TITLE

Evaluation of LRRK2 I1371V mutation on cellular pathogenesis of Parkinson's disease using patient-specific induced pluripotent stem cells of Indian ethnicity

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INTRODUCTION

Parkinson's disease (PD) is the second most age related neurodegenerative disease affecting approximately 1% of the world population above 60 years. The disease is commonly associated with bradykinesia, impaired ability of voluntary movements and resting tremors. Pharmacological strategies for PD revolve around increasing the levels of dopamine in brain (Birtwistle et al., 1998) and carry the risks of side-effects like psychotic side-effects in PD (Bohlega et al., 2013). Although such drug-induced Parkinsonism or psychosis are slightly reduced with the advent of second generation anti-psychotic drugs, problems pertaining to side -effects of medications continue to be reported (Caligiuri et al., 2010). The classical immunopathological feature of PD patient's brain is occurrence of Lewy bodies, majorly composed of α -synuclein & ubiquitin. Aberrant level of α -synuclein is cited in familial as well as idiopathic PD subjects (McCormack et al. 2016).

Mutations in Leucine Rich Repeat Kinase 2 (LRRK2) contribute significantly to large number of familial as well as sporadic cases of PD. LRRK2 is a large 2527 amino acid protein consisting of several functional domains including a Ras-like small GTPase domain (ROC), a carboxy-terminal of Roc (COR) domain, and a kinase domain3. The various mutations in LRRK2 involved in PD, are R1441C, R1441G, I1371V in the Roc domain, Y1699C in the COR domain, and G2019S, I2020T in the kinase domain. Research on PD related LRRK2 mutations have been focused majorly on the mutations located in the kinase domain, particularly on the G2019S mutation, which increases the kinase activity. G2019S mutation is predominant in the Caucasian PD patients. The prevalence of this mutation in the Indian population is very rare with less than 0.1% harboring this mutation (Vijayan et al., 2011; Punia et al., 2006).

On the other hand clinical studies for LRRK2 mutation in Indian population for PD have shown in the I1371V allele, specifically in the East Indian population (Sadhukhan et al.,

2012; Paisán-Ruíz et al., 2005). The I1371V SNP is located in the Ras of complex proteins (ROC) domain that may act as a GTPase to regulate its protein kinase activity (Cookson et al., 2007; Cookson, 2010). The ROC domain has a dimeric fold; the I1371V SNP is located at the interface of two monomers (Kondo et al., 2011). This mutation partially disrupts the tertiary structure of the protein at the dimer interface, which results in the decrease of the GTP hydrolysis and therefore prolongs GTP-mediated activation of the kinase (Tysnes et al., 2017). Impaired GTPase activity leads to more accumulation of the GTP bound form of LRRK2. Mutations in the GTPase domain are not explored as extensively as the kinase domain counterparts. There are yet no PD patient-specific iPSCs reported with I1371V allele mutation for LRRK2. In vitro studies have shown that LRRK2 mutation (G2019S allele) is associated with α -synuclein aggregation and phosphorylation and its release. For the GTPase domain mutations of R1441C, R1441G, R1441H allele has been shown to lead to impaired phosphorylation of LRRK2 (Tong et al., 2009; Paisán-Ruiz et al., 2013) but there are yet no

studies on its association with ontogeny of dopaminergic neurons and α-synuclein phosphorylation machinery.

While post-mortem samples provide valuable information on end-stage of PD, an understanding of the early stage molecular mechanisms holds the key in developing therapeutics to prevent or halt PD progression. Cell line models such as PC12, SH-SY5Y, HEK or transgenic and primary rodent neuronal cultures fail to recapitulate the required DA neuron subtype, while animal models fail to replicate human ethnicity variations. It is well established that humans and mice have considerable developmental, genetic and physiological differences (Mouse genome sequencing consortium et al., 2002), and that genetic-mutations for human PD do not replicate the disease-phenotype in mice. Pluripotent stem cells are thus attractive candidates to study the progression of PD in vitro via directed differentiation into neuronal progenitors and subsequent neuronal types such as DA neurons. iPSCs greatly help in circumventing the ethical issues associated with the use of embryonic stem cells. Additionally, iPSC-derived neuronal cells provide a patient-specific perspective on PD, of particular relevance considering that majority of the PD cases are idiopathic.

The rapidly growing knowledge of efficient iPSC generation and various differentiation protocols for diverse cell types has brought the promise of personalized medicine from the into reality. It is increasingly known that in complex neurodegenerative diseases, the clinical heterogeneity with respect to the severity of the disease, the stage, drug responsiveness and gene environment interaction are prevalent. IPSCs offer a unique opportunity to study these

complex disease-associated differences varying from patient to patient. Woodard et al in 2014, demonstrated the effect of a single glucocerebrosidase N370S mutation in iPSC-derived mDA neurons of twins clinically discordant for PD. Nguyen et al in 2011, showed the effect of G2019S mutation in the LRRK2 gene in multiple clones of a single iPSC line derived DA neurons which lead to increased susceptibility under 6OHDA stress. The effect of synuclein locus triplication to increased susceptibility of neurons to oxidative stress and synuclein accumulation was revealed by Byers et al in 2011, using iPSCs derived from a 48 year old male PD patient in comparison to iPSC derived from the unaffected sister. The varying responses of patients to the L-Dopa treatment coupled with the complex heterogeneity of PD cases indicate that differences in the biochemical pathways play major role in the drug responsiveness. Patient specific genetic differences largely influence the metabolic pathways governing the drug uptake and activity. (Stoddard-Bennett et al, 2019)

