CHAPTER VI

To study the microRNA expression profile in custom peptides-treated cultured *Caenorhabditis elegans*

6.1 Results

6.1.1 Identification of mouse 2.5 S-NGF/custom peptide (HNP)-regulated miRNAs in *C. elegans* by miRNA sequencing analysis

The miRNA microarray analysis showed differential expression of twenty miRNAs in the NGF group of *C. elegans* (Table 6.1A). Among them, 10 miRNAs were upregulated, and 10 were downregulated in the NGF group of *C. elegans* compared to the CT group of *C. elegans*. Similarly, the microarray analysis revealed the differential regulation of 13 miRNAs in the HNP group of *C. elegans* (Table 6.1B); ten miRNAs were upregulated, whereas three miRNAs were downregulated compared to the CT group *C. elegans*. cel-miR-255-3p, cel-miR-1-3p, cel-miR-62, cel-miR-85-3p, cel-miR-71-3p, cel-miR-83-3p, cel-miR-84-5p, and cel-miR-4936 were found to be in common in mouse 2.5 S-NGF and HNP treated groups of *C. elegans*. Among them, the expressions of cel-miR-1-3p and cel-miR-255-3p were markedly increased in both the NGF and HNP groups of *C. elegans*. The annotated pathways of potential miRNA targets in the NGF and HNP groups of *C. elegans* are listed in Table 6.1C.

Table 6.1A. The miRNA microarray analysis data shows the fold changes of differentially regulated miRNA in the *C. elegans* treated with 50 µg/mL of mouse 2.5 S-NGF (NGF) for 2 h with respect to 1X PBS-treated *C. elegans* (CT) at 20°C.

Types of miRNAs	**logFC (CT vs NGF)**
cel-miR-1-3p	2.69
cel-miR-255-3p	2.21
cel-miR-83-3p	1.93
cel-miR-85-3p	1.92
cel-miR-59-3p	1.87
cel-miR-62	1.72
cel-miR-84-5p	1.60
cel-miR-794-5p	1.54
cel-miR-795-3p	1.51
cel-miR-71-3p	1.30
cel-miR-4936	-3.08
cel-miR-67-3p	-3.14
cel-miR-41-5p	-3.27
cel-miR-5545-3p	-3.33
cel-miR-8211-5p	-3.79
cel-miR-8204-5p	-3.88
cel-miR-67-5p	-4.12
cel-miR-8205-3p	-4.81
cel-miR-2217b-3p	-5.04
cel-miR-8202-5p	-5.10

Table 6.1B. The miRNA microarray analysis data shows the fold changes of differentially regulated miRNA in the *C. elegans* treated with 50 µg/mL of custom peptide (HNP) for 2 h compared to 1X PBS-treated *C. elegans* (CT) at 20°C.

Types of miRNAs	**logFC (CT vs HNP)**
cel-miR-255-3p	3.47
cel-miR-87-5p	3.13
cel-miR-1-3p	1.87
cel-miR-1820-5p	1.54
cel-miR-62	1.53
cel-miR-358-3p	1.50
cel-miR-85-3p	1.38
cel-miR-71-3p	1.30
cel-miR-83-3p	1.40
cel-miR-84-5p	1.55
cel-miR-791-3p	-1.36
cel-miR-789-5p	-1.44
cel-miR-4936	-1.55

Table 6.1C. Pathways annotation of potential miRNA target genes in only HNP/mouse 2.5 S-NGF-treated *C. elegans*.

Pathways of potential miRNA target genes	**The p-Value of HNP-treated *C. elegans***	**The p-Value of mouse 2.5 S-NGF treated *C. elegans***
BINDING: ATP		0.463
CARBOHYD: N-linked (GlcNAc) asparagine	0.183	0.239
cel01100: Metabolic pathways		0.000
cel01200: Carbon metabolism	0.006	0.004
cel01230: Biosynthesis of amino acids	0.001	0.000
GO:0000166~nucleotide binding	0.299	0.198
GO:0000287~magnesium ion binding	0.009	0.006
GO:0004888~transmembrane signaling receptor activity	0.255	0.209
GO:0005216~ion channel activity	0.367	0.321
GO:0005230~extracellular ligand-gated ion channel activity	0.249	0.214
GO:0005524~ATP binding	0.425	0.308
GO:0005739~mitochondrion	0.024	
GO:0005886~plasma membrane	0.226	0.055
GO:0006811~ion transport	0.055	0.039
GO:0006813~potassium ion transport	0.069	0.060
GO:0006937~regulation of muscle contraction	0.032	0.028
GO:0007165~signal transduction	0.070	0.109
GO:0007268~chemical synaptic transmission	0.348	0.314
KW-0407~Ion channel		0.481
GO:0016301~kinase activity	0.198	0.279
KW-0460~Magnesium		0.207

