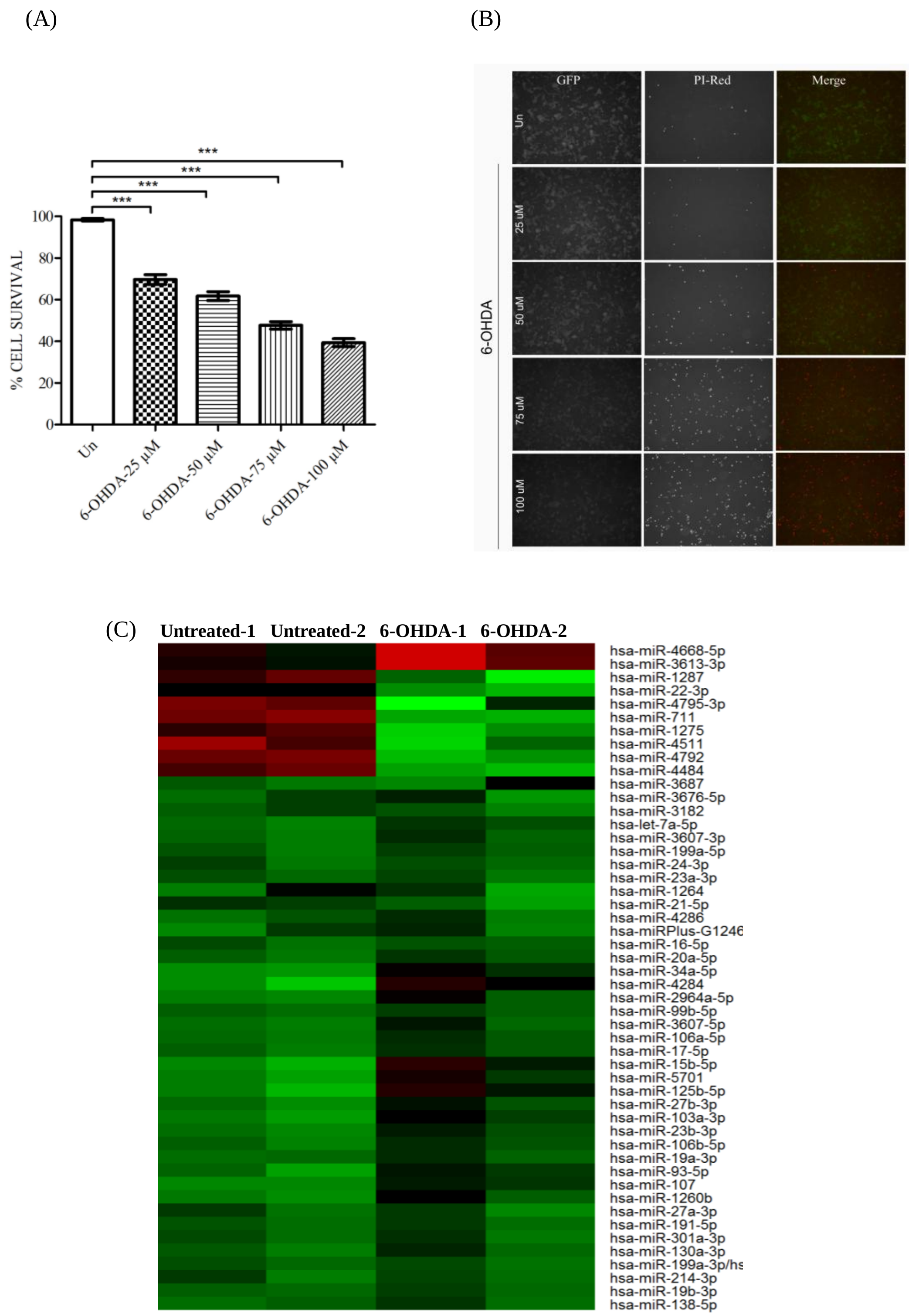
8. miRNAs levels are altered in 6-OHDA induced toxin model of Parkinson’s disease

8.1 miRNAs levels are altered in 6-OHDA induced PD stress model of SH-SY5Y.

6-hydroxydopamine (6-OHDA) is a neurotoxicant which induce PD like conditions in experimental animal models (Luthman *et al.*, 1989) and induce degeneration of DAergic neurons. SH-SY5Y cells express tyrosine hydroxylase, dopamine transporter and can preferentially uptake 6-OHDA (Berndt *et al.*, 2013). It accumulates in the cytosol and induces cell death by inhibiting the mitochondrial respiratory chain complexes I and IV activity and inducing ROS (Berndt *et al.*, 2013). In this study, SH-SY5Y cells were treated with different concentration of 6-OHDA for 24 hrs and cell survival was analyzed using MTT (Fig 8.1A) and Acridine/Propidium Iodide staining (Fig 8.1B). It was observed that low dose (25μM and 50μM) of 6-OHDA induced modest cell death in 24 hours. On the other hand, 100μM of 6-OHDA induced high level of cell death (necrotic) in 24 hours. The concentration of 75μM of 6-OHDA, induced slow and moderate cell death, hence was selected for further studies.

We analyzed the miRNAs expression in 6-OHDA stress condition and untreated condition using miRCURY™ array that contained all miRNAs present in the miRBase 18.0. The clustering was performed on all samples, and on the top 50 miRNAs with highest standard deviation (Fig 8.1C). The miRNAs were broadly classified into two categories: upregulated and down regulated upon 6-OHDA treatment (Fig 8.1D). The microarray results were further evaluated using real time polymerase chain reaction (RT–PCR) Time PCR. It was observed that miR-5701, miR-34a, miR-125a-5p and miR-92a-3p was down-regulated significantly (Fig 8.1E) whereas the levels of miR-1287 significantly increased in the presence of 6-OHDA (Fig 8.1F). The miRNAs identified here have not yet been characterized for their role in PD pathogenesis.

