Peer Review File

Article information: https://dx.doi.org/10.21037/jtd-23-1145

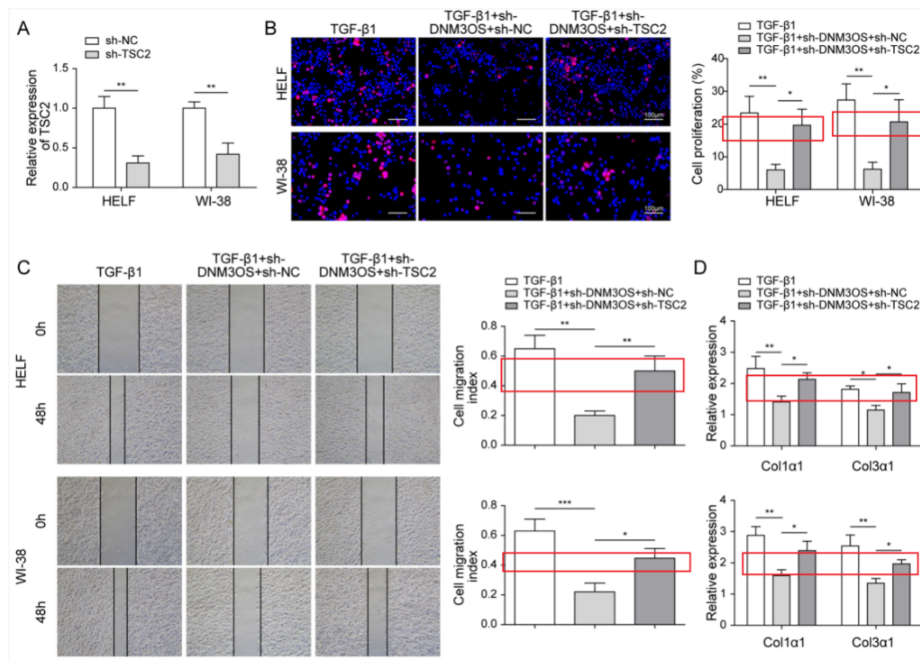
Reviewer A

In this manuscript Lv et al describe a novel mechanism by which the lncRNA DNM3OS may contribute to pulmonary fibrosis. This lncRNA has already been described as a TGFb inducible pro-fibrotic lncRNA but the mechanism was attributed to the three distinct profibrotic mature miRNAs (i.e., miR-199a-5p/3p and miR-214-3p), which influence SMAD and non-SMAD components of TGF-b signaling (PMID: 30964696). Lv et al instead described a mechanism in which DNM3OS binds EZH2 to recruit it to the TSC2 promoter to silence it.

Major criticisms

For knockdown experiments throughout the manuscript only a single shRNA and single control shRNA were used. It is important that multiple shRNAs be used for each target to confirm any major findings.
Reply: Thanks very much for your suggestion. We provide additional results in Figure 3 using a total of 3 different shRNA targeting DNM3OS, of which 2 shRNAs exhibited significant knockdown efficacy of DNM3OS. Therefore, these 2 shRNAs were used for the following experiments.
With the additional shRNA, we showed similar effect of DNM3OS targeting shRNA in regulating cell proliferation and migration, as well as the expression of EMT-related markers.
Please refer to Page 13, Line 124-136 and Figure 3 in the marked manuscript for detail.

Given that DNM3OS has already been shown to promote fibrosis through a miRNA-based mechanism the authors need to rule out the function of miRNAs in their system.
Reply: Thanks very much for your comments. LncRNA has shown its versatile functions through various mechanisms including RNA-RNA, RNA-DNA, and RNA-protein interactions (PMID: 36596869). As shown in Figure 5B-5D, knockdown of TSC2 partially reversed the effects of sh-DNM3OS, but could not completely restore to the levels in TGF-β1 treatment, insinuating that there were additional pathways involved in lncRNA DNM3OS-mediated lung fibrosis. For instance, prior study reported the role of lncRNA DNM3OS in regulating lung fibrosis through three distinct profibrotic mature miRNAs (PMID: 30964696). Furthermore, lncRNA DNM3OS modified H3K27 methylation of TIAM1 promoter in liver cancer by interacting with KDM6B (PMID: 33472090). Therefore, lncRNA DNM3OS, like other lncRNAs, exerts its functions in many ways. It is difficult for us to exclude the possible involvement of miRNA or other mechanisms in the current study, but we would like to pursue it in the future. We also added this information in the Discuss section, please refer to Page 16, Line 200-209 in the marked manuscript.



