nature portfolio

Peer Review File

Protein kinase N promotes cardiac fibrosis in heart failure by fibroblast-to-myofibroblast conversion

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript deals with the role of Protein kinase N (PKN) in cardiac fibroblast activation and fibrosis. There is no literature on the role of PKN in fibroblast (FB) activation or fibrosis; therefore, the findings presented in this paper are highly novel. I have the following comments.

1. Fig. 1 E-G, The phosphorylation residue of PKN1 and PKN2 is not noted on the figures, figure legend, or result description. This info needs to be included.
2. Fig. 1 H, the quantification of normalized PKN1 and PKN2 deletion must be shown as a bar diagram.
3. Fig. 1 H, a sketch showing the experimental timeline of mouse age, tamoxifen protocol, any washout period, and the specific day of deletion check needs to be presented.
4. Typo, page 8, line 12, "protected mice form cardiac dysfunction"-"Form" should be from.
5. Fig. 5B-C, although TGF-β1 is known to activate both SMAD-2 and SMAD-3, but the profibrotic effects of TGF-β1 signaling in the heart have been primarily attributed to SMAD-3 not SMAD2 (PMID: 28891814). Therefore, it is unclear why the authors focused on SMAD2 only and ignored SMAD3. Given the context of the paper, SMAD3 must be investigated here.
6. Fig 6A, result description Page 13, lines 9-10, "no difference was observed in fractional shortening, which indicates the presence of cardiac systolic function." This statement is factually incorrect. Actually, no difference in FS suggests comparable systolic function.
7. The authors introduced the 2 hit HFpEF model at the end of the paper and performed the cardiac phenotyping. However, author did not present the fibroblast/myofibroblast, fibrosis, or profibrotic signaling data with this model. This line of finding can not be just presumed for such a high-profile publication. This reviewer requests the authors to provide this line of data for a comprehensive paper.
8. The phosphorylation residue of all kinases must be included in the paper.
9. Finally, the mechanism aspect is still weak, especially since the proposed mechanism could be simply associative rather than driving/causative. Given the timeline of the manuscript, this reviewer understands that suggesting to generating FB-specific PKN1/2-TG mice and performing a rescue experiment with a p38 deletion/inhibitor is not reasonable. Therefore, to further strengthen the mechanistic findings, I would suggest performing rescue experiment in an isolated FB cell culture model. Specifically, overexpressing PKN1/2 to induce profibrotic signaling and FB activation and rescuing these with a p38 inhibitor. This line of investigation will establish the driving role of the proposed mechanism.
10. Finally, the discussion section (6-7 pages long) is very unfocused. A lot of it is a review kind of writing rather than truly discussing the paper's findings and implications. Some sentences are background/rationale, e.g., "The human kinome consists of over 500 protein kinases, and PKN belongs to the cAMP-dependent, cGMP-dependent and protein kinase C (AGC) kinase family, comprising evolutionarily related protein kinases. Each AGC kinase is involved in different pathologies, affecting human health issues, including cancer, metabolic disorders, and cardiovascular diseases." This kind of background knowledge may be appropriate for the "introduction" section but not for the discussion. This reviewer requests the authors to revise the discussion entirely to present a focused, concise discussion of presented findings and their implications.

Reviewer #2 (Remarks to the Author):

Protein kinase N (PKN) 1 and PKN2 are essential for mesoderm (and heart) development, and contribute to adaptation to pressure overload. Less is known about the role of PKN1 / 2 in cardiac fibroblasts. The authors show that PKN1 and PKN2 are both elevated by TGF-b1, an inducer of fibroblast activation and fibrosis. Subsequently, they delete PKN1 and PKN2 using a Pdgfra-dependent fibroblast Cre line in mice. They do not observe differences in AngII treatment, but describe subtle, but significant improvement in cardiac function in ischemia/reperfusion (I/R) model and a HFpEF model. In vitro experiments suggest that PKN1/2 is upstream of p38 signaling that induces a pathologic fibroblast response. The manuscript is well organized and logical and suggest PKN1/2 contribute to fibroblast activation in I/R and HFpEF. However, fairly superficial investigation of the phenotype is reported; procedures are insufficiently described; and considerable questions remain about the adequacy of the timepoints chosen for various experiments.

Major comments:
1. The replicate number and variability is incredibly small for an I/R in vivo cardiac physiology study. Further, all endpoints are 7 days post- I/R, which is quite early, and prior to the robust scar that forms in this model. It would be more informative to investigate readouts of fibroblast activity and scar formation, including cardiac physiology, at 28 days post-I/R.
2. The authors investigate fibroblast migration upon Pkp2 knockdown in Fig. 3. 6 hours is too early to convincingly demonstrate no change in migration. It is curious that th authors document 40% migration, given the images do not show any obvious migration at 6 hrs.
3. Immunostaining and Western blot analysis needs more details, especially antibody concentrations. How is Collagen I and Collagen III levels quantified using polarized imaging? No ColI or ColII antibodies are listed, so is this from the picrosirius red staining? Again, insufficient procedures are listed for this method that is not typically used to quantify ColI and ColIII. Red vs green bi-refringence typically marks new and mature collagen strands, not ColI vs III.
4. Two subsequent siRNA transfections are reportedly necessary to obtain knockdown of Pkn1/2; however, the methods do not describe this atypical procedure. What is the efficiency of knockdown with a single transfection; when were the transfections conducted?; etc. Further, what passages were used for cardiac fibroblast cutulres; most experients describe a prolonged culture (necessary for two transfections, and other manipulations), however the details are not provided.
5. Apart from identifying p38 as downstream of PKN1/2 in fibroblasts (based on prior literature) no novel insight is provided about fibroblast activation.

Reviewer #3 (Remarks to the Author):

The authors examined PKN1/2 distribution in the heart and the role of PKN1/2 in two models of heart disease, MI & HFpEF, in mediating fibrosis and the transition of fibroblasts to myofibroblasts. They demonstrate that PKN1 and 2, but not 3 are found in cardiac fibroblasts. They also compared WT and Pdgfra-PKN1/2 KO mice and concluded that the absence of PKN1/2 (PKN1/2 KO) reduces fibrosis and the transition to myofibroblasts (alpha SMA positivity). There are some comments to be addressed

Major comments

1. The authors need to make it clear which model is being studied and not present different models in the same paragraph or Figure. For example, Fig. 2B examines 4-week treatment with AngII, which is a model of HFpEF [Regan et al. Am J Physiol Heart Circ Physiol 2015;309:H771–H778, Dulce et al. Cardiovasc Res. 2023 118:3586-3601], but Figure 2 focuses on MI and the absence of PKN1/2 had no effect on fibrosis. In contrast, the authors state that fibrosis was reduced in the HFD+L-NAME HFpEF model in Pdgfra-PKN1/2 KO mice, but do not show those data.
2. As the authors mention, PKN1& 2 are not completely eliminated in fibroblasts (Fig 1H) from floxed, tamoxifen-treated mice. Please show quantification of signal intensity (particularly since the GAPDH band appears lighter in the flox + Tamoxifen lane).
3. Western blots are generally overexposed, which would make quantification with Image J difficult. Please provide Western blots with shorter exposure times. Also, please provide the full-size gels in the Supplemental results.
4. For cell culture experiments, the authors reduce PDK1/2 using siRNA. Why did they not isolate cardiac fibroblasts from Pdgfra-PKN1/2 KO mice for these studies?
5. The initial HFD+L-NAME study used a 5-week treatment. Why did the authors continue for 10 weeks?
6. While women are pre-disposed to HFpEF more than men, the HFD+L-NAME regimen does not replicate this sex bias (Tong et al. Circulation. 2019;140:1769–1771). Please adjust this part of the Discussion

Minor:
1. The introduction should be shortened and the Discussion also seems long

A letter to the Reviewers

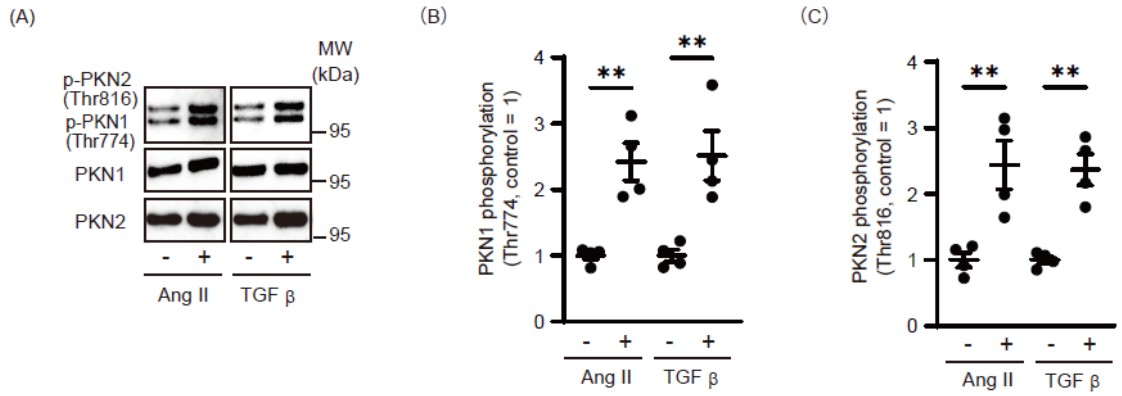
First of all, we would like to thank the Editor and Reviewers for their constructive comments and suggestions, which have improved the quality of our manuscript. I apologize that it has taken so much time to prepare the revised manuscript. All revisions have been made taking into consideration their valuable input.

Please note that comments from the Reviewers are shown below in bold italics followed by our point-by-point responses. In the manuscript, the revised text following the Reviewers' suggestions are underlined.

