Matrix stiffness regulates α-TAT1-mediated acetylation of α-tubulin and promotes silica-induced epithelial–mesenchymal transition via DNA damage

Gengxu Li, Si Chen, Yi Zhang, Hong Xu, Dingjie Xu, Zhongqiu Wei, Xuemin Gao, Wenchen Cai, Na Mao, Lijuan Zhang, Shumin Li, Fang Yang, Heliang Liu and Shifeng Li
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Original submission

First decision letter

MS ID#: JOCES/2019/243394

MS TITLE: Matrix stiffness regulates α-TAT1/Ac-α-Tub in A549 cells, leading to silica-induced epithelial-mesenchymal transition via replication stress and DNA damage

AUTHORS: Gengxu Li, Si Chen, Yi Zhang, Hong Xu, Dingjie Xu, Zhongqiu Wei, Xuemin Gao, Wenchen Cai, Na Mao, Lijuan Zhang, Shumin Li, Fang Yang, Heliang Liu, and Shifeng Li
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, there is considerable enthusiasm for the question of how silica affects cells and for the specific conclusions in the manuscript. However, all three reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors are interested in mechanisms by which silicosis damage occurs in the epithelium. Specifically they seek to understand if tubulin acetylation by a-TAT1 by promoting microtubule stability prevent EMT and DNA damage. Using a combination of a rat model of silicosis and a lung cancer cell line, A549 cells, the authors have shown that silica exposure and matrix stiffening or a combination of both lead to downregulation of acetylated-tubulin and subsequent DNA damage and replication stress. These effects are abrogated by the increase in acetylated tubulin. Overall, the paper was a nice demonstration of mechanisms by which silica lead to lung injury and potential epithelial to mesenchymal transition. However, there are several points that needs to be addressed:

Major
1. The authors rely heavily on the use of A549 cells, which is an immortalized cancer cell line where cell cycle regulation is likely to be altered. In addition to the animal model, additional proof of mechanism in primary lung epithelium would be important, since they are now commercially available.
2. In Figure 1, panels A, it would be helpful to have an image of a lung lobe, to better identify whether these nodules are alveolar or closer to the airways. Also, of note, peak injury seems to occur by 8 weeks.
3. In Fig 2, although only a few cells show 53BP1, the entire field shows vimentin. So, areis DNA damage really leading to EMT?
4. In figure 4 D, it would be helpful to see the corresponding brightfield images, to bettwe visualize cells shape. In addition, close up images of the H&E would be important to see if there are corresponding differences in epithelial structure.
5. Although the authors have implicated cell matrix stiffness, based on 4F, it is not clear that it makes a huge difference in the trajectory. Further discussion of this would be justified.
6. The authors have implicated microtubule stability as a protective mechanism. However, acetylation of tubulin is also time dependent- it is more likely to be acetylated if it is around longer. As a result, there seems to be a bit of circular logic- if the cells are replicating more, there is less likely to be a lot of acetylted tubulin.
Fig. 5 does address this a bit by showing an independent role of acetylated tubulin, but in addition, it would be good to have a few markers of cell death earlier to show they are not just dying and that is why there isn't tubulin acetylation

Comments for the author

1. Fig1 labeling is confusing. In the merged image, it isn't clear what the magenta labeling is.

Reviewer 2

Advance summary and potential significance to field

EMT diff'n in lung caused by silicate as studied extensively in vitro identifying DNA damage as a possible initiator.

Comments for the author

Differentiation has many inducers, potentially including DNA damage but such studies are few and mechanisms unclear. This is a very interesting study of EMT-differentiation in lung (& A549 culture mode) caused by silicate particles (relevant to inhalation) that are known to cause fibrosis in vivo. The authors propose a mechanism involving a MT regulator, and ultimately provide evidence of rescue. The mix of in vivo data and more extensive culture data is generally compelling, but a few issues temper enthusiasm:

1. Fig.2A,C are missing Control conditions.
Imaging of Vim in Fig.2F will not replace the needed immunoblot, and the imaging of 53BP1 is in too few cells to be convincing of any relationship to differentiation. Imaging of gH2AX is standard for the field and should be added.

2. Fig.3A shows A549s are initially sparse but at 48h are dense and contact inhibited, and so the same expts need to be repeated in Control conditions, with quantitation of cell density vs time. The concern is that gH2AX is higher in cycling cells due to replication stress.

3. The rapid loss of Col1 on soft matrix after 12h is an odd observation. If MMPs are inhibited does Col1 remain high and aSMA increase?

4. In many past studies (e.g. PMID: 26168347), the effects of stiff matrix on differentiation are suppressed by inhibiting myosin-II. The authors should at least repeat the measurements of aSMA and Ecad for the condition (48h on stiff +/- SiO2) +/- blebbistatin.

5. Trend for gH2AX in Fig.5B (for NC) seem inconsistent with Fig.2B. Fig.5C is too qualitative to be conclusive.

6. What are the cell densities in Fig.8?

7. Given the DNA damage, measures of apoptosis are needed.

Minor:
1. More details of the SiO2 is needed: particles? size?
2. Were the PAAm gels coated with matrix?

Reviewer 3

Advance summary and potential significance to field

The interplay of silica exposure and matrix stiffness is poorly understood.

