9. Summary and Conclusion

9.1 SUMMARY

9.1.1. Association of small ncRNA with mitochondria: A Systematic approach

The nuclear encoded non coding RNA and proteins are associated with human mitochondria. The systematic characterization of association of ncRNA specifically miRNA with the mitochondria of HEK293 and HeLa was performed.

- The mitochondrial RNA was prepared, libraries generated and deep sequencing was performed.
- The sequences from both the libraries were submitted to GEO with accession numbers GSM797669 and GSM797670.
- 10% of the unique sequences corresponding to 95% of unique sequences were common in both mitochondrial libraries.
- The small ncRNA included rRNA, tRNA, snRNA, piRNA, snoRNA, repeat associated RNAs and miRNAs.

9.1.1.1. Association of miRNA with mitochondria:

- 428 and 327 miRNA; 65 and 60 miRNA* were associated with human mitochondria of HEK293 and HeLa respectively.
- 193 and 13 sequences were predicted to be putative novel miRNAs in small RNA libraries of HEK293 and HeLa respectively.
- 37 miRNAs were common amongst the two libraries.
- The association of let-7b, let-7g, miR-107, miR-181b, miR-221 and miR-320a with mitochondria in both cell lines were further validated by qPCR.

9.1.1.2. Association of miRISC components with mitochondria:

The mature miRNA are bound with miRISC component, Ago2.

- Ago2 and Ago3 co-localized with mitochondria as observed by confocal microscopy.

9.1.1.3. Association of mRNAs with mitochondria:

The miRNA bound to Ago2 search for the cognate 3'UTR of target mRNA and fine tunes the protein levels.

Target mRNAs were associated at the outer membrane of mitochondria, suggesting that mitochondrial outer membrane may serve as a platform for the fine tuning of mRNA levels in narrow physiological range.

9.1.2. Association of novel miRNA with mitochondria: miR293m80059

- The association of putative novel miRNA with mitochondria was established in HEK293 and HeLa.
- The mimic of miR-23m80134, miRNA with highest count in HEK293 small RNA library was transfected
- miR-23m80134 had no effect on ATP production, ROS levels or cell viability was observed.

9.1.2.1 miR293m80059 targets mitochondrial genome to regulate its function:

- The seed sequences of 16 novel miRNA aligned to mitochondrial genome.
- miR-293m80059, was having highest counts amongst protein coding genes
- miR-293m80059 was predominantly present in mitochondria.
- miR-293m80059 undergoes canonical biogenesis and processing.

The expression of miR-293m80059:

- Decrease in mitochondrial ATP
- mitochondrial complex-1 assembly activity and ROS levels.
- Increased mature ND2 transcript levels
- Decreased ND2-COX1 processing intermediate

- miR-293m80059 associates with RNA granule component, FASTKD5 within mitochondria

9.1.2.2 miR293m80059 potentiates cell death and inhibits proliferation:

The expression of miR-293m80059 mimic:

- Potentiates cell death
- Decreases proliferation rates
- Cell death is ROS mediated

9.1.3. Mitochondrial DNA aligned miRNA: miR-4485

- The small RNA libraries were aligned to mitochondrial genome to anticipate the impact of identified miRNAs on mitochondrial genome.
- 4 known miRNA sequences aligned at various sites of mitochondrial genome.
- The seed sequences of 13 novel miRNAs aligned at various positions of mitochondrial genome.
- hsa-miR-4485 aligned at 16S rRNA region of mitochondrial genome.

9.1.3.1. hsa-miR-4485 affects mitochondrial genome encoded proteome and mitochondrial functions:

- miR-4485 was found to be enriched in mitochondria while its precursor was processed in cytoplasm and followed canonical miRNA processing.
- Its levels decreased in mitochondrial DNA depletion suggesting implication on mitochondrial DNA.
- Its expression resulted in accumulation of 16S rRNA-ND1 processing intermediate, decrease in 16S rRNA levels, mitochondrial DNA encoded transcripts and proteins.
- Its expression declined energy production, membrane potential and enhanced ROS.