Reviewer #1 (Remarks to the Author):

This manuscript deals with the role of Protein kinase N (PKN) in cardiac fibroblast activation and fibrosis. There is no literature on the role of PKN in fibroblast (FB) activation or fibrosis; therefore, the findings presented in this paper are highly novel. I have the following comments.

1. ***Fig. 1 E-G, The phosphorylation residue of PKN1 and PKN2 is not noted on the figures, figure legend, or result description. This info needs to be included.***
   Response: Following the reviewer's suggestion, we added the phosphorylation sites in the revised figures and figure legends. (**Reviewer Fig. 1-1 A-C** and **revised Fig. 1e-g**).

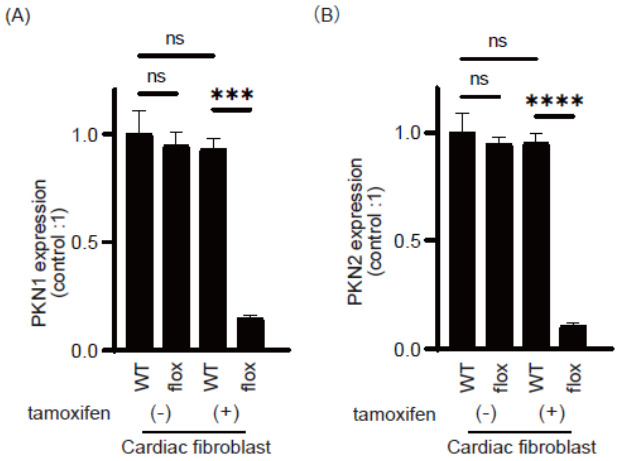


Reviewer Figure 1-1. Western blot analysis for PKN1 (Thr774) and PKN2 (Thr816) phosphorylation in cardiac fibroblasts after treatment with AngII (100 nM) or TGF-β (10 ng/mL). Data are presented as the mean ± SEM and analyzed with an unpaired

Student t test (B and C). ns, not significant; **p < 0.01.

2. ***Fig. 1 H, the quantification of normalized PKN1 and PKN2 deletion must be shown as a bar diagram.***

   Response: Following the reviewer's suggestion, we have introduced the following bad diagram in the revised figures (**Reviewer Fig. 1-2 A, B** and **Revised Fig. 2c, d**).



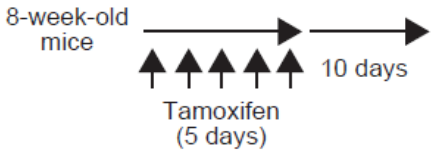
Reviewer Figure 1-2. Quantification of normalized deletion of PKN1 (A) and PKN2 (B) in tamoxifen-induced Pdgfra-PKN1/2 deficiency mice (*n* = 3). Data are presented as the mean ± SEM and analyzed using two-way ANOVA followed by the Tukey's post hoc test (A and B). ns, not significant; ****p < 0.0001.

3. ***Fig. 1 H, a sketch showing the experimental timeline of mouse age, tamoxifen protocol, any washout period, and the specific day of deletion check needs to be presented.***

   Response: Following the reviewer's suggestion, we have introduced the following text in the revised manuscript and showed the experimental timeline **(Reviewer Figure 1-3 and Revised Fig. 2a**).

Page 5, lines 10-14

"To induce Cre-mediated recombination of floxed PKN1/2 alleles, animals were treated with intraperitoneal injections of 1 mg tamoxifen on 5 consecutive days at age 8 weeks, and the efficiency of recombination was determined 10 days later by Western blot analysis (**Fig. 2a**)."



Reviewer Figure 1-3. Animals were treated with intraperitoneal injections of tamoxifen for 5 consecutive days, and the efficiency of recombination was determined 10 days later.

4. ***Typo, page 8, line 12, "protected mice form cardiac dysfunction "Form" should be from.***

   Response: Following the reviewer's suggestion, we have revised the text in the revised manuscript.

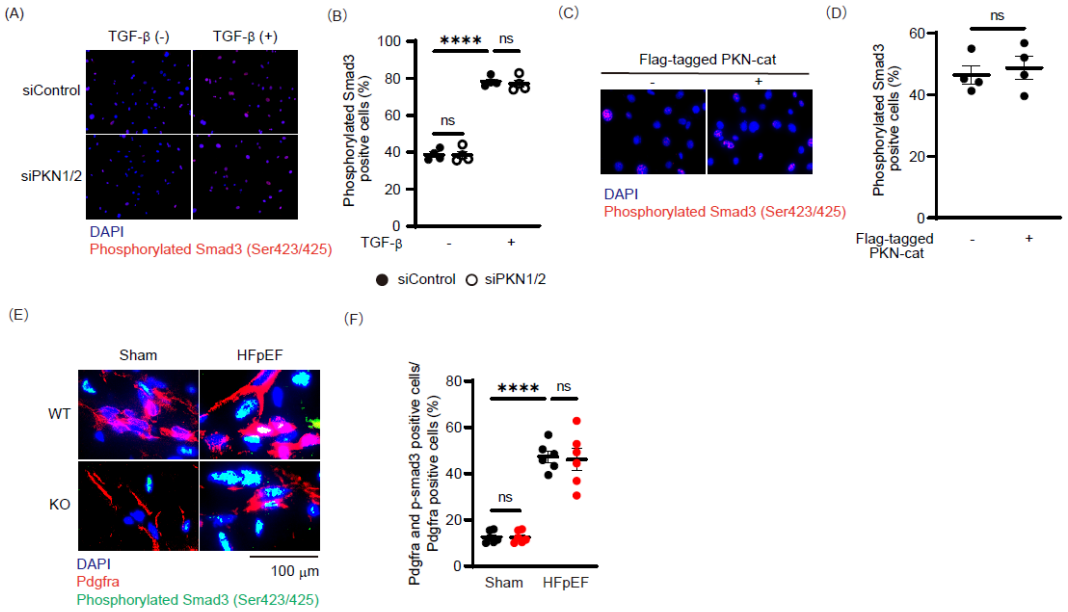
   Page 7, lines 10-12

   "These data suggest that PKN1/2 deficiency in cardiac fibroblast protected mice from cardiac dysfunction induced by both MI with IR and permanent coronary artery ligation."

5. ***Fig. 5B-C, although TGF-β1 is known to activate both SMAD-2 and SMAD-3, but the profibrotic effects of TGF-β1 signaling in the heart have been primarily attributed to SMAD-3 not SMAD2 (PMID: 28891814). Therefore, it is unclear why the authors focused on SMAD2 only and ignored SMAD3. Given the context of the paper, SMAD3 must be investigated here.***

   Response: Thank you for providing these important suggestions. Following the reviewer's suggestion, we examined Smad3 activation by Western blot. However, the quality of Western blot using anti-phospho SMAD-3 antibody (#9520, Cell Signaling Technology, Danvers, MA, USA) was inadequate to analyze Smad3 activation. Thus, instead of Western blot, we used an immunostaining assay to investigate Smad3 phosphorylation with anti-phospho SMAD3 antibody (ab52903, Abcam, Cambridge, UK). In isolated cardiac fibroblasts, TGF-β treatment increased Smad3 phosphorylation, and the TGF-β-induced Smad3 phosphorylation was unaffected following PKN1/2 deletion using siPKN1/2 treatment (**Reviewer Fig. 1-4 A, B** and **Revised Fig. 6e, f**). We also investigated whether overexpression of flag-tagged PKN catalytic domain (PKN-cat) increases Smad3 phosphorylation. Flag-tagged PKN-cat expression did not phosphorylate endogenous Smad3 in 3T3 cells (**Reviewer Fig. 1-4 C, D**). Moreover, we examined Smad3 phosphorylation in fibroblasts in the heart

after a 10-week combination exposure to high-fat diet (HFD) and L-NAME (a nitric oxide synthase inhibitor). The combination exposure to HFD and L-NAME increased Smad3 phosphorylation in fibroblasts (Pdgfra-positive cells) in the heart; however, Pdgfra-PKN1/2 KO was not affected by HFD- and L-NAME-induced increase in Smad3 phosphorylation (**Reviewer Fig. 1-4 E, F** and **Revised Fig. 9c**). These data suggest that PKN is not a key molecule for mediating Smad3 phosphorylation in cardiac fibroblasts.

