Author's response to reviews

Title: MicroRNA and mRNA expression profiling in rat acute respiratory distress syndrome

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Author's response to reviews: see over
Dear Dr. Sleiman;

Enclosed please find our revised manuscript entitled “MicroRNA and mRNA expression profiling in rat acute respiratory distress syndrome”. We have revised the manuscript according to the comments of the reviewers. The changes in the revision are highlighted by red font. The following is the point-to-point response.

**Reviewer 1: Wenbo Mu**

**Major Compulsory Revisions**

1) *Is there any enriched pathway in dysregulated mRNAs?*

**Response:** We have performed KEGG pathway enrichment analysis of the dysregulated mRNAs in rat ARDS using STRING software and the results are included in Table 6 in the revision.

2) *Histopathological changes were measured and graded, do the authors conduct any research on the relationship between gene/miRNA expression pattern and histopathological changes?*

**Responses:** This is an important and complex question to be answered. However, in the current studies, we only have two conditions (control and ARDS), which do not allow us distinguish various parameters of pathological changes. If we have different models to distinguish pathological changes, e.g. neutrophils, edema and hyaline membranes, we can correlate these changes with gene/miRNA expression patterns.

3) More advanced network analysis among genes should be conducted.

**Response:** Additional network analysis has been performed using STRING software. STRING is a web tool to explore Protein-Protein Interactions, KEGG
pathway, and Go annotation. The results are shown in the revised Figure 4, Table 6 and Table 7.

Minor Essential Revisions
1) The author should provide a list of miRNA and DNA on the in-house printed microarray in supplementary table.

Response: These have been submitted to the GEO database (Access numbers: GSE57223 and GSE57011). See the Results section (page 5).

2) Table 5. Are the P values raw p value or p value after FDR control involved in functional annotation? GO annotations with large p values don’t mean anything in the functional annotation.

Response: This is a good comment. The raw p values were used in functional annotation. We did GO annotation on the up-regulated and down-regulated mRNAs separately. The small number of mRNAs, involved in the annotation, causes a relative large p-value. When performed GO annotation on all the dys-regulated mRNAs, the p values were much smaller (data not presented here).

3) Figure 3. Missing label in legend of the sixth figure.

Response: This has been corrected.

4) In abstract, “miR-346, miR-135b, miR-30a/b, miR-344, and miR-18a had more than one mRNA target. Gabrb1, Sod2, Eif2ak1, Fbln5, and Tspan8 were targeted by multiple miRNAs.” This is not a convincing result in this paper. It is obvious fact that many miRNAs have more than one target and many genes are targeted by multiple miRNAs.
Response: We apologize for the confusion. We meant multiple interactions among the dys-regulated mRNAs and dys-regulated miRNAs. We have revised the abstract accordingly.

Reviewer 4: Sabarinathan Radhakrishnan

Reviewer's report:
The work by Huang and colleagues presents an analysis of expression profiling of miRNA and mRNA in rat acute respiratory distress syndrome (ARDS). The major finding of this study includes the identification of dys-regulated miRNAs and mRNAs in rat ARDS through microarray analyses, and the correlation between the identified dys-regulated miRNAs and mRNAs which has been predicted through computational approaches. This manuscript is interesting as it begins to reveal how dys-regulated miRNA-mRNA pairs may be involved in the pathogenesis of rat ARDS.

Discretionary Revisions
1) Are there any specific reasons for the choice of these two programs, TargetScan and miRanda, in particular for miRNA-mRNA interaction prediction?

Response: These two programs are commonly used and we have used them in the past.

2) It would be interesting to cross-check if the predicted miRNA-mRNA pairs have already been validated experimentally (using the information available from databases, for example, TarBase/mirRecords).

Response: Thanks for the excellent suggestion. We have cross-checked the predicted miRNA-mRNA pairs in Tarbase/mirRecords database. We found that miR-26a-APC pair has been reported (Chi SW, et al. Nature, 460: 479-486, 2009). This is added to the revision (page 10)
Minor essential revision

3) In Table 6, some of the miRNA-mRNA pairs have been predicted by both TargetScan and miRanda programs, here the authors need to mention whether the predicted miRNA binding site in the target mRNA is in the same location or it varies between the two programs.

Response: All the predicted miRNA binding sites in the target mRNAs are in the same location between the two programs. However, miRanda predicted two rno-miR-128b binding sites and TargetScan only predicted one in the 3’-UTR of Gabrb1. This statement was added to the revision (page 8).

4) In the title of Table 7, the word "predictd" is misspelled.

