Induced Pluripotent Stem Cell Models of Progranulin-Deficient Frontotemporal Dementia Uncover Specific Reversible Neuronal Defects

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SUMMARY

The pathogenic mechanisms of frontotemporal dementia (FTD) remain poorly understood. Here we generated multiple induced pluripotent stem cell lines from a control subject, a patient with sporadic FTD, and an FTD patient with a novel heterozygous GRN mutation (progranulin [PGRN] S116X). In neurons and microglia differentiated from PGRN S116X induced pluripotent stem cells, the levels of intracellular and secreted PGRN were reduced, establishing patient-specific cellular models of PGRN haploinsufficiency. Through a systematic screen of inducers of cellular stress, we found that PGRN S116X neurons, but not sporadic FTD neurons, exhibited increased sensitivity to staurosporine and other kinase inhibitors. Moreover, the serine/threonine kinase S6K2, a component of the phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways, was specifically downregulated in PGRN S116X neurons. Both increased sensitivity to kinase inhibitors and reduced S6K2 were rescued by PGRN expression. Our findings identify cell-autonomous, reversible defects in patient neurons with PGRN deficiency, and provide a compelling model for studying PGRN-dependent pathogenic mechanisms and testing potential therapies.

INTRODUCTION

Frontotemporal dementia (FTD), the second most common form of presenile dementia before the age of 65, is associated with focal atrophy of the frontal or temporal lobes and deficits in cognition, behavior, and language (Boxer and Miller, 2005). Mutations that cause FTD have been identified in several genes, including those encoding valosin-containing protein (VCP; Watts et al., 2004), charged multivesicular body protein 2B (CHMP2B; Skibinski et al., 2005), progranulin (PGRN; Baker et al., 2006; Cruts et al., 2006), and chromosome 9 open reading frame 72 (C9ORF72; DeJesus-Hernandez et al., 2011; Renton et al., 2011). It is not known how these diverse mutations cause similar clinical manifestations, and no effective treatment is available.

The secreted glycoprotein PGRN has been implicated in cell growth and survival, inflammation, synaptic functions, and other cellular functions (He and Bateman, 2003; Yin et al., 2010; Tapia et al., 2011). Although most (if not all) pathogenic mutations in GRN lead to pathological changes in FTD due to PGRN haploinsufficiency (Baker et al., 2006; Cruts et al., 2006), the underlying molecular mechanism is unknown. PGRN mutations are a common cause of FTD. However, no robust pathological phenotype has been found in Grn−/− mice, and selective neuronal cell loss is limited even in Grn knockout mice (Ahmed et al., 2010; Ghoshal et al., 2012; Petkau et al., 2012; Yin et al., 2010). Thus, a more suitable model for dissecting the pathogenic mechanisms that underlie PGRN haploinsufficiency is needed.

The ability to generate human induced pluripotent stem cells (iPSCs) offers an unprecedented opportunity to analyze the molecular consequences of pathogenic mutations in the context of the unique genetic background of individual patients (Yamana, 2007). Indeed, iPSCs have been generated from patients with different neurodegenerative diseases (e.g., Dimos et al., 2008; Ebert et al., 2009; Soldner et al., 2009; Nguyen et al., 2011; Israel et al., 2012). In this study, we generated multiple FTD-patient-specific iPSC lines and established a human neuronal model of PGRN haploinsufficiency. From studies of human postmitotic neurons derived from these lines, we identify
Figure 1. Generation and Characterization of FTD Patient-Specific iPSCs

(A–C) Total and endogenous (Endo) levels of the reprogramming factors in control, sporadic, and PGRN S116X iPSC lines relative to the values in H9, as assessed by qRT-PCR. Values are mean ± SEM.

(D) Genomic DNA sequencing of the heterozygous PGRN S116X mutation g.4627C > A (p.S116X: nonsense mutation) in PGRN S116X iPSCs.

(E) Methylation status of the OCT4 promoter for control iPSC line 20, sporadic iPSC line 9, and PGRN S116X iPSC line 26. B, unmethylated CpG dinucleotides; C, methylated CpG dinucleotides.