9.1.3.2 hsa-miR-4485 regulates ROS mediated cell death and tumorigenicity:

- The expression of miR-4485 inhibits the ROS mediated cell survival and clonogenic ability.
- It is down regulated in breast cancer tissue.
- The tumors harboring miR-4485 regress in size and volume in mouse xenograft models.

9.1.4. Association of miRNA with mitochondria is stress sensitive: MiR-320a

- Association of Ago2 with mitochondria altered in stress.
- The association of 5 novel miRNAs with high count, 11 novel miRNAs aligned mitochondrial genome and 10 known miRNAs was monitored in oxidative stress, mitochondrial stress, ER stress and upon TNF stimulation.
- The level of association varied in different cellular stress conditions.
- The association of hsa-miR-320a was highest in the presence of TNF.

9.1.4.1. hsa-miR-320a associates at outer surface of mitochondria:

- The association of miR-320a was further characterized by striping of outer membranes and outer membrane associated proteins. The levels of miR-320a were diminished in such preparations, suggesting its association at outer surface.
- miR-320a was found to be associated with Ago2, the core component of miRISC, Ago2 was immuno-precipitated from mitochondrial fractions.

9.1.4.2. hsa-miR-320a regulates NDUFA10 to modulate complex-1 assembly:

- The putative target of miR-320a included NDUFA10, a mitochondrial super complex-1 assembly factor.
- The expression of hsa-miR-320a mimic decreases mitochondrial super complex assembly factor and NDUFA10.
- NDUFA10 mRNA is transport by CLUH at mitochondria, a conserved RNA binding protein involved in transport of nuclear genome transcribed RNA of mitochondrial proteins (nmRNAmp).
- Ago2 mediated down regulation of NDUFA10 occurs at mitochondrial outer surface.

9.1.4.3. hsa-miR-320a regulates ROS mediated cell death and tumorigenicity:

The expression of miR-320a mimic

- inhibits cell survival
- clonogenic ability
- migration potential of the cancer cells.
- It is down regulated in breast cancer tissue.

