Science and Medicine,

UT Southwestern Medical Center, Dallas, TX

Research interests: Nucleocytoplasmic transport in neural development and neurological disorders.

2010-2016 Postdoctoral Fellow

Departments of Physiology and Neurobiology, University of Massachusetts Medical School, Worcester, MA

Research topic: Molecular Mechanisms of Gene Expression in Neurodevelopment and nuclear mRNA export.

2005 —2010 Graduate Research Assistant

Department of Comparative Biomedical Science, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA

Research topic: The Molecular Mechanisms of Transcription Coupled DNA Repair in Eukaryotic cells. (Ph.D. Dissertation: http://etd.lsu.edu/docs/available/etd-04072010-012548/unrestricted/Ding_diss.pdf)

2004 —2005 Research Associate

Department of Biological Sciences, Louisiana State University, Baton Rouge, LA

Research topic: The Biogenesis and Functions of Iron-Sulfur Cluster proteins.

2002 —2004 Research Assistant

The Institute of Cellular and Molecular Medicine, Wenzhou Medical College, Wenzhou, Zhejiang, P. R. China

Research topic: Isolation, Purification and Screening of Effective Components from Natural Products.

2000 —2001 Intern Doctor

Affiliated Hospital of Medical College of Qingdao University, Qingdao, P. R. China

SELECTED PUBLICATIONS (*Dr. Baojin Ding is the corresponding author)

<https://www.ncbi.nlm.nih.gov/myncbi/baojin.ding.1/bibliography/public/>

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6. **Ding B**, Dobner PR, Mullikin-Kilpatrick D, Wang W, Zhu H, Chow CW, Gronostajski RM and Kilpatrick DL. (2018). BDNF Activates an NFI-Dependent Neurodevelopmental Timing Program By Sequestering NFATc4. *Mol Biol Cell*. 2018 Apr 15; 29(8):975-987
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8. Li Y, Hassinger L, Thomson T, **Ding B**, Ashley J, Hassinger W and Budnik V. (2016). Lamin Mutations Accelerate Aging via Defective Export of Mitochondrial mRNAs through Nuclear Envelope Budding. *Curr Biol*. 2016 Aug 8;26(15):2052-9
9. **Ding B**, Cave HW, Dobner PR, Kilpatrick DM, Bartsokis M, Zhu H, Chow CW, Gronostajski RM and Kilpatrick DL. (2016) Reciprocal Auto-Regulation by NFI Occupancy and ETV1 Promotes the Developmental Expression of Dendrite-Synapse Genes in Cerebellar Granule Neurons. *Mol Biol Cell*. 2016 May 1;27(9):1488-99
10. Packard M, Jokhi V, **Ding B** and Budnik V. (2015) Nucleus to Synapse Nesprin Railroad Tracks Direct Synapse Maturation through RNA localization. *Neuron*. 86(4):1015-28).
11. **Ding B**, Wang W, Selvakumar T, Xi HS, Zhu H, Chow CW, Horton JD, Gronostajski RM and Kilpatrick DL. (2013) Temporal Regulation of Nuclear Factor One Occupancy by Calcineurin/NFAT Governs a Voltage-Sensitive Developmental Switch in Late Maturing Neurons. *J Neurosci*. 33(7):2860-2872.
12. **Ding B** and Kilpatrick DL. (2013) Lentiviral Vector Production, Titration, and Transduction of Primary Neurons. *Methods Mol Biol*. 1018:119-31. Chapter 12.
13. **Ding B** and Kilpatrick DL.(2013). Chromatin Immunoprecipitation Assay of Brain Tissue Using Percoll Gradient-Purified Nuclei. *Methods Mol Biol*. 1018:199-209. Chapter 19.
14. **Ding B**, Lejeune D and Li S. (2010) The C-terminal Repeat Domain of Spt5 Plays an Important Role in Suppression of Rad26-independent Transcription Coupled Repair. *J Biol Chem*. 285 (8): 5317-5326.
15. Chen X, **Ding B**, Lejeune D, Ruggiero C and Li S. (2009) Sumoylation of Rpb1 in Response to UV Radiation or Impairment of Transcription Elongation in Yeast. *PLoS One*. 4 (4) e5267.
16. **Ding B**, Ruggiero C, Chen X and Li S. (2007) Tfb5 is Partially Dispensable for Rad26 Mediated Transcription Coupled Nucleotide Excision Repair in Yeast. *DNA Repair (Amst)*. 6 (11): 1661- 1669.

