nature portfolio

Peer Review File

Adipocyte associated glucocorticoid signaling regulates normal fibroblast function which is lost in inflammatory arthritis

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors investigated the mechanism by which homeostatic function of synovial fibroblasts, such as lubrication, is regulated under healthy conditions. They identified two synovial fibroblast populations enriched in healthy synovium by single cell RNA-seq. These synovial fibroblast populations were characterized by programs of enhanced fatty acid metabolism and lipid transport. In vitro experiment showed that cortisol and fat-conditioned media induced the expression of APOD, NNMT and CEBPD, which are highly expressed in healthy synovial fibroblasts. Glucocorticoid receptor gene (NR3C1) deleted synovial fibroblasts failed to upregulate these genes in vitro. Depletion of adipocytes in mice resulted in reduction of Hsd11b1, which is necessary to generate active cortisol, as well as cortisol responsive genes in the synovial tissue. Using 3D synovial fibroblasts organoids, they found that cortisol signaling in fibroblasts is important for adipogenesis and stimulation with TNFα- and TGFβ repressed cortisol signaling and adipogenesis. However, most of the experiments are done in vitro. In addition, they did not show the phenotype of synovial fibroblasts in adipocyte-depleted mice or synovial fibroblast-specific NR3C1 KO mice. Moreover, they did not examine whether these mice are resistant to inflammatory diseases, such as rheumatoid arthritis. Thus, they did not show the physiological role of the adipocyte-cortisol axis in homeostatic function of synovial fibroblasts in vivo. Taken together, although their findings are potentially interesting, I think this MS is not suitable for publication in its current form.

Major points

1. To elucidate physiological role of the adipocyte-cortisol axis in homeostatic function of synovial fibroblasts in vivo, they need to show whether and how composition and phenotypes of synovial fibroblast populations are changed in joint of adipocyte-depleted mice by sc-RNAseq etc.

2. Related to 1, the authors need to show whether and how composition and phenotypes of synovial fibroblast populations are changed in the joint of synovial fibroblast-specific NR3C1 KO mice by sc-RNAseq etc.

3. The authors need to show whether adipocyte-depleted mice are resistant to inflammatory diseases using arthritis models.

4. Related to 3, The authors need to show whether synovial fibroblast specific NR3C1 KO mice are resistant to inflammatory diseases using arthritis models.

5. They need to show whether adipocytes are the main cellular source of Hsd11b1 in joints at a mRNA level

6. They need to provide molecular mechanisms on whether and how active cortisol is upregulated in healthy joints.

Reviewer #2 (Remarks to the Author):

The manuscript by Faust et al performed single cell RNA sequencing analysis in synovial samples of osteoarthritis, Rheumatoid arthritis, and healthy controls, and found characteristic gene expressions featuring lipid metabolism. They further recapitulated the key gene signature with fat-cultured media in cultured fibroblasts. Using fractionation and mass spectrometry techniques they identified cortisol played a key role in driving this healthy fibroblast pjhenotype. This study is well designed and executed. The insights from this study are likely to shed new light into the role of interactions between fibroblasts adipocytes in maintaining tissue homeostasis and in inflammatory diseases related to joints. There are only some minor comments and suggestions to further improve this manuscript. First of all, a graphic scheme to summarize the key findings of this study will help readers to better grasp the key messages. Secondly, the title of this manuscript does not appear to reflect the major findings of this work. All the data shown in this study are related to inflammatory diseases in the joints, specifically OA, and RA. Some of the conclusions in the abstract appear to be somewhat overstated for the scope of this study.

1. Line 71, the unit for the ORO staining should be micrometer (μm) instead of micro molar (uM).

2. Line 277-278, it is unclear why the authors used ELISA to determine the levels of aldosterone instead of mass spectrometry.

Reviewer #3 (Remarks to the Author):

The authors present a well written and novel manuscript focusing on synovial tissue adipocyte-fibroblast interactions in the heathy synovium and transition to the disease state. They identify cortisol signalling as a key regulator of the homeostatic synovial fibroblast function.

Strengths

The manuscript provides a novel and insightful analysis of the healthy synovial state including of the first time an analysis of the potential role of adipocytes in maintaining a healthy fibroblast cell state in the joint. It is proposed that the disruption of this interaction and signalling pathway is fundamental to the transition to a diseased state.

Weaknesses

While the authors focus on comparisons of a disease state (OA/RA) and make some useful in vitro observations to support their conclusions, functional in vivo data that would underpin the impact loss of cortisol signalling in transition from a healthy to diseased state is missing.

Main points

1. Have the authors considered the induction of inflammatory arthritis in mice following adipocyte depletion – does this lead to chronicity and failure to resolve, does cortisol injection locally restore functionality of the fibroblasts, are adipocyte depleted joints primed to respond to joint inflammation – ie more severe inflammatory arthritis on induction.

2. Are HSD11b KO mice more susceptible to developing severe arthritis in response to an inflammatory challenge? If so is this fibroblast mediated?

3. I was confused by the liptox are the authors quantifying adipoicytes or lipid containing fibroblasts – perhaps they could clarify ? if fibroblasts, but not adipocytes – then how do they quantify loss of adipocytes (currently labelled as fibroblasts)

4. I don't see why the analysis on ACM is included in this manuscript. It identifies a synthetic compound (DEX) as the active compound, something that would have little relevance in understanding the biology of adipose tissue on regulating fibroblast.

5. Figure 6A – Why does fat conditioned media not have the same affect as cortisol? Is cortisol not in fat conditioned media?

6. Have the authors had the opportunity to examine the synovia of patients with active RA on steroids is there a partial defect in cortisol signalling / haemostatic fibroblasts?

7. Have the authors considered where the adipocytes are derived from in the joint and how they are re-populated with resolution of inflammation?

Minor points

Figure 1b- clearer axis labels on flow histograms and need to demonstrate gating strategy (can be in supplementary).

Figure 1C- quantification of this is essential.

Figure 1 Eb – the UMAP is not clear and would benefit from splitting the UMAP per disease state for clarity

Figure 1 – all images need scale bars

Figure 1 – statistical tests and n numbers need to be included in the legend

Figure 2 – statistics and n numbers need to be included in the legend

Line 135- Ref to Fig 2c should be Fig 2sc

Figure 2E- does each dot here represent a sample? Need clarification

Figure 2F, S2D- More details on how DEGs were computed. Which conditions were compared? Where the samples pseudobulked or is this DE at the cell levels? Although he feature plots show a robust difference, some mention of p values would be needed. Volcano plots here would be useful.

Lines 150-153, refs are needed here to back up the statements about CEBPD and APOD.

Figure 3 – statistical tests and n-numbers need to be included in the legend

I don't see any results for NNMT is fig S4b (as mentioned in the text), is this a mistake?

Line 302, I don't get the link to TGFb here, this need to be clearer.

Figure S12F, needs quantification.

Reference or study ID? AMP phase 2

Figure 1C – Colour legends needs to be clearer

Line 109-142 – some genes names are defined more than once. Define the first time described and no need to define again

Line 170 – what fibroblasts? Healthy knee synovial fibroblasts? Not clear

Line 174-175 – generation of abdominal or synovial fat conditioned media. The author needs to refer to the figure here as it is not clear to the reader what this is. It is explained in the figure but not referenced.

