Increasing progranulin levels and blockade of the ERK1/2 pathway: Upstream and downstream strategies for the treatment of progranulin deficient frontotemporal dementia

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Abstract
Frontotemporal lobar degeneration (FTLD) is a neurodegenerative disorder marked by mild-life onset and progressive changes in behavior, social cognition, and language. Loss-of-function progranulin gene (GRN) mutations are the major cause of FTLD with TDP-43 protein inclusions (FTLD-TDP). Disease-modifying treatments for FTLD-TDP are not available yet. Mounting evidence indicates that cell cycle dysfunction may play a pathogenic role in neurodegenerative disorders including FTLD. Since cell cycle re-entry of postmitotic neurons seems to precede neuronal death, it was hypothesized that strategies aimed at preventing cell cycle progression would have neuroprotective effects. Recent research in our laboratory revealed cell cycle alterations in lymphoblasts from FTLD-TDP patients carrying a null GRN mutation, and in PGRN deficient SH-SY5Y neuroblastoma cells, involving overactivation of the ERK1/2 signaling pathway. In this work, we have investigated the effects of PGRN enhancers drugs and ERK1/2 inhibitors, in these cellular models of PGRN-deficient FTLD. We report here that both restoring...
1. Introduction

The term frontotemporal lobar degeneration (FTLD) refers to a group of progressive brain rare diseases, which involve shrinkage of specific areas of the brain that regulate behavior, personality, and language. The onset of symptoms usually occurs before the age of 60 years, accounting for 5–10% of dementia patients (Graff-Radford and Woodruff, 2007; Ratnavalli et al., 2002). The course of this disease is progressive with mortality within 6–8 years. FTLD patients can be classified into three clinical syndromes depending on the early and predominant symptoms: a behavioural variant (bvFTD) and two language variants; semantic dementia (SD) and primary progressive non-fluent aphasia (PPNFA) (Neary et al., 1998; Rabinovici and Miller, 2010). Each clinical variant is associated with a distinct regional pattern of brain atrophy and, to a varying degree, a characteristic histopathology. Additionally, other neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), progressive supranuclear palsy (PSP), parkinsonism with frontotemporal dementia (FTDP) or corticobasal degeneration syndrome (CBS) are closely related to FTLD (Burrell et al., 2011; Deng et al., 2011; Lomen-Hoerth, 2004; Van Langenhove et al., 2012).

Pathologically, these disorders share deposits of abnormal proteins in neuroectodermic cells, and severe cell loss with atrophy of vulnerable cortical and subcortical structures. Histochemically, FTLD can be categorized according to the major component of the cellular inclusions deposited in the brain. In the majority of cases, the pathological protein is either the microtubule-associated protein tau or the transactive response DNA-binding protein TDP-43 (FTLD-tau, FTLD-TDP respectively), a small number of cases present inclusions of fused in sarcoma (FUS) protein (FTLD-FUS) (Cairns et al., 2007; Mackenzie et al., 2011a, 2011b).

A positive family history of FTLD is present in 25–50% of cases and the transmission is usually autosomal dominant (Goldman et al., 2007; Rademakers et al., 2012; Sieben et al., 2012). A few genes have been associated with familial FTLD including microtubule-associated protein tau (MAPT) (Roorka et al., 1998; Spillantini et al., 1998), progranulin (GRN) (Baker et al., 2006; Cruts et al., 2006), transactive response (TAR) DNA-binding protein-43 (TARDBP) (Gitcho et al., 2009), chromatin-modifying 2B protein (CHMP2B) (Holm et al., 2007), valosin-containing protein (VCP) (Watts et al., 2004), and chromosome 9 open reading frame 72 (C9ORF72) (DeJesus-Hernandez et al., 2011). Recently, two new rare mutations associated with FTLD and ALS have been identified in the sequestosome1/p62 (SQSTM1) (Le Ber et al., 2013) and Ubiquilin-2 (UBQLN2) (Deng et al., 2011; Vengoechea et al., 2013) genes.

A large number of FTLD-TDP patients have been identified to harbor loss-of-function mutations (including null mutations) in the gene encoding progranulin (GRN) (Baker et al., 2006; Cruts et al., 2006). Up to now more than 60 different mutations in GRN have been described associated with the etiology of the disease (www.molgen.ua.ac.be/FTDmutations/) (Gijselink et al., 2008). Most of the pathogenic mutations result in null allele, suggesting that FTLD in these families results from progranulin (PGRN) haploinsufficiency (Cruts et al., 2006; Cruts and Van Broeckhoven, 2008).

Current treatment options for FTLD associated to PGRN deficiency remain very limited, mainly involving therapy for the mood and behavioral symptoms (Kirshner, 2010). Identification of molecular targets to slow or hopefully prevent the neurodegenerative process relies in a better understanding of both the biological functions of PGRN, and the role of protein haploinsufficiency in the development of dementia. For this purpose, familial forms of the disease with known pathogenic mutations provide an opportunity to get inside in FTLD-TDP pathogenesis and for fast-track development of new therapies for PGRN-deficient FTLD.

Cell cycle-related events are now considered as an important pathogenic mechanism for neurodegenerative disorders including Alzheimer disease (AD) (Mosch et al., 2007; Yang et al., 2001), Parkinson disease (PD) (Hoglinger et al., 2007), ALS and FTLD (Husseman et al., 2000). In these studies, it was suggested that cell cycle signaling might affect neuronal death pathway. The cell cycle is associated with the phase specific expression or modification of defined sets of regulatory genes that control proliferation, differentiation or entry into a quiescent state (Ross, 1996). However, re-entry of quiescent, post-mitotic neurons into the cell cycle may result in a mitotic catastrophe and cell death (Copani et al., 2001; Herrup et al., 2004; Zhu et al., 2004). Therefore, understanding the molecular pathways underlying this cell cycle-mediated neurodegeneration may be important to find new therapeutic targets to slow or prevent the onset and progression of FTLD.

