Summary

1. **FMRpolyG formed via RAN translation of CGG repeats at 5'-UTR of *FMR1* gene is pathogenic inFXTAS.**
    - Expanded CGG repeats driven by ATG(canonical) and ACG(non-canonical) codon were expressed in different cell lines and transgenic mice model.
    - Expression of expanded CGG repeats in different cell lines altered cellular metabolism as observed by MTT/XTT staining and LDH assay.
    - Increased activated caspases levels and PARP cleavage suggest initiation of apoptotic cell death due to expression of FMRpolyG in FXTAS condition.

2. **FMRpolyG protein forms small cytosolic aggregates that dynamically interacts with mitochondria and alters mitochondrial functions.**
    - Apart from large nuclear aggregates, FMRpolyG also tends to form small cytosolic aggregates.
    - Confocal microscopy and subsequent immunoblotting results confirmed the interaction of cytosolic FMRpolyG aggregates with mitochondria in cells and transgenic mice model.
    - Expression of premutation allele showed altered mitochondrial respiratory chain assembly and activity as evident by Blue Native PAGE and ingel activity assay.

3. **Expression of expanded CGG repeats affects miRNA expression profile in FXTAS.**
    - Sequestration of DROSHA/DGCR8 by FMRpolyG leads to altered miRNA expression in HEK293 cells transfected with premutation plasmids.
    - Small RNA isolation from mitochondrial fraction followed by NGS analysis confirmed the altered trafficking of miRNA to mitochondria upon expression of expanded CGG repeats.
    - Interestingly, a specific population of miRNAs is enriched in mitochondrial fraction in CGG repeats expressing cells.
    - List of candidate mito-miRs was prepared on the basis of bioinformatics tool to predict their putative mRNA targets.

- The targets of candidate miRNAs were annotated and clustered into meaningful groups by using DAVID platform.

4. **CGG repeats expressing cell showed enhanced translocation miR-320a and Ago2 to mitochondria.**
   - We validated mitochondrial enrichment of candidate mito-miRs in FXTAS condition by qPCR.
   - One of the mito-miR, miR-320a having targets related to mitochondria and apoptotic pathway was further characterized for its functional implication in FXTAS.
   - miR-320a localizes in mitoplast and its level gets enhanced in mitoplast under expression of CGG repeats.
   - miR-320a has putative seeding sequence for mitochondrial DNA encoded transcripts which suggests its role in regulation of mitochondrial translation.

5. **Exogenous expression of miR-320a improves mitochondrial function and has cytoprotective effect in FXTAS condition.**
   - miR-320a along with Ago2 is highly enriched in mitoplast; however, it fails to form functional RISC like complex as other subunits of RISC complex is not present in mitochondria.
   - This suggests probability of other mechanism to regulate mitochondrial transcripts in FXTAS.
   - Transfection of mimic of miR-320a rescues mitochondrial transcript levels and enhances complex IV activity.
   - Exogenous miR-320a decreases the cellular ROS levels and cellular cytotoxicity caused due to expression of CGG repeats.