Figure 8.1


(A)
(B)
***
***
***
***
100
80
60
40
20
0
% CELL SURVIVAL
Un
6-OHDA-25 µM
6-OHDA-50 µM
6-OHDA-75 µM
6-OHDA-100 µM
GFP
PI-Red
Merge
Un
25 uM
50 uM
75 uM
100 uM
6-OHDA
(C)
Untreated-1
Untreated-2
6-OHDA-1
6-OHDA-2
hsa-miR-4668-5p
hsa-miR-3613-3p
hsa-miR-1287
hsa-miR-22-3p
hsa-miR-4795-3p
hsa-miR-711
hsa-miR-1275
hsa-miR-4511
hsa-miR-4792
hsa-miR-4484
hsa-miR-3687
hsa-miR-3676-5p
hsa-miR-3182
hsa-let-7a-5p
hsa-miR-3607-3p
hsa-miR-199a-5p
hsa-miR-24-3p
hsa-miR-23a-3p
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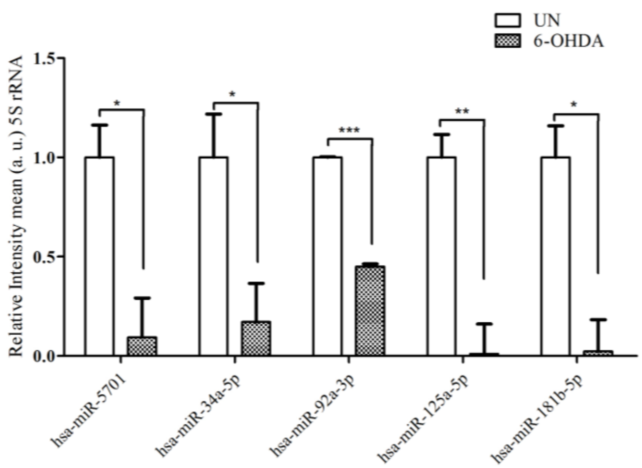
hsa-miR-21-5p
hsa-miR-4286
hsa-miRPlus-G1246
hsa-miR-16-5p
hsa-miR-20a-5p
hsa-miR-34a-5p
hsa-miR-4284
hsa-miR-2964a-5p
hsa-miR-99b-5p
hsa-miR-3607-5p
hsa-miR-106a-5p
hsa-miR-17-5p
hsa-miR-15b-5p
hsa-miR-5701
hsa-miR-125b-5p
hsa-miR-27b-3p
hsa-miR-103a-3p
hsa-miR-23b-3p
hsa-miR-106b-5p
hsa-miR-19a-3p
hsa-miR-93-5p
hsa-miR-107
hsa-miR-1260b
hsa-miR-27a-3p
hsa-miR-191-5p
hsa-miR-301a-3p
hsa-miR-130a-3p
hsa-miR-199a-3p/hs
hsa-miR-214-3p
hsa-miR-19b-3p
hsa-miR-138-5p

(D)

Expression	Name of miRNA
Up-regulated	hsa-miR-**4792**, hsa-miR-**4511**, hsa-miR-**711**, hsa-miR-**1287**, hsa-miR-**22-3p**
Down regulated	hsa-miR-**5701**, hsa-miR-**181b-5p**, hsa-miR-**125a-5p**, hsa-miR-**34a**, hsa-miR-**92a-3p**

(E)



(F)

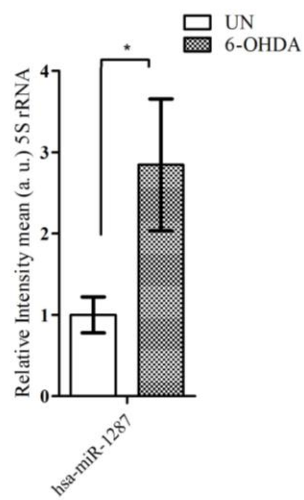


Figure 8.1: miRNAs levels are altered in 6-OHDA induced Parkinson's disease stress model of SH-SY5Y

(A) SH-SY5Y cells were treated with different concentration of 6-OHDA for 24 hours and MTT assay was performed for cell survival analysis. (B)SH-SY5Y cells were treated with different concentration of 6-OHDA for 24 hours and Acridine orange/Propidium Iodide staining was performed to monitor different types of cell death. (C) The clustering was performed on all samples, and on the top 50 miRNAs with highest standard deviation. (D) List of differentially expressed miRNAs in 6-OHDA (75 µM/ml for 24 hours) treated conditions analyzed from microarray data. (E & F) The expression levels of miRNAs which downregulated/upregulated in microarray analysis were determined using 5S rRNA as endogenous control by qPCR. Asterisk (*) indicates that p value <0.05, for SEM.

8.2 Potential targets of miR-5701 are involved in mitochondrial quality control and lysosomal degradation pathways.

To study the putative targets of validated miRNAs and their biological significance, we applied combinatorial approach using miRNAs function tools of starBase v.2.0 (Yang *et al.*, 2011; Li *et al.*, 2014c). The putative targets of up-regulated miRNAs were involved in regulation of neurotropin signaling pathway, axonal guidance, calcium signaling pathway (Fig 8.2.1A). Similarly, the putative targets of downregulated miRNAs included neurotropin signaling pathway, mTOR signaling pathway, cell cycle, RNA degradation (Fig 8.2.1B). The several cellular processes as described above targeted by miRNAs have important implications in the progression of PD.

The starBase analysis also revealed that miR-5710 may target mitochondrial quality control and lysosomal degradation pathways. (Table 8.2.1 and Table 8.2.2). The putative targets of miR-5701 were involved in autophagosome assembly, mitophagy, protein ubiquitination, mitochondrial fission, endocytosis, synapse assembly and mitochondrial transport (Table 8.2.2 & Table 8.2.3). This also clustered in basic cellular functional like brain development, neuron projection morphogenesis, axon guidance, neuron projection guidance, cell morphogenesis involved in neuron differentiation, nervous system development and regulation of neuron differentiation (Table 8.2.3). Cellular localization of putative targets of miR-5701 was found in neuron projection, neuron part, axon, cell cortex, synapse and neuronal cell body (Table 8.2.4).

The expression of putative targets of miR-5701 was validated in miR-5701 mimic transfected in SH-SY5Y cells. The quantitative PCR analysis showed that transcript levels of VCP, LAPTM4A and ATP6V0D1 decreased significantly in the presence of miR-5701 mimic (Fig 8.2.2 A& B&C). SH-SY5Y cells were transfected with miR-5701 mimic and levels of VCP were analyzed by western blotting. The level of VCP significantly decreased in the presence of miR-5701 mimic as compare to control (Fig 8.2.2D). To confirm that VCP is a direct target of miR-5701, RNA immunoprecipitation using biotin-labelled-miR-5701 was performed. The transfection of biotin-labelled-miR-5701 showed the significant decrease of VCP transcript as compared to control suggesting that biotin labeled miR-5701 may bind to target mRNAs (Fig 8.2.2E). RNA immunoprecipitation experiment was performed from miR-5701 mimic transfected SH-SY5Y cells. VCP was significantly enriched in streptavidin beads of miR-5701 as compared to negative control (Fig 8.2.2F). Above result suggests that VCP is the novel direct target of the miR-5701 and negatively regulates its expression.

