Journal of Cell Science

AP-1 signaling modulates cardiac fibroblast stress responses

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DOI: 10.1242/jcs.261152

Editor: Arnoud Sonnenberg

Review timeline
Original submission: 9 March 2023
Editorial decision: 5 June 2023
First revision received: 24 October 2023
Editorial decision: 10 November 2023
Second revision received: 15 November 2023
Accepted: 15 November 2023

Original submission

First decision letter

MS ID#: JOCES/2023/261152

MS TITLE: Modulation of Cardiac Fibroblast Stress Responses through an AP-1-NFkB-GATA5 Signaling Axis

AUTHORS: Alexander J Whitehead, Hamza Atcha, James D Hocker, Bing Ren, and Adam J Engler
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

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

Reviewer 1

Advance summary and potential significance to field

Whitehead and co-authors use existing bulk RNA and scATAC sequencing data sets derived from regenerative (early postnatal) and nonregenerative (late postnatal) mice to identify an enrichment in NF-kappa B and TNF signaling as well as accessibility-associated motifs from the AP-1 family. In induced pluripotent stem cell-derived cardiac fibroblasts (iCFs), AP-1 and NFkB activate sequentially in response to several fibroinflammatory agonists. NFkB pathway inhibition reduces fibronectin deposition/assembly, preventing the adherence of monocytes to decellularized matrices from iCFs. The authors hypothesize that SNPs in the long non-coding RNA ANRIL at the 9p21 locus work in conjunction with AP1 to orchestrate these transcriptional programs. The study also demonstrates in iCFs from patients with the 9p21 risk haplotype that AP-1 inhibition reduces Cx43 mRNA, GATA5 protein, fibronectin deposition/assembly, and monocyte adhesion to the deposited matrix. Most of these pathways have been well characterized in fibroblasts and inflammation/fibrosis including the lncRNA Anril. The impactful information comes from the experiments performed in cardiac fibroblasts obtained from patients with SNPs in ANRIL, but overall the broad mechanistic impact of the findings is limited.

Comments for the author

1. The evidence for activation of NFkB by AP-1 is not sufficiently strong to support a sequential pathway, such as the one drawn in Figure 5. The authors show that p65 (NFkB) phosphorylation occurs after cJun (AP-1) phosphorylation, but both are already well documented in the literature to activate concurrently, and potentially modulate one another (Fan et. al AJP Heart Circ, 2002; Fujioka et. al Molecular and Cellular Biology, 2004). The representative blot showing attenuated p65 phosphorylation in response to AP-1i is not particularly convincing compared to the quantitation. The compound used is well documented to reduce Il1-beta secretion, which could explain the attenuation of NFkB activation. (A) Is inhibiting AP-1s binding to DNA directly altering NFkB phosphorylation or is this an autocrine effect? (B) Without data directly showing the interaction of the two transcription factors, the authors need to moderate the types of conclusions implied by the pathway diagram in 5.

2. The image analysis in figures 3B and 4G is lacking methodological detail required to assess its rigor. (A) How many fields of view/cells were analyzed? (B) For figure 4G were all patient lines imaged and then was the data aggregated by technical replicate? (C) The y-axes on quantification are labeled as intensity per cell, yet the methods suggest that the fluorescent intensity across an image was normalized to the number of nuclei in the image; would this make one technical replicate in the violin plot a field of view? (D) Since actin and nuclei are included in these images, it is possible that cellprofiler could be used with relative ease to obtain single-cell measurements, and additionally quantify the interesting fibronectin phenotype seen with NFkB inhibition in 3B where inhibition seems to inhibit assembly/deposition around cell adhesions but not the perinuclear fibronectin.

3. It is not clear whether AP-1 pathway inhibition is inhibiting fibronectin transcription/translation/secretion or some other aspect of its fibrillogenesis. For example with AP-1 inhibition in 4G there are still bright punctae of fibronectin but less fiber deposition. (A) Does AP-1 inhibition actually reduce Fn-EDA transcription? (B) Is the effect indirect and related to secretion/assembly?

Minor:

- Figure 1A – consider changing the axis labels to logFC(PN1 vs PN28) or similar to match the PN1/28/60 notation in 1B/C.

- Figure 2C – If you do PCA on genes that are statistically differentially expressed between particular conditions, isn't it a given that the PCs will separate the differences? These plots seem tautological and could probably be removed outright (or replaced with a PCA on global gene expression).

- Figure 1B/C/D are called out of order in the main text.

- Figure 2A – increase the brightness of the actin channel.

- Figure 2B/2C – On the flipside, housekeeping bands in some of the other blots seem overexposed, such as in 2B/2C – this is worrisome as if they were overexposed in the raw data quantified it would preclude quantitation.

- Figure 2C – the “Actin” channel appears to show an overlay, with some green pixels overlapping from the p65 bands, which is odd because the p65 bands do not seem to have overlapping red pixels. The authors should either show an overlay as one image with all bands on it or only show one color per image, in which case it may make sense to invert and display the bands in greyscale for easier quantification of band density by eye for the reader.

- Line 191 – Some additional discussion of the patient lines would be useful here for readers unfamiliar with the 9p21 locus to help understand what constitutes a risk line. The citation in the methods, which I assume is for the Cell paper by Lo Sardo et al, is cited numerically whereas the citation style for the rest of the paper is Author-Date.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Whitehead et al. examines the role of an AP-1 dependent signalling pathway in the fibrotic responses of cardiac fibroblasts derived from pluripotent stem cells. The studies combine bioinformatic of new and existing data sets along with in vitro experiments to propose a model in which inflammatory signalling through AP-1 and NFKb promotes ECM deposition and monocyte adhesion. The authors also provide evidence that SNPs in a long non-coding RNA, ANRIL, interfere with this response. The studies are generally well constructed and clearly presented. Although AP-1 has previously been implicated in cardiac fibrosis, this study provides some new molecular insights. However, some of the conclusions are over-stated and some additional experiments/analysis are needed to confirm the key findings. Specific comments are listed below.

