Peer Review File

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Reviewer A

Major points

The authors speculate that ER stress of AEC2 is the main regulator of EMT, and the author`s hypothesis is that Nogo-B directly regulate ER-stress of AEC2 and secondary regulate EMT of AEC2. The authors should show these evidences of papers in the manuscript.

Reply: Thank you for your valuable comments, which are being appreciated; moreover, these suggestions provide us a more precise research focus in our future study. Previous studies have shown that ERS plays a significant role in fibrotic conditions, through increasing α-SMA protein expression and promoting fibroblast-like morphologic changes in primary alveolar epithelial cells (Curr. Opin. Rheumatol,2012; Am. J. Respir. Cell Mol. Biol, 2011). Recently, ERS has been demonstrated to regulate EMT in a number of tissues (Mol Med Rep, 2020) and is involved in EMT of alveolar epithelial cells (Int J Mol Sci, 2019). As a member of the reticular protein family, Nogo-B has been verified to participate in cardiac fibrosis and hepatic cirrhosis (Biomed Pharmacother, 2018; Hepatol Res, 2015); moreover, it is recognized as an inducer of EMT in some cells such as NSCLC, HCC, breast cancer cells (Cancer Lett, 2018; Life Sci, 2018; J Proteomics, 2015). As mentioned above, Nogo-B is an endoplasmic reticulum-residential protein, involved in regulating protein binding, folding and homodimerization activity; meanwhile ERS is triggered by perturbations in ER function such as those caused by protein misfolding or by increases in protein secretion. However, role of Nogo-B in constructing the association between ERS and EMT in lung fibrosis remains obscure. In the study, we firstly observed that Nogo-B expression was up-regulated in lung tissues of fibrosis model mice and alveolar epithelial cells; in the view of Nogo-B upregulation, we knocked Nogo-B expression down in vivo and in vitro, and examined the expressions of ERS-related key sensors including PERK, IRE1α, ATF6 as well as E-cad and N-cad, vimentin in purified AECIIs and cultured murine lung epithelial cells and concluded that Nogo-B plays an important role in regulating ERS (particularly for PERK branch) or EMT in lung epithelial cells. Furthermore, to demonstrate whether PERK is involved in Nogo-B-mediated EMT or not, we knocked down the expression of PERK in lung epithelial cells with Nogo-B overexpression and found, after PERK knockdown, Nogo-B-induced the upregulation of E-cad and the downregulation of N-cad and vimentin were significantly inhibited. These results suggested at some extent that dysregulated ERS may affect EMT in lung, in which Nogo-B was involved, and finally contributed for lung fibrogenesis. In fact, there are several limitations in our current study. For example, we had not elucidated the exhaustive molecular mechanism for explaining the regulation of Nogo-B in the expressions of ERS- and EMT-markers at transcriptional or post-transcriptional levels, which consequently leading to lung fibrogenesis. Moreover, exact role of Nogo-B in constructing the association between ERS and EMT in lung fibrosis is still to be verified. Nevertheless, from the available data, we are able to reveal that

Nogo-B functions as a mediator between EMT and ERS in promoting lung fibrogenesis. Of course, your thoughtful suggestion will make our future study goals clearer.

Changes in the text: ……There are several limitations in our study. We had not elucidated the exhaustive molecular mechanism for explaining the regulation of Nogo-B in the expressions of ERS- and EMT-markers at transcriptional or post-transcriptional levels, which consequently leading to lung fibrogenesis. Moreover, exact role of Nogo-B in constructing the association between ERS and EMT in lung fibrosis is still to be verified. In addition, the current study is purely a murine study without human samples. Nevertheless, from the available data, we are able to reveal that Nogo-B functions as a mediator between EMT and ERS in promoting lung fibrogenesis, from mouse model in vivo and cell lines model in vitro;…… (see Page 16-17, line 389-395) .

Minor points

1. (p2. line 27) Change ER stress as long form and provide abbreviation.
Reply: Thank you for the comment. Because ERS appears the first time, we provided the full-name.
Changes in the text: endocytoplasmic reticulum stress (ERS) sensors (see Page 2, line 29).