GO:0016310~phosphorylation	0.186	0.282
GO:0022848~acetylcholine-gated cation-selective channel activity	0.017	0.014
GO:0022857~transmembrane transporter activity	0.278	0.083
GO:0030054~cell junction	0.165	0.119
GO:0030594~neurotransmitter receptor activity	0.286	0.247
GO:0034220~ion transmembrane transport	0.146	0.123
GO:0040011~locomotion	0.039	0.154
GO:0042391~regulation of membrane potential	0.241	0.215
GO:0043005~neuron projection	0.254	0.189
GO:0045211~postsynaptic membrane	0.063	0.048
GO:0046662~regulation of oviposition	0.056	0.049
GO:0050877~neurological system process	0.226	0.201
GO:0055085~transmembrane transport	0.424	0.191
GO:0060078~regulation of postsynaptic membrane potential	0.002	0.001
GO:0060079~excitatory postsynaptic potential	0.021	0.018
GO:0090326~positive regulation of locomotion involved in locomotory behaviour	0.014	0.012
IPR002394: Nicotinic acetylcholine receptor	0.009	0.007
IPR006029: Neurotransmitter-gated ion-channel transmembrane domain	0.118	0.102
IPR006201: Neurotransmitter-gated ion-channel	0.172	0.149
IPR006202: Neurotransmitter-gated ion-channel ligand-binding	0.176	0.154
IPR011701: Major facilitator superfamily	0.157	0.137
IPR018000: Neurotransmitter-gated ion-channel, conserved site	0.116	0.100
KW-0067~ATP-binding	0.138	0.044
KW-0325~Glycoprotein	0.259	0.265
KW-0406~Ion transport	0.089	0.103
KW-0418~Kinase	0.063	0.119
KW-0443~Lipid metabolism	0.485	
KW-0472~Membrane	0.244	0.124
KW-0496~Mitochondrion	0.084	0.121
KW-0547~Nucleotide-binding	0.050	0.012
KW-0628~Postsynaptic cell membrane	0.031	0.021
KW-0770~Synapse	0.133	0.094
KW-0808~Transferase	0.132	0.153
KW-0809~Transit peptide	0.078	0.221
KW-0812~Transmembrane	0.066	0.177
KW-0813~Transport	0.419	0.472
KW-0965~Cell junction	0.098	0.060
KW-1003~Cell membrane	0.030	0.009
KW-1015~Disulfide bond	0.233	0.108
KW-1071~Ligand-gated ion channel	0.035	0.030
KW-1133~Transmembrane helix	0.233	
NP_BIND: ATP	0.375	0.146
R-CEL-112314~Neurotransmitter receptors and postsynaptic signal transmission	0.294	0.281

R-CEL-112316~Neuronal System	0.419	0.400
R-CEL-181431~Acetylcholine binding and downstream events	0.074	0.070
R-CEL-195721~Signaling by WNT		0.486
R-CEL-212436~Generic Transcription Pathway	0.224	0.425
R-CEL-3858494~Beta-catenin independent WNT signaling		0.143
R-CEL-556833~Metabolism of lipids	0.327	
R-CEL-622323~Presynaptic nicotinic acetylcholine receptors	0.011	0.010
R-CEL-622327~Postsynaptic nicotinic acetylcholine receptors	0.074	0.070
R-CEL-629594~Highly calcium permeable postsynaptic nicotinic acetylcholine receptors	0.074	0.070
R-CEL-629597~Highly calcium permeable nicotinic acetylcholine receptors	0.011	0.010
R-CEL-6798695~Neutrophil degranulation		0.493
R-CEL-71387~Metabolism of carbohydrates	0.031	0.112
R-CEL-73857~RNA Polymerase II Transcription	0.418	
R-CEL-74160~Gene expression (Transcription)	0.489	
R-CEL-8878171~Transcriptional regulation by RUNX1	0.196	
TOPO_DOM: Cytoplasmic	0.062	0.096
TOPO_DOM: Extracellular	0.103	0.072
TRANSIT: Mitochondrion	0.059	0.189

The volcano plot (p-values vs. fold change) displayed the differential gene expression between (i) NGF and CT groups (Fig. 6.1A) and (ii) HNP and CT groups (Fig. 6.1B). The heatmap analysis exhibited a disparity in gene expression between NGF and HNP groups compared to the CT group (Fig 6.1C-D). The list of possible targets of differentially expressed miRNAs was predicted. It was observed that probable target genes of upregulated miRNAs were involved in the PI3K pathway, Wnt/β-catenin signaling, TGF signaling, Axon development, transcriptional regulation, and cellular development in the NGF and HNP groups of *C. elegans*. The target genes of downregulated miRNAs were implicated in apoptotic pathways, p53 pathways, neurological diseases, Ras pathways, Innate immune response pathways, etc., in NGF and groups of *C. elegans* (data not shown). The role of some of the upregulated miRNAs in different stages of neuronal development in NGF and HNP groups of *C. elegans* is shown in Fig 6.2.

