9. miR-5701 modulates mitochondrial-lysosomal cross talk and regulates cell death

9.1 miR-5701 regulates mitochondrial function.

The direct target of miR-5701, VCP is known to regulate mitochondrial function and quality control (Bartolome *et al.*, 2013; Fang *et al.*, 2015) hence we further analyzed the role of miR-5701 in the regulation of mitochondrial function. SH-SY5Y cells were transfected with miR-5701 mimic and analyzed different mitochondrial parameters. The levels of ATP decreased significantly in the presence of miR-5701 mimic (Fig 9.1.1A). miR-5701 transfection increased ROS levels in untreated as well as in 6-OHDA treated condition (Fig 9.1.1B). The activity of mitochondrial respiratory complexes regulates bioenergetics capacity and ROS levels in the cells (Stowe and Camara, 2009). The dysregulation of mitochondrial complex-I results in leakage of electrons and is one of the primary sources of ROS. The cells were transfected with miR-5701 mimic and complex-I activity was determined by BN-PAGE and spectrophotomertically. The mitochondrial complex-I activity decreased in the presence of miR-5701 as observed by in gel activity staining (Fig 9.1.1C) as well as by spectrophotomertically as compared to control (Fig 9.1.1D).

The defect in mitochondrial function leads to its degradation by mitophagy (Ashrafi and Schwarz, 2013) and thereafter mitochondrial biogenesis is initiated for maintaining a healthy population of mitochondria and cellular homeostasis. Further experiments were done to study the effect of miR-5701 on mitochondrial number and analysis of mitochondrial DNA encoded transcripts. Interestingly, the transfection of miR-5701 significantly increased all mitochondrial DNA encoded transcripts except COX1 (Fig 9.1.2A&C) and was further enhanced in the presence of 6-OHDA (Fig 9.1.2B). mtDNA level was analyzed by quantification of a unique mitochondrial fragment relative to a single copy region of the nuclear gene RNaseP (Malik *et al.*, 2011). The unique region of the human mitochondrial sequence between positions 241 and 390 has been used in the study, which was had been previously shown to found ‘least similar’ to any human nuclear genomic region (Malik *et al.*, 2011). Primers were designed to RNaseP and B2 M genes and the primers and amplicons were tested using blast to ensure that they were present only once in the nuclear genome and did not show similarity to any other regions

in the genome. The cells were transfected with miR-5701 and mtDNA level was monitored by qPCR. The mtDNA levels increased significantly in presence of miR-5701 as compare to mimic control (Fig 9.1.2D).

