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A cellular model for sporadic ALS using patient-derived induced pluripotent stem cells

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Abstract

Development of therapeutics for genetically complex neurodegenerative diseases such as sporadic amyotrophic lateral sclerosis (ALS) has largely been hampered by lack of relevant disease models. Reprogramming of sporadic ALS patients' fibroblasts into induced pluripotent stem cells (iPSC) and differentiation into affected neurons that show a disease phenotype could provide a cellular model for disease mechanism studies and drug discovery. Here we report the reprogramming to pluripotency of fibroblasts from a large cohort of healthy controls and ALS patients and their differentiation into motor neurons. We demonstrate that motor neurons derived from three sALS patients show *de novo* TDP-43 aggregation and that the aggregates recapitulate pathology in postmortem tissue from one of the same patients from which the iPSC were derived. We configured a high-content chemical screen using the TDP-43 aggregate endpoint both in lower motor neurons and upper motor neuron like cells and identified FDA-approved small molecule modulators including Digoxin demonstrating the feasibility of patient-derived iPSC-based disease modelling for drug screening.

Keywords

ALS; iPS; TDP-43; Digoxin; drug screening

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Author Contributions: A.J. conceived of and led the project, developed MN differentiation method, and wrote the manuscript. A.J. designed the experiments with M.F.B. and F.J.M. M.F.B. and F.J.M. completed the experiments. D.V. developed image processing and data analysis algorithms. C.R., F.J.M., M.F.B. performed cell culture and differentiation. R.M., H.G., U.S.-M. reprogrammed fibroblasts. M.G. and J.G. performed qPCR. S.W., R.B., C.J., I.G.-P., E.V. carried out small molecule screening. V.D. carried out electrophysiology. S.I., B.C., L.N., J.B. developed CN differentiation method. J.L. established robotics and automation routines.

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset genetically complex progressive neurodegenerative disease marked by degeneration of upper and lower motor neurons (MN) (Pasinelli and Brown, 2006). About 10% of cases are familial (fALS) and 90% are sporadic (sALS) with largely unknown genetic etiology (Pasinelli and Brown, 2006). In addition, ALS patients differ significantly in presentation of clinical symptoms including site of onset, rate of progression, presence of cognitive dysfunction, and co-morbidity with Frontotemporal lobar degeneration (FTLD) (Lomen-Hoerth, 2011). While animal models have been developed for a form of familial ALS caused by mutations in the SOD1 gene, no models of sALS exist and no effective treatments that can extend the lives of patients beyond a few months are available.

The most prominent form of pathology present across sALS patients includes changes in TAR DNA-binding protein 43 (TDP-43, *TARDBP*) expression and subcellular localization (Arai et al., 2006; Mackenzie et al., 2007; Neumann et al., 2009; Neumann et al., 2007; Neumann et al., 2006). Histological studies of postmortem tissue have shown that TDP-43 pathology is also present in patients with other neurodegenerative diseases including FTLD, Lewy body dementia, and Alzheimer's disease (AD) suggesting its potential central role in neurodegenerative diseases (Amador-Ortiz et al., 2007; Arai et al., 2006; Higashi et al., 2007; Nakashima-Yasuda et al., 2007; Neumann et al., 2007). A minority of familial ALS and FTLD patients carry mutations in TDP-43 possibly explaining the formation of TDP-43 pathology in these patients (Kabashi et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008). However, nearly all sporadic ALS patients show signs of TDP-43 pathology and the cause of TDP-43 pathology in sporadic ALS, FTLD, and AD remains elusive (Kabashi et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008).

Different forms of TDP-43 pathology reported in ALS and FTLD patient central nervous system (CNS) include cytoplasmic mis-localization; hyperphosphorylated, intranuclear and cytoplasmic TDP-43 positive aggregates; TDP-43 and ubiquitin-positive aggregates; and nuclear clearing of TDP-43 (Arai et al., 2006; Arai et al.; Inukai et al., 2008; Neumann et al., 2007; Neumann et al., 2006). While cytoplasmic inclusions are the predominant forms of TDP-43 pathology, neuronal intranuclear TDP-43 inclusions have also been reported in postmortem tissue from some patients (Arai et al., 2006; Cairns et al., 2007; Collins et al., 2012; Neumann et al., 2009; Neumann et al., 2007; Neumann et al., 2006).

Sporadic ALS patient induced pluripotent stem cell (iPSC)-neurons that show *de novo* TDP-43 pathology would be invaluable for gaining insight into biology of wild type TDP-43 aggregation and drug discovery. Here we report that iPSC-MN derived from three sporadic ALS patients showed spontaneous intranuclear and hyperphosphorylated TDP-43 aggregates. We also report that postmortem brain and spinal cord tissue from one of the patients from which we derived the iPSC showed similar intranuclear TDP-43 aggregates therefore directly connecting our phenotype in iPSC-MN back to the same patient's pathology. We used iPSC-MN from one of these patients in a high-throughput screen of small molecules and identified previously-approved drugs with known targets that modulate TDP-43 aggregates.