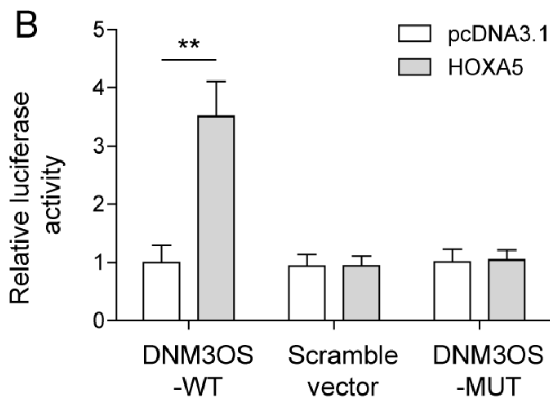
No work has been done in primary cells or patient samples to confirm that this is not a cell line phenomenon. Does TSC2 expression correlate with fibrosis?

Reply: Thanks very much for your comment. We examined lncRNA DNM3OS expression in the blood from 8 patients with idiopathic pulmonary fibrosis and found that DNM3OS expression was elevated (Supplementary Figure S1A). Please refer to Page 12, Line 98-101 and Supplementary Figure1A in the marked manuscript.

Moreover, results of figure 5D revealed that TGF-β1-induced expression of Col1α1 and Col3α1 was suppressed by sh-DNM3OS but partially rescued by further knockdown of TSC2 (Figure 5D), suggesting the role of TSC2 in regulating fibrosis, which is consistent with prior reports (PMID: 28041914, 31461347, 33634104). These evidences indicate that the expression of TSC2 is associated with fibrosis.

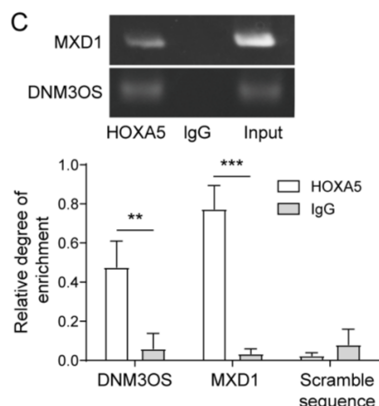
Luciferase assay in figure 2 is lacking proper controls. A vector containing a scrambled binding site should also be included.

Reply: Thanks very much for your suggestion. According to the suggestion, we supplemented a vector control containing a random sequence and conducted the experiment again. The result showed that the luciferase activity in DNM3OS-WT expressing cells were significantly increased, whereas cells expressing scramble vector or DNM3OS-MUT didn't show an elevation of luciferase activity after HOXA5 overexpression. Please refer to Page 12, Line 112-115 and figure 2B in the marked manuscript.



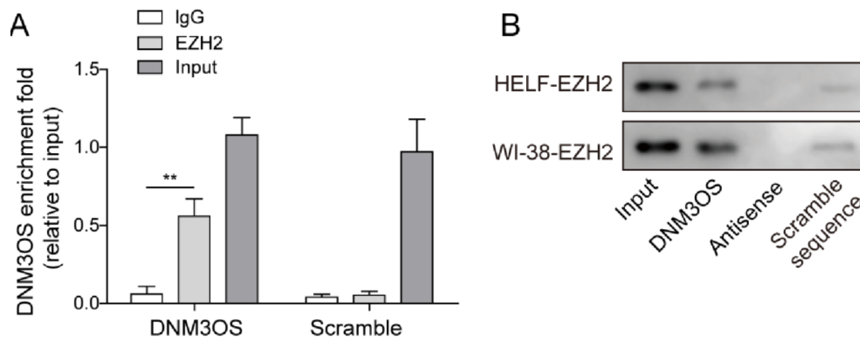
ChIP experiment in figure 2 is lacking proper controls. Should amplify both a known binding site and a non-bound sequence from another active gene.

Reply: Thanks very much for your suggestion. According to the suggestion, we supplemented a positive control MXD1 (PMID: 36167790), a negative control with random sequence and conducted the experiment again. The result showed that HOXA5 antibody enriched a large amount of MXD1 and DNM3OS, but not the scramble control, while IgG control failed to pull down any targets examined. Please refer to Page 12, Line 115-119 and figure 2C in the marked manuscript.



