Author's response to reviews

Title: Transgenically-expressed secretoglobin 3A2 accelerates resolution of bleomycin-induced pulmonary fibrosis in mice

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Author's response to reviews: see over
Dear Editor:

We wish to submit our revised manuscript entitled “Transgenically-expressed secretoglobin 3A2 accelerates resolution of bleomycin-induced pulmonary fibrosis in mice” for publication in the BMC Pulmonary Medicine.

First of all, we would like to thank the expert reviewers for their thorough readings of our originally submitted manuscript and many valuable comments and critiques. In the revised manuscript, we feel that we have addressed most of the questions/comments. The point-to-point details are as described below.

I sincerely hope that our manuscript is now suitable for publication in the BMC Pulmonary Medicine.

Sincerely,
Shioko Kimura

Reviewer 1's comments:

Major comments:
This is a report by Cai et al examining how transgenically-expressed secretoglobin 3A2 (SCGB3A2) accelerates resolution of pulmonary fibrosis in mice using the bleomycin-model. This is an interesting study as SCGB3A2 has been shown previously to have anti-fibrotic activities. Their research shows that transgenic mice express SCGB3A2 protein approximately 5 fold higher than WT mice and interestingly, while the transgenic mice had exacerbated fibrosis 3 weeks post challenge, they more rapidly resolved inflammation by 6 weeks when compared to WT mice. Overall, this is a wonderful investigation using a novel mouse that furthers our understanding of SCGB3A2 and provides a need to explore its potential as a therapeutic for IPF.

Minor comments:
1. Please describe where SCGB3A2 antibody is from (line 162).

R: The antibody was previously produced in our laboratory, and the purified IgG was used for current experiments. The details were now added to the revised manuscript (line 165-167).

2. Lines 204-205 grammar should be corrected to be something more like this “….using methods previously described (ref).”

R: The English grammar was modified according to this suggestion. The section including this sentence is now in the Supporting Materials section (line 16).
3. The section starting at line 362 is very long and should be divided up into smaller paragraphs.

R: This section was divided into two shorter sections as recommended; one mainly describing characteristics based on histological findings, while the second describes changes in gene expression patterns.

4. wording in lines 387-388 is awkward and should be adjusted.

R: The wording was adjusted so that the sentence reads more smoothly (line 400-403).
Reviewer 2's comments

General overview:
In this manuscript, Cai and co-authors describe a transgenic mouse that overexpresses Scgb3a2 under control of the human Surfactant Protein C promoter. While this mouse has no observed phenotype under homeostatic conditions, the authors show that Scgb3a2-overexpressing mice have an altered response to intratracheal bleomycin. At three weeks post-bleomycin, transgenic mice have increased inflammation and fibrosis, while at 6 and 9 weeks post-bleomycin the transgenic mice have decreased fibrosis and collagen deposition compared to wild type controls. Lastly, the authors use microarray analysis of transgenic mice to suggest that Scgb3a2 overexpression may alter cellular homeostasis and regulation of inflammation, thus making mice potentially more susceptible to the early effect of bleomycin treatment. Overall, the experiments are thorough with appropriate controls. This is an interesting report with potentially useful therapeutic implications for pulmonary fibrosis. Specific comments and questions are stated below:

Major Compulsory Revisions

1. It is unclear why the authors chose a constitutively-active overexpression of Scgb3a2, compared to an inducible system. Additionally, it is unclear why the authors chose to overexpress Scgb3a2 in alveolar epithelial cells, rather than airway epithelium. Additional explanation in the text would be useful.

R1: The frequently used lung-specific inducible system uses tetracycline (Tichelaar, J.W., et al., J Biol Chem, 275, 11858-11864, 2000) as the agent to regulate genes of interest. However, we avoided using this system because 1) the method for producing bleomycin-induced pulmonary fibrosis in mice has been established in our laboratory, and 2) whether and/or how tetracycline affects formation and/or resolution of bleomycin-induced pulmonary fibrosis are not known thus complicating interpretation of our results. Further in our previous study using the bleomycin-induced pulmonary fibrosis model, SCGB3A2 was administered to mice through the tail vein, which resulted in decreased fibrogenesis (Kurotani, R, et al, J Biol Chem, 286, 19682-19692, 2011; Cai, Y., et al., Am J Physiol Lung Cell Mol Physiol, 306, L10-L22, 2014). In the latter system, we believe that SCGB3A2 reached the blood circulation via the distal as well as proximal areas of lungs at similar levels. Accordingly, we chose to use overexpression of SCGB3A2 in alveolar epithelial cells to particularly determine whether and/or how overexpression of SCGB3A2 in alveolar areas affects fibrosis. Accordingly, the additional sentences were added to clarify this issue in the Discussion (line 477-487).

2. While it is clear that there is no observed homeostatic phenotype, the microarray analysis suggests that there are clearly cellular signaling alterations induced by continuous Scgb3a2 overexpression, in particular cellular metabolism and control of inflammation. The authors should discuss this apparent
discrepancy, and also describe whether any metabolic or inflammatory abnormalities have been identified in transgenic mice to support the microarray data.