Results

Reprogramming Of Patient Cells And Differentiation

We reprogrammed skin fibroblasts from 10 healthy subjects, 8 familial and 16 sporadic ALS patients into iPSCs using retroviruses carrying *KLF4*, *SOX2*, *OCT4*, and *cMYC* (Dimos et al., 2008; Takahashi and Yamanaka, 2006) (Table S1). We analyzed 92 iPSC clones from 34 patients for morphology consistent with human pluripotent cells, DNA fingerprint matching corresponding fibroblasts, presence of normal karyotype, silencing of exogenous factors, and expression of pluripotency markers (Fig. 1A, Table S1, Figs. S1). Four of the clones were also confirmed for differentiation into three germ layers by *in vitro* embryoid body formation and *in vivo* teratoma assay (data not shown). We neuralized up to five iPSC clones from each patient by inhibition of SMAD signalling and patterned these into motor neurons (Fig. 1C and 1D) (Chambers et al., 2009; Dimos et al., 2008). We found that iPSC-derived motor neurons expressed motor neuron markers ISLET1 and HB9 (Fig. 1). We also found that neurons were electrically active within 2 weeks after co-culturing with primary human astrocytes or after 3 months without astrocytes (Figs. 1 and S2). Excitability of neurons was confirmed by whole-cell patch clamp recording as well as by calcium imaging of spontaneous calcium transients that were blocked by Tetrodotoxin (Figs. 1 and S2).

TDP-43 Aggregates In Patient-Derived Motor Neurons

All cells in the iPSC-MN cultures from healthy and ALS patients stained positive for TDP-43 with nuclear localization consistent with its reported ubiquitous nuclear expression pattern (Sephton et al.) (Fig. 2A, Fig. S3). Motor neurons had higher levels of TDP-43 expression as compared to other cells in culture such as progenitors (Fig. S2B). We identified 3 sporadic ALS patients' iPSC-MNs with abnormal TDP-43 staining, hereon referred to as TDP-43 aggregates (Figs. 2 and S3, Table S1). TDP-43 aggregates formed under basal conditions and without the use of additional stressors indicative of *de novo* TDP-43 aggregation in patient iPSC-MNs. Motor neurons from these iPSC clones reproducibly showed TDP-43 aggregates in all differentiations experiments (N = 5 per clone). In iPSC-MN from these three patients, neurons had striking TDP-43-positive apparently round structures that were morphologically similar to intranuclear inclusions previously reported in ALS and FTLN patient postmortem CNS (IPRN.0048: 2 of 5 clones tested; IPRN.015: 2 of 3 clones tested; and IPRN.0360: 4 of 5 clones tested) (Arai et al., 2006; Neumann et al., 2007; Neumann et al., 2006) (Fig. 2A–B, arrows). We observed one aggregate per neuron and aggregates were 1–2 μ m in diameter. To determine if any of the 3 sALS patients might have mutations that could result in TDP-43 pathology in their iPSC-MN, we sequenced several genes that are known to be associated with TDP-43 pathology (Cairns et al., 2007; Neumann et al., 2007). No mutations in *GRN*, *TDP-43*, *VCP*, *VAPB*, or expansion in hexanucleotide repeats in *c9ORF72* or CAG repeats in *ATXN2* were found in the sporadic ALS patients with TDP-43 aggregates, suggesting an uncharacterized genetic or epigenetic alteration as the initiator of aggregate formation in these three sALS patients. TDP-43 aggregates were absent from fibroblasts from patients (Fig. 2A, bottom panel).

Supplementary Material includes 4 figures and 2 tables

Optical sectioning of co-stained cells with nuclear envelope marker LAMIN-A revealed that TDP-43 aggregates were intranuclear suggesting recapitulation of this subtype of TDP-43 pathology (Figs. 2 A–B and S3).

We designed custom algorithms to quantify TDP-43 aggregates in confocal images of iPSC-MN cultures (Fig. S3C). TDP-43 aggregates specific to sALS samples were present two times more frequently in motor neurons (30.7% of ISLET1 or HB9-positive cells combined as compared to 16.2% of ISLET1/HB9-negative cells in sALS iPSC-MN cultures) compared to other cells in the iPSC-MN cultures in neurons derived from patient IPRN.0048 clone 1 (Fig. 2C). For neurons derived from other iPSC clones, the frequency of cells with TDP-43 aggregates varied between 2–5% (Fig. S3). The preferential presence of TDP-43 aggregates in ISLET1/HB9-positive cells mimics preferential presence of TDP-43 aggregates in ALS patient motor neurons observed postmortem and indicates sALS patient-derived iPSC-MN can model neuronal intranuclear TDP-43 pathology.

To determine whether TDP-43 aggregates in iPSC-MN are posttranslationally modified, we stained the cultures with ubiquitin and phosphoserine 409/410 specific TDP-43 antibodies. We did not find ubiquitinated TDP-43 aggregates under these conditions however TDP-43 aggregates were phosphorylated consistent with observations in patient postmortem CNS where mislocalized cytoplasmic TDP-43 aggregates have been found to be phosphorylated (Fig. 2D) (Inukai et al., 2008; Neumann et al., 2009; Neumann et al., 2006). These data suggest that phosphorylation may be a feature in common of mislocalized TDP-43 to the cytoplasm and to intranuclear aggregates. It also suggests that this model represents an early stage disease phenotype and that TDP-43 phosphorylation may also precede ubiquitination in ALS. We also co-stained neurons with markers of splicing machinery including SMN and GEMIN as well as of the proteasome but did not find any co-localization with aggregates. ALS iPSC-MN with TDP-43 aggregates did not have significantly higher levels of TDP-43 as compared to healthy controls (Fig. S2B).