17. Li S, **Ding B**, LeJeune D, Ruggiero C, Chen X and Smerdon MJ. (2007) The Roles of Rad16 and Rad26 in Repairing Repressed and Actively Transcribed Genes in Yeast. **DNA Repair (Amst)**. 6 (11): 1596-1606.
18. Li S, **Ding B**, Chen R, Ruggiero C and Chen X. (2006) Evidence that Transcription Elongation Function of Rpb9 is Involved in Transcription Coupled DNA Repair in *Saccharomyces cerevisiae*. **Mol Cell Biol**. 26 (24): 9430-9441.
19. **Ding B**, Smith ES, and Ding H. (2005) Mobilization of Iron Center in IscA for Iron-sulfur Cluster Assembly in IscU. **Biochem. J**. 389 (Pt 3):797-802.

TEACHING

- **Fall Semester (2018- present)**
BIOL-457(G), Advanced Cell Biology, CRN 224133, 3 Credits Lecture
This course aims to examine life at its most fundamental level, including mechanisms and pathways responsible for membrane transport, metabolism, gene expression, protein synthesis and secretion, membrane trafficking, cytoskeleton dynamics, and cell signaling.
BIOL-458(G), Advanced Cell Biology Lab, 2 Credits, 4 Hours Lab
This class is intended to immerse students in the process of performing scientific experiments, through which students will master some experimental skills, acquire the capabilities for data analysis and result interpretation. The model system will be cultured mammalian cells and/or fixed cells on slides.
BIOL-410, 1-6 Credit(s) Individual Project
Cellular and Molecular Neuroscience - from Basic Knowledge to Advanced Techniques
- **Spring Semester (2019-present)**
BIOL-472(G), Neurophysiology, 3 Credits Lecture
This course aims to understand neurophysiological activities at cellular and molecular levels and their linkage to human neurological diseases.
BIOL-473(G), Neurophysiology Lab, 1 Credit Lab
BIOL-410, 1-6 Credit(s) Individual Project
Cellular and Molecular Neuroscience - from Basic Knowledge to Advanced Techniques

STUDENTS/POSTDOCS MENTORED (*co-authors of publication)

Postdoc Researcher/Visiting Scholar Mentored

1. *Dr. Masood Sepehrimanesh (Ph.D., D.V.M., Shiraz University of Medical Sciences, Shiraz, Iran. Postdoctoral Research Associate, 03/2020- present)
2. Dr. Ameneh Zare-Shahabadi (M.D. Tehran University of Medical Sciences, Iran. Research Assistant, 2018-2019, UL Lafayette. Current: Medical Doctor imain advisorn Cincinnati, OH)

Graduate Students (Main Advisor)

1. *Masuma Akter (PHD, 08/2019-, UL Lafayette)
2. Jacob Stagray (PHD, 08/2019-, UL Lafayette)
3. Haochen Cui (PHD, 01/2020-, UL Lafayette)
4. Casey Cottee (MSci, 08/2020-, UL Lafayette)
5. Abir Hosain (PHD, 01/2021-, UL Lafayette)
6. Makaila Mitchell (MSci, 01/2019-12/2019, UL Lafayette)

Graduate Students (Committee Member)

1. Santosh Paudel (PhD, UL Lafayette, 2018-)
2. Brittany M. Pitrucha (PhD, UL Lafayette, 2018-)
3. Chukwunonso Chukwudozie (MSc, UL Lafayette NIRC, 2018-2020)
4. Alex Esteve, (MSc. UL Lafayette, 2018-)

