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Mutant induced pluripotent stem cell lines recapitulate aspects of TDP-43 proteinopathies and reveal cell-specific vulnerability

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Transactive response DNA-binding (TDP-43) protein is the dominant disease protein in amyotrophic lateral sclerosis (ALS) and a subgroup of frontotemporal lobar degeneration (FTLD-TDP). Identification of mutations in the gene encoding TDP-43 (\textit{TARDBP}) in familial ALS confirms a mechanistic link between misaccumulation of TDP-43 and neurodegeneration and provides an opportunity to study TDP-43 proteinopathies in human neurons generated from patient fibroblasts by using induced pluripotent stem cells (iPSCs). Here, we report the generation of iPSCs that carry the TDP-43 M337V mutation and their differentiation into neurons and functional motor neurons. Mutant neurons had elevated levels of soluble and detergent-resistant TDP-43 protein, decreased survival in longitudinal studies, and increased vulnerability to antagonism of the PI3K pathway. We conclude that expression of physiological levels of TDP-43 in human neurons is sufficient to reveal a mutation-specific cell-autonomous phenotype and strongly supports this approach for the study of disease mechanisms and for drug screening.

disease modeling | reprogramming | motor neuron disease | Lou Gehrig disease

Cytoplasmic accumulation of detergent-resistant transactive response DNA-binding (TDP-43) is a pathological hallmark of both sporadic and inherited FTLD-TDP (a subgroup of frontotemporal lobar degeneration) and ALS (except superoxide dismutase 1 (SOD1) and fused in sarcoma (FUS) cases) and suggests a common pathological mechanism (1–4). Mutations in the gene encoding TDP-43 (\textit{TARDBP}) have been identified in familial and sporadic ALS (5–8). Several in vitro and in vivo models established the toxicity of ALS-associated TDP-43 mutations, although the underlying mechanism is unclear (9, 10). Most cellular and animal models of ALS and FTLD-TDP pathogenesis involve overexpression of TDP-43 in nonneuronal or nonhuman cells and cannot be used to investigate the selective vulnerability of neurons or key molecular events that are unique to human cells. By contrast, induced pluripotent stem cells (iPSCs) (11–14) coupled with defined in vitro differentiation protocols (15–20) offer a model system to investigate disease mechanisms in a more physiological context. Here, we report the pathological effects of endogenous mutant TDP-43 in iPSC-derived human neurons from an ALS patient carrying the M337V mutation.

\textbf{Results}

\textbf{Generation of iPSCs from TDP-43 M337V Fibroblasts.} iPSC lines were established by using cells from a 56-y-old man with ALS who had the TDP-43 M337V mutation (8, 21) and from two healthy controls. Fibroblasts (Fig. 1A) were transduced with retroviral vectors encoding the reprogramming factors \textit{OCT4}, \textit{SOX2}, \textit{KLF4}, and \textit{c-MYC} as described in ref. 13. Colonies with compact human ES cell (hESC)-like morphology were expanded, and clonal lines were established for both genotypes (Fig. 1B). All iPSCs used in the study exhibited silencing of the four transgenes and activation of endogenous \textit{OCT4}, \textit{SOX2}, and \textit{KLF4} (Fig. 1A). All iPSCs expressed the protein markers of pluripotency—\textit{OCT4}, \textit{SOX2}, and \textit{TRA-1-60} (Fig. 1B)—and had comparable levels of \textit{OCT4}, \textit{SOX2}, \textit{c-MYC}, \textit{KLF4}, and \textit{NANOG} expression, as determined by quantitative RT-PCR (qRT-PCR) (Fig. 1G and Fig. S1C). Pluripotency was confirmed by teratoma formation (Fig. S1D) and bisulfite sequencing of the \textit{OCT4} promoter (13) (Fig. 1F). The genetic mutation was confirmed by direct sequencing of exon 6 in \textit{TARDBP} in all lines (Fig. 1E). All clones were maintained a normal karyotype for more than 40 passages.