9.1.5. Pictorial Summary:

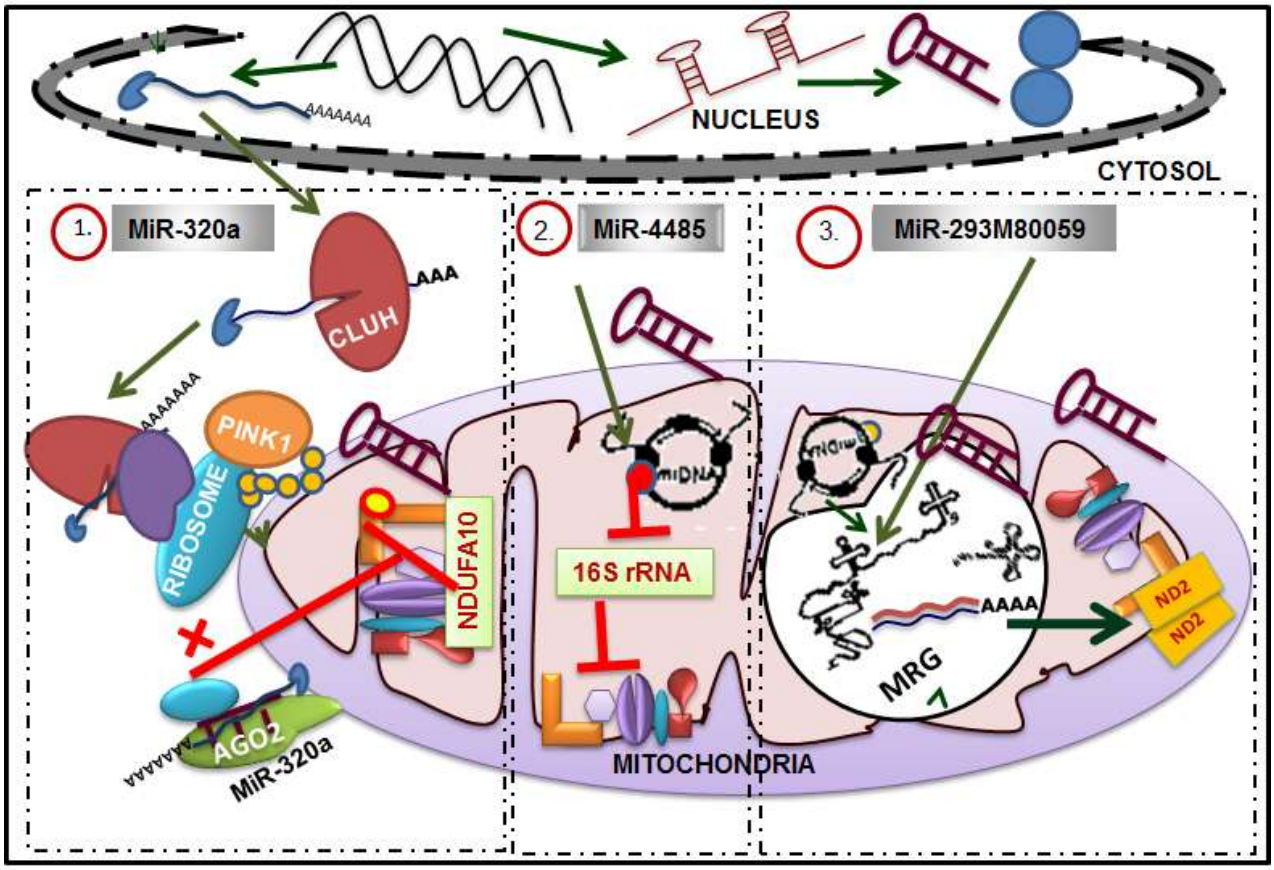

Figure 8.1 Schematic representations of different pathways regulated by three mitochondrial miRNAs among many identified in the study. *miRNA associates with mitochondria and regulates its function. The miRNAs studied are highlighted in grey boxes and the target in green boxes. Their localisation and mode of action are depicted in the cartoon. 1. hsa-miR-320a regulates NDUFA10 bound with CLUH at mitochondrial surface, affecting super-complex-1 assembly. 2. miR-4485 has sequence similarity at 16S rRNA and globally decreases all mtDNA encoded transcripts and proteins. 3. miR-293m80059, a novel miRNA forms a part of mitochondrial RNA granule to enhance ND2 levels and increase complex-1. The three pathways potentiate cell death and decreases tumorigenicity in cancer cell lines.*

9.2 CONCLUSION

miRNAs, the components of RISC and nascent mRNAs (encoded from both nuclear and mitochondrial genomes) are either associated or enriched within the mitochondria dynamically in various stimuli.