177-178 – Abdominal fat-conditioned media upregulated healthy fibroblast genes. Does this mean this phenomenon is not restricted to the joint and can occur where ever fat cells and fibroblast exist together?

Sub title – "Search for active molecules using fractionation and lipidomics" – search for should be "Identification".

Methods:

a better description as to where in the coding region the gRNA targets is required.

Line 684-689Add in version of Seurat, Harmony and R used. The specific functions used to process the data is required, such as ScaleData() etc.

Stats- what type of post-hoc test was applied to the one way anova?

Would a 2 way ANOVa not be more appropriate for the analysis in Figure S11 and Figure 5C?

General comments to reviewers: We thank the reviewers for their comments. We have performed additional experiments which are detailed in the point-by-point responses. This has resulted in a new main figure and additional panels in the supplemental figures. Please note the updated figure numbers in the responses below.

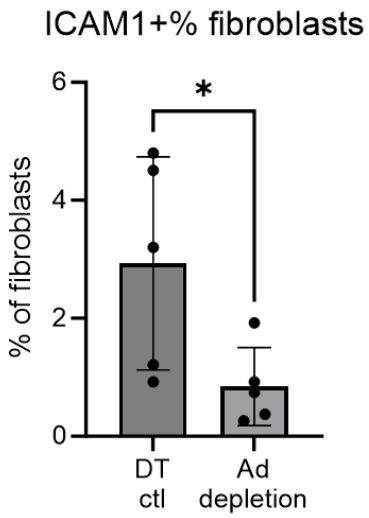
Reviewer #1 (Remarks to the Author):

In this manuscript, the authors investigated the mechanism by which homeostatic function of synovial fibroblasts, such as lubrication, is regulated under healthy conditions. They identified two synovial fibroblast populations enriched in healthy synovium by single cell RNA-seq. These synovial fibroblast populations were characterized by programs of enhanced fatty acid metabolism and lipid transport. In vitro experiment showed that cortisol and fat-conditioned media induced the expression of APOD, NNMT and CEBPD, which are highly expressed in healthy synovial fibroblasts. Glucocorticoid receptor gene (NR3C1) deleted synovial fibroblasts failed to upregulate these genes in vitro. Depletion of adipocytes in mice resulted in reduction of Hsd11b1, which is necessary to generate active cortisol, as well as cortisol responsive genes in the synovial tissue. Using 3D synovial fibroblasts organoids, they found that cortisol signaling in fibroblasts is important for adipogenesis and stimulation with TNFα- and TGFβ repressed cortisol signaling and adipogenesis. However, most of the experiments are done in vitro. In addition, they did not show the phenotype of synovial fibroblasts in adipocyte-depleted mice or synovial fibroblast-specific NR3C1 KO mice. Moreover, they did not examine whether these mice are resistant to inflammatory diseases, such as rheumatoid arthritis. Thus, they did not show the physiological role of the adipocyte-cortisol axis in homeostatic function of synovial fibroblasts in vivo. Taken together, although their findings are potentially interesting, I think this MS is not suitable for publication in its current form.

Major points
1. To elucidate physiological role of the adipocyte-cortisol axis in homeostatic function of synovial fibroblasts in vivo, they need to show whether and how composition and phenotypes of synovial fibroblast populations are changed in joint of adipocyte-depleted mice by sc-RNAseq etc.

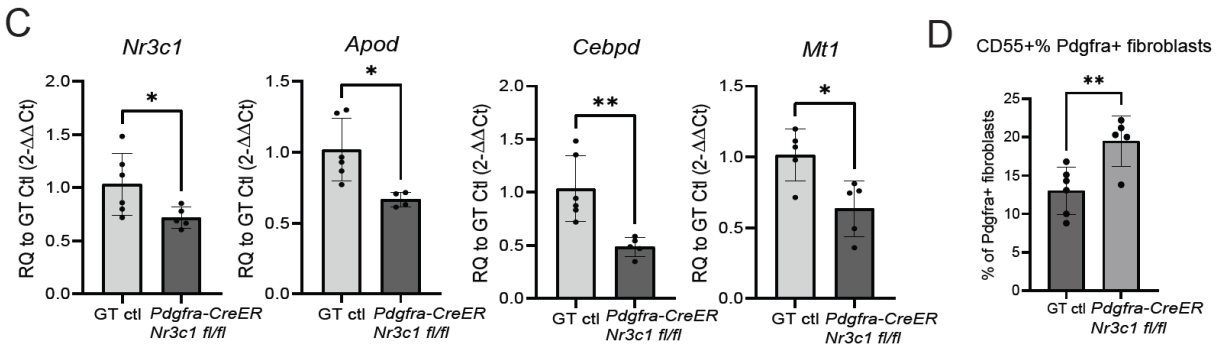
We agree that performing in-depth analyses of synovial fibroblasts from adipocyte depleted vs non-depleted joints would enrich our study. We attempted to sort synovial fibroblasts for single cell RNA sequencing to address your question. However, we were not able to sort enough live cells to merit running the experiment. The low cell number and high ECM content of healthy synovium renders sorting and culture of these cells difficult. We do include qPCR on knee synovium in the manuscript, which shows that cortisol responsive genes are decreased after adipocyte depletion.

In our paper, we show that diseased states such as OA and RA have fewer preadipocyte-like cells, which we hypothesized was due to a lack of cortisol-generating adipocytes. We drew on single cell studies on mouse visceral and subcutaneous adipose tissue, including those from Emont, Schwalie, and Merrick, to nominate a few surface markers to represent specific stromal cell subsets for flow cytometry (Emont, M. et al., A single-cell atlas of human and mouse white adipose tissue, *Nature* (2022). Schwalie, P. C. et al. A stromal cell population that inhibits adipogenesis in mammalian fat depots. *Nature* 559, 103–108 (2018). Merrick, D. et al. Identification of a mesenchymal progenitor cell hierarchy in adipose tissue. *Science* 364, eaav2501 (2019)). These include CD55 for universal progenitor cells, ICAM1 for committed preadipocytes, and F3 for Adipocyte regulatory cells. To further address your question, we performed another adipocyte depletion experiment in the knee joint. We found similar total numbers of fibroblasts among depleted and non-depleted control joints, with similar percentages of F3+ Aregs and DPP4+ progenitors, but lower percentages of ICAM1+ cells in adipocyte depleted joints. This suggests that adipocytes support commitment of progenitors to becoming preadipocytes (ICAM1+ population). In a repeat experiment, ICAM1 was again found in lower levels in adipocyte depleted joints, showing that adipocyte depletion leads to a loss of preadipocyte-like cells.



2. Related to 1, the authors need to show whether and how composition and phenotypes of synovial fibroblast populations are changed in the joint of synovial fibroblast-specific NR3C1 KO mice by sc-RNAseq etc.