Comments for the author

This paper from Li et al examines an interesting application of lung silica exposure and attempts to link it to stiffness. While interesting, a number of control experiments and/or other assays are missing. These should be done first to ensure that the strong statements from the authors are correct.

From the outset, the abstract is confusing in its logic. If silicosis results in lung stiffening AND alpha-TAT1 promotes microtubule stability in response to stiffness, then shouldn't stiffer substrates increase alpha-TAT1 expression and its downstream acetylated-tubulin? The abstract says that these are decreased in silicosis which doesn't seem to make sense logically.

To prove the universality of EMT that the authors observe in A549 cells, it would be advisable to show this in a different cell line. Perhaps the A549s just have a genetic background that predisposes them to show more robust EMT changes in response to silica exposure?

Figure 3A lacks an untreated control at 1 and 60 kPa. Its okay to show the time course, which is interesting but we don't know what the default growth and morphologies should be. The individual cell changes are also hard to see with respect to morphology.

Since it occurs in multiple instances, it is important to show that total amounts of tubulin do not change as AC-Tub does. This occurs in Figures 4, 5 and 7 at least.

To actually establish causal evidence, the authors need to measure stiffness of silica-treated mouse lungs.

It is also important to note that the images of the blots are generally overexposed and with excessive contrast.. Some simultaneously also appear to have their background washed out, e.g. Figure 3D, col I blot Figure 6D 53BP1 bands, etc. It is worth revisiting the original images and potentially running some new blots to capture the data with greater dynamic range. It is possible that this issue arose during image compression.

Minor:
Figure 3C and 3E needs error bars.

First revision

Author response to reviewers' comments

October 11, 2020

Dear Dr. Andrew Ewald,

Thank you very much for extending the time of our revision, and for your comments on our manuscript entitled, "**Matrix Stiffness Regulates α-TAT1/α-Ac-Tub in A549 Cells, Leading to Silica-Induced Epithelial-Mesenchymal Transition via Replication Stress and DNA Damage**" (MS ID#: JOCES/2019/243394). The comments were valuable for revising and improving our paper, and provided important guidance for our research. We have studied the comments carefully, and the edits are in red font in the revised manuscript. We hope that the revised manuscript meets with your approval.

Reviewer 1 Advance Summary and Potential Significance to Field

The authors are interested in mechanisms by which silicosis damage occurs in the epithelium. Specifically, they seek to understand if tubulin acetylation by a-TAT1 by promoting microtubule stability prevent EMT and DNA damage. Using a combination of a rat model of silicosis and a lung cancer cell line, A549 cells, the authors have shown that silica exposure and matrix stiffening or a combination of both lead to downregulation of acetylated-tubulin and subsequent DNA damage and replication stress. These effects are abrogated by the increase in acetylated tubulin. Overall, the paper was a nice demonstration of mechanisms by which silica lead to lung injury and potential epithelial to mesenchymal transition. However, there are several points that need to be addressed:

Responses: Thank you very much for your recognition. We have revised the content to make the manuscript more scientific and rigorous.

Reviewer 1 Comments for the Author.

Comment NO.1 The authors rely heavily on the use of A549 cells, which is an immortalized cancer cell line where cell cycle regulation is likely to be altered. In addition to the animal model, additional proof of mechanism in primary lung epithelium would be important, since they are now commercially available.

Response: Thank you very much for your suggestion. The A549 cell lines have been used in many studies to study the mechanisms of epithelial interstitial transformation in fibrosis by our group and others (Respir Res. 2018,19(1):111.1-3; Toxicol Appl Pharmacol. 2019,369:17-29). We now see the limitations of A549 cells derived from cancer, and supplemented mouse lung type II epithelial cells (MLE-12) to study the mechanisms, as shown in revised Fig. 2E and Fig. S4.

Comment NO.2 In Figure 1, panels A, it would be helpful to have an image of a lung lobe, to better identify whether these nodules are alveolar or closer to the airways. Also, of note, peak injury seems to occur by 8 weeks.

Response: We have provided an image of the lung lobe in the revised manuscript (Fig. 1A), which indicates that the silicotic nodules are mainly alveolar. Many studies and ours have reported the DNA double-strand breaks (DSBs) mainly occur in alveolar epithelial cells (AECs) in both *in vivo* and *in vitro* models of silicosis, and are often accompanied by phenotypic transformations including apoptosis, senescence, and the epithelial-mesenchymal transition (EMT) (Toxicol Appl Pharmacol. 2019; 369:17-29; Toxicol Appl Pharmacol. 2018; 350:1–10). Studies of scRNA-seq from the lung tissue of bleomycin mice also found that in addition to the apoptosis of AECs, there was abnormal activation of AECs, which can play a role in injury repair and may also be involved in the progression of fibrosis through abnormal activation of EMT or other signal transduction pathways (Cell. 2020;180(1):107-121.e17). In this study, we found that since the 8th week of silica exposure, the number of γH2AX-positive cells in alveoli increased significantly (Fig. 1A and 1D), and γH2AX is co-expressed with SP-D (Fig. 1C), indicating epithelial cell injury. Thereafter, the expression of injury marker was consistently high. In the revised manuscript, we have also added this explanation of the results to the "**Discussion**" section (see lines 247-257).

Comment NO.3 In Fig 2, although only a few cells show 53BP1, the entire field shows vimentin. So, are is DNA damage really leading to EMT?

