Journal of Cell Science

Expression analysis and function of mitochondrial genome-encoded microRNAs

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DOI: 10.1242/jcs.258937

Editor: Michael Way

Review timeline
Original submission: 21 May 2021
Editorial decision: 6 July 2021
First revision received: 24 December 2021
Editorial decision: 24 January 2022
Second revision received: 11 March 2022
Accepted: 11 March 2022

Original submission

First decision letter

MS ID#: JOCES/2021/258937

MS TITLE: Expression analysis and function of mitochondrial genome encoded microRNAs

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ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.organd click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, your MS received very mixed reviews. In particular, the reviewers raise a number of substantial criticisms concerning the text and presentation of the data, which they feel lacks many controls and does not fully support your conclusions. Nevertheless, they do feel the study is potentially interesting and that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all text changes made in the revised manuscript in a different colour. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In the paper entitled "Expression analysis and function of 1 mitochondrial genome encoded microRNAs", the authors report differential expression of several mitochondria-encoded miRNAs in breast cancer lines from different origin (luminal Vs TNBC). Considering that this is an hot topic, if better explained/reported and supported, this piece of work would be very interesting for the cancer and mitochondrial community.

Comments for the author

-The writing of the manuscript is very superficial. In the introduction no effort have been made to explain how different breast cancer subtypes may be linked to different metabolic and mitochondrial profiles. The references are also incomplete, and old (I don't want to undermine the Work of Warburg, but....).
Further readings about ncRNA and mitochondria that can help shaping the intro and the discussion can be found here:
doi: 10.15252/embj.201695546 doi: 10.1038/s41594-018-0143-4 doi: 10.1038/nature17161 The results are also lacking a proper description. In particular, it is not clear why specific experiments are performed and how they have been performed (see also legends; for example Fig.1c what is the scale here??).
-As for the first figure is not clear whether these miRNA have been reported before, wether the authors have overlapping results with previously published studies or not.
-Linked to the above comment, in the discussion the authors announce that they intend to enlarge their cohort. However, probably these data can be also mined from publicly available datasets, with minor efforts.
-In the AGO IPs both the target mRNA and the miRNA should be measured. Ago IP should be performed upon KD and mitomir transfection to prove a direct effect of mitomiR-5

Reviewer 2

Advance summary and potential significance to field

In their manuscript Kuthethur et al. have shown the importance of mitochondria encoded microRNAs in mammalian breast cancer cells. This is an interesting manuscript that have explored some newly identified miRNAs for their role in mitochondrial metabolism. However, the data to support the conclusion is not strong enough to warrant its publication in the current form in JCS. There are several limitations that I can see and these are the suggestions that may make the revised manuscript a better fit for the journal.

Comments for the author

Comments to Authors:
1. To confirmation of the length and abundance in specific cellular compartment for these identified miRNAs must be validated by Northern blot data. Without that It is not convincing for the reviewer to accept their existence as canonical miRNAs.

2. In Fig2 the authors have tried the expression of the mitomiRs in different cell types and in different grade tumors. I wonder what happens with other non-mitoMiRs in this context and what about this mito miRs expression in non-breast cancer cells?
3. The conclusion on the sensitivity of these mitomiRs to agents that have detrimental effect on mitochondrial function is interesting. However it could be due to mitochondrial degeneration and the effect should be measured on mitochondrial enriched nuclear encoded miRs to conclude on the specificity of the effect observed.
4. In Fig4, the authors have tried the Ago2 IP from different cellular fractions to conclude on Ago2 association of these mitomiRs in the mitochondrial enriched fraction. There is a little amount of Ago2 in mitochondrial fraction and that too could be contributed by ER contamination of the mitochondrial fraction. Proper marker of ER should be used to rule it out that the Ago2 in mitochondrial fraction is not coming from the associated ER fraction.
5. The authors claim that the mitomiR-5-5p regulate mitochondrial DNA replication but rather than showing the mechanism of its action they went on to explore the regulation of mitochondrial encoded genes by mito-miRs! This rather defocused the story. I suggest the authors to explore mechanistically why and how the miR-miR-5p regulate the mitochondrial DNA replication in the revised manuscript.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Kuthethur et al reports the finding of 13 novel mtDNA-encoded miRNAs (mito-miRs) through initial bioinformatic analysis. The findings are interesting, adding to the previously described mitochondrial-encoded miRNAs.
However, several minor and major issues must be met.