We did not observe TDP-43 aggregates in iPSC-MN from healthy individuals unrelated to patients nor from fALS patients with SOD1 mutations consistent with previous postmortem reports showing lack of TDP-43 aggregates in these patient types (Mackenzie et al., 2007; Maekawa et al., 2009) (Figs. 2 and S3, Table S1). We also did not observe intranuclear TDP-43 aggregates in iPSC-MN cultures from a fALS patient with TDP-43 A315T mutation (IPRN.0270) even after 2 months in culture.

To determine whether TDP-43 aggregates would be present in upper motor neuron-like cells (layer 5 cortical neurons), that also degenerate in ALS, we differentiated iPSC clones from one of the patients (IPRN.0048, clone 1) and two healthy controls into forebrain cortical neuron lineage (here-on referred to as iPSC-CN) by dual SMAD inhibition (Figs. 3 and S4) (Arlotta et al., 2005). ALS patient iPSC-CN, but not healthy iPSC-CN, had intranuclear TDP-43 aggregates that appeared similar in size and shape to those found in iPSC-MNs (Fig. 3 A & B). TDP-43 aggregates were 3 times more frequently observed in disease-relevant CTIP2-positive neurons (Fig. 3 A–F & I).

Validation of TDP-43 Aggregate Phenotype in Patient Postmortem Pathology

To probe the comparison between iPSC models and patient pathology, we analyzed postmortem brain and spinal cord tissue available from one of the patients, who proceeded to autopsy, with the TDP-43 phenotype in iPSC-MN (patient IPRN.0360, Figure S3) for TDP-43 pathology. The presence of TDP-43 pathology was confirmed by a pathologist and was indicated in the autopsy report. Postmortem tissue from the remaining two patients was not available. We found that anterior horn neurons of the spinal cord as well as cortical neurons from this patient had round intranuclear TDP-43 aggregates that stained with higher intensity and were morphologically similar to those found in iPSC-MN derived *in vitro* from this patient (Fig. 3 G–H). These aggregates were present in cervical, lumbar, and thoracic spinal cord as well as frontal lobe neurons (Fig. 3 G–H and data not shown). Some anterior horn neurons showed additional forms of TDP-43 pathology such as cytoplasmic staining (Fig. 3G arrowhead). Postmortem tissue from a control subject stained alongside this tissue showed no intranuclear TDP-43 pathology (Fig. S3). These data establish a direct phenotypic correlation between iPSC-MN and postmortem pathology of intranuclear TDP-43 aggregates in a sporadic ALS patient. The data also indicates that this patient is a subtype of ALS that prominently demonstrates intranuclear aggregates compared to the more common cytoplasmic aggregates and nuclear clearing.

High-Content Screening to Identify TDP-43 Aggregation Inhibitors

We developed a high-content assay around TDP-43 aggregates to investigate the mechanisms underlying TDP-43 aggregate formation and to test the utility of patient iPSC-MN for high-content drug screening. We screened 1757 bioactive compounds on iPSC-MN from one sALS patient (IPRN.0048, clone 1) in the TDP-43 aggregate assay using a custom-built automated screening system with robotics. We treated the iPSC-MN cultured in 384-well plates with compounds at single concentrations of 20 μ M for 48 hours, stained with ISLET1 and TDP-43 antibodies, imaged using automated confocal microscopy, and analyzed the resulting images for presence of aggregates using custom algorithms. We identified 38 hits in this primary screen that reduced the percentage of cells with aggregates. These hits did not reduce overall TDP-43 expression level. As a confirmation and secondary screen, we tested the hits from the motor neuron screen on iPSC-derived cortical neurons from the same sALS patient in 10 doses for 48 hours. We found four classes of compounds that reduced percent cells with TDP-43 aggregates in a dose-dependent manner in sALS patient iPSC-CN: cyclin-dependent kinase inhibitors, c-Jun N-terminal kinase inhibitors (JNK), Triptolide, and FDA-approved cardiac glycosides, Digoxin, Lanatoside C, and Proscillaridin A (Fig. 4 and Table S2).

Discussion

Sporadic ALS is a complex disease and patients are diverse with respect to genetics, clinical symptoms (i.e. site of onset and rate of progression) and types of TDP-43 pathology. While cytoplasmic TDP-43 aggregates are more common than intranuclear aggregates, intranuclear TDP-43 aggregates have been reported in FTLN patients with GRN mutations, fALS patients with VCP mutations, and in some sALS patients (Arai et al., 2006; Cairns et al., 2007; Collins et al., 2012; Neumann et al., 2009; Neumann et al., 2007; Neumann et al.,