Response: Corrected in the revised manuscript (Table 9 in the revision).

5) In the footnote of Table 4 and 5, the closing round bracket at the end of the text is placed without its counterpart.

Response: Corrected in the revised manuscript.

6) In places where the software called as "MIRpath" should be replaced as "DIANA-mirPath" for consistency.

Response: Changed in the revised manuscript.

7) In Figure 4, the details of the different colors used for the nodes can be explained in the legend for better understanding.

Response: Done as suggested (Figure 5 in the revision).

8) In Figure 5 legend, the word “Go” needs to be changed as “GO”.
Response: Changed in the revised manuscript (Figure 6 in the revision)

Reviewer 5: Djanybek Adyshev

Reviewer’s report:
I think author’s findings are novel and potentially important for understanding the epigenetic factors involves in ARDS. However the paper would be improved if the authors could provide additional in vitro experimental data to confirm inverse correlation between selected predicted targets and miRNAs (i.e. down regulation by miRs of the luciferase reporter, on mRNA & protein level).

Response: We thank the reviewer for the good suggestion. However, we feel that experimental validation of the identified miRNA-mRNA pairs is beyond the scope of the current manuscript and will be included in our future studies. As suggested by the reviewer 4, we have cross-checked our miRNA-mRNA pairs with two databases, TarBase/mirRecords, which collect experimentally validated miRNA-mRNA interactions. We found that miR-26a-APC pairs have been verified (Chi SW, et al. Nature, 460: 479-486, 2009).

Also would be useful, if authors provide actual miRNA and mRNA expression profiling figures in the Results section of manuscript.

Response: miRNA and mRNA expression data has been submitted to a public database, GEO (access numbers: GSE57223 and GSE57011). Furthermore, the identified miRNAs and mRNAs changed in ARDS were listed in Table 2 and Table 3.

Furthermore, beside direct degradation of mRNA (inverse correlation), mRNAs can inhibit translation of the target without mRNA degradation, should be explained.

Response: We have discussed this point in the Discussion (see page 10).
Also I would like to express, that ARDS characterized by profound lung inflammation, increased lung vascular permeability. MiRNAs & target genes, involved in these processes should be identified.

Response: We have discussed this point in the Discussion section (page 11).

Some minor typographical and grammatical errors should be corrected (for example dys-regulation).

Response: We have carefully proof-read the manuscript and corrected any typographical and grammatical errors we found. Dys-regulated or dys-regulation has been changed to altered or alteration.

Reviewer 3: Tamas Dolinay

Reviewer's report:

Huang et al. discuss a novel approach of discovery of gene-products that play a role in the mechanism of acute lung injury (ALI). The authors performed simultaneous microRNA (miRNA) and messenger RNA (mRNA) profiling on rat lungs that were subjected to the combined treatment surfactant depletion and mechanical ventilation. Results of gene expression were compared to animals who received no surfactant depletion (sham). Their findings suggest that there is a strong correlation between miRNA and mRNA expression changes in ALI/ARDS. The strength of the manuscript is that it highlights the importance of post-transcriptional modification of gene expression in the mechanism of ALI. It uses a reasonable animal model and it follows a sound methodology. However in its current format the manuscript has major weaknesses and requires further work. Please see see below:

Major Compulsory revisions:
1. Animal model:
   A. Controls. It is not discussed what intervention is performed on the sham
animals. This is an important point because the animals in the treatment group receive low, than high tidal volume ventilation, finally they undergo lavage. While this seems to be a reasonable approach to induce lung injury, it is important to know what is called sham. The authors should provide a cartoon that describes the time and length of interventions.

Response: The controls were non-lavaged and non-ventilated rats. We have included this statement in the Methods section (page 13). A cartoon for the experimental procedure is added to the revision (Figure 7).

B. Lung injury is only expressed as histology findings. Please include at least one more form of quantitative measurement of lung injury. I recommend Evans-Blue dye extravasation studies as this will not be altered by lavage of the lungs during the experiment.

Response: Since this model was previously established (Ref 25, 58), we did not measure other parameters of lung injuries.

C. How do the authors measure animal well being during the experiment? A major concern is hypotension in lavaged animals, which can be a confounder that contributes to lung injury. Please describe, if animals become hypotensive during the experiment.

Response: We did not monitor changes in pulmonary arterial pressures. In the original paper that reported this model, PaO2 was monitored. PaO2 decreased during the early postlavage period (up to 30 min) (Ref 25, 38).

