nature portfolio

Peer Review File

Deletion of ASPP1 in myofibroblasts alleviates myocardial fibrosis by reducing p53 degradation

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Nature Communications manuscript NCOMMS-23-59316

A. What are the noteworthy results?

In the study of Li et al. 'Myofibroblast-specific inhibition of ASPP1 alleviates myocardial fibrosis by enhancing p53 degradation' the authors propose that a selective loss of ASPP1 in myofibroblasts in a mouse model is protective against myocardial fibrosis by increasing p53 levels and inhibiting myofibroblast proliferation. At the molecular level, the study shows that in myofibroblasts ASPP1 interacts with deubiquitinase OTUB1 to prevent p53 deubiquitination, and thus promotes p53 degradation.

B. Will the work be of significance to the field and related fields?

In my assessment, the report of ASPP1 activity in myofibroblasts promoting p53 degradation via interaction with deubiquitinase OTUB1 and the implications for myocardial fibrosis are novel. The discussed opposing activities of ASPP1 on p53 in myofibroblasts versus its previously reported pro-apoptotic activities in cardiomyocytes and tumor cells are particularly interesting, if fully validated.

Cell type specific differences in the modes of p53 regulation and in the roles of p53 in tissue physiology are an important direction of study in the research field.

C.

- Does the work support the conclusions and claims, or is additional evidence needed?

- Are there any flaws in the data analysis, interpretation, and conclusions?

- Is the methodology sound? Does the work meet the expected standards in your field?

Here are technical questions that need to be addressed for the data to fully support the conclusions of the study.

C1.1. Do we know that the protective effects of ASPP1-loss in Figure 3D are myofibroblast specific? How strong is the evidence that the activity of the tamoxifen-inducible Postn promoter-driven MerCreMer transgene (PostnMCM), used in Figure 3, is specific to myofibroblasts? While the reduction in ASPP1 levels in total heart tissue is convincing in Figure 3C, are we sure the effect is restricted to myofibroblasts? Similarly for Figure 5A - right panel, measuring p53 levels in this model, is there evidence that this effect is restricted to myofibroblasts?

C1.2. On a related note, a comparison between ASPP1fl/fl PostnMCM and ASPP1+/+ PostnMCM groups, instead of the ASPP1fl/fl Cre-negative control group, would be ideal in Figure 3. This would rule out that background Cre expression and activity has any effects on the phenotype, independently of the ASPP1 gene inactivation.

C2. It is recommended to further explore the effects of ASPP1-loss and downstream increase in p53 levels on myofibroblasts to clarify the following issues.

C2.1. While the shifts in cell cycle phase of cardiac fibroblasts with the siRNA-knockdown or over-expression of ASPP1 are significant, is this sufficient to conclude that cell proliferation is affected? This is especially so, as the effects are numerically small and the S- and G2-phases show opposite effects. Can the authors add data that directly measures cell proliferation in these models to prove their conclusions? If the authors can further demonstrate that the changes in cell proliferation are p53-depedent, this would especially strengthen the conclusions of the study.

C2.2. The proposed model indicates that the loss of ASPP1 stabilizes p53, which can be expected to result in a global activation of p53 stress response transcriptional programs. However, the only well-known p53-target gene the expression of which is measured to support this model is Cdk1na. The opposite effects on the Ccn, Cdk, Col1, and Fn1 genes shown in Figure 5 is interesting but doesn't directly show p53 transcriptional activity. Do the authors think Ccn/Cdk transcriptional changes are also under the direct control of p53, acting as a transcriptional repressor? Are Col1 and Fn1 known to be directly p53-regulated or are these transcriptional changes induced via indirect mechanisms? Given the previously reported role of ASPP1 in cardiomyocytes and tumor cells in regulating pro-apoptotic p53 target genes, should pro-apoptotic p53-target genes or other classical p53-target genes apart from Cdkn1a be measured in the AASP1 knockdown/overexpression myofibroblast models?

C2.3. If any of the readouts presented in Figures 5-6, apart from the total p53 protein levels, can be measured in myofibroblasts from the in vivo mouse myocardial fibrosis model, instead of the TGF-beta-treated myofibroblasts in culture, this would further strengthen the conclusions of the study.

C3. It is recommended to further explore (and/or discuss) the mechanisms through which ASPP1 modulates p53 levels in myofibroblasts to complement and strengthen the studies in Figure 6.

C3.1. A cycloheximide chase assay to measure p53 half-life in control, ASPP1-knockdown, and ASPP1-overexpressing myofibroblasts could strengthen the conclusion that the primary mechanism is regulation of p53 protein stability.

C3.2. Can the authors speculate why the effects of ASPP1 on p53 activity are distinct between myofibroblasts and other cell types? While perhaps this can't be addressed in full right now, can some hints be provided in the current study? For example, is ASPP1/OTUB1 interaction specific to myofibroblasts over other cell types where ASPP1 was previously shown to cooperate with rather than antagonize p53 activity?

C3.3. In the Discussion the authors state that 'inhibition of myofibroblast ASPP1 holds the therapeutic potential in cardiac remodeling of infarct hearts'. Can the authors mention if an ASPP1 inhibitor exists or if it's feasible to develop one? Would this approach have benefits over alternative existing modes of p53 activation through nutlin MDM2 inhibitor, for example? Would stabilization of p53 in myofibroblasts with nutlin have equivalent effects to ASPP1 inhibition? I understand that in cardiomyocytes ASPP1 inhibition is preferred because of its expected anti-apoptotic effects. Existing pharmacological tools to stabilize p53 should be mentioned and the advantages of ASPP1 inhibition over these tools explained to the reader.

D. Is there enough detail provided in the methods for the work to be reproduced?

- Are all the mouse strains used in the study on C57BL/6J or another single inbred genetic background? If mouse strains of different genetic backgrounds are crossed and used this is problematic.

- I would have liked more explanation about the origins, genetic background, and any previously reported phenotypes of the ASPP1-KO mouse model used in Figure 2. This should be concisely incorporated into the manuscript, either in the Introduction or in Results before Figure 2 data is presented. This information is only accessible when following a citation from the Methods section and then going to supplemental materials of the cited study: Circ Res. 2023 Jan 20;132(2):208-222.

Other clarity and formatting issues:

- Title: 'Myofibroblast-specific inhibition of ASPP1 alleviates myocardial fibrosis by enhancing p53 degradation'. Doesn't the study in fact argue that inhibition (loss) of ASPP1 inhibits p53 degradation? You can see in Figure 5A-B that the levels of p53 go up with ASPP1-loss and go down with ASPP1 over-expression.

- I also don't like the use of 'inhibition' in the title as this can suggest to the reader that an inhibitor was developed and tested.

- Why does Figure 2B seem to present a 12-week experiment, when all the data in the Figure is from weeks 2 and 4? This is similar for Figure 3B also.

- lines 153-154: Do you mean 'knockdown and overexpression efficiency of p53, rather than OTUB1, are shown in Figures S1A and S1B'?

- Figure 5C: Please indicate on the Figure that p53 transcript is measured. I know this information is found in the Legend, but it will be much easier for the reader if this is also stated in the Figure itself.

Reviewer #2 (Remarks to the Author):

General synopsis

The authors of the current paper have assembled a paper that addressed how ASPP1 protein influences fibroblast p53 trafficking and in doing so, compared the effects of ASPP1 knock out in mice. In their approach, they utilized both global and myofibroblast specific KO mice. ASPP1 KO was associated with beneficial changes in cardiac function, fibrosis and remodeling of post MI hearts. Of considerable interest is the finding that ASPP1 KO yielded an effect which is opposite to that of cardiac myocytes in that p53 levels and cell fates. In other words knockdown of ASPP1 is currently reported to yield an increase in p53 levels in cardiac myofibroblasts and also inhibited “the activity” of cardiac myofibroblasts. They also showed that IF staining in TGFb stimulated cells was associated with ASPP1 accumulation in the cytoplasm of fibroblasts while the level of p53 was reduced while inhibition of ASPP1 increased p53 level and promoted p53 nuclear translocation (nicely depicted in Figure 7). They demonstrate that ASPP1 may bind to deubiquitinase OTUB1 and prevents binding to p53, preventing its degradation. This paper deals with a novel protein and the dataset supports their conclusions with a conservative summary. With the following points addressed in a categorical manner, the impact of this paper will be improved.

1. The dataset shows both mRNA and proteins of key genes assessed, which is a strength. In addition, all data points are shown, another point of transparency and clarity. However it is difficult to see all six data points in the tightly grouped controls, perhaps the histograms could be enlarged to provide for easier understanding. Figures 1-6 are affected.

2. Figure 1. Why was 4 weeks chosen as the MI duration in these animals? Why not 1 week or 2 weeks? Did the authors collect data from those time points (Figure 1)? It may provide some additional insight into the response of the ASPP1 gene following MI.

3. Figure 2G – It appears that while ventricular geometry is preserved in the post-MI hearts from ASPP1-KO mice, when compared to WT post-MI mice. Despite this the infarct scar is still very prominent. Do the authors feel that the preservation of cardiac performance as shown in panel E may be simply due to the preservation of ventricular geometry? If not, why? In the same line of thinking why is there no light microscopy with something like Masson's trichrome stain to offer a visual confirmation of the data in panel H?