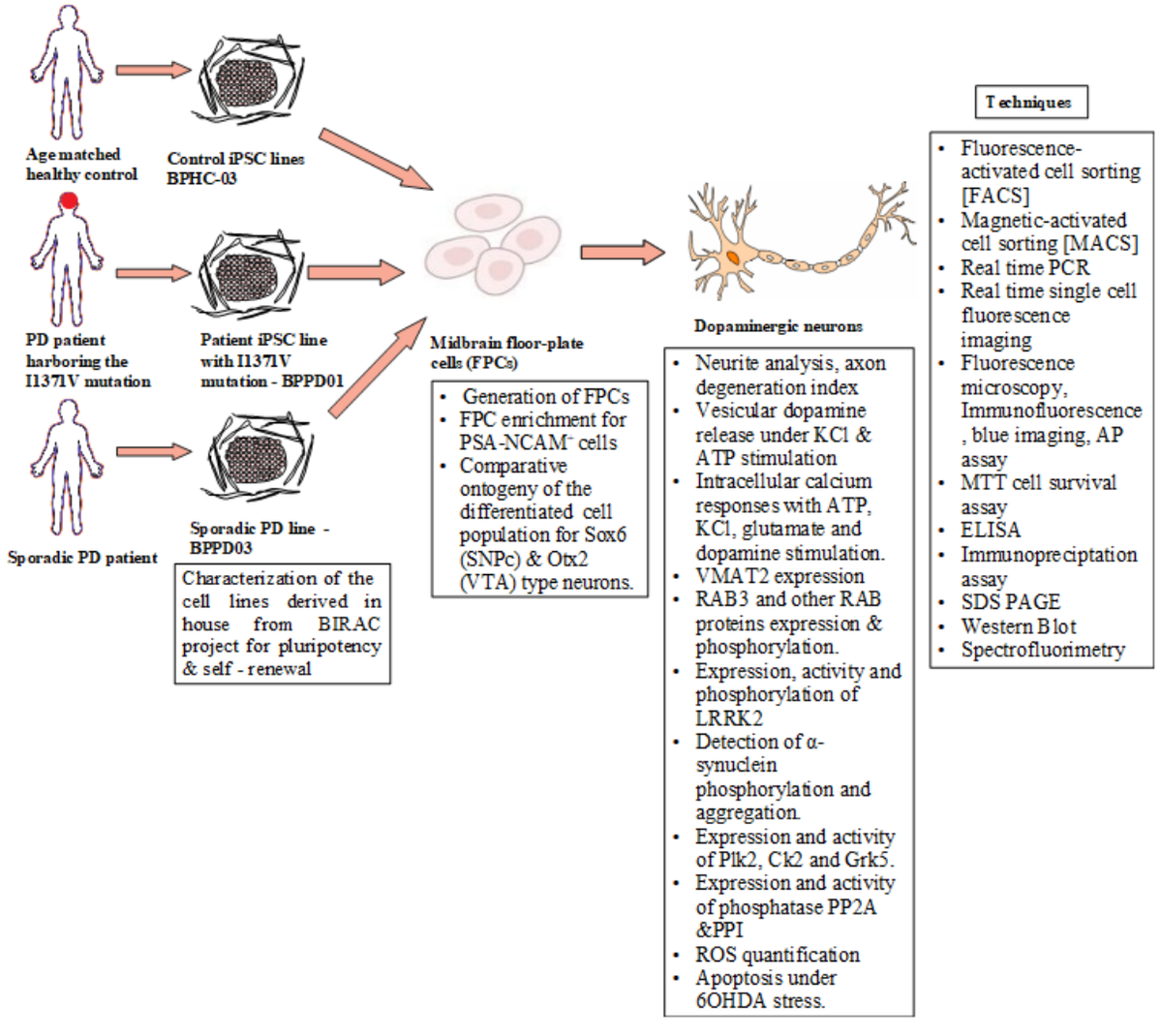
Hence in this PhD work, we aim to study the LRRK2 I1371V allele mutation on cellular pathogenesis of Parkinson's disease along with a comparative analysis in sporadic PD case using patient-specific induced pluripotent stem cells. In the present study we will be generating midbrain floor plate cells (FPCs) from iPSC lines harboring the I1371V mutation , a sporadic PD line along with iPSC derived from age-matched healthy control. The FPCs will be characterized for their commitment to mature dopaminergic neurons. The intracellular Ca2+ and vesicular dopamine release from these neurons under physiological stimuli will also be detected through real time fluorescence imaging, FACS and ELISA. Neurite analysis, axon degeneration index, along with synaptic vesicle release proteins, synapsin, VMAT2 and RAB protein expression and phosphorylation will be determined. Along with the cellular and biophysical differences, the differential ontogeny of the FPC population into substantia nigra pars compacta (SNPc) and ventral tegmental area (VTA) type neurons as the SNPc dopamine

neurons exhibit increased degenration compared to VTA type neurons with PD progression (Brichta et al, 2014). Enzyme expression and activity and phosphorylation of LRRK2, PLK2, CK2 and GRK5 (involved in α -synuclein phosphorylation), phosphatase (PPI and PP2A) will be assessed. The susceptibility to 6-OHDA stress of these neurons will be assessed through ROS generation and apoptosis.

OBJECTIVES OF THE RESEARCH

Objective 1: To generate and characterize midbrain floor-plate cells and their commitment to mature dopaminergic neurons from iPSCs of LRRK2 I137V mutation PD subject and healthy donor.
Objective 2: Monitoring the difference in function of dopaminergic neurons derived from the PD and healthy subject iPSCs with respect to intracellular calcium response and vesicular dopamine release under physiological stimuli.
Objective 3: To assess α-synuclein phosphorylation, α-synuclein aggregation and expression and activity of enzymes involved in phosphorylation and de-phosphorylation of LRRK2 and α-synuclein.
Objective 4: To assess the susceptibility of these dopaminergic neurons to 6-OHDA stress through ROS generation and apoptosis.

MATERIALS AND METHODOLOGY



Schematic of the methodology followed

Objective1: To generate and characterize midbrain floor-plate cells and their commitment to mature dopaminergic neurons from iPSCs of LRRK2 I137V mutation PD subject and healthy donor.

Methodology

A. Screening of patients: The patients were screened at NIMHANS for Parkinson's disease and the clinical diagnosis was performed by using the Unified Parkinson's disease rating Scale - III. The stage, severity and confirmation of PD onset was confirmed by MRI reports and 6-fluoro(^{18}F)-L-dopa (F-DOPA) PET scan. Blood samples were withdrawn from patients where the onset of PD was confirmed and PBMCs were isolated. The isolated PBMCs were used for reprogrammed to generate iPSC lines using the CytoTune-iPS 2.0 Sendai Reprogramming Kit from ThermoFisher. The blood samples were also used for targeted gene

sequencing for a panel of 13 genes identified as risk factors or mutations associated with PD pathogenesis. A patient carrying the I1371V mutation in the LRRK2 gene was identified through targeted gene analysis and the line generated was denoted at BPPD-01. Another case of sporadic PD was used for iPSC derivation and labelled as BPPD-03. The age-matched healthy control was recruited and the iPSC line denoted as BPHC-03. Due to ethical considerations, the F-DOPA PET and MRI scan for the control sample was not performed.