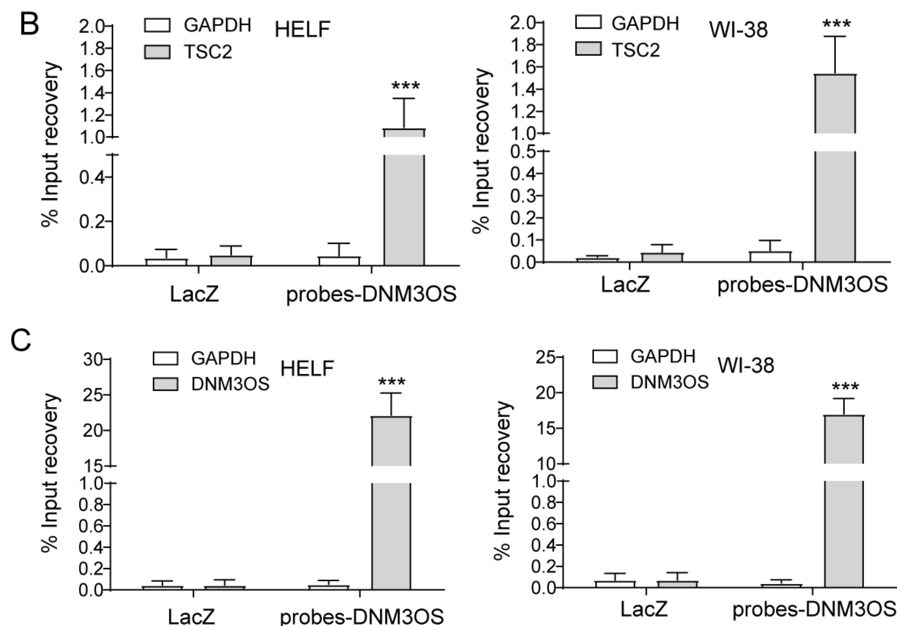
RIP pull down in figure 4 is lacking proper controls. RNAs that do and do not bind to EZH2 should be included as controls.

Reply: Thanks very much for your suggestion. We added a vector control containing a random sequence and conducted the experiment again in Figure 4A-B according to the suggestion. For instance, the result of Figure 4A showed that EZH2 specifically bound to DNM3OS but not the scramble control, the IgG control didn't bind to DNM3OS or the scramble control. Please refer to Page 13-14, Line 139-144 and figure 4A-B in the marked manuscript.



In figure 4 a ChIRP assay to test whether DNM3OS in bound to the TSC2 promoter would be strengthen the proposed mechanism.

Reply: Thanks very much for your suggestion. We performed a ChIRP assay in Supplementary Figure 1B-C. Result revealed that DNM3OS effectively enriched the promoter region of TSC2, demonstrating that there is the interaction between DNM3OS and TSC2 promoter. Please refer to Page 14, Line 146-147 and Supplementary Figure 1B-C in the marked manuscript.



The conclusion from figure 5 that DNM3OS promotes fibrosis is overstated. Fibrosis was not tested and so this conclusion cannot be made.

Reply: Thanks very much for the reviewer’s comment. We apologize for the overstating description. According to the suggestion, we have changed “fibrosis” to “fibrosis-related genes”. We have carefully checked our manuscript and modified accordingly. Moreover, we found that the expressions of fibrosis-related genes (Col1α1 and Col1α1) were downregulated after knockdown of DNM3OS in figure 5D. Furthermore, with all the experiments were performed in human embryo lung fibroblast (HELF and Wi-38) and treatment with TGF-β1. IPF patients showed significant higher production of TGF-β1 compare to healthy controls (PMID: 26921108, 29126797). TGF-β1 is known to be a crucial pro-fibrosis factor in IPF (PMID: 37643008, 34013369), and treatment with TGF-β1 promotes the expression

of fibrosis-related genes (PMID: 37710230). Inhibition of TGF-β production prevents IPF progression (PMID: 35069531). Remarkably, our study analyses of 8 patients with IPF also provided additional verification of the role of DNM3OS in pulmonary fibrosis. We added the information in the Discussion section. Please refer to Page 15, Line 181-188 in the marked manuscript.

Similar over statements are made in the discussion.
-"our study revealed that HOXA5 promotes IPF via inducing the transcription of the lncRNA DNM3OS." There were no IPF models in the manuscript.
-"The HOXA5-DNM3OS-EZH2-TSC2 axis 238 is essential for fibrosis progression, and targeting this axis holds great potential to treat lung fibrosis." Fibrosis progression was not tested in this manuscript.
Reply: Thanks very much for the reviewer's comment and we apologize for the inappropriate description. We have revised the description about IPF model and pulmonary fibrosis for the whole manuscript. Please refer to the revised manuscript.
Moreover, results of figure 3D and 5D revealed that downregulation of fibrosis-related genes Col1α1 and Col1α1 after knockdown of DNM3OS. With all the experiments were performed in human embryo lung fibroblast (HELF and Wi-38), our results established the connection between DNM3OS and fibrosis. In addition, compare to healthy controls, IPF patients showed significant higher production of TGF-β1 (PMID: 26921108, 29126797), a crucial pro-fibrosis factor in IPF (PMID: 37643008, 34013369). Treatment with TGF-β1 promotes the expression of fibrosis-related genes (PMID: 37710230), while inhibition of TGF-β production prevents IPF progression (PMID: 35069531). The analyses of 8 patients with IPF also provided additional verification of the role of DNM3OS in pulmonary fibrosis. We added the information in the Discussion section. Please refer to Page 15, Line 181-188 in the marked manuscript.