Comments for the author

Minor observations:
1. English must be extensively reviewed, I recommend the help of a professional editing service or a native speaker. For example, the title should corrected to:
"Expression analysis and function of mitochondrial genome-encoded microRNAs
(hyphenated).
2. For better clarity, the scheme in Fig 1B and/or Table 2B should specify if each of the 13 novel pre-mito-miRs is transcribed from the light or heavy chain. Or whether they are sense or antisense regarding the heavy chain, for example. Also it would be interesting to mention if any of these novel mito-miRs overlap with any of the previously described mtDNA-encoded mito-miRs, such as miR-4485, miR-4484, miR-4484, miR-1973, etc.
3. Line 248-249 states that "MitomiR expression profile was significantly high in triple negative breast cancer cell lines", which is true for MDA-MB-468, but the levels in MDA-MB-231, the other TNBC line, are lower than MCF7 and non-tumor MCF10A cells. Please comment on this.
4. Line 251-252 states "qRT-PCR based expression analysis in matched normal and breast tumor tissue specimens showed mitomiR-5 is overexpressed in tumor tissue mitochondria". Is this data actually from tissue isolated mitochondria? Or from total tissue RNA? The legend does not mention mitochondria-isolated RNA for this analysis.
5. Lines 268-269: Why does knockdown of the mitochondrial transcription factor decrease mtDNA copy number (and to a higher extent than TFAM decrease) and mitochondrial mass? Please discuss.
6. Fig. 5 and Fig. 5 legend. For clarity, please maintain the same name for PPARGC1a.
7. There is no mention of Fig. S4 in the text.
8. Bioinformatic analysis cannot unequivocally identify miR targets, therefore some sentences should be rephrased: Line 290: instead of "...were identified as direct targets...", should say
"..were predicted to be direct targets..." or
"...were identified as putative direct targets...". Same for Fig. S4 legend,
lines 294-295, line 315, and other parts in the text where it should apply.
9. Fig. S2, referenced in lines 291-292: only mitomiR-5-3p shows a conserved seed region among species, regarding canonical 5' seed. mitomiR-5-5p shows no conservation on the 5' side of the sequence, only on the 3' region. Is it possible that this miR has a 3' seed? Please discuss. And, in this context, why did the authors decide to study the mitomiR without the conserved 5' seed?

10. Fig. S3: Please confirm that the miRNA inhibitors are RNA and not DNA oligos the methods section is not clear on this.
11. Fig. S6: please maintain the order of the cell lines for clarity.
12. Lines 298-299 only mentions higher levels of PPARGC1a in MCF10A, but doesn't mention any other result shown in the graph. Authors should mention the fact that mitomiR-5-5p and PPARGC1a expresion seem to be inverse in the different cell lines.
13. In Fig. 5 A, C, D and E, the - sign seems to suggest an abscence of any additions, the graphs should specifiy that these are control mimics and control inhibitors. Same for Fig. 6.
14. Fig. 6C and 6E: Luciferase assays are not mentioned in the main text. Also the figure legend says something completely different, mentioning mito-miR-5-3p which is nowhere to be seen in the figure

Major observations:
1. Fig. 4: authors do not mention whether mitoplasts were externally treated with RNase A before lysis. Therefore, it is important to present the determination of purity of cytosolic ad mitochondrial fractions by RT-qPCR instead of (or in addition to) Western blot.
2. Fig. 4C: How could the authors conclude that the bands seen in lanes 2 and 4 correspond to mito-miR-5-5p? This could be determined by Northern blot but what is shown is a simple agarose gel electrophoresis.
3. Fig. S3 should be repeated, it lacks the necessary controls (control mimic and control inhibitor). Also, the graph shows no decrease with the inhibitor.
4. Fig. 5: In order to corroborate unequivocally a direct miRNA binding to the 3'UTR, the same experiments must be performed with 3'UTR constructs containing mutated putative miRNA-binding sites (mutated seeds).
5. Lines 318-319: this conclusion is not fundamented in any way, since there is no evidence showing that the synthetic transfected mimics or inhibitors actually reached the inside of mitochondria. If this is what they are trying to show, there should be an assay proving incorporation of the mimics/inhibitors into mitochondria.

First revision

Author response to reviewers' comments

Response to reviewers' comments
Reviewer 1 Advance Summary and Potential Significance to Field:
In the paper entitled "Expression analysis and function of 1 mitochondrial genome encoded microRNAs", the authors report differential expression of several mitochondria-encoded miRNAs in breast cancer lines from different origin (luminal Vs TNBC). Considering that this is a hot topic, if better explained/reported and supported, this piece of work would be very interesting for the cancer and mitochondrial community.

Response: We sincerely thank the reviewer for recognizing the novelty and importance of our work. We have now performed additional experiments to substantiate our previous data to show the mitochondrial genome encoded miRNAs (mitomiRs) function in regulation of mitochondrial DNA content in breast cancer cells. The new results and discussion have been updated and added to the revised manuscript.

Reviewer 1 Comments for the Author:

The writing of the manuscript is very superficial. In the introduction no effort have been made to explain how different breast cancer subtypes may be linked to different metabolic and mitochondrial profiles. The references are also incomplete, and old (I don't want to undermine the Work of Warburg, but....). Further readings about ncRNA and mitochondria that can help shaping the intro and the discussion can be found here: doi: 10.15252/embj.201695546, doi: 10.1038/s41594-018-0143-4, doi: 10.1038/nature17161

Response: We thank you for your constructive suggestion to improve the introduction section of our manuscript. We have re-written the introduction section of the manuscript accordingly and have incorporated necessary references highlighting the contribution to the mitochondrial metabolism in different breast cancer subtypes in the revised manuscript.

The results are also lacking a proper description. In particular, it is not clear why specific experiments are performed and how they have been performed (see also legends; for example, Fig.1c what is the scale here??).
-As for the first figure is not clear whether these miRNA have been reported before, whether the authors have overlapping results with previously published studies or not.