2. Gene expression studies:
A. While the manuscript raises the importance of posttranscriptional modification of gene expression in ALI it fails to answer whether it is biologically important. The authors should provide biochemical evidence (co-immunohistochemistry or chemical inhibition) of miRNA targets to show that mRNA expressions change
with them. Otherwise it will be hard to convince the audience that their findings contribute to changes on protein level. A potential exciting target is: let miRNA family. A good example of such work cited by the authors: Vaporidi et al. Am J Physiol Lung Cell Mol Physiol. 2012 August 1; 303(3): L199–L207.

Response: We thank the reviewer for the excellent suggestion. We feel that these experiments are beyond the scope of this manuscript and will be performed in our future studies.

B. It is hard to draw any meaningful conclusion from study linking miRNA to singling pathways (Fig 7). I believe the better approach would be to identify one miRNA that changes mRNA expression and analyze what are the potential other targets (genes and pathways). This could significantly elevate the relevance of this work.

Response: Once again, this is an excellent suggestion, but we feel that it is beyond the scope of this manuscript.

Minor Essential Revisions:

1. Abstract:
   A. Line 1. ARDS is a severe form of injury.

Response: “a more severe form of acute lung injury” has been deleted per the suggestion of the reviewer 2.

B. Line 4. ARDS provide insights.

Response: Changed.

C. Line 18. The use of “dys-regulation” is very confusing throughout the Manuscript because it suggests that somehow this expression change is not
“well-regulated”. I would continue with the expression “regulated”. In the same sentence: rat model of VILI. ARDS is preserved for human disease.

**Response:** We changed “dys-regulated” or “dys-regulation” to “altered” or alteration. We changed rat ARDS to a rat model of ARDS since the rat model we used is surfactant depletion plus ventilation, which is different from VILI.

2. **Background:**
   A. The first 2 paragraphs needs to be re-written because its current form is superficial and does not explain why the investigation is done.

   **Response:** Rewritten.

   B. In the context of the manuscript, I would focus on introducing ARDS as a disease severe inflammation and cell damage.

   **Response:** Modified.

   C. Please explain the role of animal modeling of ARDS (complex and heterogeneous mechanisms that are hard to discern from human samples).

   **Response:** Added (page 4).

   D. If the authors wish to use human outcome data, please use more recent epidemiology as ARDS morbidity and mortality has improved since the sited reference.

   **Response:** We cited a more recent paper.

   E. Reference 19 is not a miRNA but mRNA-based study.
Response: Corrected

F. The authors cite 22 classes miRNAs, but articles talk about several hundreds of miRNA (see Transl Res. 2011 April; 157(4): 180–190). How does this compare?

Response: “22 nucleotide” means the length of microRNAs. We have modified this sentence to make it clear. “MicroRNAs (miRNAs) are a class of non-coding small RNAs with approximately 22 nucleotides in length” (page 3).

G. Paragraph 3: I would separate MRNA and miRNA profiling. In the current format it is confusing and does not help the reader to learn why it is important to perform both analyses. The key here is that they have additional value and miRNA can alter the post transcriptional level of mRNA.

Response: Revised

H. Last paragraph. The current content belongs to results section. The authors should discuss the goals of the study here: 1. perform simultaneous miRNA and mRNA profiling in a non-infectious model of VILI. 2. Identify potential interactions between miRNA and mRNA expression. 3. Impact of these interactions on the mechanism of ARDS.

Response: Revised as suggested.

3. Results
A. Animal model. As detailed above, Table 1 needs to include data from controls.

Response: Based on the scoring system (see the Methods section, page13), all of the parameters are 0 for control rats. Thus, we did not include controls in the table. However, we have added a statement in the text (Page 5).
B. Second paragraph. References do not belong to the Results section. It should be moved to the Discussion.

Response: Moved.

C. MiRNA and mRNA profiles section: Why did the authors use a different threshold for the miRNA array results than for the mRNA? If this was because of loosening the criteria to find more genes, the authors should discuss it in the Methods section.

Response: The reviewer is correct. We used a fold change of 1.5 in miRNA profiling and a fold change of 2 in mRNA profiling in order to find more miRNAs. This point is discussed in the Methods (page 15).

4. Methods
A. Please explain the abbreviation SAM and provide reference.

Response: Done as suggested (page 15)

B. Bioinformatics analysis. Since the manuscript heavy relies on available software package a brief description why the particular package was used is warranted.

Response: A brief description of software packages are added to the Method section (page 17).