Undergraduate students

1. Catherine Wertz (Scholarship, Biology major, UL Lafayette. 01/2020-)
2. Bennett Garbarino (Scholarship, Biology major, UL Lafayette. 08/2018-)
3. Matthew Authement (Scholarship, Biology major, UL Lafayette. 08/2018-)
4. Casey A. Coutee (Undergraduate Researcher, Biology major, UL Lafayette. 01/2019-05/2020)
5. Bao Doan (BIOL410, Biology major, UL Lafayette. Fall 2019, Spring 2020)
6. Audrey Lumpki (BIOL410, Biology major, UL Lafayette. Fall 2019)
7. Erin Miller (BIOL410, Biology major, UL Lafayette. Fall 2019)
8. Joy Aniede (STARS Summer Research Program, 2017, UT Southwestern Medical Center)
9. Adrienne Lemieux (Worcester State University, 2016, UMass Medical School)
10. Alexandra D'Ordine (Worcester Polytechnic Institute, Class of 2017; 2015 at UMass Medical School)
11. Emily Vancor (NIH, MIT 2014; 2013 at UMass Medical School) *Ms. Emily Vancor obtained the second place of the summer research presentation. This research experience made her stand out from many applicants and got acceptance by Yale School of Medicine in 2014.*
12. *Marina Bartzokis (2012-2014, UMass Medical School)
13. Salvador Esparza (NIH, MIT 2014, 2012 at UMass Medical School)
14. Robert Lumley (The 2011 Summer Research Fellowship Program at UMass Medical School)
15. Amy Corron (The 2011 Summer Research Fellowship Program at UMass Medical School)
16. Tatenda Mujeni (HHMI, Bennett College For Women, 2009, Louisiana State University)

HONORS AND AWARDS

James H. Harper South Louisiana Mid-Winter Fair Association/BORSF Professorship (2020-2023)
DoD Peer Reviewed Medical Research Program (PRMRP) Discovery Award (2020)
Lahaye Faculty Development Grant (2019, 2020)
Alzheimer's Disease Research Award from Friends of the Alzheimer's Disease Center (2017)
American Association of Anatomists (AAA) Travel Award (2013, 2014)
American Society for Microbiology Postdoctoral Research Fellowship Program (2010, declined)
Guang Hua Scholarship (2002, 2003)

PROFESSIONAL ACTIVITIES

Professional Services

Associate Editor

Frontiers in Molecular Neuroscience (2018-)

Editorial Board

American Journal of Psychiatry and Neuroscience (AJPN) (2020-);
Austin Neurology (2016-2018);
Journal of Autism & Related Disabilities (2016-2018);
Journal of Down Syndrome & Chromosome Abnormalities (2015-2017);
Frontiers in Molecular Neuroscience (2013-2018)

Grant Reviewer

European Research Council (ERC) grant review (2016)

Ad Hoc Reviewer: *Neurobiology of Disease; Scientific Reports; Journal of Cellular Biochemistry; Journal of Cellular and Molecular Medicine; Frontiers in Molecular Neuroscience, Analytica Chimica Acta, Frontiers in Computational Neuroscience, Journal of Neurosciences in Rural Practice, Neural Regeneration Research etc.*

Scientific Member:

Member of American Society for Cell Biology (ASCB) (2017-)
Member of Society for Neuroscience (2014-)
Member of American Association of Anatomists (AAA) (2012-2016)
Member of American Association for the Advancement of Science (AAAS) (2009-2013)
Member of American Society for Microbiology (ASM) (2009-2012)
Member of Environmental Mutagenesis Society (EMS) (2009-2010)

University Services

Faculty Search Committee for Physiologist (2019-2020)
Pre professional Committee (2020- present)
-Review applicants for professional school and write letters of support.