2006). The pathological significance of TDP-43 to ALS remains unclear. However, mislocalization of TDP-43 to the cytoplasm has been linked to increased neuronal death (Barmada et al., 2010). Mislocalization of TDP-43 may lead to its loss of function in the nucleus causing neuronal dysfunction. Similarly, aggregation of TDP-43 in the nucleus could also result in reduced levels of 'functional TDP-43' and loss of function of this protein. Furthermore, aggregation of TDP-43 may cause the sequestering of other important interacting proteins and/or RNA in the nucleus and their loss of function. Subtypes of TDP-43 pathology may be mechanistically different and patients have previously been categorized based on subtype of TDP-43 pathology and varying clinical symptoms that are correlated with TDP-43 subtypes (Rohrer et al., 2010). Differences in genetic background or complex interaction of multiple low-penetrant mutations in ALS and FTLT patients may contribute to the heterogeneity of clinical symptoms and varying forms of TDP-43 pathology in patients and in our iPSC-neurons. Of the 16 sporadic ALS patients we studied, we found three patients (~20%) whose iPSC-MN show spontaneous TDP-43 pathology. These three patients likely represent a subset of the sALS patient population that show intranuclear TDP-43 aggregation and have unique underlying genetic and epigenetic underpinnings. Our observation that TDP-43 aggregates are present in only a subset of motor neurons in the iPSC-MN culture could be due to the relatively short time (up to two months) that these neurons were cultured. It is possible that in ALS patients, environmental factors such as oxidative damage, neuroinflammation, and mitochondrial damage lead to formation of TDP-43 pathology in larger number of neurons because of accumulated damage over many years. All of these factors could influence the phenotype. Furthermore our findings that only a subset of neurons shows TDP-43 aggregates is consistent with previous reports where detailed examination of postmortem spinal cord from ALS patients also indicate that TDP-43 pathology is present in a subset of neurons (Bodansky et al. 2010). It is also possible that patients such as the three described here, may harbour as-of-yet unidentified epigenetic or genetic lesions that are not fully penetrant. One explanation for the lack of TDP-43 aggregates in some of the iPSC clones may be that in some clones, important epigenetic memory related to ALS pathology may have been lost during reprogramming. Future investigation of DNA methylation in patient cells could shed light on epigenetic changes that take place in ALS neurons.

While we observed that TDP-43 aggregates were hyperphosphorylated at Ser 409/410, we did not observe ubiquitination of TDP-43 in aggregates. These data suggests hyperphosphorylation may occur prior to ubiquitination and that ubiquitination of TDP-43 may take place at a later stage in disease.

In one instance where postmortem tissue was available for pathology, we were able to show that intranuclear TDP-43 aggregates were present in brain and spinal cord of the patient thereby validating our findings in iPSC-MN. To our knowledge this is the first report of corroboration of an iPSC-disease phenotype with pathology from the corresponding patient from whom the reprogrammed cells were derived and validates the utility of iPSC disease modelling for an adult onset-genetically complex disease such as ALS.

In previous studies, iPSC-derived neurons from fALS patients with TDP-43 mutations (Q343R, M337V, and G298S) have been reported to have decreased survival and altered

neurite development (Bilican et al., 2012; Egawa et al., 2012). Some iPSC lines from these patients were also reported to have cytoplasmic TDP-43 aggregates (Egawa et al., 2012). One difference between our study and these previous studies is that we focused our efforts on sporadic ALS patients where the cause of TDP-43 aggregation is not known. In addition, we investigated iPSC from a large cohort, a total of 92 iPSC clones, in order to identify sALS patient subtypes that reveal a phenotype. We investigated TDP-43 aggregates in one fALS patient with a TDP-43 mutation but did not find a significant number of intranuclear aggregates compared to controls. One explanation may be that our patient had a different mutation (TDP-43 A315T) that that described in Egawa et al, 2012 that may be less prone to aggregation *in vitro*. In addition, intranuclear TDP-43 aggregates have not been reported in patients with TDP-43 mutations and therefore would not be expected in iPSC-MN from fALS patients with TDP-43 mutation.

By screening a small molecule library that contained FDA-approved drugs, we aimed to identify known drugs that could give insight into the biology of TDP-43 aggregation and potentially be repurposed for ALS and fast-tracked to the clinic. Cardiac glycosides are inhibitors of the Na^+/K^+ ATPase pump and can alter Ca^{++} influx into cardiac cells and subsequently affect Ras, IP3, and NF- κ B signalling which have been shown to be involved in ALS (Prassas and Diamandis, 2008). More importantly, cardiac glycosides have been shown to be neuroprotective, inhibit SOD1 aggregates, and prevent polyglutamine-induced cell death (Corcoran et al., 2004; Piccioni et al., 2004; Prassas and Diamandis, 2008). Our findings are consistent with these reports and suggest inhibition of the Na^+/K^+ ATPase pump may reduce TDP-43 aggregation. The Na^+/K^+ ATPase pump is a significant ATP-sink in cells and demands a high level of energy. One potential mechanism by which cardiac glycosides may reduce aggregates is that inhibition of the Na^+/K^+ ATPase pump could increase ATP levels and improve metabolic function in ALS neurons (Pasinelli and Brown, 2006). Our hits also included inhibitors of cyclin-dependent kinase (CDK). Consistent with our findings, a recent study identified kenpaullone, an inhibitor of GSK3 and CDK to rescue ALS patient iPSC-derived motor neuron cell death suggesting mechanisms that lead to protein aggregation may also be involved in neuronal death (Yang et al., 2013). It will be important to test these compounds for their neuroprotective properties in future studies. While we did not measure neuronal survival in these experiments, reports from other groups have correlated mislocalization of TDP-43 with poor neuronal survival (Barmada et al., 2010; Bilican et al., 2012; Egawa et al., 2012).

