Manuscript WJSC 63568

"Comprehensive immunohistochemical evaluation of human tendon repair following injection of autologous, unmodified stem cells: a first-in-human case report"

by E.U. