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Dr. Michal Duliban, PhD
Assistant Editor *IJMS*

Dear Dr Duliban,

I have just submitted our revised article **manuscript ID: ijms-2919284** *'*β1-integrin mediated uptake of chondrocyte extracellular vesicles regulates chondrocyte homeostasis.*'* by Mohammed Tayab Hussain, Shani Austin-Williams, Thomas Dudley Wright, Umesh Kumar Dhawan, Andreia L Pinto, Dianne Cooper, Lucy V Norling. We thank the handling Editors and Reviewers for their valuable comments on our manuscript and have addressed the specific questions point by point below:

Reviewer: 1
Comments to the Author:
The manuscript presents interesting data on the role of chondrocyte derived EVs in a 3D culture model. However, several key issues need to be addressed before the manuscript can be considered for publication:

Major comments:

1. TGF-β treatment increased the yield of EVs from 3D cultures but not from 2D cultures, and unexpectedly also induced the expression of catabolic genes (MMP13 and ADAMTS5) in these cultures. Can the authors comment on the mechanism(s) by which TGF-β treatment increases EV release only in 3D cultures? Is this related to the increased presence of ECM or other factors specific to the 3D microenvironment?

We thank the reviewer for raising this interesting point. We agree that the increased yield of EVs in 3D but not 2D cultures following TGF-β treatment is likely related to an increase in ECM. We have performed additional experiments to determine the ECM accumulation in chondrocytes grown in 3D culture with or without TGF-β treatment using Alcian blue staining (Supplementary Figure 1). TGF-β stimulated 3D cultures had a greater proteoglycan content compared with vehicle treatment. This is in line with our other data which demonstrates that chondrocyte EVs are sequestered within the cartilage matrix. The discussion has been updated appropriately (page 12, lines 333-338).

2. The authors observed that TGF-β treatment increased the expression of catabolic genes alongside the increase in EV release in 3D cultures. Are these EVs promoting or inhibiting catabolic activity in recipient cells?

We did observe an increase in catabolic gene expression in 3D chondrocyte cultures treated with TGF-β. We believe this response demonstrates a remodelling phenotype, which is

supported by the literature. Indeed, the following review indicates the synergetic increase in both TGF-β and ADAMTS5 are essential for efficient pericellular matrix turnover (Plaas *et al*., PMID: 21624477). Given the increased proteoglycan content that we detected, we think this is also the case in our settings and have added this to the discussion (page 11, lines 315-321). Whilst we have not directly assessed the effect of chondrocyte EVs from TGF-β stimulated cultures we would hypothesise that they promote anabolism, however this is the subject of future work.

3. The authors claim that most EVs are sequestered within the 3D cartilage ECM. However, they don't provide convincing evidence to distinguish between EVs released from the cells and EVs trapped within the matrix from the beginning of the experiment. Did the authors perform any experiments to distinguish between these two possibilities?

As the ECM accumulates over time from chondrocytes grown in 3D cultures the matrix at the beginning of the experiment when the pellets are initially created is minimal. We have analysed the EVs in the supernatant of 3D pellets (where we detected barely any) and following dissociation of EV pellets after 96h. We have now updated the legend and y-axis titles in Figure 1 for clarity.

4. The authors should discuss their findings in the context of existing literature on chondrocyte EVs and osteoarthritis (OA). How do these findings align with previous studies, and what are the potential clinical implications?

We have now added an additional section to the manuscript as requested (page 13, final paragraph).

5. The study does not delve into the mechanisms that could be involved in the increased production of EVs in 3D cultures and with TGF-β. Has the role of the endosomal machinery in this process been evaluated?

Whilst we have not evaluated this, it is possible that the endosomal machinery is heavily involved in both processes. There is a large wealth of data in a variety of cell types where 3D culture methods have substantially elevated EV production. However, mechanistic insight into this phenomenon directly is entirely under studied. However, the literature demonstrates that membrane tension strongly regulates intraluminal vesicle (ILV) formation and endosomal trafficking. Additionally, the authors demonstrate that epidermal growth factor has a direct effect on inducing ILV formation, and this was accompanied by changes in membrane tension (Mercier *et al.,* Nature Cell Biology 2020; PMID: 32753669). It is likely that the biomechanical differences between 2D and 3D culture have similar effects on membrane tension, which may potentially go on to influence EV generation dynamics, but this remains outside the scope of this work.

6. The manuscript would benefit from a more concise and focused presentation of the results.

We have modified the figure legends to improve readability as highlighted.