Here we report a cellular model for TDP-43 proteinopathy in sporadic ALS patients and provide a direct link between an iPSC model and postmortem patient pathology. Our findings demonstrate the importance of characterization and differentiation of a large number of patient specific iPSCs into multiple cell types to study the heterogeneity of a sporadic disease such as ALS. Our model of human iPSC-derived TDP-43 proteinopathy is amenable to drug discovery and offers promise for discovery of novel disease-modifying therapeutics for ALS and other TDP-43 proteinopathies.

Experimental methods

Patient biopsies and fibroblasts derivation

Patients were recruited with proper consent and approval of institutional review board (IRB) for each clinical site. Biopsies from healthy subjects, sALS and fALS patients with SOD1 and FUS mutations were collected at Johns Hopkins University in Baltimore, MD; biopsy from fALS patient with TDP-43 was collected at Washington University in St. Louis, MO; and biopsies from sALS patient samples were collected at California Pacific Medical Center in San Francisco, CA. A 3 mm skin punch biopsy was taken and fibroblasts were derived in vitro.

Cell culture and human iPSC induction

Adult human fibroblasts were cultured in DMEM supplemented with 20% Medium 199 containing Earle's salts, 10% fetal calf serum, 2 mM GlutaMAX and 50 μ M 2-mercaptoethanol (Invitrogen). Control and ALS human fibroblasts were reprogrammed with retroviruses expressing the transcription factors *OCT4*, *SOX2*, *KLF4*, and *cMYC*, according to published methods (Dimos et al., 2008). Human fibroblasts were infected in human ES media. Colonies were manually passaged onto matrigel (Becton Dickinson) and grown in mTeSR media (Stemcell Technologies). Later passages were split using Accutase (Invitrogen). Retroviral vectors, bearing human KLF4, OCT4, SOX2 and cMYC, based on murine leukemia viral vector pMX were produced and concentrated according to standard protocol published by Dimos et al 2008.

DNA fingerprinting and karyotyping

Live parental fibroblasts and their derivative iPSC lines were sent to Cell Line Genetics (Wisconsin, USA) for fingerprinting and karyotype analysis.

qRT-PCR

RNA was isolated using an RNeasy kit (Qiagen) and reverse-transcribed using SuperScript VILO cDNA Synthesis Kit (Invitrogen) followed by preamplification with PreAmp Master Mix (Applied Biosystems) according to manufacturers' protocols. Quantitative RT-PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems) on the Biomark 96.96 Dynamic Array system (Fluidigm).

Embryoid body differentiation

To form embryoid bodies (EBs), confluent undifferentiated iPSC were transferred to 100 mm, low attachment plates in serum-free differentiation medium consisting of DMEM/F12, supplemented with N2, B27 and non-essential amino acids (all Invitrogen) containing 10 μ M Rock inhibitor (Y-27632, Cayman Chemical). Cells were allowed to aggregate and grow in suspension for 5–6 days in the absence of Rock inhibitor. The EBs were then plated on gelatin-covered plates and cultured in DMEM with 10% FBS for additional 5–6 days before being fixed and processed for ICC.

Differentiation of human iPSC into motor neurons

Human iPSCs were differentiated according to a modified version of Chambers et al. The following modifications were made: Dorsomorphin dihydrochloride (Tocris) at 1 μ M was used as SMAD inhibitor instead of recombinant hNOGGIN, cells were dissociated at day 18 instead of day 11 and maturation of motor neurons was carried out in Maturation Media: D-MEM/F12 + GLUTAMAX, 2% N-2 Supplement, 4% B-27 Serum-Free Supplement (Invitrogen), 9.0 mM D-Glucose, 0.1 mM Ascorbic Acid in addition to 2ng/mL each of Ciliary Neurotrophic Factor (CNTF), Brain-Derived Neurotrophic Factor (BDNF), and Glial Cell-Derived Neurotrophic Factor (GDNF). The neurons were ventralized with 200 nM Smoothened Agonist (EMD Biosciences) instead of recombinant SHH. Following plating, cells were maintained in Maturation Media and 1.5 μ M Retinoic Acid, 200 nM Smoothened Agonist, 2 μ M DAPT for a period of 4 days. Media was changed daily. We analyzed iPSC-MN for neuronal markers at two or more time points including 25 and 32 days after neural induction. Cells in iPSC-MN cultures had processes that stained positive for axonal protein neurofilament and expressed lineage markers of motor neurons that innervate the limb: ISLET1 and HB9. For co-cultures, iPSC-MN were purified using MACS Cell Separation system (Miltenyi Biotec) using antibody to L1CAM (Anti-Human CD171 antibody, eBioscience) and plated onto primary human astrocytes (Lonza). The co-cultures were fed with 1:1 Maturation Media and astrocyte media (Lonza) for 2 days and then switched to Maturation Media.