4. Figure 2H. Extracellular Matrix is misspelled.

5. As in comment #3, the same questions apply to Figure 3.

6. Sometimes the terms fibroblast and myofibroblast are interchanged without any specificity. This is distracting but some editing would correct the problem. I suspect that the main target of function for ASPP1 lies in the activated myofibroblast, and so the paper should consistently reflect this view.

Responses to Reviewer's Comments

We thank the reviewers for their positive and constructive comments and suggestions on our work, which are very important for us to improve the quality of the work. We have performed a series of additional experiments to strengthen the conclusion of the study.

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A. What are the noteworthy results?

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Reply: In the present study, we mainly demonstrated that ASPP1 promoted cell proliferation and myocardial fibrosis following myocardial infarction (MI). Both global and Postn promoter driven deletion of ASPP1 mitigated cardiac fibrosis and remodeling by suppressing cell proliferation through augmenting p53-mediated transcription of cell cycle target genes. In addition, silencing ASPP1 promoted the binding of p53 to the deubiquitinase OTUB1, inhibiting p53 ubiquitination degradation, enhancing p53 transcriptional activation, and thus alleviating myocardial fibrosis caused by MI. These

findings reveal a previously unrecognized function of ASPP1 and identify a potential therapeutic target.

B. Will the work be of significance to the field and related fields?

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Cell type specific differences in the modes of p53 regulation and in the roles of p53 in tissue physiology are an important direction of study in the research field.

Reply: It has been demonstrated that the same molecule or pathway may exert different or opposing biological functions in different cell or tissue types. It has been reported that ubiquitin specific peptidase 7(USP7) yields multiple functions through interacting with p53 in cellular processes. USP7 promotes colorectal cancer cell apoptosis by directly stabilizing p53[1, 2]. However, USP7 induces fibroblasts activation and myocardial fibrosis by binding to and stabilizing MDM2, leading to p53 degradation[3]. We previously reported that p53 exacerbates ischemia-reperfusion injury by upregulating Puma, Bax, and Noxa to promote myocardial cell apoptosis[4]. Interestingly, in the current study, p53 mainly upregulates Cdkn1a and inhibits cell cycle progression, eventually leading to cell cycle arrest rather than apoptosis, thereby preventing abnormal proliferation of cardiac fibroblasts and reducing the burden on the heart[3, 5]. Our findings provide a new insight that ASPP1 is a novel regulator in cardiac fibrosis, and, more importantly, the same genetic manipulation strategy for silencing ASPP1, which inhibits p53 in cardiomyocytes while promotes p53 in fibroblasts, can produce cardiac protective effects as reflected by the reduced cardiac fibrosis and increased cardiomyocyte survival through differential regulatory mechanisms of p53 expression. Different cells or tissues exhibit different biological functions and responses to the

stimulation/stress, which primarily depend on different cell-specific components and signaling pathways. The different response of cell/tissue to one stimulation confers the significant potential and implication in the clinical research and treatment. The relevant information has been included in the Discussion of the revised manuscript **(Page 13).**

C.

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Reply: Thank you for the insightful comment. As periostin (Postn) is also expressed in other cell types such as vascular smooth muscle cells, endothelial cells, mesenchymal cells[6]. It cannot be ruled out the potential contribution of ASPP1 deficiency in these cells to cardiac protection.

The $Postn^{MCM}$ mouse (Strain #:029645) utilized in our study was described in the work by Kanisicak[7]. They confirmed that the Postn allele effectively recognized all myofibroblasts in the heart. We employed the same transgenic strategy for effective gene expression of in myofibroblasts as Bugg et al. In addition, studies also demonstrated that Postn promoter driven MerCreMer transgenic ($Postn^{MCM}$) with

tamoxifen specifically knocked out target genes in myofibroblasts need more clear description [8, 9, 10]. Notably, Postn is significantly upregulated in remodeling caused by long-term stress overload stimulation and myocardial infarction[11]. In addition, Onur et al. analyzed the cells in the injury area after myocardial infarction using lineage tracing and fluorescent-activated cell sorting (FASC), and found that Postn positive cells significantly appeared in myofibroblasts and originated from fibroblasts, rather than endothelial cells, immune/myeloid cells, or smooth muscle cells[7]. As myofibroblasts are the major contributor to cardiac fibrosis development, it can be speculated that ASPP1 deficiency driven by $Postn^{MerCreMer}$ is predominately restricted in myofibroblasts to alleviate cardiac fibrosis. The *in vitro* data also support the contribution of ASPP1 in cardiac fibrosis.

We cannot conclude that the genetic model employing $Postn^{MCM}$ technique to delete ASPP1 can induce myofibroblast-specific effects, as ASPP1 can be concomitantly deleted in vascular smooth muscle cells, endothelial cells, mesenchymal cells[6]. Therefore, the original description of **"Myofibroblast-specific ASPP1 deletion"** in the manuscript is not accurate. We have exchanged "Myofibroblast-specific ASPP1 deletion" to "Myofibroblast ASPP1 deletion" when describing deletion of ASPP1 in myofibroblast throughout the manuscript.

C1.2. On a related note, a comparison between $ASPP1^{fl/fl}$ $Postn^{MCM}$ and $ASPP1^{+/+}$ $Postn^{MCM}$ groups, instead of the $ASPP1^{fl/fl}$ Cre-negative control group, would be ideal in Figure 3. This would rule out that background Cre expression and activity has any effects on the phenotype, independently of the ASPP1 gene inactivation.

Reply: Thank you for the valuable comment. Accordingly, we performed the experiments with $ASPP1^{+/+}Postn^{MCM}$ and $ASPP1^{fl/fl}$ $Postn^{MCM}$ mice as recommended. After the establishment of MI model in $ASPP1^{+/+}Postn^{MCM}$ and ASPP1-CKO mice, tamoxifen was administered intraperitoneally immediately to induce activation of $Postn^{MCM}$ gene initiation. Mice were continued to feed until 4 weeks after MI **(Figure R1A and B)**. We observed that the survival rate of $ASPP1^{+/+}Postn^{MCM}$ mice was significantly lower than that of ASPP1-CKO mice after MI. The echo data showed that

the EF and FS were significantly lower in ASPP1$^{+/+}$Postn^{MCM} mice than in ASPP1-CKO mice **(Figure R1D, E)**. After MI, the infarct area and heart weight to body weight ratio were significantly lower in ASPP1$^{+/+}$Postn^{MCM} mice than in ASPP1-CKO mice **(Figure R1F, G)**. ASPP1-CKO mice also had significantly lower fibrosis than ASPP1$^{+/+}$Postn^{MCM} mice after MI **(Figure R1H, I)**. We have replaced the ASPP1$^{fl/fl}$ element with ASPP1$^{+/+}$Postn^{MCM} in **Figure 3** of the manuscript.