B. Maintenance of iPSC cultures: The iPSC cultures were observed and maintained in complete iPSC media -DMEM/F-12 supplemented with 20% KOSR, 1X penicillin-streptomycin, glutamax, non-essential aminoacids (NEAA), 0.1mM 2-mercaptoethanol and 20µg FGF2. Daily media change will be given to the iPSC cultures. By day 15 to 21, colonies would reach appropriate size for transfer. The iPSC colonies with distinct borders were subcloned by manual picking.

C. Characterization of the iPSC cell lines: Till passage 4, the iPSC lines were screened for the expression of endogenous pluripotency markers *viz.*Oct4, Sox2, SSEA4, Nanog, Ki67 as well the viral exogeneous markers for cMyc, KOS, Klf4, Oct4 & Sox2 by PCR. Expression of the pluripotency markers was also checked by immunostaining the cells for Nanog, Oct4, Sox2, SSEA4 and Tra-1-60. Additionally, alkaline phosphatase and blue cell imaging was performed for validation of pluripotency.

For immunofluorescence, pluripotent stem cells were fixed with 4% paraformaldehyde for 20 min at 4ºC. The cells were washed twice with PBST 1X PBS and 0.05 % tween permeabilized in 0.1 Triton X-100 except for cell surface markers-SSEA4, TRA-1-60 for 15min and subsequently blocked with 3 BSA in PBS for 30min at room temperature. PBST wash was given and cells were incubated with specific primary antibodies diluted in PBS, overnight at 4ºC in moist chamber. Primary antibodies 1:200 dilution for pluripotent markers like OCT 4, Sox2, Nanog, SSEA4 and TRA-1-60 were used to characterize expression in iPSCs. Appropriate secondary antibodies, anti-mouse IgG-FITC and anti-rabbit IgG-FITC were used for visualization. Cells were incubated in secondary antibody 1:400 dilution for 45 min at room temperature in dark and counter-stained with 4, 6-diamidino-2-phenylindole dihydro-Q3chloride DAPI 300nM for 3min. Then PBS-Tween PBST washes were given. A drop of DABCO was added onto the slide and coverslips were transferred and mounted. It was observed under fluorescent microscope and images were taken.

D. Embryoid body (EB) formation and tri-lineage differentiation: iPSC colonies were mechanically cut and plated on EB media. On Day 2.5, the aggregates were transferred to a fresh non-adherent dish to remove any residual MEFs. The EBs formed were screened through phase contrast microscope for typical round morphology and characterized for all 3 derm-lineage markers through RT-PCR and immunofluorescence.

The EBs were differentiated into hepatocyte (endoderm), cardiomyocte (mesoderm) and neuronal cells (ectoderm) according to the protocols established in the PI's lab.

E. Generation and Maintenance of Floor Plate Cells (FPCs): EBs were cultured for additional 2 -3 days with agitation in neural induction media NIM consisting of DMEM/F12, 1xNEAA, 0.5x L -Glu, and freshly made and sterile filtered N2, which contained 1.55 g/l glucose, 2g/l sodium bicarbonate, 100µM putrescine, 30nM sodium selenite, 20 nM progesterone, 0.1 mg/ml transferrin, 0.025 mg/ml insulin and FGF2 20ng/mL. Cell culture plates were coated with Geltrex, media changed every day. Neural rosettes were formed in 2–5 days in adherent culture. To obtain a pure population of FPCs, rosettes were manually isolated using No. 15 scalpel cutting in squares with distance to edges of colonies. Dissected pieces of rosettes were lifted using a pipette, replated onto Geltrex coated culture dishes and maintained in FPC media containing neurobasal media , 1xNEAA, 1xL-Glu, 1xP/S, 1xB27 supplement, and FGF2 20ng/mL. Manual isolation of rosettes as described above was repeated once to obtain more pure population of FPCs. Approximately 5–10 pieces of rosettes were dissociated into single cells using Accutase. Cells were treated with Accutase for 2–5 minutes until cells became round in shape, then the cells were collected, centrifuged, re-suspended in FPC media, and plated onto one 96-well coated with Geltrex and cultured at 37◦C and 5%CO_2. When confluent, FPCs were split at a ratio of 1:2 in single wells with larger surface area such as 48-well, 24-well, 12-well, and so forth in FPC media.

F. FPC Enrichment Using Anti PSA-NCAM Magnetic sorting: For magnetic bead sorting, FPCs were treated with Accutase, collected, and passed through 30μm nylon mesh pre-separation filters, 30 μm, Miltenyi Biotec . The total cell number was kept at 10 million. Cell suspension was centrifuged at 300×g for 10min. Supernatant was aspirated completely and cell pellet was re-suspended in 60μl of buffer 1x PBS, 2mM EDTA and 0.5 albumin from bovine serum Sigma. Cells were mixed well and incubated for 10min in the refrigerator 2−8◦C . Then, 20μl of anti-PSA -NCAM microbeads Miltenyi Biotech were added to the mixture, mixed well with pipetting up and down, and incubated for 15min in the refrigerator 2−8ºC . Cells were washed by adding 2ml of buffer and centrifuged at 300×g for 10min. Supernatant was aspirated completely and cell pellet was re-suspended up to 10^8cells in 500μl of buffer. A MS column from Miltenyi Biotec, was placed in the magnetic field of a miniMACS separator, rinsed with 500μl of buffer three times. Cell suspension was applied onto the column. The column was then washed with 500μl of buffer three times again. New buffer was added when the column reservoir is empty. The column was removed from the separator and placed on a 15ml BD Falcon conical tube BD Bioscience. One ml of buffer was added onto the column and magnetically labeled cells were flushed out by firmly pushing the plunger into the column. The eluted fraction was directly enriched over a second column and the magnetic separation procedure was repeated once by using a new MS column. One ml of FPC media was added onto the column to flush out the magnetically labeled cells. Then another 1ml of FPC media was added and cell suspension was transferred to a 35mm Geltrex-coated culture dish. The purified population was assessed by flow cytometry for the expression of neural progenitor markers such as Ki67, Musashi12, Sox2, S100β,nestin along with PSA-NCAM.
The pilot data shows enrichment of NP markers in the positively sorted population. This method has been standardized in the lab and I have performed it effectively.