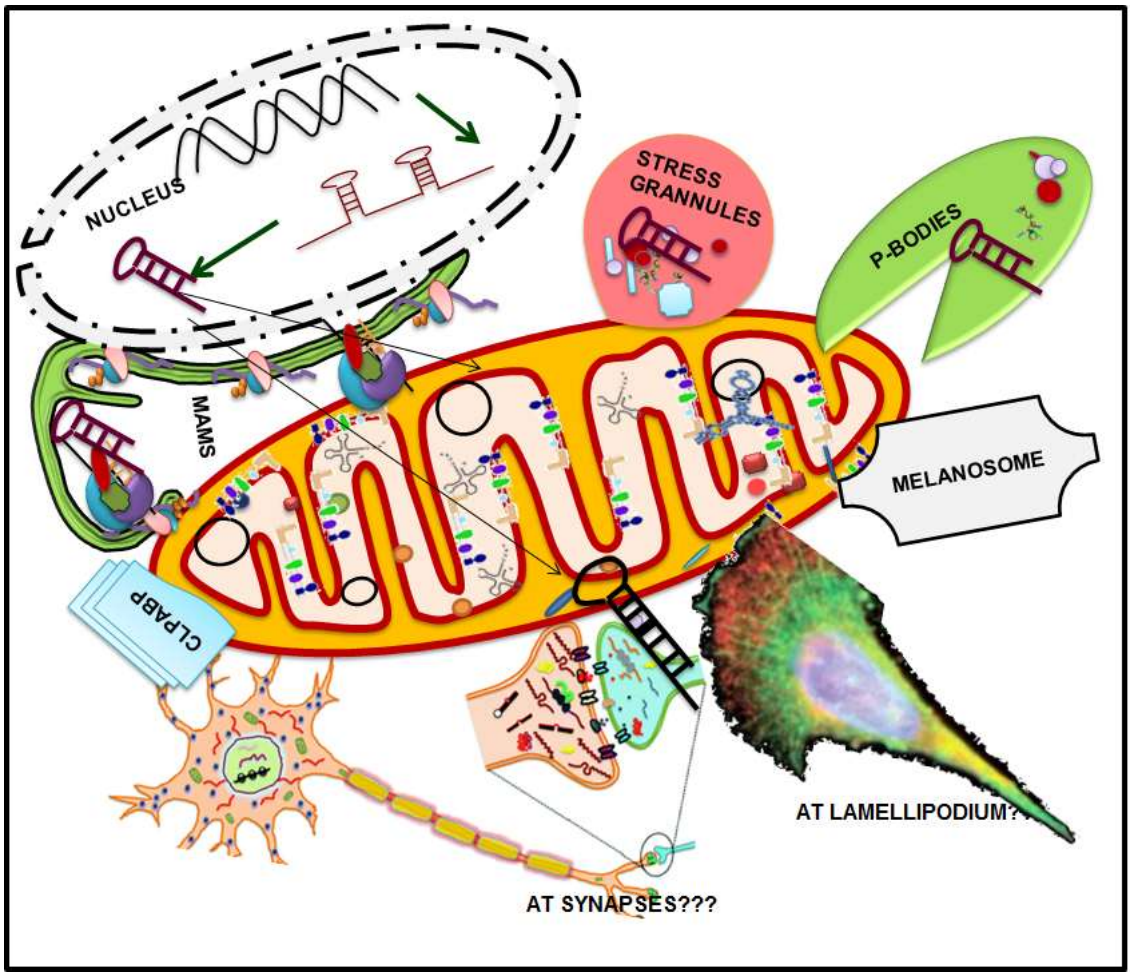

Figure 8.2.Possible mechanisms of mitochondrial associated miRNAs and their role in mitochondrial functions: *The miRNA are synthesized in nucleus, processed in cytoplasm, associates at or are imported inside the mitochondria along with the RNA binding proteins. The cognate target levels are fine tuned in these sites to yield various physiological pathways.*

These miRNAs along with RNA binding proteins, screen cognate mRNA to regulate site specific fine tuning. These novel pathways may have important implications in mitochondrial function, cellular survival and tumorigenicity.

Mitochondria appear to serve as carrier for transport of nascent/ repressed mRNAs. It may emerge as novel platform for site specific fine tuning of protein levels at narrow physiological range to meet the immediate response. The miRNA in turn are associated/enriched in mitochondria in specific conditions to regulate nuclear encoded mitochondrial proteins at mitochondrial surface and mitochondrial genomic message within.

9.3 LIMITATIONS OF THE STUDY

1. All non coding RNAs identified in sequencing could not be addressed
2. All the pathways could not be validated in animals.
3. The dynamic process of miRNA, nascent mRNA and associated RNA binding protein should have been studied with better resolution techniques.
4. The site specific local gene regulation at mitochondria in critical cellular sites like axonal branches, synapses and migrating cancer cells couldn't be addressed.
5. The mitochondrial genome derived miRNAs should be further investigated.

9.4 FUTURE PROSPECTIVES

The study in this direction will definitely decipher the role of mitochondria associated miRNAs in the inter organellar cross talk and implications in various pathophysiological conditions.

1. Impact and dynamics of association of piRNA and other ncRNAs with mitochondria of cancerous cells will help to understand many unknown mechanisms.
2. Dynamics of association of miRISC components in cellular stress and stimuli will further help to develop the RNA silencing mechanisms.
3. Role of miRNA association with mitochondria (mitomiRs) in pathological conditions and their impact can be extrapolated to design biomarkers.
4. mitomiRs in body fluids and their impact in exosomal pinch offs and cellular communications: a systematic impact can be elucidated.
5. mitomiRs in regulation of metabolic pathways and cellular survival.
6. The impact of mitomiRs in physiologically crucial sites can be elucidated.