(F) Immunofluorescence analysis of pluripotency markers in control iPSC line 20, sporadic iPSC line 9, and PGRN S116X iPSC line 26, and their respective normal karyotypes. Cell nuclei were counterstained with DAPI (blue). Scale bar, 50 μm.

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cell-autonomous and reversible defects in specific signaling pathways that are compromised in PGRN-deficient neurons.

**RESULTS**

**Generation and Characterization of FTD-Patient-Specific iPSCs**

The two FTD patients under investigation in this study were part of a longitudinal dementia research program at the Memory and Aging Center, University of California, San Francisco. Both had an 8-year history of behavioral changes and memory impairment at the time of tissue collection for this study. One patient, a 67-year-old male with sporadic FTD, tested negative for mutations in GRN, MAPT, and C9ORF72. The other patient, a 64-year-old male with a significant family history of dementia, had behavioral variant FTD. MRI in this patient demonstrated severe bifrontal and temporal atrophy associated with gliosis in the frontal lobes (greater on the right). One year later, MRI scans showed progression of atrophy and gliosis. Genetic testing revealed a novel nonsense mutation in GRN, p.S116X (g.4627C > A, c.347C > A), which is predicted to result in a premature stop codon. Both FTD patients had parkinsonism, which is typical of all FTD which is predicted to result in a premature stop codon. Both lines maintained a normal karyotype after reprogramming (Figures 1F and S1C) and could spontaneously differentiate into cell types of all three germ layers in vitro (Figures 1G and S1C). Moreover, representative iPSC lines from the subjects (control line 20, sporadic line 9, and PGRN S116X line 26) transplanted into severe combined immunodeficiency (SCID) mice gave rise to teratomas in vivo (Figure 1G). These findings confirm the successful reprogramming and generation of FTD-patient-specific iPSC lines, and demonstrate that these lines are similar to those in controls in terms of both their expression of stem cell markers and their pluripotency.

**Differentiation of FTD-Patient-Specific iPSCs into Neurons**

Next, we differentiated three fully reprogrammed iPSC lines at passages 20–26 from each subject into postmitotic neurons, using a protocol available in our lab (Delaloy et al., 2010). The differentiation starts with neural induction, which is followed by expansion of the neural progenitor cells and neural maturation. The first step, induction of multilineage differentiation and embryoid body (EB) formation, was inefficient when iPSCs were maintained on feeder cells. Adaptation of iPSCs to feeder-free conditions allowed robust and reliable formation of EBs (Figures 2A and 2B). After 5–6 days in suspension, neural induction was initiated with basic fibroblast growth factor and N2 supplement, and rosettes (elongated cells arranged in circular structures) appeared (Figure 2C). Ten days later, the rosettes were isolated, expanded in suspension as neurospheres for 3–4 weeks (Figure 2D), and dissociated into single cells. Terminal differentiation was induced with glial-cell-line-derived neurotrophic factor, brain-derived neurotrophic factor, ascorbic acid, and cyclic AMP. Two weeks later, the cells displayed typical neuronal morphology (Figure 2F). Both FTD and control iPSCs differentiated at similar rates.

We then sought to determine whether the disease and/or the mutation affected the percentage of neurons obtained with this protocol. After 2 weeks, ~80% of cells in culture were positive for the neuronal marker microtubule-associated protein 2 (MAP2) and had neuronal morphology (Figure 2G), and <4% of cells were positive for the glial marker glial fibrillary acidic protein, regardless of the genetic mutation of the iPSC line used (Figures 2G and 2K). Thus, the PGRN S116X mutation did not

(G) In vitro (EB formation) and in vivo (teratoma formation) differentiation of control iPSC line 20, sporadic iPSC line 9, and PGRN S116X iPSC line 26 into cells of all three germ layers. For in vitro studies, cells were immunostained with α-fetoprotein (AFP, endoderm), desmin (mesoderm), III-l-Fil (ectoderm), and DAPI (nuclei). Hematoxylin/eosin staining of teratoma sections showed neuron/rosette structures (ectoderm), smooth muscle (mesoderm), and ducts (endoderm).