G. Fluorescence Activated Cell sorting for midbrain FPC population ontogeny: The FPC population obtained from the iPSC lines was assessed for the differential expression of SNPc and VTA markers such as Sox6, Otx2, DOC2B, CLIC5 (chloride intracellular channel 5) by real time PCR, immunofluorescence and flow cytometry.
Cells were harvested and fixed with 2% paraformaldehyde for 30 min at 4°C. Next cells will be permeabilized using 0.1% triton X-100 for 15 min followed by blocking in 3% BSA for 1 h and incubated with primary antibody for Sox6, Otx2, DOC2B, CLIC5 (1:100) for 45 min. Cells were washed with PBS containing 0.01% sodium azide (Sigma) and incubated with secondary antibodies for 30 min at room temperature. Flow cytometry was performed on BD Caliber flow cytometer (BD Biosciences) and analyzed using FACS CELLPRO QUEST software (BD Biosciences). The appropriate secondary antibodies were used as an isotype control. Percentage immunopositive for Sox6, Otx2, DOC2B, CLIC5 population was plotted as mean ± SEM for n = 6.

H. Dopaminergic Differentiation of FPCs: The FPCs were primed in FGF8 media containing 1X neural induction supplement 25ng/ml of FGF8 & Neurobasal media for 4 days on Geltrex coated plates. After 48 hours media change was given. The cells were passaged & replated on Geltrex & 1μg/ml laminin was added while plating. On day 7, 5ng/ml SHH, 50nM dbcAMP 0.2mM Ascorbic Acid & 1X N2B27 supplement was added in the media containing Advanced F12 & Neurobasal in 1:1 ratio. Media change was given every 48 hours & while media change it was observed that air exposure is not given to the cells. After 2 media changes with 5ng/ml of SHH, the concentration of SHH was reduced to 1 ng/ml. By day 10-12 DA neurons can be taken for downstream experiments.

Objective 2: Monitoring the difference in function of dopaminergic neurons derived from the PD and healthy subject iPSCs with respect to intracellular calcium response and vesicular dopamine release under physiological stimuli.

Methodology

A. Intracellular calcium response: The basal $[Ca^{2+}]i$ was monitored for the control as well as the PD patient lines. The lines were assessed using Ca^{2+} sensitive fluorescent probe Fluo-4AM. Cells will be loaded with 3 μM Fluo-4 acetoxymethylesters (Fluo-4AM; Molecular Probes) in HBSS

containing 0.08% pluronic acid (Molecular Probes) for 30 min at 37°C. After two washes with HBSS, cells were kept in dark for 20 min at room temperature for de-esterification. Cell permeant Fluo-4AM was cleaved by cytosolic esterases to membrane impermeant Fluo-4, that exhibits increase in fluorescence intensity on binding Ca^{2+}. The fluorescence intensity of Fluo-4 (ex/em-485 nm/535 nm) was measured using a microplate reader (Infinite®M200, TECAN, Switzerland). The basal and the peak intensity after stimulation with KCl (56 mM) and ATP (100 μM) was measured and plotted as mean ± SEM for n = 6.

B. Vesicular dopamine release: Dopamine release was quantified using an enzyme-linked immunosorbent assay (ELISA) kit obtained from LDN, Nordhorn, Germany, as per the manufacturer's instructions. This was earlier used by the PI for previous experiments as cited in her publications. In brief, the cell culture supernatants of control and induced cells were collected on days 0, 3, 6, and 9, respectively and their dopamine release will be estimated. For depolarization, cells were stimulated with 56mM KCl and 100mM ATP for 5 and 1 min, respectively, in 4- 2-hydroxyethy1 -1-piperazineethanesulfonic acid HEPES -buffered salt solution HBSS 130mM Na^{+}, 5.4mM K^{+}, 0.8mM Mg^{2+}, 1.8mM Ca^{2+}, 130.6mM Cl^{-}, 20mM HEPES, and 15mM Glucose at 37°C to measure the inducible release of dopamine. The absorbance was subsequently measured at 450nm in a multi-label counter TECAN . Culture supernatants from SH-SY5Y cells for both constitutive and inducible releases of dopamine, were be used as positive control.

C. Epi-fluorescence microscopy for neurite analysis: Neurite analysis will be performed on re-plated iPSC-derived neurons at 30-35 DIV. The Molecular Probes® Neurite Outgrowth Staining Kit will be used for neurite outgrowth analysis. The kit allows for quick and simple measurement of neurite outgrowth and cell viability in the same sample. It includes three fluorescent dyes: a cell viability indicator, a cell membrane stain, and a background suppression reagent. A 1x staining solution will be prepared by diluting the Cell Membrane Stain and Cell Viability Indicator dyes provided at 1000x together in either PBS or Live Cell Imaging Solution Life Technologies . Next, the growth medium will be removed from the wells and replaced by adding 1x staining solution 100 μl/well, 96-well format to the wells. Assay plates will be incubated at either room temperature or 37°C, for 10–20 minutes or until sufficient green fluorescence from the converted Cell Viability indicator will be detected. Following the incubation step, the stain solution will be removed and replaced with 1x Background Suppressor provided at 100x , also diluted in PBS or Live Cell Imaging Solution. Neurite outgrowth will be monitored via bright orange-red staining of outer cell membrane surfaces. Neuronal cell health will be assessed simultaneously by means of a cell-permeable viability indicator dye that is converted by live cells to emit green fluorescence. The number of neurites extending from the soma of at least 100 single DsRed-labelled neurons per sample will be determined. Neuronal cell health will be assessed simultaneously by means of a cell-permeable viability indicator dye that is converted by live cells to emit green fluorescence. Neurite length will be estimated by manually tracing the length of all neurites on DsRed-labelled neurons using the NeuronJ plugin of ImageJ NIH.

D. Epi-fluorescence microscopy for Axon degeneration index: Analysis of axonal degeneration will be performed by immunostaining for βIII-tubulin TUJ1 in iPSC-derived healthy and PD neuronal cultures. The cultures will be either untreated or be in the presence of different concentrations of 6-OHDA. The number of TUJ1+ spots in blebbed or fragmented axons will be counted manually ImageJ on twenty randomly selected fields and the ratio between the number of spots and the total TUJ1+ staining area ImageJ will be defined as axon degeneration index.

E. Fluorescence Activated Cell sorting for VMAT2 immunopositive population: Levels of the VMAT2 protein will be assessed by immunofluorescence & FACS. Cells will be harvested and fixed with 2% paraformaldehyde for 30 min at 4°C. Next cells will be permeabilized using 0.1% triton X-100 for 15 min followed by blocking in 3% BSA for 1h and incubated with primary antibody VMAT2 (1:100) for 45 min. Cells will be washed with PBS containing 0.01% sodium azide (Sigma) and incubated with secondary antibody (FITC conjugated anti-mouse IgG) (Chemicon) for 30 min at room temperature. Flow cytometry will be performed on BD Caliber flow cytometer (BD Biosciences) and analyzed using FACS CELLPRO QUEST software (BD

Biosciences). Mouse/Rabbit IgG1-FITC (Chemicon) will be used as an isotype control. Percentage immunopositive for VMAT2 population will be plotted as mean ± SEM for n = 6.