We thank the reviewer for this suggestion. We purchased floxed *Nr3c1* KO mice and have spent 6 months breeding them to a *Pdgfra*-cre ERT mouse to generate the *Nr3c1* fibroblast specific knockout mice needed for this experiment. We deleted *Nr3c1* by injecting tamoxifen, and then waited 4 weeks to perform qPCR and flow cytometry on synovium from either *Nr3c1* fl/fl *Pdgfra* cre *ER*+ or littermate control mice. By flow cytometry, we found that lack of glucocorticoid signaling in synovial fibroblasts resulted in increased CD55 expression (a marker of universal progenitor fibroblasts), suggesting that lack of Nr3c1 signaling leads to de-differentiation of fibroblasts, likely from a pre-adipocyte-like state to a less defined state. By qPCR on synovial tissue, we found that *Nr3c1* expression was decreased in *Nr3c1 fl/fl Pdgfra cre ER+* mice, as well as several glucocorticoid responsive genes, including *Cebpd, Cidec, Apod*, and *Mt1*. This data shows that synovial fibroblasts do respond to endogenous glucocorticoids at rest, and the lack of Nr3c1 signaling leads to changes in their cellular phenotype. We have included this data in **figure 5 c and d,** and we have added the methods to the methods section. The data is pasted below as well:

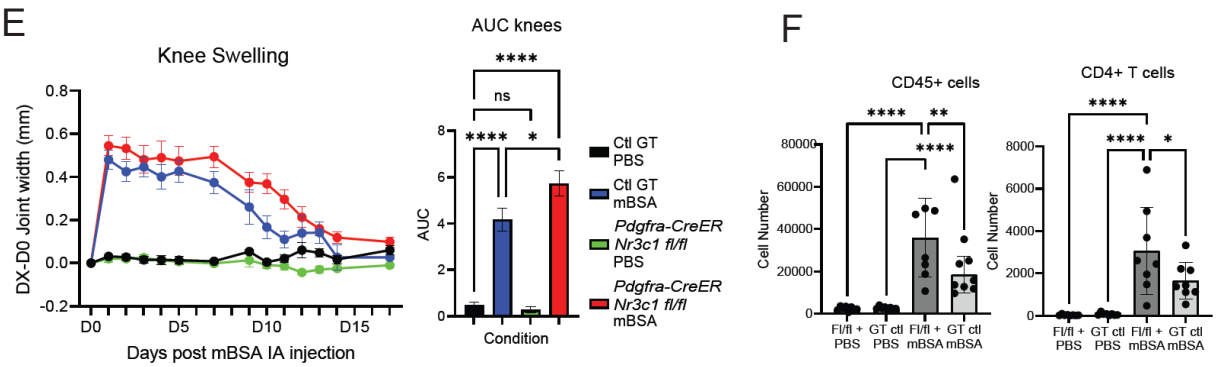


3. The authors need to show whether adipocyte-depleted mice are resistant to inflammatory diseases using arthritis models.

We thank the reviewer for this suggestion. We depleted adipocytes from the ankles of male mice for 1 month. After this, we utilized the serum transfer (STIA) model of inflammatory arthritis by injecting 50uL of KbXN mouse serum to induce a moderate level of arthritis severity. We assessed ankle clinical scores and swelling via daily caliper measurements. Just past the peak of arthritis, we harvested mice and collected ankle synovium for flow cytometry to profile fibroblast numbers and levels of FAP and PDPN. We did not detect any differences in arthritis severity among adipocyte depleted mice and diphtheria injected controls. We think that the multiple functions of adipocytes mask the effects of lowered cortisol generation resulting from adipocyte depletion. For example, certain adipokines have been implicated in arthritis pathogenesis (Adipokines and Autoimmunity in Inflammatory Arthritis, Neumann, Cells 2021). However, we did get a positive result using Nr3c1 depleted mice as described below.

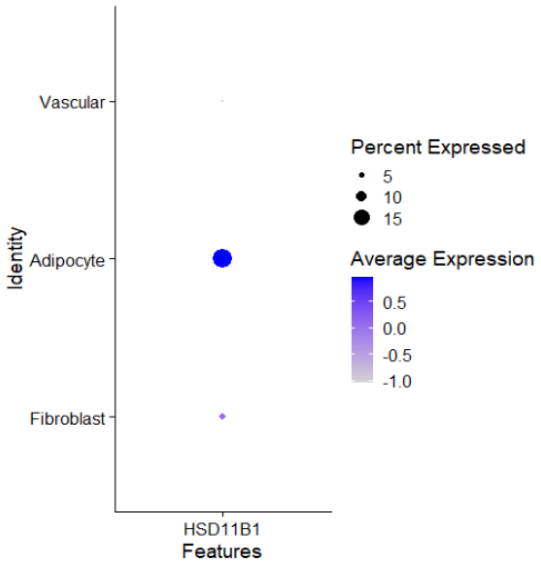
4. Related to 3, The authors need to show whether synovial fibroblast specific NR3C1 KO mice are resistant to inflammatory diseases using arthritis models.

We thank the reviewer for this suggestion. As mentioned above, we purchased floxed *Nr3c1* KO mice and after 6 months breeding them to a *Pdgfra*-cre ERT mice we generated the *Nr3c1* fibroblast specific knockout mice needed for this experiment. We deleted Nr3c1 by injecting tamoxifen, and then waited 2 weeks to begin antigen induced arthritis (AIA) model experiments. We primed mice with methylated bovine serum albumin (mBSA)+complete Freund's adjuvant (CFA), and after 2 weeks, injected mice intra-articularly with 50ug mBSA. We measured knee joint swelling by caliper for up to 17 days. We performed the experiment 3 times and pooled the results to obtain n=11 genotype control and n=13 *Nr3c1 fl/fl Pdgfra CreER*+ mice. We observed that lack of cortisol signaling in fibroblasts results in prolonged and slightly elevated knee swelling, quantified by area under the curve. We also performed flow cytometry and found that, on day 11, *Nr3c1 fl/fl Pdgfra CreER*+ mice have higher numbers of total CD45+ immune cells and CD4 T cells. Flow cytometry was done in 2 independent experiments with a total n of 8. These results demonstrate that mice lacking glucocorticoid signaling in fibroblasts have worsened arthritis, which supports our human data. **This data has been added in figure 5 e-f** in the revised manuscript. The panel is included here:



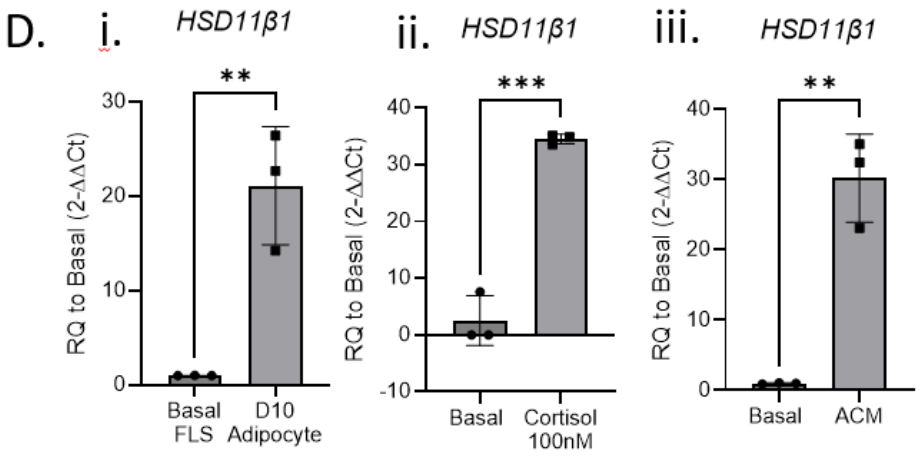
5. They need to show whether adipocytes are the main cellular source of Hsd11b1 in joints at a mRNA level

