7. TNF-α regulates miRNAs expression in SH-SY5Y cells

7.1 TNF-α regulates miRNAs expression targeting mitochondrial functions.

The evidences in the current study strongly suggest that mitochondrial function is compromised in the presence of TNF-α and leads to cell death. The optimal functioning of mitochondria needs more than 1000 proteins which are nuclear encoded and translocated to the mitochondria. The import/association of non coding RNA including miRNA to mitochondria is emerging (Bandiera *et al.*, 2011; Mercer *et al.*, 2011; Sripada *et al.*, 2012; Zhang *et al.*, 2014). The miRNA targets both nuclear encoded and mitochondria encoded mRNA transcripts. Hence, we hypothesized that increased levels of TNF-α observed in PD conditions may alter the levels of miRNA which in turn may regulate mitochondrial functions and cell death. The differentially expressed miRNA in SH-SY5Y in presence of TNF-α were selected from previous reports (Culpan *et al.*, 2011). The expression level of identified miRNA was analyzed by real time PCR. It was observed that hsa-miR-let-7b, hsa-miR-let-7g, hsa-miR-103, hsa-miR-155, hsa-miR-16-5p, hsa-miR-17, hsa-miR-204, hsa-miR-27 and hsa-miR-98 were up-regulated significantly whereas the levels of hsa-miR-let-7a, hsa-miR-128, hsa-miR-145, hsa-miR-181a, hsa-miR-23a, hsa-miR-23b and hsa-miR-320a significantly decreased in the presence of TNF-α (Fig 7.1.1).

The putative targets of validated miRNAs and their biological significance were determined by combinatorial approach using miRNA 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 neuronal differentiation, axonal guidance and nerve projection development (Table 7.1B). The targets of up-regulated miRNAs were also clustered in basic cellular functional areas like cell cycle, mRNA transport, proteasomal ubiquitin dependent protein catabolism, mRNA transport and stabilization, protein phosphorylation, response to mechanical stress, acetyl CoA synthesis, ubiquitination, ubiquitin proteasome system and mitochondrial import. These processes are important for the mitochondrial homeostasis and functions (Table 7.1C). Similarly, the putative targets of down regulated miRNA regulates mitotic cell cycle, positively regulates protein phosphorylation, negatively regulates ubiquitination, inflammatory genes, neuronal projections, mRNA transport, cAMP reponse, translation, transcription, protein stabilization and negatively regulates cellular migration (Table 7.1D). The several cellular processes as described above targeted by miRNAs have important implication in the progression of PD.

The starBase analysis also revealed that some of the validated miRNAs may target nuclear encoded subunits of mitochondrial complex-I to complex-V (Table 7.1A). It was observed that NDUFV1, NDUFA7, NDUFAB1, NDUFB5, NDUFC1 and NDUFS4 subunits of mitochondrial complex-I are putative targets of hsa-miR-23a, hsa-miR-23b, hsa-miR-27a, hsa-miR-27b, hsa-miR-107 and hsa-miR-103 respectively. Since, the mitochondrial complex-I was compromised in the presence of TNF-α, miRNAs targeting nuclear encoded mitochondrial complex-1 subunit were specifically analyzed by real time PCR. The levels of miR-27a, hsa-miR-17, hsa-miR-103 and hsa-miR-155 significantly increased in the presence of TNF-α (Fig 7.1.2A). The target mRNA transcripts, NDUFS4 (target of hsa-miR-27a) and NDUFVI (target of hsa-miR-103) decreased significantly in the presence of TNF (Fig 7.1.2B). This suggests that TNF-α regulates miRNAs that target mitochondrial complex-I subunits which is critical for neuronal function. Similarly, ATP5G3, a subunit of mitochondrial complex-V is putative target of miR-155 and miR-27a. It was observed that the levels of both miRNAs (miR-155 and miR-27a) were high and the levels ATP5G3 (target of miR-155 and miR-27a) transcripts, decreased significantly in the presence of TNF-α (Fig 7.1.2B). This suggests that TNF-α alters the expression of miRNAs that target transcripts encoding mitochondrial complex-I and complex-V subunits leading to mitochondrial defects and decline in ATP production during neuronal cell death in PD. The evidence in the current study suggest that hsa-miR-155 target mitochondrial functions and previous reports (Nederlof *et al.*, 2014) suggest its implication in cell death hence further experiments were done to confirm its role in TNF-α induced cell death. SH-SY5Y cells were transfected with anti-miR-155 and cell survival was monitored in presence of TNF-α. The cell death significantly decreased in the presence of TNF-α in anti-miR-155 transfected cells as compared to control (Fig 7.1.2C). These evidences suggest that TNF-α alters the expression of miRNAs that target transcripts encoding mitochondrial complex-I and complex-V subunits leading to mitochondrial defects and decline in ATP production during neuronal cell death in PD.