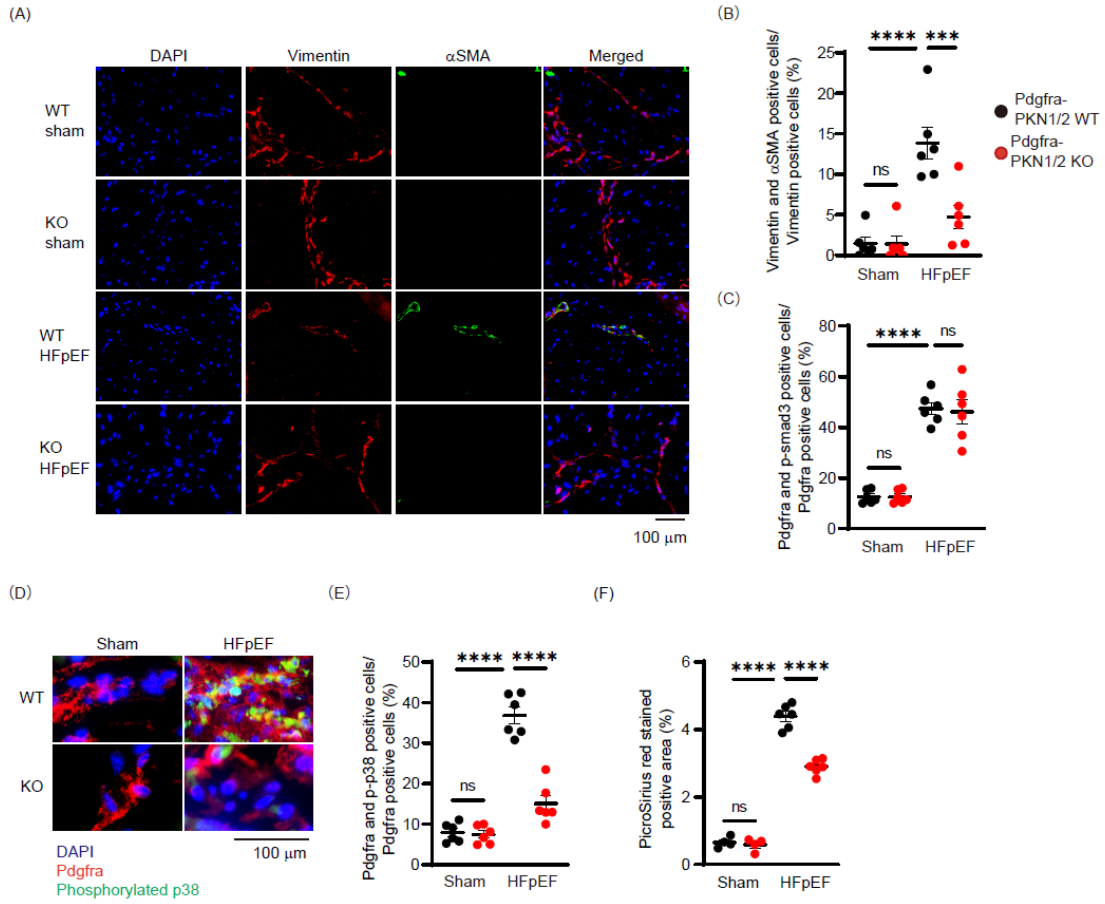


Reviewer Figure 1-4. (**A**, **B**) Immunofluorescent images (**A**) and quantification of the number (**B**) of cardiac fibroblasts with phosphorylated Smad3 (red) after siRNA-mediated PKN1/2 knockdown and TGF-β treatment (n = 4). (**C**, **D**) Immunofluorescent images (**C**) and quantification of the number (**D**) of 3T3 cells with phosphorylated Smad3 (red) after overexpression of flag-tagged PKN catalytic domain (PKN-cat) (n = 4). (**E**, **F**) Immunofluorescent staining of fibroblasts and myofibroblasts in the heart that were immunostained for Pdgfra (red), Smad3 phosphorylation (green) and nuclei (blue) at 10 weeks after a combination exposure to HFD and L-NAME. Quantification of vimentin-positive cells is shown as a percentage of DAPI-positive cells (**F**; n = 6). Data are presented as the mean ± SEM and analyzed using an unpaired Student t test (D) and two-way ANOVA followed by the Tukey's post hoc test (B, F). ns, not significant; ****p < 0.0001.

6. ***Fig 6A, result description Page 13, lines 9-10, "no difference was observed in fractional shortening, which indicates the presence of cardiac systolic function." This statement is factually incorrect. Actually, no difference in FS suggests comparable systolic function.***
Response: Following the reviewer's suggestion, we introduced the following text in the revised manuscript.

Page 13, lines 2-4
"In Pdgfra-PKN1/2 WT and KO male mice, no difference was observed in fractional shortening, suggesting comparable systolic function (**Fig. 8c**)."

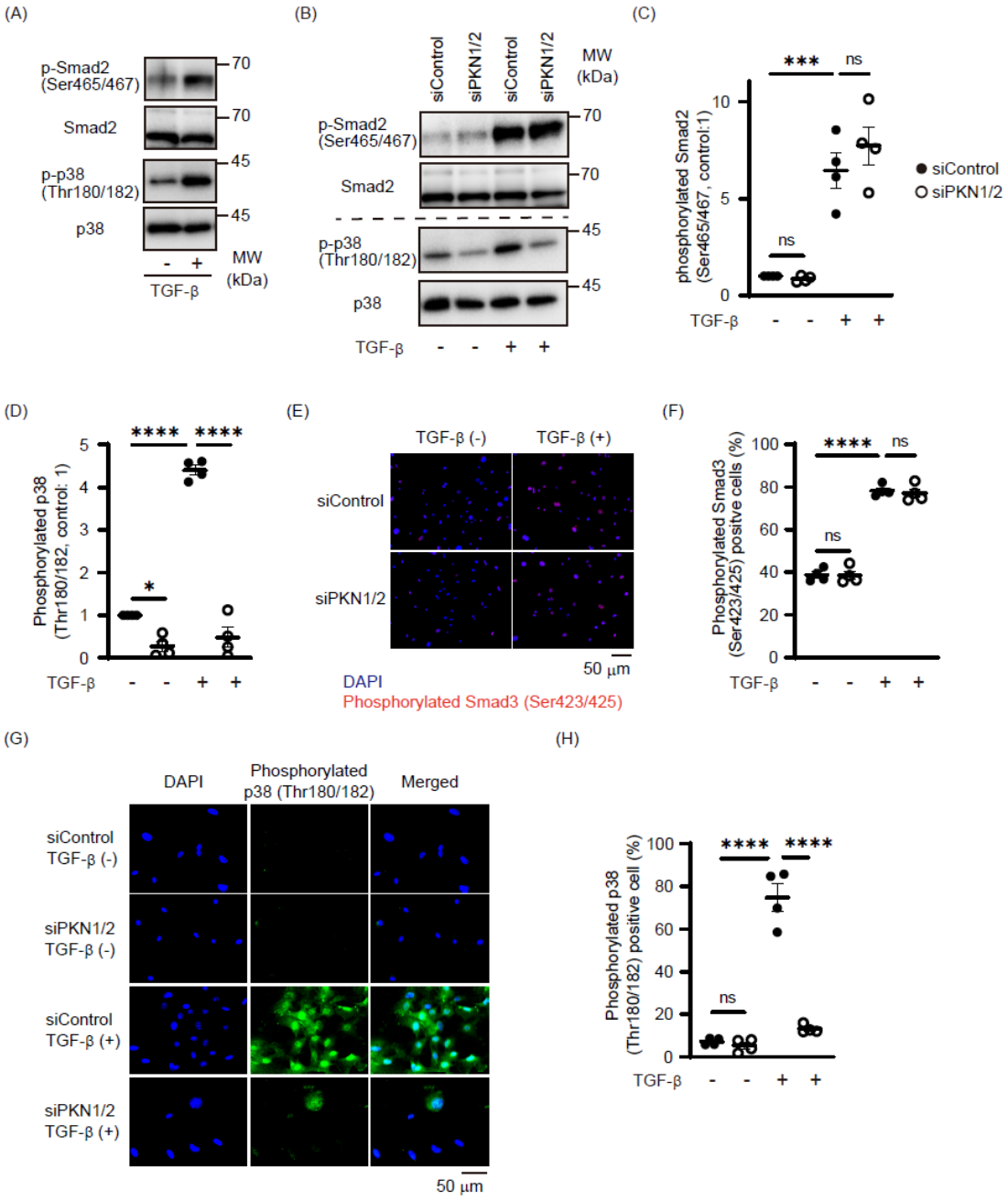
7. ***The authors introduced the 2 hit HFpEF model at the end of the paper and performed the cardiac phenotyping. However, author did not present the fibroblast/myofibroblast, fibrosis, or profibrotic signaling data with this model. This line of finding can not be just presumed for such a high-profile publication. This reviewer requests the authors to provide this line of data for a comprehensive paper.***
Response: Following the reviewer's suggestion, we investigated whether PKN1/2 mediates the conversion of fibroblasts into myofibroblasts in the heart in the HFD/L-NAME-induced HFpEF model *in vivo*. In Pdgfra-PKN1/2 WT mice, the intensity of αSMA in vimentin-positive cells increased after the 10-week HFD and L-NAME exposure (**Reviewer Fig. 1-5A** and **Revised Fig. 9a**). PKN1/2 deficiency decreased HFD/L-NAME-induced-induced increase of vimentin and αSMA double-positive cells (**Reviewer Fig. 1-5B** and **Revised Fig. 9b**). To investigate whether the combination of HFD and L-NAME activates Smad and non-Smad cascades, we examined the phosphorylation of Smad3 and p38 via an immunostaining assay. No differences were observed in the number of Pdgfra- and phosphor-Smad3-positive cells in the heart between Pdgfra-PKN1/2 WT and KO mice (**Reviewer Fig. 1-5C** and **Revised Fig. 9c**). The combination of HFD and L-NAME increased p38 phosphorylation in Pdgfra-positive cells in the heart; however, Pdgfra-PKN1/2 KO significantly decreased the HFD/L-NAME-induced p38 phosphorylation in fibroblasts (**Reviewer Fig. 1-5D, E** and **Revised Fig. 9d, e**). Finally, we evaluated the cardiac fibrosis using Picrosirius red staining after the 10-week HFD and L-NAME exposure. The cardiac fibrotic area induced by the combination was significantly reduced in Pdgfra-PKN1/2 KO mice (**Reviewer Fig. 1-5F** and **Revised Fig. 8a**). These data suggest that PKN1/2 contributes to the conversion of fibroblasts into myofibroblasts through p38 activation in HFD- and L-NAME-induced heart failure.



