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Metformin inactivates the cGAS-STING pathway through autophagy and suppresses senescence in nucleus pulposus cells

Chenghao Ren, Jie Jin, Chenchao Li, Jianwei Xiang, Yaosen Wu, Yifei Zhou, Liaojun Sun, Xiaolei Zhang and Naifeng Tian
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Original submission

First decision letter

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MS TITLE: Metformin inactivates the cGAS-STING pathway through autophagy and suppresses senescence in nucleus pulposus cells

AUTHORS: Naifeng Tian, Chenghao Ren, Jie Jin, Chenchao Li, Jianwei Xiang, Yaosen Wu, Yifei Zhou, Liaojun Sun and Xiaolei Zhang
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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. 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 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

In this article, the authors report that metformin, a pharmacological activator of autophagy, can inhibit intervertebral disc degeneration (IVDD) by inhibiting the cGAS/STING pathway. The cytoplasmic DNA sensor, cGAS, can trigger inflammation upon sensing the damaged DNA. Metformin can cause the removal of this damaged DNA the inducers of the cGAS/STING pathway, to prevent the inflammatory responses. As a result, metformin inhibits the excessive senescence of the nucleus pulposus cells (NPCs). The study is well designed, with complementary approaches, and the results largely support the conclusions. However, the interpretations of some results are not acceptable, and the authors have not followed up on several key observations, which support the mechanisms, as noted below.

Comments for the author

The authors consistently noted that metformin treatment reduces STING and cGAS protein expression, which were interpreted as inhibition of cGAS/STING activation.
However, they have not examined whether the autophagy-lysosome pathway degraded these proteins? In addition to DNA damage inhibition, the loss of these key signaling proteins may add to the mechanism of metformin action.
The loss of gH2AX protein may also be associated with the metformin-mediated autophagy-degradation pathway. The use of autophagy inhibitors or genetic ablation of autophagy genes can help.
The basis for TBHP-mediated enhancement of STING, cGAS protein expression has not been followed up. Why does TBHP upregulate these proteins?
The study is entirely based on one cell type; this is acceptable for mechanistic purposes. However, are these results validated in other cells?

Reviewer 2

Advance summary and potential significance to field

This manuscript focuses on senescence in nucleus pulposus cells, which is thought to be a factor in intervertebral disc degeneration. The authors previously found that metformin can activate autophagy to inhibit senescence of nucleus puposus cells. In this study, the authors explored the relationship between metformin-activated autophagy and the cGAS-STING pathway.

The authors show that TBHP induces senescence based on p16 and p21 expression and senescence associated beta-galactosidase positive cell percentage. Treating with Metformin reduced the induction of senescence in a dose-dependent manner.

The cGAS/STING/phosphop65 showed a similar pattern with an increase in TBHP-treated cells. Treatment with metformin reversed the increase in cGAS/STING/phospho-p65 in a dose-dependent manner.

TBHP treatment increased p65 nuclear localization. Knocking down String or treating with Met inhibited the ability of THP to induce nuclear localization of p65. Overexpressing STING could reverse the inhibitory effects of metformin resulting in nuclear p65 localization.

The role of STING in the ability of metformin to prevent senescence was tested by knocking down STING and monitoring the effects of metformin on senescence endpoints.

Metformin treatment increased LC3-II and reduced p62. Autophagy induction by either rapamycin or metformin reduced cGAS/STING/p16/p21/phospho-p65, while 3-MA partially reversed this effect.

TBHP induced DNA damage over time, and this effect was reversed by metformin in a dose-dependent manner.

In a model of intervertebral disc degeneration based on disc puncture, metformin was found to reduce the impact of the procedure on degeneration of the disc and disc height.

Overall, this is an interesting paper that lays out a clear model for the molecules involved in TBHP-induced senescence. The findings suggest that metformin could protect against senescence in an invertebral disc degeneration model, and the data support this hypothesis.

The data are clear and mostly convincing, but I do have some questions below.