Interestingly, it seems that dysfunction of the cell cycle in neurodegenerative disorders is a general phenomenon affecting cells other than neurons (Nagy et al., 2002; Stieler et al., 2012).
Therefore, peripheral cells from patients, mainly lymphocytes or fibroblasts, have been extensively used as a model to study pathogenic molecular mechanisms and to evaluate therapeutic strategies aimed at blocking cell cycle progression (Bialopiotrowicz et al., 2012; de las Cuevas et al., 2003; Munoz et al., 2008; Nagy et al., 2002; Sala et al., 2008; Zhang et al., 2003). We have previously reported alterations in cell survival/death mechanisms in immortalized lymphocytes from FTLD-TDP patients carrying a loss-of-function mutation in the GRN gene (c.709-1G>A) (Alquezar et al., 2012a, b). This mutation was previously described in a small number of families of Basque descent (Lopez de Munain et al., 2008; Moreno et al., 2009). We found that PGRN deficit increased cell cycle activity in lymphoblasts from FTLD-TDP patients. This effect was associated with enhanced levels of cyclin-dependent kinase 6 (CDK6) and phosphorylation of retinoblastoma protein (pRb), resulting in a G1/S regulatory failure. Moreover, we demonstrated that activation of the CDK6/pRb is the consequence of increased stimulation of the ERK1/2 signaling, induced by PGRN haploinsufficiency (Alquezar et al., 2014). The present work was undertaken to evaluate whether restoring PGRN levels or targeting the ERK1/2 cascade would normalize the cell cycle alterations found in lymphoblasts harboring the c.709-1G>A GRN mutation. We investigated the effects of already approved drugs for use in clinical practice or in on-going clinical trials for unrelated conditions, which should make it easier to move quickly to human FTLD trials. PGRN levels were modulated by suberoylanilide hydroxamic acid (SAHA) that increases GRN mRNA expression levels (Cenik et al., 2011) or by chloroquine (CQ) that prevents proteolytic degradation of the protein (Capell et al., 2011). The blockade of ERK1/2 activation was achieved by using Selumetinib (AZD6244) and MEK162 (ARRY-438162). Both compounds are small molecules ATP-uncompetitive highly selective inhibitors of MEK1/2 (Mitogen-activated protein kinase kinase or ERK kinase 1 and 2), the upstream activator of ERK1/2 (Davies et al., 2007; Yeh et al., 2007).

We report here that increasing PGRN levels or alternatively by preventing the overactivation of the CDK6/pRb cascade, unravelling molecular targets to design novel therapeutic approaches in the FTLD linked to PGRN haploinsufficiency.

2. Experimental procedures

2.1. Lymphoblastic cell lines

Blood samples were obtained from 29 individuals: 19 carriers of the c.709-1G>A GRN gene mutation (7 of them patients of FTLD-TDP and 12 asymptomatic) and 10 control individuals without sign of FTLD. Asymptomatic and control individuals were relatives of patients. All patients were diagnosed as FTD in the Donostia Hospital by applying consensus criteria as published elsewhere (McKhann et al., 2001). Demographic information for control and GRN mutation carriers is presented in Table 1.

All study protocols were approved by the Donostia Hospital and the Spanish Council of Higher Research Institutional Review Board and are in accordance with National and European Union Guidelines. In all cases, peripheral blood samples were taken after written informed consent of the patients or their relatives to determine the presence of the c.709-1G>A GRN mutation and to establish the lymphoblastoid cell lines.

Establishment of lymphoblastic cell lines was performed in our laboratory as previously described (Barretta et al., 1998), by infecting peripheral blood lymphocytes with the Epstein Barr virus (EBV). Cells were grown in suspension in T flasks in an upright position, in approximately 8 ml of RPMI-1640 medium (Life technologies, Barcelona, Spain) that contained 2 mM L-glutamine, 100 µg/ml streptomycin/penicillin and 10% (v/v) fetal bovine serum (FBS) (Life technologies, Barcelona, Spain) and maintained in a humidified 5% CO2 incubator at 37°C. Fluid was routinely changed every three days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

2.2. GRN knockdown neuroblastoma SH-SY5Y cell lines culture

Stable GRN knockdown neuroblastoma SH-SY5Y cells (Clone # 207) (gift from Drs. Drs. Joselin and Wu) were grown in high glucose DMEM (Dulbecco’s modified Eagle’s medium) (Life-Technologies, Barcelona, Spain) containing 10% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich, Tres Cantos, Spain) and 1% penicillin/streptomycin (Life-technologies, Barcelona, Spain) at 37°C in 5% CO2. GRN knockdown was achieved by using pSUPERIOR RNAi constract as previously described (Gao et al., 2010). The target sequence of 19 nucleotides targeted against nucleotides 207-226

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<th>Table 1</th>
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Control: Individuals without sign of neurological degeneration; c.709-1G>A: GRN mutation carriers; n: number of subjects. Values are expressed as mean ± SEM. CBS: Cortico basal syndrome; FTD-bv: Frontotemporal dementia (behaviour).
The human GRN mRNA was designed. The 64 nt short hairpin RNA sense and antisense primer sequences were: 5'-gatccggccgccttgcttggatatcgcagagtttgctgca-3' and 5'-agcttttccaaagaagctgccggcctgtcttggataaa-3'.