F. Fluorescence Activated Cell sorting for Co-immunophenotyping of RAB3 protein with VMAT2: RAB3 and other RAB protein expression and phosphoylation will be detected with co-immunophenotyping with VMAT2 and measured using FACS.

For FACS, cells will be harvested and collected in PBS/EDTA (5 mM EDTA in PBS) followed by centrifugation at 1,800 rpm for 5 min. These cells will be then fixed in 2% paraformaldehyde for 20 min at RT, permeabilized using 0.1% Triton X-100 for 15 min followed by blocking with 3% BSA for 1 h. Cells will be then incubated with anti-RAB3 and anti-VMAT2 primary antibody overnight at 4°C followed by staining with appropriate secondary antibodies for 30 min, at room temperature. FACS analysis will be performed using BD FACS Verse and evaluated by BD FACSuite software application (BD Biosciences). Cells will be identified by light scatter for 10,000 gated events. Dual analysis will be introduced using quadrant density plot and corresponding histograms were obtained to assess the total FITC and total TRITC immunopositive population.

Objective 3: To assess α-synuclein phosphorylation, α -synuclein aggregation and expression and activity of enzymes involved in phosphorylation and de-phosphorylation of LRRK2 and α -synuclein.

Methodology

A. Fluorescence Activated Cell sorting for detection of α-synuclein phosphorylation and aggregates: The occurrence of α-synuclein phosphorylation and aggregates (control vs. PD) will be detected through FACS by co-labeling the cells with α-synuclein and ubiquitin, as reported earlier from the PI's lab (Ganapathy *et al.*, 2016). Quantification of aggregation and basal ratio of phosphorylated α-synuclein to total α-synuclein will be estimated by immunophenotyping followed by FACS analysis. The single immunopositive population of cells for α-synuclein and ubiquitin will also be measured through this procedure. The aggregation of α-synuclein will be further evaluated through western blot and co-immunoprecipitation of α-synuclein and ubiquitin.

For western blot, protein extraction from control and treated cells will be performed by incubating the harvested cells for 10 min in cold lysis buffer of pH 8. Lysis buffer will contain of 150 mM NaCl, 1% triton X-100, 2 mM Dithiothreitol, 1 mM EDTA, 50 mM Tris HCl, 1 mM Phenylmethylsulfonyl fluoride, and 1% protease inhibitor cocktail. Cells will be sonicated by short sonication bursts of 10 pulses, 30% duty cycle. Cell lysate will be then centrifuged at 14,000 rpm for 15 min and the supernatants will be collected. Proteins separated by SDS–PAGE will be transferred onto polyvinylidene difluoride membrane (Millipore) by semi-dry transfer method. Membranes will be washed with TBST (TBS with 0.05% Tween 20) followed by blocking with 3% BSA for 1 h at RT. Each membrane will be probed separately with rabbit polyclonal anti phosphorylated α-synuclein antibody, and anti β tubulin III antibody at 4°C, overnight. HRP-tagged secondary antibodies will be used at 1:5000 dilution for 1 h at RT. Bands will be visualized by enhanced chemiluminescence (ECL) super signal pico chemiluminescent substrate (Pierce, Thermo Scientific™) using G:BOX imaging system (Syngene) and ImageJ software. Densitometric analysis for α-synuclein will be normalized for β tubulin III and represented as relative density (mean ± SEM) for n = 3.

For quantification of basal ratio of phosphorylated α-synuclein to total α-synuclein across the lines, FACS will be performed by co-labelling the cells with anti-phosphorylated α-synuclein

B. Phosphorylation machinery of α-synuclein: Expression and activity of PLK2, GRK5 and CK2, the enzymes involved in synuclein phosphorylation, will be checked by western blot and kinase activity assay. The kinase activity shall be assessed using the cell lysates. In brief, the cells will be plated in a 96-well plate at 1.5 to 2 million cells/well in growth medium. Incubate plate in a 37°C incubator with a humidified atmosphere of 5% CO2 for 20–24 hours. Growth medium will be removed and cells washed once with PBS. Cells will be trypsinized and lyzed for 10 min in cold lysis buffer of pH 8 containing150 mM NaCl, 1% triton X-100, 2 mM Dithiothreitol, 1 mM EDTA, 50 mM Tris HCl, 1 mM Phenylmethylsulfonyl fluoride, and 1% protease inhibitor cocktail. Cells will be sonicated by short sonication bursts of 10 pulses, 30% duty cycle. Cell lysate will be then centrifuged at 14,000 rpm for 15 min and the supernatants will be collected.

The CK2 and PLK2 activity shall be assessed using Cyclex Kinase Assay kit from Medical and Biological Laboratories Co, Ltd according to the manufacturer's instructions. For the assay, reaction mixture will be 95µl Kinase buffer, 5µl 20X ATP. 100µl of reaction mixture will be added to the wells, followed by incubation for 30min at 30ºC. Wells will be washed

with PBS, add 100µl of HRP conjugated anti-phosphorylated form CK2 and PLK2 specific antibody and incubated for 30min at room temperature. 100µl of substrate reagent will be added, followed by 100µl stop solution addition. Absorbance at dual wavelength of 450/540nm on a Fluorescence plate reader will be measured. Absorbance values for the samples as well as the triplicate sample values will be averaged. For kinetic values, mean absorbance values vs the time points (in minutes) will be plotted. Comparative analysis will be performed between the mutant PD line, sporadic PD line and the age matched healthy control lines.

The GRK5 activity will be assessed using the ADP-Glo™ Kinase Assay from Promega according to the manufacturer's instructions. The standard graph for the GRK5 is generated using concentrations of 200ng, 100ng, 50ng, 25ng, 12.5ng, 6.3ng, 3.1ng and 1.6ng of purified GRK5 supplied along with the kit. In brief, the cell lysate, substrate and ATP shall be diluted in kinase buffer consisting of 40mM Tris,7.5; 20mM MgCl2; 0.1mg/ml BSA; 50µM DTT.. The diluted components will be added to wells of 384 low volume plate: 2 µl of cell lysate and 2 µl of substrate/ATP mix. The mixture will be incubated at R.T. for 120min in dark. After the incubation, 5 µl of ADP-Glo™ Reagent will be added to the wells. The plate will further be incubated at R.T. for 40 minutes. 10 µl of Kinase Detection Reagent will be added to the wells and incubated for 30 minutes. Luminescence will be recorded (Integration time 0.5-1second) and the GRK5 activity will be determined by comparing it with the standard graph.