Comments for the author

Comments:

1. Are there differences in AP-1 genes themselves in either of the Wang 2019 or 2020 data sets? This could provide some further insight into whether the activation of AP-1 is transcriptionally regulated.

2. In Figure 2B, the western blots should include total p65 and cJun. Similar to the previous Comment this information would provide insight into whether activation of the pathway involves an overall upregulation of expression or a phopho-signalling event.

3. How do AP-1 factors signal to the NFKb so rapidly (p65 is only activated 30 min later than cJun) if their primary function is as transcription factors rather than kinases? It would be good to provide a clear potential mechanism with citations in the discussion.

4. The concept that the deposited Fn is not just increased in amount but also adhesivity is intriguing, but there could be many reasons for this. It would be good to confirm the proposed hypothesis of functional changes in the cell adhesion site by staining the matrices with RGD-specific monoclonal Abs.

5. What was the sample size in Fig 3B,C? How many cells over how many experiments? Please specify this in the caption.

6. The readability of Figure 4 could be significantly improved. A schematic of the experimental and analytical strategy is likely to help here. Perhaps also consider splitting this into two figures with more comprehensive labelling.

7. Much of the proposed signalling mechanisms are based on motif analysis and inhibition with AP-1 inhibitors, but there is no evidence that there are actual changes in AP-1 binding or

transcriptional activity. Further support for this mechanism could be provided by ChIP-PCR experiments or transcriptional reporter assays.

8. The authors should be careful about including GATA-5 in the signalling axis that regulates the fibrotic response. While the data indicate that AP-1 and NFK-b regulate ECM deposition and AP-1 regulates GATA-5, the functional role of GATA-5 has not been directly tested here. It is recommended that the title and abstract are amended accordingly, unless further experimental evidence is provided.

Reviewer 3

Advance summary and potential significance to field

In this manuscript, Whitehead et al, make use if iPS-derived fibroblasts to understand how cardiac fibroblasts respond to stress responses present in the injured myocardium. They show that the inflammatory environment result in the deposition of a fibronectin rich matrix via the AP-1-NFkB-GATA5 axis, providing a substrate for enhanced immune cell adhesion. The data presented in this manuscript is based on previous where they demonstrated that lncRNA SNPs at the 9p21 locus act through JNK, a kinase upstream of AP-1, interfere with cardiomyocyte function. In this paper the authors move to another important cell type in the heart, the cardiac fibroblast. Using ES en iPS derived cardiac fibroblasts, the cellular responses steered by inflammation and the impact of SNPs at the 9p21 locus is studied. Whitehead identified AP-1 and NFkB as critical drivers of fibroblast induced matrix production, making use of existing databases comparing regenerative and non-regenerative mouse hearts. This is not unexpected based on prior art, however they are the first to link this effect to GATA-5 and the lncRNA SNPs at the 9p21 locus, providing an interesting new path for future interventions.

Comments for the author

1. The first link is the age dependent inflammatory differences.
Differences in inflammation may be linked to differences in immune cell type or differences in the fibroblasts to respond to the same inflammatory stimuli. Can the authors, based on their results, discuss what is the chicken or the egg?
2. In the manuscript there is quite some emphasis on the LncRNA ANRIL, as well as in the text as in the summary figure. The authors show that ANRIL modulates stress responses via AP-1 and GATA5. However, they also show that it is mainly the SNP and not the LncRNA driving the GATA5 promoter. Can the authors explain this difference in observation. It is not clear how the authors envision ANRIL to be involved.
3. The data presented in figure 3 and figure 4 are cells treated with TGFβ, LMW HA, 646 and ANGII. As many of the cellular responses by these factors are depending on the microenvironment, and there signaling pathways can influence each other, it is not clear what the trigger is. Are indeed all factors needed to observe the reported response?
4. Since LMW HA and ANGII influence TGFb and v.v. the impact of co-stimulation on the level of downstream signal activation needs to be shown.
5. While the abstract focusses on the response of CF on inflammatory signals, there is no reference towards e.g. ANRIL, nor to the inflammatory cytokines present in the infarcted myocardium. It would be appreciate if the abstract better reflects the content of the manuscript.
6. Work by Hinz et al has shown that TGFb induced modulation of the matrix via Smad3 phosphorylation influence the adhesion of immune cells. How does the data presented in this manuscript relate to this study? What is the receptor used by the immune cells to adhere to the matrix?
7. There is no verification in the manuscript that indeed e.g. TGFb results in activating the Smad or non-Smad pathway. These controls need to be included.
8. The immune cells used are not monocytes as they have been differentiated towards a macrophage phenotype. This has to be made more clear in the paper. Are the M1 or M2 as this will have impact on the response observed. Is the same phenotype observed when not using this lymphoma cell line but primary macrophages?

9. Figure 2 lacks controls as only the phosphorylated forms are shown and it is important for the understanding of the mechanism what the ratio is to the total protein.
10. The cartoon showing the data is confusing as not all that is represented in the figure is based on data presented in the paper.