Scale bar, 50 μm.

See also Figure S1.
affect the percentage of neurons generated with the differentiation protocol. Approximately 40% of the MAP2+ cells were presumably glutamatergic and expressed VGLUT1 (Figure 2H), and <10% of cells were GABA+ inhibitory neurons or tyrosine hydroxylase (TH)+ dopaminergic neurons (Figures 2I and 2J). Again, the percentages of neurons differentiated from control and FTD-patient-specific iPSC lines were indistinguishable. Additional analysis at the messenger RNA (mRNA) level indicative of glutamatergic (VGLUT1), GABAergic (GAD67) and dopaminergic (TH) neuronal subtypes or postsynaptic density (PSD95) detected no significant differences across the different lines (Figures S2A–S2D). Thus, the PGRN S116X mutation did not affect neural differentiation of iPSCs into specific type of neurons.

We next performed whole-cell voltage-clamp recordings and measured membrane properties and synaptic transmission on...
neurons differentiated from two iPSC lines (control line 20 and PGRN S116X line 26; Figures 2L–2N). Most cells in culture were capable of inducing tetrodotoxin-sensitive action potentials (control: 79.2%; PGRN S116X: 75%), which is consistent with the finding that ∼80% of cells are MAP2-positive neurons (Figure 2G). The resting membrane potential between two cell lines does not show a statistically significant difference (control neurons: 62.5 ± 1.5 mV; PGRN S116X: 60.0 ± 1.9 mV; n = 24, p = 0.17). To address whether these cells can form functional synaptic connections, we found that PSD95 puncta were present on dendrites of these neurons (Figure S2E), and also measured AMPA-type glutamate receptor-mediated miniature excitatory postsynaptic currents (mEPSCs). Neurons differentiated from PGRN S116X iPSCs showed synaptic connections indistinguishable from those of control neurons (mEPSC amplitude of control neurons: 12.1 ± 1.7 pA, PGRN S116X: 14.37 ± 1.7 pA, p = 0.36; frequency of control neurons: 3.2 ± 0.6 pA, PGRN S116X: 2.2 ± 0.2 pA, n = 10, p = 0.12). These results indicate that postmitotic neurons differentiated from control and PGRN S116X iPSCs are functional.

A Human Neuronal Model of PGRN Haploinsufficiency

To establish a human neuronal model of PGRN haploinsufficiency, we first examined the expression levels of PGRN in fibroblasts from each subject by qRT-PCR. GRN mRNA levels were similar in cells from the control subject and sporadic FTD patient (Figure 3A), but in cells from the FTD patient with the PGRN S116X mutation, the mRNA level was only ∼30% of that found in the control (Figure 3A). This observation is consistent with the substantially lower average plasma PGRN levels in FTD patients harboring GRN mutations compared with those without such mutations (Coppola et al., 2008; Finch et al., 2009). However, after reprogramming, the GRN mRNA was 50% lower in all three PGRN S116X iPSC lines (Figure 3B), as expected. Moreover, the relative expression levels of GRN mRNA in all control or sporadic FTD iPSCs showed little variation (Figure 3B). Correspondingly, PGRN S116X iPSCs secreted 50% less PGRN than iPSCs from the control subject and sporadic FTD patient (Figure 3C).

Upon differentiation, GRN mRNA levels were ∼41% lower in PGRN S116X neurons than in control and sporadic FTD neurons differentiated from multiple iPSC lines (Figure 3D). The levels of both intracellular and secreted PGRN in these neurons were also correspondingly reduced, as measured by ELISA (Figures 3E and 3F). Thus, we established a patient-specific human neuronal model of PGRN haploinsufficiency. We were also able to differentiate these iPSCs into microglia as shown by expression of the microglia-specific marker Iba1 (Figures S3A and S3B). PGRN secretion from these cells was also ∼50% lower than in control and sporadic FTD cells (Figures S3C and S3D).
PGRN S116X Neurons Are More Sensitive to Cellular Stress Induced by Inhibitors of the Phosphatidylinositol 3-Kinase/Akt and MEK/Mitogen-Activated Protein Kinase Signaling Pathways