Differentiation of human iPSC into cortical neurons

Human iPSCs were differentiated using dual Smad inhibition similar to a method described by Chambers et al. for derivation of neurons expressing forebrain markers. Briefly, high density iPSCs were cultured on Matrigel and differentiated for 10 days in differentiation media supplemented with 1.5 μ M Dorsomorphin and 10 μ M SB431542 with daily media changes (DM, 50:50 mixture of D-MEM/F12: Neurobasal media supplemented with 5ml/L N2 Supplement, 10 ml/L B-27 without Vitamin A, Glutamax, Penicillin/Streptomycin, 5 μ g/ml human recombinant Insulin, 100 μ M non-essential and 100 μ M β -mercaptoethanol). From Day 11 to day 14 cells were fed with DM media alone. Cells were then fed with DM media supplemented with 0.05 μ M retinoic acid from day 15 to 19. At day 20 cultures were dissociated using enzyme-free cell dissociation buffer and replated in DM + 2ng/mL BDNF + 2ng/mL GDNF + 0.05 μ M retinoic acid at 15×10^6 cells per PLL-Laminin coated 10cm dish. Cultures were fed every other day from day 21 to day 45 using DM + 2ng/mL BDNF + 2ng/mL GDNF + 0.05 μ M retinoic acid. At day 45 cells were either passaged onto new PLL-Laminin coated plates or frozen for subsequent use.

Immunohistochemistry

Immunofluorescence was carried out according to Dimos et al. The following antibodies were used ISLET1 at 1:1000 (Abcam, ab86501), phospho-S409/S410-TDP-43 (Sigma), LAMIN-A at 1:200 (Cell Signaling, 4777), MNR2/HB9 at 1:100 (Developmental Studies Hybridoma Bank, 81.5C10), SMI31 at 1:1000 (Covance, SMI-31R) and/or TARDBP at 1:500 (ProteinTech, 10782-2-AP), CTIP2 at 1:500 (Abcam). Each iPSC-MN culture differentiated from iPSC clones derived from 30 healthy and ALS patients were fixed,

stained and visually evaluated for abnormal TDP-43 subcellular localization on a Leica or Zeiss wide field fluorescent microscope by at least two individuals and at least one investigator was blinded to the sample. Presence of TDP-43 aggregates in the three sALS patient iPSC-MN cultures was confirmed in at least four sets of subsequent differentiation experiments for each sALS patient that showed TDP-43 aggregates.

For iPSC and fibroblasts, the following antibodies were diluted in 10% goat serum with 0.01% Triton X-100 NANOG at 1:100 (Cell Signaling Technologies, 4777), TE-7 at 1:200 (Millipore, CBL271), TRA-1–60 at 1:200 (Millipore, MAB4360), TRA-1–81 at 1:200 (Millipore, MAB4381), SSEA-3 at 1:200 (Millipore, MAB4303), SSEA-4 at 1:200 (Millipore, MAB4304), TARDBP at 1:500 (ProteinTech, 10782-2-AP). Primary antibodies were allowed to incubate overnight at 4°C. Following incubation, the primary antibody solution was removed and the cells were rinsed 5 times for 10 minutes at room temperature with DPBS and 0.01% Triton. Cells were incubated with the secondary antibody solution for 1 hour at room temperature and then washed 5 times for 10 minutes at room temperature with DPBS and 0.01% Triton. Nuclei were labeled for 10 minutes with Hoechst 33342 at a concentration 1 µM. Fluorescence images were acquired using a Nikon C1 confocal microscope equipped with an ELWD 40xC objective.

Custom image analysis

Algorithms were implemented in Matlab (Mathworks Inc., Natick, MA). Algorithms were developed to automatically locate and quantify nuclear markers in dense, heterogeneous populations of cells, and to determine the fractions of nuclei having at least one aggregate, separately for motor neurons and other cell types, as determined by ISLET/HB9 markers. To locate and quantify individual cells standard algorithms of image processing based on grayscale morphology were employed. First marker controlled watershed segmentation was applied in DNA channel (DAPI) to recognize nuclei, to address the problem of partial overlaps the procedure was repeated iteratively. Once nuclei had been identified, average intensity of TDP-43 and ISLET/HB9 markers in red and green channels were quantified respectively. In addition, TDP-43 marker was utilized and Gaussian blur was applied followed by TopHat morphological filter to locate aggregates and their average intensity was quantified together with relative contrast with respect to neighborhood outside of an aggregate, and size. Finally information obtained from the previous steps was combined to quantify various subpopulations of cells, that is ISLET/HB9 positive (or negative), aggregate positive (or negative) as well as double positive (both ISLET/HB9 and having at least one aggregate) subpopulations of cells.

Flow Cytometry

iPSC's were treated with Accutase (Invitrogen) for approximately 5 minutes. After dissociation cells were placed on ice in FACS buffer (Ca^{2+} and Mg^{2+} free DPBS supplemented with 2% FBS, 20 mM D-Glucose and 100 units/mL penicillin/streptomycin) in 96 well plates. Individual wells were treated with antibodies against one of the following antigens at the manufacturer's recommended concentration. SSEA-1 FITC (555401, Becton Dickinson (BD)), SSEA-3 FITC (560237, BD), SSEA-4 Alexa 647 (560796, BD). TRA-1–60 FITC (560380, BD), TRA-1–81 PE (560161, BD) followed by 30 minutes incubation.