First revision

Author response to reviewers' comments

We would like to thank the Editor and Reviewers of JOCES/2023/261152 for their helpful comments to this revision. Below and in the manuscript, a second round of reviewer concerns are in **BOLD**, edits to the manuscript are shown in RED and our comments are shown in BLUE.

Reviewer #1: The evidence for activation of NFkB by AP-1 is not sufficiently strong to support a sequential pathway, such as the one drawn in Figure 5. The authors show that p65 (NFkB) phosphorylation occurs after cJun (AP-1) phosphorylation, but both are already well documented in the literature to activate concurrently, and potentially modulate one another (Fan et. al AJP Heart Circ, 2002; Fujioka et. al, Molecular and Cellular Biology, 2004). The representative blot showing attenuated p65 phosphorylation in response to AP-1i is not particularly convincing compared to the quantitation. The compound used is well documented to reduce Il1-beta secretion, which could explain the attenuation of NFkB activation. (A) Is inhibiting AP-1s binding to DNA directly altering NFkB phosphorylation or is this an autocrine effect? (B) Without data directly showing the interaction of the two transcription factors, the authors need to moderate the types of conclusions implied by the pathway diagram in 5.

> We thank the reviewer for their overall insight and comments, and we agree that the overall connection between NFkB and AP-1 was previously unclear and convoluted the proposed signaling mechanism. As a result, we have performed additional experiments utilizing mass spectrometry on p-NFκB immunoprecipitation lysates to uncover molecules bound to the transcription factor. When all identified proteins were paired with known NFκB and AP-1 signaling molecules within an association network through the STRING database, we see interconnectivity between our samples and the two pathways. The complex nature of these associations makes a single interaction difficult to isolate and identify. However, we have reorganized the manuscript to focus primarily on the role of AP-1 in modulating cardiac fibroblast activation. In doing so, the effects of NFkB inhibition have now been moved to the supplementary data and text modified to soften previous claims regarding sequential transcription factor activation. The pathway diagram has also been modified to focus primarily on the effects of AP-1. We hope that these modifications provide a clear and concise mechanism into cardiac fibroblast mediated fibrotic responses.

The image analysis in figures 3B and 4G is lacking methodological detail required to assess its rigor. (A) How many fields of view/cells were analyzed? (B) For figure 4G were all patient lines imaged and then was the data aggregated by technical replicate? (C) The y-axes on quantification are labeled as intensity per cell, yet the methods suggest that the fluorescent intensity across an image was normalized to the number of nuclei in the image; would this make one technical replicate in the violin plot a field of view? (D) Since actin and nuclei are included in these images, it is possible that cellprofiler could be used with relative ease to obtain single-cell measurements, and additionally quantify the interesting fibronectin phenotype seen with NFkB inhibition in 3B where inhibition seems to inhibit assembly/deposition around cell adhesions but not the perinuclear fibronectin

> We have now updated our methods section to include more detail regarding the quantification of the immunofluorescence images. The following is copied from the newly written “Immunofluorescence imaging and analysis” section found starting at **line X:**

"All image analysis was performed using FIJI[26]. For transcription factor analysis, image intensity for each color was calculated and normalized to the number of nuclei in each image, a total of three randomized fields of view were analyzed across three-four biological replicates. For monocyte adhesion, the number of nuclei was normalized to the area imaged, five randomized fields of view were analyzed across three biological replicates. All other image analyses involved calculating the mean fluorescence intensity per cell. Briefly, a minimum of 150 cells were outlined and analyzed across five randomized fields of view and three biological replicates."

Additionally, the caption of Figure 6 (Figure 4G prior to revision) has been amended to specify that imaging was performed on 1-5 (RR) and 1-9 (RKO) cells. The new text can be found starting **line X**:

"Representative immunofluorescent images of 1-5 RR and 1-9 RKO iCFs treated with TGFβ, LMW HA, and ANGII for 72 hours (left) and quantification of mean fibronectin signal per cell (right), * $p < 0.05$ as determined by nonparametric Kruskal-Wallis test."

Finally, the manuscript has been reorganized to place more emphasis on AP-1 and its role in modulating cardiac fibroblast fibrotic responses. As a result, much of the NFkB inhibition data has been moved to the supplementary documents.

It is not clear whether AP-1 pathway inhibition is inhibiting fibronectin transcription/translation/secretion or some other aspect of its fibrillogenesis. For example, with AP-1 inhibition in 4G there are still bright punctae of fibronectin but less fiber deposition. (A) Does AP-1 inhibition actually reduce FN-EDA transcription? (B) Is the effect indirect and related to secretion/assembly?

We thank the reviewer for this comment. We have now included a new Figure 3 which evaluates the expression and assembly of fibronectin in response to AP-1 inhibition. We find that AP-1 inhibition significantly reduces fibronectin expression, as measured using both Western blots and immunofluorescence (Figure 3A-B). In addition, our monocyte adhesion assay shows that the matrix produced is functional and AP-1 inhibition results in suppressed monocyte adhesion, thus suggesting a role for AP-1 in direct regulation of fibronectin expression. The above discussed data can be found starting at **line x**.

Figure 1A - consider changing the axis labels to logFC(PN1 vs PN28) or similar to match the PN1/28/60 notation in 1B/C.

Figure 1C - If you do PCA on genes that are statistically differentially expressed between particular conditions, isn't it a given that the PCs will separate the differences? These plots seem tautological and could probably be removed outright (or replaced with a PCA on global gene expression).

Figure 1B/C/D are called out of order in the main text.

The axis labels in Figure 1A-C have been modified according to the reviewer recommendation.

As a result of the reorganization of the manuscript, Figure 1A-D have been moved to the supplementary data and the text modified accordingly.