Figure 8.2.1

(A) (B)

Figure 8.2.1: KEGG pathways enriched for miRNAs

(A) Up/ (B) down regulated upon 6-OHDA treatment. The targets of miRNAs down regulated upon 6-OHDA treatment were determined by StarBase and clustered into KEGG pathways using the DAVID gene annotation tool

Table 8.2.1

Target Score[1]	miRNA Name[2]	Gene Symbol[3]	Gene Description[4]
98	hsa-miR-5701	VSNL1	Visinin-like 1
92	hsa-miR-5701	TRIM23	Tripartite motif containing 23
87	hsa-miR-5701	SLC25A24	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 24
79	hsa-miR-5701	ATP6V0D1	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1
77	hsa-miR-5701	LAPTM4A	Lysosomal protein transmembrane 4 alpha
75	hsa-miR-5701	MRPL35	Mitochondrial ribosomal protein L35
61	hsa-miR-5701	IMMP2L	IMP2 inner mitochondrial membrane peptidase-like (S. cerevisiae)
58	hsa-miR-5701	SLC25A31	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 31
57	hsa-miR-5701	DNM3	Dynamin 3
56	hsa-miR-5701	VCP	Valosin containing protein
55	hsa-miR-5701	MRPL37	Mitochondrial ribosomal protein L37
51	hsa-miR-5701	RB1CC1	RB1-inducible coiled-coil 1

Table 8.2.1: The putative targets of miR-5701 are involved in mitochondrial quality control and lysosomal degradation pathway.

The putative target of miR-5701 was selected using 5 target prediction tools and minimum stringency in starBase v2.0. The putative targets were tabulated. [1.] Target Score [2.] miRNA Name [3.] Gene Symbol. [4.] Gene Description.

Figure 8.2.2

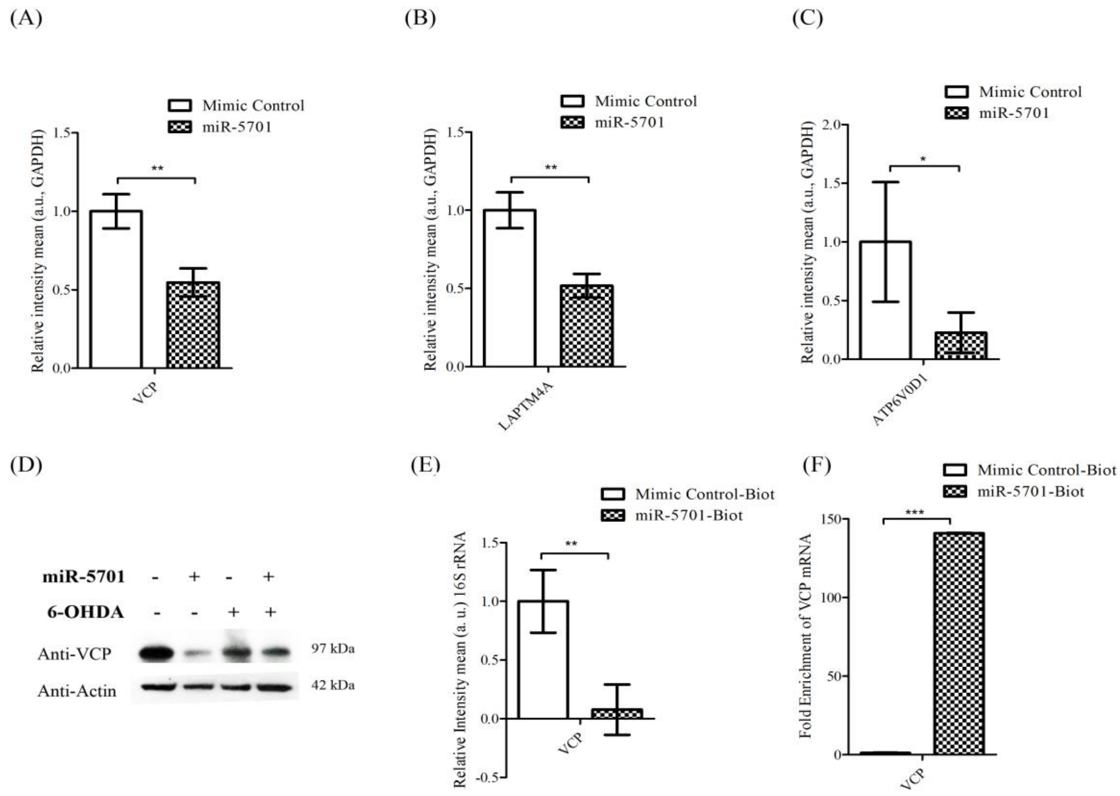


Figure 8.2.2: miR-5701 inhibits the expression of genes involved in lysosomal function and degradation pathways

(A & B & C) SH-SY5Y cells were transfected with miR-5701 mimic and the expression levels VCP, LAPTM4A and ATP6V0D1 were determined by real time PCR using GAPDH as endogenous control. (D) SH-SY5Y cells were transfected with miR-5701 mimic and level of VCP was determined by western blotting. (E) The levels VCP in presence of biotin labeled miR-5701 was determined by real time PCR using 16sRNA as endogenous control. (F) Biotin tag miR-5701 were transfected in SH-SY5Y and RNA IP was performed as describe in method. Fold enrichment analysis of VCP in biotin labeled miR-5701 was determined by qPCR. Asterisk (*) indicates that p value <0.05, for SEM.

Table 8.2.2

Gene Name[1]	Functional annotation [2]	Description [3]
Visinin like 1 (VSNL1)	COG_ONTOLOGY	Signal transduction mechanisms / Cytoskeleton / Cell division and chromosome partitioning / General function prediction only,
	GOTERM_BP_DIRECT	positive regulation of insulin secretion involved in cellular response to glucose stimulus, positive regulation of exocytosis, negative regulation of insulin secretion,
	GOTERM_CC_DIRECT	cytosol, membrane,
	GOTERM_MF_DIRECT	calcium ion binding, protein binding,
	INTERPRO	EF-hand domain, EF-hand-like domain, EF-Hand 1, calcium-binding site,
	SMART	EFh,
	UP_KEYWORDS	Calcium, Complete proteome, Lipoprotein, Metal-binding, Myristate, Polymorphism, Proteomics identification, Reference proteome, Repeat,
	UP_SEQ_FEATURE	calcium-binding region:1, calcium-binding region:2, calcium-binding region:3, chain:Visinin-like protein 1, domain:EF-hand 1, domain:EF-hand 2, domain:EF-hand 3, domain:EF-hand 4, lipid moiety-binding region:N-myristoyl glycine, sequence conflict, sequence
	GOTERM_BP_DIRECT	small GTPase mediated signal transduction, viral process, protein ubiquitination, positive regulation of catalytic activity,
	GOTERM_CC_DIRECT	Golgi membrane, nucleus, lysosomal membrane, extracellular exosome,
	GOTERM_MF_DIRECT	GTPase activity, ubiquitin-protein transferase activity, protein binding, GTP binding, enzyme