Response: We thank you for the suggestion. We have included all the experimental details in the methods and supplementary methods in the revised manuscript. All the figures have been updated with more details provided in the figure legends included Fig. 1c in the revised manuscript. We have analyzed all the mitomiRs with previously published mitochondria associated noncoding RNAs. We have found that pre-mitomiR-4 shows partial sequence similarity with precursor sequence of miR-4485 and miR-1973 (Fig. S9). None of the other mitomiRs showed sequence similarity with nuclear encoded miRNAs or mitochondrial noncoding RNAs previously reported to be present inside mitochondria. We have included this in the discussion section of the revised manuscript.

-Linked to the above comment, in the discussion the authors announce that they intend to enlarge their cohort. However, probably these data can be also mined from publicly available datasets, with minor efforts.

Response: We thank you for your suggestion. We have performed expression analysis of our mitomiRs in publicly available datasets (methods section, page number: 6, line number: 162-171) and included them in the revised manuscript in the supplementary data section (Fig. S3).

-In the AGO IPs both the target mRNA and the miRNA should be measured. Ago IP should be performed upon KD and mitomiR transfection to prove a direct effect of mitomiR-5

Response: We thank you for your suggestion. We have now performed the Ago2-IP in 1) cytoplasmic and mitochondrial fraction of breast cancer cells, 2) whole cell lysate from mitochondria targeted exogenously expressed Ago2 breast cancer cells and 3) TFAM-KD breast cancer cells (Fig. 4). We have also measured target gene abundance through qRT-PCR in both cytoplasmic and mitochondrial RISC complex by analyzing their expression from respective Ago2-IP elutes (Fig. 4). This has been included in the revised manuscript.

Reviewer 2 Advance Summary and Potential Significance to Field:
In their manuscript Kuthethur et al. have shown the importance of mitochondria encoded microRNAs in mammalian breast cancer cells. This is an interesting manuscript that have explored some newly identified miRNAs for their role in mitochondrial metabolism. However, the data to support the conclusion is not strong enough to warrant its publication in the current form in JCS. There are several limitations that I can see and these are the suggestions that may make the revised manuscript a better fit for the journal.

Response: We sincerely thank you for your critical comments and suggestions to improve our manuscript.

Reviewer 2 Comments for the Author:

Comments to Authors:

1. To confirmation of the length and abundance in specific cellular compartment for these identified miRNAs must be validated by Northern blot data. Without that It is not convincing for the reviewer to accept their existence as canonical miRNAs.

Response: We sincerely thank the esteemed reviewer for the critical comment on the manuscript to improve the data and support the MitomiRs as canonical miRNAs inside mitochondria. We have designed custom miRNA TaqMan assays (ThermoFisher Scientific) for mitomiR-5-5p and mitomiR-5-

3p. They are generated by stem-loop reverse transcription from the RNA followed by PCR amplification, providing PCR amplicon of approximately 65bp (Chen, 2005), to ensure the specificity of quantified microRNA.

References:

Chen, C. (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.* **33**, e179–e179.

We have also performed immunofluorescence using mitomiR-5-5p mimic tagged with 5´Biotin and co-stained with MitoTracker Red to confirm their localization inside mitochondria. This confirmed the mitomiRs localization inside mitochondria. This data has been included in the revised manuscript (Fig. 4A).

2. In Fig2 the authors have tried the expression of the mitomiRs in different cell types and in different grade tumors. I wonder what happens with other non-mitoMiRs in this context and what about this mito miRs expression in non-breast cancer cells?

Response: We thank the reviewer for their excellent suggestion. We have performed mitomiR-5-5p and mitomiR-5-3p expression analysis in several non-breast cancer cell lines as per reviewer's suggestions namely, cervical cancer cell lines (SiHa, HeLa, Caski), prostate cancer cell line (PC3), hepatocellular cancer (HepG2), lung cancer cell line (A549), neuroblastoma cells (IMR-32 and SHSY-5Y), leukemia (THP1) and non-cancerous cells such as HEK293, primary cells such as PBMC cells, cultured fibroblast cells. The data has been included in the revised manuscript (Fig. S2).

3. The conclusion on the sensitivity of these mitomiRs to agents that have detrimental effect on mitochondrial function is interesting. However, it could be due to mitochondrial degeneration and the effect should be measured on mitochondrial enriched nuclear encoded miRs to conclude on the specificity of the effect observed.

Response: We sincerely thank you for your suggestion. We have performed mitomiR-5 expression analysis in MCF-7 cells treated with mitochondrial ETC inhibitors Antimycin A and mitochondrial translation inhibitor Tetracycline, along with nuclear miR-146a as positive control. All the inhibitor concentrations were selected from published articles for sub-lethal dose to IC50 concentration to observe the gradual change. Interestingly, mitomiR-5 expression was downregulated when breast cancer cells were treated with known mitochondrial respiratory chain inhibitor antimycin A (complex III). This could be due to perturbed mtDNA replication and transcription as antimycin A is known inducer of mitochondrial ROS and mtDNA damage. Tetracycline is a well-known translation inhibitor of mitochondria, induces proteotoxic stress and mito-nuclear protein imbalance in mitochondria. Sub-lethal doses of tetracycline reduced mitomiR-5 levels suggesting the possibility of the nuclear-encoded mitochondrial protein function in the biogenesis of mitomiRs. We have included this data in the revised manuscript (Fig. 3K-L).