The sense and antisense primer pairs were annealed and ligated into the pSUPERIOR vector (OligoEngine) according to manufacturer’s instructions. The vector control was also stably introduced into SH-SY5Y cells to generate the control cell line.

2.3. Drugs and treatments

The PGRN enhancer drugs, suberoylanilide hydroxamic acid; Vorinostat (SAHA) (Cayman Chemical company, Ann Arbor, MI, USA), or chloroquine diphosphate salt (CQ) (Sigma-Aldrich, Tres Cantos, Spain) were used. SAHA was prepared in dimethylsulfoxide (DMSO) and used to a final concentration of 0.25 and 1 µM. In the other hand, CQ was dissolved in water and used to a final concentrations between 2.5 and 20 µM. Progranulin (human) (recombinant) (rhPGRN) (Enzo, Zandhoven, Belgium) was utilized as positive control for its proven efficacy in normalizing PGRN levels and was used in a final concentration of 100 ng/ml.

For blocking the ERK1/2 activity, the MEK1/2 inhibitors selumetinib (AZD6244) (Selleck Chemicals, Houston, TX, USA), MEK162 (ARRY-439162) (AdooQ Bioscience, Irvine, CA, USA), and PBD8059 (Calbiochem, Darmstadt, Germany) were used. All three drugs were prepared in DMSO and were used to a final concentration between 0.25 and 2.5 µM for Selumetinib, between 0.5 and 1 µM for MEK162, and 20 µM for PBD8059.

Elastase from human leukocytes (Sigma-Aldrich, Tres Cantos, Spain) was diluted in water and used a final concentration of 0.01 U/ml.

2.4. Determination of cell proliferation

Cell proliferation was determined by total cell counting, using a TC10 Automated Cell Counter from Bio-Rad Laboratories, S.A. (Madrid, Spain). EBV-immortalized lymphocytes from control and GRN mutation carriers were seeded at an initial cell concentration of 1 x 10^6 cells x ml^-1. Cells were enumerated everyday thereafter. In some experiments, cell proliferation was assessed by the 5-bromo-2-deoxyuridine (BrdU) incorporation method using an enzyme-linked immunosassay kit procured from Roche (Madrid, Spain) or by the MTT assay (Mitsiades et al., 2002) obtaining similar results.

2.5. Cell cycle analysis

Exponentially growing cultures of cell lines were seeded at an initial concentration of 1 x 10^6 cells x ml^-1. Cell cycle analysis was performed using a standard method (Krishan, 1975). Cells were fixed in 75% ethanol for 1 h at room temperature. Subsequent centrifugation of the samples was followed by incubation of cells in PBS containing 1 mg/ml of RNase at room temperature for 20 min and staining with propidium iodide (PI; 25 µg/ml). Cells were analyzed in an EPICS-XL cytometer (Coulter Científica, Móstoles, Spain). Estimates of cell cycle phase distributions were obtained by computer analysis of DNA content distributions.

2.6. Preparation of whole-cell extracts and subcellular fractionation

To prepare whole-cell extracts, cells were harvested, washed in PBS and then lysed in ice-cold buffer (50 mM Tris pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40), containing 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate and protease inhibitor Complete Mini Mixture (Roche, Madrid, Spain).

To separate the cytosolic and nuclear fractions, cells were harvested, washed in PBS and then lysed in ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM PMSF and protease inhibitor mixture). After extraction on ice for 15 min, 0.5% Nonidet P-40 was added and the lysed cells were centrifuged at 4000 rpm for 10 min. Supernatants containing cytosolic proteins were separated and after extraction on ice for 30 min, the samples were centrifuged at 15,000 rpm for 15 min at 4 °C. Antibody against α-tubulin was used to assess the purity of the cytosolic fraction.

The protein content of the extracts was determined by the Pierce BCA Protein Assay kit (Thermo Scientific).

2.7. Immunoblotting analysis

Cells were harvested, washed in PBS and then lysed in ice-cold lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40), containing 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM sodium pyrophosphate and protease inhibitor Complete Mini Mixture (Roche, Mannheim, Germany). 50-100 µg of protein from cell extracts were fractionated on a SDS polyacrylamide gel, and transferred to PVDF membrane. The membranes were then blocked with 1% Bovine Serum Albumin (BSA) (Sigma) and incubated, overnight at 4 °C, with the following primary antibodies: CDK6 (1:1000), pRB (1:500), β-actin (1:500) and α-tubulin (1:1000) obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Anti-Progranulin (1:500) from Abcam (Cambridge, UK). Phospho-p44/42 MAPK (Erk1/2) (XP) (1:2000) and p44/42 MAP Kinase (ERK1/2) (1:1000) obtained from Cell Signaling (Danvers, MA, USA) and Anti-TDP-43 (1:500) from ProteinTech Group (Manchester, UK). The specificity of the antibodies used in this work was checked by omitting the primary antibody in the incubation medium. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Bio-Rad) and detected with a chemiluminescent substrate detection system ECL. Protein band densities were quantified using Image J software (National Institutes of Health, Bethesda, Maryland, USA) after scanning the images with a GS-800 densitometer from Bio-Rad.