Reviewer 2: Maneesh Bhargava
Reviewer’s report:
This is a well-written manuscript in an important area of research. Systems level approach for identifying mechanisms that are involved in pathogenesis of ARDS
will improve our understanding of this disease and also result in targets for therapy. However, I have several concerns about this manuscript

**Major Concern**

1. What are the controls that are shown in Figure 1? Were these rats with ventilation by normal tidal volume or rats that were maintained at room air? Ideally room air controls rats and mechanically ventilated rats should be included in the study.

**Response:** The controls were non-lavaged, non-ventilated rats, which were maintained at room air. The statement was added to the Methods (page 13).

2. Please describe how the rat lungs were fixed using formalin. Was formalin injected into the trachea with a plunger or was certain amount of pressure used for instillation into the lung? In Table 1, for hyaline membranes, the median value for ARDS is outside the range. The mean value is also below the minimum value. This is not statistically possible. How many rats were used to generate this table. Please provide statistics that compare the means and/or medians in the two comparison groups. I would also favor using standard deviation to demonstrate the variance instead of SEM.

**Response:** The detailed procedures for fixing the rat lungs are added to the revision (Page 13). The typographic errors for hyaline membrane in Table 1 have been corrected. 19 rats were used to generate this table. Statistic comparison between control and ARDS groups is not possible because all of the values for control groups are 0 based on the scoring system (see page 13).

3. Please provide wet to dry lung weight in the groups compared.

**Response:** Unfortunately, we did not measure wet or dry lung weights.

Response: Since this model was previously established, we did not measure other parameters of lung injuries (Ref 25, 58).

5. Purity of RNA isolation is marginal.

Response: The RNA quality was assessed by agarose gel electrophoresis and spectrophotometer.

6. Is the miRNA microarray modified since its development? Similarly what was the reason to choose in-house printed DNA microarray

Response: No, we have not updated our arrays. The reason to use these arrays is economic consideration.

7. What are the backgrounds used for the DAVID GO enrichment analysis? This is very important the null hypothesis test implements hypergeometric distribution, which is sensitive to the background or ‘N’, used for the analysis.

Response: We used DAVID default population (Rattus norvegicus) background in enrichment calculation. This point has been added to the revision (page 6).

8. On table 4 and 5 list the genes that mapped to the various GO annotations. If the purpose was to determine the GO terms why are PIR and SEQ mapping shown in Table 4 and 5. Typically GO has each gene has only 3 annotations-biological process, cell compartment and molecular function.

Response: DAVID functional annotation clustering uses a novel algorithm to determine relationships among the annotation terms based on the degrees of their co-association genes. The similar, redundant, and heterogeneous annotation contents from the same or different resources were clustered into
annotation groups due to their similar biological meaning. However, STRING GO enrichment is typically GO analysis including 3 annotations-biological process, cell compartment and molecular function. The information is provided in the Results section (Pages 6 and 7). Also new results using STRING GO enrichment analysis is shown in the revised Table 6.

9. Show the genes that were mapped to the individual annotation term in DAVID.

Response: Please see the supplementary file 1.

10. The statistical cutoff is a major concern with methods. For Functional annotation clustering in DAVID, several cluster with and enrichment score of < 1.3 are shown. By the DAVID algorithm enrichment > 1.3 should be used for a cluster to be statistically significant. This will be even more relevant depending on the appropriateness of the ‘universe of genes’ for the background. Also for figure 5, an alpha of 0.05 is not appropriate as multiple comparisons are done. It is not clear if any correction is used for multiple hypothesis testing. What are the statistical cutoffs for miRpath analysis?

Response: Very good comments. As suggested, we deleted the clusters with the DAVID algorithm enrichment score < 1.3 in Table 4 and Table 5. For Figure 5, we did not do multiple comparisons. p<0.01 was the statistical cutoffs for miRpath analysis, which is added to the table legend (the revised Table 8).

11. Was the functional annotation clustering done with default parameters or with modifications? What was the stringency used for the clustering?

Response: The functional annotation clustering was done with default parameters. Classification stringency was set as medium. These statements were added to the revision (page 6).

12. Why use targetscan and miRanda
Response: These two programs are commonly used and we have used them in the past.

13. Text and table 6 for Let does not match

Response: Changed.

14. In context of Berlin definition of ARDS, it is best to avoid using the term ‘Acute Lung Injury’

Response: deleted

15. Please include a paragraph regarding the limitations of this study

Response: The limitation of this study is discussed in the Discussion section (pages 9, 10).

We thank the reviewer for their excellent suggestions. We hope that the revised manuscript is improved and is acceptable for BMC.

Sincerely;

Lin Liu, Ph.D.
Professor