Peer Review File

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Reviewer Comments

The study by Fu et al. investigates how miR-146a-3p and miR-134-5p regulates TNF-a-induced cell death and CMH in COPD. There are a few points to consider

1. In the intro, the authors fail to explain why they are looking specifically at the cytokine TNF-a. What is the reasoning behind choosing this particular cytokine and not another inflammatory cytokine such as IL-1B for e.g?

Response: Thank you very much for your comments on our article. According to your suggestions, we have supplemented several descriptions here and corrected several mistakes in our previous draft. We have made extensive revisions to our draft. The detailed point-by-point responses are listed below.

Although several inflammatory cytokines (IL-1β, IL-5, IL-13, etc.) have been demonstrated to produce MUC5AC expression *in vitro* and *in vivo* [1, 2], the effect of TNF-α on airway mucus hypersecretion has not been widely investigated, and thus TNF-α was used to establish the cell model of airway mucus hypersecretion based on our preliminary experiments. Please find the description in INTRODUCTION in the revised manuscript. Thank you very much.

2. In addition to the first comment, did the authors perform any dose experiments to inform the choice of the concentration of TNF-a used? This should be provided in the manuscript.

Response: 16HBE cells were treated with TNF-α (R&D Systems, Minneapolis, MN, USA; 10 ng/ml) for 24 h to construct airway mucus hypersecretion cell model according to previous reports [3, 4] and our preliminary experiments (Supporting Figure S1). You can find the information in ***Cell culture and TNF-α treatment*** section in the revised manuscript. Thank you.

3. The authors also fail to provide a wide variety of publications to back the reason why they are looking at miR-146a and miR-134. Is it solely due to the paper of Tasena et al? Aren't there other papers showing the roles of these miRNAs in COPD that could also be cited and explained to provide a more rounded reason for the study?

Response: miR-134-5p is a potential marker for the clinical diagnose of acute exacerbation of COPD [5]. Down-regulated miR-146a-5p is involved in the pathogenesis of COPD through inducing more proinflammatory phenotype [6-8]. The information has been described and corresponding references have been cited. You can find the information in INTRODUCTION in the revised manuscript. Thank you very much.

4. Did the authors do any dose experiments to inform the concentration of the mimic they used? How physiologically relevant is the over-expression of the miRNAs in the study? As in, will those miRNAs ever be increased to the level they were increased in the human body?

Response: 16HBE cells were transfected with 40 nmol/L miR-134-5p, miR-146a-5p mimic, or NC mimic with Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) and Opti-MEM-reduced serum medium (Solarbio) according to previous reports and our preliminary experiments. You can find the information in ***miRNA mimics*** section in the revised manuscript. Mounting studies have demonstrated that miRNAs play a significant biological function *in vitro* after transfecting 20-100nM of miRNA. The present study has also shown that the expression level of miR-134-5p and miR-146a-5p is markedly increased in 16HBE cells after transfecting 40nM of miRNA mimics (Figure 1G and H).

In the study, the role of miR-134-5p and miR-146a-5p was only investigated *in vitro*. The clinical application of these miRNAs (in other word, their physiologically relevant) will be explored in the future. This is a good idea. Thank you.

5. In the results the authors introduce us to new miRNAs which they did not talk about

in the Intro? why are they now assessing these other miRNAs in addition to miR-146a and 134? In line with this comment, there was increased expression of Let7f in the panel of miRs in fig 1C. Why was this not studied also?

Response: Indeed, it is confused to introduce new miRNAs which we did not talk about in the Introduction. For contextual unity, we only described the association of TNF-a with miR-134 and miR-146a expression (Figure 1F).

6. All western blot data should have densitometry graphs to go along with the images shown. Showing the images of western blots is not quantitative.

Response: The quantitative analysis of all western blot data (Figure 1E, 2E and F, 3E, and 4) has been carried out according to your advice. You can find the revision in the revised version. Thank you very much.

7. In the mimic experiments, where are the Reagent Controls?

Response: Thank you for your reminding. The Reagent Controls in the mimic experiments are miRcont. Sometime, the miRcont was not labeled in some figures. In the revised version, the miRcont has been labeled in all figures. Please check all figures to affirm whether all figures are correct. Thank you very much.

8. Figure 2A does not show that forced expressing of miR-134+146a inhibits muc5AC more than 134 and 146A separately. The graph is wrong and must be changed.

Response: I am sorry for the mistake. The wrong image was placed in Figure 2A. According to your reminding, we have corrected the mistake in the revised version. Please check whether it is correct. Thank you very much.

9. Why are graphs 2B and 3A not having the same outlook since they are the same experiments but with different miRs. Why do the authors have NC, TNF-a, TNF-a+miR134 in Fig 2B and only TNF-a+miRcont and TNF-a+miR146a in Fig 3A?

Response: Figure 2B and Figure 3A have been adjusted the same outlook.

10. When the authors talk about the miR-146a mimic experiments they say miR-146a overexpression upregulated muc5AC. However, this is not what they show in their graphs. The authors could do well to read their paper very carefully and correct all these basic mistakes.

Response: In the studies about miRNAs, the "miRNA overexpression" was commonly described by using miRNA mimics.

According to your suggestion, we have carefully checked the manuscript and revised the description.

11. In the discussion the authors should talk about the relation of inflammation with mucus hypersecretion and why this was important to model in the current study.

Response: COPD is chronic pulmonary disorder, characterized by persistent inflammatory response to inhaling cigarette smoke [9]. Bronchial epithelial cells and macrophages are mainly responsible for chronic inflammatory response [9]. These cells could discharge proteases which lead to elastin degradation and emphysema, and thus facilitate inflammatory response [10, 11]. Epithelial cells also release TGF-β to induce tissue remodeling [12]. Based on these facts, we established a cell model of mucus hypersecretion using the human bronchial epithelial cells (16HBE) by TNF-α treatment. As expected, TNF-α treatment increased the mRNA and protein level of MUC5AC in 16HBE cells. The information has been described in DISCUSSION in the revised manuscript according to your suggestion. Thank you very much.

12. One can also argue that to study mucus expression, the authors should have stained cells to show the muc5AC expression in the cells themselves instead of western blots. It probably will have been better to use ALI's for this. However, I believe they can get away with at least mentioning the fact that using an ALI would have been better and discuss this dynamic in the Discussion.

Response: As described previously [3, 13-16], it is acceptable to assess muc5AC expression in the cells using qPCR and western blot analysis. According to your advice, we will assess the muc5AC expression in the cells using an ALI in the future work.

Thank you very much for your kind reminding.

Minor.

There are several places in the manuscript where some words are missing or the sentence doesn't form quite well. I will suggest that the authors find a native English speaker to carefully read and correct their manuscript before resubmission.

Response: The full manuscript has been carefully revised to avoid grammar or syntax error. Thank you.

[1] Busse PJ, Zhang TF, Srivastava K, Lin BP, Schofield B, Sealfon SC, et al. Chronic exposure to TNF-alpha increases airway mucus gene expression in vivo. The Journal of allergy and clinical immunology. 2005;116:1256-63.

[2] Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. The Journal of clinical investigation. 1999;103:779-88.

[3] Song KS, Lee WJ, Chung KC, Koo JS, Yang EJ, Choi JY, et al. Interleukin-1 beta and tumor necrosis factor-alpha induce MUC5AC overexpression through a mechanism involving ERK/p38 mitogen-activated protein kinases-MSK1-CREB activation in human airway epithelial cells. The Journal of biological chemistry. 2003;278:23243-50.

[4] Hauber HP, Daigneault P, Frenkiel S, Lavigne F, Hung HL, Levitt RC, et al. Niflumic acid and MSI-2216 reduce TNF-alpha-induced mucin expression in human airway mucosa. The Journal of allergy and clinical immunology. 2005;115:266-71.

[5] Peng L, Han L, Li XN, Miao YF, Xue F, Zhou C. The Predictive Value of microRNA-134 and microRNA-1233 for the Early Diagnosis of Acute Exacerbation of Chronic Obstructive Pulmonary Disease with Acute Pulmonary Embolism. International journal of chronic obstructive pulmonary disease. 2020;15:2495-503.

[6] Osei ET, Florez-Sampedro L, Tasena H, Faiz A, Noordhoek JA, Timens W, et al. miR-146a-5p plays an essential role in the aberrant epithelial-fibroblast cross-talk in COPD. The European respiratory journal. 2017;49.

[7] Li N, Li S, Wu Y, Xiong L, Li T, Xing D, et al. Dexmedetomidine targets miR-146a and participates in the progress of chronic obstructive pulmonary disease in vivo and in vitro. Genes & genomics. 2021.

[8] Sato T, Liu X, Nelson A, Nakanishi M, Kanaji N, Wang X, et al. Reduced miR-146a increases prostaglandin E(2)in chronic obstructive pulmonary disease fibroblasts. American journal of respiratory and critical care medicine. 2010;182:1020-9.

[9] Aghasafari P, George U, Pidaparti R. A review of inflammatory mechanism in

airway diseases. Inflammation research : official journal of the European Histamine Research Society [et al]. 2019;68:59-74.
[10] Suki B, Lutchen KR, Ingenito EP. On the progressive nature of emphysema: roles of proteases, inflammation, and mechanical forces. American journal of respiratory and critical care medicine. 2003;168:516-21.
[11] Lane N, Robins RA, Corne J, Fairclough L. Regulation in chronic obstructive pulmonary disease: the role of regulatory T-cells and Th17 cells. Clin Sci (Lond). 2010;119:75-86.
[12] Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. Nature reviews Immunology. 2008;8:183-92.
[13] Lee SU, Sung MH, Ryu HW, Lee J, Kim HS, In HJ, et al. Verproside inhibits TNF-alpha-induced MUC5AC expression through suppression of the TNF-alpha/NF-kappaB pathway in human airway epithelial cells. Cytokine. 2016;77:168-75.
[14] Sikder MA, Lee HJ, Mia MZ, Park SH, Ryu J, Kim JH, et al. Inhibition of TNF-alpha-induced MUC5AC mucin gene expression and production by wogonin through the inactivation of NF-kappaB signaling in airway epithelial cells. Phytotherapy research : PTR. 2014;28:62-8.
[15] Bae CH, Choi YS, Na HG, Song SY, Kim YD. Interleukin (IL) 36 gamma induces mucin 5AC, oligomeric mucus/gel-forming expression via IL-36 receptor-extracellular signal regulated kinase 1 and 2, and p38-nuclear factor kappa-light-chain-enhancer of activated B cells in human airway epithelial cells. American journal of rhinology & allergy. 2018;32:87-93.
[16] Chen Y, Garvin LM, Nickola TJ, Watson AM, Colberg-Poley AM, Rose MC. IL-1beta induction of MUC5AC gene expression is mediated by CREB and NF-kappaB and repressed by dexamethasone. American journal of physiology Lung cellular and molecular physiology. 2014;306:L797-807.