Reviewer Figure 1-5. PKN1 and PKN2 deletion suppresses the differentiation of cardiac fibroblasts in the HFpEF model. (**A**) Immunofluorescent staining of fibroblasts and myofibroblasts at 10 weeks after the combination of HFD and L-NAME in Pdgfra-PKN1/2 WT and KO male mice (*n* =6; red, vimentin; green, αSMA; blue, nuclei). (**B**) Quantification of dual-positive cells for vimentin and αSMA is shown as a percentage of vimentin-positive cells (n = 6). (**C**) Quantification of the number of cardiac fibroblasts with phosphorylated Smad3 (Ser423/425) at 10 weeks after combination exposure to HFD and L-NAME in Pdgfra-PKN1/2 WT and KO male mice. (**D**, **E**) Immunofluorescent images (**D**) and quantification of the number (**E**) of cardiac fibroblasts with phosphorylated p38 (Thr 180/182) at 10 weeks after combination exposure to HFD and L-NAME in Pdgfra-PKN1/2 WT and KO male mice (*n* =6, red, Pdgfra; green, phosphorylated p38; blue, nuclei). (**F**) Schematic diagram showing PKN1/2-mediated cardiac fibrosis. Data are presented as the mean ± SEM and analyzed using two-way ANOVA followed by the Tukey's post hoc test (B, C, and E). ns, not significant; ***p < 0.001; ****p < 0.0001.

8. ***The phosphorylation residue of all kinases must be included in the paper.***

Response: Following the reviewer's suggestion, we added the phosphorylation sites in the revised text and figures (**Reviewer Figure 1-6** and **Revised Fig. 6**).

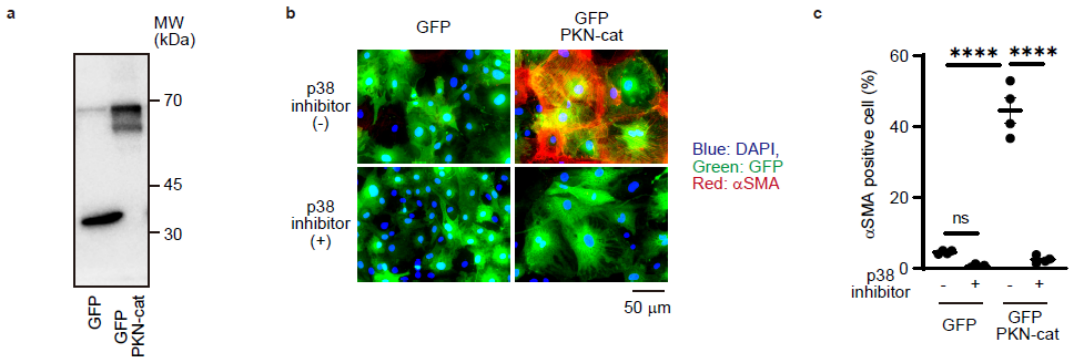


Reviewer Figure 1-6. PKN1/2 knockdown significantly reduced TGF-β-mediated p38 phosphorylation. (**A**) Western blot analysis for TGF-β-mediated phosphorylation of Smad2 (Ser465/467) and p38 (Thr180/182) in cardiac fibroblasts. The results are representative of three independent experiments. (**B-D**) Western blot analysis (B) and quantification for TGF-β-mediated phosphorylation of endogenous

Smad2 (C) and p38 (D) in cardiac fibroblasts after siRNA-mediated knockdown of PKN1/2 (n = 4). (**E**, **F**) Immunofluorescent images (E) and quantification of the number (F) of cardiac fibroblasts with phosphorylated Smad3 (Ser423/425; red) after siRNA-mediated PKN1/2 knockdown and TGF-β treatment (n = 4). (**G**, **H**) Immunofluorescent images (G) and quantification of the number (H) of cardiac fibroblasts with phosphorylated p38 (green) after siRNA-mediated PKN1/2 knockdown and TGF-β treatment (n = 4). Data are presented as the mean ± SEM and analyzed using two-way ANOVA followed by the Tukey's post hoc test (C, D, F, and H). ns, not significant; *p < 0.05; ***p < 0.001; ****p < 0.0001.

9. ***Finally, the mechanism aspect is still weak, especially since the proposed mechanism could be simply associative rather than driving/causative. Given the timeline of the manuscript, this reviewer understands that suggesting to generating FB-specific PKN1/2-TG mice and performing a rescue experiment with a p38 deletion/inhibitor is not reasonable. Therefore, to further strengthen the mechanistic findings, I would suggest performing rescue experiment in an isolated FB cell culture model. Specifically, overexpressing PKN1/2 to induce profibrotic signaling and FB activation and rescuing these with a p38 inhibitor. This line of investigation will establish the driving role of the proposed mechanism.***

Response: Following the reviewer's suggestion, we examined whether p38 inhibitor treatment rescues the PKN1/2-induced profibrotic signaling. After GFP-tagged PKN catalytic domain (PKN-cat) was overexpressed in cardiac fibroblasts isolated from murine hearts using a lentiviral vector system, the fibroblasts were treated with a p38 inhibitor. Immunofluorescent images showed that overexpressing PKN increased the number of αSMA-positive cells and that the treatment with the p38 inhibitor significantly decreased PKN-mediated αSMA expression (**Reviewer Fig. 1-7 a-c** and **Revised Fig. 7a-c)**.



Reviewer Figure 1-7. Treatment with a p38 inhibitor decreased PKN-mediated increase in αSMA-positive cells. (**a**) Western blot analysis for GFP-PKN-cat expression in cardiac fibroblasts. The results were representative of three independent experiments. (**b**, **c**) Immunofluorescent images (**b**) and quantification of the number (**c**) of αSMA-positive cardiac fibroblasts after overexpression of GFP-PKN-cat and treatment with a p38 inhibitor (10 μM, $n = 4$). Data are presented as the mean ± SEM and analyzed using two-way ANOVA followed by Tukey's post hoc test (c). ns, not significant; ****$p < 0.0001$.

10. ***Finally, the discussion section (6-7 pages long) is very unfocused. A lot of it is a review kind of writing rather than truly discussing the paper's findings and implications. Some sentences are background/rationale, e.g., "The human kinome consists of over 500 protein kinases, and PKN belongs to the cAMP-dependent, cGMP-dependent and protein kinase C (AGC) kinase family, comprising evolutionarily related protein kinases. Each AGC kinase is involved in different pathologies, affecting human health issues, including cancer, metabolic disorders, and cardiovascular diseases." This kind of background knowledge may be appropriate for the "introduction" section but not for the discussion. This reviewer requests the authors to revise the discussion entirely to present a focused, concise discussion of presented findings and their implications.***

Response: Thank you for providing these important suggestions. We have introduced the following text in the revised introduction and discussion manuscript.

Introduction (**page 3**, **line 14- page 4, line 4**)

"Additionally, other signaling cascades, including the small GTPase RHOA, have been implicated in cardiac fibroblast activation.[17] Protein kinase N (PKN), an effector protein kinase of RHOA, belongs to the cAMP-dependent, cGMP-dependent, and protein kinase C (AGC) kinase family.[18-20] RHOA and other RHOA-mediated signaling proteins play crucial physiological and pathological roles in cardiovascular diseases.[21] Recent in vivo analyses showed that PKN physiologically contributed to the vascular tone of hypertension.[22] Moreover, PKN pathologically contributes to cardiac hypertrophy leading to heart failure[23] and tumor invasion of pancreatic cancer.[24] However, the role of PKN in fibrosis remains unclear."

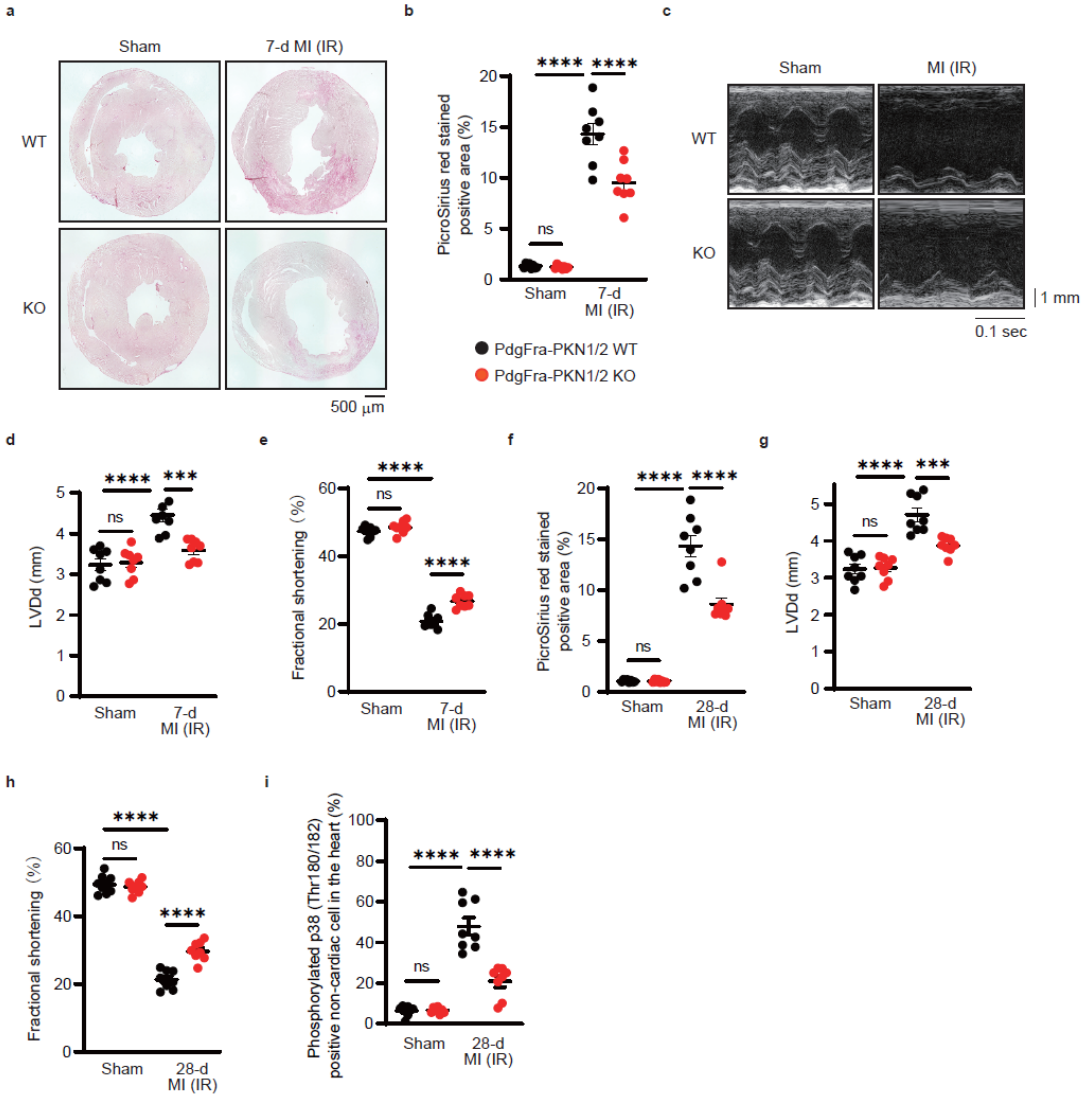
Reviewer #2 (Remarks to the Author):