We thank the reviewer for this pertinent question which can provide insight into the importance of adipocytes in this pathway. First, we attempted Nuclear sequencing (nucseq) of healthy synovial tissue in order to obtain adipocytes to answer this question (as adipocytes are lost during flow sorting and traditional droplet capture scRNAseq). However, due to the low cell density and high amount of ECM present, the cells we obtained were of low quality and not suitable for analysis. Thus, we decided to address this question using publicly available data and *in vitro* experiments. We analyzed a publicly available single cell atlas on human adipose tissue from Emont (Nature 2022), which included nuclear-sequencing. We found that adipocytes express *HSD11β1* 1.6x higher than vascular cells (p<1e-305) and 1.2x higher than fibroblasts (p=2.73E-132), see dot plot below. We only compared adipocytes, vascular cells (endothelial cells, pericytes) and fibroblasts since these are the primary cell types we found present in healthy synovial tissue. This data supports that adipocytes are the highest source of *HSD11β1* in adipose tissues, but not the only source.



To complement this data, we performed *in vitro* cell culture. We differentiated preadipocytes into mature adipocytes for 10 days, then isolated RNA and performed qPCR analysis for *HSD11β1*. We also isolated RNA from cultured synovial RA fibroblast cells lines and found that cultured adipocytes express *HSD11β1* at >20-fold higher levels than cultured synovial fibroblasts. Additionally, we stimulated synovial fibroblasts with cortisol or adipocyte conditioned media and found that either addition stimulated fibroblasts to substantially upregulate their expression of *HSD11β1* (~30-fold). This has been added to **fig. S7c** and included below.

This suggests that at baseline, adipocytes are a major source of *HSD11β1*, which can help generate more cortisol that can then act on local fibroblasts to increase their expression of *HSD11β1*. This would imply that adipose-rich tissues contain fibroblasts which express higher levels of *HSD11β1*. Indeed, when we go back to our single cell data, we observe increased *HSD11β1* expression in healthy synovial fibroblasts compared to RA and OA synovial fibroblasts by ~2.3x fold (p<0.00001).



6. They need to provide molecular mechanisms on whether and how active cortisol is upregulated in healthy joints.

In the normal synovium, we did not mean to suggest that the levels of cortisol are upregulated, but rather they are homeostatically available. We think the levels are disrupted during arthritis via the proposed mechanisms of inflammatory cytokine blockade. We show that cytokines can suppress cortisol signaling, and adipogenesis, which we believe leads to decreased *HSD11β1* expression. It has been shown that adipose tissue expresses *HSD11β1*, and that adipocytes greatly contribute to this expression (Weight loss increases 11β-hydroxysteroid dehydrogenase type 1 expression in human adipose tissue, Tomlinson, 2004, J Clin Endocrinol Metab.). Extensive literature has shown that the mechanism of cortisol activation is via the enzyme HSD11β1, and we assume this is the mechanism of the activation in the synovium.

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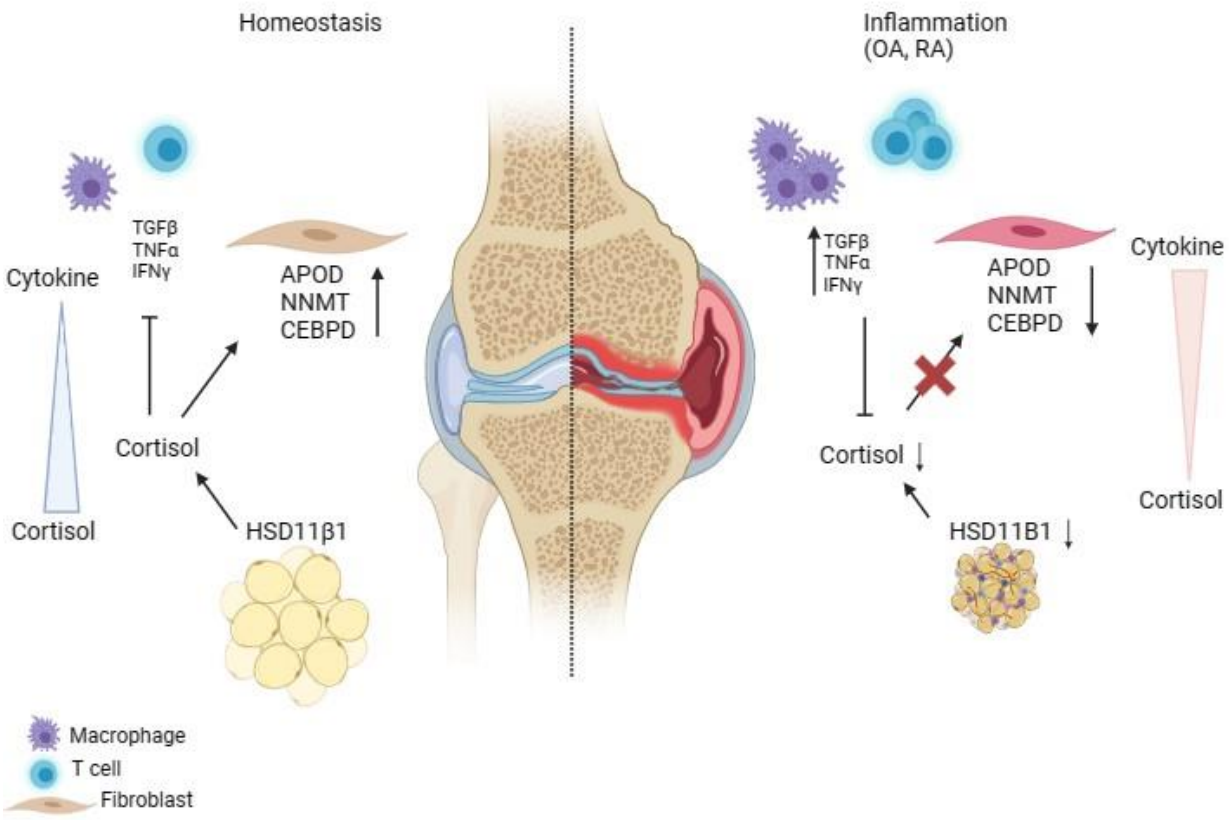
1. Line 71, the unit for the ORO staining should be micrometer (μm) instead of micro molar (uM).
2. Line 277-278, it is unclear why the authors used ELISA to determine the levels of aldosterone instead of mass spectrometry.

We thank the reviewer for these comments. Below, we have included a graphic summary of the work as suggested. Nature Communications does not allow graphical abstracts, however we have included this graphic summary in supplementary figure 14. Additionally, we have revised the title and abstract to be as accurate as possible and not overstate the findings of our studies.



Regarding point 1, thank you for pointing out this mistake in the µm units. We have corrected this in the manuscript.