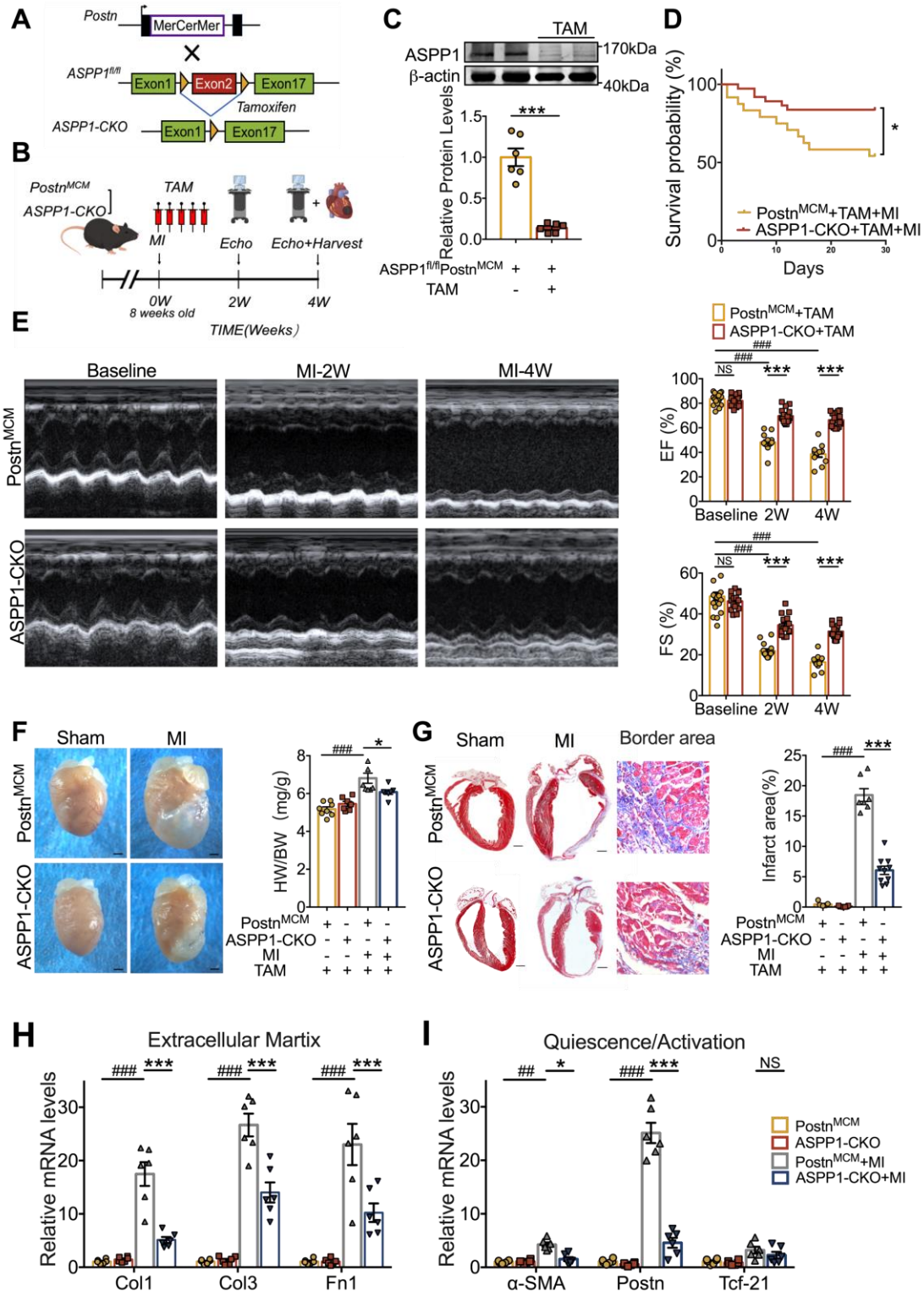

Figure R1. ASPP1 deletion prevents cardiac fibrosis in mice. A. Schematic diagram for the construction of ASPP1 knockout in mouse myofibroblasts. ASPP1$^{fl/fl}$ mice were crossed with Postn^{MCM} to obtain ASPP1$^{fl/fl}$ Postn^{MCM} mice**. B.** Schematic diagram of the experimental design. ASPP1$^{+/+}$Postn^{MCM} (Postn^{MCM}) and ASPP1$^{fl/fl}$ Postn^{MCM}

(ASPP1-CKO) mice were given tamoxifen continuously for 5 days after MI by intraperitoneal injection. $Postn^{MCM}$, $ASPP1^{+/+}$ $Postn^{MCM}$; ASPP1-CKO, $ASPP1^{fl/fl}$ $Postn^{MCM}$; TAM, tamoxifen. **C.** ASPP1 protein level in the heart of ASPP1 deletion mice by Western blot. n = 6. ***p < 0.001 vs. $ASPP1^{fl/fl}$ **D.** Kaplan–Meier analysis of the survival of $ASPP1^{+/+}Postn^{MCM}$ ($Postn^{MCM}$) and ASPP1-CKO mice 4 weeks post MI. n=24 in $Postn^{MCM}$ +MI group, n=37 in ASPP1-CKO+MI group. **p<0.01. **E.** Echocardiographic measurement of cardiac function. n=16-18, $^{###}p$ < 0.001 vs. $Postn^{MCM}$, NS. p>0.05 vs. $Postn^{MCM}$, ***p <0.001 vs. $Postn^{MCM}$ +MI. **F.** Representative images of the hearts and the ratio of heart weight (HW) to body weight (BW) in four groups of the mice as indicated. n = 7-12 per group. $^{###}p$ < 0.001 vs. $Postn^{MCM}$, **p < 0.01 vs. $Postn^{MCM}$ +MI. Scale bar = 1 mm. **G.** Images of Masson's trichrome staining and statistical analysis of fibrotic area by Image-Pro Plus. n = 7-12. $^{###}p$ < 0.001 vs. $Postn^{MCM}$, ***p < 0.001 vs. $Postn^{MCM}$ +MI. Scale bar = 1 mm. **H.** Transcriptional level of genes encoding extracellular matrix and myofibroblast markers in left ventricular tissue 4 weeks after MI by qRT-PCR. n= 6 per group. $^{#}p$<0.05 vs. $Postn^{MCM}$, $^{###}p$<0.001 vs. $Postn^{MCM}$, *p < 0.05 vs. $Postn^{MCM}$ +MI, ***p < 0.001 vs. $Postn^{MCM}$ +MI, NS p>0.05 vs. $Postn^{MCM}$ +MI. Data are represented as mean ± SEM. Statistics: Student's t test was used to calculate the presented P values in **C**. Log-rank (Mantel-Cox) test (for **D**). 2-way ANOVA followed by Tukey post hoc test (for **E**). One-way ANOVA, followed by Tukey post hoc multiple comparisons test (for **F, G**, **H** and **I**).

C2. It is recommended to further explore the effects of ASPP1-loss and downstream increase in p53 levels on myofibroblasts to clarify the following issues.

C2.1. While the shifts in cell cycle phase of cardiac fibroblasts with the siRNA-knockdown or over-expression of ASPP1 are significant, is this sufficient to conclude that cell proliferation is affected? This is especially so, as the effects are numerically small and the S- and G2-phases show opposite effects. Can the

authors add data that directly measures cell proliferation in these models to prove their conclusions? If the authors can further demonstrate that the changes in cell proliferation are p53-depedent, this would especially strengthen the conclusions of the study.

Reply: We appreciate your insightful comment. Accordingly, we performed EdU test for cell proliferation measurement to further examine the role of ASPP1 and p53 in cell proliferation. The knockdown of ASPP1 significantly reduced the number of EdU^+ fibroblasts treated with TGF-β1, and silencing of p53 significantly hindered the inhibitory effects of ASPP1 knockdown on cell proliferation reflected by EdU assay **(Figure R2A)**. Overexpression of ASPP1 dramatically increased EdU^+ fibroblasts, while overexpression of p53 canceled the promoting effects of ASPP1 on cell proliferation **(Figure R2B)**. We also evaluated the expression of genes related to cell cycle. The results revealed that knockdown or overexpression of p53 reversed the alterations of cell-cycle associated genes induced by ASPP1 knockdown or overexpression at mRNA level, respectively **(Figure R2C, D)**. These findings collectively suggest that ASPP1 regulates fibroblast proliferation in a p53 dependent manner. The detailed results are incorperated in **Supplementary Figure 3C-F** of the revised manuscript.

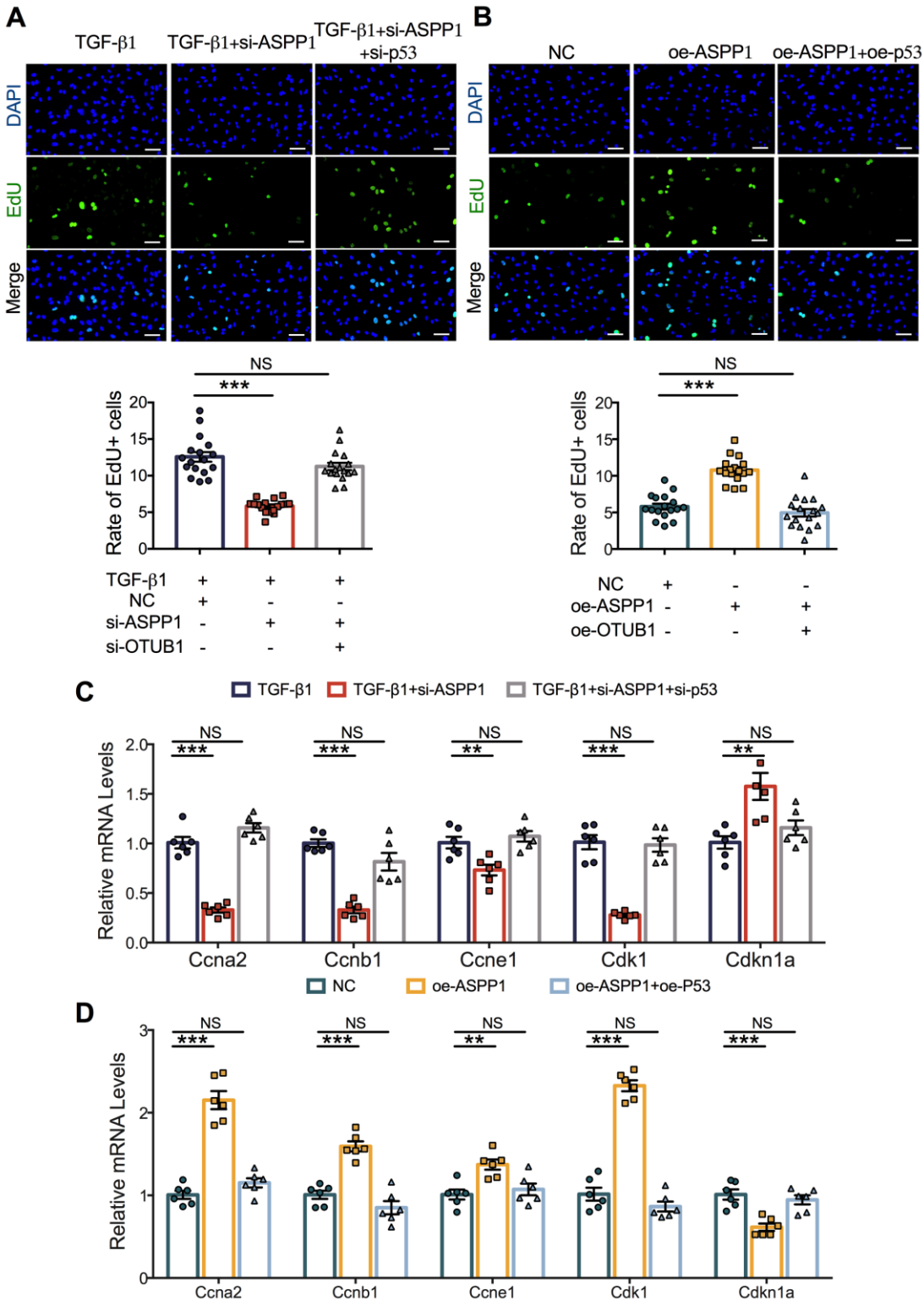

Figure R2. (A) and **(B)** Representative images and statistical results of EdU positive cells with silence or overexpression of ASPP1, and simultaneous knockdown or overexpression of both ASPP1 and p53 as indicated. n=17. NS, p >0.05 vs. TGF- β1 or NC, ***p < 0.001 vs. TGF-β1 or NC. Scale bar= 50μm. **(C)** and **(D)** qRT-PCR assay

was used to evaluate the expression of genes related to cell cycle regulation in PMCFs in the indicated groups. n=6. $^{**}p < 0.01$ vs. TGF- β1or NC, $^{***}p < 0.001$ vs. TGF- β1or NC. Data are represented as mean ± SEM. Statistics: One-way ANOVA, followed by Tukey post hoc multiple comparisons test (for **A - D**).