Figure 2A - increase the brightness of the actin channel.

Figure 2B/2C - On the flipside, housekeeping bands in some of the other blots seem overexposed, such as in 2B/2C - this is worrisome as if they were overexposed in the raw data quantified it would preclude quantitation.

Figure 2C - the "Actin" channel appears to show an overlay, with some green pixels overlapping from the p65 bands, which is odd because the p65 bands do not seem to have overlapping red pixels. The authors should either show an overlay as one image with all bands on it or only show one color per image, in which case it may make sense to invert and display the bands in greyscale for easier quantification of band density by eye for the reader.

All comments related to image adjustments have been made.

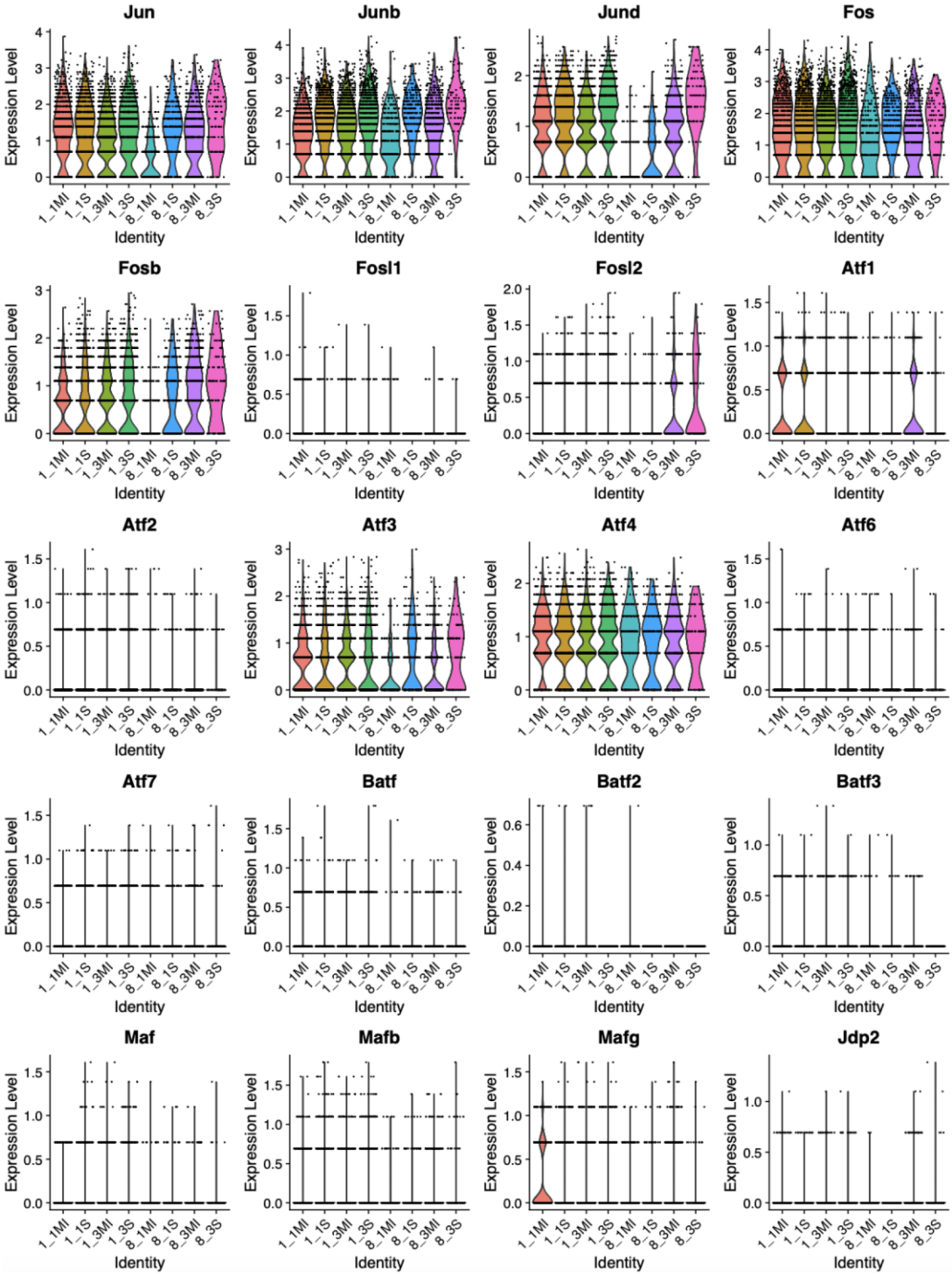
Line 191 - Some additional discussion of the patient lines would be useful here for readers unfamiliar with the 9p21 locus to help understand what constitutes a risk line. The citation in the methods, which I assume is for the Cell paper by Lo Sardo et al, is cited numerically whereas the citation style for the rest of the paper is Author-Date.

We apologize for the differences in citation styles, this has now been corrected. Moreover, additional discussion on what constitutes a risk line has been added within the Results section starting at line **X**. This discussion has been copied below for convenience:

"Our group has previously demonstrated that presence of these SNPs alters cardiomyocyte responses to stiffness, resulting in differences in gap junction assembly and suggesting that the ANRIL non-coding RNA may be stress induced (Kumar et al., 2019). To probe this hypothesis, we generated 6 lines of iCFs in triplicate, spanning two patients. For each homozygous risk (RR) line, which contains SNPs within ANRIL, a TALEN knockout (RKO) of the SNP-containing region was performed, generating three isogenic clonal comparisons (**Fig. 4A**)."