2. (p3. line 56 and 59) Change endoplasmic reticulum stress to ERS
Reply: Thank you for the comment. We have changed endoplasmic reticulum stress to ERS.
Changes in the text: which is associated with the PERK branch of ERS pathway. (see Page 2, line 36).

3. (p14. line 275) Incomplete sentence.
Reply: Thank you. We have rewritten the sentence.
Changes in the text: epithelial cells experienced EMT and transformed into fibroblasts, accounting for about 50% of the total number of fibroblasts (see Page 14, line 324-325).

4. Provide the day when these data were evaluated in Figure 1 and 2.
Reply: Thank you. We have added the detailed description in the Figure legends.
Changes in the text: Representative Images of Masson staining and Nogo-B immunohistochemical staining control lung tissues and lung tissues at the 28th day from a pulmonary fibrosis mouse model at a magnification of ×200 and then representative fields were chosen for presentation (see Page 21, line 421-422). And "……control lung tissue and lung tissue at the 7th and 28th day during constructing a pulmonary fibrosis model of Nogo-B knockout mice……." (see Page 21, line 429-430).

5. (Figure legends, Fig.2 I-L) p PERK / PERK was not described.
Reply: Thank you. We have added the detailed description in the Figure legends.

Changes in the text: ……ERS-related markers including p-PERK/PERK, p-IRE1α/IRE1α and cleaved ATF6 (c-ATF6)…… (see Page 22, line 504-505).

6. Provide the cell lines used in Figure 3 and 4.

Reply: Thank you. We have added the detailed description in the Figure legends.

Changes in the text: ……lung epithelial cells MLE-12 and TC-1 JUH-1 treated with…… (see Page 23, line 511-512).

7. Insert spaces after Nogo-B in figure legends.

Reply: Thank you. We have inserted spaces after Nogo-B in the whole Figure legends section.

Reviewer B

The paper entitled 'Nogo-B promotes epithelial mesenchymal transition in lung fibrosis via PERK branch of the endoplamic reticulum stress pathway' by Zhu et al examines the role of Nogo-B in EMT in a murine model of fibrosis and two murine epithelial cell lines.

At present, there are some major issues that require addressing before recommending approval for publication:

Major issues:

1. Outline the ethical approval for the murine study

Reply: Thank you. We have supplemented and outlined the animal ethical approval.

Changes in the text: All animal studies were approved by the Institutional Animal Care and Use Committees of the Seven Medical Center of PLA General Hospital; studies were performed in adherence with the international Guide for the Care and Use of Laboratory Animals (see Page 5, line 111-113).

2. Migration assay missing from methods section

Reply: Thank you. We have supplemented the method description for migration assay.

Changes in the text: The migration assay was performed using Transwell plates (Costar, USA). During this assay, lung epithelial cells (5×104 in 100 μL serum-free medium) were placed on the upper layer of a cell culture insert with permeable membrane and a complete medium was placed below the cell permeable membrane. The chamber was incubated in a humidified environment with 5% CO2 at 37°C for 24 h. Cells that had not migrated through the membrane were removed from the upper surface with a cotton swab; then migrated cells on the lower membrane surface were fixed and stained with crystal violet; then counted and photographed in five random fields (see Page 9, line 197-205).

3. Statistical analysis: It is not stated by the authors how normality was tested for. This needs to be added to justify why a t-test & ANOVA test were used.

Reply: Thank you for the suggestion. We have supplemented the text description about how and why performing these statistical analyses.

Changes in the text: The normal distribution of data was firstly identified by using the Kolmogorov-Smirnov test. Then, for quantitative variables with normal distribution, comparison among them was performed by using one-way analysis of variance (ANOVA) and post hoc Tukey test; for quantitative variables without normal distribution, the non-parametric test (Kruskal–Wallis) was used. The qualitative variables using the Chi-square test and the Student's t-test. The association between the various factors was determined using Pearson's correlation. There was a statistically significant difference in data at $P<0.05$ (see Page 9, line 209-215).