TRIM23		activator activity, zinc ion binding, ligase activity, GDP binding, identical protein binding,
	INTERPRO	Zinc finger, B-box, Zinc finger, RING-type, B-box, C-terminal, Small GTP-binding protein domain, Small GTPase superfamily, ARF/SAR type, Zinc finger, C2H2, Zinc finger, RING/FYVE/PHD-type, Zinc finger, RING-type, conserved site, Small GTPase superfamily, ARF type, P-loop containing nucleoside triphosphate hydrolase,
	SMART	RING, BBOX, BBC,
	UP_KEYWORDS	Alternative splicing, Coiled coil, Complete proteome, Golgi apparatus, GTP-binding, Host-virus interaction, Ligase, Lysosome, Membrane, Metal-binding, Nucleotide-binding, Polymorphism, Reference proteome, Ubl conjugation pathway, Zinc, Zinc-finger,
	UP_SEQ_FEATURE	chain:GTP-binding protein ARD-1, mutagenesis site, nucleotide phosphate-binding region:GTP, region of interest:ARF-like, sequence variant, splice variant, zinc finger region:B box-type, zinc finger region:RING-type,
	BIOCARTA	Stathmin and breast cancer resistance to antimicrotubule agents,
	GOTERM_BP_DIRECT	autophagosome assembly, mitophagy, liver development, positive regulation of protein phosphorylation, transcription, DNA-templated, regulation of transcription, DNA-templated, autophagy, cellular response to nitrogen starvation, cell cycle, heart development, macroautophagy, pexophagy, positive regulation of cell size, positive regulation of JNK cascade, reticulophagy, glycophagy, negative regulation of extrinsic apoptotic signaling pathway,
	GOTERM_CC_DIRE	cytoplasm, cytosol, nuclear membrane, pre-

RB1 inducible coiled-coil 1(RB1CC1)	CT	autophagosomal structure membrane, ATG1/ULK1 kinase complex,
	GOTERM_MF_DIRECT	protein binding, protein kinase binding, protein complex scaffold,
	INTERPRO	Autophagy-related protein 11,
	OMIM_DISEASE	Breast cancer, somatic,
	UP_KEYWORDS	Alternative splicing, Autophagy, Cell cycle, Coiled coil, Complete proteome, Cytoplasm, Nucleus, Phosphoprotein, Polymorphism, Proteomics identification, Reference proteome, Transcription, Transcription regulation, Tumor suppressor,
	UP_SEQ_FEATURE	chain:RB1-inducible coiled-coil protein 1, compositionally biased region:Poly-Thr, modified residue, sequence conflict, sequence variant, short sequence motif:Nuclear localization signal,
Dynamin 3(DNM3)	GOTERM_BP_DIRECT	mitochondrial fission, dynamin polymerization involved in mitochondrial fission, endocytosis, synapse assembly, receptor internalization, filopodium assembly, positive regulation of filopodium assembly, negative regulation of dendritic spine morphogenesis, membrane fusion,
	GOTERM_CC_DIRECT	photoreceptor inner segment, Golgi apparatus, microtubule, postsynaptic density, axon, mitochondrial membrane, synaptic cleft, dendritic spine, dendritic spine head, perinuclear region of cytoplasm, extracellular exosome,
	GOTERM_MF_DIRECT	GTPase activity, protein binding, GTP binding, microtubule binding, type 1 metabotropic glutamate receptor binding, type 5 metabotropic glutamate receptor binding, nitric-oxide synthase binding,
	INTERPRO	Dynamin central domain, Dynamin, GTPase domain, Pleckstrin homology domain, Dynamin GTPase effector, Pleckstrin

		homology-like domain, Dynamin, GTPase region, conserved site, GTPase effector domain, GED, Dynamin, P-loop containing nucleoside triphosphate hydrolase,
	KEGG_PATHWAY	Endocytosis, Synaptic vesicle cycle, Endocrine and other factor-regulated calcium reabsorption, Bacterial invasion of epithelial cells,
	SMART	DYNc, PH, GED,
	UP_KEYWORDS	3D-structure, Alternative splicing, Complete proteome, Cytoplasm, Cytoskeleton, Endocytosis, GTP-binding, Hydrolase, Microtubule, Motor protein, Nucleotide-binding, Phosphoprotein, Proteomics identification, Reference proteome,
	UP_SEQ_FEATURE	chain:Dynamin-3, domain:GED, domain:PH, modified residue, nucleotide phosphate-binding region:GTP, sequence conflict, splice variant,
Inner mitochondrial membrane peptidase subunit 2(IMMP2L)	GOTERM_BP_DIRECT	ovarian follicle development, signal peptide processing, protein processing involved in protein targeting to mitochondrion, superoxide metabolic process, cellular response to DNA damage stimulus, spermatogenesis, brain development, blood circulation, respiratory electron transport chain, ovulation, mitochondrial respiratory chain complex assembly, cerebellum vasculature development,
	GOTERM_CC_DIRECT	membrane, integral component of membrane, mitochondrial inner membrane peptidase complex,
	GOTERM_MF_DIRECT	serine-type endopeptidase activity, peptidase activity, serine-type peptidase activity,
	INTERPRO	Peptidase S26A, signal peptidase I, Peptidase S24/S26A/S26B/S26C, Peptidase S26A, signal peptidase I, conserved site, Peptidase S24/S26A/S26B,
	KEGG_PATHWAY	Protein export,
	UP_KEYWORDS	Alternative splicing, Complete proteome,

		Hydrolase, Membrane, Mitochondrion, Mitochondrion inner membrane, Protease, Proteomics identification, Reference proteome, Transmembrane, Transmembrane helix,
	UP_SEQ_FEATURE	chain:Mitochondrial inner membrane protease subunit 2, splice variant, transmembrane region,
Mitochondrial ribosomal protein L35 (MRPL35)	GOTERM_BP_DIRECT	translation, mitochondrial translational elongation, mitochondrial translational termination,
	GOTERM_CC_DIRECT	mitochondrion, mitochondrial inner membrane, mitochondrial ribosome, mitochondrial large ribosomal subunit, ribosome,
	GOTERM_MF_DIRECT	structural constituent of ribosome,
	INTERPRO	Ribosomal protein L35, mitochondrial, Ribosomal protein L35,
	KEGG_PATHWAY	Ribosome,
	UP_KEYWORDS	3D-structure, Alternative splicing, Complete proteome, Mitochondrion, Polymorphism, Proteomics identification, Reference proteome, Ribonucleoprotein, Ribosomal protein, Transit peptide,
	UP_SEQ_FEATURE	chain:39S ribosomal protein L35, mitochondrial, sequence variant, splice variant, transit peptide:Mitochondrion,
Mitochondrial ribosomal protein L37 (MRPL37)	GOTERM_BP_DIRECT	translation, mitochondrial translational elongation, mitochondrial translational termination,
	GOTERM_CC_DIRECT	mitochondrion, mitochondrial inner membrane, mitochondrial ribosome, mitochondrial large ribosomal subunit, ribosome,
	GOTERM_MF_DIRECT	structural constituent of ribosome, poly(A) RNA binding,
	INTERPRO	Ribosomal protein L37/S30,
	UP_KEYWORDS	3D-structure, Complete proteome, Mitochondrion, Polymorphism, Proteomics identification, Reference proteome,