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Figure 7.1.1

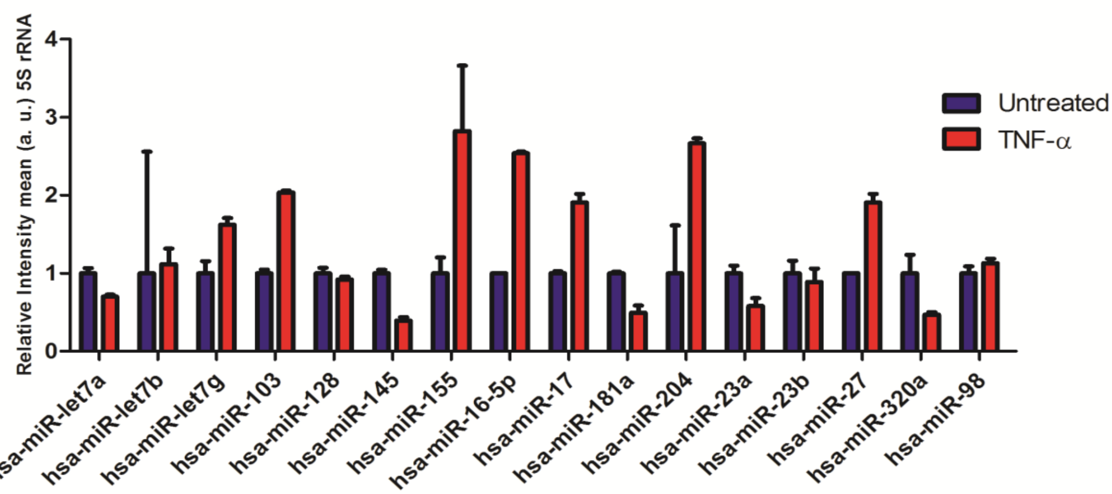

Figure 7.1.1: TNF-α regulates miRNAs levels.

The panel of miRNA that were altered in presence of TNF-α were selected from literature. The expression levels of miRNAs were determined using 5S rRNA as endogenous control by real time PCR.

Table 7.1 A

Sr. No.	miRNA[1]	Target[2]	Complex[3]
1	hsa-miR-103	NDUFV1	Cx-I
2	hsa-miR-128	NDUFS4, COX7b, ATP5G3	Cx-I, Cx-IV, Cx-V
3	hsa-miR-155	NDUFV1, UQC, COX7B, ATP5G3	Cx-I, Cx-III, Cx-IV, Cx-V
4	hsa-miR-17	NDUFAB1, NDUFB5	Cx-I
5	hsa-miR-181a	UQCR11, UQCRH, ATP5J	Cx-III, Cx-V
6	hsa-miR-204	COX6B1	Cx-IV
7	has-miR-221	NDUFA1	Cx-I
8	hsa-miR-23a	NDUFA7, NDUFC1	Cx-I
9	hsa-miR-23b	NDUFA7, NDUFC1	Cx-I
10	hsa-miR-27a	NDUFS4, ATP5G3	Cx-I, Cx-V
11	hsa-miR-27b	NDUFS4, ATP5G3	Cx-I, Cx-V

Table 7.1 A: The miRNAs target nuclear encoded mitochondrial subunits.

The miRNAs that target mitochondrial subunits were determined by starBase v2.0. The

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putative targets from mitochondrial subunits for the altered miRNAs were tabulated. [1.] miRNA which are altered in the presence of TNF. [2.] The putative nuclear encoded mitochondrial subunit as target as determined by starBase v2.0 with minimum stringency. [3.] The mitochondrial electron transport complex of which the subunit is integral part.