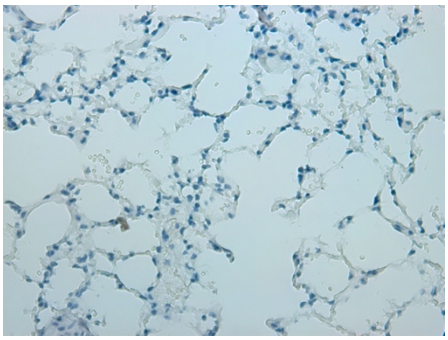
4. line 44: formation of cellulite? Clarify this, very unclear.

Reply: Thank you for the comment. We have deleted the words in order to make the description clearer and more accurate, after reviewing the reference [1].

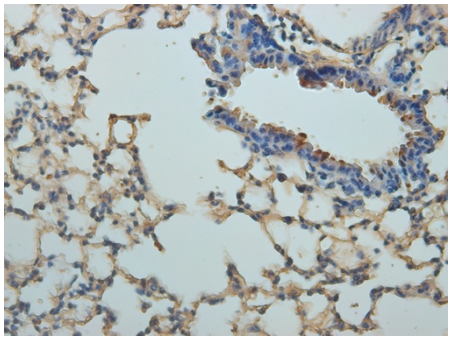
Changes in the text: Idiopathic pulmonary fibrosis (IPF) is an irreversible, progressive, and fatal chronic pulmonary fibrosis disease, characterized by diffuse pulmonary interstitial fibrosis with

mild inflammation, appearance of fibroblastic foci and deposition of matrix… (see Page 3, line 49-51).

5. Immunohistochemistry: Differing magnifications reported: 20x magnification in methods, 200x magnification in the results. No scale bars for both low and higher magnification images. Need scale bars inserted in both figure 1 & 2. Also, need positive and negative controls for Nogo-B antibody. Figure 1: Insert into figure legend re NC = normal control. Clarify at what day the 'model' images are taken at for figure 1 (i.e. 7 or 28).
"Infiltrated inflammatory cells were increased" – need to clarify WHAT cells were increased and WHERE.
Figure 4I: scale bars missing.
Reply: Thank you for the comment. We apologized for the mislabeling in the methods; moreover, revised the associated text and supplemented the scale bars in the figures including scale bars and indicated days, according to your suggestions. Moreover, when performing an IHC assay every time, we added the negative and positive controls for Nogo-B antibody as a technical reference; representative control images are presented below. In addition, we described the infiltrated inflammatory cells with more details- infiltrated inflammatory cells including neutrophils, lymphocytes, and eosinophils were increased, mainly in the interalveolar septa regions.
Changes in the text: Representative Images of Masson staining and Nogo-B immunohistochemical staining control lung tissues and lung tissues at the 28th day from a pulmonary fibrosis mouse model at a magnification of ×200 and then representative fields were chosen for presentation. NC: normal control. Scale bars show 50 μm. …… (see Page 22, line 486-489).
Representative images of Masson staining and Nogo-B immunohistochemical staining control lung tissue and lung tissue at the 7th and 28th day during constructing a pulmonary fibrosis model of Nogo-B knockout mice at a magnification of ×200 for presentation. NC: normal control. Scale bars show 50 μm…….(see Page 22, line 494-497).
……infiltrated inflammatory cells including neutrophils, lymphocytes, and eosinophils were increased, mainly in the interalveolar septa regions…… (see Page 10, line 229-231).



Negative control:
primary antibody is substituted by PBS



Positive control
Proven positive expression of Nogo-B in pilot experiment.

6. Western blotting for only one EMT marker, loss of E-cadherin and gain of N-cadherin. Paper would be strengthened with blotting for another EMT marker, e.g. vimentin? Quality of western

blotting is quite poor, e.g. figure 3A p-PERK WB is very blurred and difficult to interpret, thereby I question the accuracy of the densitometry in figure 3B. Poor quality alpha-SMA blot in figure 3G. Figure 3G and 3I: separate blots for control and shRNA-NogoB: quality of experiments would be strengthened if all run simultaneously.

Reply: Thank you for the comment. We revised and updated the associated text and figures according to your suggestions. Firstly, we supplemented the blotting data for another EMT marker-vimentin. Secondly, we updated the poor qualified blotting images for p-PERK and α-SMA. Finally, limited by stored cell extract amount, we had not integrated the blots for control and shRNA-Nogo-B, but the quantification of western blots was analyzed densitometrically related to the internal sampling reference-β-actin. Hence, the results are credible and strong.