\textbf{TDP-43 M337V Does Not Prevent Differentiation and Functional Maturation of Motor Neurons (MNs).} To assess the effects of the TDP-43 M337V mutation on neural differentiation and function, we generated caudalized neuronal populations containing spinal MNs from iPSCs as described previously (21–23). Feeder-free iPSCs were differentiated into neuroectoderm by dual-SMAD signaling inhibition (21) (Fig. 2A and Fig. S2A). Replating generated >90% \textit{NESTIN}\textsuperscript{+} and \textit{SOX1}\textsuperscript{+} cells (Fig. 2B and C). \textit{PAX6}\textsuperscript{+} neuroepithelial cells (Fig. S2B) were treated with retinoic acid and purmorphamine to generate \textit{NKX6.1} and \textit{OLIG2} ventral spinal progenitors (Fig. 2A and Fig. S2C), which differentiated into MNs expressing \textit{Hb9}, \textit{β3-tubulin}, \textit{SMI-32}, and choline acetyltransferase (ChAT) (Fig. 2D–F and Fig. S2D) (23–25). Current-clamp recordings from ChAT\textsuperscript{+} MNs derived from M337V and control iPSCs revealed tetrodotoxin-sensitive action potentials.
M337V Increases Soluble and Detergent-Resistant TDP-43 Protein Levels. A defining biochemical feature of diseased ALS and FTLD-TDP neural tissue is the accumulation of detergent-resistant ~43-kDa and C-terminal fragments of TDP-43 (29). We next examined the biochemical profile of TDP-43 in undifferentiated iPSCs. As determined by qRT-PCR, M337V and control iPSC lines expressed similar levels of TARDBP, the pluripotency markers TERT and REX1, and histone deacetylase 6 (HDAC6) mRNA (Fig. 3A), the latter of which is regulated by TDP-43 (30). Western blotting revealed that two independent M337V iPSC clones had higher levels of full-length TDP-43 in the soluble fraction and C-terminal TDP-43 fragments in the detergent-resistant fraction than controls (Fig. 3B). Importantly, TDP-43 fragmentation was not associated with conventional indices of apoptosis [cleaved poly(ADP-ribose) polymerase (PARP) (Fig. 3B) and annexin V/propidium iodide profiling (Fig. 3C)] (31–33).

Before establishing the biochemical profile of TDP-43 in MN-containing neuronal populations, we first verified that MN cultures derived from M337V and control 2 (Cont-2) had comparable expression levels of TARDBP, HB9, 

\[ \text{ChAT} \]

+ and REX1, and HDAC6 as determined by qRT-PCR analysis (Fig. S3A). Furthermore, MN cultures derived from both M337V and controls showed similar percentages of SMI-32\(^\dagger\) cells by quantitative immunohistochemistry (Fig. S3B), altogether indicating comparable differentiation potential and composition of MN cultures at the point of assay. Immunoblot analysis revealed that M337V MN cultures from two independent clones had up to fourfold higher levels of soluble and detergent-resistant TDP-43 than Cont-2 MN cultures (Fig. 4A and B). Difference in TDP-43 levels in MN-containing populations was also evident with Cont-1 MN cultures (see control lanes in Figs. S3D and S5B). To determine whether this observation was independent of neuronal cell type and differentiation protocol, we generated caudalized neuronal populations that did not contain MNs and compared the amount of TDP-43 in cultures derived from mutant or control iPSCs (Fig. S4A and B). Again, M337V neurons had higher levels of soluble (2.6 ± 0.46 vs. 1.0 ± 0.13) and insoluble TDP-43 than controls (Fig. S4 C and D).

We next investigated the subcellular distribution of TDP-43. Densitometric analysis revealed that strongly SMI-32\(^\dagger\) neurons had significantly higher levels of nuclear TDP-43 than SMI-32\(^\dagger\) cells (118.89% ± 2.6% vs. 100% ± 2.9%, P < 0.001) (Fig. 4). However, the predominant nuclear localization of TDP-43 did not differ in M337V and control lines (Fig. 4C). Subtle granular cytoplasmic staining was also detected in SMI-32\(^\dagger\) and ChAT\(^+\) M337V neurons (Fig. 4 E and F and Fig. S4 E and F).