2.8. Quantitative real-time PCR

Total RNA was extracted from cell cultures using Trizol reagent (Invitrogen, Alcobendas, Madrid, Spain). RNA yields were quantified spectrophotometrically and RNA quality was checked by the A260/A280 ratio and on a 1.2% agarose gel to determine the integrity of 18S and 28S ribosomal RNA. RNA was then treated with DNase I. Following the manufacturer’s protocol. Primers were designed using the Universal ProbeLibrary for Human (Roche Applied Science, Madrid, Spain) and used at a final concentration of 20 µM. The sequences of the forward and reverse primers used are the following: for GRN, 5'-ctgctgtctgctgctggacccttg-3' and 5'-agggtcactgcctgtcgtc-3'; for β-actin, 5'-ccaaagccggaagaatgga-3' and 5'-ccagaggctcagaggtatgc-3'. Real time quantitative PCR was performed in the Bio-Rad IQ5 system using a thermal profile of an initial 5-min melting step at 95°C followed by 40 cycles at 95°C for 10 s and 60°C for 60 s. Relative messenger RNA (mRNA) levels of the genes of interest were normalized to β-actin expression using the simplified comparative threshold cycle delta-delta CT method (2^ΔΔCT PGRN -ΔΔCT β-actin).
2.9. Statistical analysis

Statistical analyses were performed on GraphPad Prism 5 for Macintosh (La Jolla, CA, USA). All the statistical data are presented as mean ± standard error of the mean (SEM). Statistical significance was estimated by analysis of variance (ANOVA) followed by the Bonferroni’s test for multiple comparisons. Differences were considered significant at a level of \( p < 0.05 \).

Figure 1  Proliferative activity of lymphoblasts from control and c.709-1G>A GRN mutation carriers individuals. (A) Immortalized lymphocytes from control and c.709-1G>A GRN mutation carriers, asymptomatic or FTLD-TDP patients, were seeded at an initial density of \( 1 \times 10^6 \times \text{ml}^{-1} \) and were incubated in RPMI medium containing 10% FBS, 24 h later cells were harvested for cell extracts preparation. PGRN levels were determined by Western blotting. Box plots represent the PGRN content in lymphoblasts form all individuals enrolled in this studio (\( ** p < 0.01 \), significantly different form control cells). (B) Proliferative response of control and PGRN deficient cells. 100,000 cells/well were seeded in 96-well plates for 24 h and pulsed with 10 \( \mu \text{M} \) BrdU for an additional period of 4 h. DNA synthesis was assessed by BrdU incorporation method according to the manufacturer’s instructions. Proliferation was expressed as absorbance of stimulated minus that of non-stimulated cultures. Each bar represents the mean ± SEM of seven independent experiments performed in triplicate. (C) Lymphobastoid cell lines were seeded at an initial density of \( 1 \times 10^6 \times \text{ml}^{-1} \) and were incubated for 72 h with rhPGRN (100 ng/ml) alone or preincubated with elastase (0.1 U/ml) for 3 h. Cell proliferation was determined everyday by counting the cells excluding trypan blue using a TC10™ Automated Cell Counter. Values shown are the mean ± SEM of all cell lines used in this work (*\( p < 0.05 \); **\( p < 0.01 \) significantly different from control cells). (D) rhPGRN (100 mg/ml) was incubated in the absence and presence of 0.1 U/ml of elastase for 3 h and the levels of PGRN protein were determined by Western blot. (E) Representative immunoblots showing the effects of rhPGRN on pERK1/2, CDK6 levels and pRb phosphorylation status.
3. Results

3.1. Proliferative activity of lymphoblasts from control or c.709-1G>A GRN mutation carriers

Figure 1 summarizes the distinct response of EBV-immortalized lymphocytes bearing a loss-of-function GRN mutation c.709-1G>A to serum stimulation. As expected, PGRN content of mutant lymphoblasts, either from asymptomatic or FTLD-TDP patients, was significantly reduced when compared with the protein content of control cells (Figure 1A) (**p < 0.01 significantly different from untreated cells). (C, D) Effect of the treatment with SAHA 1 μM (C) and CQ 10 μM (D) on proliferation in control and PGRN deficient lymphoblasts. Aliquots were taken for cell counting 72 h after the drug administration. Data shown are the mean ± SEM of ten determinations from SAHA and seven for CQ carried out with different cell lines (**p < 0.01 significantly different from control cells.

3.2. Effects of SAHA and chloroquine on proliferation of lymphoblasts from control or c.709-1G>A GRN mutation carriers individuals

Figure 2 Effects of SAHA and chloroquine on the proliferation of lymphoblasts from control and c.709-1G>A GRN mutation carriers individuals. Immortalized lymphocytes from control and c.709-1G>A GRN mutation carriers, asymptomatic or FTLD-TDP patients, were seeded at an initial density of 1 x 10⁶/ml and were incubated in medium containing 10% FBS, in the absence or presence of increasing doses of SAHA (0–2 μM) (A) and CQ (0–20 μM) (B) for 72 h. A total of 100,000 cells per well were seeded in a 96-well plate for the MTT assay. Results represent the % of cell survival of treated cells referred to untreated ones (†p < 0.05 and ††p < 0.01 significantly different from untreated cells). (C, D) Effect of the treatment with SAHA 1 μM (C) and CQ 10 μM (D) on proliferation in control and PGRN deficient lymphoblasts. Aliquots were taken for cell counting 72 h after the drug administration. Data shown are the mean ± SEM of ten determinations from SAHA and seven for CQ carried out with different cell lines (**p < 0.01 significantly different from control cells.

Targeting PGRN levels and ERK1/2 activity in peripheral cells from FTLD patients

Figure 2 Effects of SAHA and chloroquine on the proliferation of lymphoblasts from control and c.709-1G>A GRN mutation carriers individuals. Immortalized lymphocytes from control and c.709-1G>A GRN mutation carriers, asymptomatic or FTLD-TDP patients, were seeded at an initial density of 1 x 10⁶/ml and were incubated in medium containing 10% FBS, in the absence or presence of increasing doses of SAHA (0–2 μM) (A) and CQ (0–20 μM) (B) for 72 h. A total of 100,000 cells per well were seeded in a 96-well plate for the MTT assay. Results represent the % of cell survival of treated cells referred to untreated ones (†p < 0.05 and ††p < 0.01 significantly different from untreated cells). (C, D) Effect of the treatment with SAHA 1 μM (C) and CQ 10 μM (D) on proliferation in control and PGRN deficient lymphoblasts. Aliquots were taken for cell counting 72 h after the drug administration. Data shown are the mean ± SEM of ten determinations from SAHA and seven for CQ carried out with different cell lines (**p < 0.01 significantly different from control cells.