7. Line 233 and 234: if increase in alpha-SMA was only seen in MLE-12 cells, needs explanation of why it was not observed in TC-1 JHU-1 cells before making the suggestion that inhibition of Nogo-B is involved in fibrogenesis in lung epithelial cells.

Reply: Thank you very much for the comment. When Nogo-B existed, TGF-β1 resulted in the decrease of ZO-1 and SPC as well as the increase of α-SMA and MMP4 in MLE-12 and TC-1 JHU-1 cells; after Nogo-B knockdown, TGF-β1-induced decrease of ZO-1, SPC and increase of α-SMA, MMP4 were not observed in these cells. Of note, we abandoned blurred α-SMA band images and performed a western blotting; then re-quantified a densitometrical data from α-SMA band of MLE-12 cell extract using QuantityOne software and performed a statistical analysis and got an updated P value (0.0513; previously 0.0492). Accordingly, we updated the result description and discussion. Although genetic heterogeneity exists among cell lines, MLE-12 and TC-1 JHU-1 still posed a consistent performance.

Changes in the text: however, after Nogo-B knockdown, TGF-β1-induced decrease of ZO-1, SPC and increase of α-SMA, MMP4 were not observed in MLE-12 and TC-1 JHU-1 cells (Fig. 3G-J), suggesting the inhibition of Nogo-B in fibrogenesis in lung epithelial cells (see Page 12, line 308-310).

8. EMT references in Discussion (lines 255 – 256) are a cystic fibrosis paper and a review paper. Need EMT references which refer to Interstitial lung diseases, ? accuracy of 50% of total number of myofibroblasts.

Reply: Thank you for the comment. We updated the associated reference according to your suggestion.

Changes in the text: Kage H and Borok Z. EMT and interstitial lung disease: a mysterious relationship. Curr Opin Pulm Med. 2012;18:517-23 (see Page 17, line 443-444).

9. Discussion section needs a LIMITATIONS paragraph, outlining the limitations of the current study, particularly the fact that it is purely a murine study with no human samples.

Reply: Thank you for the suggestion. We added the associated description for discussing the limitation of the current study.

Changes in the text: There are several limitations in our study. We had not elucidated the exhaustive molecular mechanism for explaining the regulation of Nogo-B in the expressions of

ERS- and EMT-markers at transcriptional or post-transcriptional levels, which consequently leading to lung fibrogenesis. Moreover, exact role of Nogo-B in constructing the association between ERS and EMT in lung fibrosis is still to be verified. In addition, the current study is purely a murine study without human samples (see Page 15, line 389-395).

10. Study would be greatly strengthened with a figure 5 with some human data.
Reply: Thank you for the comment. We discussed the issue in the LIMITAIONS of the DISCUSSION section. Further study will be performed according to your suggestion.
Changes in the text: more detailed studies in human samples or disease models are needed to determine the feasibility and therapeutic benefit of targeting Nogo-B in pulmonary fibrotic diseases in the future. (see Page 15, line 399-401).

Other MINOR issues:
Line 101 – 102: Primary antibody for Nogo-B: more details required including Company, code number, host species etc
Reply: Thank you for the comment. Information including company, code number and host species about antibodies used in the study were supplemented in the Methods.
Changes in the text: ……membranes were incubated with a primary antibody including sheep anti-mouse Nogo-B (1:1000; AF6034), rabbit anti-mouse total or phospho-PERK (all, 1:1000; #3192 and #3179), rabbit anti-mouse total or phospho-IRE1α (1:1000; #3294 and PA1-16927), rabbit anti-mouse c-ATF6 (1:1000; ab203119), rabbit anti-mouse ZO-1 (1:1000; ab96587), rabbit anti-mouse SPC (1:1000; ab196677), rabbit anti-mouse α-SMA (1:1000; ab108424), rabbit anti-mouse MMP4 (1:500; ab51074), sheep anti-mouse E-cadherin (1:1000; sc-1500), rabbit anti-mouse N-cadherin (1:1000; SC-7939) or rabbit anti-mouse vimentin (1:1000; sc-5565) respectively from R&D system, Cell Signaling Technology Company, Thermo Scientific™, Abcam and Santa Cruz Biotechnology Company, overnight at 4°C. (see Page 8, line 182-190).