Protein kinase N (PKN) 1 and PKN2 are essential for mesoderm (and heart) development, and contribute to adaptation to pressure overload. Less is known about the role of PKN1 / 2 in cardiac fibroblasts. The authors show that PKN1 and PKN2 are both elevated by TGF-b1, an inducer of fibroblast activation and fibrosis. Subsequently, they delete PKN1 and PKN2 using a Pdgfra-dependent fibroblast Cre line in mice. They do not observe differences in AngII treatment, but describe subtle, but significant improvement in cardiac function in ischemia/reperfusion (I/R) model and a HFpEF model. In vitro experiments suggest that PKN1/2 is upstream of p38 signaling that induces a pathologic fibroblast response. The manuscript is well organized and logical and suggest PKN1/2 contribute to fibroblast activation in I/R and HFpEF. However, fairly superficial investigation of the phenotype is reported; procedures are insufficiently described; and considerable questions remain about the adequacy of the timepoints chosen for various experiments.

Major comments:

1. ***The replicate number and variability is incredibly small for an I/R in vivo cardiac physiology study. Further, all endpoints are 7 days post- I/R, which is quite early, and prior to the robust scar that forms in this model. It would be more informative to investigate readouts of fibroblast activity and scar formation, including cardiac physiology, at 28 days post-I/R.***

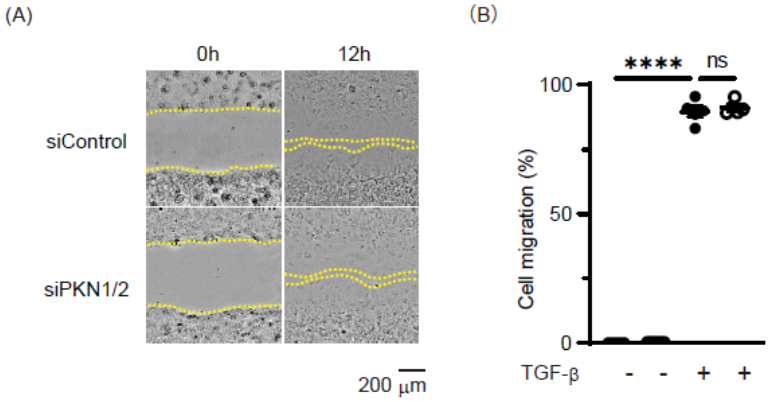
   Response: Thank you for providing these important suggestions. Following the reviewer's suggestion, we conducted an *in vivo* study at 7 days and 28 days post-I/R (n = 8; **Reviewer Fig. 2-1 a-h** and **revised Fig. 3**). Moreover, we examined whether PKN1/2 mediates p38 phosphorylation to investigate fibroblast activity. The phosphorylation of p38 was increased in the border area's fibroblasts in the MI accompanied by 28-d IR model, and Pdgfra-PKN1/2 KO decreased p38 phosphorylation (**Reviewer Fig. 2-1 i** and **revised Fig. 7g**).



Reviewer Figure 2-1. PKN1 and PKN2 deletion in cardiac fibroblasts suppresses cardiac fibrosis after myocardial infarction. (**a**, **b**) Fibrotic changes in the left ventricles after 7 days of MI with ischemia-reperfusion (IR) model were assessed using Picrosirius red staining ($n = 8$). (**c**-**e**) Echocardiogram analysis of left ventricular end-diastolic diameter (**d**; LVDd) and fractional shortening (**e**) was performed before and 7 days after MI accompanied by IR ($n = 8$). (**f**-**h**) Fibrotic changes in the left ventricles (**f**), LVDd (**g**), and fractional shortening (**h**) were examined after 28 days of MI with IR. (**i**) Immunofluorescent-based quantification of dual-positive cells for PDGFRα and phosphorylated p38 shown as a percentage of PDGFRα-positive cells in the control and ischemia-reperfusion hearts at 28 days ($n = 8$) after injury. Data are presented as the mean ± SEM and analyzed using two-way ANOVA followed by the Tukey's post hoc test (**b**, and **d**-**i**). ns, not significant; *p < 0.05; ***p < 0.001; ****p < 0.0001.

2. ***The authors investigate fibroblast migration upon Pkp2 knockdown in Fig. 3. 6 hours is too early to convincingly demonstrate no change in migration. It is curious that th authors document 40% migration, given the images do not show any obvious migration at 6 hrs.***

Response: Following the reviewer's suggestion, we examined fibroblast migration after 12-h treatment with TGF-β (**Reviewer Fig. 2-2 A-B** and **Revised Fig. 4d-e**). The scratch wound closure rate of TGF-β-treated fibroblasts was unaffected following by PKN1/2 deletion.



Reviewer Figure 2-2. Scratch wound healing assay after 12-h treatment with TGF-β. Scratch wound healing assay (**A**) and quantification (**B**) after 12-h treatment with TGF-β (n = 5). Data are presented as the mean ± SEM and analyzed using two-way ANOVA followed by the Tukey's post hoc test (A and B); ns, not significant; ****p < 0.0001.

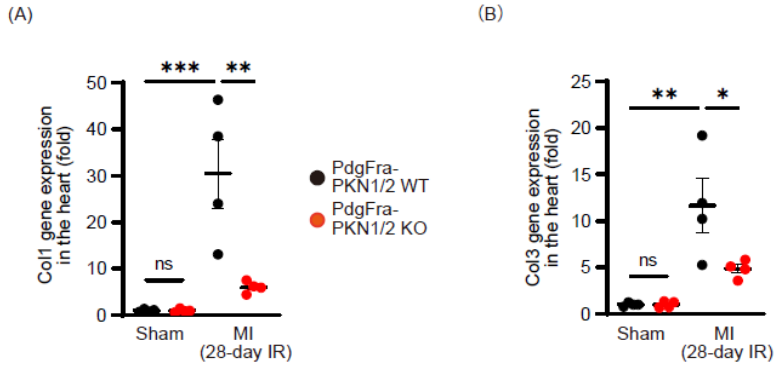
3. ***Immunostaining and Western blot analysis needs more details, epecially antibody concentrations. How is Collagen I and Collagen III levels quantified using polarized imaging? No ColI or ColII antibodies are listed, so is this from the picrosirius red staining? Again, insufficient procedures are listed for this method that is not typically used to quantify ColI and ColIII. Red vs green bi-refringence typically marks new and mature collagen strands, not ColI vs III.***

Response: Following the reviewer's suggestion, we included the antibody concentration in the Revised method.

Page 20, lines 6-17

"Antibodies to vimentin (1:1000, ab45939), Ki67 (1:1000, ab15580) and phospho-Smad3 (1:100, ab52903) were obtained from Abcam (Cambridge, UK). PKN1(1:1000, sc-1842) and MRTFA (1:1000, sc-21558) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies to CD45 (1:100, #70257), PKN2 (1:1000, #2612S), phospho-PKN1/2 (1:1000, #2611S), phospho-p38 MAPK (1:1000, #4511), p38 MAPK (1:1000, #8690), phospho-MKK3/6 (1:1000, #12280), phospho-SMAD2 (1:1000, #18338), and smad2/3 (1:1000, #8685) were obtained from Cell Signaling Technology (Danvers, MA, USA). We used antibodies to CD31 (1:200, #553370, BD Biosciences, San Jose, CA, USA), PDGFRα (1:1000, AF1062, R &D Systems, Minneapolis, MN, USA), αSMA (1:1000, C6198, Sigma-Aldrich, St. Louis, MO, USA), and DAPI (1:1000, #340-07971, Dojindo, Kumamoto, Japan). An antibody to phospho-MRTFA (1:200) was purified as previously described.[23]"