Figure 9.1.1

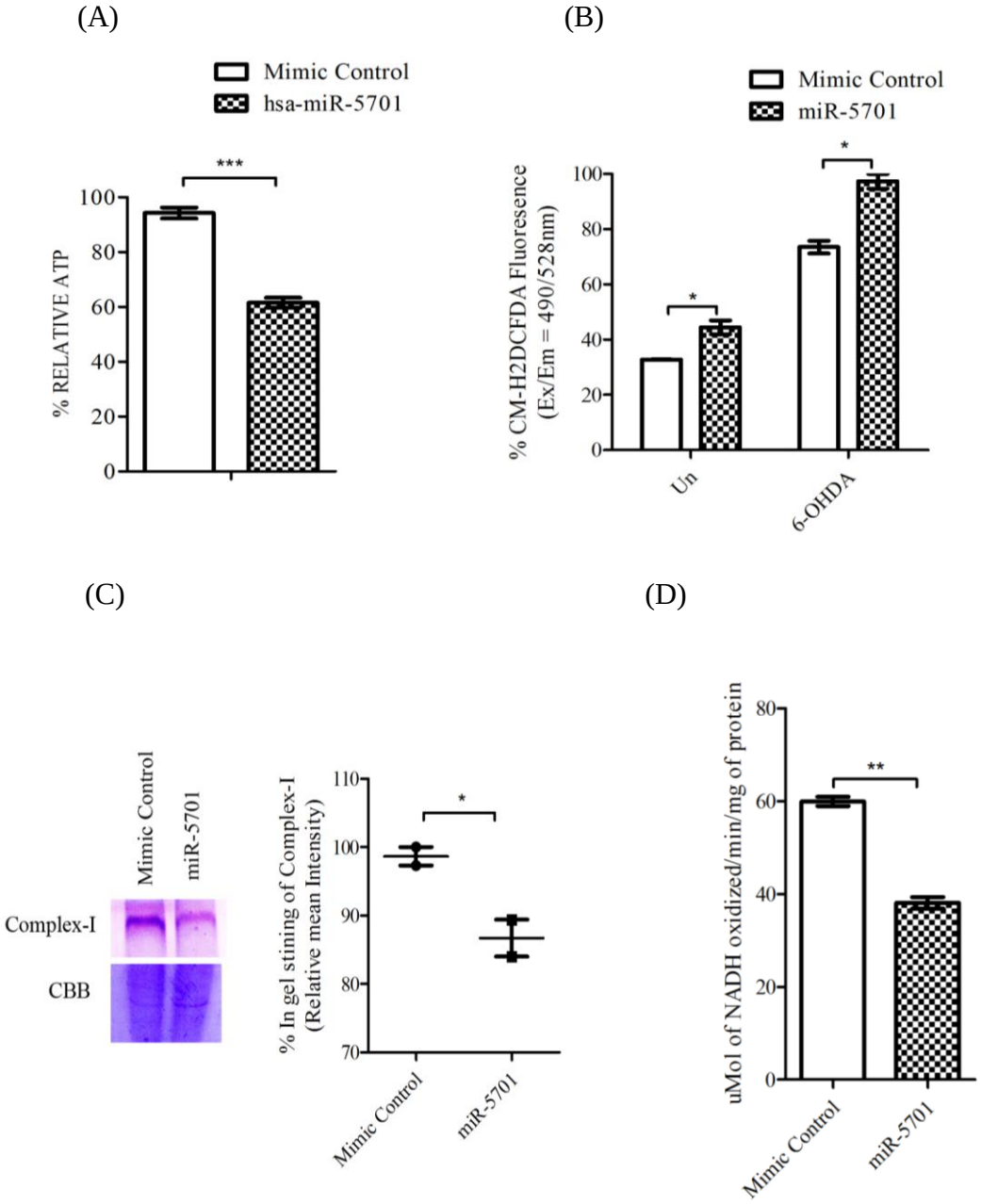

Figure 9.1.1: miR-5701 regulates mitochondrial function

(A) SH-SYSY cells were transfected with miR-5701 and ATP levels was measured by ATP-dependent luciferase activity. (B) SH-SYSY cells were transfected with miR-5701. After 24 hrs of transfection treated with 6-OHDA (75 µM/ml) for 12 hrs and ROS levels were analyzed using CM-H2DCFDA by spectrofluorometry. Complex-I activity was measured after 24 hrs of miR-5701 transfection (C) by in gel staining of Blue Native page of complex-I (D) by monitoring the decrease in absorbance at 340 nm. Asterisk (*) indicates that p value <0.05, for SEM.