4. In Fig4, the authors have tried the Ago2 IP from different cellular fractions to conclude on Ago2 association of these mitomiRs in the mitochondrial enriched fraction. There is a little amount of Ago2 in mitochondrial fraction and that too could be contributed by ER contamination of the mitochondrial fraction. Proper marker of ER should be used to rule it out that the Ago2 in mitochondrial fraction is not coming from the associated ER fraction.

Response: We thank the reviewer for their excellent suggestion. We have generated MCF7 cells stably expressing Ago2, targeted to mitochondria using pCMV/myc/mito (Addgene #71542) plasmid that carries N-terminal mitochondria targeting signal (MTS) as well as C-terminal myc-tag. We have performed IP in MCF7 cells expressing mitochondrial Ago2, and empty vector group using anti-myc tag antibody to ensure the specificity of mitochondrial miRNAs association with Ago2 in the mitochondria. We have included this data in result section and updated figures in the revised manuscript (Fig. 4).

5. The authors claim that the mitomiR-5-5p regulate mitochondrial DNA replication but rather than showing the mechanism of its action they went on to explore the regulation of mitochondrial

encoded genes by mito-miRs! This rather defocused the story. I suggest the authors to explore mechanistically why and how the miR-miR-5p regulate the mitochondrial DNA replication in the revised manuscript.

Response: We thank the reviewer for their excellent suggestion. We have shown that mitomiR-5 target *PPARGC1a* and downregulate *PPARGC1a* expression in breast cancer cells (Fig. 6A). Immunofluorescence analysis of mitomiR-5 mimic transfected cells showed reduced mtDNA content while mitomiR-5 inhibitor treated cells showed increased mtDNA content (Fig. 6E). We have generated mitomiR-5 sponge expression cells which blocks the binding of mitomiR-5 with target mRNAs (Fig. 6F-I). MitomiR-5 sponge transfected cells showed increased expression of *PPARGC1a* (Fig. 6J) and its downstream target gene *TFAM* (Fig. 6K). *PPARGC1a* regulates mitochondrial biogenesis and mtDNA transcription by increasing the expression of *TFAM*. We observed that along with *PPARGC1a* and *TFAM* overexpression, there is an increase in mtDNA copy number in mitomiR-5 sponge transfected cells when compared with sponge control cells (Fig. 6L). We have added this result in the revised manuscript.

Reviewer 3 Advance Summary and Potential Significance to Field:

The manuscript by Kuthethur et al reports the finding of 13 novel mtDNA-encoded miRNAs (mito-miRs) through initial bioinformatic analysis. The findings are interesting, adding to the previously described mitochondrial-encoded miRNAs.
However, several minor and major issues must be met.

Response: We sincerely thank you for your critical comments and suggestion to improve our manuscript.

Reviewer 3 Comments for the Author:

Minor observations:

1. English must be extensively reviewed, I recommend the help of a professional editing service or a native speaker. For example, the title should corrected to:
"Expression analysis and function of mitochondrial genome-encoded microRNAs (hyphenated).

Response: We thank the esteemed reviewer for their comments on improving the manuscript writing. We have made extensive corrections throughout the manuscript for possible grammatical errors including the changes to the title. We have rewritten the entire introduction, added new results and discussion and updated the supplementary files in the revised manuscript.

2. For better clarity, the scheme in Fig 1B and/or Table 2B should specify if each of the 13 novel pre-mito-miRs is transcribed from the light or heavy chain. Or whether they are sense or antisense regarding the heavy chain, for example. Also, it would be interesting to mention if any of these novel mito-miRs overlap with any of the previously described mtDNA-encoded mito-miRs, such as miR-4485, miR-4484, miR-4484, miR-1973, etc.

Response: We thank the esteemed reviewer for the suggestion. As per our observation through small RNA sequencing data analysis, majority of the reads were aligned to heavy chain sense strand (Fig. 1E). We observed that H strand specific reads are primarily coming from sense strand whereas L strand specific reads are coming from antisense strand (Fig. 1E). Analysis of our predicted MitomiRs with the previously published non-coding RNAs from mitochondria showed sequence similarity between pre-mitomiR-4 with miR-4485 and miR-1973. This could be possible as miR-4485 and miR-1973 are originated from nuclear mitochondrial pseudogene *MTRNR2L8* (Fig. S9). This has been included in the result and discussion section of our revised manuscript.

3. Line 248-249 states that "MitomiR expression profile was significantly high in triple negative breast cancer cell lines", which is true for MDA-MB-468, but the levels in MDA-MB-231, the other TNBC line, are lower than MCF7 and non-tumor MCF10A cells. Please comment on this.

Response: We thank the reviewer for the important suggestion. We have now rewritten this paragraph as “Among the mitomiRs, mitomiR-5 was significantly upregulated in MDA-MB-468 cell

lines when compared with MDA-MB-231 cells. Differential expression of mitomiRs among TNBC cell lines suggests their association with mitochondrial and metabolic requirements in breast cancer where MDA-MB-231 cell line harbors high glycolytic and low OXPHOS metabolic properties compared to its counterpart" in the results section of the revised manuscript.

4. Line 251-252 states "qRT-PCR based expression analysis in matched normal and breast tumor tissue specimens showed mitomiR-5 is overexpressed in tumor tissue mitochondria". Is this data actually from tissue isolated mitochondria? Or from total tissue RNA? The legend does not mention mitochondria-isolated RNA for this analysis.