C2.2. The proposed model indicates that the loss of ASPP1 stabilizes p53, which can be expected to result in a global activation of p53 stress response transcriptional programs. However, the only well-known p53-target gene the expression of which is measured to support this model is Cdk1na. The opposite effects on the Ccn, Cdk, Col1, and Fn1 genes shown in Figure 5 is interesting but doesn't directly show p53 transcriptional activity. Do the authors think Ccn/Cdk transcriptional changes are also under the direct control of p53, acting as a transcriptional repressor? Are Col1 and Fn1 known to be directly p53-regulated or are these transcriptional changes induced via indirect mechanisms? Given the previously reported role of ASPP1 in cardiomyocytes and tumor cells in regulating pro-apoptotic p53 target genes, should pro-apoptotic p53-target genes or other classical p53-target genes apart from Cdkn1a be measured in the ASPP1 knockdown/overexpression myofibroblast models?

Reply: Thank you for your valuable comment. According to the findings of Engeland et al., there is no direct evidence supporting the transcription of Ccn/Cdk being directly controlled by p53 that does not bind to their promoter[12]. The indirect transcriptional inhibition of p53 on Ccn/Cdk requires cyclin dependent kinase inhibitors Cdkn1a[12, 13, 14, 15, 16]. Cdkn1a as a direct target of p53 blocks the activity of several cyclin-CDK complexes, which indicates that p53-dependent transcriptional repression on Ccn/Cdk is indirect [12]. To evaluate the binding capacity of p53 to the promoter regions of Col1 and Fn1, we analyzed the ChIP peaks dataset of mouse fibroblasts from the Cistrome database and identified potential target genes associated with these peaks, as well as any overlap with gene promoters. According to the score of target genes in the database, p53 does not show enrichment for binding in the promoter regions of Fn1 and Col1

(Figure R3C). Additionally, the existing literature does not report direct regulation of Col1 and Fn1 by p53. Collectively, Col1 and Fn1 alterations should be regulated by p53 indirectly.

We conducted additional experiments to investigate the expression of various established target genes of p53, such as apoptosis-related Puma, Bax, and several others. Our results revealed that the levels of pro-apoptotic p53-target genes, along with other conventional p53-target genes like TIGAR, GLS, PML, PAI, MGMT, ERCC5, and XPC, remained unaltered in myofibroblast with ASPP1 knockdown/overexpression **(Refer to Figure R3A, B)**. These findings have been documented in **Supplementary Figure 2E and 2F**.

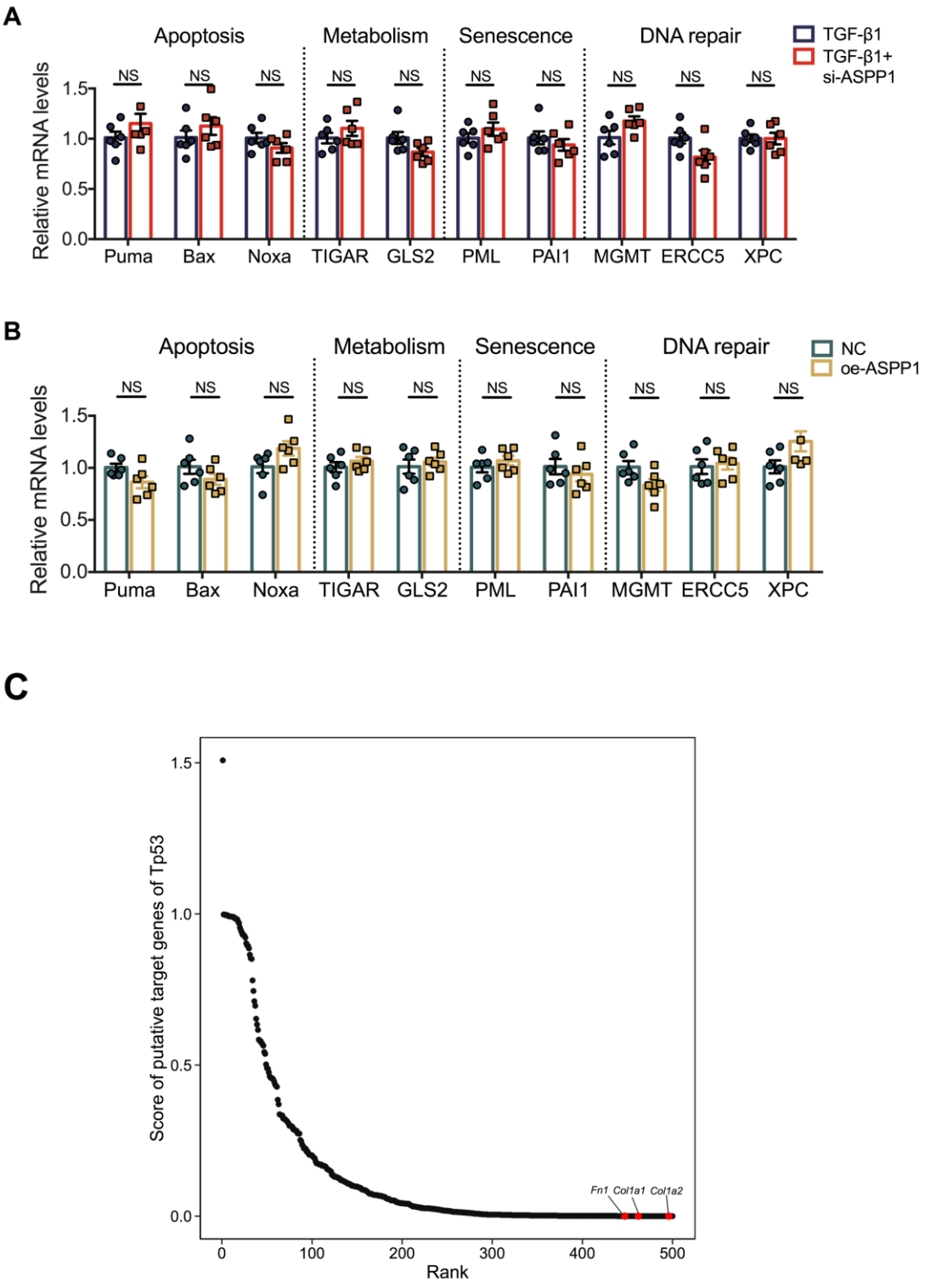

Figure R3. qRT-PCR was used to evaluate the expression of p53 target genes in TGF-β1-treated PMCFs with silencing of ASPP1 **(A)** or overexpression of ASPP1 **(B)**. n=6. NS, $p > 0.05$ vs. TGF-β1 or NC. **(C)** Chromatin immunoprecipitation (ChIP) peak datasets were publicly available and intersections of binding peaks and gene promoter

regions to get p53 putative target genes in Cistrome. Data are represented as mean ± SEM. Statistics: Multiple t test was used to calculate the presented P values.

C2.3. If any of the readouts presented in Figures 5-6, apart from the total p53 protein levels, can be measured in myofibroblasts from the in vivo mouse myocardial fibrosis model, instead of the TGF-beta-treated myofibroblasts in culture, this would further strengthen the conclusions of the study.