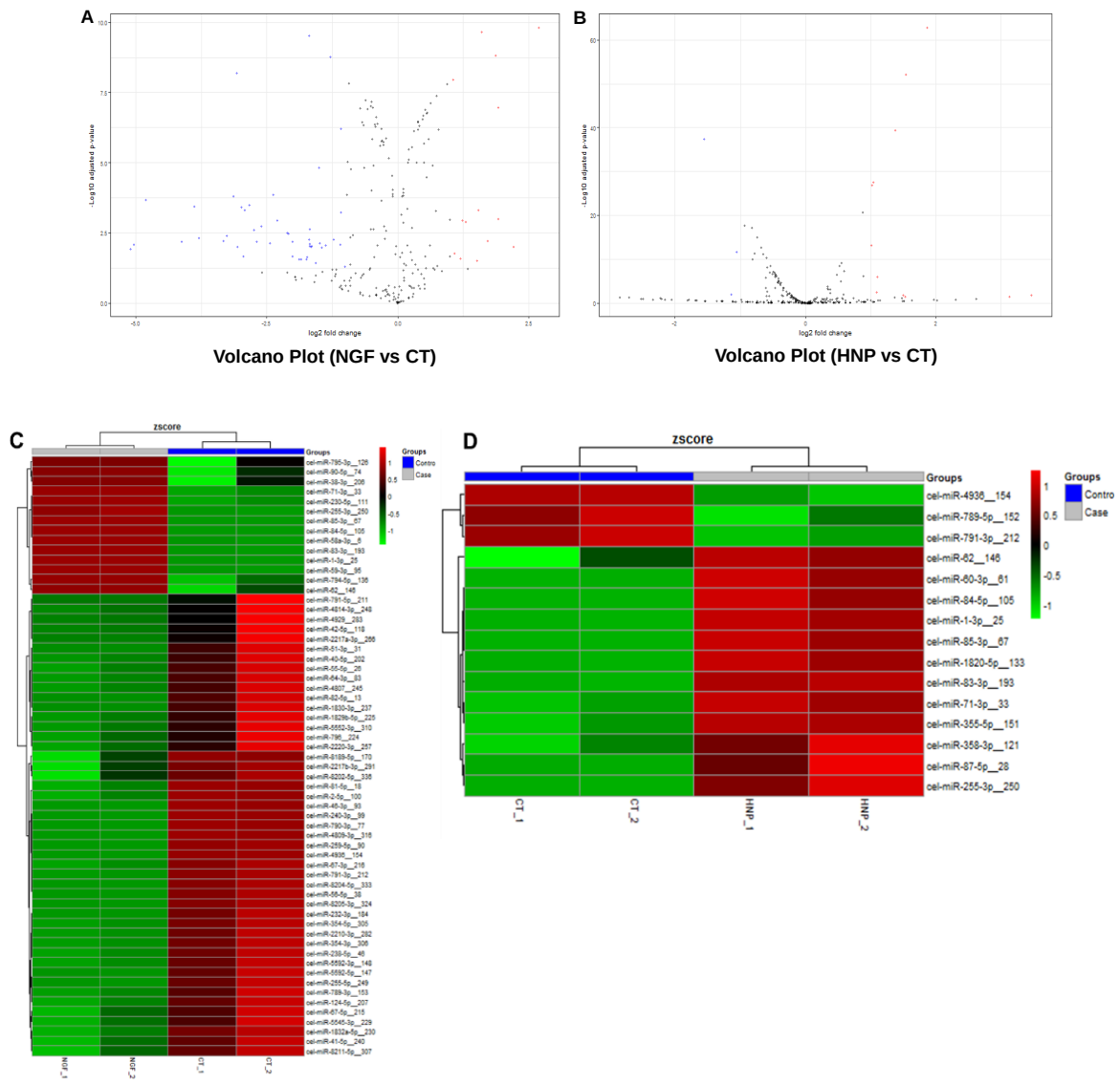

Fig 6.1 Differential expression of miRNAs between mouse 2.5S-NGF/peptide HNP-treated group of *C. elegans* and 1X PBS-treated (control) group of *C. elegans*. (A) Volcano plot (p-value v/s log Fc) for mouse 2.5S-NGF-treated (NGF group) versus 1X PBS-treated (CT group) *C. elegans*. (B) Volcano plot (p-value v/s log Fc) for custom peptide HNP-treated (HNP group) versus 1X PBS-treated (CT) *C. elegans*. Heat map showing the differential expression of the upregulated and downregulated genes among (C) mouse 2.5S-NGF-treated (NGF group) versus 1X PBS-treated (CT group) *C. elegans* and (D) custom peptide HNP-treated (HNP group) versus 1X PBS-treated (CT) *C. elegans*.