Regarding point 2, we initially performed ELISA to detect cortisol and aldosterone. Since cortisol levels were detected at the ng/mL level, we then used mass spectrometry to determine the cortisol concentrations more quantitatively. Since the levels of aldosterone were much lower by ELISA (in the pg/mL range) we decided it was unlikely to be contributing significantly to the signaling through NR3C1 and did not measure it by mass spectrometry.

We have since examined our existing data for the mass of aldosterone (m/z 361.2015) to see if we did pick up any aldosterone in the FCM we tested. However, there were no major peaks present, only some random and very broad signals in the spectra, which indicated that any small organic molecule with this mass could be detected in the spectra. As we did not run a standard for aldosterone, we cannot determine if any of these peaks represent aldosterone.

Reviewer #3 (Remarks to the Author):

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The manuscript provides a novel and insightful analysis of the healthy synovial state including of the first time an analysis of the potential role of adipocytes in maintaining a healthy fibroblast cell state in the joint. It is proposed that the disruption of this interaction and signaling pathway is fundamental to the transition to a diseased state.
Weaknesses
While the authors focus on comparisons of a disease state (OA/RA) and make some useful in vitro observations to support their conclusions, functional in vivo data that would underpin the impact loss of cortisol signaling in transition from a healthy to diseased state is missing.

Main points
1. Have the authors considered the induction of inflammatory arthritis in mice following adipocyte depletion – does this lead to chronicity and failure to resolve, does cortisol injection locally restore functionality of the fibroblasts, are adipocyte depleted joints primed to respond to joint inflammation – ie more severe inflammatory arthritis on induction.
We thank the reviewer for this suggestion. We depleted adipocytes from the ankles of male mice for 1 month. After this, we utilized the STIA model by injecting 50uL of KbXN serum to induce a moderate level of arthritis severity. We assessed ankle clinical scores and swelling via daily caliper measurements. Just past the peak of arthritis, we harvested mice and collected ankle synovium for flow cytometry to profile fibroblast numbers and levels of FAP and PDPN. We did not detect any differences in arthritis severity among adipocyte depleted mice and diphtheria injected controls. We think that the multiple functions of adipocytes mask the effects of lowered cortisol generation resulting from adipocyte depletion. For example, certain adipokines have been implicated in arthritis pathogenesis (Adipokines and Autoimmunity in Inflammatory Arthritis, Neumann, Cells 2021). Note below that we show positive results on arthritis using *HSD11β1* deficient mice.

Regarding your comment on cortisol injection and fibroblast functionality, Koenen et al (2017, Ann Rheum Dis) has shown that dexamethasone (DEX) treatment was less effective in relieving arthritis in mice with impaired fibroblast GC signaling. This supports the idea that cortisol injection does restore functionality of fibroblasts in an arthritis setting.

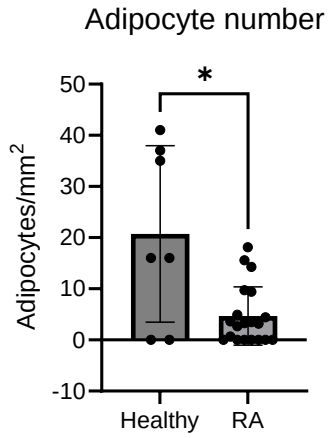
2. Are HSD11b KO mice more susceptible to developing severe arthritis in response to an inflammatory challenge? If so is this fibroblast mediated?

We thank the reviewer for this suggestion. Performing arthritis experiments in floxed *HSD11β1* fibroblast specific knockout mice would be informative, we could not find any commercially available floxed *HSD11β1* mice. However, towards answering your question, we did purchase floxed *Nr3c1* KO mice and breed them to a *Pdgfra*-cre ERT mouse to generate *Nr3c1* fibroblast specific knockout mice. Although this model does not directly show whether the ability to convert inactive cortisone to active cortisol impacts arthritis, it does tell us whether the ability of glucocorticoids to signal through *Nr3c1* in fibroblasts impacts arthritis pathogenesis. We think that this is highly relevant to the reviewer's question and hope this strategy is sufficient to address the reviewer's question.

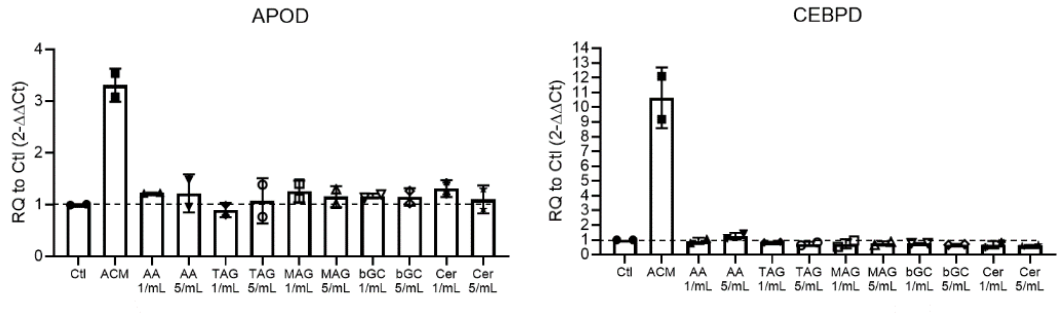
Please see our response to reviewer 1, point 4 for details on the experimental set-up and positive results obtained. The results also are found in the new Figure 5.

3. I was confused by the lipidtox are the authors quantifying adipocytes or lipid containing fibroblasts – perhaps they could clarify? if fibroblasts, but not adipocytes – then how do they quantify loss of adipocytes (currently labelled as fibroblasts)

The lipidtox analysis is only performed on fibroblasts since adipocytes lyse when run through the flow cytometer. Figure 1b is quantifying the loss of lipids contained in fibroblasts. We did not perform a formal analysis on the loss of adipocytes in human synovium during arthritis but have added an analysis to address your question. Since adipocytes cannot survive flow cytometry analysis, we quantified adipocytes (identified by morphology) on H&E images of healthy and RA synovium. Synovial tissue can be heterogenous depending on the area imaged, so in patients with multiple fragments of synovium imaged, we calculated a total adipocyte number among all the fragments per donor and have displayed the result as number of adipocytes per mm squared. Utilizing this method, we were able to show that RA has 4.66 adipocytes per mm^2, while healthy synovium has 20 adipocytes per mm^2. We have included this quantification in **fig. S1d** and below:



4. I don't see why the analysis on ACM is included in this manuscript. It identifies a synthetic compound (DEX) as the active compound, something that would have little relevance in understanding the biology of adipose tissue on regulating fibroblast. Adipocyte conditioned medium (ACM) is included because we performed extensive, unbiased HPLC-QToF-MS analyses which identified IBMX and indomethacin, but also several fatty acids as candidates which were enriched in the active fraction (acetone). We tested these fatty acid candidates (Arachidonic acid (AA), Monoacylglycerol (MAG), β-glusocylceramide (βGC), and Ceramide). We tested multiple concentrations of each lipid as well as a control lipid, TAG (triacylglycerol), and found that none of these lipids induced *APOD*, *NNMT*, or *CEBPD* expression. Due to the importance and relevance of ruling out other potential active factors which could be secreted by adipocytes, we kept in the ACM analysis and performed follow-up studies in fat conditioned medium (FCM) to confirm cortisol as the active compound. However, we did not repeat the unbiased screen in FCM due to time and cost. We had removed the lipid testing (below), but have added them here for your reference: AA= arachidonic acid, TAG= triacylglycerol, MAG= monoacylglycerol, bGC= β-Glucosylceramide, Cer= ceramide



5. Figure 6A – Why does fat conditioned media not have the same affect as cortisol? Is cortisol not in fat conditioned media?