Comments for the author

1. I would expect that if STING were important for the ability of metformin to affect senescence, then knocking down STING would result in a situation in which metformin treatment does not result in reduced p16/p21. But the data in Fig 2A and 2B show that a similar reduction in p16 and p21 is observed with and without STING knockdown.
2. Similarly, metformin seemed to have a similar effect of increasing p16 and p21 when an empty vector or STING protein was introduced. This would suggest that STING is not part of the pathway through which metformin affects senescence. Similarly, metformin still has a strong effect on senescence induction based on senescence-associated beta galactosidase with STING overexpression or STING knockdown. This also seems to suggest that STING is not in the pathway between metformin and senescence.
3. The images in Fig 3C don't allow the reviewer to observe that met treatment facilitated autophagosome-lysosome fusion as reported by the authors.
4. The data in Fig 3D with TEM would need to be quantified from multiple cells to support a conclusion.
5. Fig 4E, the effects of 3-MA on γ-H2AX are difficult to perceive in this blot.
6. The quantification of the images in Fig 5E is based on the fraction of cells that are positive for staining, but the effects look more like an impact on the intensity of staining rather than the fraction of cells that are positive for staining.

Minor: Line 298 western "blot"

Reviewer 3

Advance summary and potential significance to field

The authors of this manuscript have shown a novel mechanism of metformin suppressing senescence through the cGAS-STING pathway, and this has been implicated to be occurring by autophagy. The mechanism has been directly related to the disease model of Intervertebral Disc Degeneration (IVDD) where such complex processes including senescence of nucleus pulposus (NPCs) is major factor.

Comments for the author

While the authors have addressed the hypothesis with several experiments and data there are some major and minor comments below that need to be added to the manuscript:

Major corrections:
- Metformin showed inhibition of the senescence of nucleus pulposus cells and activation of the cGAS-STING pathway. Were any other analogs used, like phenformin? Please show some preliminary data in supplementary figures.
- While cellular senescence markers including p16 and p21 have been shown, is there a direct/indirect effect through the p53 or LKB1 pathway? Please show the expression levels of p53, LKB1 and/or AMPK.

- 3-MA has been used as an autophagy inhibitor in this study. Were other drugs tested, including hydroxychloroquine and/or specific inhibitors like Lyso5? Please show some preliminary results using these drugs.
- Were any inflammatory cytokines or oxidative stress markers measured? Please state some information in the discussion, and add data to support the hypothesis.
- Figure 1: It has been stated using previous studies, it would be appropriate to show cell viability of NPCs upon treatment with TBHP and/or Metformin, and then the western blot images.
- All western blot images (y-axis): Please state in the y-axis if the graphs are normalized to GAPDH (or the ratio of protein expression of X and Y, state X, Y)
- Figure 5: Puncture-induced rat IVDD model: Why weren't Met and 3-MA groups not shown in the figure? It would be appropriate to show all the tested groups or justify in the results section for not using/testing these groups.
- Figure 5C: Please show IHC for p62.

Minor corrections:
- Methods Section 2.4 (line 116): please correct 'METFORMIN' was pretreated with metformin...
- Methods Section 2.6 (line 134-135): Advisable to add 'purified': Forty nanograms of 'purified' protein were separated...
- Methods Section 2.9 (line 169): 30 'percent' confluence
- Methods Section 2.10 (line 197): what locomotor activities were noted? Weight changes? Any results? Please add in the figures.
- Results section 3.1 (line 240): It would be more appropriate to add 'current experiment' or 'current hypothesis' instead of 'next experiment'.

First revision

Author response to reviewers' comments

To reviewer 1
The study is well designed, with complementary approaches, and the results largely support the conclusions. However, the interpretations of some results are not acceptable, and the authors have not followed up on several key observations, which support the mechanisms, as noted below:
1. The authors consistently noted that metformin treatment reduces STING and cGAS protein expression, which were interpreted as inhibition of cGAS/STING activation. However, they have not examined whether the autophagy-lysosome pathway degraded these proteins? In addition to DNA damage inhibition, the loss of these key signaling proteins may add to the mechanism of metformin action.