Response: Immunofluorescence (IF) staining is a good method for observing the co-localization of two proteins. However, it is easily interfered with by many factors, such as non-specific fluorescence and the intensity of excitation light. Therefore, we changed the IF staining image to the immunohistochemical staining images of γH2AX and α-SMA in Fig. 2D to 2F.

Comment NO.4 In figure 4 D, it would be helpful to see the corresponding brightfield images, to better visualize cells shape. In addition, close up images of the H&E would be important to see if there are corresponding differences in epithelial structure.

Response: As mentioned above, although immunofluorescence staining is a good method for observing the co-localization of two proteins, it is easily interfered with by many factors, such as non-specific fluorescence and the intensity of excitation light. Therefore, we changed the IF staining image to the immunohistochemical staining images in Fig. 4F to better examine the expression of Ac-α-Tub and α-SMA and visualize the cell shape.

Comment NO.5 Although the authors have implicated cell matrix stiffness, based on 4F, it is not clear that it makes a huge difference in the trajectory. Further discussion of this would be justified.

Response: It has been established that cell mechanosensitivity is dependent upon the acetyltransferase activity of α-tubulin acetyltransferase (α-TAT1), which when absent, leads to a decrease in cellular elasticity (Elife. 2016;5:e20813). We previously revealed the decrease of α-TAT1 and Ac-α-Tub levels during silicosis. Quantitative promotion of microtubule acetylation may be a target for overcoming EMT and fibrotic diseases (Sci Rep. 2016 Aug 31;6:32257.) (Cell Mol Life Sci. 2020 Jan 7). In our study, Ac-α-Tub was inversely correlated with the substrate stiffening in A549 cells *in vitro* and the progress of rat silicosis *in vivo*, accompanied by α-SMA increase. The current results support that the stiffening of ECM inhibits α-TAT1-mediated acetylation of α-tubulin and destroys the stability of MTs. We have reorganized the text to explain our results in the discussion section (see lines 307–320).

Comment NO.6 The authors have implicated microtubule stability as a protective mechanism. However, acetylation of tubulin is also time dependent- it is more likely to be acetylated if it is around longer. As a result, there seems to be a bit of circular logic- if the cells are replicating more, there is less likely to be a lot of acetylted tubulin. Fig. 5 does address this a bit by showing an independent role of acetylated tubulin, but in addition, it would be good to have a few markers of cell death earlier to show they are not just dying and that is why there isn't tubulin acetylation.

Response: To explain whether the acetylation loss is associated with cell proliferation or apoptosis, we measured the viability and apoptosis of epithelial cells grown on soft and stiff substrate by the

CCK-8 assay and TUNEL staining in Fig. S1.

Minor:

Comment NO.1 Fig1 labeling is confusing. In the merged image, it isn't clear what the magenta labeling is.

Response: Thank you for carefully reviewing our article. The magenta fluorescence is the merged red with blue fluorescence, and the yellow fluorescence is the merged red with green fluorescence. We have described the merged fluorescence in the figure legends to better illustrate the results see in line 626-628. *"The magenta fluorescence is the merge of red with blue fluorescence. And the yellow fluorescence is the merge of red with green fluorescence."*.

Reviewer 2 Advance Summary and Potential Significance to Field

EMT diff'n in lung caused by silicate as studied extensively in vitro, identifying DNA damage as a possible initiator.
Differentiation has many inducers, potentially including DNA damage, but such studies are few and mechanisms unclear. This is a very interesting study of EMT-differentiation in lung (& A549 culture mode) caused by silicate particles (relevant to inhalation) that are known to cause fibrosis in vivo. The authors propose a mechanism involving a MT regulator, and ultimately provide evidence of rescue. The mix of in vivo data and more extensive culture data is generally compelling, but a few issues temper enthusiasm:

Response: Thank you very much for your recognition. We have revised the content to make the manuscript more scientific and rigorous.

Reviewer 2 Comments for the Author.

Comment NO.1 Fig.2A,C are missing Control conditions. Imaging of Vim in Fig.2F will not replace the needed immunoblot, and the imaging of 53BP1 is in too few cells to be convincing of any relationship to differentiation. Imaging of gH2AX is standard for the field and should be added.

Response: Figure 2A and 2C show the temporal effects of silica stimulation on DNA damage and EMT in epithelial cells, and the time point of 0 h served as the control (Chem Biol Interact. 2020;319:109024.). However, as you mentioned, the dose relationship for DNA damage is needed. In the revised manuscript, we treated A549 cells with 0.1–10 μg/cm^2 of silica particles. As shown in Fig. S2A-S2C, exposure to silica for 6, 24, and 48 h led to the dose-dependent phosphorylation of histone H2AX (γH2AX), a marker of DNA damage. We also show the expression of α-SMA and DNA injury marker γH2AX in Figure 2F to show the relationship between DNA damage and differentiation.

Comment NO.2 Fig.3A shows A549s are initially sparse, but at 48h are dense and contact inhibited, and so the same experiments need to be repeated in Control conditions, with quantitation of cell density vs time. The concern is that γH2AX is higher in cycling cells due to replication stress.