Fat conditioned media and cortisol have similar effects, they both reduce the amount of ECM remodeling performed by fibroblasts in the presence of cytokine. FCM has a slightly lower reduction of ECM remodeling, likely because there is more pure cortisol added in the cortisol condition than there is present in the FCM. We did not try to match the exogenous cortisol to the biological cortisol levels present in FCM because we wanted to see the effect of cortisol at a relatively high level (1uM) to ensure we would not miss a potentially important biological effect. Please note this is now figure 7 in the manuscript.

6. Have the authors had the opportunity to examine the synovia of patients with active RA on steroids is there a partial defect in cortisol signalling / haemostatic fibroblasts?

This is a very interesting and relevant question. In the AMP phase 2 dataset (from which we analyzed naïve RA and OA samples from, reference: Zhang et al.), 28% of RA patients are taking low dose steroids (<7.5mg oral prednisone, which is similar to the amount of endogenous cortisol generated daily, see sources below) and none had received intra-articular injection of steroids. However, this study was not designed to test the difference of non-steroid vs steroid treated patients, and the low levels of steroid administration make it unlikely we would observe a robust difference between users and non-users. To adequately answer your question, we would need to perform more single cell RNA sequencing on a new cohort of patients with a high dose of steroids, and this is not feasible for us to perform in the context of this revision.

Sources:

1. Kraan, "The Daily Cortisol Production Reinvestigated in Healthy Men. The Serum and Urinary Cortisol Production Rates Are Not Significantly Different." *The Journal of Clinical Endocrinology & Metabolism*, Volume 83, Issue 4, 1 April 1998, Pages 1247–1252, https://doi.org/10.1210/jcem.83.4.4694
2. Mdcalc Steroid Conversion Calculator created by Bryan D. Hayes, PharmD

7. Have the authors considered where the adipocytes are derived from in the joint and how they are re-populated with resolution of inflammation?

We hypothesize that adipocytes are derived from a subset of the resident fibroblast populations, which may also be referred to as mesenchymal stem cells. Fibroblasts are quite plastic, and it seems that a significant proportion of them can differentiate into adipocytes with the proper stimulus (as in figure 7D and E, with adipocyte differentiation media added). Based on our in vitro assays and RNA sequencing data, we think that when inflammation resolves in the joint, cortisol signaling is restored (i.e, cortisol

signaling is no longer suppressed by cytokines) and can then participate in stimulating fibroblasts to undergo adipogenesis.

Minor points

Figure 1b- clearer axis labels on flow histograms and need to demonstrate gating strategy (can be in supplementary).

Thank you, we have added a gating strategy for Figure 1b in **fig. S1c**.

Figure 1C- quantification of this is essential. To address this, we quantified the number of CD45+ cells and intensity of PRG4 and PDPN staining from n=3 healthy, n=4 OA, and n=3 RA tissues. We used "analyze->measure" in ImageJ to obtain intensity measures. This quantification confirmed significant increases in PDPN staining in OA and RA tissues. PRG4 intensity was not significantly altered, so we edited the text to: "Staining for the synovial lining marker lubricin (PRG4) showed that healthy synovium has a strong and well-defined border of PRG4 staining in the lining, which appears more irregular in OA and RA lining." Additional images which were used for quantification are now added in **fig. S1e**. Healthy synovium had the lowest average number of CD45+ staining cells, but it was only significantly lower than the number quantified in the RA tissue, so we edited the text to: "Next, we performed immunofluorescence staining, which revealed a smaller number of $CD45^{+}$ cells in healthy synovium compared to RA samples (Fig. 1c)." The panel added in fig. S1e is included here for your reference:

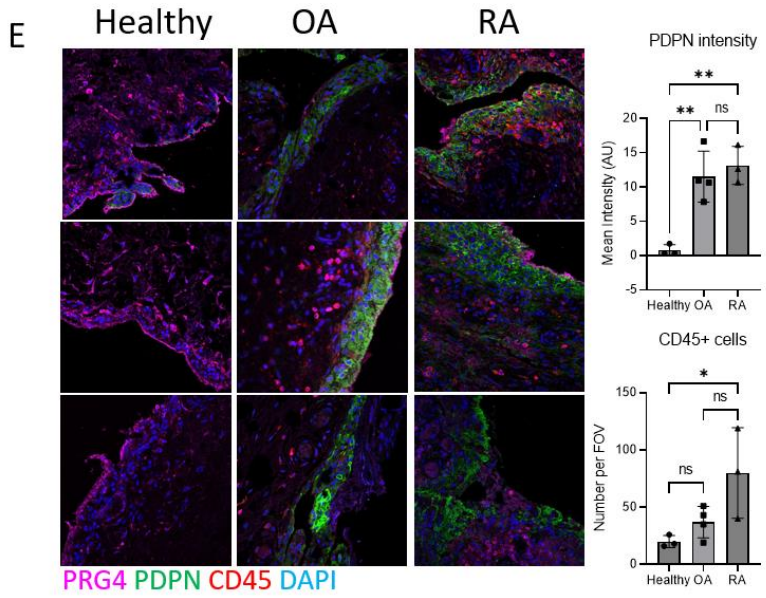


Figure 1 Eb – the UMAP is not clear and would benefit from splitting the UMAP per disease state for clarity. We agree that it would be helpful to view the UMAP separated by disease state. We have added this to **fig. S1h**, and pasted it below as well:

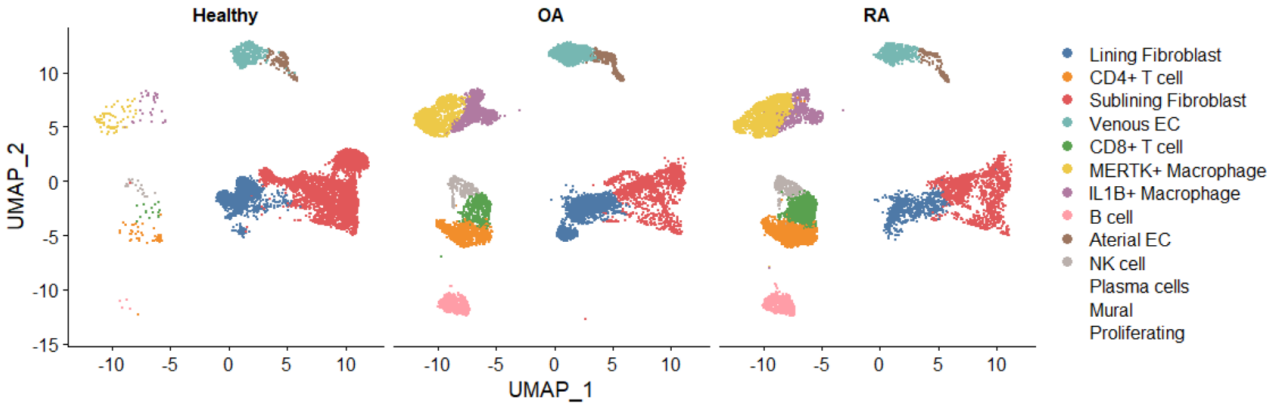


Figure 1 – all images need scale bars. Thank you for pointing this out. We have added scale bars to all images in figure 1.
Figure 1 – statistical tests and n numbers need to be included in the legend
Figure 2 – statistics and n numbers need to be included in the legend
Figure 3 – statistical tests and n-numbers need to be included in the legend
For these figures, the statistical tests have been outlined in the methods section, and Data File S8 contains all p-values, f-values, and n. However, we have gone back and added statistical tests and n for each figure in the figure legends.