TDP-43 M337V Mutation Confers Selective Cellular Vulnerability to MNs. We next analyzed the survival of M337V and control MNs under basal conditions by longitudinal fluorescence microscopy. To specifically focus on MNs, we transfected M337V and control cultures with a plasmid encoding enhanced GFP under the control of the MN-specific HB9 promoter described previously (HB9::GFP, Fig. 5A) (34). Quantification of GFP\(^+\) neurons per well confirmed no difference between M337V and control MN cultures (Fig. S5A). Automated fluorescence microscopy, a technique that can image thousands of fluorescently labeled neurons for extended periods of time, was then used to track individual neurons and determine their time of death. Neuronal death was marked by abrupt loss of fluorescence or dissolution of the cell itself, criteria that have proved equally as sensitive as traditional markers of cell death (35, 36). Kaplan–Meier survival analysis was used to plot cumulative hazard curves depicting the risk of death for MNs derived from M337V and control iPSCs (Fig. 5B). The relative risk associated with the M337V mutation was calculated by Cox proportional hazards analysis as 1.84, indicating that M337V MNs have an 84% increased risk of death compared to control MNs.

potentials (Fig. 2 G and H). Whole-cell voltage–clamp recordings revealed functional NMDA, AMPA, and GABA receptors that typify MNs (Fig. S2 E and F). Mature MN cultures also exhibited spontaneous excitatory postsynaptic currents (EPSCs) that were blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNOX) (Fig. 2F). Thus, both M337V and control iPSC lines generated phenotypically and functionally comparable MNs (26–28).
analysis, demonstrating a hazard ratio of 2.76, indicative of a 276% increase in the risk of death in M337V compared with control MNs.

We next determined whether the M337V mutation increased neuronal vulnerability to antagonism of key signaling pathways necessary for neuronal survival (37). MN-containing cultures were treated with U0126, a selective inhibitor of MAPK signaling; LY294002, a selective inhibitor of PI3K; or tunicamycin, an endoplasmic reticulum stressor. After 48 h of treatment, neuronal death was measured by lactate dehydrogenase (LDH) release. M337V and control MN cultures did not differ in their response to MAPK inhibition or tunicamycin (Fig. S5 C and D). However, M337V mutant lines 1 and 2 were significantly more vulnerable than controls were to LY294002 (Fig. 5 C), suggesting an intrinsic susceptibility to PI3K inhibition in neurons derived from M337V iPSCs.

**Discussion**

This paper describes an iPSC model of a TDP-43 proteinopathy in human MNs derived from a patient carrying a pathogenic TDP-43 mutation. Detailed cell-lineage and functional studies showed that the TDP-43 M337V mutation does not affect the functional maturation of neurons, including MNs. However, neurons carrying the mutation had increased levels of soluble and detergent-resistant TDP-43 and reduced survival under basal conditions, suggesting an inherent vulnerability. Mutant neurons were also more susceptible than control neurons to inhibition of PI3K, an essential signaling pathway in neurons (38). Thus, the mutant cells recapitulate key aspects of TDP-43 proteinopathies in vitro, including cell-autonomous neuronal degeneration and the accumulation of insoluble TDP-43. This model will facilitate mechanistic studies, the identification of therapeutic targets, and eventually the development of treatments for ALS and FTLD-TDP.
stability of mutant TDP-43 proteins had only been observed in isogenic transformed cell lines (41). Our findings suggest that differences in TDP-43 protein levels result from a posttranslational mechanism rather than from transcriptional differences. In addition, the mutant proteins do not appear to interfere with the proposed autoregulatory feedback mechanism proposed for the control of TDP-43 mRNA levels (42, 43). The dominant missense mutations located in the C-terminal domain of TDP-43 might inhibit the turnover of the mutant protein or constrain protein quality-control pathways.