Response: Thank you for the good question and suggestion. The morphological changes visualized by inverted phase-contrast microscopy revealed that the A549 cells became spindle-like at 48 h of SiO2 stimulation on stiff matrix (Fig. 3A), and the accompanied changes in EMT markers (COL I, α-SMA, vimentin, and E-cadherin) confirmed this phenotypic transformation. And studies have shown that the hallmarks of EMT include loss of contact inhibition (Dev Cell. 2015;34(4):421-434), which could be induced by DNA damage and replication stress (Oncogene. 2018;37(33):4518-4533.). But we did ignore the control conditions. In the revised manuscript, the corresponding control conditions were included in (Fig. 3A, B). And cell viability was also added in Fig. S1 to rule out the status of cells reaching contact inhibition.

Comment NO.3 The rapid loss of Col1 on soft matrix after 12h is an odd observation.
If MMPs are inhibited, does Col1 remain high and α-SMA increase?

Response: In normal damage repair, the secretion and degradation of the ECM are in equilibrium, as seen in the soft matrix gel, with the level of Col1 reduced at 48 h after the withdrawal of SiO2 stimulation. However, when A549 was cultured on stiff substrate, the EMT and collagen secretion continued even withdrawal the SiO2 stimulation (Fig. 4D-F), which is similar to our previous findings that MMP decrease and extracellular matrix accumulation during silicosis (Mol Med Rep. 2018;17(6):7467-7476.).

Comment NO.4 In many past studies (e.g. PMID: 26168347), the effects of stiff matrix on differentiation are suppressed by inhibiting myosin-II. The authors should at least repeat the measurements of α-SMA and E-cadherin for the condition (48h on stiff +/- SiO2) +/- blebbistatin.

Response: Thanks for the suggestion. As in the study (e.g. PMID: 26168347), culture of primary MSCs on stiffer substrates promotes myosin-dependent migration and cell tension—which α-SMA reveals—contributing significantly to injured tissue stiffness. Also, Rho-associated coiled coil-forming protein kinase (ROCK) directly phosphorylates myosin light chain (MLC) and increases the contractile force generated by cross-linking with actin filaments. Moreover, it has been reported that ROCK inhibits the activity of α-TAT1 (eNeuro. 2018;5(1):ENEURO.0240-17.) and increases the activity of HDAC6 (J Biol Chem. 2013;288(11):7907-7917.), followed by a decrease in α-Tub acetylation, and promotes cell proliferation and migration. We have also reported that treatment of fibroblasts with and Y-27632 (a ROCK inhibitor) caused Ac-α-Tub to be redistributed, and it attenuated the up-regulation of α-SMA, Col I, and myosin phosphatase-1 (MYPT1) induced by Ang II (Sci Rep. 2016 Aug 31;6:32257.). In the revised manuscript, we have measured the α-SMA and E-cadherin for the condition (48 h on stiff +/- SiO2) +/- Y-27632 (Fig. 3B, C).

Comment NO.5 Trend for γH2AX in Fig.5B (for NC) seem inconsistent with Fig.2B. Fig.5C is too qualitative to be conclusive.

Response: To assess matrix stiffness regulating microtubules, which in turn influence DNA repair, A549 cells were cultured on soft and stiff substrate with SiO_2 incubation for 48 h, and we found stiffness-induced MT disruption (Fig. 4F) synergized with SiO_2-induced DNA damage and EMT (Fig. 2D-F). As a study has shown that microtubule-targeting agents augment the toxicity of DNA-damaging agents by disrupting intracellular trafficking of DNA repair proteins (Proc Natl Acad Sci U S A. 2015;112(5):1571-1576.), in the additional experiment, A549 cells cultured on soft and stiff substrate were stimulated with SiO2 for 12 h; subsequently, the SiO2 was washed out and cells were maintained for an additional 3, 6, 12, 24, or 48 h to monitor the levels and rate of disappearance of γH2AX. As expected, Fig. 4D shows A549 cells lacking the α-TAT1 and Ac-α-Tub in the stiff matrix. And the stiff matrix resulted in γ-H2AX levels that were maintained above basal levels longer compared with the results in stiff matrix cells (Fig. 3D-E), supporting the necessity for intact MTs to facilitate intracellular trafficking of DNA repair proteins to the nucleus. We have reorganized the text in the results section.

Comment NO.6 What are the cell densities in Fig.8?

Response: In the revised manuscript, we provide more details about cell densities and how to make slides in "**Immunohistochemistry and immunofluorescence**" section (see line 386–388). *"A549 cells were cultured at a density of 6 × 10^3 cells/well in a four-well chamber slide for 12 h to 50% confluence and then treated as described above."*

Comment NO.7 Given the DNA damage, measures of apoptosis are needed.

Response: It is true, and we have reported that silica stimulation incurs DNA damage epithelial cell apoptosis (Toxicol Appl Pharmacol. 2019;369:17-29.). In this study, we found that EMT is another outcome of DNA damage. In the revised manuscript, we measured the viability and apoptosis of epithelial cells grown on soft and stiff substrate by CCK8 and TUNEL staining to rule out the acetylation loss affected by cell proliferation or apoptosis (Fig. S1).

Minor:

Comment NO.1 More details of the SiO_2 is needed: particles? size?

Response: The particles size of SiO_2 is 1–10 μm in diameter. We have provided the relative information in **"Cell culture and treatment"** section (see line 342).

Comment NO.2 Were the PAAm gels coated with matrix?