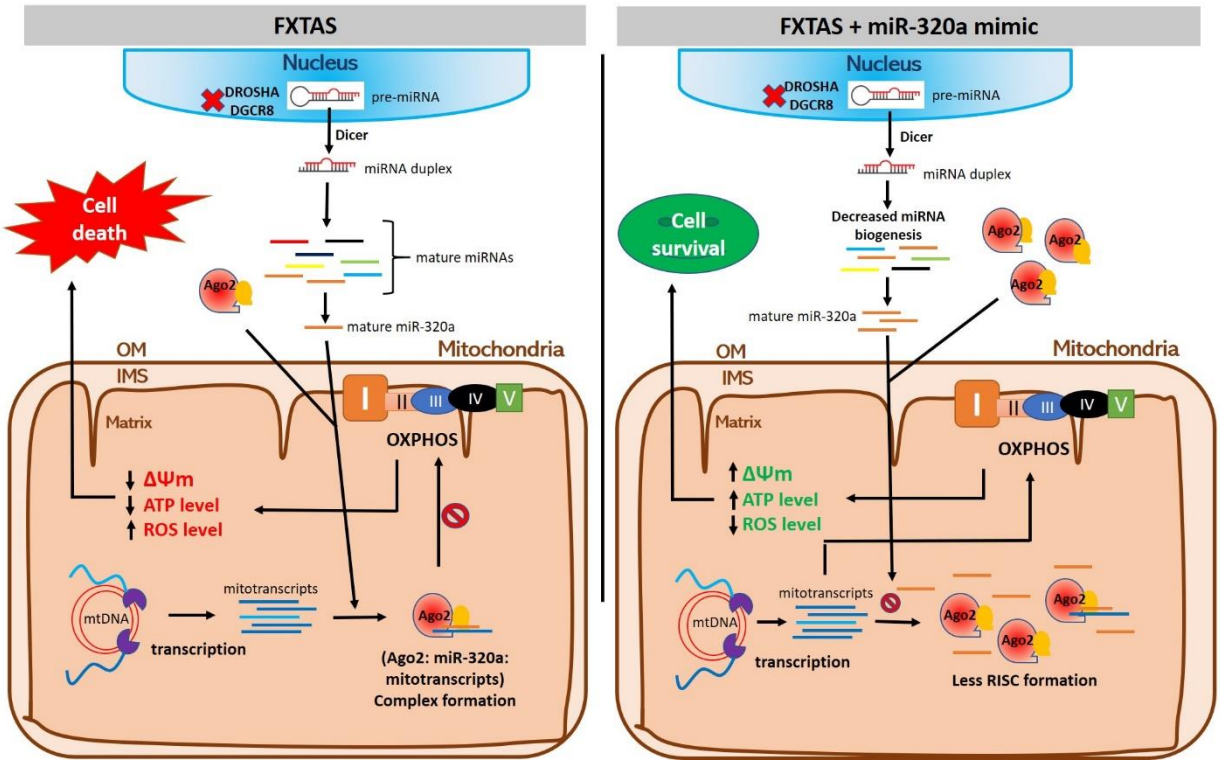

Figure: schematic representation of role of mito-miR, miR-320a in FXTAS condition.

Conclusion

The major problems associated with premutation condition is presence of FMRpolyG and less availability of the functional FMRP. FMRP has crucial role in regulation of translation of mRNA via interacting with Ago2 and Dicer[1]. The experiments here suggest the involvement of probably two complimentary mechanisms. Firstly, RNA gain of function due to expanded CGG repeat in FMR sequestration of DROSHA/DGCR8 in FXTAS condition[2]–[6] which decreases the miRNA level in the cell. The decreased level of miRNA may also affect other sub organellar functions like mitochondria, which is key organelle in several neurodegenerative conditions. The reports from our lab also suggest that miRNAs can localize to various subcellular organelles, specifically it can associate with mitochondria and affect mitochondrial functions [7]–[9]. Thus, dysregulation in miRNA biogenesis as well as sequestration of various RNA binding proteins cumulatively affect localization of miRNA on mitochondria in FXTAS.