Figure 7.1.2

(A) (B) (C)

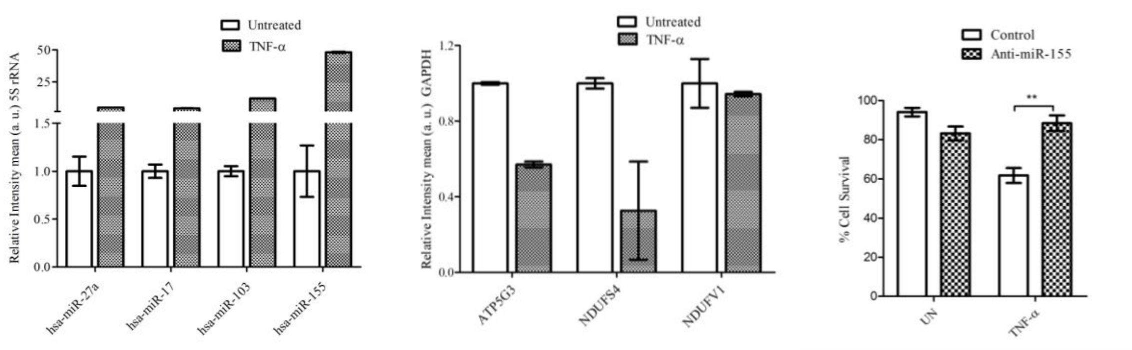

Figure 7.1.2: TNF-α regulates miRNAs expression targeting mitochondrial functions and modulate cell death.

(A) The expression levels of miRNAs which putatively targets mitochondrial complex-I subunits were determined using 5S rRNA as endogenous control using qPCR (B) The putative targets were determined by starBase v.2.0. The expression levels of targets comprising nuclear encoded mitochondria complex-I subunits were determined by real time PCR using GAPDH as endogenous control. (C) SH-SY5Y cells were transfected withanti-miR-155 and control. Cell survival was monitored using MTT assay in presence of TNF-α. Asterisk (*) indicates that p value b 0.05, for SEM.

Table 7.1 B

Sr. No	Term Id[1]	Term Name[2]	Hit Gene Number[3]	P-Value[4]
1	P00018	EGF receptor signaling pathway	6	0.00049992
2	P04398	p53 pathway feedback loops	4	0.00069093

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3	P00059	p53 pathway	5	0.00078965
4	P00048	PI3 kinase pathway	4	0.00088871
5	P00021	FGF signaling pathway	5	0.00236291
6	P00049	Parkinson disease	4	0.00771688

Table 7.1 B: The pathways affected by up-regulated miRNA in presence of TNF.

The miRNAs were quantified by real time PCR. The up-regulated miRNAs were selected in miRfunction tool with combination of 5 target prediction tools and minimum stringency in starBase v2.0 to determine their putative roles. The biological pathways were extracted from PANTHER Pathway v1.2 and tabulated. 1. Identification number of the pathway from PANTHER Pathway database. 2. The nomenclature of pathway identifier. 3. P-value of the pathway prediction.

Table 7.1 C

Sr. No.	**Term Id[1]**	**Term Name[2]**	**Gene Number in Term[3]**	**P-Value[4]**
1	GO:0051574	positive regulation of histone H3-K9 methylation	2	8.33E-05
2	GO:0038026	reelin-mediated signaling pathway	3	0.00024838
3	GO:0046579	positive regulation of Ras protein signal transduction	14	0.000254579
4	GO:0021766	hippocampus development	36	0.000317344
5	GO:0050731	positive regulation of peptidyl-tyrosine phosphorylation	67	0.000369518
6	GO:0045664	regulation of neuron differentiation	16	0.000386422
7	GO:0000083	regulation of transcription involved in G1/S phase of mitotic cell cycle	19	0.000655294
8	GO:0007256	activation of JNKK activity	5	0.000817991
9	GO:0007049	cell cycle	219	0.000938113
10	GO:0001934	positive regulation of protein phosphorylation	85	0.00109987
11	GO:0051028	mRNA transport	51	0.00121033
12	GO:0021517	ventral spinal cord development	6	0.00121961
13	GO:0048752	semicircular canal morphogenesis	6	0.00121961
14	GO:0070507	regulation of microtubule cytoskeleton organization	6	0.00121961
15	GO:2000727	positive regulation of cardiac muscle cell differentiation	6	0.00121961