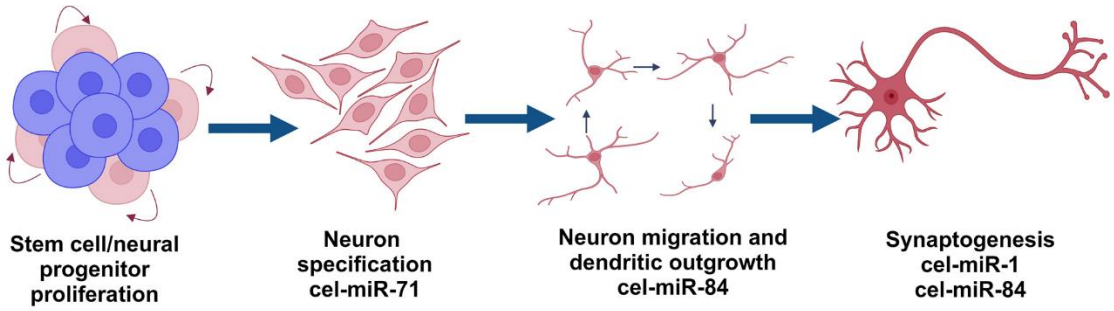

Fig 6.2 Roles of mouse 2.5S-NGF and HNP-induced miRNAs involved in different stages of neuronal development in *C. elegans*.

6.1.2 A comparison of the differential expression of global miRNA between paraquat-treated *C. elegans* and pre-treatment of *C. elegans* with mouse 2.5 S-NGF/ HNP followed by paraquat treatment

Novel miRNAs were identified in all the treated groups. The list of differentially expressed miRNAs with their respective logFC values in the PT group, compared to the CT group and their restoration with the treatment of mouse 2.5S-NGF and custom peptide HNP treatment in *C. elegans*, is shown in Table 6.2A and Table 6.2B, respectively. The principal component analysis (PCA) score plot showed the clustering between group 1 consisting of CT, PT, PNGF, and NGF groups of *C. elegans* (Fig 6.3A). The clustering between group 2, comprising CT, PT, PHNP, and HNP groups of *C. elegans* (Fig 6.3B), and the clustering between group 1 and group 2 is shown in Fig 6.3C. Similarly, the correlation plot (red indicates the positive, and blue indicates the negative correlation) was constructed within different groups as described previously (Fig 6.3D-F). The volcano plots (p-values vs. fold change) show the differential expression of the miRNAs in (i) PNGF vs. PT group of *C. elegans* (Fig 6.3G) and (ii) PHNP vs. PT group of *C. elegans* (Fig 6.3H).