		Ribonucleoprotein, Ribosomal protein, Transit peptide,
	UP_SEQ_FEATURE	chain:39S ribosomal protein L37, mitochondrial, modified residue, sequence variant, transit peptide:Mitochondrion,
Solute carrier family 25 member 24 (SLC25A24)	COG_ONTOLOGY	Signal transduction mechanisms / Cytoskeleton / Cell division and chromosome partitioning / General function prediction only,
	GOTERM_BP_DIRECT	translation, mitochondrial transport, regulation of cell death, ATP transport, cellular response to oxidative stress, transmembrane transport, cellular response to calcium ion,
	GOTERM_CC_DIRECT	mitochondrion, mitochondrial inner membrane, integral component of membrane,
	GOTERM_MF_DIRECT	structural constituent of ribosome, ATP transmembrane transporter activity, calcium ion binding,
	INTERPRO	EF-hand domain, Mitochondrial carrier protein, Graves disease carrier protein, EF-hand-like domain, Mitochondrial substrate/solute carrier, EF-Hand 1, calcium-binding site, Mitochondrial carrier domain,
	SMART	EFh,
	UP_KEYWORDS	3D-structure, Acetylation, Alternative splicing, Calcium, Complete proteome, Membrane, Metal-binding, Mitochondrion, Mitochondrion inner membrane, Proteomics identification, Reference proteome, Repeat, Transmembrane, Transmembrane helix, Transport,
	UP_SEQ_FEATURE	calcium-binding region:1, calcium-binding region:2, calcium-binding region:3, chain:Calcium-binding mitochondrial carrier protein SCaMC-1, domain:EF-hand 1, domain:EF-hand 2, domain:EF-hand 3, domain:EF-hand 4, modified residue, repeat:Solcar 1, repeat:Solcar 2, repeat:Solcar 3, sequence conflict, splice variant, topological domain:Mitochondrial intermembrane,

		topological domain:Mitochondrial matrix, transmembrane region,
Solute carrier family 25 member 31 (SLC25A31)	GOTERM_BP_DIRECT	translation, transmembrane transport,
	GOTERM_CC_DIRECT	nucleus, mitochondrion, mitochondrial inner membrane, integral component of membrane, motile cilium,
	GOTERM_MF_DIRECT	structural constituent of ribosome, transporter activity,
	INTERPRO	Mitochondrial carrier protein, Adenine nucleotide translocator 1, Mitochondrial substrate/solute carrier, Mitochondrial carrier domain,
	KEGG_PATHWAY	Calcium signaling pathway, cGMP-PKG signaling pathway, Parkinson's disease, Huntington's disease, HTLV-I infection,
	UP_KEYWORDS	Cell projection, Cilium, Complete proteome, Flagellum, Membrane, Mitochondrion, Mitochondrion inner membrane, Polymorphism, Reference proteome, Repeat, Transmembrane, Transmembrane helix, Transport,
	UP_SEQ_FEATURE	chain:ADP/ATP translocase 4, modified residue, repeat:Solcar 1, repeat:Solcar 2, repeat:Solcar 3, transmembrane region,
Valosin containing protein(VCP)	GOTERM_BP_DIRECT	DNA repair, double-strand break repair, ubiquitin-dependent protein catabolic process, NADH metabolic process, ER to Golgi vesicle-mediated transport, autophagy, activation of cysteine-type endopeptidase activity involved in apoptotic process, cellular response to DNA damage stimulus, positive regulation of mitochondrial membrane potential, protein ubiquitination, protein N-linked glycosylation via asparagine, viral genome replication, translesion synthesis, ER-associated ubiquitin-dependent protein catabolic process, endoplasmic reticulum unfolded protein

Valosin containing protein(VCP)		response, retrograde protein transport, ER to cytosol, positive regulation of protein complex assembly, positive regulation of proteasomal ubiquitin-dependent protein catabolic process, protein hexamerization, ERAD pathway, regulation of apoptotic process, proteasome-mediated ubiquitin-dependent protein catabolic process, establishment of protein localization, positive regulation of protein catabolic process, ATP metabolic process, protein homooligomerization, aggresome assembly, error-free translesion synthesis, ER-associated misfolded protein catabolic process, flavin adenine dinucleotide catabolic process, autophagosome maturation, positive regulation of protein K63-linked deubiquitination, positive regulation of Lys63-specific deubiquitinase activity, regulation of aerobic respiration, positive regulation of oxidative phosphorylation, positive regulation of ubiquitin-specific protease activity, positive regulation of ATP biosynthetic process,
	GOTERM_CC_DIRECT	proteasome complex, nucleus, nucleoplasm, cytoplasm, endoplasmic reticulum, endoplasmic reticulum membrane, lipid particle, cytosol, VCP-NPL4-UFD1 AAA ATPase complex, site of double-strand break, Derlin-1 retrotranslocation complex, myelin sheath, intracellular membrane-bounded organelle, perinuclear region of cytoplasm, extracellular exosome, VCP-NSFL1C complex,
	GOTERM_MF_DIRECT	protein binding, ATP binding, lipid binding, hydrolase activity, ATPase activity, protein phosphatase binding, protein domain specific binding, polyubiquitin binding, ubiquitin protein ligase binding, deubiquitinase activator activity, K48-linked polyubiquitin binding, MHC class I protein binding, identical protein binding, ADP binding, ubiquitin-like protein