Secondly, FMRpolyG produced due to RAN translation forms smaller cytosolic aggregates before forming predominant nuclear ubiquitin positive inclusion. In this study, for the first time we have shown that these smaller aggregates can interact with mitochondria and may dysregulate mitochondrial functions. We have also explained mitochondrial dysfunctions associated with premutation pathology in terms of altered mitochondrial super complexes assembly and defect in activity of individual complexes in cells and transgenic mice model of FXTAS. As mentioned earlier the sequestration of miRNA biogenesis protein may alters the miRNA expression profile and their translocation to mitochondria. To prove this hypothesis, we combined novel approaches of mitochondrial fractionation followed by mitochondrial small RNA sequencing using Next Generation Sequencing to systematically investigate the translocation of miRNAs to mitochondria in HEK293 cells mimicking premutation condition. Interestingly, we identified specific population of miRNAs which is highly enriched in mitochondrial fraction upon transfection of expanded CGG repeats. Among such candidate mito-miRNAs, miR-320a was selected for its functional validation. Strikingly, increased miR-320a levels in mitoplast showed enhanced mitochondrial functions and rescue in cell death in FXTAS condition. ***In conclusion, we have systematically addressed, and analysed the array of mitochondrial dysfunctions involved in FXTAS pathology. We also identified and characterized the functional role of mito-miR, miR-320a in regulation of mitochondrial functions and cell death. This study strongly suggests the complimentary role of RNA gain of function and FMRpolyG mediated toxicity to explain mitochondrial dysfunctions associated with premutation pathology.***

Future perspectives

The control of gene expression in neurons at distant sites is essential for neuronal connectivity and other functions. Recent evidences suggest that subcellular localization of miRNAs helps in maintaining correct transcript levels at different cellular locations[10]–[13] Interestingly, mitochondrial function is important for the local neuronal branching, synaptic plasticity and other functions. We and others have previously reported that miRNAs can translocate to mitochondria under certain stimulus and cell type specific manner. However, the patho-physiological significance of miRNAs translocation into mitochondria in neuronal cells, and its role in modulation of mitochondrial functions in FXTAS and other neurodegenerative disorders is not known. Hence, the understanding of this aspect will be a key to design novel miRNA

based biomarkers and therapeutic approaches. Further, this study identified the altered association miRNAs with mitochondria in FXTAS. As, mitochondrial associated miRNAs play crucial role in modulating mitochondrial functions by regulating its transcription and surface translation, identification of candidate miRNAs and their functional role in FXTAS may lead to another important clue to understand its neuro pathology. It is known that expression profile of miRNAs is disease specific. Hence, the unique pattern of miRNA translocation to mitochondria may also serve as novel prognosis marker for the disease progression.

There is currently no treatment for FXTAS. Our results along with the earlier reports have confirmed mitochondrial dysfunction as key pathology involved in FXTAS. Enhancement of mitochondrial functions by using antioxidants and drugs which specifically targets mitochondria may have broad implications in terms of stopping or delay in disease progression. The advancement of this study will lead to the development of miRNA-based therapeutics. Adding on to this, recent developments in antisense oligonucleotide chemistry render them suitable for therapy of human pathologies through blockage (fully modified RNA oligonucleotides) or RNase H driven degradation (gapmer oligonucleotides constituted of a core DNA bordered by modified RNA) of the targeted pathogenic mRNA[14]. This can be very important in case of elimination of pathogenic mRNAs and protecting the pool of RBPs from getting sequestered. Finally, the study here implies that miRNAs translocation to mitochondria can rescue some of the mitochondrial deficits and can be emerging therapeutic modalities in FXTAS and related neurodegenerative disorders.

Limitations of the study

1. Pathogenicity induced by expanded CGG repeats was validated in cell lines; however, its validation in mice model and patient derived neurons or fibroblasts will further strengthen the hypothesis.
2. The results of decreased mito-transcripts levels in FXTAS patients could have been repeated in large cohort to generalized it as specific pathology associated with premutation condition.
3. The question of how dynamic interaction of cytosolic FMRpolyG aggregates with mitochondria lead to mitochondrial dysfunctions remained unanswered.

4. Effect of FMRpolyG on nuclear-mitochondrial crosstalk has not been addressed. This can be an important clue to understand transport defects of nuclear encoded mitochondrial proteins to mitochondria.
5. The dynamic process of miR-320a, mitotranscripts and associated RISC components should have been studied with better resolution techniques.
6. Role of miR-320a should also be validated in iPSCs derived neuron from FXTAS patient.