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16	GO:0000910	Cytokinesis	59	0.002083
17	GO:0034644	cellular response to UV	28	0.00208563
18	GO:0045651	positive regulation of macrophage differentiation	8	0.00224932
19	GO:0043161	proteasomal ubiquitin-dependent protein catabolic process	61	0.00235489
20	GO:0007411	axon guidance	317	0.00262398
21	GO:0000278	mitotic cell cycle	319	0.00273632
22	GO:0035235	ionotropic glutamate receptor signaling pathway	9	0.00287462
23	GO:0031175	neuron projection development	66	0.0031395
24	GO:0009636	response to toxic substance	68	0.00349784
25	GO:0007596	blood coagulation	461	0.00351747
26	GO:0007257	activation of JUN kinase activity	35	0.00397633
27	GO:0042692	muscle cell differentiation	35	0.00397633
28	GO:0006468	protein phosphorylation	341	0.00424148
29	GO:0043434	response to peptide hormone stimulus	36	0.00430896
30	GO:0043488	regulation of mRNA stability	11	0.00433926
31	GO:0016024	CDP-diacylglycerol biosynthetic process	12	0.00517592
32	GO:0010510	regulation of acetyl-CoA biosynthetic process from pyruvate	12	0.00517592
33	GO:0045860	positive regulation of protein kinase activity	40	0.00580567
34	GO:0034613	cellular protein localization	42	0.00665665
35	GO:0009612	response to mechanical stimulus	42	0.00665665
36	GO:0010744	positive regulation of macrophage derived foam cell differentiation	14	0.00705135
37	GO:0000122	negative regulation of transcription from RNA polymerase II promoter	508	0.00718142
38	GO:0015031	protein transport	371	0.00725746
39	GO:0002092	positive regulation of receptor internalization	15	0.00808755
40	GO:0032722	positive regulation of chemokine production	15	0.00808755
41	GO:0070527	platelet aggregation	15	0.00808755
42	GO:0045672	positive regulation of osteoclast differentiation	15	0.00808755
43	GO:0031668	cellular response to extracellular stimulus	15	0.00808755
44	GO:0032008	positive regulation of TOR signaling	16	0.00918771

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		cascade		
45	GO:0061098	positive regulation of protein tyrosine kinase activity	16	0.00918771
46	GO:0060037	pharyngeal system development	16	0.00918771

Table 7.1 C: The biological process modulated by upregulated miRNAs in presence of TNF-α.

The upregulated miRNAs were selected in miR function tool with combination of 5 target prediction tools and minimum stringency in starBase v2.0. The putative targets were clustered according to the biological processes 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.

Table 7.1 D

Sr. No.	Term Id[1]	Term Name[2]	Gene Number in Term[3]	P-Value[4]
1	GO:0000083	regulation of transcription involved in G1/S phase of mitotic cell cycle	19	2.71E-05
2	GO:0031397	negative regulation of protein ubiquitination	23	4.90E-05
3	GO:0001934	positive regulation of protein phosphorylation	85	0.000143632
4	GO:0033137	negative regulation of peptidyl-serine phosphorylation	14	0.000852532
5	GO:0045648	positive regulation of erythrocyte differentiation	19	0.00158602
6	GO:0060412	ventricular septum morphogenesis	21	0.00193996
7	GO:0019048	virus-host interaction	316	0.00302819
8	GO:0048706	embryonic skeletal system development	28	0.00344332
9	GO:0030501	positive regulation of bone mineralization	30	0.00394673
10	GO:0032024	positive regulation of insulin secretion	32	0.00448222
11	GO:0002053	positive regulation of mesenchymal cell proliferation	35	0.00534471
12	GO:0045727	positive regulation of translation	38	0.00627712
13	GO:0051591	response to cAMP	38	0.00627712
14	GO:0031398	positive regulation of protein	46	0.00909525

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		ubiquitination		
15	GO:0010976	positive regulation of neuron projection development	49	0.0102727
16	GO:0051028	mRNA transport	51	0.0110933
17	GO:0090263	positive regulation of canonical Wnt receptor signaling pathway	51	0.0110933
18	GO:0001938	positive regulation of endothelial cell proliferation	51	0.0110933
19	GO:0030509	BMP signaling pathway	58	0.0141832
20	GO:0043154	negative regulation of cysteine-type endopeptidase activity involved in apoptotic process	63	0.0165916
21	GO:0030512	negative regulation of transforming growth factor beta receptor signaling pathway	64	0.0170929
22	GO:0045893	positive regulation of transcription, DNA-dependent	484	0.0173976
23	GO:0050821	protein stabilization	68	0.0191616
24	GO:0030336	negative regulation of cell migration	71	0.0207788

Table 7.1 D: The biological process modulated by down regulated miRNAs in presence of TNF-α.