Levels of the phosphatase PP2A will also be checked in neurons derived from all the lines by western blot as described before.

C. Phosphorylation machinery of LRRK2: Expression and phosphorylation of LRRK2 in the DA neurons will be assessed by western blotting and immunofluorescence. Alteration in the LRRK2 activity will be checked by LanthaScreen™ Kinase Assay kit from ThermoFisher according to the manufacturer's instructions.

The cells will be plated in a 6-well plate at 1.5 to 2 million cells/well in growth medium. Once the cells reach the required confluency growth medium will be removed and cells will be washed once with PBS with Ca^{2+} and Mg^{2+}. 1.5 mL addition of PBS with Ca^{2+} and Mg^{2+} to each well, will be followed by addition of 0.5 mL BacMam LRRK2-GFP reagent to 25% (v/v) final. The plate will be incubated at room temperature in the dark with gentle rocking for 3–4 hours. The virus/PBS solution will be removed and 2 mL/well of growth medium

containing 0.3X BacMam Enhancer Solution will be added. The plate will be incubated in a 37°C incubator with a humidified atmosphere of 5% CO2 for 20–24 hours. After transduction, cells will be harvested and resuspended in Assay Medium at the following density (U-2 OS: 0.5 × 10_6 cells/mL; HEK293: 0.75 × 10_6 cells/mL; SH-SY5Y: 1 × 10_6 cells/mL). Cells will then be plated on 384-well assay plate in 20 µL/well volume. The plate will be incubated in a 37°C incubator with a humidified atmosphere of 5% CO2 for 20–24 hours.

Complete 6X Lysis Buffer will be prepared by adding protease inhibitor and phosphatase inhibitor cocktails to LanthaScreen® 6X Lysis Buffer, at a 1:33 dilution of 100X stock (e.g., 30 µL of 100X stock inhibitors per 1,000 µL of LanthaScreen® 6X Lysis Buffer) and add LanthaScreen® Tb-anti-LRRK2 [pSer935] Antibody to the 6X Lysis Buffer at a concentration of 12 nM. Mix gently by inversion. Store on ice until use. 5 µL/well of Complete LanthaScreen® 6X Lysis Buffer will be added followed by centrifugation briefly at 300 × g and incubated at room temperature in the dark for 2–3 hour

20 µl/well of HIGH Control will be added to empty assay plate wells for 384-well format with a minimum of 3 replicates. 20µl/well of the LOW Control will be added to empty assay plate wells for 384-well format with a minimum of 3 replicates. The plate will be read in a Fluorescence plate reader. For each well, the TR-FRET Emission Ratio (e.g., 520 nm/495 nm) is calculated by dividing the acceptor emission value (e.g., 520 nm) by the donor emission value (e.g., 495 nm). Averaging the Emission Ratios for the HIGH Control and separately averaging the Emission Ratios for the LOW Control will be done. The HIGH to LOW fold-change will be determined by dividing the average Emission Ratio for the HIGH by the average Emission Ratio for the LOW.

Objective 4: To assess the susceptibility of these dopaminergic neurons to 6-OHDA stress through ROS generation and apoptosis.

Methodology

A. Fluorescence Activated Cell sorting for Cell survival: The comparative cell survival analysis of DA neurons in in response to 6OHDA and H_2O_2 stress will be estimated by MTT assay for cytotoxicity and annexin-PI staining measured through flow cytometry.

Cells will be incubated with 1 mg/mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) at 37°C and 5% CO2 for 2 h. MTT medium will be carefully aspirated from the wells, and the formazan crystals will be dissolved using dimethyl sulphoxide. Absorbance will be measured using a spectrophotometer (VICTOR3TM, Perkin Elmer, MA) at a wavelength of 560 nm; data was analyzed with respect to control. The results will be presented as mean ± SEM from n = 6 in duplicates.

B. Fluorescence Activated Cell sorting for co-labeling of apoptotic population with α-synuclein: FACS analysis of annexinV co-stained with α-synuclein will be conducted at different time points, as mentioned in the PI's publication (Ganapathy *et al.*, 2016). Control and treated cells will be co-immunolabeled with apoptosis marker annexin–V conjugated with FITC and α-synuclein.

Apoptosis will be measured using Annexin-V FITC assay kit (Molecular probes, Invitrogen). The cells along with floaters will be harvested and collected in PBS/EDTA (5 mM EDTA in PBS). Cell pellet will be suspended in 0.5 mL of annexin binding buffer with 5 µL of annexin-V and 1 µL of propidium iodide (PI) per 1 × 106 cells for 30 min at room temperature. Fluorescence will be measured using BD FACS Caliber flow cytometer and analyzed using Cell-QuestPro software. Dual analysis will be introduced using quadrant density plot. Early apoptotic cells will be identified as those stained by FITC but not PI, late apoptotic cells stained by both FITC and PI, necrotic cells will be those stained by PI alone and live cells will be identified as those which are not stained for both the dyes. The number of cells in each category will be expressed as a percentage (mean ± SEM, n = 6) of total number of cells.

C. ROS level measurement: Reactive oxygen species in 6-OHDA treated cells will be determined using 2′, 7′-Dichlorofluorescin diacetate (H2DCF.DA; molecular probes, Invitrogen). Cells plated at density 1.5 × 10_5 cells/well, in a 24 well-plate will be treated with different

concentrations of 6-OHDA for 48 h. Cells will be washed and then equal number of live-cells collected for each 6-OHDA treatment and incubated with 5 µM H2DCF.DA at 37°C for 15 min. H2DCF. DA diffuses through the cell membrane and is hydrolyzed by intracellular esterases to the non-fluorescent form of dichlorofluorescein (H2DCF). ROS generated by cells oxidizes the non-fluorescent form into a highly fluorescent compound, that is, 2′, 7′-dichlorofluorescein (DCF). H_2O_2 induced ROS will be used as positive control for H2DCF.DA in the cell lines. The fluorescence intensity of DCF (ex/em-485 nm/535 nm) will be measured using a spectrophotometer (VICTOR, Perkin-Elmer) and results are presented as mean ± SEM for n = 6 samples.