Figure 9.1.2

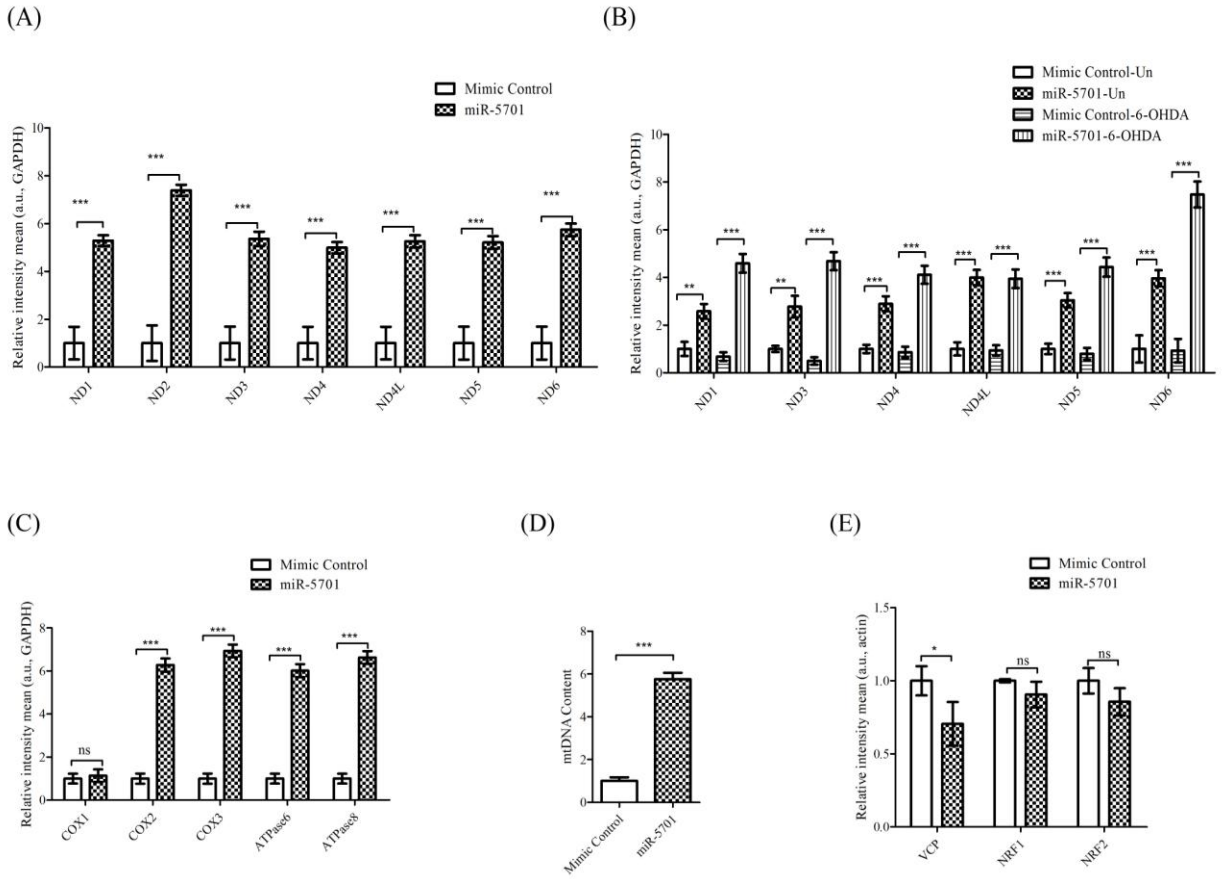

Figure 9.1.2: Expression of miR-5701 increases mitochondrial DNA and transcripts
(A & B & C) SH-SYSY cells were transfected with miR-5701 and treated with 6-OHDA. Relative expression level of mitochondrial DNA encoded transcripts were measured by qRTPCR. (D) SH-SYSY cells were transfected with miR-5701 and mtDNA was monitored

by qRTPCR (E) SH-SYSY cells were transfected with miR-5701 and relative expression level of NRF1 and NRF2 were measured by qRTPCR. Asterisk (*) indicates that p value <0.05, for SEM.

Increase mtDNA number and transcript in presence of miR-5701 suggest either mitochondrial biogenesis is initiated or mitophagy is inhibited leading to accumulation of defective mitochondria. To confirm this, we further analyzed the levels of transcription factors like NRF1 and NRF2, which regulate mitochondrial biogenesis. There was no change in the transcript of both transcriptional factors NRF1 and NRF2 in presence of miR-5701 (Fig 9.1.2E). These evidence suggest that increased level miR-5701 in neuronal cells leads to accumulation of defective mitochondria.

9.2 miR-5701 regulates the fusion of auotphoagosme with lysosome.