3.2. Effects of SAHA and chloroquine on proliferation of lymphoblasts from control or c.709-1G>A GRN mutation carriers individuals

Given that PGRN deficit seems to be causally associated with neurodegeneration in FTLD-TDP patients (Baker et al., 2006; Mackenzie et al., 2011a), treatments to increase the
PGRN levels were envisioned as promising therapies. For this reason, we considered interesting to evaluate the efficacy of drugs known to increase PGRN content, such as SAHA, a histone deacetylase (HDAC) that increases the GRN expression (Cenik et al., 2011) or chloroquine, an alkalizing reagent able to prevent the proteolytic degradation of PGRN (Capell et al., 2011), to restore the normal response of PGRN-deficient peripheral cells from FTLD patients. These drugs are already US Food and Drug Administration-approved drugs for the treatment of cutaneous T-cell lymphoma and malaria respectively (Duvic and Vu, 2007; Mann et al., 2007; Pullman et al., 1948).

To determine the dose-response effects of SAHA and CQ on cell proliferation, lymphoblasts from control and c.709-1G>A GRN mutation carriers, asymptomatic or FTLD-TDP patients were incubated in the absence or in the presence of escalating concentrations of SAHA (0-2 μM) or CQ (0-20 μM) for 72 h and cell proliferation were determined by the MTT assay. As shown in Figure 2A, SAHA suppressed the cell proliferation in a dose-dependent manner (Figures 2A) (F0.2 μM SAHA x individuals (8,86) = 0.7851, p = 0.6170). Similar results were obtained with increasing concentrations of CQ (Figure 2B) (F0.20 μM CQ x individuals (8,60) = 1.173, p = 0.3303). Maximal effects of SAHA or CQ were obtained at 1 μM or 10 μM, respectively. At these doses, both SAHA and CQ were able to abrogate the enhanced proliferative response of PGRN-deficient lymphoblasts without affecting the proliferation of control cells (F1 μM SAHA x individuals (2,27)= 13.27, p < 0.0001; F10 μM CQ x individuals (2,29)= 3.224, p = 0.0497). The effect of these drugs on cell proliferation mimicked the addition of rhPGRN (see Figure 1).

Since the change in cell number depends on the balance between cell proliferation and cell death, we tested whether SAHA or CQ treatment induced cell death by necrosis/apoptosis. For this purpose, we analyzed the distribution of cell cycle phases. We did not observe significant changes in the proportion of sub-G0/G1 hypodiploid cells, characteristic of apoptosis/necrosis, in control and PGRN deficient lymphoblasts after SAHA or CQ treatment (Figure 3). SAHA was more effective in decreasing the percentage of cells in S/G2M phases than CQ. Taken together, these results suggest that the decreased cell number in cultures of GRN mutation c.709-1G>A carriers in the presence of SAHA or CQ truly reflects a decrease in cell proliferation.

The analysis of mRNA and protein levels of PGRN in control and c.709-1G>A GRN mutation carriers lymphoblasts revealed that, at the concentrations used, both drugs were effective in increasing the cellular levels of protein (Figure 4A) (F1 μM SAHA x individuals (2,32)= 6.105, p = 0.0057; F10 μM CQ x individuals (2,32)= 4.637, p = 0.0170) and, in consonance with the distinct mechanism of action of these drugs,
3.4. Effects of ERK1/2 cascade inhibitors on proliferation of lymphoblasts from control or c.709-1G>A GRN mutation carriers individuals

Providing that ERK1/2 activity alterations underlie the enhanced proliferative response of PGRN deficient lymphoblasts, we were interested in testing the effects of selective ERK inhibitors, such as selumetinib (AZD6244) and MEK162 (ARRY-438162) (Davies et al., 2007; Yeh et al., 2007) that eventually could be used as therapeutic drugs in FTLD. These drugs, orally available, are being investigated for the treatment of various types of cancer including lung cancer and gynecologic malignancies (Akinleye et al., 2013; Miller et al., 2014).

The sensitivity of lymphoblastoid cell lines from control or carriers of the c.709-1G>A GRN mutation individuals to selumetinib is shown in Figure 6A. Control cells were moderately sensitive to selumetinib treatment (up to 2.5 μM); however this drug inhibited the proliferation of PGRN deficient cells in a dose-dependent manner (Figure 6B) (F1 μM Selumxindividuals (4,42)=2.995., p=0.0291). For comparison, the effect of the known pharmacological MEK inhibitor PD98059 (20 μM) is also shown. After 72 h of cell treatment with 1 μM selumetinib, it was observed that the drug abrogated the serum-mediated increased proliferation of PGRN deficient cells, without affecting the proliferation of control cells (Figure 6C) (F1 μM Selumxindividuals (4,49)=2.824., p=0.0343).

In Figure 7, we summarized the results obtained with the ERK1/2 inhibitor MEK162. Maximal effect of this drug was observed at 0.1 μM concentration (F0.1 μM MEKxindividuals (10,44)=1.05., p=0.4199) (Figure 7A). As observed in Figure 7B, treatment with 0.1 μM of MEK162 induced a significant decrease in the levels of pERK1/2 in both control and PGRN-deficient lymphoblasts (F0.1 μM MEKxindividuals (2,16)=4.003., p=0.0389).