Reply: Thank you for the good comment. We performed the experiments to measure the expression of p53 and cell cycle genes in MI heart tissues of mice. The data showed that there is no significant difference in p53 mRNA level in either ASPP1 global knockout or Postn driven conditional knockout of ASPP1 in mice compared with controls (**Figure R4 A**). Global deletion of ASPP1 significantly inhibited the expression of Ccna2, Ccnb1, Ccne1, CDK1, and upregulated Cdkn1a. Postn driven conditional deletion of ASPP1 in significantly inhibited the expression of Ccna2, Ccnb1, CDK1, and upregulated Cdkn1a (**Figure R4 B**). These data were added in the **Supplementary Figure 2A - D.**

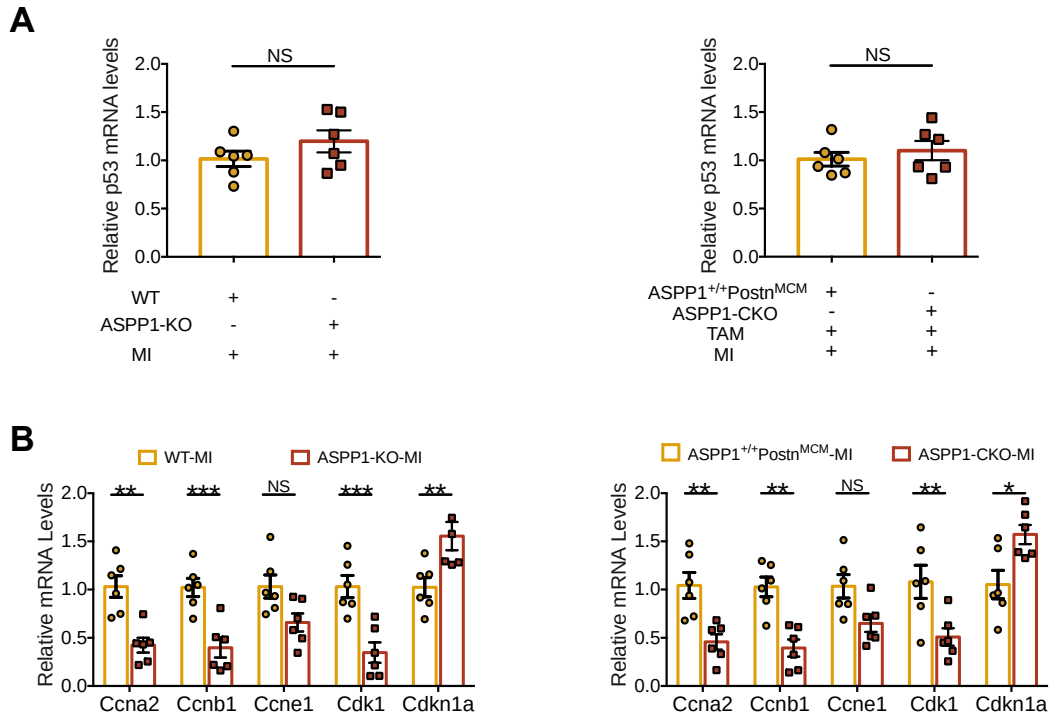

Figure R4. **The expression of p53 and cell cycle related target genes in ASPP1-KO and ASPP1-CKO mice. (A)** qRT-PCR was used to evaluate p53 mRNA level in ASPP1-KO and ASPP1-CKO mice. **(B)** Expression of Cell-cycle related genes in ASPP1-KO and ASPP1-CKO mice. n=6. *$p < 0.05$ vs. WT-MI group or $Postn^{MCM}$ +TAM group, **$p < 0.01$ vs. WT-MI group or $Postn^{MCM}$ +TAM group, ***$p < 0.01$ vs. WT-MI group or $Postn^{MCM}$ +TAM group, NS, $p > 0.05$ WT-MI group or $Postn^{MCM}$ +TAM group. Data are represented as mean ± SEM. Statistics: Student's t test was used to calculate the presented P values.

C3. It is recommended to further explore (and/or discuss) the mechanisms through which ASPP1 modulates p53 levels in myofibroblasts to complement and strengthen the studies in Figure 6.

C3.1. A cycloheximide chase assay to measure p53 half-life in control, ASPP1-knockdown, and ASPP1-overexpressing myofibroblasts could strengthen the conclusion that the primary mechanism is regulation of p53 protein stability.

Reply: Thank you for your insightful insights. We carried out cycloheximide chase assay to measure the half-life of p53 under conditions of ASPP1 overexpression or knockdown. The results showed that the half-life of p53 was significantly increased following ASPP1 knockdown by its siRNA, whereas ASPP1 overexpression substantially shortened the half-life of p53 **(Figure R5)**. These data were added in the **Supplementary Figure 4A and 4B.**

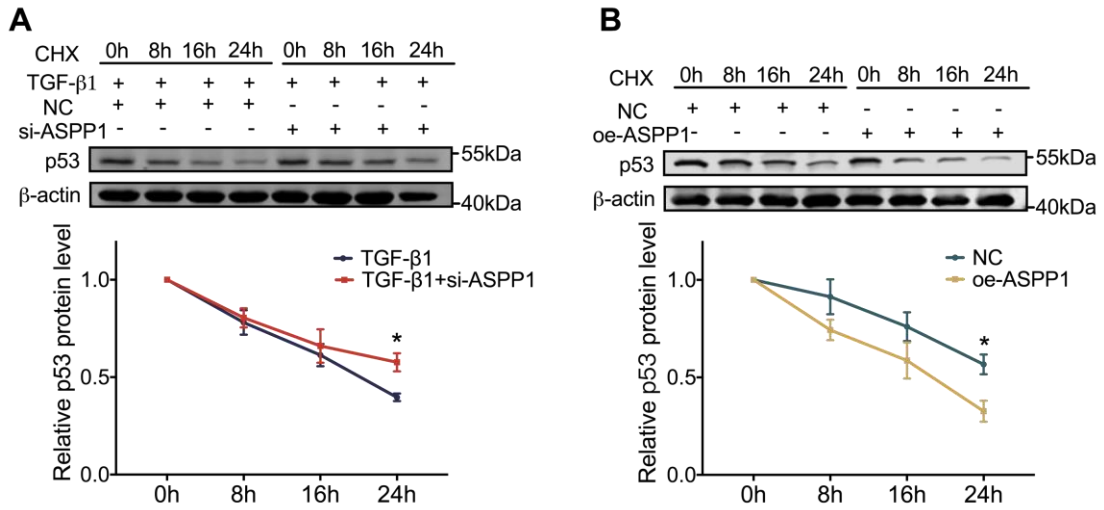

Figure R5. Effects of ASPP1 on p53 stability. (A) The half-life of p53 was prolonged by silencing of ASPP1 in PMCFs. n=3. *p < 0.05 vs. TGF-β1. **(B)** Overexpression of ASPP1 shortened the half-life of p53 in PMCFs. n=3. *p < 0.05 vs. NC.

C3.2. Can the authors speculate why the effects of ASPP1 on p53 activity are distinct between myofibroblasts and other cell types? While perhaps this can't be addressed in full right now, can some hints be provided in the current study? For example, is ASPP1/OTUB1 interaction specific to myofibroblasts over other cell types where ASPP1 was previously shown to cooperate with rather than antagonize p53 activity?

Reply: Thank you for the insightful comments. Firstly, we examined the expression level of OTUB1 in cardiomyocytes and fibroblasts, and found no difference in OTUB1 expression in both cells **(Figure R6A),** indicating that the expression level of OTUB1 was not involved in the distinct effects of ASPP1 on p53 activity in myofibroblasts and cardiomyocytes.

It has been reported that a same molecule can have distinct functions in different types of cells. In different cells, USP7 plays an opposite role in regulating p53. USP7 enhances apoptosis in colorectal cancer cells by directly stabilizing p53[1, 2]. However, USP7 triggers the activation of fibroblasts and the development of cardiac fibrosis by binding to and stabilizing MDM2, which results in the degradation of p53[3]. In our previous study, we found that ASPP1 promotes cardiomyocyte apoptosis by

upregulating p53 and promoting p53 nuclear translocation, upregulating Puma, Bax, and Noxa. In the present study, it is noteworthy that inhibit of ASPP1 upregulates p53, results in the increasing the expression of Cdkn1a and hinders the progress of the cell cycle. This leads to an arrest in cell cycle instead of apoptosis, which in turn prevents excessive growth of cardiac fibroblasts and reduces the strain on the heart. Due to the different specific components and signaling pathways of cells themselves, cells or tissues have different biological effects and responses to stimuli or stress.

We next examined the interaction between OTUB1 and ASPP1 in both cardiomyocytes and fibroblasts. Our findings revealed that more binding between ASPP1 and OTUB1 was observed in fibroblasts compared with cardiomyocytes, which in turn results in different expression level of p53 **(Figure R6B, C)**. This data may partially explain the distinct role of ASPP1 through regulating expression level of p53 in fibroblasts and cardiomyocytes. It is speculated that there are other identified molecular mechanisms involved in this special effects of ASPP1 on p53 in fibroblasts.