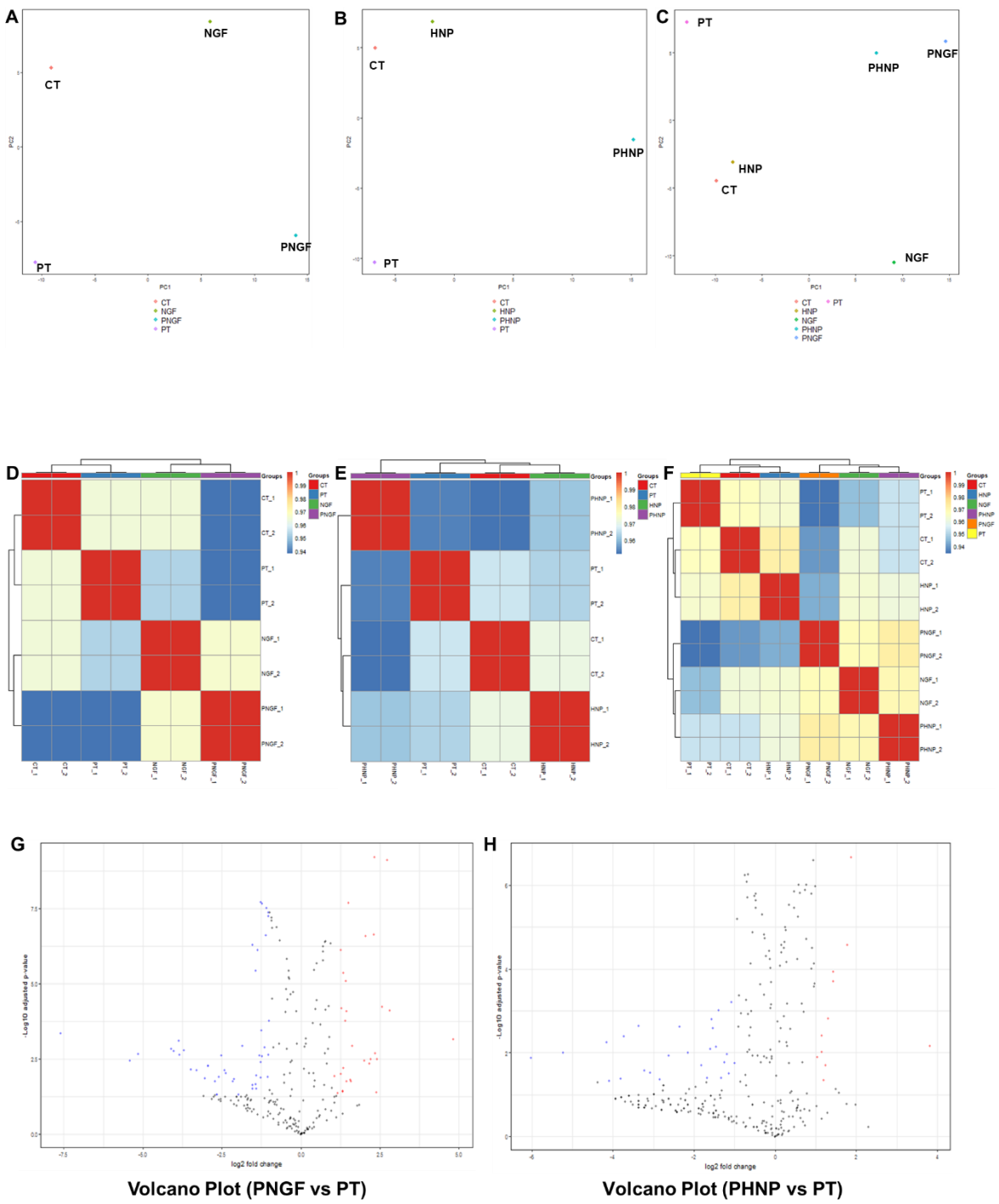

Fig 6.3 Differential expression of miRNAs between different treated groups of *C. elegans*. (A-C) PCA score plot showing the gene expression variability between the groups of *C. elegans* and within the biological replicates. (D-F) Correlation plot showing a correlation between treated groups of *C. elegans*. (G) Volcano plot (p-value v/s log Fc) for PNGF group versus PT group and (H) Volcano plot (p-value v/s log Fc) for PHNP group versus PT group of *C. elegans*. CT: untreated worms, PT: PT treated worms,

PHNP: custom peptide HNP pre-treatment followed by PT treatment, PNGF: mouse 2.5S-NGF pre-treatment followed by PT treatment HNP: custom peptide HNP treated worms, NGF: mouse 2.5S-NGF treated worms.

Table 6.2A. The miRNA sequencing analysis data compares the fold changes in the differential expression of miRNA in the different treatment groups.

miRNAs	**Log FC (CT vs PT)**	**logFC (PNGF vs PT)**	**Function**	**References**
cel-miR-77-5p	-3.17	1.32	Embryogenesis	[1]
cel-miR-4813-3p	-3.05	3.65	Degradation of alpha-synuclein	[2,3]
cel-miR-34-3p	-1.95	1.58	Increasing autophagic flux against oxidative stress	[4]
cel-miR-236-3p	-1.39	2.80	Phosphoinositide 3-kinase (PI3K) pathways for cell growth and development (promote longevity)	[5]
cel-miR-46-3p	-1.09	1.36	Development	[6,7]
cel-miR-786-3p	1.39	-1.16	Fatty acid metabolism	[8]
cel-miR-1820-5p	1.43	-1.54	Innate immune response	[9]
cel-miR-83-3p	1.75	1.35	Loss of miR-83 extends lifespan	[10]
cel-miR-795-3p	2.17	-1.44	Dauer formation	[11]
cel-miR-57-3p	3.23	-2.38		
cel-miR-8207-3p	4.31	-7.62		

Table 6.2B. The miRNA sequencing analysis data compares the fold changes in the differential expression of miRNA in the different treatment groups. For comparison of differential expression of the miRNA, the *C. elegans* were subjected to the following treatments at 20°C: (a) 1X PBS (control) treated *C. elegans* (CT), (b) 10 mM paraquat (PT) treatment for 1 h, (c) pre-treatment with 50 µg/mL custom peptide for 2 h followed by 10 mM paraquat treatment (PHNP) for 1 h.

miRNA	**logFC (CT vs PT)**	**logFC (PHNP vs PT)**	**Function**	**References**
cel-miR-77-5p	-3.17	1.18	Embryogenesis	[1]

cel-miR-62	-3.00	3.82	Regulate stress response and increase the lifespan	[5]
cel-miR-4813-3p	-3.05	1.20	Degradation of alpha-synuclein	[3]
cel-miR-4936	-2.59	1.57	Activation of heat shock factor-1 (HSF-1) to protect the cell from organelle dysfunction	[12]
cel-miR-236-3p	-1.39	1.77	Phosphoinositide 3-kinase (PI3K) pathways for cell growth and development (promote longevity)	[5]
cel-miR-83-3p	1.75	1.03	Loss of miR-83 extends lifespan	[10]
cel-miR-57-3p	3.23	-3.23	Unknown	
cel-miR-8207-3p	4.31	-6.03	Unknown	
cel-miR-354-5p	4.60	-5.24	The transforming growth factor beta (TGF -β) signaling pathway shows a protective response	[13]

The heat map analysis compared the expression profile of miRNA within different treatment groups (Figs 6.4A-B). Further, gene network analysis revealed the involvement of downstream mRNA targets, which modulate multiple classes of proteins (Appendix Table A2). The miRNA and the target mRNA networks for PNGF (Fig 6.5A) and PHNP (Fig 6.5B) groups of *C. elegans* were constructed.