Compared with many other neurodegenerative diseases, the cellular defects associated with FTD remain poorly defined. Human neurons derived from patient-specific iPSCs are an excellent system in which to examine disease-gene-specific cellular phenotypes. To conduct such an examination, we first used two iPSC lines from each patient and differentiated them into postmitotic neurons. Under normal culture conditions, PGRN S116X and control neurons show similar viability. As a late-onset disease, FTD likely results from damage that accumulates over time rather than from an acute effect of the reduced PGRN levels. Very little is known about the cellular defects caused by PGRN haploinsufficiency in human neurons. Thus, to identify pathways that might be compromised in PGRN S116X neurons, we performed a systematic screen with the cell viability assay, PGRN S116X neurons showed greater caspase-3 activity in both PGRN S116X and sporadic FTD neurons, whereas tunicamycin increased caspase-3 activity. Consistent with the results of the cell viability assay, PGRN S116X neurons showed greater caspase-3 activity than control or sporadic FTD neurons (Figure 4B). This finding suggests that PGRN deficiency affects kinase pathways involved in cell survival, causing them to be more susceptible to inhibition of such pathways.

To further explore PGRN-dependent cellular defects in FTD neurons, we also tested the effect of staurosporine, a broad-spectrum kinase inhibitor that induces apoptosis (Figure 4B). Interestingly, PGRN S116X neurons were more sensitive to staurosporine than control or sporadic FTD neurons (Figure 4B). This finding suggests that PGRN deficiency affects kinase pathways involved in cell survival, causing them to be more susceptible to inhibition of such pathways.

To validate the findings of the cell viability assay, we also measured the activation of caspase-3, a well-studied mediator of apoptotic cell death. Consistent with the results of the cell viability assay, PGRN S116X neurons showed greater caspase-3 activation in response to staurosporine than control or sporadic FTD neurons, whereas tunicamycin increased caspase-3 activity in both PGRN S116X and sporadic FTD neurons (Figure 4D). Because TDP-43 pathology is a hallmark in the brains of FTD patients with PGRN deficiency (Neumann et al., 2006), and increased caspase-3 activity leads to enhanced cleavage and mislocalization of TDP-43 (Zhang et al., 2007), we also analyzed the cellular distribution of TDP-43 under stress to confirm our initial findings. After exposure to staurosporine, the percentage of neurons with redistribution of TDP-43 from the nucleus to the cytoplasm was significantly higher in PGRN S116X neurons than in control or sporadic FTD neurons (Figure S4D). This result is consistent with previous findings showing that PGRN haploinsufficiency causes a late-onset disease, FTD likely results from damage that accumulates over time rather than from an acute effect of the reduced PGRN levels. PGRN S116X neurons were more susceptible to staurosporine and tunicamycin than control or sporadic FTD neurons (Figure 4C and S4), suggesting that PGRN deficiency affects kinase pathways involved in cell survival.

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Because staurosporine is a broad-spectrum kinase inhibitor that affects several signaling pathways, we next tested more specific kinase inhibitors to identify specific pathways affected by reduced PGRN levels. PGRN S116X neurons were more susceptible than control or sporadic FTD neurons to wortmannin (Figure 4C) and LY294002 (data not shown), two phosphatidylinositol 3-kinase (PI3K) inhibitors, and PD98059, an MEK inhibitor (Figure S4E). These findings suggest that PGRN deficiency impairs the PI3K/Akt and MEK/mitogen-activated protein kinase (MAPK) signaling pathways in human neurons.