Reviewer 2:

The authors address a very interesting issue – the impact of 3D cell culture and paracrine signaling of chondrocytes on their function. Although the authors indicate in the title that β1 is crucial for this process their results seem not to be strong enough to fully support such a strong anchorage of integrin in the title of the manuscript. After several updates and additional (rather simple) experiments, this work could be very

interesting and beneficial in the context of understanding skeletal system homeostasis. I appreciate addressing integrin's role in this process, but it should be more precisely described and investigated.

1. I would suggest authors rewrite the abstract because it's written in a little chaotic way. For instance I would suggest changing “These EVs were assessed for their effects on chondrogenic gene expression…” to “We assessed the effect of EVs on chondrogenic…” It could make readers more interested in this work.

The abstract has now been updated in line with the reviewers’ suggestions to improve ease of reading as highlighted.

2. The figure caption regarding the impact of trapping EVs in a 3d cell culture matrix seems to be a little bit confusing. I understand it after reading the text but the authors should think about more clear description also of Figure captions (especially 1A vs 1C)

Figure captions have now been amended to provide more clarity to the reader.

3. Could authors provide deeper discussion and more critical interpretation of TEM images of EVs?

We thank the author for the suggestion, we have added more information regarding the shape and integrity of the EVs as seen via TEM in the main text (page 3, lines 119-120).

4. The caption of Figure 2 is bolded. Please revise if it shouldn’t be only the title of the figure caption bolded.

Figure captions have been amended so that only the appropriate words are in bold.

5. Figure 3 D – gene name on the graph is missing

Figure 3D is not gene expression/ PCR data, it depicts the concentration of EVs isolated from each condition. A title has been added to reflect this to avoid any confusion for the reader.

6. Why supplementary figure 1 is located in the main text of the manuscript?

Supplementary data is now in an independent word document entitled Hussain et al IJMS supplementary figures.

7. Figure 4 – please precise what is marked in red.

Both Figures 4 and 5 have been updated to reflect the staining represented in the respective images and captions have been updated appropriately as highlighted.

8. Authors assessed only one integrin – β1, while integrins are dimer proteins. It would be valuable to test particular dimers – at the list by using RGD peptide, which is biding to multiple integrins dimers (10.3390/cancers13071711) – i.e. α5β1.

We thank the reviewer for their interesting idea. This is also something we have considered for future studies, but it is currently out the scope of the current body of work. Indeed, we chose β1-integrin as the focus here as it is commonly expressed in the various αβ1 dimers, the β1 integrin being the key integrin expressed in chondrocytes (Loeser, Matrix Biology 2014 PMID: 25169886). Therefore, we viewed it as a sufficient starting point and bottle neck to

begin to understand the role of β1 integrin dimers using a very straightforward assay. Future work aims to further interrogate α-β1 dimers to elucidate their role in chondrocyte EVs in cartilage homeostasis.

9. It should be underlined that addressing the significance of integrins appears to be an important part of this work in any case. It would be beneficial to discuss more precisely both integrins roles in chondrocytes' homeostasis as well as in general discuss the impact of the 3D condition on the function of cells responsible for skeletal system functioning (several reviews and interesting works might be found in this field i.e. 10.18388/abp.2019_2893)

Both points have now been addressed within the discussion (Pages XXX, Lines 301-305 and Lines 361-376).

10. Authors address the role of integrins (which as underlined by authors on page 13, line 348 is crucial in focal adhesion establishment) it would be beneficial to analyze how evs affects the cytoskeleton (at least actin) organization of chondrocytes. Such an experiment should not be too much challenging since requiring only the staining of cells with phalloidin. Several interesting quantitative parameters depicting the impact of EVs on cellular cytoskeleton organization and morphology might be obtained from such studies.

We thank the reviewer for their interesting suggestion, phalloidin staining was carried out following treatment with EVs or not, data can be found in supplementary figure 3. We did not observe any changes in the amount of staining for F-actin in this experiment on a per cell basis (supplementary figure 3). The results and discussion are updated to reflect this (page 9, lines 240-242 and page 12 lines 374-377).

We thank the reviewers for the useful suggestions and hope that you agree that our revised manuscript makes a conceptual advance on the understanding of chondrocyte EVs. We remain for any further information you and your colleagues may have. Thank you again for considering our revised manuscript.

Sincerely,



Lucy Norling, PhD, FHEA.

Senior Research Fellow Versus Arthritis.