Author response:
Thank you very much for your comments, which helped us a lot. There is a close link between cGAS-STING signaling and autophagy, Liang and collaborators found that cytoplasmic DNA can trigger autophagy to degrade cGAS proteins by promoting cGAS interaction with beclin-1 (Liang et al., 2014). In addition, Prabakaran and collaborators found that DNA fragments stimulated activation of the cGAS-STING pathway simultaneously triggers phosphorylation of p62, which targets STING for autophagosomal degradation (Prabakaran et al., 2018). These studies illustrate that autophagy can suppress excessive activation of cGAS-STING signaling by degrading cGAS and STING protein, thereby preventing excessive immune responses to maintain homeostasis.
In the present study, we mainly investigated the effects of metformin on disc degeneration, in which cellular damage occurs and thereby releases large amounts of DNA fragments, which are the main substances that activate cGAS-STING signaling. After activation of autophagy by metformin, autophagy mainly inhibits the activation of cGAS-STING signaling by degrading damaged DNA fragments in cells, in addition, autophagy may directly act on cGAS and STING protein to mediate the degradation of cGAS and STING protein, thereby collectively inhibiting the activation of downstream signaling, reducing inflammatory responses, and ultimately inhibiting the excessive senescence of cells.

2. The loss of gH2AX protein may also be associated with the metformin-mediated autophagy-degradation pathway. The use of autophagy inhibitors or genetic ablation of autophagy genes can help.

Author response:
Thank you very much for your comments, which helped us a lot. As shown in Fig4-C, the expression of γH2AX decreased as the concentrations of metformin increased, indicating that metformin inhibited the expression of γH2AX. Subsequently in Fig4-E, we found that the protein expression of γH2AX decreased in the metformin group, while increased after the addition of 3-MA, a classical autophagy inhibitor, to inhibit the autophagic effect of metformin. In addition, this result was also further confirmed by the immunofluorescence expression of γH2AX, the fluorescence intensity of γH2AX in the nucleus decreased after the addition of metformin, while the addition of 3-MA inhibited metformin induced autophagy reversed this phenomenon. These results illustrate that loss of γH2AX is associated with metformin mediated autophagic degradation.

3. The basis for TBHP-mediated enhancement of STING, cGAS protein expression has not been followed up. Why does TBHP upregulate these proteins?

Author response:
Thank you very much for your comments, which helped us a lot. As shown in Fig1-G, the proteins of both cGAS and STING were elevated under TBHP. In addition, the cGAS-STING signaling pathway is a key mediator in the alleviation of cellular stress and tissue damage, is mainly composed of the synthase for the second messenger cyclic GMP-AMP (cGAS) and the cyclic GMP-AMP receptor stimulator of interferon genes (STING), the activation of which is first by the cGAS protein recognizing a wide range of DNA fragments of foreign or self-origin, but the decisive role in the activation of STING and the initiation of downstream signals to exert its effects, So in this study, we mainly studied STING protein and investigated the mechanism.
TBHP is a known inducer of oxidative stress and cell damage. Studies have shown that TBHP treatment can be used as a model of cellular senescence (Wedel et al., 2020). Therefore, in this study, we used TBHP to stimulate nucleus pulposus cells to model the degeneration of intervertebral discs in vitro. In addition, TBHP-induced oxidative stress can cause DNA damage, and cGAS-STING signaling is a DNA sensing mechanism to detect DNA damage, so there must be an inevitable connection between the two. Finally, previous experiments in our group have confirmed that TBHP can activate cGAS-STING signal, and targeted knockdown of STING protein can alleviate TBHP-induced intervertebral disc degeneration (Guo et al., 2021), so the expression of these proteins is up-regulated after TBHP stimulation

4. The study is entirely based on one cell type; this is acceptable for mechanistic purposes. However, are these results validated in other cells?