Response: We deeply regret for the typographical error occurred. We have modified the statement to "tumor tissue specimen", as the expression analysis was performed using total RNA isolated from normal and tumor tissue specimen, not from mitochondrial RNA (Fig. 2). We have updated the results section along with figure legends in the revised manuscript.

5. Lines 268-269: Why does knockdown of the mitochondrial transcription factor decrease mtDNA copy number (and to a higher extent than TFAM decrease) and mitochondrial mass? Please discuss.

Response: We thank the reviewer for their suggestion. As mitochondrial mass in every cell type varies according to the energy requirement of the respective tissue origin, so does the mtDNA copy number per every mitochondrion. Factors affecting the mitochondrial biogenesis regulate mtDNA copy number as an upstream regulator, followed by overall mitochondrial mass, which in turn termed as regulation of mitochondrial biogenesis (Clay Montier et al., 2009). This could explain the observation in our study where TFAM, mitochondrial transcription factor, involved in mitochondrial biogenesis through maintenance of mtDNA copy number show higher effect on mtDNA copy number than mitochondrial mass, upon its knock-down. In the revised manuscript, we have repeated the experiment and re-analyzed the qRT-PCR data with the formula $2^{-\Delta C_T}$, to get true mtDNA copy number than fold change (as plotted earlier through formula $2^{-\Delta\Delta C_T}$). We have included the new results in the revised manuscript (Fig. 3H-I).

Clay Montier, L. L., Deng, J. J. and Bai, Y. (2009). Number matters: control of mammalian mitochondrial DNA copy number. J. Genet. Genomics Yi Chuan Xue Bao 36, 125–131.

6. Fig. 5 and Fig. 5 legend. For clarity, please maintain the same name for PPARGC1a.

Response: We thank you for highlighting the error. We have now maintained PPARGC1a in figure 4, 5 and 6 as suggested along with figure legends throughout the revised manuscript to maintain the clarity.

7. There is no mention of Fig. S4 in the text.

Response: We deeply regret for missing the important figure of the study in the manuscript. We have now included the same in the results and discussion sections of the revised manuscript.

8. Bioinformatic analysis cannot unequivocally identify miR targets, therefore some sentences should be rephrased: Line 290: instead of "...were identified as direct targets...", should say "..were predicted to be direct targets..." or "...were identified as putative direct targets...". Same for Fig. S4 legend, lines 294-295, line 315, and other parts in the text where it should apply.

Response: We sincerely thank the reviewer for suggestions. Accordingly, we have made the necessary changes in the revised manuscript as suggested.

9. Fig. S2, referenced in lines 291-292: only mitomiR-5-3p shows a conserved seed region among species, regarding canonical 5' seed. mitomiR-5-5p shows no conservation on the 5' side of the sequence, only on the 3' region. Is it possible that this miR has a 3' seed? Please discuss. And, in this context, why did the authors decide to study the mitomiR without the conserved 5' seed?

Response: We sincerely thank the reviewer for their critical question. Multiple Sequence Alignment performed to analyze the conservation status of mitomiR-5 from lower invertebrates such as *C. remanei* and *Drosophila melanogaster* to humans. The extent of conservation for mitomiR-5 from

such lower phylum to humans seem to be evolutionarily significant. This was one of the reasons for us to choose mitomiR-5 for our experimental characterization. However, subsequent experimental observation involving Ago2-IP showed significantly higher mitomiR-5-5p association, than mitomiR-5-3p with Ago2 protein in RISC complex in cytoplasm and in mitochondrial fraction (Fig. 4D). *In-silico* analysis and experimental validation with wild type and mutant 3'-UTR construct showed stable mitomiR-5-5p 5' seed interaction with target genes (*PPARGC1a*, *MT-CO1* and *MT-CO2*) in our study.

10. Fig. S3: Please confirm that the miRNA inhibitors are RNA and not DNA oligos, the methods section is not clear on this.

Response: We thank the reviewer for the suggestion. Both miRNA mimics and inhibitors are RNA oligos, procured from ThermoFisher Scientific (USA): Custom mirVana miRNA Mimics (#4464068) and Custom mirVana miRNA Inhibitors (#4464086).

11. Fig. S6: please maintain the order of the cell lines for clarity.

Response: We thank the reviewer for highlighting the error. We deeply regret for the error that had occurred. We have made necessary changes to maintain the clarity in the revised manuscript (Fig. S7).

12. Lines 298-299 only mentions higher levels of PPARGC1a in MCF10A, but doesn't mention any other result shown in the graph. Authors should mention the fact that mitomiR-5-5p and PPARGC1a expresion seem to be inverse in the different cell lines.

Response: We thank the reviewer for the excellent suggestion. We have observed that PPARGC1a expression was highest in MDA-MB-231 cells when compared to MCF-7 and MDA-MB-468. While mitomiR-5-5p expression was lowest in MDA-MB-231 when compared to MCF-7 and MDA-MB-468. The expression profile of mitomiR-5-5p showed inverse correlation with *PPARGC1a* expression in MDA-MB-468 cells (Fig. S7). We have now rewritten the sentence in the results and discussion section in the revised manuscript.