Response: The PAAm gels were coated on slides, which is based on a previous article (Mol Biol Cell. 2012;23(5):781-791.), and the details of PAAm gels are depicted in the **"Polyacrylamide gel preparation"** section.

Reviewer 3 Advance Summary and Potential Significance to Field...

The interplay of silica exposure and matrix stiffness is poorly understood. This paper from Li et al examines an interesting application of lung silica exposure and attempts to link it to stiffness. While interesting, a number of control experiments and/or other assays are missing. These should be done first to ensure that the strong statements from the authors are correct.

Response: Thank you. We revised the content to make the manuscript more scientific and rigorous.

Reviewer 3 Comments for the Author...

Comment NO.1 From the outset, the abstract is confusing in its logic. If silicosis results in lung stiffening and α-TAT1 promotes microtubule stability in response to stiffness, then shouldn't stiffer substrates increase alpha-TAT1 expression and its downstream acetylated-tubulin? The abstract says that these are decreased in silicosis, which doesn't seem to make sense logically.

Response: It has been established that cell mechanosensitivity is dependent upon the acetyltransferase activity of α-tubulin acetyltransferase (α-TAT1), which when absent leads to a decrease in cellular elasticity (Elife. 2016;5:e20813). And we have previously reported that rat lacking α-TAT1 and Ac-α-Tub resulted in silicosis (Sci Rep. 2016;6:32257.). In the present study, Ac-α-Tub was found reverse related to the substrate stiffen in A549 cells *in vitro*, indicating microtubule acetylation was a target for overcoming EMT and fibrotic diseases (Cell Mol Life Sci. 2020;10.1007/s00018-019-03412-x.). In the revised manuscript, we have reorganized the relevant content in the discussion section (see lines 307–320).

Comment NO.2 To prove the universality of EMT that the authors observe in A549 cells, it would be advisable to show this in a different cell line. Perhaps the A549s just have a genetic background that predisposes them to show more robust EMT changes in response to silica exposure?

Response: In this study, we explained the mechanisms of DNA damage on EMT, and suggest that DNA damage participates in phenotypic transition. To prove the universality of DNA damage on phenotypic transition, human embryonic lung fibroblast cells (MRC-5) was also examined, and we found similar results (Fig. 2D-F).

Comment NO.3 Figure 3A lacks an untreated control at 1 and 60 kPa. Its okay to show the time course, which is interesting, but we don't know what the default growth and morphologies should be. The individual cell changes are also hard to see with respect to morphology.

Response: We apologize for the absence of control conditions. In the revised manuscript, the corresponding control conditions of cells cultured on soft and stiff substrates at 48 h have been added to Fig. 3A-B and Fig. S4.

Comment NO.4 Since it occurs in multiple instances, it is important to show that total amounts of tubulin do not change as AC-Tub does. This occurs in Figures 4, 5 and 7 at least.

Response: The total amounts of tubulin have been added to the revised manuscript.

Comment NO.5 To actually establish causal evidence, the authors need to measure stiffness of silica-treated mouse lungs.

Response: Thank you for the good suggestion. However, it is a pity that the data cannot be provided, because both the tissue fixed in paraformaldehyde or cryopreservation are not suitable for stiffness measurement of silica-treated mouse lungs. It would take at least 6 months to build a new rat model, or even longer due to the epidemic situation. We will collect the relative data in further studies.

Comment NO.6 It is also important to note that the images of the blots are generally overexposed and with excessive contrast. Some simultaneously also appear to have their background washed out, e.g. Figure 3D, Col I blot, Figure 6D 53BP1 bands, etc. It is worth revisiting the original images and potentially running some new blots to capture the data with greater dynamic range. It is possible that this issue arose during image compression.

Response: The blots have been rerun and some blots have a light background.

Minor:

Comment NO.1 Fig. 3C and 3E needs error bars.

Response: The error bars are provided in the revised figures.

Thank you very much for your kind work and consideration on publication of our paper. On behalf of my co-authors, we would like to express our great appreciation to the editor and reviewers. We look forward to hearing from you at your earliest convenience.

Thank you and best regards,

Sincerely,
Shifeng Li PhD. & MD.
Hebei Key Laboratory for Organ Fibrosis Research, School of Public Health, North China University of Science and Technology.
No. 21 Bohai Road, Tangshan city, Hebei province 063000, China.
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Second decision letter

MS ID#: JOCES/2019/243394

MS TITLE: Matrix stiffness regulates α-TAT1/Ac-α-Tub and promotes silica-induced epithelial-mesenchymal transition via DNA damage

AUTHORS: Gengxu Li, Si Chen, Yi Zhang, Hong Xu, Dingjie Xu, Zhongqiu Wei, Xuemin Gao, Wenchen Cai, Na Mao, Lijuan Zhang, Shumin Li, Fang Yang, Heliang Liu, and Shifeng Li
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. Specifically, Reviewer 2 raises some issues with the lack of a relevant ECM and suggests some citations and Reviewer 3 identifies what appears to be a significant

problem with some of the Western blots and requires that they be rerun. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors are interested in mechanisms by which silicosis damage occurs in the epithelium. Specifically they seek to understand if tubulin acetylation by a-TAT1 by promoting microtubule stability prevent EMT and DNA damage. Using a combination of a rat model of silicosis and a lung cancer cell line, A549 cells as well as MLE-12 cells, the authors have shown that silica exposure and matrix stiffening or a combination of both lead to downregulation of acetylated-tubulin and subsequent DNA damage and replication stress. These effects are abrogated by the increase in acetylated tubulin.
Overall, the paper was a nice demonstration of mechanisms by which silica lead to lung injury and potential epithelial to mesenchymal transition.