Data was acquired on an Accuri C6 flow cytometer. Analysis and plotting was performed using FlowJo (Treestar).

Calcium imaging

After approximately 90 days of long term culture, motor neurons were dissociated and replated in 96 well plates. After 7–14 days in 96 well plates, motor neurons were prepared for calcium imaging: cells were stained in 2 μ M Oregon Green 488 BAPTA-1 AM (O6807, Invitrogen) prepared with HEPES buffered, phenol red free DMEM/F12 (11039-021, Invitrogen). Motor neurons were incubated with the dye for 45 minutes at 37°C. Plates were loaded into a stage top incubation system with humidity, CO₂, and temperature control (Pathology Devices). Imaging was performed on a Nikon Eclipse TE2000 inverted microscope with an ELWD 40xC (Nikon) objective. Images were acquired using an Andor DU-885 EMCCD camera. Frames were binned 2×2 at a resolution of 500×502 pixels and acquired at a rate of 29 frames per second for two minutes. For Tetrodotoxin (TTX) inhibition experiments, fields were first imaged without TTX for two minutes. Wells were then spiked to a final concentration of 500nM TTX (Tocris). TTX was allowed to diffuse through the well, and then the same fields were reimaged. Image segmentation and analysis was performed using custom routines written in Matlab (Mathworks) and R (R project).

Electrophysiology

Whole cell patch clamp recording from iPS derived motor neurons or cortical neurons cultured on monolayer of primary human astrocytes (Lonza) using patch pipette (2–5 MOhm) filled with solution containing (mM): K-methyl-sulfate (140), NaCl (10), CaCl₂ (1), Mg-ATP (3); Na-GTP (0.4), EGTA (0.2), HEPES (10) with adjusted pH= 7.3 and mOsm = 300. Neurons were perfused (1–2 ml/min) with artificial cerebral spinal fluid containing (mM): NaCl₂ (140), KCl (2.5), MgCl₂ (2) CaCl₂ (2), Na-Hepes (10), D_Glucose (10), sucrose (20). Adjusted pH= 7.4. Resting membrane potentials (–52±4 mV (n=7) were determined soon after whole cell access in current clamp configuration with pClamp 10.3 (Molecular Devices) using MultiClamp 700B (Axon Instrument; Foster City CA) by removing holding current applied to maintain –60 – –70 mV membrane potentials. Recordings were conducted at 34–37 degree C.

Screening of compounds

iPSC-MN screen—On day 18 of motor neuron differentiation, motor neurons were dissociated and plated into black, 384 well plates (6007460, Perkin Elmer) at 10,000 cells per well. Cell plating and media exchanges were performed using an EL406 combination washer/dispenser (Biotek). Cells were grown in maturation media supplemented with 1.5 μ M RA, 200 nM SAG, and 2 μ M DAPT. Media was replaced at 3 days and 5 days following replating. Compound treatment was performed 5 days after replating. Compounds were dissolved to the limit of solubility in DMSO.

After a 48 hour incubation period, treated plates were fixed and stained for ISLET1 and detected with an Alexa 488 secondary, TDP-43 was detected with an Alexa-549 antibody as described above. Automated immunostaining was performed using an EL406 washer/dispenser (Biotek). Fluorescence imaging was performed using an ImageXpress Ultra

(Molecular Devices) high throughput imaging system. Using a 20x objective, 2 fields of view were acquired in each well. Nuclei, ISLET1 positive cells, and TDP-43 aggregates were detected and quantified as described above. All experiments were carried out in duplicate and replicates were averaged. Hits were defined based on ability to reduce the percent cells with aggregates by at least two standard deviations from the median.

iPSC-CN screen—iPSC were differentiated into cortical lineage as described above. After 75 days in culture, iPSC-CN were plated into 384-well plates and treated with hits from the iPSC-MN screen. A 10-point, two fold dilution series was prepared in 384 well plates using a Hamilton Microlab STAR. Compound addition was achieved using an Agilent Bravo Workstation equipped with a 384 sample pin tool (V&P Scientific). Cells were grown in 384 plates for 10 days prior to treatment. Cells were treated for 48 hours in triplicates and 4 areas per well were imaged and analyzed as described.

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Abbreviations

ALS	amyotrophic lateral sclerosis
iPSC	induced pluripotent stem cells
TDP-43, TARDBP	TAR DNA binding protein
SOD1	superoxide dismutase 1
FTLD	fronto-temporal lobar degeneration
iPSC-MN	induced pluripotent stem cell-derived motor neurons
iPSC-CN	induced pluripotent stem cell-derived cortical neurons
HTS	high-throughput screening

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Figure 1. Patient-specific iPSC and iPSC-derived motor neurons