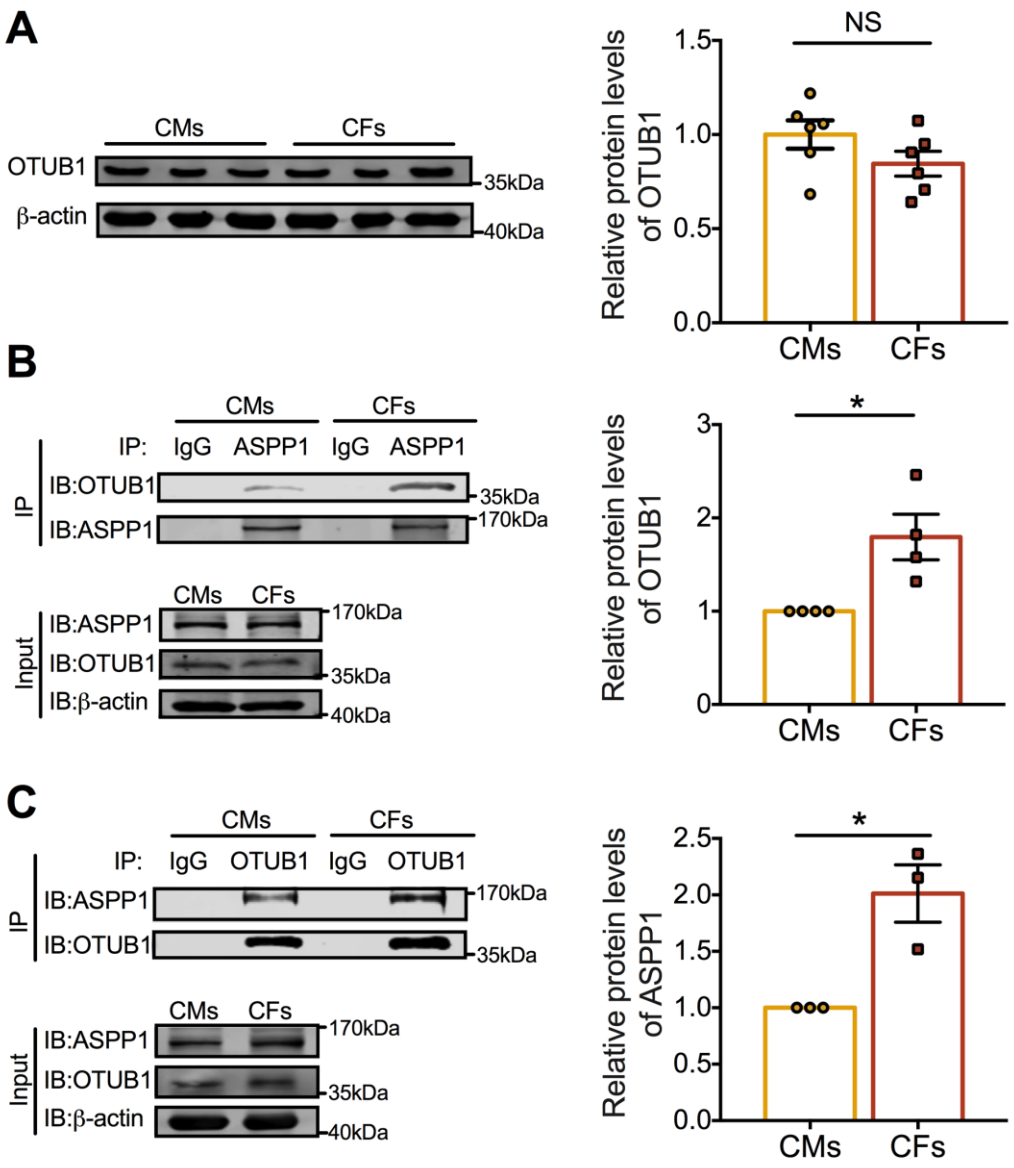

Figure R6. The interaction between ASPP1 and OTUB1 is stronger in fibroblasts than in cardiomyocytes. (A) The expression of OTUB1 in cardiomyocytes and fibroblasts by Western blot. n=3. NS > 0.05 vs. CMs. **(B)** Binding ability of ASPP1 to OTUB1 in cardiomyocytes and fibroblasts. n = 4. *p < 0.05 vs. CMs. **(C)** Binding ability of OTUB1 to ASPP1 in cardiomyocytes and fibroblasts. n = 3. *p < 0.05 vs. CMs. Data are represented as mean ± SEM. Statistics: Student's t test was used to calculate the presented P values.

C3.3. In the Discussion the authors state that 'inhibition of myofibroblast ASPP1 holds the therapeutic potential in cardiac remodeling of infarct hearts'. Can the authors mention if an ASPP1 inhibitor exists or if it's feasible to develop one? Would this approach have benefits over alternative existing modes of p53 activation through nutlin MDM2 inhibitor, for example? Would stabilization of p53 in myofibroblasts with nutlin have equivalent effects to ASPP1 inhibition? I understand that in cardiomyocytes ASPP1 inhibition is preferred because of its expected anti-apoptotic effects. Existing pharmacological tools to stabilize p53 should be mentioned and the advantages of ASPP1 inhibition over these tools explained to the reader.

Reply: Thank you for the good suggestion. Currently, there is no small molecular inhibitors for ASPP1 available except of its siRNA or shRNA. It is a good idea or strategy to develop a pharmacological inhibitor of ASPP1 to evaluate its beneficial effects. A number of approaches such as the bioinformatic technique are available to screen or design a small molecule to inhibit or activate a target molecule.

Our findings revealed that inhibiting ASPP1 in both cardiomyocytes and cardiac myofibroblasts produced beneficial effects on cardiac function in mice. Thus, suppressing ASPP1 could be an excellent strategy for protecting heart function against myocardial infarction through reducing cardiac remodeling. It is interesting to investigate whether the beneficial effects achieved by ASPP1 inhibition utilizing genetic manipulation technique are comparable with p53 activation through nutlin MDM2 inhibitor in the ischemic hearts.

The p53 stabilizers, such as the nutlin MDM2 inhibitor, prevents the degradation of p53 and results in an increase of p53 protein in both cardiomyocytes and myofibroblasts. It can be speculated that cardiac fibrosis is suppressed due to the inhibition of fibroblast activity by p53, while cardiomyocyte apoptosis may be promoted due to pro-apoptotic function of p53. However, inhibition of ASPP1 may suppress fibrosis by increasing p53 in myofibroblasts and alleviate heart injury by

decreasing p53 in cardiomyocytes. Thereby, inhibition of ASPP1 produces beneficial effects from both fibroblasts and cardiomyocytes and is likely a better strategy than only inhibition of p53 degradation by nutlin an MDM2 inhibitor. However, this needs to be demonstrated with experimental data. We have added the description in the **Discussion** of the revised manuscript **(Page 15)**.

D. Is there enough detail provided in the methods for the work to be reproduced?

- Are all the mouse strains used in the study on C57BL/6J or another single inbred genetic background? If mouse strains of different genetic backgrounds are crossed and used this is problematic.

Reply: Thank you for the good comment. All the animals used in this study were C57BL/6J strain.

- I would have liked more explanation about the origins, genetic background, and any previously reported phenotypes of the ASPP1-KO mouse model used in Figure 2. This should be concisely incorporated into the manuscript, either in the Introduction or in Results before Figure 2 data is presented. This information is only accessible when following a citation from the Methods section and then going to supplemental materials of the cited study: Circ Res. 2023 Jan 20;132(2):208-222.

Reply: Thank you for the insightful comment. In our study, we employed ASPP1 conventional knockout (KO) mice (C57BL/6 background) (7-8 weeks old, 22-25 g). The ASPP1-KO mice were generated by Cyagen Biosciences Co., Ltd (China) and constructed by CRISPR/Cas9 strategy. The ASPP1 KO mice showed normal morphology and growth. In our previous work, following the cardiac ischemia/reperfusion, ASPP1-KO mice exhibited improved cardiac function, decreased infarct size, and reduced cardiomyocyte apoptosis.[17] Additionally, cardiomyocytes absent of ASPP1 exhibited a notable decrease in the level of p53 in the nucleus. We have added the description in the **Introduction (Page 4)** and **Results (Page 6)** section of the manuscript.

Other clarity and formatting issues:

- Title: 'Myofibroblast-specific inhibition of ASPP1 alleviates myocardial fibrosis by enhancing p53 degradation'. Doesn't the study in fact argue that inhibition (loss) of ASPP1 inhibits p53 degradation? You can see in Figure 5A-B that the levels of p53 go up with ASPP1-loss and go down with ASPP1 over-expression.

- I also don't like the use of 'inhibition' in the title as this can suggest to the reader that an inhibitor was developed and tested.

Reply: Thank you for the good comment. We changed the title to: Deletion of ASPP1 in myofibroblast alleviates myocardial fibrosis by reducing p53 degradation.

- Why does Figure 2B seem to present a 12-week experiment, when all the data in the Figure is from weeks 2 and 4? This is similar for Figure 3B also.

Reply: Thank you for the good comment. We apologize for the misunderstanding description. In Figure 2B, myocardial infarction model was established using 8-week old mice. The mice were then examined with echocardiography at 2 and 4 weeks after MI. It was a time course of 12 weeks for the tested mice. In Figure 3B, myocardial infarction model was also established using 8-week old mice. After model, tamoxifen was administered to the mice to induce deletion of ASPP1 in myofibroblasts. The cardiac function of the mice was assessed at weeks 2 and 4 following MI. All tested mice in Figure 2 and 3 had the same time course of 12 weeks in the experimental protocol.

We have reconstructed the schematic graph to show clear elucidation of the experimental design in **Figure2B** and **Figure3B.**

- lines 153-154: Do you mean 'knockdown and overexpression efficiency of p53, rather than OTUB1, are shown in Figures S1A and S1B'?

Reply: Thank you for pointing out the mistake. It should be written as “the expression efficiency of p53”, but not OTUB1. We have corrected this mistake and the previous Figures S1A and S1B have been incorperated to **Supplementary Figure 3 in the revised manuscript.**

- Figure 5C: Please indicate on the Figure that p53 transcript is measured. I know this information is found in the Legend, but it will be much easier for the reader if this is also stated in the Figure itself.