Community services

Louisiana Region VI Science and Engineering Fair Judge 2019
Massachusetts State Middle School Science and Engineering Fair Judge (2014-2016)
Reuters Health
<http://www.psychcongress.com/article/tiny-brain-organoids-show-neuron-differentiation-autism-23405>

ORAL PRESENTATIONS AND INVITED TALKS (Selected from >20)

1. January 23, 2020. *Impaired Nucleocytoplasmic Transport in Neurological Diseases*. Department of Biological Sciences, University of Texas at Dallas, Dallas, TX (Host: Dr. Heng Du)
2. July 19, 2019. *Nucleocytoplasmic Transport in Neurodegenerative Diseases*. National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, Hunan Province, China. (Host: Dr. Yu Tang)
3. May 7, 2018. *Spatiotemporal Regulation of Gene Expression*. Department of Biology, University of Louisiana at Lafayette, Lafayette, LA. (Host: Dr. Karl H. Hasenstein)
4. April 26-30, 2014. *Auto-Regulatory Interactions Between NFI Occupancy and ETV1 Direct the Timing of Gene Expression in Late Maturing Neurons*. Experimental Biology Annual Meeting. San Diego, CA.
5. August 7, 2013. Department of Neurology, University of Massachusetts Medical School. Worcester, Massachusetts (Host: Dr. Fen-Biao Gao)

6. July 30, 2013. Department of Microbiology & Immunobiology, New England Primate Research Center, Harvard Medical School. (Host: Dr. Min Dong)
7. July 5, 2013. Gene Therapy Center, University of Massachusetts Medical School. Worcester, Massachusetts (Host: Dr. Guangping Gao)
8. April 8, 2010. Stowers Institute for Medical Research, Kansas City, Missouri (Host: Dr. Jerry Workman)

POSTER PRESENTATIONS (Selected from 15)

1. **Ding B.**, Yu T. and Zhang C-L. *Dysregulated nuclear LMNB1 and impaired nucleocytoplasmic transport in Dystonia patient-derived neurons*. The American Society for Cell Biology (ASCB) Annual Meeting. December 2-16, 2020. ASCB/EMBO Virtual Meeting.
2. K Selvam, **B Ding**, R Sharma and S Li. *Promotion of error-free transcriptional bypass of DNA lesions is essential for Rad26 to facilitate transcription coupled DNA repair*. ENVIRONMENTAL AND MOLECULAR MUTAGENESIS 59, 71-71, 2018.
3. **Ding, B.**, Dobner PR. and Kilpatrick DL. *Nuclear Factor One (NFI)-Dependent Developmental Program Directs the Timing of Gene Expression in Maturing Neurons*. The American Society for Cell Biology (ASCB) Annual Meeting. December 2-6, 2017. Philadelphia, Pennsylvania.
4. **Ding B.**, Mirza A.M., Budnik V. and Munson M. *An alternative nuclear export pathway in Saccharomyces cerevisiae*. The American Society for Cell Biology (ASCB) Annual Meeting. December 3-7, 2016. San Francisco, California.
5. **Ding, B.**, Cave J. and Kilpatrick DL. *Auto-Regulatory Interactions Between NFI Occupancy and ETV1 Direct the Timing of Gene Expression in Late Maturing Neurons*. American Association of Anatomists (AAA) Annual Meeting. April 26-30, 2014. San Diego, California. (Also accepted as oral presentation)
6. **Ding, B., and** Kilpatrick DL. *Nuclear factor one controls a voltage-sensitive developmental switch required for late neuronal maturation*. American Association of Anatomists (AAA) Annual Meeting. April 20-24, 2013. Boston, Massachusetts. (Postdoctoral Poster Presentation Award Finalist)
7. **Ding, B.**, LeJeune, D and Li, S. *Phosphorylation of Yeast Spt5 C-terminal Repeat by Bur1 Kinase Is Involved in the Suppression of Transcription Coupled DNA Repair*. Environmental Mutagen Society 40th Annual Meeting. October 24-28, 2009. St. Louis, Missouri.
8. **Ding, B.**, Chen X., Ruggiero, C., LeJeune, D. and Li, S. *Spt4 and Spt5 cooperatively suppress transcription coupled DNA repair through binding to RNA polymerase II in the absence of Rad26*. ASBMB (American Society for Biochemistry and Molecular Biology) annual meeting. April 18-22, 2009, New Orleans, Louisiana.

LINKS

Ding Lab

www.DingLabBiomed.com

Google Scholar Profile

<https://scholar.google.com/citations?user=gGuQKNIAAAAJ&hl=en>