13. In Fig. 5 A, C, D and E, the - sign seems to suggest an abscence of any additions, the graphs should specifiy that these are control mimics and control inhibitors. Same for Fig. 6.

Response: We sincerely regret for the inadvertent mistake. We have now updated Fig. 5 along with figure legend explaining the "+" and "-" sign in the revised manuscript.

14. Fig. 6C and 6E: Luciferase assays are not mentioned in the main text. Also, the figure legend says something completely different, mentioning mito-miR-5-3p, which is nowhere to be seen in the figure

Response: We thank the reviewer for highlighting the mistake. We have now updated the respective figure (Fig. 5) and the figure legend in the revised manuscript.

Major observations:
1. Fig. 4: authors do not mention whether mitoplasts were externally treated with RNase A before lysis. Therefore, it is important to present the determination of purity of cytosolic ad mitochondrial fractions by RT-qPCR instead of (or in addition to) Western blot.

Response: We sincerely thank the reviewer for the excellent suggestion. We have indeed treated the isolated mitochondria and mitoplast fractions with RNase A before lysis and isolation of mitochondrial RNA for all our experiments (Methods section, page number: 6-7, line number: 189-196). To confirm the purity, we have performed RT-PCR using *GAPDH* and *MT-CYB* specific primer using RNA from cytosolic and mitochondrial fraction (Fig. S1). The mitochondrial RNA did not show *GAPDH* amplification confirming the absence of nuclear/cytoplasmic RNA contamination in the mitochondrial preparation.

2. Fig. 4C: How could the authors conclude that the bands seen in lanes 2 and 4 correspond to mito-miR-5-5p? This could be determined by Northern blot but what is shown is a simple agarose gel electrophoresis.

Response: Thank you for the comment and suggestion. Figure 4E showed the qRT-PCR amplification products mitomiR-5-5p in the cytoplasmic and mitochondrial RNA associated with Ago2. We have designed custom miRNA TaqMan assays (ThermoFisher Scientific) for mitomiR-5-5p and mitomiR-5-3p. They are generated by stem-loop reverse transcription from the RNA followed by PCR amplification, providing PCR amplicon of approximately 65bp (Chen, 2005). We have also designed biotin tagged mitomiR-5-5p mimic which showed mitochondrial localization by immunofluorescence assay (Fig. 4A).

References:
Chen, C. (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.* **33**, e179–e179.

3. Fig. S3 should be repeated, it lacks the necessary controls (control mimic and control inhibitor). Also, the graph shows no decrease with the inhibitor.

Response: We thank the reviewer for suggestion. We have performed qRT-PCR based expression analysis of mitomiR-5-5p in cells transfected with scrambled mimic (mimic control) and inhibitor control in MDA-MB-468 cells along with mitomiR-5-5p mimic and inhibitor (Fig. S4). However, the relative quantity (RQ) was plotted by normalizing the mimic and inhibitor groups (shown in the figure) to their respective control oligos using the formula $2^{-\Delta\Delta C_T}$. mitomiR-5-5p inhibitor (custom mirVana miRNA inhibitor), is a single stranded anti-sense RNA molecule complementary to mitomiR-5-5p. They function by binding directly to the mitomiR-5-5p, rendering it functionally unavailable for interacting with the target mRNA. Hence, they do not inhibit the expression of the miRNA but competes with the target mRNA for the seed region of the miRNA.

4. Fig. 5: In order to corroborate unequivocally a direct miRNA binding to the 3'UTR, the same experiments must be performed with 3'UTR constructs containing mutated putative miRNA-binding sites (mutated seeds).

Response: We sincerely thank the reviewer for suggesting this experiment. We have now performed the luciferase assay with wild type and mutant 3'UTR of *PPARGC1a*, *MT-CO1* and *MT-CO2*. We observed that only wild type 3UTR construct, not the mutant construct showed mitomiRs specific binding to its nuclear and mitochondrial target gene (Fig. 5A-C). This data has been included in the revised manuscript.

5. Lines 318-319: this conclusion is not fundamented in any way, since there is no evidence showing that the synthetic transfected mimics or inhibitors actually reached the inside of mitochondria. If this is what they are trying to show, there should be an assay proving incorporation of the mimics/inhibitors into mitochondria.

Response: We thank the esteemed reviewer for the suggestion. We have performed immunofluorescence imaging showing the localization of biotin tagged mitomiR-5-5p mimic in the mitochondria (Fig. 4A) in the revised manuscript.

Second decision letter

MS ID#: JOCES/2021/258937

MS TITLE: Expression analysis and function of mitochondrial genome-encoded microRNAs

AUTHORS: Raviprasad Kuthethur, Vaibhav Shukla, Sandeep Mallya, Divya Adiga, Shama Prasada Kabekkodu, Lingadakai Ramachandra, PU Prakash Saxena, Kapaettu Satyamoorthy, and Sanjiban Chakrabarty
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.organd click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the two reviewers who were willing to re-review the MS have very different opinions. On balance I think that reviewer 2 raises some very valid concerns that will need to be address before we can accept the MS. If you think that you can deal satisfactorily with the issues that have been raise, I would be pleased to see a revised manuscript.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 2