Representative images of: (A) iPSC colonies from one control and one ALS patient in phase-contrast. (B) iPSC colonies stained with antibodies for pluripotency markers NANOG and TRA-1-60. (C) iPSC-MN cultures stained with nuclear marker DAPI (blue) and antibodies to motor neuron markers ISLET1 (red) and HB9 (green). (D) iPSC-MN cultures stained with antibody to axonal marker Neurofilament (SMI31). iPSC and iPSC-MN shown are from healthy control IPRN.0013 and fALS patient IPRN.0028. (Scale bars a-b: 200 μ m, c-d: 75 μ m). (E) (i-ii), Representative trace showing electrical activity of iPS-MN from a healthy individual (Scale bars: 100 mV; 100 ms. 8 neurons recorded from fired robust and repetitive action potentials (bursts) to depolarizing current steps. (Iii) Example trace

showing action potential elicited by the rebound depolarization following 300 pA hyperpolarizing step for 100 ms (Scales bars: 100 mV; 100 ms) and example trace of spontaneous bursts of action potentials and spontaneous post-synaptic potentials from iPS derived motor neurons (50 mV; 3 Sec). (Insets) Examples of EPSP (scales: 3 mV; 250 ms), and IPSP (scales: 30 mV, 100 ms). (F) (i) Sporadic ALS iPSC-MN responses -10, 0, and 10 pA current steps for 300 msec (scale: 25 mV). (ii) APs elicited by rebound depolarization following hyperpolarizing current step (300 pA; 300 msec). (iii) Spontaneous activity of sALS iPSC-MN (scale bars; 50 mV; 500 msec) (insets example of sEPSP and sIPSP (scale bars: 5 mV; 200 msec))(n = 7).

Figure 2. TDP-43 localization in patient iPSC-derived motor neurons

(A) iPSC-MN stained for TDP-43 shows TDP-43 is ubiquitously expressed and nuclear in all cells including in ISLET1-positive neurons from control (left panel) and ALS (right panel) patients. ISLET1-positive cells from sporadic ALS iPSC-MN have nuclear staining as well as nuclear aggregates that stain with higher intensity for TDP-43 (arrowheads, right panel) but aggregates are not present in healthy controls (left panel). Fibroblasts from healthy control and sALS patients do not show nuclear aggregates. Scale bar: 30 μ m. (B) Sporadic ALS patient-derived iPSC-MN cells stained with nuclear envelope marker LAMIN-

A (green) and TDP-43 (red) shows TDP-43 aggregates are inside the nuclear envelope; scale bar: 20 μ m. (C) Quantification of healthy control IPRN.0013 and sALS IPRN.0048 clone 1 iPSC-MN cultures shows TDP-43 aggregation is present in higher fraction of ISLET1 or HB9-positive cells compared to negative cells. (30.7% of ISLET1/HB9-positive motor neurons as compared to 16.2% of ISLET1/HB9-negative cells in sALS iPSC-MN cultures. Bars are standard deviation 9% and 8% respectively; $P < 2.2 \times 10^{-16}$ Welch t-test; data from three differentiations, N= 458 and 466 images for control and ALS respectively, with each image containing on average 400 cells to account for more than 180,000 cells counted per group). (D) Phospho-serine 409/410 TDP-43 antibody labels TDP-43 aggregates in patient iPSC-MN and co-localizes with aggregates stained with pan-TDP-43 antibody, from left to right: DAPI, Pan-TDP-43, Phospho-TDP-43 antibody, and merged images (scale bar = 60 μ m). iPSC-MN cultures shown were derived from healthy control, IPRN.0013, and sALS patient, IPRN.0048.

Figure 3. TDP-43 aggregates are present in iPSC-cortical neurons and in patient postmortem CNS

(A) Healthy control IPRN.0013 and (B) ALS patient IPRN.0048 iPSC-CN cultures stained with MAP2 (green) and TDP-43 (red) show intranuclear TDP-43 aggregates are present in ALS patient cells (B) but not in healthy control cells (A). (C & E), Control and (D & F) ALS iPSC-CN stained for corticospinal neuron marker CTIP2 (green in C & D) and TDP-43 (red in C & D). (C–F), CTIP2 positive neurons have TDP-43 aggregates (arrows in D & F). (G–J), Intranuclear TDP-43 aggregates in patient (IPRN.0360) postmortem CNS stained with TDP-43 (brown) and Luxol fast blue. (G & I), Spinal cord anterior horn motor neurons have intranuclear TDP-43 aggregates (arrow) and cytoplasmic mislocalization (arrowhead). (H & J), frontal lobe neurons have intranuclear TDP-43 aggregates (arrow). (K) 11% of CTIP2-positive and 3% of CTIP2-negative cells had TDP-43 aggregates. Error bars are standard deviation (4 and 1 respectively; total cells counted = 1548). Scale bars (A–F)= 20 μ m, (G–J) 10 μ m.

Figure 4. sALS patient iPSC-CN treated with cardiac glycosides

(A–C), sALS patient iPSC-CN control DMSO-treated for 48 hours and stained for TDP-43. (D–F), iPSC-CN treated with Digoxin at 3 μ M, Lanatoside C at 1.5 μ M, and Proscillaridin at 1.5 μ M for 48 hours and stained for TDP-43. (G–I), Dose response curves for the three compounds on iPSC-CN show dose dependent reduction in percent cells containing TDP-43 aggregates. (G–I), total cell count for compound-treated iPSC-CN shows that cell number does not decrease with increasing dose and compounds are not cytotoxic.