Valosin containing protein(VCP)		ligase binding, poly(A) RNA binding, BAT3 complex binding, ubiquitin-specific protease binding,
	INTERPRO	CDC48, N-terminal subdomain, AAA+ ATPase domain, ATPase, AAA-type, core, ATPase, AAA-type, conserved site, CDC48, domain 2, ATPase, AAA-type, CDC48, Aspartate decarboxylase-like domain, Vps4 oligomerisation, C-terminal, P-loop containing nucleoside triphosphate hydrolase,
	KEGG_PATHWAY	Protein processing in endoplasmic reticulum, Legionellosis,
	OMIM_DISEASE	Inclusion body myopathy with early-onset Paget disease and frontotemporal dementia 1, Amyotrophic lateral sclerosis 14, with or without frontotemporal dementia, Charcot-Marie-Tooth disease, type 2Y,
	SMART	AAA, SM01072, SM01073,
	UP_KEYWORDS	3D-structure, Acetylation, Amyotrophic lateral sclerosis, ATP-binding, Autophagy, Charcot-Marie-Tooth disease, Complete proteome, Cytoplasm, Direct protein sequencing, Disease mutation, DNA damage, DNA repair, Endoplasmic reticulum, Hydrolase, Lipid-binding, Methylation, Neurodegeneration, Neuropathy, Nucleotide-binding, Nucleus, Phosphoprotein, Polymorphism, Proteomics identification, Reference proteome, Transport, Ubl conjugation, Ubl conjugation pathway,
	UP_SEQ_FEATURE	chain:Transitional endoplasmic reticulum ATPase, modified residue, mutagenesis site, nucleotide phosphate-binding region:ATP, region of interest:Interaction with UBXN6, sequence conflict, sequence variant,
	GOTERM_BP_DIRECT	vacuolar transport, vacuolar acidification, brain development, insulin receptor signaling pathway, ATP hydrolysis coupled proton transport, proton transport, regulation of

ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1 (ATP6V0D1)		macroautophagy, transferrin transport, ion transmembrane transport, IRE1-mediated unfolded protein response, cilium assembly, cilium morphogenesis, phagosome acidification,
	GOTERM_CC_DIRECT	lysosomal membrane, early endosome, centrosome, synaptic vesicle, endosome membrane, membrane, apical plasma membrane, vacuolar proton-transporting V-type ATPase complex, phagocytic vesicle membrane, proton-transporting V-type ATPase, V0 domain, plasma membrane proton-transporting V-type ATPase complex, axon terminus, extracellular exosome,
	GOTERM_MF_DIRECT	protein binding, hydrogen-exporting ATPase activity, phosphorylative mechanism, hydrogen ion transmembrane transporter activity, protein complex binding, proton-transporting ATPase activity, rotational mechanism,
	INTERPRO	ATPase, V0 complex, c/d subunit, ATPase, V0 complex, subunit d,
	KEGG_PATHWAY	Oxidative phosphorylation, Metabolic pathways, Lysosome, Phagosome, Synaptic vesicle cycle, Collecting duct acid secretion, Vibrio cholerae infection, Epithelial cell signaling in Helicobacter pylori infection, Tuberculosis, Viral carcinogenesis, Rheumatoid arthritis,
	PIR_SUPERFAMILY	V-type ATPase, subunit d,
	UP_KEYWORDS	Cilium biogenesis/degradation, Complete proteome, Hydrogen ion transport, Ion transport, Membrane, Phosphoprotein, Proteomics identification, Reference proteome, Transport,
	UP_SEQ_FEATURE	chain:V-type proton ATPase subunit d 1, modified residue, sequence conflict,
	GOTERM_BP_DIRECT	transport,

lysosomal protein transmembrane 4 alpha (LAPTM4A)	GOTERM_CC_DIRECT	Golgi apparatus, membrane, integral component of membrane,
	INTERPRO	Lysosomal-associated transmembrane protein 4/5, Lysosomal-associated transmembrane protein, 4A/5,
	KEGG_PATHWAY	Lysosome,
	UP_KEYWORDS	Acetylation, Complete proteome, Membrane, Reference proteome, Signal, Transmembrane, Transmembrane helix, Transport,
	UP_SEQ_FEATURE	chain:Lysosomal-associated transmembrane protein 4A, compositionally biased region:Poly-Pro, modified residue, transmembrane region,

Table 8.2.2: The functional annotation of putative targets of miR-5701.

The putative target of miR-5701 describe in Table1 were uploaded DAVID platform and annotated into respective subclasses and tabulated. [1.] Gene Name [2.] Functional Annotation [3.] Description.

Table 8.2.3:

Sr. No	**Term Id[1]**	**Term Name[2]**	**Hit Gene Number[3]**	**P-Value[4]**
1	GO:0008361	regulation of cell size	9	4.37E-04
2	GO:0030308	negative regulation of cell growth	8	0.002017
3	GO:0022603	regulation of anatomical structure morphogenesis	22	0.002799
4	GO:0032535	regulation of cellular component size	11	0.003808
5	GO:0034645	cellular macromolecule biosynthetic process	69	0.005023
6	GO:0045930	negative regulation of mitotic cell cycle	8	0.007737
7	GO:0043280	positive regulation of cysteine-type endopeptidase activity involved in apoptotic process	6	0.007873
8	GO:0001558	regulation of cell growth	11	0.009215

9	GO:2001056	positive regulation of cysteine-type endopeptidase activity	6	0.010596
10	GO:0001525	angiogenesis	11	0.010821
11	GO:0001944	vasculature development	14	0.010828
12	GO:0071158	positive regulation of cell cycle arrest	5	0.011643
13	GO:0045926	negative regulation of growth	8	0.012466
14	GO:0048514	blood vessel morphogenesis	12	0.013189
15	GO:0060041	retina development in camera-type eye	6	0.013504
16	GO:0022604	regulation of cell morphogenesis	13	0.01503
17	GO:0016049	cell growth	11	0.015316
18	GO:0048646	anatomical structure formation involved in morphogenesis	21	0.015433
19	GO:0010950	positive regulation of endopeptidase activity	6	0.015572
20	GO:0031344	regulation of cell projection organization	13	0.015798
21	GO:0001568	blood vessel development	13	0.016596
22	GO:0043281	regulation of cysteine-type endopeptidase activity involved in apoptotic process	7	0.017781
23	GO:0006490	oligosaccharide-lipid intermediate biosynthetic process	3	0.017842
24	GO:0006488	dolichol-linked oligosaccharide biosynthetic process	3	0.017842
25	GO:0060627	regulation of vesicle-mediated transport	11	0.018406
26	GO:0072359	circulatory system development	18	0.018415
27	GO:0072358	cardiovascular system development	18	0.018415
28	GO:0060560	developmental growth involved in morphogenesis	7	0.019699
29	GO:0044773	mitotic DNA damage checkpoint	5	0.020036
30	GO:0019408	dolichol biosynthetic process	2	0.020555
31	GO:1901991	negative regulation of mitotic cell cycle phase transition	6	0.02191
32	GO:0010952	positive regulation of peptidase activity	6	0.022441
33	GO:0061387	regulation of extent of cell growth	5	0.022768
34	GO:2000112	regulation of cellular macromolecule biosynthetic process	54	0.023687
35	GO:0007093	mitotic cell cycle checkpoint	6	0.024733