Comments for the author

The authors have adequately addressed the previous concerns. The modified figures as submitted do not raise further concerns.

Reviewer 2

Advance summary and potential significance to field

per previous

Comments for the author

The authors were reasonably responsive, but a few issues remain:

1. The authors did not seem to attach a specific matrix to their gels. The cited reference Leight et al (2012) studied cells undergoing EMT on multiple types of ECM proteins and found differences, which highlights the importance of attached matrix. The authors therefore must repeat their experiments with a specific and relevant matrix.

2. Given Fig.8 links Matrix Stiffness to DNA damage and replication, one paper that should be cited is Cho et al (Dev Cell 2019): it showed cell cycle and DNA damage are modulated by cell and tissue forces in another process of differentiation (cardiogenesis).

3. Because the authors find a-SMA becomes stabilized on stiff substrates, the authors should cite the previously mentioned PMID: 26168347 with a similar observation based on a molecular mechanism relevant to lung (as cited therein).

Reviewer 3

Advance summary and potential significance to field

The interplay of silica exposure and matrix stiffness is poorly understood. This paper from Li et al examines an interesting application of lung silica exposure and attempts to link it to stiffness.

Comments for the author

The authors have addressed all of my concerns.

However, to my 4th comment: "Since it occurs in multiple instances, it is important to show that total amounts of tubulin do not change as AC-Tub does. This occurs in Figures 4, 5 and 7 at least," the authors respond:
"The total amounts of tubulin have been added to the revised manuscript."

Yet what it appears that they did in each figure panel is NOT to run the same samples and stain for total tubulin to identify what fraction was acetylated, but rather that they just dropped the protein name "GAPDH"
in each blot and replace it with "alpha-Tubulin." This is not acceptable and manipulates the reader in to thinking that they are actually looking at a different protein. At best this is sloppy. At worst, this is misconduct. All the authors need to do is re-run the same (or equivalent)
samples for figures 4A, 6A (old 5A),
and 7A to see that tubulin concentration does not change but the acetylation does consistent with their other data. If concentrations are changing, rather, that would suggest a different conclusion.

(note from editorial staff - the ethics team have investigated this, and the authors have provided new versions of figures 2, 4, 5 and 7 which are now uploaded as source files - 27/08/2020)

Second revision

Author response to reviewers' comments

Dear Dr. Andrew Ewald and Reviewers,

Thank you for your comments on our manuscript entitled **"Matrix stiffness regulates α-TAT1/Ac-α-Tub and promotes silica-induced epithelial-mesenchymal transition via DNA damage"** (MS ID#: JOCES/2019/243394). The comments were valuable for revising and improving our paper, and provided important guidance for our research. We have studied the comments carefully, and the edits are in red font in the revised manuscript. We hope that the revised manuscript meets with your approval. We have also uploaded a formatted PDF of the Response as Supplementary Information.

Figure Labelling

Please check the labelling of your figures. One of the reviewers was concerned that Figures 5 and 6 may be mislabelled.

Responses: We have checked and revised the figure labeling.

Reviewer 1 Advance Summary and Potential Significance to Field

The authors are interested in mechanisms by which silicosis damage occurs in the epithelium. Specifically, they seek to understand if tubulin acetylation by a-TAT1 by promoting microtubule stability prevent EMT and DNA damage. Using a combination of a rat model of silicosis and a lung cancer cell line, A549 cells, the authors have shown that silica exposure and matrix stiffening or a combination of both lead to downregulation of acetylated-tubulin and subsequent DNA damage and replication stress. These effects are abrogated by the increase in acetylated tubulin.

Overall, the paper was a nice demonstration of mechanisms by which silica lead to lung injury and potential epithelial to mesenchymal transition.

Responses: Thank you very much for your recognition.

Reviewer 1 Comments for the Author.

The authors have adequately addressed the previous concerns. The modified figures as submitted do not raise further concerns.

Response: Thank you.

Reviewer 2 Advance Summary and Potential Significance to Field

per previous

Response: Thank you. We have revised the content to make the manuscript more scientific and rigorous.

Reviewer 2 Comments for the Author.

Comment NO.1 The authors did not seem to attach a specific matrix to their gels. The cited reference Leight et al (2012) studied cells undergoing EMT on multiple types of ECM proteins and found differences, which highlights the importance of attached matrix. The authors therefore must repeat their experiments with a specific and relevant matrix.