RESULTS

1. Generation and characterization of iPSC lines:

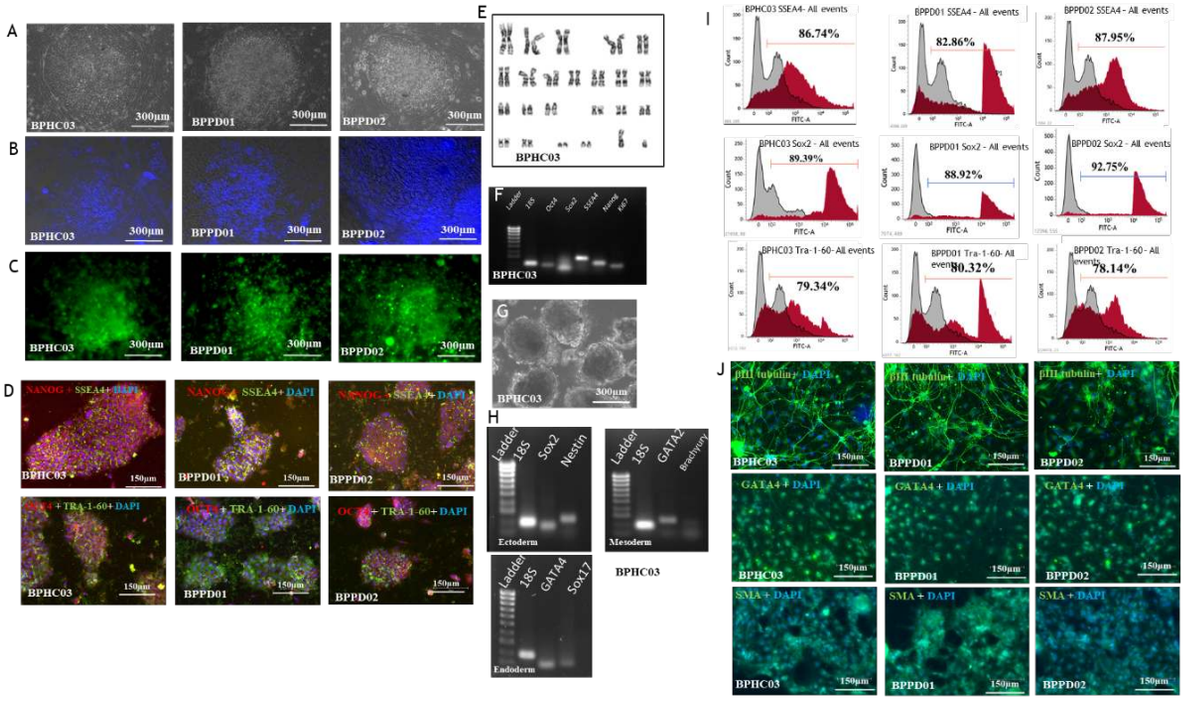


Fig. 1. The iPSC lines from healthy subject (BPHC03), PD patient carrying the LRRK2 I1371V mutation (BPPD01) and sporadic PD patient (BPPD02) ***A)*** *showed formation of Embryonic stem cell like colonies which exhibited* ***B)*** *retinyl ester associated blue fluorescence and* ***C)*** *expression of alkaline phosphatase characteristic of pluripotent stem cells. The iPSC lines were characterized for the expression of stemness and pluripotent markers by* ***D)*** *Immunofluorescence (IF),* ***F)*** *gene expression by PCR and* ***I)*** *flow cytometry.* ***G)*** *Embryoid body formation and trilineage differentiation into endoderm, ectoderm and mesoderm was checked by* ***H)*** *PCR and* ***J)*** *IF.*

2. Differentiation and characterization of the FPC (Floor plate cells)

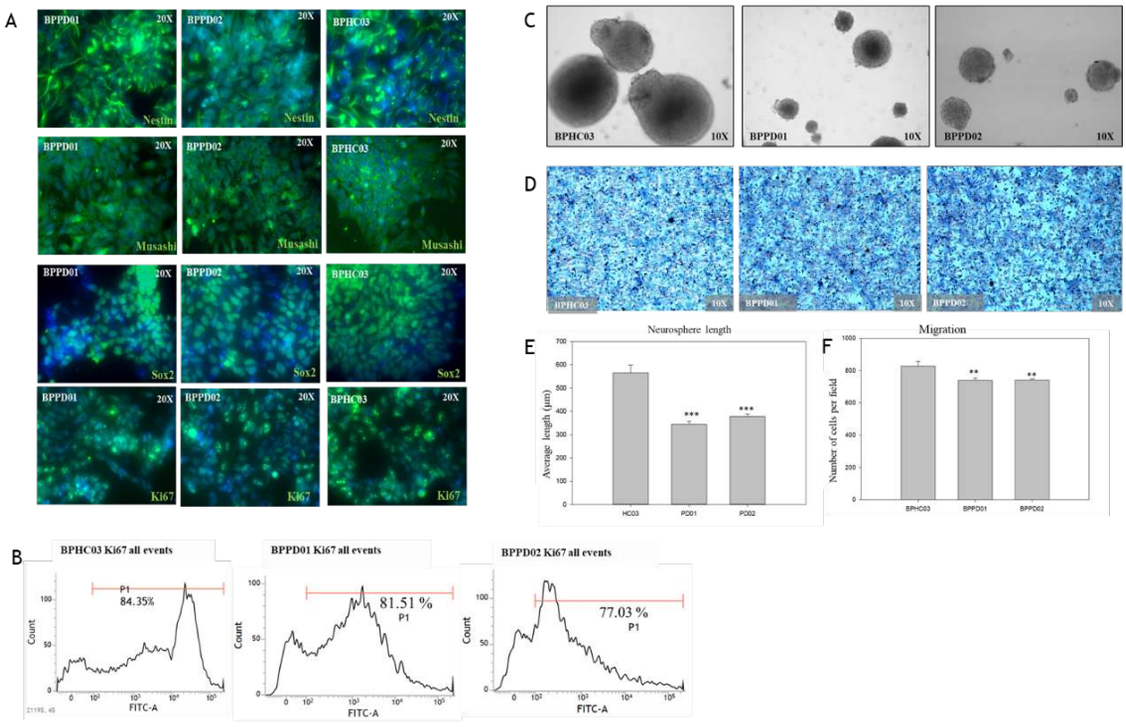


Fig. 2. The iPSC lines were successfully differentiated into FPCs which expressed FPC specific markers Nestin, Musashi, Sox2 and Ki67 confirmed by ***A)*** *IF and* ***B)*** *Flow cytometry. PD-iPSC derived FPCs exhibited* ***C,E)*** *smaller neurosphere formation and* ***D,F)*** *reduced migration*