The earlier reports suggest that VCP plays role in autophagosome–lysosome fusion (Ju *et al.*, 2009; Tresse *et al.*, 2010; Dargemont and Ossareh-Nazari, 2012). As we observed here that VCP is the direct target of miR-5701, hence autophagy was monitored in the presence of miR-5701. LC3 is a known marker protein of autophagy pathway. This is conjugated to maturing autophagosome membrane with the help of other ATG proteins and degraded in lysosome (Zhou *et al.*, 2013). The BFA is specific V-ATPase inhibitor act as an inhibitor of autophagosome and lysosome fusion and increases the pH of lysosome (Crider *et al.*, 1994) leading to inhibition of lysosomal degradation. The level of LC3-II increased significantly in presence of miR-5701 and was similar to the levels observed in presence of BFA (Fig 9.2A). The autophagy flux was further confirmed by analyzing the turnover of p62 using tandem mCherry-p62-GFP constructs. Interestingly, the number of orange puncta of p62 increased in presence of miR-5701 as compared to mimic control (Fig 9.2B & C).These observations suggest that increased level of miR-5701 inhibit the fusion of autophagosome to lysosome or lysosomal activity itself. The recent study, autophagosome-lysosome fusion has been shown to play an essential role in the regulation of the lysosomal function (Zhou *et al.*, 2013). We monitored the lysosomal function in presence of miR-5701 using LysoSensor staining. The LysoSensor staining decreased in the presence miR-5701 as compared to control (Fig 9.2D). These data

collectively shows that miR-5701 have regulatory role act on the maturation of autophagolysosome, hence lysosomal function.