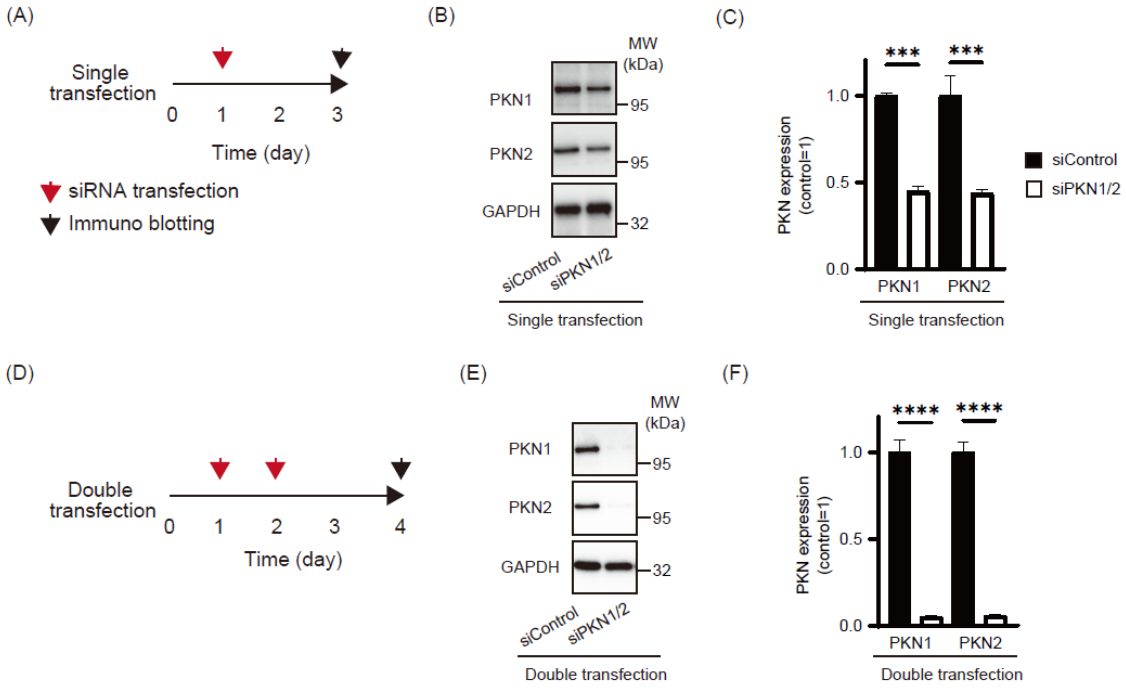
Response: We showed the Col1 and Col3 area using polarized imaging. We tried to perform immunostaining analysis using anti-Col1 (#72026, Cell Signaling Technology, Danvers, MA, USA) and anti-Col3 (ab7778, Abcam, Cambridge, UK), but it did not work. Thus, instead of immunostaining, we examined Col1 and Col3 gene expression in the heart at 28 days post I/R using quantitative real-time polymerase chain reaction (**Reviewer Fig. 2-3** and **Revised Fig. 4h and i**).



Reviewer Figure 2-3. Col1 and Col3 gene expression after MI. (**A**, **B**) Gene expression of collagen isoforms 1 and 3 (determined by quantitative real-time polymerase chain reaction; $n = 6$) in the infarct area after 28 days of an MI with the ischemia-reperfusion (IR). Data are presented as the mean ± SEM and analyzed using two-way ANOVA followed by the Tukey's post hoc test (A and B); ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4. ***Two subsequent siRNA transfections are reportedly necessary to obtain knockdown of Pkn1/2; however, the methods do not describe this atypical procedure. What is the efficiency of knockdown with a single transfection; when were the transfections conducted?; etc. Further, what passages were used for cardiac fibroblast cutulres; most experients describe a prolonged culture (necessary for two transfections, and other manipulations), however the details are not provided.***

Response: We showed the siPKN1/2 efficiency with a single transfection (**Reviewer Fig. 2-4 A-C**) or double transfection (**Reviewer Fig. 2-4 D-F** and **Revised Fig. 4a-b**).



Reviewer Figure 2-4. siPKN1/2 transfection experiment. (**A**) Schematic diagram showing single siRNA transfection. (**B**) Efficiency of PKN1 and PKN2 knockdown in cardiac fibroblasts as shown via western blot analysis. (**C**) Statistical evaluation of B ($n = 6$). (**D**) Schematic diagram showing double siRNA transfection. (**E**) Efficiency of PKN1 and PKN2 knockdown in cardiac fibroblasts as shown via western blot analysis. (**F**) Statistical evaluation of E ($n = 6$). Data are presented as the mean ± SEM and analyzed using an unpaired Student t test (C and F); ***$p < 0.001$; ****$p < 0.0001$.

Response: We used one-passage murine cardiac fibroblasts for all experiments. We indicated this in the revised methods.

Page 22, line 5

"One-passage murine cardiac fibroblasts were used for all experiments."

5. ***Apart from identifying p38 as downstream of PKN1/2 in fibroblasts (based on prior literature) no novel insight is provided about fibroblast activation.***

Response: We completely agree that the mechanism of PKN-mediated cardiac fibrosis is still unclear. Compared to other AGC superfamily protein kinases (e.g., PKC) and RHOA-mediated molecules (e.g., RHOK), the substrates and physiological and pathological roles of PKN have remained unclear since PKN was first identified in the 1990s (Thumkeo D, et al. *Eur J Cell Biol. 2013*; 92: 303–315). Approximately 10 proteins have been identified as substrates for PKNs. Although PKC- and RHOA-mediated molecules play a key role in cell proliferation and migration, the physiological and pathological relevance of their phosphorylation by PKNs in the heart is yet to be elucidated (Marrocco V, et al. *J Mol Cell Cardiol. 2019*; 128: 212–226). We recently identified MRTFA as a novel substrate of PKN in cardiomyocytes (Sakaguchi T, et al., *Circulation 2019*; 140: 1737-1752); however, PKN did not phosphorylate MRTFA in cardiac fibroblasts (**Revised Fig. S1**). Moreover, PKN did not mediate cell proliferation and migration (**Revised Fig. 4c-e**). These observations make it difficult to identify the role of PKN.

The current study found that PKN mediated cardiac fibrosis in heart failure through fibroblast-to-myofibroblast conversion (**Revised Fig. 5**). Following the suggestion of the Editor and Reviewer 1, we examined whether PKN-mediated Smad3 was a novel substrate of PKN in cardiac fibroblasts. However, PKN did not increase Smad3 phosphorylation *in vitro* and *in vivo* (**Revised Fig. 6 e, f**, and **Revised Fig. 9c**).

Transforming growth factor β (TGF-β) is a central cytokine involved in fibroblast activation and a clear therapeutic target for preventing or slowing fibrosis in patients (Tallquist MD, et al, *Nat. Rev. Cardiol. 2017*; 14: 484-491). TGF-β inhibitors have not exerted therapeutic effects because TGF-β inhibition in regulatory T cells can exacerbate autoimmune disease (Meng et al., *Nat. Rev. Nephrol. 2016*; 12:325-338). Myofibroblast deletion following MI results in ventricular rupture by reducing collagen production and scar formation (Kanisicak et al., *Nat. Commun. 2016*; 7:12260). Although p38 deficiency in fibroblasts decreases cardiac fibrosis 28 days after MI, cardiac rupture increases within 1 week after MI (Molkentin, J.D, et al., *Circulation 2017*; 136: 549-561). These observations suggest that direct inhibition of TGF-β and p38 may be inappropriate for cardiac fibrosis in heart failure. ECM deposition initially strengthens tissue integrity in MI; however, extensive and

sustained fibrosis impairs heart function (Tallquist MD, Annu. Rev. Physiol. 2020; 82: 63-78). The current study found that PKN1/2 deletion suppressed cardiac fibrosis without affecting cardiac rupture rates (**Revised Fig. 3i**). p38 cascades are regulated in response to many stimuli and can be activated through at least a dozen kinases (Chang L., et al., *Nature 2001*; 410: 37-40). Although further studies are needed to identify PKN1/2-phoshorylated molecules, the findings support that PKN1/2 is a potential therapeutic target for cardiac fibrosis in heart failure.

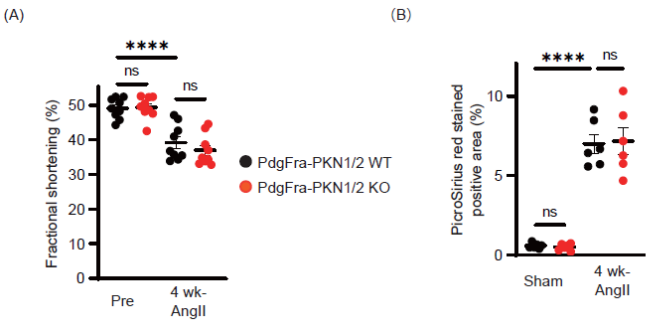
Reviewer #3 (Remarks to the Author):

The authors examined PKN1/2 distribution in the heart and the role of PKN1/2 in two models of heart disease, MI & HFpEF, in mediating fibrosis and the transition of fibroblasts to myofibroblasts. They demonstrate that PKN1 and 2, but not 3 are found in cardiac fibroblasts. They also compared WT and Pdgfra-PKN1/2 KO mice and concluded that the absence of PKN1/2 (PKN1/2 KO) reduces fibrosis and the transition to myofibroblasts (alpha SMA positivity). There are some comments to be addressed