Reviewer #2: Are there differences in AP-1 genes themselves in either of the Wang 2019 or 2020 data sets? This could provide some further insight into whether the activation of AP-1 is transcriptionally regulated.

We thank the reviewer for their recommendation. The Wang data alone does not suggest any specific member of the AP-1 family as being unique to the infarcted sample (see below). After performing RNA-seq on our own samples and identifying JunD as the candidate AP-1 protein regulating cardiac fibroblast stress response, however, we also confirm a differential regulation of JunD in the Wang data. A dysregulation of JunD upon infarction of nonregenerative cardiac fibroblasts supports our claim of this transcription factor's importance.



In Figure 2B, the western blots should include total p65 and cJun. Similar to the previous Comment, this information would provide insight into whether activation of the pathway involves an overall upregulation of expression or a phopho-signaling event.

We thank the reviewer for their insight and have now included total and phospho-cJun blots within the main figures and total as well as phospho-p65 blots within the supplementary data. No differences were observed in total transcription factor expression; therefore, agonist stimulation enhances phosphorylation of AP-1 and NFkB. The following sentences were included to reflect these observations:

"Next, we measured phosphorylation of the AP-1 pathway (via cJun) we previously predicted to drive matrix formation. We found that all three agonists drove a peak

in phosphorylation of cJun after 30 minutes of stimulation, while total transcription factor expression remained constant (**Fig. 2B**)."

How do AP-1 factors signal to the NFKb so rapidly (p65 is only activated 30 min later than cJun) if their primary function is as transcription factors rather than kinases? It would be good to provide a clear potential mechanism with citations in the discussion.

We thank the reviewer for their comment, and similar to Reviewer #1's comment, we agree that the overall connection between NFkB and AP-1 was unclear. As a result, we have reorganized the manuscript to focus primarily on the role of AP-1 in modulating cardiac fibroblast activation. In doing so, the effects of NFkB inhibition have now been moved to the supplementary data and text modified to soften previous claims regarding sequential transcription factor activation. The pathway diagram has also been modified to focus primarily on the effects of AP-1.

The concept that the deposited Fn is not just increased in amount but also adhesivity is intriguing, but there could be many reasons for this. It would be good to confirm the proposed hypothesis of functional changes in the cell adhesion site by staining the matrices with RGD-specific monoclonal Abs.

We appreciate the reviewer's comment and would like to respectfully point out that the monocyte adhesion assay is a direct measure of ECM adhesivity. Monocytes are usually grown in suspension and do not adhere to glass or tissue culture treated plastic. However, monocytes do express a variety of RGD binding integrins (Chung and Kao, 2009; Gao et al., 2021) that further explain their adhesion to decellularized matrices. To further highlight these attributes, we have now included additional text to highlight the ability of monocytes to adhere to ECM, this can be found at **line X** and is copied below:

"Activation of this pathway in response to stress stimuli resulted in enhanced matrix production, which was abrogated with pharmacological AP-1 inhibition. Matrix production was not only enhanced intracellularly but we also observed an increase in extracellular assembled and functional matrix (**Fig. 3B**). This ECM allowed for the adhesion of U937 monocytes, which are non-adherent cells that would otherwise be grown in suspension, through integrin mediated interactions (**Fig. 3C**, **Fig. 2S**) (Chung and Kao, 2009; Gao et al., 2021; Harris and Ralph, 1985)."

What was the sample size in Fig 3B,C? How many cells over how many experiments? Please specify this in the caption.

We have updated our methods section to include more detail regarding the quantification of the immunofluorescence images. The following is copied from the newly written "Immunofluorescence imaging and analysis" section found starting at **line X**:

"All image analysis was performed using FIJI[26]. For transcription factor analysis, image intensity for each color was calculated and normalized to the number of nuclei in each image, a total of three randomized fields of view were analyzed across three-four biological replicates. For monocyte adhesion, the number of nuclei was normalized to the area imaged, five randomized fields of view were analyzed across three biological replicates. All other image analyses involved calculating the mean fluorescence intensity per cell. Briefly, a minimum of 150 cells were outlined and analyzed across five randomized fields of view and three biological replicates."

The readability of Figure 4 could be significantly improved. A schematic of the experimental and analytical strategy is likely to help here. Perhaps also consider splitting this into two figures with more comprehensive labelling.

We thank the reviewer for their suggestion. We have now split up Figure 4 into two separate figures (Figures 4 and 6). Additionally, we have included a schematic detailing the experimental cell lines used. We hope these changes enhance the overall strengths of the study.

Much of the proposed signaling mechanisms are based on motif analysis and inhibition with AP-1 inhibitors, but there is no evidence that there are actual changes in AP-1 binding or transcriptional activity. Further support for this mechanism could be provided by ChIP-PCR experiments or transcriptional reporter assays.

We apologize for any confusion caused, the inhibitor used prevents AP-1 family proteins from binding to the DNA (Tsuchida et al, 2004). Therefore, combined our data show that AP-1 motif enrichment is enhanced and that inhibition of AP-1 binding prevents cardiac fibroblast activation in response to agonist treatment. Additionally, we also performed RNA-seq on RR and RKO cell lines and found that JUND is highly expressed and could be involved in cardiac fibroblast activation. Interestingly, JUND is known to play a role in GJA1 expression (Mitchell and Lye, 2005) and has been shown to bind within the gene body of connexin-43 (*GJA1*). For more clarity, the role of T-5224 and its associated publications have been cited and discussed within the main text, this can be found at line **X** or copied below for convenience:

"To elucidate the role of AP-1 in modulating fibroblast matrix responses, we used T-5224 (AP-1i) to pharmacologically inhibit the binding of AP-1 factors to DNA (Tsuchida et al., 2004)."