Figure 9.2

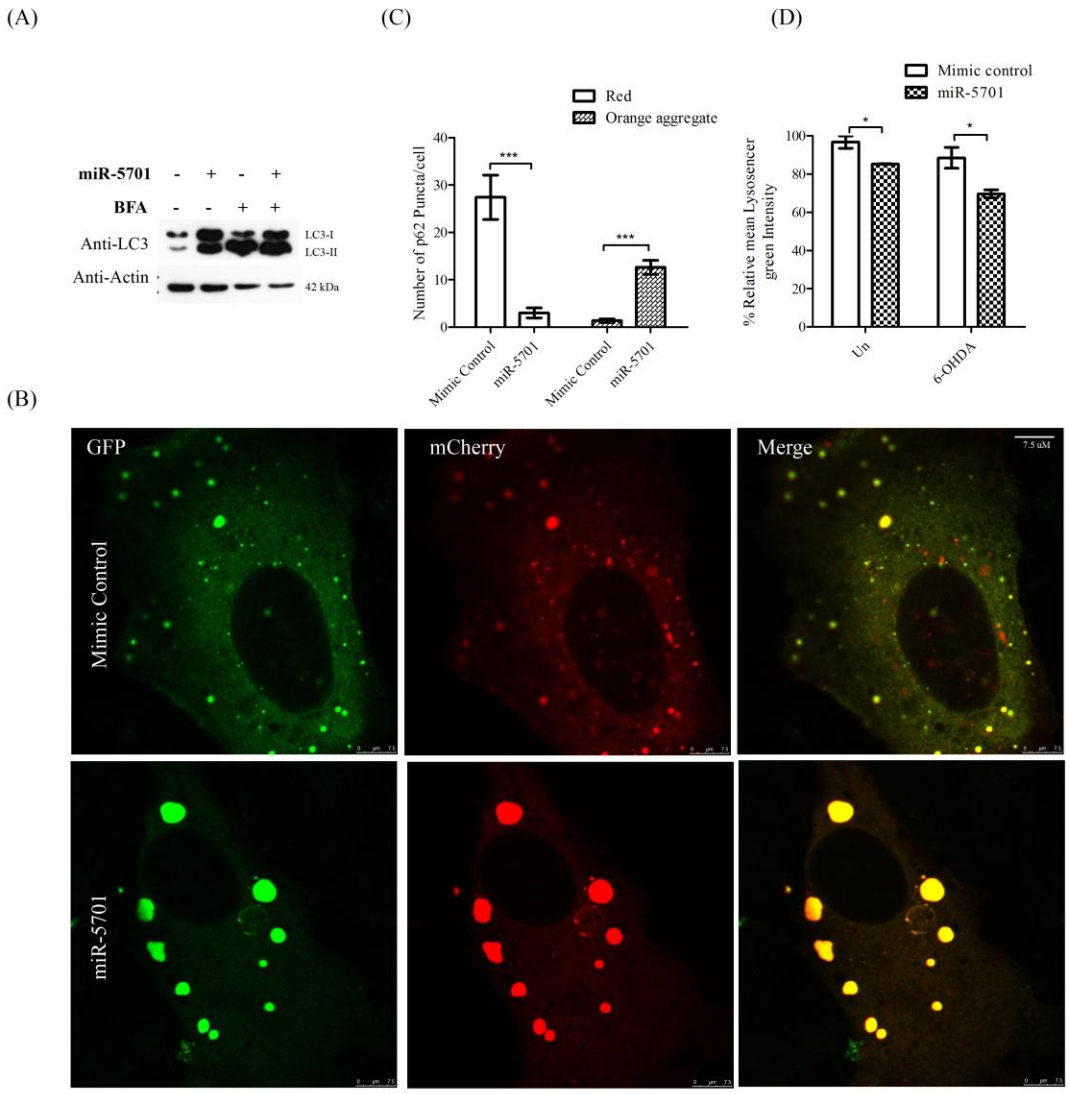

Figure 9.2: miR-5701 regulate the fusion of auotphoagosme with lysosome

(A) SH-SYSY cells were transfected with miR-5701. After 24 hrs of transfection treated with BAF for 4 hrs and LC3 level was measured by western blotting. (B) SH-SY5Y were co-transfected miR-5701 and with mCherry-p62-GFP and cells were monitored under confocal microscopy for red and orange puncta. Scale bars, 7.5 µm. (C) Graphical representations of the numbers red and orange p62 puncta in miR-5701 with control. (D) SH-SYSY cells were transfected with miR-5701 mimic and lysosomal acidification was

checked using LysoSensor Green staining. Asterisk (*) indicates that p value <0.05, for SEM.

9.3 miR-5701 sensitizes 6-OHDA induced cell death.

All the above experiments suggest the role of miR-5701 in the regulation of mitochondrial and lysosomal cross talk to modulate mitophagy. The accumulation of defective mitochondria may sensitize 6-OHDA induced cell death. To study the effect of the miR-5701 on cell death, SH-SY5Y cells were transfected with miR-5701 with mimic control, treated with 6-OHDA and cell survival was analyzed by MTT assay. The expression of miR-5701 sensitized 6-OHDA induced cell death as compared to mimic control transfected cells (Fig 9.3A). This was further confirmed by Acridine orange/Propidium Iodide staining. The Acridine orange/Propidium Iodide positive cells increased significantly in miR-5701 transfected cells as compared to control mimic in presence of 6-OHDA, suggesting cell death through apoptosis (Fig 9.3B). The induction of apoptosis was confirmed by monitoring caspase-3 activation by western blotting. The increased level of 19kDa/17kDa band corresponding cleaved subunit of caspase-3 was observed in miR-5701 transfected as compared to mimic control in the presence of 6-OHDA (Fig 9.3C). Caspase 3/7 activity was also monitored by caspase 3/7 luciferase assay in the presence of miR-5701. The transfection of miR-5701 significantly increased caspase 3/7 activity in presence of 6-OHDA (Fig 9.3D). As observed above that transfection of miR-5701 increases ROS level in normal and 6-OHDA stress condition, we further confirmed if ROS is essential for miR-5701 mediated cell death. SH-SY5Y cells were transfected with miR-5701 with mimic control, co-treated with 6-OHDA and NAC and cell survival was monitored in the presence of antioxidant (NAC). miR-5701 induced cell death in 6-OHDA stress condition was rescued by NAC (Fig 9.3E). Collectively, the evidence here suggests that miR-5701 can potentiate caspase activation leading ROS mediated apoptosis in PD stress condition.