Author response:
Thank you very much for your comments, which helped us a lot. Our experimental group mainly studied the direction related to disc degeneration, in which nucleus pulposus cells are the main functional cells in the intervertebral disc, so we mainly used nucleus pulposus cells in a series of studies. As for in other cells, further exploration is needed.
To reviewer 2
This is an interesting paper that lays out a clear model for the molecules involved in TBHP-induced senescence. The findings suggest that metformin could protect against senescence in an invertebral disc degeneration model, and the data support this hypothesis.
1.I would expect that if STING were important for the ability of metformin to affect senescence, then knocking down STING would result in a situation in which metformin treatment does not result in reduced p16/p21. But the data in Fig 2A and 2B show that a similar reduction in p16 and p21 is observed with and without STING knockdown. Similarly, metformin seemed to have a similar effect of increasing p16 and p21 when an empty vector or STING protein was introduced. This would suggest that STING is not part of the pathway through which metformin affects senescence. Similarly, metformin still has a strong effect on senescence induction based on senescence-associated beta galactosidase with STING overexpression or STING knockdown. This also seems to suggest that STING is not in the pathway between metformin and senescence.

Author response:
Thank you very much for your comments, which helped us a lot. Based on your confusion, we modified Figure2. First, we explored the relationship between STING and senescence. Some studies have confirmed that STING can promotes the senescence-associated secretory phenotype (SASP) leading to senescence (Dou et al., 2017). In this study, we found that knockdown of STING protein significantly inhibited the expression of TBHP-induced senescence marker proteins p16 and p21 compared with the control group, whereas STING silencing alone did not affect the expression of these proteins. However, overexpression of STING protein increased the expression levels of p16 and p21, indicating a positive correlation between STING and senescence, and STING promoted senescence (Fig2A-D). This result was further confirmed by the results of SA-ß-gal staining (Fig2E, K). Subsequently, we explored the relationship between metformin and STING protein. As shown in Fig2G, Met inhibited TBHP-induced expression of p16 and p21, but this phenomenon was reversed after overexpression of STING. SA-ß-gal staining results showed that the number of senescent cells decreased after met treatment, but senescent nucleus pulposus cells instead increased after overexpression of STING (Fig2I.J). indicating that met exerts anti-senescence effects through the regulation of STING protein. In addition, we also examined the gene expression of inflammatory factors IL-1ß and IL-6. As shown in Fig2F, the expression of inflammatory factors decreased after met treatment, but overexpressing STING inhibited the anti-inflammatory effect of met. These results illustrate that met reduces inflammatory responses by inhibiting the activation of cGAS-STING pathway and ultimately delaying senescence of nucleus pulposus cells

2. The images in Fig 3C don't allow the reviewer to observe that met treatment facilitated autophagosome-lysosome fusion as reported by the authors.

Author response:
Thank you very much for your comments, which helped us a lot. To satisfy the reviewer's observations, we have changed the images. As shown in Figure 3E, we found that Met activates autophagy and promotes autophagosome-lysosome fusion.

3. The data in Fig 3D with TEM would need to be quantified from multiple cells to support a conclusion.

Response:
Thank you very much for your comments, which helped us a lot. As shown in Figure 3D, we added a quantification plot to further support the conclusion.

4. Fig 4E, the effects of 3-MA on γ-H2AX are difficult to perceive in this blot.

Response:
Thank you very much for your comments, which helped us a lot. We re-selected representative blots for replacement as shown in Figure 4E.

5. The quantification of the images in Fig 5E is based on the fraction of cells that are positive for staining, but the effects look more like an impact on the intensity of staining rather than the fraction of cells that are positive for staining.

Response:
Thank you very much for your comments, which helped us a lot. Immunohistochemical staining was developed with a chromogen after specific binding of antigen and antibody, the positive reaction site was brownish yellow, and the nucleus was blue. In this study, tissues senescence was obvious, and the cGAS-STING signaling pathway was activated with strong positive staining reaction and dark color in the IVDD-induced model group. When metformin treatment was added, the tissues senescence was inhibited, the positive response was weakened, and the color became lighter. Subsequently we counted the positive area positive cells in the tissue, the resulting data were calculated as follows: % positive cells = positive nuclei cells/total cells nuclei × 100 (Bologna-Molina et al., 2011).