Line 135- Ref to Fig 2c should be Fig 2sc Thank you, we have changed the reference to fig. S2c.

Figure 2E- does each dot here represent a sample? Need clarification
Thank you for pointing this out. Each dot represents a patient sample. We have added "Each data point represents a patient sample." to the figure legend.

Figure 2F, S2D- More details on how DEGs were computed. Which conditions were compared? Where the samples pseudobulked or is this DE at the cell levels? Although the feature plots show a robust difference, some mention of p values would be needed. Volcano plots here would be useful.
These DEGs were calculated using the Seurat function "FindMarkers" with default settings applied. The source code specifying this has now been provided in Zenodo. The output of this analysis, including fold changes and p-values, is included in Data files 2 and 3 (for healthy sublining fibroblasts vs RA and OA sublining fibroblasts, respectively) and referenced in the text. This analysis was performed at the cell, not pseudobulk, level.

Lines 150-153, refs are needed here to back up the statements about CEBPD and APOD.
Thank you for pointing out this oversight. We have added references 12-15 to support these statements.

I don't see any results for NNMT is fig S4b (as mentioned in the text), is this a mistake?
This is a mistake, thank you for pointing that out! We have removed the text citing the effects on NNMT, in this particular experiment the FCM did not robustly upregulate NNMT, so it is not fair to say that mifepristone did not block its upregulation.

Line 302, I don't get the link to TGFb here, this need to be clearer.
We included the link to TGFβ because we later show that TGFβ can suppress cortisol signaling, so wanted to point out that OA and RA fibroblasts exhibit higher TGFβ signaling compared to healthy fibroblasts. We agree this seems out of place. We moved this information to the section titled "Inflammation" and moved the data to **fig. S13a**, where we think it's location fits better.

Figure S12F, needs quantification.
Figure S12F is quantified, the quantification is shown in Figure 7b (was Figure 6b).

Reference or study ID? AMP phase 2
This dataset is also referred to as Zhang et al. earlier in the manuscript and has been referenced (reference number 8). We missed the continuity error and see how this is confusing. We have changed the reference to "AMP phase 2" to say "Zhang et al" and have provided the reference again in the methods section.

Figure 1C – Colour legends needs to be clearer
We have increased the font size and increased the color legend brightness to help see the legend better. We hope these changes are sufficient for clear viewing.

Line 109-142 – some genes names are defined more than once. Define the first time described and no need to define again
We apologize if this is confusing in the text. We do list some genes multiple times as they are either defined or listed as a gene falling within a certain category, i.e., APOD gets defined once, but then is also listed as a gene falling under the category of apolipoprotein later. We think it is helpful to include specific gene examples within each category, so we have kept this text the same.

Line 170 – what fibroblasts? Healthy knee synovial fibroblasts? Not clear
We added "RA synovial" to specify they are synovial fibroblasts derived from RA patients.

Line 174-175 – generation of abdominal or synovial fat conditioned media. The author needs to refer to the figure here as it is not clear to the reader what this is. It is explained in the figure but not referenced.
On the next lines (175 and 177) we do reference and specify which figures implement synovial or fat conditioned media.

177-178 – Abdominal fat-conditioned media upregulated healthy fibroblast genes. Does this mean this phenomenon is not restricted to the joint and can occur where ever fat cells and fibroblast exist together?
Yes, that is exactly what we think is happening!

Sub title – "Search for active molecules using fractionation and lipidomics" – search for should be "Identification".
Thank you, we have changed the sub-title.

Methods:
a better description as to where in the coding region the gRNA targets is required.
Thank you for pointing this out. We have provided the additional information in the methods section:

"The primer coordinates are as follows: Left primer: chr5:143300617-143300639, Right primer: chr5:143300397-143300419. Primer sequences: Left primer: CTGGTGTCACTGTTGGAGGTTA, Right primer: GGGCTCACGATGATATAAAAGC. The sequence targets the following nucleotide sequence of the *NR3C1* coding region for deletion: ATGACTACGCTCAACATGTTAGG."

Line 684-689Add in version of Seurat, Harmony and R used. The specific functions used to process the data is required, such as ScaleData() etc.
We have some of this in the reporting summary, but have added the information into the methods section as well: Seurat version 4.0.1 and R version 4.1.1. Harmony only has 1 release version from February 19th 2020. We have made the code used to produce the data available on Zenodo, in which the specific functions can be found.

Stats- what type of post-hoc test was applied to the one way anova?
In figure 2e, 3c, 3d,4a,4c, 7e (originally 6e), Dunnett's multiple comparisons test was used, as each column was only compared to 1 group of interest. In figure 6c, 7a, 7b, 7c, (originally figures 5 and 6) Tukey's multiple comparisons test was used (assumes equal SDs and all groups compared to each other). In figure 4i and 6b (originally 5), Kruskal-Wallis test was performed with Dunn's Multiple comparisons test performed as the post-hoc test. In figure 4g, 7d (originally 6d), 2-way ANOVA was used with Tukey's multiple comparisons.

Would a 2 way ANOVa not be more appropriate for the analysis in Figure S11 and Figure 5C?
No, because a 2-way ANOVA is designed to determine the influence of two different independent variables on one dependent variable. In 6c (originally 5c), we are analyzing the impact of disease status on the proportion of each cell subset, so there is only one variable. The same is true for Figure S11, where we are analyzing only the effect of BMI on cell subset proportions within adipose tissue.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In the revised manuscript, they newly generated the fibroblast specific Nr3c1-knockout mice and found that arthritis was exacerbated in the fibroblast specific Nr3c1-KO mice. These newly obtained results suggest the inhibitory role of glucocorticoid signaling in synovial fibroblasts in vivo under arthritic conditions. However, the authors did not show how populations and phenotypes of synovial fibroblasts alter in the cKO mice in detail. We assume these analyses we requested are technically feasible and necessary to elucidate the importance of glucocorticoid signaling in differentiation and activation of synovial fibroblasts in vivo. In addition, they failed to show any differences in arthritis between adipocyte-depleted mice and the control mice. Thus, they did not provide any convincing evidence that adipocytes play an important role in arthritis or differentiation and activation of synovial fibroblasts. Thus, manuscript in the current status does not achieve the priority necessary for publication in Nature Communications.

Reviewer #3 (Remarks to the Author):

The authors have addressed all of my concerns and suggestions

REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

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Reviewer #1:

Comment 1: Reviewer #1 states "However, the authors did not show how populations and phenotypes of synovial fibroblasts alter in the cKO mice in detail. We assume these analyses we requested are technically feasible and necessary to elucidate the importance of glucocorticoid signaling in differentiation and activation of synovial fibroblasts in vivo."