Figure 9.3

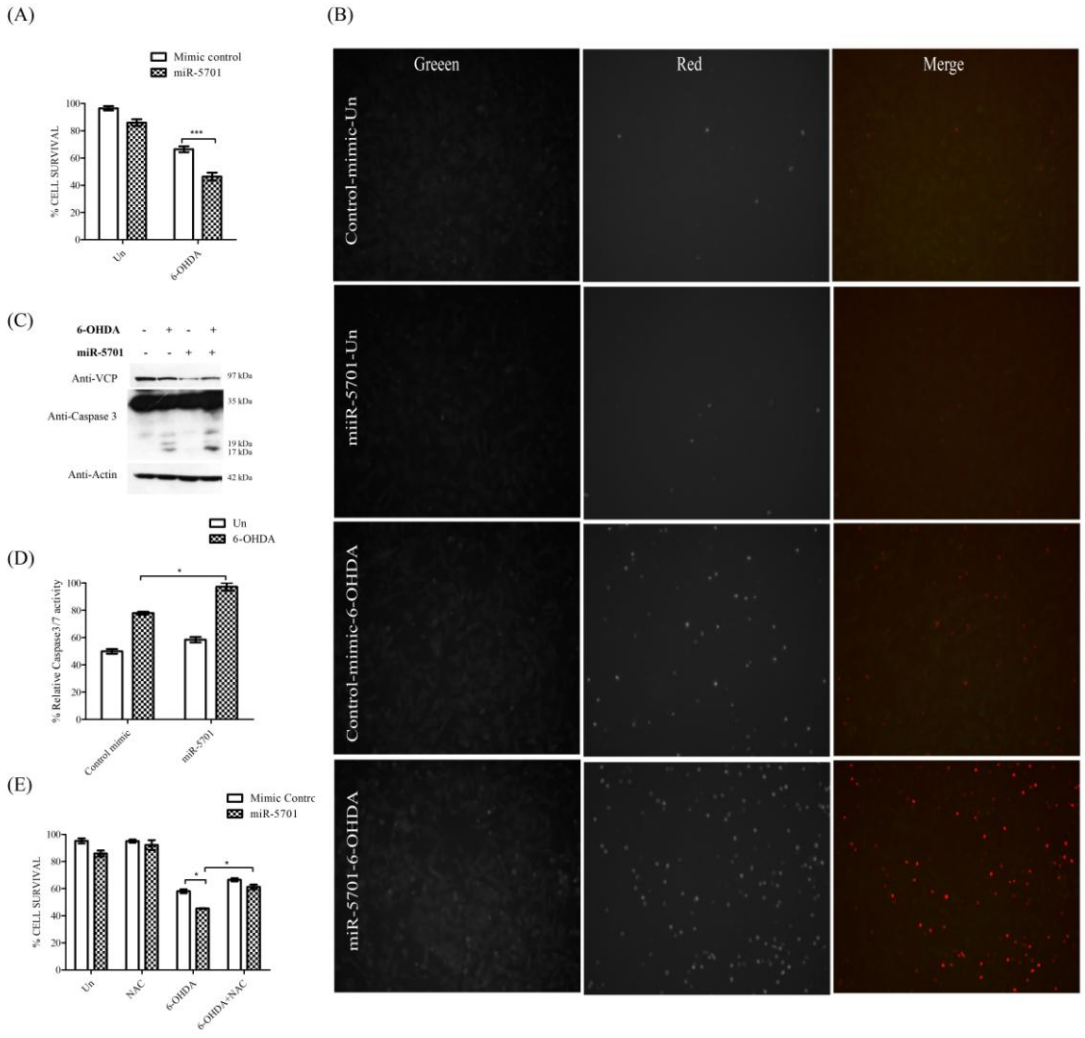

Figure 9.3: miR-5701 sensitized 6-OHDA induced cell death

(A) SH-SYSY cells were transfected with miR-5701 mimic. After 24 hrs of transfection, cells were treated with 6-OHDA for 16 hrs and MTT assay was performed. (B) miR-5701 transfected SH-SY5Y cells were treated with 6-OHDA (75 µM /ml) for 16 hrs and Acridine orange/Propidium Iodide staining was performed to monitor cell death. (C) SH-SYSY cells were transfected with miR-5701 mimic. After 24 hrs of transfection treated with 6-OHDA (75 µM/ml) for 16 hrs and caspase-3 cleavage was check using western blotting. (D) Caspase-3/7 activation assessed by caspas-3/7 Luciferase assay in miR-5701 express cell after 16 hrs of 6-OHDA treatment. (E) SH-SYSY cells were transfected with miR-5701 mimic. After 24 hrs of transfection, cells were co-treated with 6-OHDA and NAC for 16 hrs. MTT assay was performed to monitor cell survival. Asterisk (*)