The Cellular and Molecular Defects of PGRN S116X Neurons Can Be Rescued by PGRN Expression

We next examined the causal relationship between PGRN haploinsufficiency and enhanced sensitivity to cellular stress induced by inhibitors of the PI3K/Akt and MEK/MAPK pathways in PGRN S116X neurons. To that end, we used a CS-CW-GRNIG lentiviral vector to express PGRN in most (if not all) of the human neurons in culture. The decreased cell viability (Figure 4E) in staurosporine-treated PGRN S116X neurons was rescued by PGRN expression. A similar result was obtained when increased caspase-3 activation was used as the assay (Figure 4F), confirming the validity of the cell viability assay. In contrast, the increased sensitivity of PGRN S116X neurons to the ER stress induced by tunicamycin was not rescued by PGRN expression (Figure 4E). More importantly, the increased sensitivity of PGRN S116X neurons to inhibitors of the PI3K/Akt and MEK/MAPK pathways was also rescued (Figure 4E). Thus, the novel cellular defects of PGRN S116X neurons uncovered under stress are specific to PGRN deficiency.

Next, we sought to identify misregulated components in the PI3K/Akt and MEK/MAPK pathways by performing gene expression analyses on two to three replicate neuron cultures differentiated from each iPSC line and four iPSC lines per individual (30 samples total). We compared PGRN S116X neurons and sporadic FTD neurons versus control neurons, and identified a number of differentially expressed genes, both shared between PGRN S116X and sporadic FTD neurons, and specific to PGRN S116X neurons (Figure 4G). In addition, a clustering analysis showed that the expression patterns in neurons differentiated from three separate iPSC lines of the same individual were remarkably similar to each other (Figure 4G).

Among the top downregulated genes in PGRN S116X neurons (but not in control or sporadic FTD neurons) was the ribosomal protein S6 kinase beta-2 (RPS6KB2; Figure 4H). This gene encodes S6K2, a member of the S6 kinase family of serine/threonine kinases that has been shown to play an important role in both the PI3K/Akt and MEK/MAPK signaling pathways (Fenton 2006).
Figure 4. Novel Cellular and Molecular Defects of PGRN S116X Neurons Can Be Rescued by PGRN Expression

(A–C) Effects of stress inducers on human neurons. Values are expressed as a percentage of the cells exposed to DMSO (control; n = 3–4 independent cultures).

(D) Caspase-3-like activity after exposure to 10 nM staurosporine, 0.5 μM tunicamycin, or DMSO for 24 hr.

(E and F) Measurement of cell viability (E) and caspase-3 activation (F) after rescue with PGRN expression (n = 5–6 independent cultures).

(G) Heat map depicting fold changes of gene expression in two to three neuron cultures differentiated from each one of the four iPSC lines from the sporadic FTD patient (blue) or the PGRN S116X patient (fuchsia) compared with control neurons.

(H) Gene expression changes on the array for GRN and RPS6KB2. The log fold change is relative to control neurons.

(I and J) PGRN expression restores S6K2 protein levels in PGRN S116X neurons.

(P) Representative western blotting image for S6K2 (control line 17 and PGRN S116X line 1).

(Q) Quantification of S6K2 relative to GAPDH for three experiments performed on lines 17 and 20 (control), and lines 1 and 26 (PGRN S116X).

In all panels, values are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S4.
with previous findings that PGRN promotes the survival of rodent expression of PGRN in human PGRN S116X neurons, consistent
pathogenesis of FTD. This cellular defect is rescued by ectopic
 PI3K/Akt and MEK/MAPK signaling pathways in the molecular
induced by specific protein kinase inhibitors, implicating the
S116X human neurons are more prone to reduced cell viability
approach has been used recently to recapitulate some key
features of major neurodegenerative diseases in human neurons
manifest under stress conditions in culture. Indeed, this
some intrinsic vulnerabilities of human neurons are more likely
PGRN deficiency and demonstrated specific and reversible
defect appears to be PGRN-independent since PGRN expres-
levels are normal in sporadic FTD neurons. In accordance
with our findings, it was recently reported that ER stress and
unfolded protein response activation contribute to both sporadic
and familial FTD caused by MAPT mutations (Nijholt et al.,
2012). Moreover, both Aβ and increased levels of phosphory-
ated tau induce ER stress in Alzheimer disease (e.g., Hoozemans et al.,
2009), as does the accumulation of misfolded α-syn-
uclein in PD (Colla et al., 2012). Therefore, altered ER stress
responses are likely to be a general feature in a variety of neuro-
degenerative diseases.