R2: The sentence “the changes may be too subtle to affect lung homeostasis without challenge, since we did not detect any gross metabolic or abnormal inflammatory phenotypes in the transgenic mice” was added to address these comments in the Discussion (line 518-521).

3. The authors describe previous reports of Scgb3a2 anti-fibrotic activity via STAT1 phosphorylation, SMAD7 expression, and inhibition of TGF-beta and SMAD2 phosphorylation. This data should be included in the current study, as it would provide some potential cellular mechanism for the observed anti-fibrotic phenotype. Furthermore, microarray data of transgenic (vs wild type) mice treated with bleomycin would potentially be useful to evaluate a mechanism for the accelerated reversal 6 weeks after bleomycin.

R3: We agree with the reviewer that it is critical to determine whether the same mechanism involving the TGFbeta signaling is responsible for the currently observed anti-fibrotic phenotype of the Scgb3a2 transgenic mouse lungs and what the cellular mechanism is for the resolution of fibrosis and its acceleration by SCGB3A2 in post-BLM periods. Together with microarray analysis using more post-BLM time points with wild-type and transgenic mice, our future studies aim to determine the mechanism how spontaneous resolution of fibrosis occurs, and how it is accelerated by SCGB3A2 in post-BLM lungs.

4. The authors should reconcile two apparently contradictory statements – 1) that Scgb3a2 overexpression predisposes mice to a more severe phenotype 3 weeks after bleomycin, and 2) that Scgb3a2 overexpression is anti-fibrotic and accelerates recovery from bleomycin at 6 weeks after injury.

R4: As the reviewer noted, SCGB3A2 overexpression predisposes mice to a more severe phenotype 3 weeks post bleomycin, at which time SCGB3A2 level in the transgenic lungs is almost the same as that of wild-type (see Fig. 4A), suggesting that changes in gene expression patterns predominate in determination of the phenotype. However, the transgenic SCGB3A2 expression level drastically increases by 6 weeks and is significantly higher than that in wild-type lungs and reaches levels similar to those found in the pre-bleomycin treated mice. This rapid increase coincides with the accelerated recovery from bleomycin-induced injury in transgenic mice, suggesting the anti-fibrotic activity of SCGB3A2. Accordingly, more discussion was added to the revised manuscript (line 528-545, 553-556).

5. For Figure 1F, the immunohistochemistry for both SP-C and Scgb3a2 is not impressive. For the DAB staining, images with improved signal relative to hematoxylin counter-stain is needed. Additionally, co-immunofluorescence for SPC and Scgb3a2 is needed to convincingly prove co-localization.
R5: We improved the images of immunohistochemical staining in Figure 1F. We also added immunofluorescence image for co-expression of SP-C and SCGB3A2 in type II cells in Figure 1G. Accordingly the Method for “Immunofluorescence” (line 221-233) and the legend for the new figure were added.

6. For Figure S1, it is not clear why ex vivo organ culture studies are appropriate for this study. Previous studies applied exogenous Scgb3a2 to cultured embryonic lungs, while this system utilizes overexpression. Analysis of branching of embryonic lungs at different stages are appropriate, but should be done immediately after dissection. Furthermore, given that the SP-C promoter is not active at E11 (and no measurement of Scgb3a2 expression was done at this time point), E11 is not an appropriate time point for this analysis. Analysis of lung development and branching morphogenesis in transgenic mice is appropriate, but at later stages.

R6: The human SP-C promoter transgenic construct was obtained from Dr. Jeffrey Whitsett (Cincinnati Children’s Hospital Medical Center). This promoter drives transgene expression as early as E10 of mouse gestation in epithelial cells of primordial lung buds (Wert, SE. et al., Dev Biol, 156, 426-443, 1993). In our previous study using mouse embryonic lung ex vivo organ cultures (Am J Respir Crit Care Med, 78, 389-398, 2008), anti-SCGB3A2 antibody or siRNA for SCGB3A2 suppressed branching morphogenesis of embryonic lungs when added to the media or transfected into cultured lungs, suggesting that cultured embryonic lungs secrete SCGB3A2 into the media, which in turn promotes branching morphogenesis of the lung. Based on these results, we hypothesized that over-expression of SCGB3A2 would increase branching morphogenesis of embryonic lungs of transgenic mouse in ex vivo culture. However, as the reviewer pointed out, and as shown in the original Fig. S1A and B, there was very little difference in the levels of SCGB3A2 in the cultured media as well as branching degree between wild-type and transgenic mouse lungs. Apparently there was not sufficient overexpression of SCGB3A2 in the transgenic lungs as compared to wild-type at E11.5-12 that can affect branching morphogenesis. Ex vivo cultures of whole embryonic lungs at later gestation is difficult since the lung is much bigger in three dimensions, which prevents all lung cells from getting sufficient nutrients and oxygen, thus potentially producing non-reproducible results. There are no reports describing mouse embryonic lung ex vivo organ cultures using the whole lung to examine branching morphogenesis at the age older than E12.5. Accordingly, we have deleted the results shown in the original Figure S1A and B. The Results and the Discussion related to these results were also deleted from the revised manuscript.