6. Minor: Line 298 western “blot”

Response:
Thank you very much for your comments, which helped us a lot. We revised it in the manuscript. Result Section 2.4 (line 160)

To reviewer 3
While the authors have addressed the hypothesis with several experiments and data, there are some major and minor comments below that need to be added to the manuscript:
1. Metformin showed inhibition of the senescence of nucleus pulposus cells and activation f the cGAS-STING pathway. Were any other analogs used, like phenformin? Please show some preliminary data in supplementary figures.

Response:
Thank you very much for your comments, which helped us a lot. Although metformin and phenformin have similar functions, some studies have shown that phenformin has serious adverse effects leading to lactic acidosis compared to metformin, and it has been withdrawn from clinical use. Therefore, this study mainly conducted research on metformin and explored new therapeutic effects of metformin in clinic (McGuinness and Talbert, 1993).

2. While cellular senescence markers including p16 and p21 have been shown, is there a direct/indirect effect through the p53 or LKB1 pathway? Please show the expression levels of p53, LKB1 and/or AMPK.

Response:
Thank you very much for your comments, which helped us a lot. Cellular senescence is mainly controlled by the p53-p21 pathway and the p16-RB pathway (Feng et al., 2016; Wang et al., 2016), which induce excessive senescence in disc cells when activated by various stimuli. p53, p21, p16 protein expression was enhanced during nucleus pulposus cell senescence (Chen et al., 2021). In addition, previous studies have reported that metformin can inhibit the expression of senescence biomarker genes (p16, p53, p21)(Al Dubayee et al., 2021), which is consistent with the results of this study, as shown in Figure 1D, metformin inhibited the expression of senescence markers in a concentration gradient manner.
Metformin itself is an AMPK activator, and our group previously demonstrated that metformin could activate AMPK to induce autophagy(Chen et al., 2016). LKB1 is an upstream kinase of AMPK, and LKB1 encodes a serine / threonine kinase that directly phosphorylates and activates AMPK to exert its effects (Shackelford and Shaw, 2009). In addition, Shaw RJ published in Science in 2005 suggested that metformin exerts glucose lowering effects dependent on the LKB1/AMPK pathway (Shaw et al., 2005), and Xie Z subsequently discovered in 2008 that the target of metformin is inhibition of complex I of the mitochondrial respiratory chain, and inhibition of complex I leads to phosphorylation and activation of protein kinase C. Activated protein kinase C phosphorylates LKB1 and finally causes AMPK activation (Xie et al., 2008). These studies further illustrate that there are direct effects of metformin and LKB1 and that metformin can activate the LKB1 / AMPK signaling pathway

3.3-MA has been used as an autophagy inhibitor in this study. Were other drugs tested, including hydroxychloroquine and/or specific inhibitors like Lyso5? Please show some preliminary results using these drugs.

Response:
Thank you very much for your comments, which helped us a lot. Chloroquine and its derivative hydro-chloroquine, as autophagy inhibitors, have the same effect by inhibiting the fusion of autophagosomes and lysosomes and thereby blocking the occurrence of autophagy (Mauthe et al., 2018). We used chloroquine to further verify that, as shown in the supplementary figureS2, metformin inhibited TBHP-induced cGAS-STING signaling activation and the occurrence of senescence, but the addition of CQ reversed these phenomena after inhibiting metformin-induced autophagy, indicating that metformin inhibited the activation of cGAS-STING signaling mainly by activating autophagy to ultimately retard the occurrence of senescence.

4. Were any inflammatory cytokines or oxidative stress markers measured? Please state some information in the discussion and add data to support the hypothesis.