indicates that p value <0.05, for SEM. miR-5701 transfected SH-SY5Y cells were treated with 6-OHDA (75 µM /ml) for 16 hrs and Acridine orange/Propidium Iodide staining was performed to monitor cell death.

9.4 Discussion:

Interestingly, analysis of the targets of one of the novel miRNAs, miR-5701, suggest its important role in PD conditions. The putative targets of miR-5701 miRNA may target the genes involved in lysosomal biogenesis and mitochondrial quality control. We observed decreased expression of VCP, LAPTM4A and ATP6V0D1 in the presence of miR-5701. In the present study, we identify VCP as the novel target of miR-5701 and its level in the neuronal cell are negatively regulated by miR-5701.

VCP is a type II member of the ATPase protein and its mutation causes multisystem degenerative disease mainly affects the brain, muscle and bone (Kim *et al.*, 2013b). Interestingly, mutations also show Parkinsonism like conditions suggestive of substantial nigra pathology (Chan *et al.*, 2012; Majounie *et al.*, 2012). VCP regulates of mitochondrial turnover through the selective form of autophagy called as mitophagy which is an important event in the pathogenesis of PD. VCP/p97 binds to ubiquitinated proteins on outer mitochondrial membrane proteins mitofusin-1 and mitofusin-2 following their ubiquitination by the E3 ligase Parkin (Tanaka *et al.*, 2010). It not only recognizes defective mitochondria, it may further help in fusion with the lysosome (Ju *et al.*, 2009; Tresse *et al.*, 2010). The major focus of all the ongoing studies had been on mitochondrial dysfunction during PD pathogenesis. The emerging evidence suggest that mitochondrial dysfunction compromises lysosomal activity leading to defective autophagy (Baixauli *et al.*, 2015; Demers-Lamarche *et al.*, 2016). The maintenance of mitochondrial function may be important to prevent the cell death during PD stress conditions.

Interestingly the two other major targets of miR-5701 are LAPTM4A and ATP6V0D1. LAPTM4A, is known to encode the putative lysosomal protein that has four predicted transmembrane domains. The functional study of the mouse homolog suggests its role in

the transport of small molecules across endosomal and lysosomal membranes (Hogue *et al.*, 2002). Similarly, the other target miR-5701, ATP6V0D1, is a component of vacuolar ATPase (V-ATPase), a multisubunit complex that mediates acidification of eukaryotic intracellular organelles (Agarwal and White, 2000). These strongly suggest the role of miR-5701 in the regulation of lysosomal function. Interestingly, miR-5701 here also showed compromised lysosomal function and inhibition of autophagy. The evidence here demonstrate that increased level of miR-5701 in neuronal cells compromises mitochondrial function and turnover. During PD stress conditions, the expression of miR-5701, show accumulation of defective mitochondria as increased mitochondrial DNA copy number and mitochondrial transcripts was observed. This is further supported by downregulation of VCP may inhibit autophagosome and lysosome fusion hence turnover of mitochondria.

The results here convincingly demonstrate that miR-5701 regulates the levels of proteins of lysosomal involved in acidification, hence modulates the flux of autophagy. This, in turn, leads to accumulation of defective mitochondria leading to further decrease in lysosomal function. The observed decrease in miR-5701 during 6-OHDA may be the compensatory mechanism to rescue these processes to increase the chance of neuronal survival. The evidence further suggests that mitochondrial and lysosomal cross talk is important for PD pathogenesis and is regulated by miR-5701. The further evidence in the animal model and PD tissue will further strengthen its role as novel miRNA in PD pathogenesis.