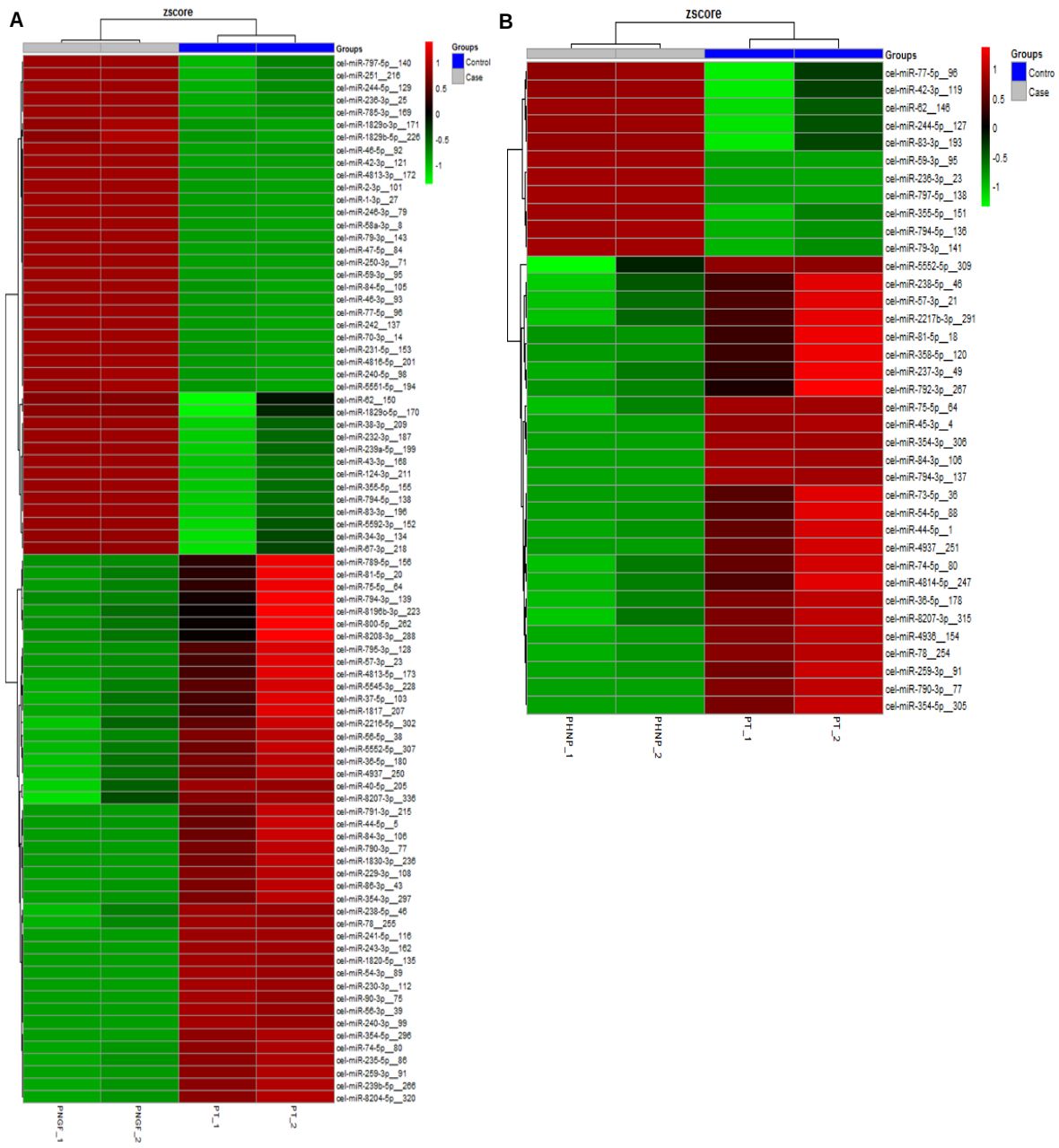

Fig 6.4 Heat map showing the differential expression of the upregulated and downregulated miRNAs among different groups of *C. elegans*. (A) PNGF group versus PT group (B) PHNP group versus PT group of *C. elegans*. CT: untreated worms, PT: PT treated worms, PHNP: custom peptide HNP pre-treatment followed by PT treatment, PNGF: mouse 2.5S-NGF pre-treatment followed by PT treatment HNP: custom peptide HNP treated worms, NGF: mouse 2.5S-NGF treated worms.