Response:
Thank you very much for your comments, which helped us a lot. Studies have reported that metformin can inhibit the expression of inflammatory cytokines (Hammad et al., 2021). In addition, studies have reported that the activation of cGAS-STING signaling can activate downstream pro-inflammatory responses, and the release of inflammatory factors promotes cellular senescence associated secretory performance (SASP) ultimately leading to cellular senescence (Dou et al., 2017). To verify the link between the two, we examined the expression of inflammatory factors IL-1 and IL-6 by PCR. As shown in Figure 2F, compared with the control group, treatment with metformin significantly decreased the expression of inflammatory factors IL-1 and IL-6, but this was reversed after overexpression of STING protein, indicating that STING is involved in the process of inhibiting the expression of inflammatory by metformin, and metformin can inhibit the expression of inflammatory factors by inhibiting the activation of STING.

5. Figure 1: It has been stated using previous studies, it would be appropriate to show cell viability of NPCs upon treatment with TBHP and/or Metformin, and then the western blot images.

Response:
Thank you very much for your comments, which helped us a lot. We supplement this part of the data as shown in Figure 1A-C.

6. All western blot images (y-axis): Please state in the y-axis if the graphs are normalized to GAPDH (or the ratio of protein expression of X and Y, state X, Y)

Response:
Thank you very much for your comments, which helped us a lot. We have made modifications in the diagram.

7. Figure 5: Puncture-induced rat IVDD model: Why weren't Met and 3-MA groups not shown in the figure? It would be appropriate to show all the tested groups or justify in the results section for not using/testing these groups.

Response:
Thank you very much for your comments, which helped us a lot. We demonstrated nucleus pulposus tissue after treatment with met and 3-MA alone in supplementary data. As shown in the supplementary figureS2, nucleus pulposus tissues showed no destruction, the annulus fibrosus was well organized and there was no difference in histological scores of the intervertebral discs. While this study was conducted through in vivo experiments mainly to verify whether the treatment with metformin could delay the progression of disc degeneration, in order to make the effect of metformin more obvious, we did not show the met and 3-MA groups in the figure.

8. Figure 5C: Please show IHC for p62.

Response:
Thank you very much for your comments. We have supplemented the figure as shown in Figure 5E

9. Methods Section 2.4 (line 116): please correct 'METFORMIN' was pretreated with metformin...

Response:
Thank you very much for your comments. We have already modified it in the manuscript. Methods Section 4.4 (line 289)

10. Methods Section 2.6 (line 134-135): Advisable to add 'purified': Forty nanograms of 'purified' protein were separated...

Response:
Thank you very much for your comments. We have already modified it in the manuscript. Methods Section 4.8 (line 334)

11. Methods Section 2.9 (line 169): 30 'percent' confluence

Response:
Thank you very much for your comments. We have already modified it in the manuscript. Methods Section 4.11 (line 367)

12. Methods Section 2.10 (line 197): what locomotor activities were noted? Weight changes? Any results? Please add in the figures.

Response:
Thank you very much for your comments. Due to an error in our formulation, we have revised it in the manuscript. Methods Section 4.12 (line 393-394)

13. Results section 3.1 (line 240): It would be more appropriate to add 'current experiment' or 'current hypothesis' instead of 'next experiment'.

Response:
Thank you very much for your comments. We have already modified it in the manuscript. Methods Section 2.1 (line 88)

Reference:

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Second decision letter

MS ID#: JOCES/2021/259738

MS TITLE: Metformin inactivates the cGAS-STING pathway through autophagy and suppresses senescence in nucleus pulposus cells

AUTHORS: Chenghao Ren, Jie Jin, Chenchao Li, Jianwei Xiang, Yaosen Wu, Yifei Zhou, Liaojun Sun, Xiaolei Zhang, and Naifeng Tian
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, reviewer 2 raised a number of criticisms that prevent me from accepting the paper at this stage. I agree with this reviewer, that at a minimum, you should quantify the SA-bGal images shown in Figure 2E, similar to what was done in Figure 3K. There are additional concerns raised by this reviewer regarding figure 2, which could be addressed through editing the conclusions of the data presented in Figure 2. 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

There are no additional concerns.

Comments for the author

There are no additional concerns.

Reviewer 2

Advance summary and potential significance to field

The main finding is that metformin suppresses senescence in nucleus pulposus cells through a pathway that involves cGAS-STING and autophagy.