Response: We were very careful not to use a global Nr3c1 knockout, but instead bred Nrc31 fl/fl mice to Pdgfra creER mice to generate fibroblast specific knockout mice. The fact that arthritis was worsened by knocking out glucocorticoid signaling in fibroblasts is evidence that glucocorticoid signaling impacts the activation and inflammatory states of synovial fibroblasts in vivo. Additionally, while we did not carry out RNA sequencing of fibroblasts in the murine model, we did carry out single cell RNA sequencing of healthy versus arthritic human synovial cells, in which we found profound differences in fibroblast cell states. We then performed bulk RNA sequencing on cultured human synovial fibroblasts stimulated with cortisol. We derived a cortisol (glucocorticoid) activation signature from this bulk RNA sequencing dataset and applied it onto our human single cell data. We found that this cortisol (glucocorticoid) activation signature was highly enriched in healthy synovial fibroblasts compared to arthritic synovial fibroblasts. This data, combined with our data that shows more severe arthritis in mice lacking fibroblast specific Nr3c1 (glucocorticoid receptor) signaling, is strong evidence that glucocorticoid signaling plays an important role in synovial fibroblast phenotype and activation. Thus, while we recognize that getting additional single cell data can be helpful, we hope that the fibroblast specific knock-out confirms our point that glucocorticoid signaling in fibroblasts is critical to their pathologic response.

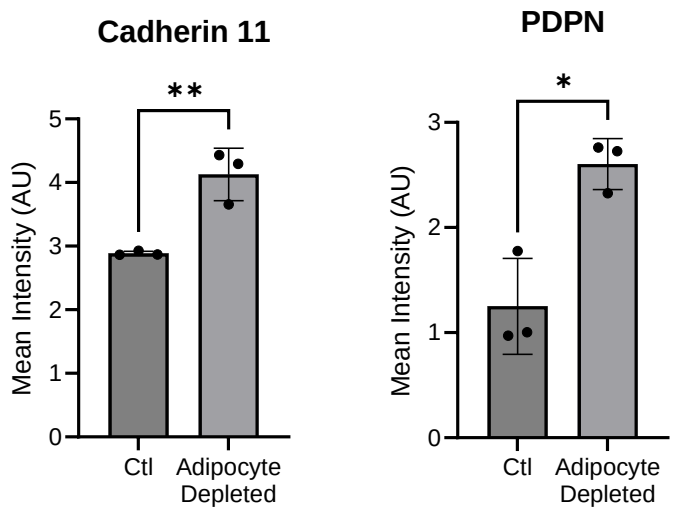
Comment 2: Reviewer #1 states that we "failed to show any differences in arthritis between adipocyte-depleted mice and the control mice".

Response: This is correct, however there are several reasons this might occur. There are multiple functions of adipocytes in addition to their role in cortisol generation. Adipocytes play a large role in controlling energy homeostasis by supplying lipids to meet nutritional needs, meaning they can provide a source of energy for infiltrating lymphocytes (Anatomical, Physiological, and Functional Diversity of adipose tissue, Zwick, Cell Metabolism 2018). Additionally, adipokines including leptin have been implicated in arthritis pathogenesis, while adiponectin is anti-inflammatory (Adipokines and Autoimmunity in Inflammatory Arthritis, Neumann, Cells 2021). Also, others have shown that adipocytes secrete adipsin, which renders mice susceptible to serum transfer arthritis (Fat-Produced Adipsin Regulates Inflammatory Arthritis, Li, Cell reports, 2019). Some of these effects worsen arthritis and others may improve arthritis, such that the effects of adipocytes are complex. Although we carried out adipocyte depletion, as the reviewer notes, we did not see a change in arthritis. This is why we undertook studies using the more selective approach relevant to the theme of our paper by using *Nr3c1* fl/fl

Pdgfra cre *ER*+ mice. This allowed us to isolate the effect of adipocytes to their cortisol-activation function on fibroblasts separate from the many other functions that adipocytes display. This knockout gave a clear clinical response in arthritis severity, supporting the thesis of our paper. We hope that the reviewer will appreciate the complexity of adipocyte depletion and the logic of our selective strategy to knockout the Nr3c1 receptor on fibroblasts.

Comment 3: Reviewer #1 states “Thus, they did not provide any convincing evidence that adipocytes play an important role in arthritis or differentiation and activation of synovial fibroblasts.”

Response: While we noted as above that adipocyte depletion results were uninformative in the arthritis experiments, we did find that adipocyte depletion under steady-state control conditions (ie not arthritis) resulted in altered fibroblast cell states. We show that at the cellular level, fibroblasts (defined by Pdgfra+ staining) in adipocyte depleted joints exhibit stronger expression of activation markers including PDPN and Cadherin 11, both of which are increased under inflammatory conditions (Astarita, 2012, Podoplanin: emerging functions in development, the immune system, and cancer, Front Immunol; and Vandooren, 2008, Tumor necrosis factor α drives cadherin 11 expression in rheumatoid inflammation, Arthritis& Rheumatism). We have added immunostaining of naïve joints which have either been adipocyte depleted or control mice which have no depletion. This data supports the importance of adipocytes in maintaining fibroblast homeostasis. This quantification appears below and has been added to supplemental figure 7d. We also highlighted the relevant text citing this figure in the manuscript and methods section.



The paper is built around our discovery that the phenotype of normal healthy fibroblasts in the synovium is dependent on glucocorticoid signaling, and that the major source of the 11β-hydroxysteroid dehydrogenase type 1 that converts inactive cortisone to cortisol is expressed in adipocytes. For this reason, we knocked out the *NR3C1* gene selectively in fibroblasts to confirm the role of this axis. While these experiments do not rule out all other explanations and constitute proof, we hope the reviewer will appreciate that they do provide strong evidence that supports the thesis of this manuscript. Further, our in vitro experiments demonstrate

convincingly that inflammatory cytokines present in inflammatory arthritis block the effects of glucocorticoid signaling on fibroblasts. Together, the data implicate glucocorticoids in the healthy fibroblast phenotype under homeostatic conditions and help explain why that homeostatic state is lost in inflammatory arthritis. Thus, we have tried to carefully make claims that our data justifies as reflected in the title of the paper “Adipocyte associated glucocorticoid signaling regulates normal fibroblast function which is lost in inflammatory arthritis.”

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

In the revised MS, The author added immunostaining of naïve joints which have either been adipocyte depleted or control mice, supporting the importance of adipocytes in maintaining fibroblast homeostasis. We understand that there are multiple functions of adipocytes besides generation of cortisol and adipocyte-depletion did not affect pathogenesis of arthritis. The author should include the data showing there are any differences in arthritis severity between adipocyte-depleted mice and the control mice in Sup Figure and discuss the possible reasons in the text since it is important to show adipocytes have multiple functions and cortisol generation is one of them under arthritic conditions.

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Response: Thank you for the additional comments. We have included the data showing that there are minimal differences in arthritis severity between adipocyte-depleted mice and the control mice in Supplemental Figure 8 (c-d). We have also added discussion of the possible reasons in the text in the results section and discussion.