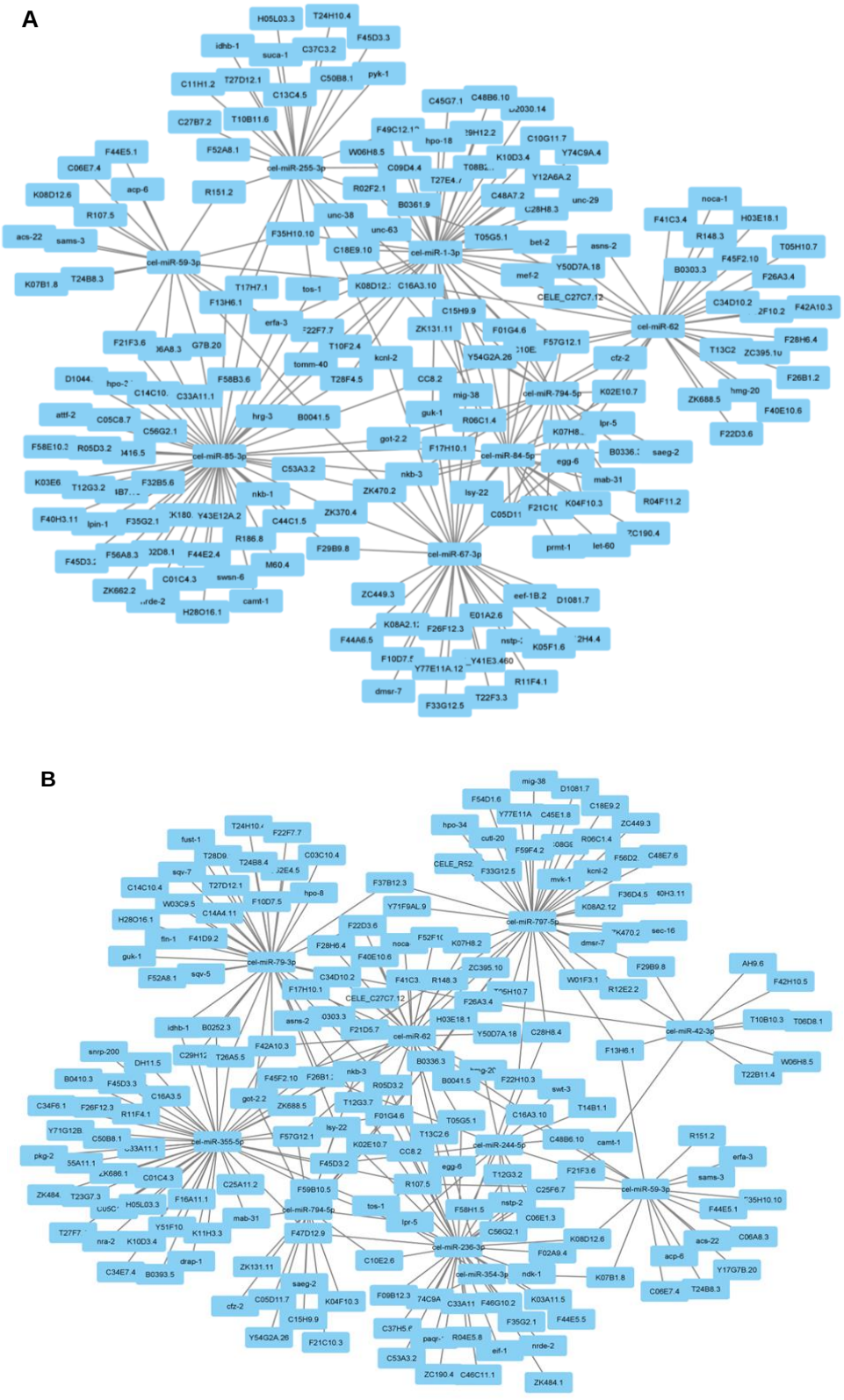

Fig 6.5 Network of miRNA and their target genes for (A) PNGF and (B) PHNP group of *C. elegans*. The interaction network miRNA and their target genes were drawn by using Cytoscape 3.9.1.

6.2. Discussion:

NDs are multifactorial diseases mainly associated with progressive loss of neuronal structure and function and neuronal cell death [14]. Since there is no effective therapy or treatment to prevent the progression of NDs such as AD and PD, which have emerged as a significant problem in neurobiology in recent years, more research efforts are being made to develop innovative neuroprotective therapeutics [14]. The potential role of miRNA in ND has encouraged finding novel therapeutic targets [15,16]. The miRNA was first discovered in *C. elegans*; fundamental research linking miRNA to crucial gene regulatory networks significant to neuronal development has been carried out using this model [13,17,18]. A clinical trial of NGF gene therapy was demonstrated because the function and degeneration of cholinergic neurons in the basal forebrain strongly depend on NGF activity. The therapeutic advantage of NGF administration to the basal forebrain has already been demonstrated [19].