Reply: Thank you for the good suggestion. We have added "p53 transcript” to Figure 5C accordingly**.**

Reviewer #2 (Remarks to the Author):

General synopsis

The authors of the current paper have assembled a paper that addressed how ASPP1 protein influences fibroblast p53 trafficking and in doing so, compared the effects of ASPP1 knock out in mice. In their approach, they utilized both global and myofibroblast specific KO mice. ASPP1 KO was associated with beneficial changes in cardiac function, fibrosis and remodeling of post MI hearts. Of considerable interest is the finding that ASPP1 KO yielded an effect which is opposite to that of cardiac myocytes in that p53 levels and cell fates. In other words knockdown of ASPP1 is currently reported to yield an increase in p53 levels in cardiac myofibroblasts and also inhibited “the activity” of cardiac myofibroblasts. They also showed that IF staining in TGFb stimulated cells was associated with ASPP1 accumulation in the cytoplasm of fibroblasts while the level of p53 was reduced while inhibition of ASPP1 increased p53 level and promoted p53 nuclear translocation (nicely depicted in Figure 7). They demonstrate that ASPP1 may bind to deubiquitinase OTUB1 and prevents binding to p53, preventing its degradation. This paper deals with a novel protein and the dataset supports their

conclusions with a conservative summary. With the following points addressed in a categorical manner, the impact of this paper will be improved.

1. The dataset shows both mRNA and proteins of key genes assessed, which is a strength. In addition, all data points are shown, another point of transparency and clarity. However it is difficult to see all six data points in the tightly grouped controls, perhaps the histograms could be enlarged to provide for easier understanding. Figures 1-6 are affected.

Reply: Thank you for the good suggestion. We have modified the related Figures to make them easier to read and understand.

2. Figure 1. Why was 4 weeks chosen as the MI duration in these animals? Why not 1 week or 2 weeks? Did the authors collect data from those time points (Figure 1)? It may provide some additional insight into the response of the ASPP1 gene following MI.

Reply: Thank you for the insightful comments. It has been widely accepted that 4-week mouse model of myocardial infarction is commonly utilized to assess the pathological changes of myocardial fibrosis[18, 19, 20, 21, 22]. The amount of collagen starts to notably rise between 4-7 days following a heart attack and reaches its highest point 3-6 weeks later[23]. Therefore, we employed 4-week MI mice to observe the role of ASPP1 in cardiac fibrosis in this study.

As suggested, we also examined the mRNA and protein of ASPP1 at 1, 2 and 4 weeks after myocardial infarction. It showed that the mRNA and protein of ASPP1 were both upregulated at 1, 2 and 4 weeks after myocardial infarction likely in a time-course manner **(Figure R7A, B)**. The data was included in **Figure** 1.

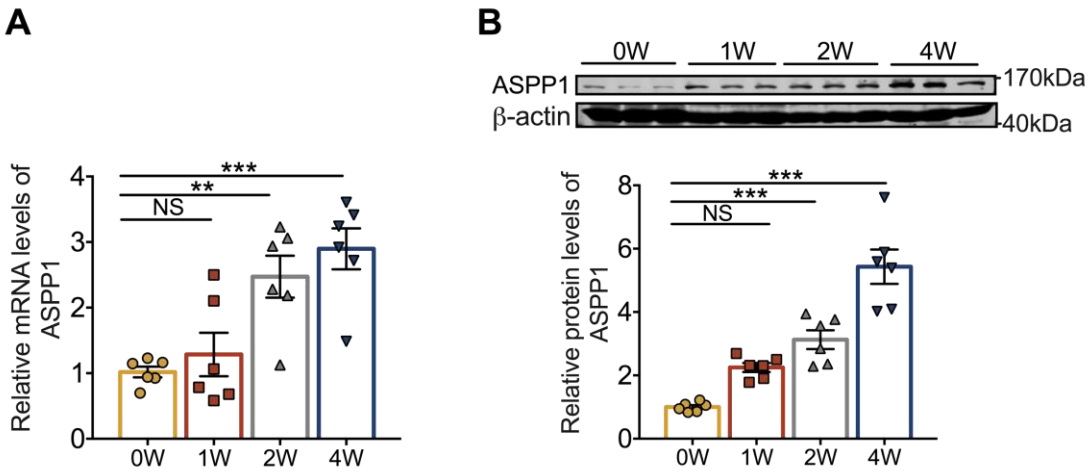

Figure R7. ASPP1 is dynamically upregulated during myocardial infarction. (A) and **(B)** The mRNA and protein of ASPP1 were elevated at 1, 2, 4 weeks of MI. n=6, NS, $p > 0.05$ vs. Sham, $^{**}p < 0.05$ vs. Sham, $^{***}p < 0.01$ vs. Sham. Data are represented as mean ± SEM. Statistics: One-way ANOVA, followed by Tukey post hoc multiple comparisons test.

3. Figure 2G – It appears that while ventricular geometry is preserved in the post-MI hearts from ASPP1-KO mice, when compared to WT post-MI mice. Despite this the infarct scar is still very prominent. Do the authors feel that the preservation of cardiac performance as shown in panel E may be simply due to the preservation of ventricular geometry? If not, why? In the same line of thinking why is there no light microscopy with something like Masson's trichrome stain to offer a visual confirmation of the data in panel H?

Reply: Thank you for the insightful comment. We completely agree that the preservation of ventricular geometry is closely associated with and is a direct cause for the preservation of cardiac performance.

We performed Masson's trichrome staining of infarct hearts of ASPP1-KO and wild type mice. The results showed that knockout of ASPP1 significantly alleviated cardiac interstitial fibrosis after MI compared wildtype mice (**Figure R8**). The data is presented in **Figure 2G.** and labeled as "Border area".

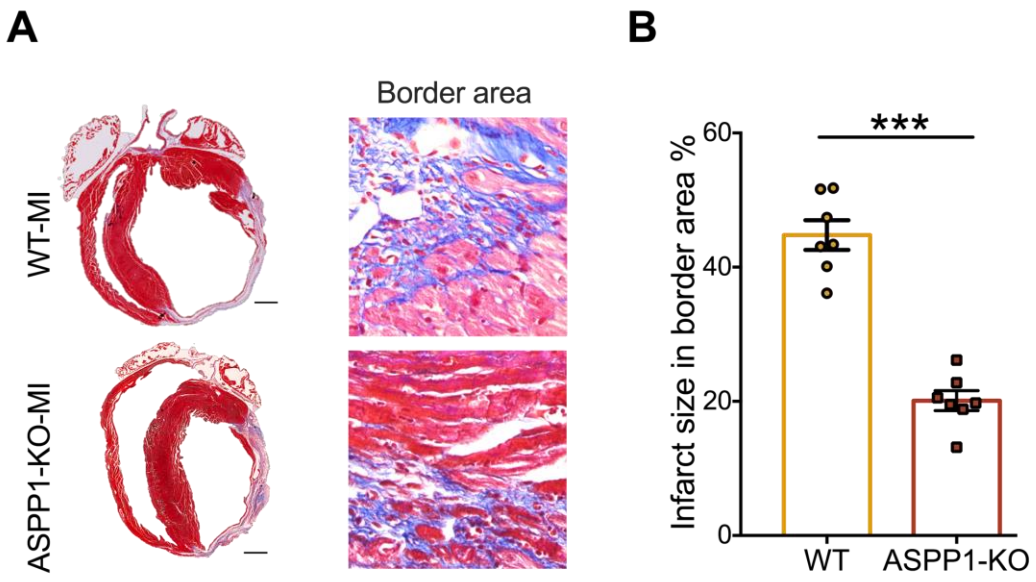

Figure R8. Masson staining of fibrotic tissue of ASPP1-KO. (A) Representative photographs and **(B)** statistical data of Masson staining in border region of infarct hearts from wild type (WT) and ASPP1-KO mice. ***$p < 0.001$ vs. WT-MI group. n=7. Data are represented as mean ± SEM. Statistics: Student's t test was used to calculate the presented P values.

4. Figure 2H. Extracellular Matrix is misspelled.

Reply: Thank you for pointing out the mistake. We have corrected it.

5. As in comment #3, the same questions apply to Figure 3.

Reply: Thank you for the good comment. We performed Masson's trichrome staining of infarct hearts. The results showed that following MI, the knockdown of ASPP1 in myofibroblasts, there was a notable decrease in fibrosis of in the infarcted hearts **(Figure R9)**. The data has been added in **Figure 3G**. and labeled as "Border area".