Despite the higher levels of TDP-43 in M337V neurons detected biochemically, we did not see more nuclear TDP-43 than in controls, as determined by immuno fluorescence densitometry. However, SMI-32+ neurons had higher levels of nuclear TDP-43 in vitro, indicating that TDP-43 protein levels can differ between neuronal subtypes. In addition, punctate TDP-43 staining in the soma and cell processes was a consistent finding. This staining pattern is compatible with the involvement of TDP-43 in nucleocytoplasmic shuttling of RNA, the association of TDP-43 with RNA granules in somatodendrites, and the presence of TDP-43 in the microsome fraction of brainstem samples, suggesting active transport of TDP-43 along the axons (44–47).

Cellular and transgenic models of TDP-43 expression established that elevated levels of WT and mutant TDP-43 can be toxic and that levels of cytoplasmic, rather than nuclear, TDP-43 correlate with cellular toxicity (9, 10, 48). As shown by longitudinal fluorescence microscopy of live MNs, the risk of death was significantly increased by the M337V mutation, suggesting an inherent cell-autonomous toxicity of the mutation in MNs. Neuronal health and function are regulated by multiple signals, including brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor, and other trophic factors that...
signal through receptor tyrosine kinases (37). We demonstrated that M337V neurons were more sensitive to PI3K inhibition than control neurons were but showed no difference in vulnerability to inhibitors of the MAPK pathway or induction of endoplasmic reticulum stress through tunicamycin. Thus, the M337V mutation confers a specific susceptibility to PI3K inhibition, highlighting the importance of trophic factor-mediated signaling in the survival of human MNs. Even though most neurotrophic factors rely on both MAPK/ERK and PI3K/AKT pathways for signal transduction, the contribution of these pathways to cell survival depends on the neuronal subtype and the combination of trophic factors (38). For instance, BDNF-induced MN survival requires the PI3K pathway (49), whereas retinal ganglion cells rely on both the PI3K and MAPK pathways in BDNF-dependent survival (50). Future studies involving the in vitro model that we established herein will focus on the contribution of different neurotrophic factors to the survival of TDP-43 M337V neurons.

In summary, our findings show that patient-derived TDP-43 M337V neurons recapitulate key biochemical aspects of TDP-43 proteinopathies and provide evidence that the M337V mutation in TDP-43 is toxic to iPSC-derived MNs, rendering them particularly susceptible to antagonism of PI3K signaling. Although this study was limited to a single patient line with subclones and controls, we identified a disease-specific phenotype in TDP-43 iPSC lines. Such lines will be useful for exploring the pathogenic mechanisms of other TDP-43 mutations and of different ALS-causing mutations under basal and stress paradigms. Finally, our findings demonstrate the utility of patient-specific iPSC lines in modeling the molecular pathogenesis of adult neurodegenerative disorders.

Materials and Methods

Generation of Human iPSCs. Fibroblasts from a normal male (56 y old; CRL-2465; ATCC), a normal female (40 y old; CRL-2524; ATCC), and from a 56-y-old male carrying the M337V TDP-43 mutation were reprogrammed as previously described (13). Details of iPSC characterization are provided in SI Materials and Methods.

Cell Culture. Human iPSC and hESC lines were maintained long term on CF-1 irradiated mouse embryonic fibroblasts, with KO-DMEM (Invitrogen) supplemented with 20% Knockout Serum Replacement (Invitrogen), 10 ng/mL basic FGF2 (PeproTech), 1 mM l-glutamine (Invitrogen), 100 mM 2-mercaptoethanol (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 1% nonessential amino acids. For the neural conversion, the cells were transitioned to feeder-free culture conditions in MTESR1 media (Stem Cell Technologies) and passaged three times before use. Details of neuronal differentiation and characterization are provided in SI Materials and Methods.

Primers and Antibodies. TaqMan and SYBR Green primer sequences are listed in Tables S1 and S2, respectively. Primary antibodies used are listed in Table S3.

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