Figure 2C & D, E & F: clarify n-number. Densitometry looks overestimated compared to subtle changes seen at western blot level.
Reply: Thank you for the comment. We performed three times repetition and chose the images close to median value; moreover, we double-checked the densitometric data and confirmed its accuracy.

Figure 2H: re-formatting of graph required
Reply: Thank you for the comment. We have finished the required reformatting of graphs according to your suggestion.

Line 214: insert ‘at 24 hours’
Reply: Thank you. We feel confused about the comment. Nogo-B knockdown in these cells were performed by using lentivirus-induced shRNA silencing and these cells harbored stable knockdown of Nogo-B.

Changes in the text: "meanwhile both in MLE-12 and in TC-1 JUH-1, the ratio of phosphor-PERK related to total PERK was significantly decreased while the levels of phosphor-IRE1α/IRE1α and cleaved ATF6 (c-ATF6) were not different". (see Page 12, line 288-290).

Line 222: TGF-beta 10ng/mL x 24 hours was chosen, justify WHY this dose
Reply: Thank you. We chose the treated dose and duration, mainly based on our previous pilot study combined with others' published study, labelled as ref [6][9].

Line 280: outline the abbreviation HGF
Reply: Thank you. We outlined the abbreviation of HGF as hepatocyte growth factor.
Changes in the text: Nogo-B can promote hepatocyte growth factor (HGF)-induced hepatocyte proliferation (see Page 15, line 365-366).

Line 289 – 295: these are RESULTS and should not be placed in the DISCUSSION section. Discussion section should outline the implications of your results & how it fits with the current available research
Reply: Thank you for the suggestion. We re-organized the associated text according to your comment.
Changes in the text: Mechanistically, Nogo-B knockdown inhibited the increase of TGF-β1-induced EMT or fibrogenesis-related markers expression; meanwhile suppressed the upregulation of TGF-β1-induced PERK branch of ERS pathway (see Page 16, line 380-383).

Line 309: in vitro and in vivo should be in italics
Reply: Thank you for the comment. We rewrote these words in italics.
Changes in the text:… from mouse model *in vivo* and cell lines model *in vitro*;… (see Page 17, line 418).

In addition, there are numerous grammatical issues, errors in spelling and excessively long sentences throughout the paper. These make the paper in its current state difficult to read and to fully interpret. Serious attention should be paid to addressing this.
Reply: Thank you for the comment. Our manuscript has been comprehensively edited by an English native-speaker, professor Peter Wong from Boston University, for twice. We expressed our acknowledgement in the revised manuscript. In addition, we also check it repeatedly and hope to avoid any grammar and spelling mistakes.

For example:
Line 21: pathological mechanisms of fibrogenesis in IPF IS still to be elucidated to 'the pathological mechanisms of fibrogenesis in IPF ARE still to be elucidated.
Reply: Thank you for the comment. We rewrote the sentence by using the correct grammar.
Changes in the text: pathological mechanisms of fibrogenesis in IPF are still to be elucidated (see Page 2, line 23).

Line 22: investigated the potential roles of Nogo-B on pulmonary fibrogenesis to 'the potential role for Nogo-B in pulmonary fibrogenesis

Reply: Thank you for your kindness. We revised the sentence.

Changes in the text: we investigated the potential role of Nogo-B in pulmonary fibrogenesis (see Page 2, line 24).

Line 51: change resulted to resulting

Reply: Thank you for your kindness. We revised the sentence.

Changes in the text: The formation of lung fibrosis is complex, resulting from a persistent irritant that……(see Page 3, line 59).

Lines 54 – 58: extremely long sentence. Consider shortening into two. Line 56: should read microRNAs and endoplasmic reticulum stress are involved in EMT. Line 58: change to in the search for new methods of fibrogenesis and for new drugs to treat IPF.

Reply: Thank you for your kindness. We revised the sentence.