Major comments

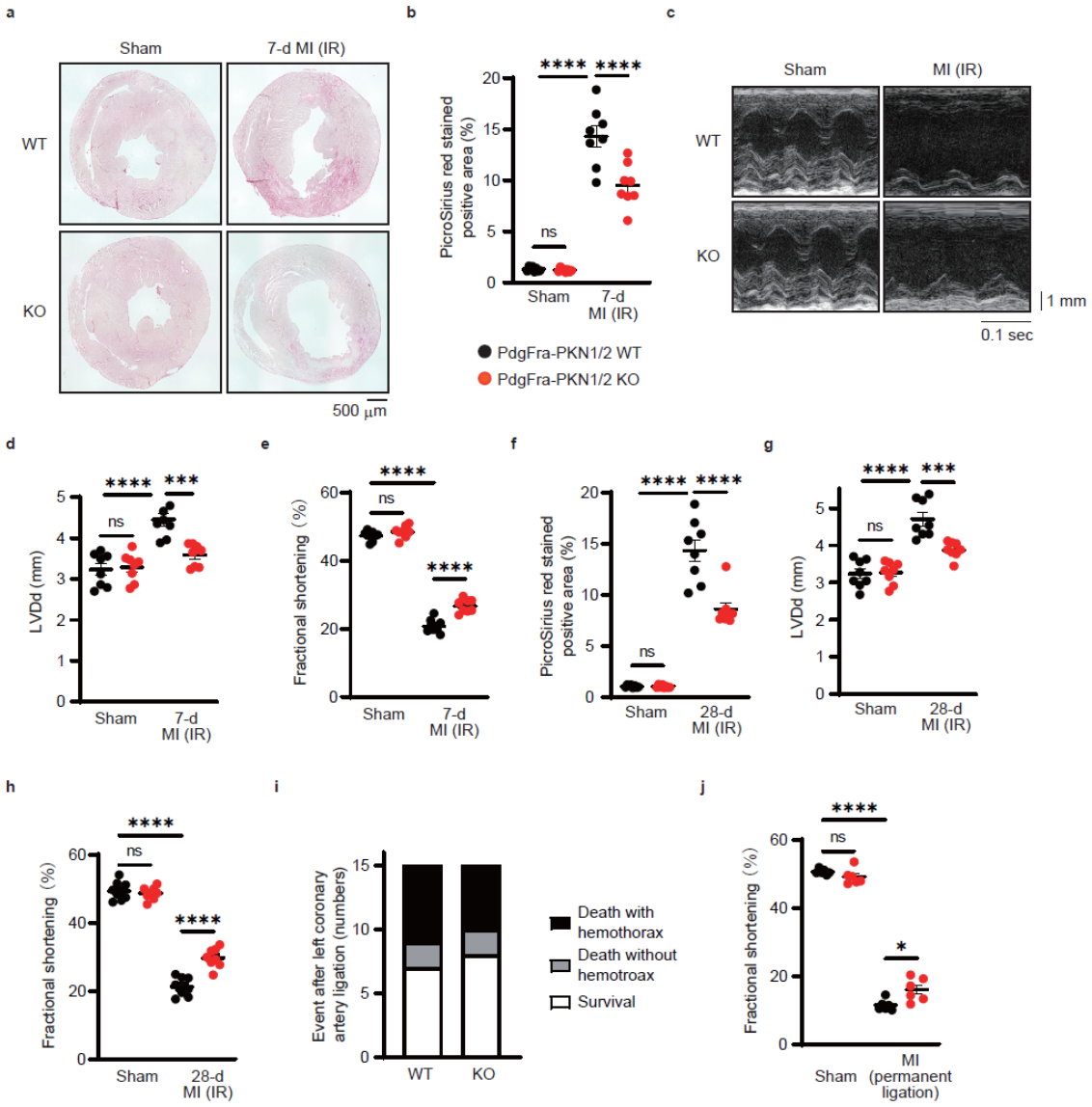
1. ***The authors need to make it clear which model is being studied and not present different models in the same paragraph or Figure. For example, Fig. 2B examines 4-week treatment with AngII, which is a model of HFpEF [Regan et al. Am J Physiol Heart Circ Physiol 2015;309:H771–H778, Dulce et al. Cardiovasc Res. 2023 118:3586-3601], but Figure 2 focuses on MI and the absence of PKN1/2 had no effect on fibrosis. In contrast, the authors state that fibrosis was reduced in the HFD+L-NAME HFpEF model in Pdgfra-PKN1/2 KO mice, but do not show those data.***

   Response: We followed the Reviewer's helpful suggestion and revised the figures accordingly. We showed the AngII and MI experiments separately (AngII experiments: **Reviewer Fig. 3-1 A**, **B** and **Revised Fig. 2f, g**; MI experiments: **Reviewer Fig. 3-2** and **Revised Fig. 3**). We also showed HFD/L-NAME-induced cardiac fibrosis (**Reviewer Fig. 3-3A, B** and **Revised Fig. 8a, b**).



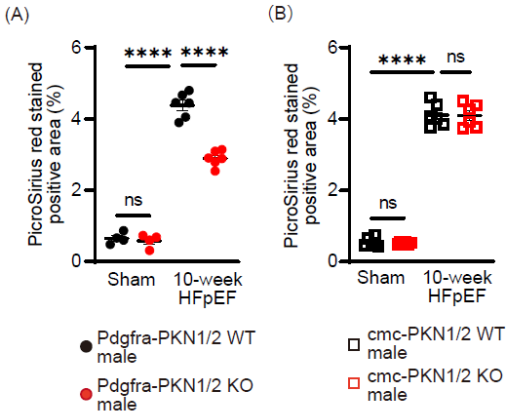
Reviewer Figure 3-1. AngII-induced heart failure model. (**A**) Left ventricular fractional shortening assessed by echocardiography (n = 8) and (**B**) fibrotic changes in the left ventricles assessed by Picrosirius red staining (n = 6) after 4 weeks of

sustained AngII infusion (100 ng·g^{-1}·d^{-1}). Data are presented as the mean ± SEM and analyzed using two-way ANOVA followed by the Tukey's post hoc test (A and B). ns, not significant; ****p < 0.0001.



Reviewer Figure 3-2. PKN1 and PKN2 deletion in cardiac fibroblasts suppresses cardiac fibrosis after myocardial infarction. (**a**, **b**) Fibrotic changes in the left ventricles after 7 days of MI with ischemia-reperfusion (IR) were assessed by Picrosirius red staining (n = 8). (**c**-**e**) Echocardiogram analysis of left ventricular end-diastolic diameter (**d**; LVDd) and fractional shortening (**e**) was performed before and 7 days after MI accompanied by IR (n = 8). (**f**-**h**) Fibrotic changes in the left ventricles (**f**), LVDd (**g**), and fractional shortening (**h**) were examined after 28 days of MI with the IR. (**i**) Number of events during 7 days of MI accompanied by permanent ligation

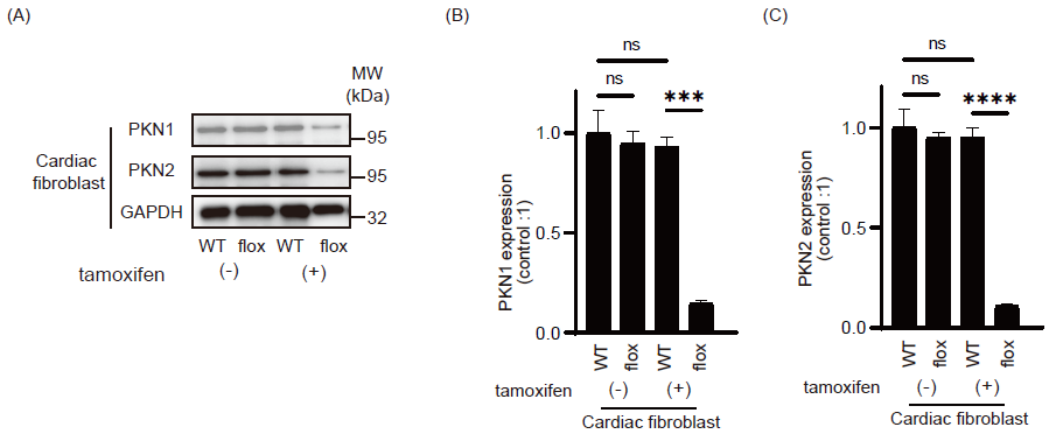
of the left coronary artery (n = 15). (**j**) Fractional shortening 7 days after permanent ligation of the left coronary artery (n = 6). Data are presented as the mean ± SEM and analyzed using two-way ANOVA followed by the Tukey's post hoc test (**b**, **d**-**h**, and **j**). ns, not significant; *p < 0.05; ***p < 0.001; ****p < 0.0001.



Reviewer Figure 3-3. Cardiac fibrosis in HFpEF. (**A**, **B**) Fibrotic changes in the left ventricles assessed by Picrosirius red staining at 10 weeks after combination exposure to HFD and L-NAME in Pdgfra-PKN1/2 WT and KO mice (**A**), and in cardiomyocyte-specific PKN1/2 (cmc-PKN1/2) WT and KO mice (**B**) (n = 6). Data are presented as the mean ± SEM and analyzed using two-way ANOVA followed by the Tukey's post hoc test (A and B). ns, not significant; ****p < 0.0001.

2. ***As the authors mention, PKN1& 2 are not completely eliminated in fibroblasts (Fig 1H) from floxed, tamoxifen-treated mice. Please show quantification of signal intensity (particularly since the GAPDH band appears lighter in the flox + Tamoxifen lane).***

Response: We followed the Reviewer's helpful suggestion and accordingly revised the figures. We also showed the quantification of signal intensity (**Reviewer Fig. 3-4 A-C** and **Revised Fig. 2 b-d**).