Additionally, details of the RNA-seq experiments can be found at line X:

"Through analysis of known AP-1 factors, JUND was found to be the most highly expressed and could therefore be responsible for downstream AP-1 activation (**Fig. 5B-C**). Interestingly, JUND is also known to be involved in Connexin 43 (*GJA1)* gene expression (Mitchell and Lye, 2005) and has been demonstrated to be able to bind within the gene body of *GJA1* as demonstrated by H1 ESC ENCODE CHIP data (**Fig. 5D**)."

The authors should be careful about including GATA-5 in the signaling axis that regulates the fibrotic response. While the data indicate that AP-1 and NFK-b regulate ECM deposition and AP-1 regulates GATA-5, the functional role of GATA-5 has not been directly tested here. It is recommended that the title and abstract are amended accordingly, unless further experimental evidence is provided.

We have now amended the title and softened our claims regarding the involvement of GATA5 in the signaling axis described in our study.

Reviewer #3: The first link is the age dependent inflammatory differences. Differences in inflammation may be linked to differences in immune cell type or differences in the fibroblasts to respond to the same inflammatory stimuli. Can the authors, based on their results, discuss what is the chicken or the egg?

We thank the reviewer for their overall insight and enthusiasm for our work. Figure 1A-D has now been moved to the supplementary data section and the discussion of these results significantly shortened. These changes were made as part of a reorganization of the manuscript by which the main mechanistic focus is now on AP-1 signaling in cardiac fibroblast activation.

In the manuscript there is quite some emphasis on the LncRNA ANRIL, as well as in the text as in the summary figure. The authors show that ANRIL modulates stress responses via AP-1 and GATA5. However, they also show that it is mainly the SNP and not the LncRNA driving the

GATA5 promoter. Can the authors explain this difference in observation. It is not clear how the authors envision ANRIL to be involved.

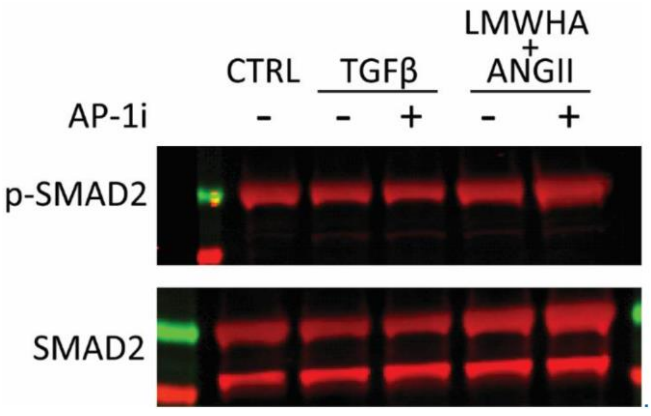
> The LncRNA ANRIL was used as an example of how changes in transcriptional regulation can impact the system. Polymorphism differences between patients can certainly drive differences in GATA5 and many other transcriptional changes. However here, to emphasize just how the lncRNA itself modulates the promotor, we focused on an isogenic comparison of cell lines with and without the entire region, i.e. WT and KO; we did not edit individual polymorphisms for reasons articulated in prior work (e.g., Lo Sardo, *Cell* 2018; Mayner, *PNAS* 2023). There are differences in splicing as a function of the polymorphisms as we've shown elsewhere (Mayner, *PNAS* 2023), but that is most relevant when comparing between genotypes rather than within an isogenic comparison as was done here.
>
> We apologize for this confusion. To mitigate it, we have updated the schematic at the end of the paper to identify where more clearly in the pathway ANRIL impacts signaling (i.e. AP-1) and its downstream modulation of Cx43.

The data presented in figure 3 and figure 4 are cells treated with TGFβ, LMW HA, and ANGII. As many of the cellular responses by these factors are dependent on the microenvironment, and the signaling pathways can influence each other, it is not clear what the trigger is. Are indeed all factors needed to observe the reported response?

> We would like to respectfully point out that the data presented in Figure 3A shows the ability of each agonist individually as well as all agonists together to elicit a fibrotic response. The degree of response is similar across all stimulation conditions suggesting that there are no additive effects in fibronectin expression with agonist stimulation. The all agonist stimulation was included to provide a robust response with minimal experimental conditions.

Since LMW HA and ANGII influence TGFb and v.v. the impact of co-stimulation on the level of downstream signal activation needs to be shown.

> We thank the review for this comment. We have reorganized our manuscript to focus primarily on AP-1 signaling with NFkB signaling now included as part of the supplementary data. In addition, we have also included Western blot data analyzing the effects of the agonist stimulation on SMAD signaling below, as per the reviewers following comments. The data shown below shows that there is significant differences in SMAD2 phosphorylation following 30 mins of agonist stimulation. While SMAD signaling has been shown to play a role in fibrosis, our data shows that AP-1 signaling is early and can also have an effect of cardiac fibroblast fibrotic responses. While interesting, we believe that the addition of another transcription factor would only convolute the signaling mechanisms proposed, a reason why NFκB data was also moved to the supplementary document.



Representative Western blot of p-SMAD2 and total SMAD2 following no stimulation (CTRL), 30 mins of stimulation with TGFB alone, or 30 mins of stimulation with LMWHA and ANGII.