36	GO:0044774	mitotic DNA integrity checkpoint	5	0.024959
37	**GO:0007420**	**brain development**	**14**	**0.025321**
38	**GO:0048812**	**neuron projection morphogenesis**	**12**	**0.02561**
39	GO:0071156	regulation of cell cycle arrest	5	0.025717
40	GO:0051345	positive regulation of hydrolase activity	17	0.028091
41	**GO:0007411**	**axon guidance**	**7**	**0.028425**
42	GO:2000116	regulation of cysteine-type endopeptidase activity	7	0.028977
43	**GO:0097485**	**neuron projection guidance**	**7**	**0.028977**
44	GO:0090066	regulation of anatomical structure size	11	0.028989
45	GO:1901988	negative regulation of cell cycle phase transition	6	0.029071
46	**GO:0070536**	**protein K63-linked deubiquitination**	**3**	**0.029314**
47	GO:0097191	extrinsic apoptotic signaling pathway	7	0.029774
48	GO:0016094	polyprenol biosynthetic process	2	0.030674
49	GO:0070085	glycosylation	8	0.030717
50	GO:0009101	glycoprotein biosynthetic process	9	0.030922
51	GO:0010972	negative regulation of G2/M transition of mitotic cell cycle	3	0.031448
52	GO:2000113	negative regulation of cellular macromolecule biosynthetic process	22	0.032308
53	**GO:0048667**	**cell morphogenesis involved in neuron differentiation**	**11**	**0.033456**
54	GO:0010468	regulation of gene expression	57	0.034098
55	GO:1902750	negative regulation of cell cycle G2/M phase transition	3	0.035893
56	GO:0017157	regulation of exocytosis	6	0.036051
57	GO:0060322	head development	14	0.036145
58	**GO:0007399**	**nervous system development**	**32**	**0.037114**
59	GO:0007423	sensory organ development	11	0.037957
60	GO:0048638	regulation of developmental growth	8	0.03802
61	GO:0017145	stem cell division	3	0.038201
62	GO:0010948	negative regulation of cell cycle process	7	0.038623
63	GO:0010556	regulation of macromolecule biosynthetic process	54	0.038973
64	GO:0044723	single-organism carbohydrate metabolic process	13	0.039751

65	GO:0014911	positive regulation of smooth muscle cell migration	3	0.040564
66	GO:0048840	otolith development	2	0.040689
67	GO:0097659	nucleic acid-templated transcription	50	0.042857
68	GO:0051641	cellular localization	36	0.043135
69	GO:0006355	regulation of transcription, DNA-templated	48	0.044803
70	GO:0006351	transcription, DNA-templated	48	0.045179
71	GO:0007435	salivary gland morphogenesis	3	0.04545
72	GO:0048588	developmental cell growth	6	0.045687
73	GO:0035966	response to topologically incorrect protein	6	0.045687
74	GO:0010977	negative regulation of neuron projection development	5	0.045913
75	GO:0000122	negative regulation of transcription from RNA polymerase II promoter	14	0.04661
76	GO:1903506	regulation of nucleic acid-templated transcription	48	0.049067
77	**GO:0045665**	**negative regulation of neuron differentiation**	**6**	**0.050117**
78	GO:0043268	positive regulation of potassium ion transport	3	0.05054
79	GO:0048755	branching morphogenesis of a nerve	2	0.050601
80	GO:0060916	mesenchymal cell proliferation involved in lung development	2	0.050601
81	GO:0009887	organ morphogenesis	17	0.051327
82	GO:2001141	regulation of RNA biosynthetic process	48	0.052994
83	**GO:0048841**	**regulation of axon extension involved in axon guidance**	**3**	**0.053159**
84	GO:0032989	cellular component morphogenesis	21	0.054242
85	GO:0009100	glycoprotein metabolic process	9	0.055497
86	GO:0007431	salivary gland development	3	0.055825
87	GO:0010467	gene expression	66	0.05702
88	**GO:1990138**	**neuron projection extension**	**5**	**0.058561**
89	GO:0051252	regulation of RNA metabolic process	49	0.05922
90	GO:0010558	negative regulation of macromolecule biosynthetic process	22	0.059484
91	GO:0051336	regulation of hydrolase activity	21	0.059932
92	GO:0019348	dolichol metabolic process	2	0.060412
93	GO:0051171	regulation of nitrogen compound	56	0.060834

		metabolic process		
94	GO:0006919	activation of cysteine-type endopeptidase activity involved in apoptotic process	4	0.06123
95	GO:1901016	regulation of potassium ion transmembrane transporter activity	3	0.061294
96	GO:0051172	negative regulation of nitrogen compound metabolic process	23	0.061338
97	GO:0043269	regulation of ion transport	11	0.061901
98	GO:0051489	regulation of filopodium assembly	3	0.064094
99	GO:0048846	axon extension involved in axon guidance	3	0.064094
100	GO:1902667	regulation of axon guidance	3	0.064094
101	GO:1902284	neuron projection extension involved in neuron projection guidance	3	0.064094
102	GO:0010975	regulation of neuron projection development	9	0.065137
103	**GO:0007409**	**axonogenesis**	**9**	**0.065137**
104	**GO:0045664**	**regulation of neuron differentiation**	**11**	**0.065535**
105	**GO:0030516**	**regulation of axon extension**	**4**	**0.066392**
106	GO:0000077	DNA damage checkpoint	5	0.067488
107	GO:0035967	cellular response to topologically incorrect protein	5	0.068735
108	GO:0007346	regulation of mitotic cell cycle	10	0.068903
109	GO:0043413	macromolecule glycosylation	7	0.070047
110	GO:0006486	protein glycosylation	7	0.070047
111	GO:1902669	positive regulation of axon guidance	2	0.070122
112	GO:0071679	commissural neuron axon guidance	2	0.070122
113	GO:0048842	positive regulation of axon extension involved in axon guidance	2	0.070122
114	GO:0046465	dolichyl diphosphate metabolic process	2	0.070122
115	GO:0006489	dolichyl diphosphate biosynthetic process	2	0.070122
116	GO:0043270	positive regulation of ion transport	6	0.070264
117	GO:0032774	RNA biosynthetic process	50	0.072165
118	GO:0031345	negative regulation of cell projection organization	5	0.074267
119	**GO:0048666**	**neuron development**	**16**	**0.074627**
120	GO:0051049	regulation of transport	26	0.075081