7. For figure S1G, the immunohistochemistry of Scgb3a2 is not clearly demonstrated. Improved staining signal is needed, and immunofluorescence would be a good alternative. Additionally, co-IF of Scgb3a2 and SPC would be useful in this figure.

R7: We now show IHC results that more clearly demonstrate the expression of
SCGB3A2 in the new Figure S2E. We further added co-IF results showing cells co-expressing SCGB3A2 and SPC.

8. For bleomycin experiments, lower-magnification images of entire lung lobes would be useful to compare overall patterns of lung fibrosis.

R8: In the revised Fig. 2B, we now present lower-magnification images of entire lung lobes to replace the high-magnification images of the lungs shown in the old Fig. 2B.

9. For bleomycin experiments, survival data is needed (either added to Figure 2 or as a supplemental figure).

R9: We have added the Supplemental Figure S4 showing the survival data. There is no difference between wild-type and Scgb3a2-trasngenic mice. This was added to the Results section (line 381-382).

10. For Figure 4B, improved quality IHC is needed, as it is difficult to appreciate the colorimetric stain presented.

R10: We now have improved the IHC results in the revised Figure 4B.

11. In the discussion (lines 485-487), the authors state that ectopic overexpression of Scgb3a2 may have perturbed lung development. Do the authors have data to support this – i.e. microarray data from pre-natal timepoints or other data to support abnormal development in transgenic mice?

R11: We have deleted the sentence stating “the ectopic overexpression of SCGB3A2 may have perturbed expression of other genes that play a role in lung development as demonstrated by microarray analysis” since we do not have data to support this conclusion.

12. In the discussion (lines 498-499), the authors state that Scgb3a2 expression is critical for maintenance of lung homeostasis. This statement should be clarified, as data presented in the supplemental figures argue that no abnormal phenotype exists in transgenic mice in the absence of injury.

R12: This sentence was deleted, and a new sentence “the changes may be too subtle to affect lung homeostasis without challenge, since we did not detect any abnormal phenotypes in the transgenic mice” was added to the revised manuscript (line 518 -521).

13. In the discussion (lines 516-518), the authors state that overexpression of Scgb3a2 occurred in the “therapeutic phase” after bleomycin. While overexpression did recover in transgenic mice, this statement is confusing, given that the transgenic mice are non-inducible and have constitutive overexpression of Scgb3a2.
R13: This sentence was rewritten to make more clear what we intended to say as follows: The present studies demonstrated that the expression of SCGB3A2 markedly increased in transgenic mice after the severity of fibrosis reached peak levels, and thus the situation may resemble that of SCGB3A2 being administered in the therapeutic phase, which likely resulted in the rapid decrease of fibrosis (line 553-556).

Minor Essential Revisions
1. Is the recovery of increased Scgb3a2 expression following bleomycin (Figure 4) related to recovery of SPC-expressing epithelium? Please comment on how overexpression is lost initially after bleomycin, and then recovers at the 6- and 9-week time points.

MR1: The phenomena that occur after BLM injury and the relation between SCGB3A2 and SP-C expression are now described in the Results section (line 430-442) and Discussion section (line 528-545), and a new Supplemental Figure S5 was added to the revised manuscript.

2. Why does secretion of Scgb3a2 into BALF in transgenic mice decrease steadily as mice age?

MR2: This is a very good question why the level of SCGB3A2 in BALF decreases as mice age, while the relative mRNA expression level more or less stays the same. We do not know the answer to this question. We hope to find some answer in future studies while we further use this mouse model.

3. N-values are needed for Figure 2 and Figure 4.

MR3: The N-value was added in the legends to Figures 2 and 4.

4. Figure S1: need better labeling of IHC – please state in the figure that this is IHC for Scgb3a2.

MR4: We labeled the IHC image with “SCGB3A2” in a new Figure S2 (old Figure S1) and more detailed explanation was added to the figure legend.

5. Do adult transgenic animals have normal numbers of ciliated cells?

MR5: Ciliated cells appear to be similar in numbers in the airways of adult transgenic mice as compared to wild-type mice. The results obtained by scanning electron microscopy are now provided in Supplemental Figure S1 and this is now mentioned in the Results section of the revised manuscript (line 337-339).

6. For Figure 3, analysis of other genes involved in bleomycin-induced fibrosis would be useful. In particular, aSMA (Acta2) and CTGF in addition to matrix remodeling genes such as MMPs would be potentially helpful.
MR6: We added the qRT-PCR results for *Acta2, Ctgf, Mmp2*, and *Mmp12* in the new Figure 3, and the results are now described in the Results section of the revised manuscript (line 416-421). Accordingly the Methods “Quantitative RT-PCR analysis” was modified (line 291-296).

7. (Line 152) “as bovine serum” should be changed to “with bovine serum”

MR7: Changed.

8. Figure legend for Figure 3: “monocygtes” is mis-spelled.

MR8: Corrected.