Changes in the text: studies show that epithelial-mesenchymal transition (EMT) is a key step of pulmonary fibrosis; meanwhile, some key signaling pathways, microRNAs and ERS are involve in EMT [5, 6].Hence fully understanding the role of EMT in the development of pulmonary fibrosis will be conducive in search for new methods and new drugs for the treatment of IPF (see Page 3, line 62-66).

Line 71. New sentence after fibrogenesis. Mechanistically, we further demonstrate

Reply: Thank you for your suggestion. We revised the sentence.

Changes in the text: ……is an important mediator for lung fibrogenesis. Mechanistically, further demonstrate that Nogo-B promotes EMT in lung fibrosis via PERK pathway……(see Page 4, line 93-94).

Line 87: typo, change BLAF to BALF

Reply: Thank you for your suggestion. We revised the sentence.

Changes in the text: …… Lung tissue and BALF were collected for IHC analysis and detection of molecular markers…… (see Page 5, line 111-112).

Line 90: 'for designed' – poor English, consider rephrasing.

Reply: Thank you for your suggestion. We deleted these words.

Line 140 – 141: This should read: The manufacturers operation manual was followed at all times ??

Reply: Thank you for your suggestion. We changed the sentence to "The assay was performed according to the manufacturer's protocol".

Changes in the text: The assay was performed according to the manufacturer's protocol. (see Page 6, line 175-176).

Line 152: from and?

Reply: Thank you for your comment. We deleted the extra word “and”.

Line 154: typo – diluted
Reply: Thank you for your kindness. We corrected the misspelling.
Changes in the text: ……diluted in TBST with 1:2000 (see Page 8, line 195).

Line 170 – 172: is it in the present or past tense? Be consistent throughout
Reply: Thank you. We revised the words tense and maintained the consistence throughout the whole manuscript.

Line 179: levels of some cytokine TGF-beta ?
Reply: Thank you. We changed “some” to “the”.
Changes in the text: Meanwhile, levels of the cytokine TGF-β and pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 were significantly increased (see Page 8, line 195).

Line 201 – 202: when absence? – change to when Nogo-B is absent?
Reply: Thank you. We revised the sentence.
Changes in the text: when Nogo-B was absent (see Page 11, line 276).

Line 202: more researches indicate? Poor grammar, needs changing
Reply: Thank you. We revised the sentence.
Changes in the text: some researches have indicated the association of EMT and ERS (see Page 11, line 2676-277)

Line 256 – 257: mixture of present and past tense, be consistent throughout
Reply: Thank you. We revised the sentence.
Changes in the text: As the number of fibroblasts increased, collagen and extracellular matrix gradually accumulated (see Page 14, line 339-340).

Line 261: use another word in place of retardment
Reply: Thank you. We changed “retardment” to “inhibition”.
Changes in the text: the lung tissue of Nogo-B knockout mice posed a significant inhibition of pulmonary interstitial fibrosis (see Page 14, line 344-345).

Line 270: change the colloquial manner of ‘so how does Nogo-B affect EMT response’. Scientific language should be maintained throughout the paper
Reply: Thank you. We revised the sentences.
Changes in the text: However, what’s the molecular mechanism of Nogo-B affecting EMT in lung fibrogenesis? This question requires us to verify through further experiments (see Page 15, line 357-3459).

Line 274: change to ‘it was recently reported that….’
Reply: Thank you. We revised the sentence according to your suggestion.
Changes in the text: It was recently reported……(see Page 15, line 362).

Line 275: delete the ‘a’
Reply: Thank you. We revised the sentence according to your suggestion.
Changes in the text: recent research demonstrated that ……(see Page 15, line 345).

Line 276: change upregulated to upregulation
Reply: Thank you. We revised the sentence according to your suggestion.
Changes in the text: recent research demonstrated that upregulating Nogo-B expression helps to inhibit ……(see Page 15, line 363-364).

Line 403: insert space bar between Nogo-B and expression
Reply: Thank you. We revised the sentence and other similar errors according to your suggestion.

Line 413: insert space bar between Nogo-B and immunohistochemical
Reply: Thank you. We revised the sentence according to your suggestion.
Changes in the text: Representative Images of Masson staining and Nogo-B immunohistochemical staining control lung tissues and lung tissues (see Page 22, line 515, 522).