While the abstract focusses on the response of CF on inflammatory signals, there is no reference towards e.g. ANRIL, nor to the inflammatory cytokines present in the infarcted myocardium. It would be appreciated if the abstract better reflects the content of the manuscript.

The abstract has been rewritten to provide a more wholistic overview of the data presented within our study. For convenience, the new abstract is copied below:

"Matrix remodeling outcomes largely dictate patient survival post-myocardial infarction. Moreover, human-restricted noncoding regulatory elements have been shown to worsen fibrosis, but their mechanism of action remains elusive. Here, we demonstrate using induced pluripotent stem cell derived cardiac fibroblasts (iCFs) that inflammatory ligands abundant in the remodeling heart after infarction activate AP-1 signaling pathways resulting in fibrotic responses. This observed signaling induces deposition of fibronectin matrix and is further capable of supporting immune cell adhesion; pathway inhibition blocks iCF matrix production and cell binding. Polymorphisms in the noncoding regulatory elements within the 9p21 locus (also referred to as ANRIL) redirect stress programs, and in iCFs, they transcriptionally silence the AP-1 inducible transcription factor GATA5. The presence of these polymorphisms modulated iCF matrix production and assembly and reduced cell-cell signaling. These data suggest that this signaling axis is a critical modulator of cardiac disease models and may be influenced by noncoding regulatory elements."

Work by Hinz et al has shown that TGFb induced modulation of the matrix via Smad3 phosphorylation influence the adhesion of immune cells. How does the data presented in this manuscript relate to this study? What is the receptor used by the immune cells to adhere to the matrix?

Inflammatory agonists are known to activate a host of molecular pathways, including AP-1, NFkB, and SMAD (Whitehead and Engler et al, 2021, Hinz, 2007; Seto et al, 2016). Our study highlights the role of one of these pathways in regulating cardiac fibroblast fibrotic responses. However, further discussion has been added to highlight the importance of other signaling pathways. This can be found starting at line **X**:

"While, other pathways have also been reported to regulate fibroblast function and fibrotic responses and monocyte adhesion, which include SMAD signaling pathways (Hinz, 2007; Szeto et al., 2016; Verrecchia and Mauviel, 2002), our data show that stress responses through AP-1 related pathways also critical and can result in the production of functional matrix as well as the adhesion of immune cells."

There is no verification in the manuscript that indeed e.g. TGFb results in activating the Smad or non-Smad pathway. These controls need to be included.

In response to this reviewer comment, we have included Western blots of SMAD2 phosphorylation as part of the rebuttal letter. We do not see significant differences in SMAD2 activation within the conditions tested. We would like to respectfully highlight that the manuscript was reorganized to make a concise AP-1 centered mechanistic study. As a result, we believe that further characterization of this pathway is outside the scope of the current work.

The immune cells used are not monocytes as they have been differentiated towards a macrophage phenotype. This has to be made more clear in the paper. Are the M1 or M2 as this will have impact on the response observed. Is the same phenotype observed when not using this lymphoma cell line but primary macrophages?

We would like to sincerely apologize for any confusion, but the cells used in our studies are indeed U937 monocytes and not macrophages. We believe the prior Supplementary Figure S2 may have been misleading and, as a result, we have removed it from the current manuscript. Additionally, details of the adhesion have been provided within the methods section starting at line **X**:

"U937 human monocytes were grown in RPMI1640 + 10% FBS and seeded onto decellularized matrix at $1x10^6$ cells per coverslip and allowed to attach for 2 hours. Coverslips were then fixed and stained as described above."

Figure 2 lacks controls as only the phosphorylated forms are shown and it is important for the understanding of the mechanism what the ratio is to the total protein.

Total and phosphorylated forms of transcription factors have now been provided.

The cartoon showing the data is confusing as not all that is represented in the figure is based on data presented in the paper.

Given the reorganization of the manuscript, the schematic figure has been adjusted accordingly. We hope the figure now better portrays our data and proposed mechanisms.

Second decision letter

MS ID#: JOCES/2023/261152

MS TITLE: AP-1 Signaling Modulates Cardiac Fibroblast Stress Responses

AUTHORS: Alexander J Whitehead, Hamza Atcha, James D Hocker, Bing Ren, and Adam J Engler
ARTICLE TYPE: Research Article

We have sent your revised manuscript to the original reviewers #2 and #3.

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

As you will see, both reviewers found that the manuscript has improved substantially and their comments have been adequately addressed. However, reviewer #3 still has a few points concerning the new data that will need your attention before the paper can be finally accepted.

I would encourage you to consider the comments of this critical reviewer and submit a final revised manuscript, along with a response in which you indicate how you have dealt with the points raised by the reviewer in the 'Response to Reviewers' box online. Please ensure that you clearly highlight all changes made in the revised manuscript.Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

Reviewer 2

Advance summary and potential significance to field

The study provides new mechanistic links between inflammatory stimuli and fibrotic responses in cardiac fibroblasts. The paper implicates AP-1 transcriptional regulation and potential interactions with NFkappaB signalling.

Comments for the author

The authors have addressed all the comments raised in the first review. The manuscript is recommended for publication.

Reviewer 3

Advance summary and potential significance to field

The authors identified a novel AP-1 signaling axis involved in the stress response of cardiac fibroblasts providing a new angle to tackle cardiac fibrosis.

Comments for the author

In this revised version of the manuscript, the authors provide additional data and clarified some concerns raised before in the text. It actually is quite a different paper, now mainly focusing on the ability of the AP-1 inhibitor to interfere with ligand induced fibroblast activation. This does warrent

The abstract now reflects the context of the manuscript.