The inhibition of ERK1/2 activity resulted in the blockade of increased proliferation of PGRN mutated carriers, without affecting that of control cells (F0.1 μM MEKxindividuals (2,39)=3.474., p=0.0409) (Figure 7C).
We next studied the effects of these ERK1/2 activity inhibitors in CDK6 levels and enzyme activity. Figure 8 shows how both treatments were effective in decreasing the content of CDK6 (F1,72 = 3.753, p = 0.0282; F0.1 μM MEK × individuals (2,65) = 4.071, p = 0.0216) (Figure 8A) and the levels of pRb (F1,37 = 4.548, p = 0.0171; F0.1 μM MEK × individuals (2,34) = 3.440, p = 0.0436) (Figure 8B), in lymphoblasts from c.709-1G>A GRN mutation carriers up to levels similar to those of control cells.

3.5. SAHA and selumenitib block pathological cytoplasmic accumulation of TDP-43 in PGRN-deficient lymphoblasts

Since one of the hallmarks in FTLD associated to GRN mutations is the presence of TDP-43 protein aggregates in the cytosol of cells from PGRN-deficient FTLD patients (Mackenzie, 2007), we sought to evaluate the impact of the PGRN enhancer drug SAHA or the inhibitor of ERK1/2 selumenitib in cytoplasmic accumulation of TDP-43 in PGRN deficient lymphoblasts. To this end, we carried out fractionation of cell extracts from control and lymphoblasts harboring the c.709-1G>A GRN mutation after the treatment with the corresponding drug, and processed them for Western blot with an anti-TDP-43 antibody. Our results show that, as expected, PGRN deficient lymphoblasts present increased levels of TDP-43 in the cytosolic fraction compared with lymphoblasts from control individuals (Figure 9). Both treatments with SAHA or selumenitib were able to decrease the levels of cytosolic TDP-43 in PGRN deficient cells without affecting TDP-34 levels in control cells. These results suggest that these treatments could ameliorate the formation of cytosolic TDP-43 aggregates in FTLD-TDP patients (Fdrugs × individuals (4,38) = 6.186, p = 0.0006).

3.6. Effects of PGRN enhancers and ERK1/2 inhibitors on PGRN deficient neuroblastoma SH-SY5Y cells

We considered interesting to validate the above-described results in lymphoblastoid cell lines by testing the effects of PGRN levels-
modifying drugs or ERK1/2 inhibitors in a neuronal cell model of PGRN deficiency. To this end, we used GRN knockdown human neuroblastoma SH-SY5Y cell line, generated in Dr. Wu lab with a pSUPERIOR RNAi construct containing the target sequence corresponding to nucleotide sequences 207 to 226 specific for human GRN gene. In agreement with a previous report from this laboratory (Alquezar et al., 2014), the KD SH-SY5Y cells showed enhanced levels of BrdU incorporation into DNA, compared with control cells. Normal rates of BrdU incorporation could be rescued by the addition of the PGRN enhancers SAHA and CQ, mimicking the effect of the addition of exogenous PGRN (rhPGRN) \( (F_{\text{drugsxindividuals}}(2,12) = 6.981, \ p = 0.0032) \) (Figure 10A).

The efficacy of these drugs altering the PGRN abundance \( (F_{\text{drugsxindividuals}}(2,12) = 6.215, \ p = 0.0140) \) and ERK1/2 activity \( (F_{\text{drugsxindividuals}}(2,42) = 12.79, \ p < 0.0001) \) in KD SH-SY5Y cells is presented in Figure 10B.

Figure 11A depicts the effects of the ERK1/2 inhibitors selumenitib and MEK162 preventing the enhanced BrdU incorporation into DNA in GRN KD SH-SY5Y cells \( (F_{\text{drugsxindividuals}}(2,12) = 6.215, \ p = 0.0140) \). In Figure 11B, it is shown that both selumenitib and MEK162 effectively reduced the levels of pERK1/2 in control and PGRN-deficient lymphoblasts \( (F_{\text{drugsxindividuals}}(2,23) = 6.517, \ p = 0.0057) \).

Taken together, these results obtained in human neuroblastoma cells, indicate that PGRN deficiency induce similar alterations in the ERK1/2 pathway and proliferative activity in neuronal and non-neuronal cells.

4. Discussion

In the last decade, remarkable progress made in the understanding of FTLD biology has unraveled it as both a set of clinically different syndromes and disorders with unique genetic and neuropathological profiles. FTLD-TDP is one of the most common subtypes of FTLD that in most of cases is associated with mutations in GRN gene. Increasing knowledge of pathogenic molecular mechanisms of FTLD has provided a rationale for designing novel therapeutic strategies. There are not yet Food and Drug Administration-approved treatments for FTLD-TDP, considered a fatal and progressive rare
neurodegenerative dementia (OMIM 607485), however the PGRN haploinsufficiency associated with GRN mutations as well as the insights into pathological processing of TDP-43 and in signaling pathways involved in FTLD-TDP open new perspectives for the identification of appropriate targets.

Previously we described a cell cycle control failure in lymphoblasts harboring a single pathogenic splicing mutation in GRN gene (c.709-1G>A). It was found that PGRN haploinsufficiency increased cell proliferation by inducing overactivation of 2 the ERK1/2/CDK6/pRb pathway (Alquezar et al., 2012a, 2014). Since cell cycle reactivation in neurons appears to underlie the development of neurodegenerative disorders including FTLD (Arendt, 2012; Herrup and Yang, 2007; Hoglinger et al., 2007; Ueberham and Arendt, 2005) and cell cycle disturbances are also found in non-neuronal cells, we considered interesting to evaluate the efficacy of PGRN enhancer drugs or EK1/2 inhibitors in the proliferative activity of PGRN deficient lymphoblasts. In addition, the effects of these drugs were evaluated in GRN KD SH-SY5Y neuroblastoma cells. To the best of our knowledge, this is the first attempt to study whether blocking cell cycle progression could restore the aberrant response of peripheral cells from FTLD patients, holding promise for new therapeutic strategies in PGRN-deficient FTLD.