In summary, we have established neuronal models of human
PGRN deficiency and demonstrated specific and reversible
defects that affect the survival of these neurons. Our findings
suggest that chronic weakening of prosurvival signaling path-
ways may render neurons more sensitive to environmental
insults in FTD patients with PGRN deficiency. Thus, in addition
to strategies to increase PGRN levels, therapeutic approaches
that generally enhance neuronal survival through growth factor
signaling may be beneficial in slowing disease progression in
these patients.

**EXPERIMENTAL PROCEDURES**

**Isolation of Primary Human Skin Fibroblasts and Generation of iPSCs**

This study was approved by the Institutional Review Board and Ethics
Committees of the University of California, San Francisco, and written
informed consent was obtained in all cases. The patient with the PGRN
S116X mutation followed the classic clinical progression for FTD and de-
developed parkinsonism, as do all FTD patients with PGRN mutations, but he did not show typical features of PD dementia. The patient with sporadic FTD
also showed parkinsonism. Skin biopsies were collected, cut into small pieces,
and placed on culture dishes to allow the fibroblasts to expand. The cells
were maintained in Dulbecco’s modified Eagle’s medium supplemented with
10% fetal bovine serum, 1X nonessential amino acids, and penicillin/strepto-
mycin (100 U/ml). iPSCs were generated as described previously (Takahashi
et al., 2007). Please see Supplemental Information for more details.

**qRT-PCR, Immunocytochemistry, Differentiation and Characterization of iPSCs, and Electrophysiology**

Most of the experiments involving qRT-PCR, immunocytochemistry, differen-
tiation and characterization of iPSCs, and electrophysiology were performed
as previously described (Delaloy et al., 2010) with minor adjustments. Please see
Supplemental Information for more details.
**PGRN Measurements**
Fresh culture medium was added to the cells 24 hr before collection. After the medium was collected, the cells were washed once with phosphate-buffered saline (PBS), lysed with NP-40 buffer, and subjected to three freeze-thaw cycles. Both the culture medium and the cell lysates were centrifuged at 12,000 rpm at 4°C for 10 min to clear cellular debris. Cell lysate supernatants were assayed for protein concentration with the BioRad reagent assay. Total cell lysates and culture medium were diluted, and the PGRN levels were determined with an ELISA kit (Alexis Biochemicals, San Diego, CA) according to the manufacturer’s instructions. Data were normalized to protein concentration.

**Stress-Induced Toxicity Assay**
Two-week-old neurons were exposed for 24 hr to the following stress inducers: tunicamycin, lactacystin, rotenone, oligomycin, hydrogen peroxide, staurosporine, wortmannin, LY294002, PD98059, or DMSO. Cell viability was determined with the WST1 cell-proliferation assay (Roche Applied Science, Penzberg, Germany) according to the manufacturer’s instructions. Caspase-3 activity assay is described in the Supplemental Information.

**PGRN Rescue Experiments**
Human GRN (NM_002087.2) was inserted into a CS-CGW lentiviral vector with Nei I and Xho I. The vector also expressed green fluorescent protein through an internal ribosome entry site. One-week-old neurons were transduced overnight with lentivirus expressing PGRN or empty vector. The next morning, the medium was doubled and thereafter replaced every other day. One week after transduction, the neurons were exposed to 10 nM staurosporine, 0.5 μM tunicamycin, 50 μM PD98059, 75 nM wortmannin, or DMSO for 24 hr. Cells were assayed for cell viability, caspase-3 activation, and S6K2 levels. A multiplicity of infection of 50 was used in all cases.

**ACCESSION NUMBERS**
Microarray data are available at the NCBI Gene Expression Omnibus database under the series accession number GSE40378.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Extended Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.09.007.

**LICENSING INFORMATION**
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Update

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Induced Pluripotent Stem Cell Models of Progranulin-Deficient Frontotemporal Dementia Uncover Specific Reversible Neuronal Defects

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In the original version of this article, published on October 11, Bruce Miller’s affiliation was incorrectly listed as the University of Massachusetts Medical School. The correct affiliation is the University of California, San Francisco, and the corrected author list appears here.

The authors regret this error.