Comments for the author

The authors addressed many of the comments. Some questions remain.
1. In Fig 2A, the authors argue that with STING knockdown, p16 and p21 levels are lower, but this is only a very small fraction of the increase in p16 with TBHP. It is not clear how important this small difference is for actual senescence. Fig 2C shows that if you dramatically overexpress STING, you get more senescence, but this does not address whether the effects of TBHP are mediated through STING. The figures in 2E are not quantified and therefore the reader cannot evaluate whether STING is important for TBHP induced senescence.
For each condition there is just one image with a very limited number of cells.
A reader would need many cells to be randomly selected and quantified to be able to draw conclusions. The authors do not show cytokine expression to support a role for STING in TBHP-mediated senescence. The cytokine data is about the effects of metformin. If the authors want to claim that TBHP affects senescence through STING knockdown, then quantification of the b-galactosidase staining is necessary.
I would consider the following to be important, but possibly not absolutely required for publication:
2. The reviewers did not address experimentally Reviewer 1's question about the autophagy pathway degrades cGAS/STING components in this system. This seems like an important aspect of the mechanism.
3. Reviewer 2 was suggesting that the authors use genetic ablation of autophagy genes, but the authors did not do this experiment.
4. The authors also don't address Reviewer 1's question about how TBHP enhances STING. They hypothesize that it is through oxidative stress but don't do the experiment.
5. The question from Reviewer 1 about whether this is just one type of cell was not addressed.
6. The authors didn't respond to the question about whether p53 is involved with any experiments or any data.

Reviewer 3

Advance summary and potential significance to field

The authors of this manuscript have shown a novel mechanism of metformin suppressing senescence through the cGAS-STING pathway, and this has been implicated to be occurring by autophagy. The mechanism has been directly related to the disease model of Intervertebral Disc Degeneration (IVDD) where such complex processes including senescence of nucleus pulposus (NPCs) is major factor.

Comments for the author

The authors have addressed all the comments that were raised in the revision, and have updated the manuscript accordingly. I recommend the manuscript be accepted in this updated version.

Second revision

Author response to reviewers' comments

To reviewer 2
The authors addressed many of the comments. Some questions remain.
1. In Fig 2A, the authors argue that with STING knockdown, p16 and p21 levels are lower, but this

is only a very small fraction of the increase in p16 with TBHP. It is not clear how important this small difference is for actual senescence. Fig 2C shows that if you dramatically overexpress STING, you get more senescence, but this does not address whether the effects of TBHP are mediated through STING. The figures in 2E are not quantified and therefore the reader cannot evaluate whether STING is important for TBHP induced senescence. For each condition there is just one image with a very limited number of cells. A reader would need many cells to be randomly selected and quantified to be able to draw conclusions. The authors do not show cytokine expression to support a role for STING in TBHP-mediated senescence. The cytokine data is about the effects of metformin. If the authors want to claim that TBHP affects senescence through STING knockdown, then quantification of the b-galactosidase staining is necessary.

Author response:
Thank you very much for your comments, which are very helpful to us. As shown in Figure 2K, we have quantified the ß-gal staining in Fig.2E and showed that the number of senescent nucleus pulposus cells was significantly decreased when STING protein was knocked down, whereas the number of senescent nucleus pulposus cells was increased when STING protein was overexpressed. Combined with the experimental results of western blot in Fig.2A and Fig.2C, knockdown of STING protein inhibited the expression of TBHP-induced senescence marker proteins p16 and p21, whereas overexpression of STING protein promoted the expression of p16 and p21, these results suggested that STING was involved in TBHP-induced senescence and STING could promote senescence.
Furthermore, as shown in Figure 2F, we also examined the gene expression of inflammatory factors IL-1ß and IL-6, which were significantly enhanced after TBHP stimulation alone but decreased after the addition of metformin, and subsequently we overexpressed STING protein, which reversed the inhibition of inflammatory factors by metformin, indicating that STING is involved in the regulation of metformin against TBHP-induced cell senescence. Metformin reduced the inflammatory response by inhibiting the activation of cGAS-STING signaling, ultimately delaying the senescence of nucleus pulposus cells.