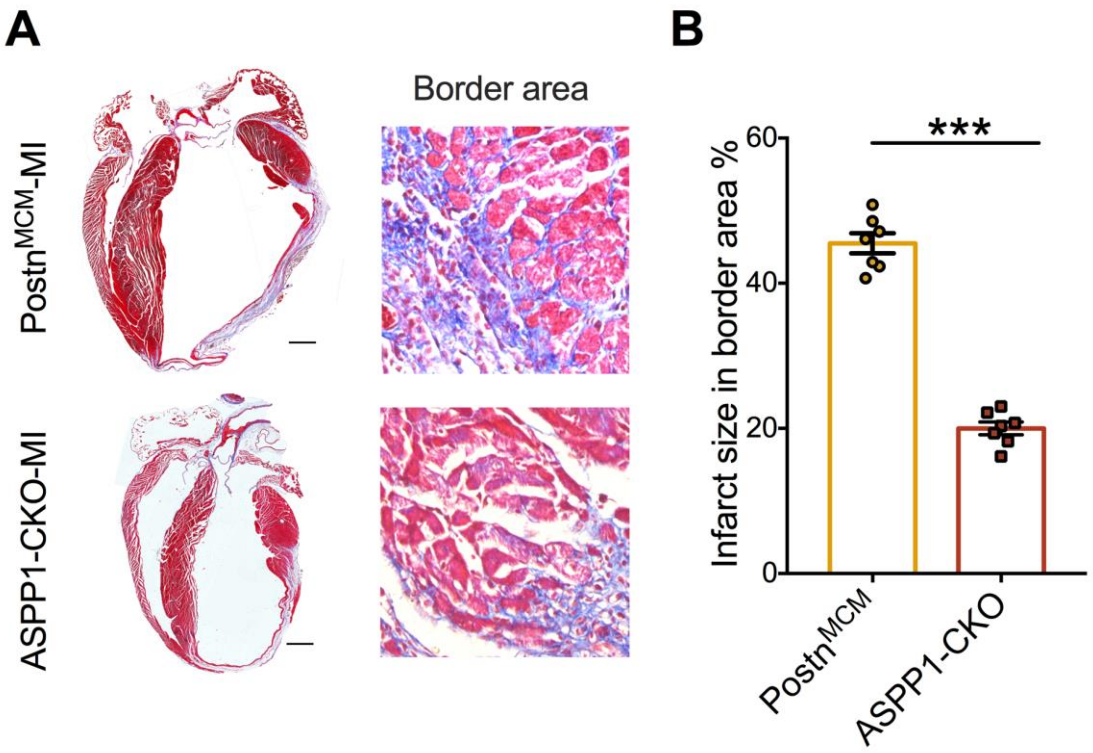

Figure R9. Masson staining of ASPP1-CKO mice. (A) Representative photographs and **(B)** statistical data of Masson staining in border region of the infarct hearts from ASPP1$^{+/+}$Postn^{MCM} (Postn^{MCM}) and ASPP1-CKO mice induced by tamoxifen. ***p < 0.001 vs. Postn^{MCM} +TAM group. n=7. Data are represented as mean ± SEM. Statistics: Student's t test was used to calculate the presented P values.

6. Sometimes the terms fibroblast and myofibroblast are interchanged without any specificity. This is distracting but some editing would correct the problem. I suspect that the main target of function for ASPP1 lies in the activated myofibroblast, and so the paper should consistently reflect this view.

Reply: Thank you for the good suggestions. We have double-checked the manuscript to make sure the proper use of "myofibroblast" and "fibroblast".

References:

1. Yang Z, *et al.* STAT3 repressed USP7 expression is crucial for colon cancer development. *FEBS Lett* **586**, 3013-3017 (2012).
2. Liu J, *et al.* The phosphorylation-deubiquitination positive feedback loop of the CHK2-USP7 axis stabilizes p53 under oxidative stress. *Cell Rep* **43**, 114366 (2024).
3. Burke RM, *et al.* Small proline-rich protein 2B drives stress-dependent p53 degradation and fibroblast proliferation in heart failure. *Proc Natl Acad Sci U S A* **115**, E3436-E3445 (2018).
4. Jiang Y, *et al.* Cytoplasmic sequestration of p53 by lncRNA-CIRPILalleviates myocardial ischemia/reperfusion injury. *Commun Biol* **5**, 716 (2022).
5. Liu X, *et al.* p53 Regulates the Extent of Fibroblast Proliferation and Fibrosis in Left Ventricle Pressure Overload. *Circ Res* **133**, 271-287 (2023).
6. Snider P, *et al.* Periostin is required for maturation and extracellular matrix stabilization of noncardiomyocyte lineages of the heart. *Circ Res* **102**, 752-760 (2008).
7. Kanisicak O, *et al.* Genetic lineage tracing defines myofibroblast origin and function in the injured heart. *Nat Commun* **7**, 12260 (2016).
8. Umbarkar P, *et al.* Fibroblast GSK-3alpha Promotes Fibrosis via RAF-MEK-ERK Pathway in the Injured Heart. *Circ Res* **131**, 620-636 (2022).
9. Umbarkar P, *et al.* Cardiac fibroblast GSK-3alpha aggravates ischemic cardiac injury by promoting fibrosis, inflammation, and impairing angiogenesis. *Basic Res Cardiol* **118**, 35 (2023).
10. Wu J, *et al.* Glutamyl-Prolyl-tRNA Synthetase Regulates Proline-Rich Pro-Fibrotic Protein Synthesis During Cardiac Fibrosis. *Circ Res* **127**, 827-846 (2020).
11. Snider P, Standley KN, Wang J, Azhar M, Doetschman T, Conway SJ. Origin of cardiac fibroblasts and the role of periostin. *Circ Res* **105**, 934-947 (2009).
12. Engeland K. Cell cycle regulation: p53-p21-RB signaling. *Cell Death Differ* **29**, 946-960 (2022).

13. Fischer M, Quaas M, Steiner L, Engeland K. The p53-p21-DREAM-CDE/CHR pathway regulates G2/M cell cycle genes. *Nucleic Acids Res* **44**, 164-174 (2016).
14. Quaas M, Muller GA, Engeland K. p53 can repress transcription of cell cycle genes through a p21(WAF1/CIP1)-dependent switch from MMB to DREAM protein complex binding at CHR promoter elements. *Cell Cycle* **11**, 4661-4672 (2012).
15. Engeland K. Cell cycle arrest through indirect transcriptional repression by p53: I have a DREAM. *Cell Death Differ* **25**, 114-132 (2018).
16. Fischer M, Steiner L, Engeland K. The transcription factor p53: not a repressor, solely an activator. *Cell Cycle* **13**, 3037-3058 (2014).
17. Yang Y, *et al.* Interdependent Nuclear Co-Trafficking of ASPP1 and p53 Aggravates Cardiac Ischemia/Reperfusion Injury. *Circ Res* **132**, 208-222 (2023).
18. Tani H, *et al.* Direct Reprogramming Improves Cardiac Function and Reverses Fibrosis in Chronic Myocardial Infarction. *Circulation* **147**, 223-238 (2023).
19. Ni C, *et al.* Flavin Containing Monooxygenase 2 Prevents Cardiac Fibrosis via CYP2J3-SMURF2 Axis. *Circ Res*, 101161CIRCRESAHA122320538 (2022).
20. Deniset JF, *et al.* Gata6(+) Pericardial Cavity Macrophages Relocate to the Injured Heart and Prevent Cardiac Fibrosis. *Immunity* **51**, 131-140 e135 (2019).
21. Garlapati V, *et al.* Targeting myeloid cell coagulation signaling blocks MAP kinase/TGF-beta1-driven fibrotic remodeling in ischemic heart failure. *J Clin Invest* **133**, (2023).
22. Ruiz-Villalba A, *et al.* Interacting resident epicardium-derived fibroblasts and recruited bone marrow cells form myocardial infarction scar. *J Am Coll Cardiol* **65**, 2057-2066 (2015).
23. Dobaczewski M, Gonzalez-Quesada C, Frangogiannis NG. The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction. *J Mol Cell Cardiol* **48**, 504-511 (2010).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors were very thorough in addressing the reviewers' comments and provided significant new data, as requested, so support their conclusions.

The following point, included in the Response the Reviews, should come across more strongly in the text of the paper. A minor edit to the text is sufficient to address this: 'As periostin (Postn) is also expressed in other cell types such as vascular smooth muscle cells, endothelial cells, mesenchymal cells. It cannot be ruled out the potential contribution of ASPP1 deficiency in these cells to cardiac protection.'

Overall, I support publication following this minor revision.

Reviewer #2 (Remarks to the Author):

The authors have spent considerable energy and time providing additional experiments to answer a few key questions about the data. In my opinion they have satisfied a large number of these problems in this reviewers queries as well as in the extensive series of questions posed by the first reviewers (including a title change to reflect an accurate representation. The impact of the current version is elevated because of all of the authors attention to previous concerns.

Responses to Reviewer's Comments

We thank the reviewers for their positive and constructive comments and suggestions on our work, which are very important for us to improve the quality of the work. We have performed a series of additional experiments to strengthen the conclusion of the study.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors were very thorough in addressing the reviewers' comments and provided significant new data, as requested, so support their conclusions.

The following point, included in the Response the Reviews, should come across more strongly in the text of the paper. A minor edit to the text is sufficient to address this: 'As periostin (Postn) is also expressed in other cell types such as vascular smooth muscle cells, endothelial cells, mesenchymal cells. It cannot be ruled out the potential contribution of ASPP1 deficiency in these cells to cardiac protection.'

Overall, I support publication following this minor revision.

Reply: Thank you for the insightful comment. We have added following relevant content in the Discussion (Page 15).

In the current study, $Postn^{MCM}$ strategy was employed to delete ASPP1 resulting in significant alleviation of myocardial fibrosis. However, Postn is also expressed in other cell types such as vascular smooth muscle cells, endothelial cells, etc. Therefore, it cannot be ruled out the potential contribution of these cells to cardiac protection in response to ASPP1 deficiency.