Response: The cited reference (e.g. PMID: 22238361, Leight et al. 2012) demonstrated *"varying matrix rigidity switched the functional response to TGF-β1. Decreasing rigidity increased TGF-β1-induced apoptosis, whereas increasing rigidity resulted in the EMT."* Furthermore, the authors found that *"compliant substrates increased TGF-β1-induced apoptosis for all ECM types"* (polyacrylamide gels conjugated with Fibronectin, re-constituted basement membrane (rBM; commercially known as Matrigel), or collagen I) although *"compliance-induced apoptosis was more marked when cells engaged with fibronectin or the Matrigel compared with collagen I, and that TGF-β-induced EMT was inhibited on compliant substrates independent of ECM subtypes."* They highlighted the *"central role for matrix mechanics in regulating the switch in the response of cells to TGF-β1 between EMT and apoptosis and showed that these two responses are independently regulated"*. Likewise, there is a widely demonstrated link between substrate stiffness and cell spreading, which is related to cell survival and EMT (Pelham and Wang, 1997; Yeung et al., 2005; Fu et al., 2010). In this study, we focused on the interplay of SiO_2 exposure and substrate stiffness in regulating EMT, which is independent of ECM subtypes as mentioned above. We found that alveolar epithelial cells undergo the EMT when they cultured on rigid gels and treated with SiO_2. During this process, cells also undergo DNA damage because of SiO_2 stimulation. Thus, EMT is an outcome of DNA damage in cells on stiff substrate with SiO_2 exposure. In the revised manuscript, we have also added the relevant explanation to the Discussion section (see lines 296–306).

Comment NO.2 Given Fig.8 links Matrix Stiffness to DNA damage and replication, one paper that should be cited is Cho et al (Dev Cell 2019): it showed cell cycle and DNA damage are modulated by cell and tissue forces in another process of differentiation (cardiogenesis).

Response: Thank you. In the revised manuscript, we have supplemented the relevant citation in the Discussion section (see line 240).

Comment NO.3 Because the authors find a-SMA becomes stabilized on stiff substrates, the authors should cite the previously mentioned PMID: 26168347 with a similar observation based on a molecular mechanism relevant to lung (as cited therein).

Response: Thank you. We have supplemented the relevant citation in the revised manuscript (see lines 159-161).

Reviewer 3 Advance Summary and Potential Significance to Field

The interplay of silica exposure and matrix stiffness is poorly understood. This paper from Li et al examines an interesting application of lung silica exposure and attempts to link it to stiffness.

Response: Thank you.

Reviewer 3 Comments for the Author

Comment NO.1 The authors have addressed all of my concerns. However, to my 4th comment: "Since it occurs in multiple instances, it is important to show that total amounts of tubulin do not change as AC-Tub does. This occurs in Figures 4, 5 and 7 at least," the authors respond: "The total amounts of tubulin have been added to the revised manuscript." Yet what it appears that they did in each figure panel is NOT to run the same samples and stain for total tubulin to identify what fraction was acetylated, but rather that they just dropped the protein name "GAPDH" in each blot and replace it with "alpha-Tubulin." This is not acceptable and manipulates the reader in to thinking that they are actually looking at a different protein. At best this is sloppy. At worst, this is misconduct. All the authors need to do is re-run the same (or equivalent) samples for figures 4A, 6A (old 5A), and 7A to see that tubulin concentration does not change but the acetylation does consistent with their other data. If concentrations are changing, rather, that would suggest a different conclusion. (note from editorial staff - the ethics team have investigated this, and the authors have provided new versions of figures 2, 4, 5 and 7 which are now uploaded as source files - 27/08/2020)

Response: We apologize for the mistake, and thank you very much for giving us the opportunity to modify the manuscript. We have provided new versions of Figures 2, 4, 5 and 7 and uploaded the source files to the editor Andrea by email on August 27, 2020.

Thank you very much for your kind work and consideration of publication of our paper. On behalf of my co-authors, we would like to express our great appreciation to the editor and reviewers. We look forward to hearing from you at your earliest convenience.

Thank you and best regards,

Sincerely,
Shifeng Li

Hebei Key Laboratory for Organ Fibrosis Research, School of Public Health, North China University of Science and Technology.
E-mail: leimengpi@163.com

Third decision letter

MS ID#: JOCES/2019/243394

MS TITLE: Matrix stiffness regulates α-TAT1/Ac-α-Tub and promotes silica-induced epithelial-mesenchymal transition via DNA damage

AUTHORS: Gengxu Li, Si Chen, Yi Zhang, Hong Xu, Dingjie Xu, Zhongqiu Wei, Xuemin Gao, Wenchen Cai, Na Mao, Lijuan Zhang, Shumin Li, Fang Yang, Heliang Liu, and Shifeng Li
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports and feel that their concerns have essentially been addressed. There is only one issue remaining- clarifying in the methods in response to Reviewer 2 whether an ECM protein was attached to the gels and, if so, which one(s). Please mark the changed text in a different color and clarify the changes for me in the cover letter. I will evaluate it myself and do not expect to return it to reviewers. I hope that you can make this change as I look forward to accepting your paper.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors are interested in mechanisms by which silicosis damage occurs in the epithelium. Specifically they seek to understand if tubulin acetylation by a-TAT1 by promoting microtubule stability prevent EMT and DNA damage. Using a combination of a rat model of silicosis and a lung cancer cell line, A549 cells as well as MLE-12 cells, the authors have shown that silica exposure and matrix stiffening or a combination of both lead to downregulation of acetylated-tubulin and subsequent DNA damage and replication stress. These effects are abrogated by the increase in acetylated tubulin.
Overall, the paper was a nice demonstration of mechanisms by which silica lead to lung injury and potential epithelial to mesenchymal transition.

Comments for the author

The authors have addressed the concerns in a satisfactory manner.