The down regulated miRNAs were selected in miR function tool with combination of 5 target prediction tools and minimum stringency in starBase v2.0. The putative targets were clustered according to the biological processes 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.

7.2 Discussion:

The emerging reports strongly suggest that mitochondrial trafficking is crucial for neuronal branching, synaptic plasticity and transmission (Cai *et al.*, 2011; Sheng, 2014). The mitochondria are immobilised at the neuronal branching point, assists site specific de novo protein synthesis and initiates axonal branching (Cai *et al.*, 2011; Sun *et al.*, 2013). This strongly suggests that mitochondria are important for the neuronal connectivity which is degenerated in different neurodegenerative diseases including PD (Solis *et al.*, 2007; Ginns *et al.*, 2014). The optimal functioning of mitochondria requires nuclear

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encoded gene that are transcribed in nucleus, translated in the cytoplasm and translocated to mitochondria (Calvo and Mootha, 2010). The recent reports including ours (Bandiera *et al.*, 2011; Mercer *et al.*, 2011; Sripada *et al.*, 2012; Zhang *et al.*, 2014) suggest that miRNA associates with mitochondria and is important for the regulation of the target in the narrow physiological range. In consonance to it, the current study demonstrated that miRNAs including mitochondria associated miRNA (miR-23a, miR-320a, miR-181, miR-let-7a, miR-let-7g a) are altered in presence of TNF-α. Intriguingly, some of these miRNAs: miR-103, miR-17, miR-181a, miR-23a, miR-23b, miR-27b and miR-320 were previously reported to be enriched at synapses (Smalheiser and Lugli, 2009; Saba *et al.*, 2012; Follert *et al.*, 2014). The role of miRNAs in regulation of synaptic functions should be also studied.

The putative targets of validated miRNA suggested that miRNA may target the genes involved in mRNA stability, transport and transcription, translation, phosphorylation and ubiquitination of proteins, cell cycle and mitochondrial complex subunits. The dysregulation of these processes and targets has been implicated in progression of PD (Ohtsuki *et al.*, 2010; Cali *et al.*, 2013; Fifel *et al.*, 2013; Soreq *et al.*, 2013; Cornelissen *et al.*, 2014; Morais *et al.*, 2014; Tenreiro *et al.*, 2014; Wang *et al.*, 2014). In consonance with the starBase analysis of miRNA and targets, the experimental results showed that TNF-α regulates miRNAs (has-miR-27a and hsa-miR-103) that target the nuclear encoded transcripts of mitochondrial complex-I subunit decreased in the presence of TNF-α. This is further supported by observation where expression of miR-27a impaired mitochondrial biogenesis, structure integrity and complex-I activity which further produced excessive ROS in human cell (Kang *et al.*, 2013). Similarly, TNF-α regulate the expression of miR-155 and miR-27a which regulate the transcript levels of ATP5G3, subunit of F1-ATPase. This helps in transport of protons across the mitochondrial inner membrane to the F1-ATPase for synthesis of ATP. This hypothesis is in consonance with recent report demonstrating the import of hsa-miR-181c in mitochondria, which targets complex-IV subunits in myocardial infract (Das *et al.*, 2014). These results strongly suggest that TNF-α may regulate several miRNAs specifically targeting nuclear encoded transcripts encoding mitochondrial targeted proteins.

The data provided here strongly suggest that mitochondrial functions were compromised in the presence of TNF-α leading to bioenergetics crisis and increased cell death in dopaminergic neurons. The study also suggests that TNF-α regulated miRNAs play important role in the regulation of mitochondrial functions suggesting its important implication in PD conditions. The innovation in sequencing technologies last few years had shown exponential increase in number of miRNAs hence it is important to systematically investigate the TNF-α regulated miRNA in dopaminergic neurons. The same needs to be validated in the tissue obtained from the patients having increased levels of TNF-α in serum and CSF and showing the symptoms of Parkinsonism. The studies in this direction will help to better understand the crosstalk of inflammation, miRNA and mitochondria and its implication in PD.

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