3. Expression of SHH receptors Ptch (Patched), Smo (Smoothened) and downstream Gli1

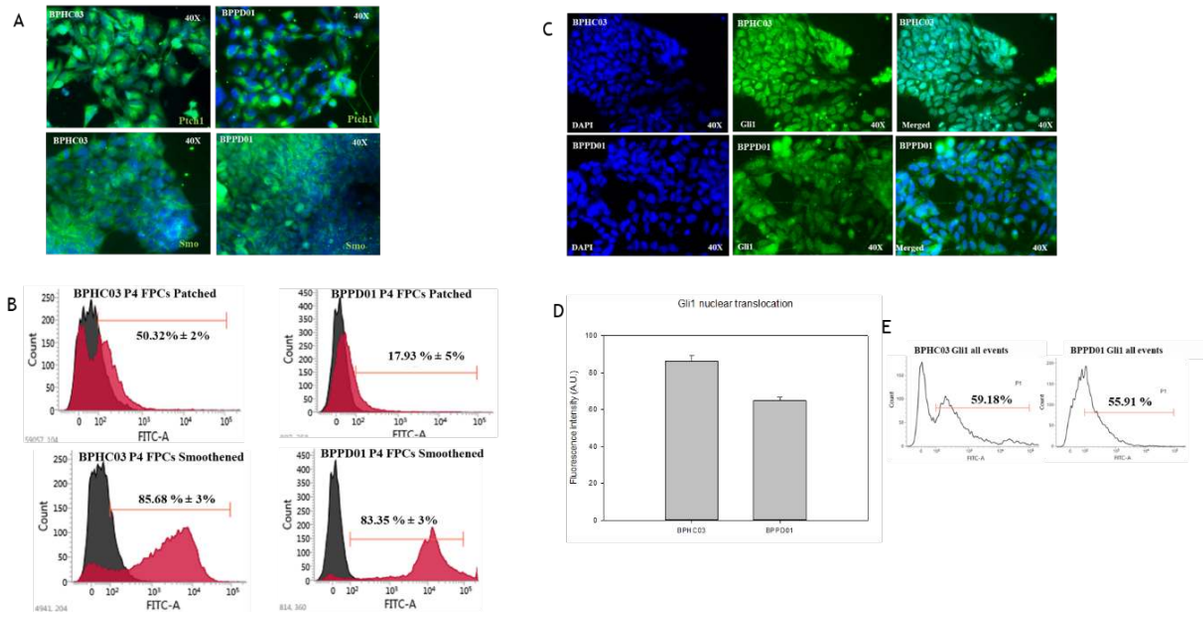


Fig. 3. Expression of Ptch and Smo in the FPCs checked by ***A)*** *IF and* ***B)*** *Flow cytometry. Gli1 nuclear translocation was checked by* ***C)****IF* ***D)****Flow cytometry and* ***E)*** *quantified.*

Results: Differentiation of FPCs into dopaminergic neurons is governed by a host of spatiotemporal as well as growth factors. Sonic Hedgehog (SHH) is the chief morphogen governing this differentiation process; therefore we assessed the expression levels of SHH receptors Ptch and Smo along with the downstream effector molecule Gli1. BPPD-01 iPSC derived FPCs exhibited reduced expression of Ptch1 as well as diminished nuclear translocation of Gli1 transcription factor

4. Dopaminergic neuron differentiation and differential intracellular calcium response

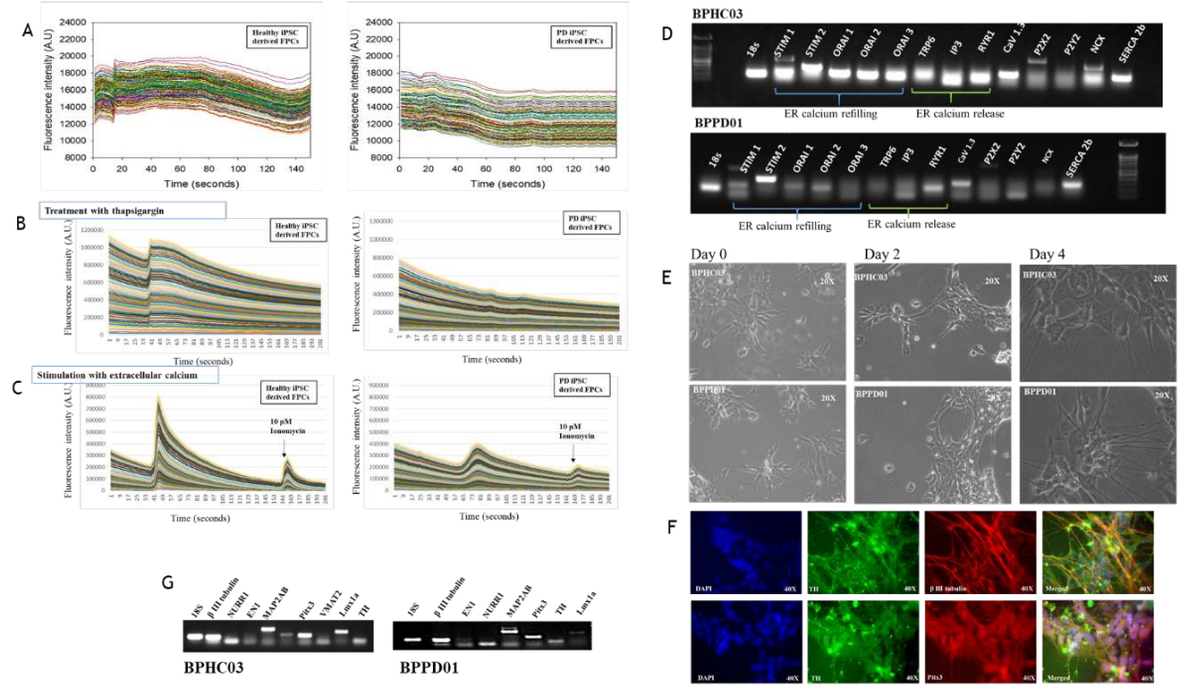


Fig. 4. Expression