Figure 7  Effects of MEK162 on proliferation and ERK1/2 activation on lymphoblasts from control and c.709-1G>A GRN mutation carriers individuals. (A) Cells were seeded in absence or presence of increasing doses of MEK162 (0-1 μM) and 72 h later the MTT assay was performed. The experimental conditions were identical to those described in the legend of Figure 6. Results represent the % of cell proliferation of treated cells referred to untreated ones (†p<0.05 and ††p<0.01 significantly different from untreated cells). (B) Efficacy of the treatment with 0.1 μM of MEK162 in the inhibition of ERK1/2 activity. The densitometric data represent the mean±SEM of seven determinations carried out in cell extracts from different individuals. (C) Proliferative activity of lymphoblast from control and GRN mutation carriers individuals 72 h after the MEK162 (0.1 μM) treatment. Data shown are the mean±SEM of seven determinations carried out with different cell lines, (*p<0.05; **p<0.01 significantly different from control cells; †p<0.05 and ††p<0.01 significantly different from untreated cells).
found that treatment of cells with drugs able to increase PGRN levels such as SAHA and CQ or with the ERK1/2 inhibitors selumetinib and MEK162 inhibited the increased proliferative activity of PGRN-deficient lymphoblast, without affecting normal basal rates of proliferation in control cells.

In agreement with previous results (Alquezar et al., 2012a, 2014), untreated lymphoblasts from individuals carrying the c.709-1G>A GRN mutation, asymptomatic or diagnosed of FTLD-TDP, showed enhanced cell proliferation when compared with cells from control subjects. The inhibition of proliferation induced by SAHA or CQ correlated with increased PGRN content. SAHA, a known histone deacetylase (HDAC) inhibitor was shown to effectively increase PGRN mRNA and protein levels in human lymphoblasts (Cenik et al., 2011) while CQ increased the PGRN content by a posttranslational mechanism (Capell et al., 2011). Both drugs were able to effectively normalize the relative abundance of PGRN in c.709-1G>A GRN mutation carrier lymphoblasts. Moreover the inhibitory effect of SAHA and CQ mimicked the effect of addition of rhPGRN to lymphoblast from GRN mutation carriers. Interestingly, the effect of exogenous PGRN is most likely due to the full-length protein, rather than to granulin peptides.

It was previously reported that PGRN haploinsufficient lymphoblasts show enhanced ERK1/2 activation following serum stimulation (Alquezar et al., 2014). Our results suggest that the mechanism underlying the anti-proliferative effects of SAHA and CQ is the capacity of these drugs to abrogate the serum-mediated stimulation of the ERK1/2 activity, which then leads to decreased CDK6 content and pRb phosphorylation, key regulators of the G1-S cell cycle transition.

SAHA demonstrated good safety profile and therapeutic potential in other neurodegenerative diseases, such as Rubinstein-Taybi syndrome, Rett syndrome, Friedreich’s ataxia, Huntington’s disease and multiple sclerosis (Kazantsev and Thompson, 2008) as well as in PD and AD (Harrison and Dexter, 2013; Meng et al., 2014). On the other hand, CQ introduced into clinical practice in 1947 for
prophylaxis treatment of malaria (Pullman et al., 1948), has been shown to alleviate the abnormal proteolytic processing of the amyloid precursor protein (APP) in a neuronal cell model of AD (Cagnin et al., 2012). Therefore, these PGRN-enhancers drugs may be useful for prevention and treatment of FTLD-TPD associated to PGRN haploinsufficiency. Encouragingly, mice overexpressing PGRN are significantly rescued from the behavioral deficits induced by middle cerebral artery occlusion (Egashira et al., 2013). In addition, the fact that SAHA was shown to induce changes in inflammatory markers in a mouse model of septic shock (Finkelstein et al., 2010) suggest that SAHA may have secondary beneficial effects in FTLD-TDP besides normalizing PGRN deficiency alleviating the chronic inflammation that is a common feature in different neurodegenerative diseases including FTLD. In the other hand, CQ could also have additional beneficial effects in FTLD patients as it can moderate the lysosomal dysfunction seen in affected brain areas (Cagnin et al., 2012; Tanaka et al., 2014). Nevertheless, caution is needed before the application of PGRN enhancer drugs therapies, since a fine tuning of PGRN levels is mandatory given its potential tumorigenic action in different tissues (Matsumura et al., 2006).