I do have some questions based on the new data presented.
1. As there are many different forms of TGFbeta and BMP, if known, please add the number of the ligand studied.
2. Is the AP-1 mediating matrix response also depending on JUND?
3. figure 5B is a bit strange in the eyes of the reviewer. Does this mean the expression of the other factors are so low, or just because of the very high expression of JUND, they are blue.
4. Figure 6b is not very convincing. It does not seem to be in agreement with the quantification.
5. Figure 6c, please include what we can see in these images. Adding all the factors at the same time, do they all equally well activate AP-1? All seems to be less efficient as one a the time (similar as in figure 3A)

Second revision

Author response to reviewers' comments

We would like to thank the Editor and Reviewers of JOCES/2023/261152 for their helpful comments to this revision. Below and in the manuscript, a second round of reviewer concerns are in **BOLD**, edits to the manuscript are shown in RED and our comments are shown in BLUE.

Reviewer #2: The authors have addressed all the comments raised in the first review. The manuscript is recommended for publication.

> We thank the reviewer for their valuable insight and comments as well as their approval of the manuscript.

Reviewer #3: As there are many different forms of TGFbeta and BMP, if known, please add the number of the ligand studied.

> We thank the reviewer for their comments. We have now clarified within the materials and methods section that TGFβ1 was used in this study. However, no BMP was used in this study. Changes to the text can be found on **line 323**, and copied below for convenience:
>
> "Angiotensin II (10 nM, 1158, Tocris 1158), low molecular weight hyaluronic acid (8-15 kDa, 30ng/mL, 40583-10MG, Millipore Sigma), and TGF-β1 (10 ng/mL, 240-B-002, RnD Systems) were used as agonists, dosed daily for 3 days and harvested 72 hours after the first dose."

Is the AP-1 mediating matrix response also depending on JUND?

The AP-1 family of transcription factors is broad and consists of many different proteins. JUND is a protein within the AP-1 family of transcription factors and as a result would be involved in the mechanism proposed. This additional point has been added to the discussion section. Changes to the text can be found on **line 242**, and copied below:

"AP-1 consist of a family of transcription factors that are composed of homodimers and heterodimers of Jun (v-Jun, c-Jun, JunB, and JunD), Fos (v- Fos, c-Fos, FosB, Fral, and Fra2), ATF (ATF2, ATF3/LRF1, B-ATF, JDP1, and JDP2), and MAF (c-Maf, MafB, MafA, MafG/F/K, and Nrl)(Angel and Karin, 1991; Hai et al., 1988; Ye et al., 2014)."

Figure 5B is a bit strange in the eyes of the reviewer. Does this mean the expression of the other factors are so low, or just because of the very high expression of JUND, they are blue.

The TPM expression of JUND is higher compared to other AP-1 factors and, as a result, the other factors are blue.

Figure 6B is not very convincing. It does not seem to be in agreement with the quantification.

We thank the reviewer for this comment and would like to apologize for the noisy Western blot. However, given the limited GATA5 reagents, the blots provided are the best we are able to generate. We have provided a clearer image of the same blot and have below provided a figure with each individual data plot linked to other conditions found within the same blot. As seen from these images the trends observed are prevalent, although the absolute differences are variable.
We hope that these changes ease this concern.

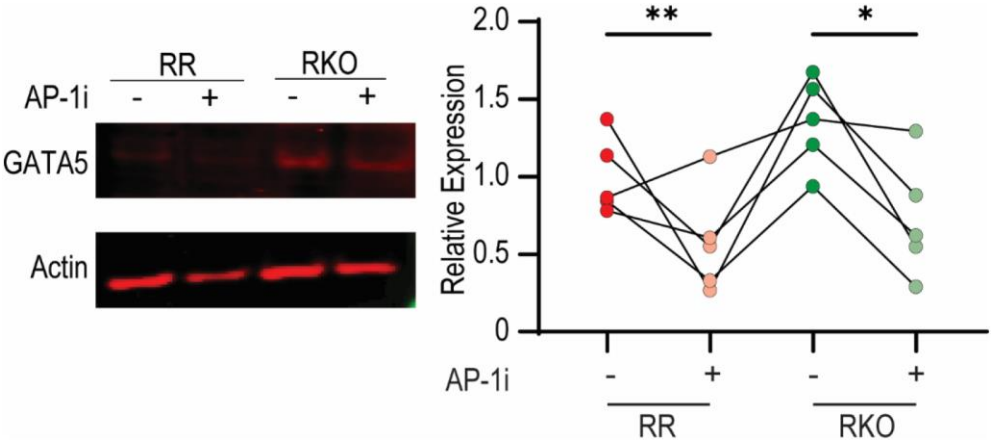


Figure 1R: GATA5 Western blot. Individual points within the same blot are connected to show trends across experiments.

Figure 6C, please include what we can see in these images.

We thank the reviewer for their comments. We have now added a legend and scale bar to the figure, we apologize for missing this earlier.

Adding all the factors at the same time, do they all activate AP-1 equally? All seems to be less efficient as one at a the time (similar as in figure 3A)

There are no statistically significant differences reported between the all-agonist condition and each agonist individually in Fig 3A. Therefore, while absolute values may seem lower, the variability in the data prevents any further conclusions from being drawn.

Third decision letter

MS ID#: JOCES/2023/261152

MS TITLE: AP-1 Signaling Modulates Cardiac Fibroblast Stress Responses

AUTHORS: Alexander J Whitehead, Hamza Atcha, James D Hocker, Bing Ren, and Adam J Engler
ARTICLE TYPE: Research Article

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