Advance summary and potential significance to field

Very important findings as far as mitochondrial miRNA is concern

Comments for the author

Although the authors have tried to address few of my concerns, two major concerns were partly answered. As these two are very important concerns related to the manuscript main conclusion, I am not supporting its publication in J cell Science.
The authors have claimed the existence of a new miRNA and to conclude on that it is necessary to confirm that the identified miRNA is of required length and exist in the specified compartment. As I have pointed it also in the initial review process, this conclusion cannot be achieved without a Northern blot data!
Also, the data is not conclusive enough to suggest the mitochondrial matrix localization of the mitomiR-5-5p mimic tagged with 5´Biotin as claimed in Figure 4A. Higher magnification/super resolution image was necessary to conclude on that.
How the authors are sure that the mitochondrial targeted Ago2 function like what Ago2 do in a physiological context particularly when Ago2 don't have such mitochondrial targeting sequence with it? This is a very artificial context and far from real-life situation where hardly any Ago2 may

be detectable in mitochondria pure fraction! Arguably, the mitochondrial targeted Ago2 may not be functional also as far as its role in miRNA repression. This need to be checked.
It is also important to see whether the mitochondrial targeted Ago2 is also binding with nuclear encoded miRNAs or not.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Kuthethur et al reports the finding of 13 novel mtDNA-encoded miRNAs (mito-miRs) through bioinformatic analysis. The findings are interesting, adding to the previously described mitochondrial-encoded miRNAs.

Comments for the author

My concerns have been met, the MS is suitable for publication in my opinion.

Second revision

Author response to reviewers' comments

Reviewer 2 Advance Summary and Potential Significance to Field:
Very important findings as far as mitochondrial miRNA is concern

Reviewer 2 Comments for the Author:
Although the authors have tried to address few of my concerns, two major concerns were partly answered. As these two are very important concerns related to the manuscript main conclusion, I am not supporting its publication in J cell Science.

The authors have claimed the existence of a new miRNA and to conclude on that it is necessary to confirm that the identified miRNA is of required length and exist in the specified compartment. As I have pointed it also in the initial review process, this conclusion cannot be achieved without a Northern blot data!

Response:

We sincerely thank the esteemed reviewer for this thought. However, we respectfully do not agree with this comment of the reviewer due the following reasons mentioned below:

To be absolutely sure that the miRNA identified, exists in the mitochondrial compartment, we always treat the isolated mitochondria and mitoplast fractions with RNase A (to specifically degrade nuclear RNA) before lysis and isolation of mitochondrial RNA for all our experiments (Methods section, page number: 17, line number: 511-520). Furthermore, to confirm the purity, we have performed RT-PCR using GAPDH primer (for nuclear RNA) and MT-CYB primer (specific for mitochondrial fraction) using RNA from cytosolic and mitochondrial fraction respectively (Fig. S1). The mitochondrial RNA fraction did not show GAPDH amplification confirming the absence of nuclear/cytoplasmic RNA contamination in the mitochondrial preparation.

Finally, to confirm the mitochondrial genome encoded miRNAs (mitomiRs), we have designed custom miRNA TaqMan assays (ThermoFisher Scientific) for mitomiR-5-5p and mitomiR-5- 3p. They are generated by stem-loop reverse transcription from the RNA (specific for miRNAs) followed by PCR amplification, providing PCR amplicon of approximately 65bp, to ensure the specificity of quantified microRNA. This is a well-established and accepted technology in the field for the detection of miRNA which can be used in lieu of Northern blot. In support of this notion, Chen et. al; 2005 (see list of references below) have demonstrated that stem-loop reverse transcription followed by TaqMan based quantitative real time PCR for miRNA expression is more sensitive than

conventional qRT-PCR. This technique has also been employed by multiple other research groups for miRNA expression analysis. Furthermore, it has also been to be a more sensitive technology than northern blot and more appropriate if the abundance of miRNA is low. Therefore, we feel that using this more recent technology is justified for the identification and expression analysis of novel mitomiR in both cell lines and more complex breast cancer patient derived tumor specimen.

Relevant references for the use of qPCR assays for microRNA detection:

1. Chen, C. (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 33, e179–e179.
2. https://www.thermofisher.com/in/en/home/life-science/pcr/real-time-pcr/real-time-pcr- assays/mirna-ncrna-taqman-assays/custom-taqman-small-rna-assays.html
3. Kramer M. F. (2011). Stem-loop RT-qPCR for miRNAs. Current protocols in molecular biology, Chapter 15, Unit15.10–15.10. https://doi.org/10.1002/0471142727.mb1510s95
4. Varkonyi-Gasic E. Stem-Loop qRT-PCR for the Detection of Plant microRNAs. Methods Mol Biol. 2017;1456:163-175. doi:10.1007/978-1-4899-7708-3_13
5. Zhang X, Zuo X, Yang B, et al. MicroRNA directly enhances mitochondrial translation during muscle differentiation. Cell. 2014;158(3):607-619. doi:10.1016/j.cell.2014.05.047

Also, the data is not conclusive enough to suggest the mitochondrial matrix localization of the mitomiR-5-5p mimic tagged with 5´Biotin as claimed in Figure 4A. Higher magnification/super resolution image was necessary to conclude on that.