The highly selective inhibitors of MEK1/2, selumenitib and MEK162, used in this work are small molecules, orally available, that have shown promising results in clinical trials, including in previously intractable cancer such as melanoma (Ascierto et al., 2013; Bennouna et al., 2011). The safety profile and tolerability of selumenitib has been evaluated in a 2-part, multicenter, ascending dose, phase I clinical study (Adjei et al., 2008). This trial showed the tolerability of selumenitib, with the most common treatment-related toxicities being rash, diarrhea, nausea, and fatigue. MEK162 is also being evaluated in phase I and II clinical trials in patients with advanced solid tumors (Ascierto et al., 2013). Our results demonstrated the efficacy of both selumenitib and MEK162 in restoring rates of proliferation of PGRN-deficient lymphoblasts to values similar to those of control cells. Although the

Figure 10 Effects of PGRN enhancers in PGRN deficient neuroblastoma SH-SY5Y cells. (A) Control and GRN KD SH-SY5Y cells (15,000 cells/well) were incubated in the absence or in the presence of rhPGRN (100 ng/ml) SAHA (1 μM) or CQ (10 μM) for 24 h. DNA synthesis was assessed by BrdU incorporation method according to the manufacturer’s instructions. Proliferation was expressed as absorbance of stimulated minus that of non-stimulated cultures. Data shown are the mean ± SEM for three individual experiments. (B) 24 h after drug administration, whole cell extracts of SH-SY5Y clones expressing either the control vector or a target sequence of human GRN mRNA were prepared to analyze by Western blotting the expression of PGRN and pERK1/2. Densitometric analyses show the mean ± SEM for four individual experiments (*p < 0.05; **p < 0.01 significantly different from control cells. †p < 0.05; ††p < 0.01 significantly different from untreated cells).
potential neuroprotective effects of these ERK1/2 inhibitors have not been explored, our results showing the blockage of increase BrdU incorporation into DNA in selumenitib and MEK162-treated GRN KD neuroblastoma cells suggest that both drugs may have beneficial effects in neurons. In this sense, it would be desirable to test the effects of these drugs in animal models of PGRN deficiency. As mentioned, these drugs have side effects that may compromise their use for FTLD patients, however, given the grave nature of FTLD, and the devastating speed at which FTLD progresses, we believe that selumenitib and MEK162 should be considered for possible treatment for PGRN-deficient FTLD-TDP.

It is worth to highlight that both the enhancer of PGRN, SAHA, and the ERK1/2 inhibitor selumenitib, were able to normalize the cytosolic levels of TDP-43 in PGRN deficient lymphoblasts. It is thought that changes in the localization of TDP-43 protein from its preferential nuclear localization to the cytosol, is responsible for increased TDP-43 hyperphosphorylation, ubiquitination, protein fragmentation, and aggregates formation in FTLD-TDP (Arai et al., 2010; Brady et al., 2008; Neumann et al., 2006). Therefore, the efficacy of SAHA and selumenitib in preventing one of the pathological hallmarks of FTLD-TDP, as it is the cytosolic accumulation of TDP-43, reinforces the use of lymphoblasts from FTLD-TDP patients to test disease-modifying drugs.

In agreement with previous work, our results show no differences in the proliferative activity and cellular content of CDK6 and ERK1/2 activity among lymphoblasts derived from GRN c.709-1G>A mutation carriers whether asymptomatic or with a clinical diagnosis of FTLD (Alquezar et al., 2012a, 2014). Neither we found differences in the effects of PGRN enhancers or ERK1/2 inhibitors (this work). Because most of the asymptomatic carriers are younger than the patients it is suggested that these features are probably early etiologically relevant events during FTLD-development. Although considered asymptomatic, the PGRN mutation carriers show poorer neuropsychological performance, and reduced thickness of the cortex (Barandiarian et al., 2012), reflecting a prodromal phase of the disease. Thus, it is possible to consider that these drugs may have beneficial effects in slowing disease progression at its early stages.

Although FTLD-TDP-associated changes detected in peripheral cells might not fully reflect those in FTLD-TDP brain, it is evident that in addition to neuronal damage, there are also peripheral aspects of the disease. Peripheral cells from patients mirror some features of brain pathology. In this regard, attention has been drawn to signaling through the ERK1/2 pathway as a system linking stress granules, containing phosphorylated TDP-43, and neuronal loss in FTLD and ALS (Ayala et al., 2011; Parker et al., 2012). Moreover, increased levels of ERK1/2 and TDP-43 can be detected in cerebrospinal fluid samples from FTLD patients (Steinacker et al., 2008) suggesting that both proteins may be released in parallel in this neurodegenerative conditions. Thus our findings showing that TDP-43 pathological changes are also associated with ERK1/2 dysfunction in PGRN-deficient lymphocytes may be another systemic manifestation of the disease. Assuming that the PGRN deficiency-induced over-activation of ERK1/2 signaling, leading to the cycle disturbances, reported here could be peripheral signs of the disease, our results suggest that neurons of c.709-1G>A mutation carriers are at high risk of entering an unscheduled cell cycle that would then drive them to death. The above considerations support the rationale of using peripheral cells from FTLD patients for preclinical studies and testing therapeutic strategies. Our results show that enhancers of PGRN content and blockers of ERK1/2 signaling prevent the cell cycle failure in PGRN-deficient lymphoblasts and neuroblastoma cells. This fact, together with that the SAHA, CQ, selumenitib and MEK162 were shown to cross the blood-brain barrier (Ascierto et al., 2013; Hirata et al., 2011; Matsuoka and Yang, 2012; Palmieri et al., 2009), suggest these drugs can be considered promising candidates for novel treatments for FTLD associated to GRN mutations.

Figure 11  Effects of ERK1/2 Inhibitors in PGRN deficient neuroblastoma SH-SY5Y cells. The experimental conditions in this figure were identical to those described in the legend of Figure 10. (A) Effect of the treatment with selumenitib (1 μM) and MEK162 (0.1 μM) on the BrdU incorporation into DNA and in the activation of ERK1/2 in control and knockdown cells. Data shown are the mean ± SEM for four individual experiments. (B) Representative immunobloting showing pERK1/2 and ERK1/2 after treatment with selumenitib and MEK162. Densitometric analyses show the mean ± SEM for four individual experiments (*p < 0.05; **p < 0.01 significantly different from control cells. †p < 0.05; ††p < 0.01 significantly different from untreated cells).
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