The reviewer would consider the following to be important, but possibly not absolutely required for publication:
2. The reviewers did not address experimentally Reviewer 1's question about the autophagy pathway degrades cGAS/STING components in this system. This seems like an important aspect of the mechanism.

Author response:
Thank you very much for your comments, which helped us a lot. There are studies reporting that autophagy can inhibit excessive activation of cGAS-STING signaling by degrading cGAS or STING proteins (Liang et al., 2014; Prabakaran et al., 2018). In addition, there are studies showing that autophagy is a major player in DNA damage (Galati et al., 2019), and autophagy can inhibit the activation of downstream STING signaling by clearing DNA fragments (Gkirtzimanaki et al., 2018). In the present study, we mainly investigated the effects of metformin on disc degeneration, senescent cells undergo damage and thereby release large amounts of DNA fragments, which are the main substances that activate cGAS-STING signaling. After activation of autophagy by metformin, autophagy mainly inhibits the activation of downstream cGAS-STING signaling by degrading damaged DNA fragments in cells, reducing the inflammatory response, and finally inhibiting the excessive senescence of cells. This study provides evidence for a possible link between autophagy and cGAS- STING signaling.

3. Reviewer 1 was suggesting that the authors use genetic ablation of autophagy genes, but the authors did not do this experiment.

Author response:
Thank you very much for your comments, which are very helpful to us. Studies have found that both genetic ablation of autophagy genes or pharmacological inhibition of autophagy (3-MA or Chloroquine) can significantly affect the effect of autophagy (Ock et al., 2017),with both having similar effects. Therefore, we mainly used autophagy inhibitors 3-MA and CQ to perform a series of experiments.

4. The authors also don't address Reviewer 1's question about how TBHP enhances STING. They hypothesize that it is through oxidative stress but don't do the experiment.

Author response:
Thank you very much for your comments, which are very helpful to us. TBHP is a known inducer of oxidative stress and cell damage, and oxidative stress is a key factor causing cell senescence (Maharajan et al., 2021), and studies have shown that TBHP treatment can be used as a model of cell senescence (Wedel et al., 2020). therefore, in this study we simulated disc degeneration in vitro by stimulating nucleus pulposus cells with TBHP. In addition, our previous study has confirmed that cGAS-STING signaling can be activated after TBHP, and targeted knockdown of STING protein can attenuate TBHP-induced disc degeneration (Guo et al., 2021). therefore, the expression of these proteins is up regulated after TBHP stimulation

5. The question from Reviewer 1 about whether this is just one type of cell was not addressed.

Author response:
Thank you very much for your comments, which are very helpful to us. We are sorry for the fact that our group mainly studied the direction related to disc degeneration.
Nucleus pulposus has been found to be the central region of the affected disc, and nucleus pulposus cells are the cells that mainly function in the disc (Wu et al., 2021). So this study mainly used nucleus pulposus cells in a series of explorations. As for in other cells, further exploration is needed.

6. The authors didn't respond to the question about whether p53 is involved with any experiments or any data.

Author response:
Thank you very much for your comments, which are very helpful to us. Cellular senescence is mainly controlled by the p53-p21 pathway and the p16-pRB pathway (Wang et al., 2016). P21 is found to be a downstream gene of p53 in the p53-p21 pathway, and its expression level is regulated by p53 (Chen et al., 2020). In addition, it has been reported that p53, p21 and p16 protein expression is enhanced during nucleus pulposus cell senescence (Chen et al., 2021). As shown in Figure.1D, we complementarily detected the expression of senescence marker p53, and found that metformin could inhibit the expression of senescence associated markers (p53, p21, p16) in a concentration gradient manner.

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Third decision letter

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AUTHORS: Chenghao Ren, Jie Jin, Chenchao Li, Jianwei Xiang, Yaosen Wu, Yifei Zhou, Liaojun Sun, Xiaolei Zhang, and Naifeng Tian
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I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.