Reviewer 2

Advance summary and potential significance to field

per previous

Comments for the author

What ECM protein(s) was attached to the gels?
I do not see the answer to this simple question in the key Methods section:

"Polyacrylamide gel preparation Polyacrylamide gels were prepared as previously described (Leight et al., 2012).
Mechanical properties of the polyacrylamide gels were controlled by varying the percentage of acrylamide and bis-acrylamide as follows: elastic modulus (% acrylamide;%bis-acrylamide), 1 kPa (3; 0.1) and 60 kPa (10; 0.5). Gels were incubated with 1% (vol/vol) ethanolamine in 50 mM HEPES, pH 8, for 30 min, and rinsed with ddH2O. ..."

Reviewer 3

Advance summary and potential significance to field

The interplay of silica exposure and matrix stiffness is poorly understood. This paper from Li et al examines an interesting application of lung silica exposure and attempts to link it to stiffness.

Comments for the author

I thank the authors for their attention to the blots in question and for resolving the issue satisfactorily. I do not have any further concerns about publication.

Third revision

Author response to reviewers' comments

Dear Dr. Andrew Ewald,

Thank you for your comments on our manuscript entitled **"Matrix stiffness regulates α-TAT1/Ac-α-Tub and promotes silica-induced epithelial-mesenchymal transition via DNA damage"** (MS ID#: JOCES/2019/243394). We have studied the comments carefully, and revised the content to make the manuscript more scientific and rigorous. The edits are in red font in the revised manuscript. We have also uploaded a formatted PDF of the Response as Supplementary Information. We hope that the revised manuscript meets with your approval.

Reviewer 1 Advance Summary and Potential Significance to Field

The authors are interested in mechanisms by which silicosis damage occurs in the epithelium. Specifically, they seek to understand if tubulin acetylation by a-TAT1 by promoting microtubule stability prevent EMT and DNA damage. Using a combination of a rat model of silicosis and a lung cancer cell line, A549 cells, the authors have shown that silica exposure and matrix stiffening or a combination of both lead to downregulation of acetylated-tubulin and subsequent DNA damage and replication stress. These effects are abrogated by the increase in acetylated tubulin. Overall, the paper was a nice demonstration of mechanisms by which silica lead to lung injury and potential epithelial to mesenchymal transition.

Responses: Thank you very much for your recognition.

Reviewer 1 Comments for the Author.

The authors have adequately addressed the previous concerns. The modified figures as submitted do not raise further concerns.

Response: Thank you.

Reviewer 2 Advance Summary and Potential Significance to Field

per previous

Reviewer 2 Comments for the Author.

Comment NO.1 What ECM protein(s) was attached to the gels? I do not see the answer to this simple question in the key Methods section: " Polyacrylamide gels were prepared as previously described (Leight et al., 2012). Mechanical properties of the polyacrylamide gels were controlled by varying the percentage of acrylamide and bis-acrylamide as follows: elastic modulus (% acrylamide;%bis-acrylamide), 1 kPa (3; 0.1) and 60 kPa (10; 0.5). Gels were incubated with 1% (vol/vol) ethanolamine in 50 mM HEPES, pH 8, for 30 min, and rinsed with ddH2O. ."

Response: Gels were covered with 140 μg/ml Matrigel. In the revised manuscript, we rearranged the description of the preparation method of polyacrylamide gels. *"The mechanical properties of the polyacrylamide gels were controlled by varying the percentage of acrylamide and bis-acrylamide as previously described (Leight et al., 2012), and the elastic modulus (% acrylamide;%bis-acrylamide) of 1 kPa (3; 0.1) and 60 kPa (10; 0.5) were used in this study. Gels were covered with 140 μg/ml Matrigel 2 h on ice. The gels were sterilized in 5% (vol/vol) isopropanol in phosphate-buffered saline (PBS) for 1 h at room temperature (RT), and rinsed two times with sterile PBS before plating with cells."*

Reviewer 3 Advance Summary and Potential Significance to Field

The interplay of silica exposure and matrix stiffness is poorly understood. This paper from Li et al examines an interesting application of lung silica exposure and attempts to link it to stiffness.

Response: Thank you.

Reviewer 3 Comments for the Author

I thank the authors for their attention to the blots in question and for resolving the issue satisfactorily. I do not have any further concerns about publication.

Response: Thank you.

Thank you very much for your kind work and consideration of publication of our paper. On behalf of my co-authors, we would like to express our great appreciation to the editor and reviewers. We look forward to hearing from you at your earliest convenience.

Thank you and best regards,
Sincerely,

Shifeng Li
Hebei Key Laboratory for Organ Fibrosis Research, School of Public Health, North China University of Science and Technology.
E-mail: leimengpi@163.com

Fourth decision letter

MS ID#: JOCES/2019/243394

MS TITLE: Matrix stiffness regulates Î±-TAT1/Ac-Î±-Tub and promotes silica-induced epithelial-mesenchymal transition via DNA damage

AUTHORS: Gengxu Li, Si Chen, Yi Zhang, Hong Xu, Dingjie Xu, Zhongqiu Wei, Xuemin Gao, Wenchen Cai, Na Mao, Lijuan Zhang, Shumin Li, Fang Yang, Heliang Liu, and Shifeng Li
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 2

Advance summary and potential significance to field

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Comments for the author

Absorption of matrigel followed by 5% alcohol sterilization doesn't seem a sound practice, but at least it is finally documented.