121	GO:0043623	cellular protein complex assembly	11	0.075213
122	GO:0040008	regulation of growth	12	0.075654
123	GO:0070646	protein modification by small protein removal	5	0.075665
124	GO:0072583	clathrin-mediated endocytosis	3	0.075708
125	GO:0019219	regulation of nucleobase-containing compound metabolic process	52	0.076738
126	GO:0071241	cellular response to inorganic substance	5	0.077005
127	GO:1903047	mitotic cell cycle process	15	0.077277
128	GO:2000134	negative regulation of G1/S transition of mitotic cell cycle	4	0.077289
129	GO:0051128	regulation of cellular component organization	32	0.078553
130	GO:0030030	cell projection organization	20	0.07913
131	GO:0010605	negative regulation of macromolecule metabolic process	32	0.079602
132	GO:0031175	neuron projection development	14	0.080984
133	GO:0031570	DNA integrity checkpoint	5	0.081425
134	GO:0051093	negative regulation of developmental process	13	0.081512
135	GO:0030850	prostate gland development	3	0.081746
136	GO:0007507	heart development	10	0.083511
137	GO:0031327	negative regulation of cellular biosynthetic process	22	0.084531
138	GO:1902807	negative regulation of cell cycle G1/S phase transition	4	0.084961
139	**GO:0007417**	**central nervous system development**	**15**	**0.085187**
140	GO:2001236	regulation of extrinsic apoptotic signaling pathway	5	0.086618
141	GO:0048639	positive regulation of developmental growth	5	0.086618
142	GO:0010629	negative regulation of gene expression	22	0.086819
143	GO:0034599	cellular response to oxidative stress	6	0.087859
144	GO:0099003	vesicle mediated transport in synapse	4	0.088915
145	GO:0000075	cell cycle checkpoint	6	0.089126
146	GO:2000794	regulation of epithelial cell proliferation involved in lung morphogenesis	2	0.089243
147	GO:0016093	polyprenol metabolic process	2	0.089243

148	GO:0000902	cell morphogenesis	19	0.089889
149	GO:0060491	regulation of cell projection assembly	5	0.090345
150	GO:0061564	axon development	9	0.090876
151	GO:0016192	vesicle-mediated transport	22	0.091041
152	GO:0035272	exocrine system development	3	0.09107
153	GO:0052548	regulation of endopeptidase activity	8	0.091536
154	GO:0050808	synapse organization	6	0.091693
155	GO:0023051	regulation of signaling	40	0.092225
156	GO:0051928	positive regulation of calcium ion transport	4	0.094987
157	GO:0009890	negative regulation of biosynthetic process	22	0.095889
158	GO:0032844	regulation of homeostatic process	9	0.096277
159	GO:0034622	cellular macromolecular complex assembly	16	0.096375
160	GO:0050770	regulation of axonogenesis	5	0.096575
161	GO:0030330	DNA damage response, signal transduction by p53 class mediator	4	0.097049
162	GO:0070493	thrombin receptor signaling pathway	2	0.098656
163	GO:0060502	epithelial cell proliferation involved in lung morphogenesis	2	0.098656
164	GO:2001233	regulation of apoptotic signaling pathway	8	0.099565

Table 8.2.3: The biological process modulated by miR-5701.

The putative target of miR-5701 was selected using 5 target prediction tools and minimum stringency in starBase v2.0. Targets were uploaded DAVID platform and clustered into the biological processes. [1.] Identification number of the GO term. [2.] The term nomenclature. [3.] Target gene number that belongs to same GO term. [4.] P-value of the GO term prediction.

Table 8.2.4:

Sr. No	Term Id[1]	Term Name[2]	Hit Gene Number[3]	P-Value[4]
1	GO:0005794	Golgi apparatus	30	14.56311
2	GO:0043005	neuron projection	19	9.223301
3	GO:0097458	neuron part	23	11.16505

4	GO:0030424	axon	10	4.854369
5	GO:0005938	cell cortex	7	3.398058
6	GO:0015630	microtubule cytoskeleton	19	9.223301
7	GO:0030667	secretory granule membrane	4	1.941748
8	GO:0015629	actin cytoskeleton	10	4.854369
9	GO:0045202	synapse	14	6.796117
10	GO:0030175	filopodium	4	1.941748
11	GO:0044297	cell body	10	4.854369
12	GO:0043025	neuronal cell body	9	4.368932

Table 8.2.4: The cellular localization of putative targets of miR-5701.

The putative target of miR-5701 was selected using 5 target prediction tools and minimum stringency in starBase v2.0. Targets were uploaded DAVID platform and clustered according to the cellular localization into GO terms. [1.] Identification number of the GO term. [2.] The term nomenclature. [3.] Target gene number that belongs to same GO term. [4.] P-value of the GO term prediction.

8.3 Discussion:

The recent studies clearly suggest that miRNAs play important role in regulation of copy number of target mRNAs in narrow physiological range to maintain the level of proteins at required physiological level. The studies from our and others strongly suggest mitochondria act as platform to translocate miRNAs at the different subcellular site in neurons (Sripada *et al.*, 2012; Vargas *et al.*, 2016) however its implication in patho-physiological is not well understood. There had been progressing in the identification of many novel miRNAs by using next generation sequencing technologies however their cellular functions and its implication in PD pathogenesis is not understood. Here we systematically identified several new miRNAs which may regulate inter-organellar cross talk and may have a role in PD pathogenesis.

The role of miRNAs is emerging in a regulation of many neuronal functions including branching and at synaptic remodeling (Dajas-Bailador *et al.*, 2012; Kaplan *et al.*, 2013; Hu *et al.*, 2014; Li *et al.*, 2014a). In the current miRBase 21.0 latest releases there are approximately 1881 precursors, 2588 mature miRNAs listed in Homo sapiens. The previous report studying the role of miRNAs in PD pathogenesis (Hoss *et al.*, 2016; Marques *et al.*, 2016; Recasens *et al.*, 2016) have used a limited number of miRNAs probe. In the current study, we used miRNA array that had 1899 capture probes, covering all human annotated in miRBase 18.0. The experiment helped us to identify many novel miRNAs that have not been yet implicated in PD disease. Interesting we observed that miR-1287 was up-regulated significantly whereas the levels of miR-5701, miR-34a, miR-125a-5p and miR-92a-3p significantly decreased in 6-OHDA conditions. Furthermore, bio-informatics analysis shows that altered expression of these miRNAs affects key pathways involved in PD pathogenesis like cell cycle, mRNA transport, proteasomal ubiquitin dependent protein catabolism, mTOR signaling pathway, Neurotrophin signaling pathway, axonal guidance, RNA degradation, calcium signaling, ubiquitination, ubiquitin proteasome system and mitochondrial import. The further studies are required to elucidate the novel role of different miRNAs in neuronal function and its role in implication to PD