Response:

We thank the reviewer for the critical comment on the manuscript. We agree that our image does not have the resolution necessary to clearly observe the mitochondrial localization of the mitomiR-5-5p. We have now included an improved high-resolution image by confocal microscopy with analysis to show the distribution in the revised version of manuscript (Fig. 4A) and updated the figure legend (Line number: 976-982) in the revised manuscript.

How the authors are sure that the mitochondrial targeted Ago2 function like what Ago2 do in a physiological context particularly when Ago2 don't have such mitochondrial targeting sequence with it? This is a very artificial context and far from real-life situation where hardly any Ago2 may be detectable in mitochondria pure fraction! Arguably, the mitochondrial targeted Ago2 may not be functional also as far as its role in miRNA repression. This need to be checked.
It is also important to see whether the mitochondrial targeted Ago2 is also binding with nuclear encoded miRNAs or not.

Response:

We thank the reviewer for the comment.

Our study characterizes novel mitochondrial genome encoded miRNAs in breast cancer model. *In-silico* identification and expression analysis in breast cancer cell lines and patient tissue specimen, we have comprehensively characterized mitomiR-5-5p as a mitochondrial genome encoded miRNA, through mtDNA-depletion, siRNA-based TFAM knock down and analyzing the sensitivity of mitomiR-5-5p expression to mitochondrial inhibitor treatments.

Argonaute-2 is an important protein of the miRNA RISC component in eukaryotes and has been previously shown to translocate to mitochondrial compartments in various cellular systems (Bandiera et al., 2011; Das et al., 2012; Jagannathan et al., 2015; Li et al., 2016; Zhang et al., 2014- see below for full list). Even though it does not have a canonical mitochondria translocation signal, Ago2 protein has identified recently by Bader et al., 2020 (screened in multiple organisms including humans, mouse, Bos taurus, Danio rerio and Drosophila melanogaster showing the evolutionary conservation of the process) to localize into mitochondrial matrix.

Furthermore, regarding the comment of the artificial translocation of Ago2 into mitochondrial compartment using plasmid-mediated MTS signal and its relevance in physiological context, we would like to cite the earlier report by Zhang et al., 2014. Here the authors have also used

mitochondria targeted Ago2 using Su9-HA-Ago2, where Su9 is a N-terminal mitochondria targeted pre-sequence and HA is the C-terminal tag to create mitochondria targeted Ago2 fusion protein. Using this system authors have rescued the mitochondrial functions of miR-1 mediated translation upregulation, in Ago2 knockout mouse embryonic fibroblasts (MEFs), suggesting the reliability and reference in using artificial translocation of ago2 protein into mitochondria (Zhang et al., 2014). These data show that although artificial, targeting Ago2 to mitochondria results in a physiological response.

In our revised manuscript, we have demonstrated that our predicted mitomiRs binds to Ago2 in the cytoplasmic compartment and inside mitochondria (Fig. 4). We have also shown mitomiR-5-5p target genes *PPARGC1a*, *MT-CO1* and *MT-CO2* are enriched in the cytoplasmic and mitochondrial Ago2 fraction.

Additionally, as suggested by the reviewer, we have performed the enrichment analysis of nuclear miRNA hsa-miR-146a, known to be localized to mitochondria, in the mitochondrial targeted Ago2 enriched fraction (Fig. S6, Page number: 9, Line number: 260-262).

References:

1. Bader, G., Enkler, L., Araiso, Y., Hemmerle, M., Binko, K., Baranowska, E., De Craene, J.-O., Ruer-Laventie, J., Pieters, J., Tribouillard-Tanvier, D., et al. (2020). Assigning mitochondrial localization of dual localized proteins using a yeast Bi- Genomic Mitochondrial-Split-GFP. Elife 9, e56649.
2. Bandiera, S., Rüberg, S., Girard, M., Cagnard, N., Hanein, S., Chrétien, D., Munnich, A., Lyonnet, S. and Henrion-Caude, A. (2011). Nuclear outsourcing of RNA interference components to human mitochondria. PLoS ONE 6, e20746.
3. Das, S., Ferlito, M., Kent, O. A., Fox-Talbot, K., Wang, R., Liu, D., Raghavachari, N., Yang, Y., Wheelan, S. J., Murphy, E., et al. (2012). Nuclear miRNA regulates the mitochondrial genome in the heart. Circulation Research 110, 1596–1603.
4. Jagannathan, R., Thapa, D., Nichols, C. E., Shepherd, D. L., Stricker, J. C., Croston, T. L., Baseler, W. A., Lewis, S. E., Martinez, I. and Hollander, J. M. (2015). Translational Regulation of the Mitochondrial Genome Following Redistribution of Mitochondrial MicroRNA in the Diabetic Heart. Circulation: Cardiovascular Genetics 8,.
5. Li, H., Zhang, X., Wang, F., Zhou, L., Yin, Z., Fan, J., Nie, X., Wang, P., Fu, X.-D., Chen, C., et al. (2016). MicroRNA-21 Lowers Blood Pressure in Spontaneous Hypertensive Rats by Upregulating Mitochondrial Translation. Circulation 134, 734– 751.
6. Zhang, X., Zuo, X., Yang, B., Li, Z., Xue, Y., Zhou, Y., Huang, J., Zhao, X., Zhou, J., Yan, Y., et al. (2014). MicroRNA directly enhances mitochondrial translation during muscle differentiation. Cell 158, 607–619.

Third decision letter

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.