Reviewer B

1. Figures:
   1) Please indicate the staining method in Figure 3B-C and Figure 5B-C legends.

Reply: Thanks very much for editor's suggestions. In Figure 3B and 5B, A fluorescent EdU probe was used to track the newly synthesized DNA. The nucleus was stained by Hoechst 33342. The wound healing assay was performed for Figure 3C and 5C, which is not needed to staining. These contents were added in the legend. Please refer to the revised manuscript for detail.

   2) Please unify the words in Figure 3D with the text and legend.

145 attenuated TGF-β1-induced Col1α1 and Col3α1 expression (**Figure 3D**). Moreover, TGF-β1-induced

Reply: We apologize for the inconsistency in the figures and texts. We now unified the words with "CoL1α1" and "CoL3α1". Please refer to the revised manuscript and figures for detail.

3) The citations of Figure 5F,5G are missing in the main text. Please check and revise.

Reply: We apologize for the errors in Figure 5. The citations of Figure 5 in the main text are correct, we have corrected the labeling in Figure 5. Please refer to the revised figures for detail.

4) You cited wrong Figure in the below sentence.

179 DNM3OS cells but was normalized in cells expressing sh-DNM3OS plus sh-TSC2 (**Figure 5D**). The

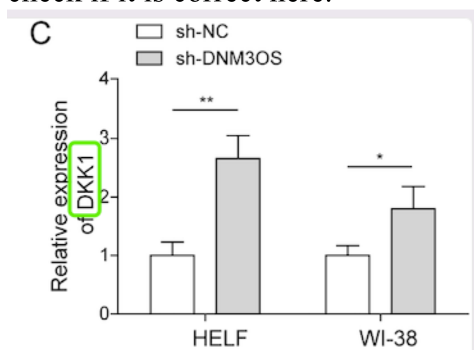
180 expression of α-SMA, vimentin, and fibronectin showed similar trends (**Figure 5E**). These data

Reply: We apologize for the errors in Figure 5. The citations of Figure 5 are correct in the main text, we have corrected the labeling in Figure 5. Please refer to the revised figures for detail.

5) The "***" is missing in Figure 4 but indicated in Figure 4 legend. Please check and revise.

Reply: We apologize for this careless error. We carefully checked the figures and legends and changed accordingly. Please refer to the revised manuscript for detail.

6) "DKK1" cannot be found in the text, but mentioned in Figure 4C. Please check if it is correct here.



Reply: We apologize for the typo in Figure 4C. The "DKK1" should be "TSC2". We have changed it in Figure 4C. Please refer to the revised figures for detail.

7) Please add the figure legends for Figure 5F, 5G.

Reply: We apologize for the errors in Figure 5. The citations of Figure 5 are correct in the main text, we have corrected the labeling in Figure 5. Please refer to the revised figures for detail.

8) The citations of "*" are missing in Figure S1. Please check and revise.

Reply: We apologize for this error. There is no "*" in Figure S1, so we deleted it from the legend. Besides, we checked all figures and legends to make sure the consistency. Please refer to the revised manuscript for detail.

9) Please provide a summarized legend for Figure S1.

Reply: We apologize for this misunderstanding. The legend for Figure S1 was provided in the previous manuscript. However, the content in Figure S1 is very different, so we couldn't provide a title for this figure. We provided detailed information for each experiment. Please refer to the revised manuscript for detail.

10) Abbreviations in all figures and legends should be explained. TGF-β, HELF and LncRNA in Figure 1 for example. Please check all your figures.

Reply: We apologize for the missing of full names for the abbreviations. We checked all legends and provided the full names for them. Please refer to the revised manuscript for detail.

2. Please use the full name and abbreviations of "EdU/TGF-β1/EZH2/TSC2" in the Abstract. Please also check through your article to ensure all the abbreviated terms have been defined when they first appear in the Abstract, highlight box and main text.

Reply: Thanks very much for editor's suggestion. We have provided the full name a of "EdU/TGF-β1/EZH2/TSC2" in the Abstract. Moreover, we have checked the article and added the full name of all the abbreviated terms when they first appear. Please refer to the manuscript for detail.