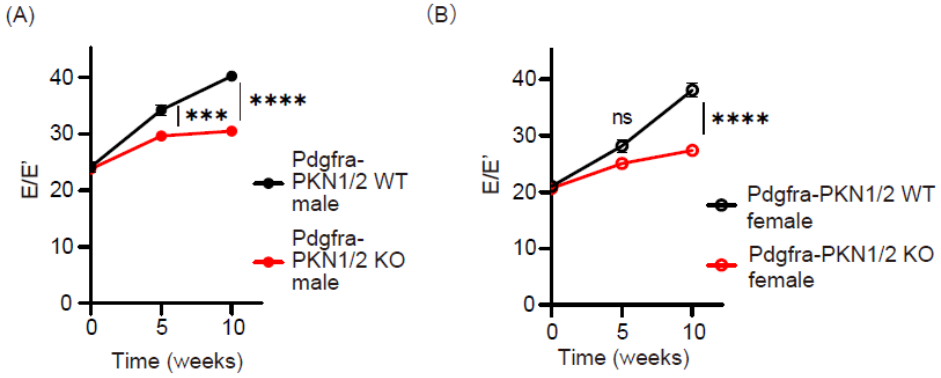
Reviewer Figure 3-4. Fibroblasts in PKN1/2-deficient mice in. (**A**) Western blot analysis for PKN1 and PKN2 from cardiac fibroblasts isolated from the heart of Pdgfra-PKN WT or KO mice after tamoxifen induction. GAPDH was used as control. (**B**, **C**) Statistical evaluation of A (n = 3). Data are presented as the mean ± SEM and analyzed using two-way ANOVA followed by the Tukey's post hoc test (B and C). ns, not significant; ****$p < 0.0001$.

3. ***Western blots are generally overexposed, which would make quantification with Image J difficult. Please provide Western blots with shorter exposure times. Also, please provide the full-size gels in the Supplemental results.***
   Response: We followed the Reviewer's helpful suggestion and performed Western blots again. The large-size image is shown (**Revised supplemental Source Data file**).

4. ***For cell culture experiments, the authors reduce PDK1/2 using siRNA. Why did they not isolate cardiac fibroblasts from Pdgfra-PKN1/2 KO mice for these studies?***
   Response: *In vivo***,** we did not find significant differences in the number of α-SMA-positive cardiac fibroblasts numbers between Pdgfra-PKN1/2 WT mice and KO mice at normal conditions. However, *in vitro*, the number of α-SMA-positive fibroblasts was noticeably decreased in the cardiac fibroblast isolated from Pdgfra-PKN1/2 KO mice without any stimulation. That is why we used siRNA experiments *in vitro*.

5. ***The initial HFD+L-NAME study used a 5-week treatment. Why did the authors continue for 10 weeks?***
   Response: This is an important question to examine whether 5-week and 10-week

HFD and L-NAME exposure affects cardiac function. In Pdgfra-PKN1/2 WT and KO male mice, PKN deficiency reduced HFD/L-NAME-induced cardiac diastolic dysfunction after 5 weeks and 10 weeks of combination exposure to HFD and L-NAME (**Reviewer Fig. 3-6 A** and **Revised Fig. 8f**). In Pdgfra-PKN1/2 WT and KO female mice, no difference was observed in cardiac diastolic function after 5-week combination exposure to HFD and L-NAME. However, PKN1/2 deficiency improved the ratio of E to E' (E/E') after 10-week combination exposure (**Reviewer Fig. 3-6 B**, and **Revised Fig. 8i**). This is why we performed echocardiography before and after the 5- and 10-week HFD feeding and L-NAME exposure.



Reviewer Figure 3-6. Ratio of the mitral E and E' waves (E/E') at 0, 5, and 10 weeks after combination exposure to HFD and L-NAME in Pdgfra-PKN1/2 WT and KO female mice.

6. ***While women are pre-disposed to HFpEF more than men, the HFD+L-NAME regimen does not replicate this sex bias (Tong et al. Circulation. 2019;140:1769–1771). Please adjust this part of the Discussion***

Response: Thank you for providing these important suggestions. We have introduced the following text in the revised discussion manuscript.

Page 17 lines 9-17

"There are marked sex differences in cardiovascular diseases, with HFpEF being more prevalent in women. In the current study, fibroblast-specific PKN1/2 deficiency started to improve cardiac diastolic function at 5 weeks after the start of combination exposure to HFD and L-NAME in male, but this was only observed after 10 weeks in female mice. PKN1/2 deficiency attenuated HFD- and L-NAME-induced cardiac diastolic dysfunction earlier in males than in females. In HFD- and L-NAME-induced HFpEF models, female mice develop

a more significantly attenuated cardiac phenotype than their male counterparts,[48] which may explain the sex differences in the current study. Further research is warranted to confirm the role of PKN1/2 in sex differences."

Minor:

1. ***The introduction should be shortened and the Discussion also seems long***
   Response: Thank you for providing these important suggestions. We have accordingly shortened these sections.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Thanks much for addressing my comments.

Reviewer #2 (Remarks to the Author):

The authors have addressed all of the prior comments with additional or improved experiments. The only remaining minor comment is related to Figures 5d and 9a, which demonstrate vimentin and SMA positive fibroblasts in heart sections. Fig. 5d shams and all the Fig. 9 images do not appear to be the morphology of fibroblasts, and instead may be blood vessels. IF the authors could select new images with more typical fibroblast morphology, this would better demonstrate the proposed phenotype.

Reviewer #3 (Remarks to the Author):

The authors adequately addressed my concerns

A letter to the Reviewers

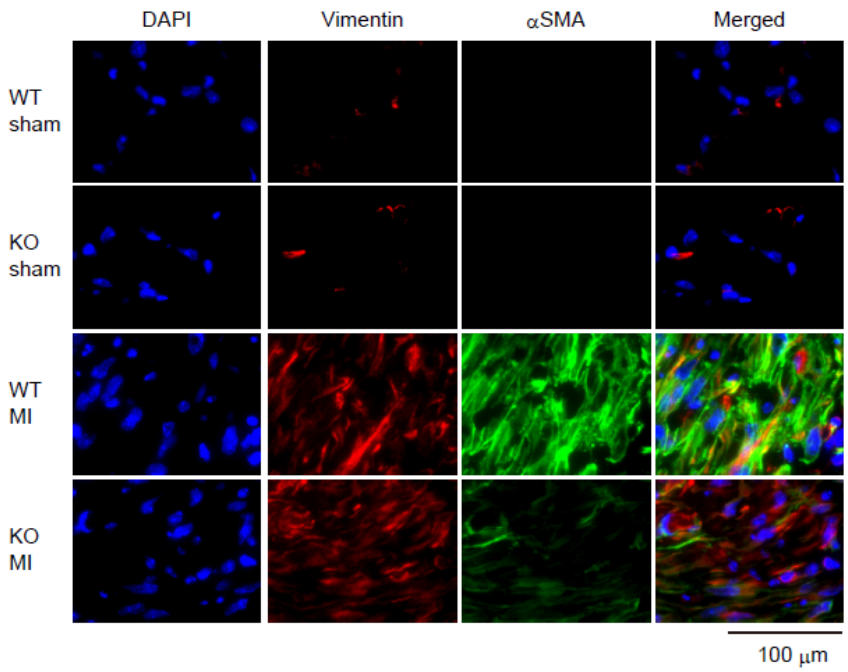
We would like to express our gratitude to the Editor and Reviewers for their constructive comments and suggestions, which have enhanced the quality of our manuscript.

Please note that comments from the Reviewer are shown below in bold italics followed by our response.

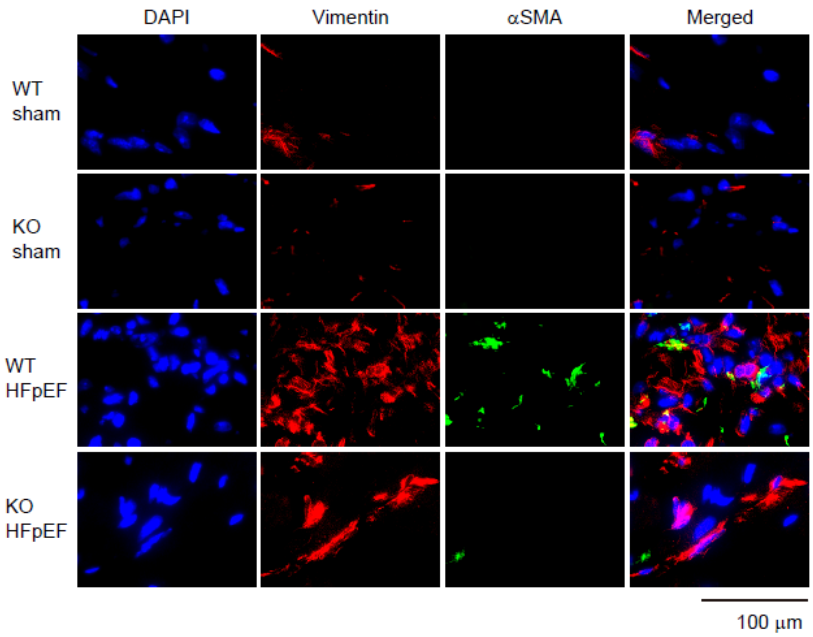
Reviewer #2 (Remarks to the Author):

The authors have addressed all of the prior comments with additional or improved experiments. The only remaining minor comment is related to Figures 5d and 9a, which demonstrate vimentin and SMA positive fibroblasts in heart sections. Fig. 5d shams and all the Fig. 9 images do not appear to be the morphology of fibroblasts, and instead may be blood vessels. IF the authors could select new images with more typical fibroblast morphology, this would better demonstrate the proposed phenotype.

Response: Thank you for your valuable suggestions. Following the reviewer's suggestion, we have prepared new images for Figures 5d and 9a.



Revised Figure 5d: Immunofluorescent staining of fibroblasts and myofibroblasts in the cardiac infarction area 7 days after injury. The fibroblasts and myofibroblasts were immunostained for vimentin (red), αSMA (green), and nuclei (blue).



Revised Figure 9a: Immunofluorescent staining of fibroblasts and myofibroblasts at 10 weeks after combination exposure to HFD and L-NAME in Pdgfra-PKN1/2 WT and KO male mice (red, vimentin; green, αSMA; blue, nuclei).