Since neurotrophic factors cannot cross the blood-brain barrier (BBB) and must be administered intracerebrally, their use in treating central nervous system dysfunction is even more complicated. However, small synthetic peptides can be used as an alternative method with several advantages over large protein molecules in treating neuronal disease [20]. Some peptides named MIM-D3 (TrkA agonist) ameliorate corneal injury in rats with dry eye syndrome [21]. Similarly, GK-2 (NGF mimetics) improved neurological disorders such as AD, PD, and brain ischemia with zero side effects [22,23]. Our previous findings demonstrated the neuroprotective potential of synthetic custom peptides (TNP and HNP) in *in vitro* PC-12 cells and *in vivo C. elegans* PT-induced PD models. This study identified and compared the differential miRNA expression profile of mouse 2.5 S-NGF-treated *C. elegans* with the custom peptide HNP-treated *C. elegans*, where some miRNAs were common. The human brain is associated with a high degree of complexity, and working with the human brain requires ethical permission; therefore, *in vivo* model organisms such as *C. elegans* can be used for understanding the mechanism and function of the miRNAs in the neuronal developmental and functional processes [24].

Studies have reported that cel-miR-71, cel-miR-84, and cel-miR-1 are involved in the different stages of neuronal development [24]. In this study, these miRNAs' expression was highly upregulated in both the NGF and HNP groups of *C. elegans*. These results

underlie that the custom peptides have neurorestorative and neurotrophic potential. The potency of the peptides is found to be comparable with the mouse 2.5 S-NGF. The target genes of the cel-miR-1-3p and cel-miR-255-3p and their roles are further investigated due to their robust expression in response to mouse 2.5 S-NGF and custom peptide HNP treatment in *C. elegans*. The target genes of cel-miR-1-3p are mef-2, unc-63, unc-29, and unc-38, involved in the oxidative stress response pathways, mitogen-activated protein kinase (p38/MAPK) pathways, and nicotinic acetylcholine receptor signaling pathways (RefSeq, miRBase, ENA, WormBase). Similarly, the G protein signaling pathway, metabolic pathways (pyruvate metabolism), and integrin signaling pathways are regulated by cel-miR-255-3p target genes (kcnl-2, idhb-1, suca-1, pyk-1, and tos-1) (RefSeq, miRBase, ENA, WormBase). The p38/MAPK pathways play a crucial role in neuronal differentiation and it has been reported from our lab that the mouse 2.5 S-NGF induced these pathways for neuronal differentiation and growth [25-27]. Because the custom peptide in this study was developed from the TrkA binding region of snake venom NGF, it also retains the property of neuronal differentiation and growth [28]. This study shows that similar to mouse 2.5 S-NGF, the HNP also upregulates cel-miR-1-3p miRNA, eventually regulating p38/MAPK pathways for neuronal differentiation in *C. elegans*.

Aggregation of α-synuclein is a hallmark for the progression of PD, also observed in the PT-induced PD model [29]. Removal of aggregation of this protein is a required mechanism for improving PD [30]. Studies have reported that cel-miR-4813-3p regulates protein homeostasis, associated with the clearance of α-synuclein deposition in neuronal cells [2,3]. The neuroprotective potential of HNP has already been demonstrated in our lab [28]. cel-miR-4813-3p is downregulated in the PT-treated group of *C. elegans*, which results in the deposition of α-synuclein and the progression of PD. Further upregulation of the cel-miR-4813-3p in the PHNP and PNGF group of *C. elegans* univocally suggests the peptide's therapeutic role by regulating miRNAs.

The phosphoinositide 3-kinase (PI3K) pathway is reported to promote longevity, is responsible for growth and neuronal development, and is regulated by cel-miR-236-3p [5]. The PT treatment downregulates the expression of this miRNA, which is further upregulated in the PHNP and PNGF group of *C. elegans*, thus confirming the neuroprotective role of HNP via miRNA regulation. Two novel miRNAs, cel-miR-8207-

3p and cel-miR-57-3p, are upregulated with the PT treatment and downregulated with the mouse 2.5 S-NGF and HNP pre-treatment. The function of these two miRNAs is unknown and needs to be explored.

6.3. Conclusion

In the PT group of *C. elegans*, the downregulated miRNAs target genes involved in the developmental process during embryogenesis; genes regulate the longevity of *C. elegans*, α-synuclein degradation, and oxidative stress response. Moreover, upregulated miRNAs target genes in the PT group involved in apoptotic pathways, decrease lifespan, innate immune response, and metabolic pathways are reported to increase the progression of NDs [3,13,31]. Pre-treatment with mouse 2.5 S-NGF and custom peptide HNP restored the expression of altered miRNAs induced by PT treatment and played a crucial role against NDs.

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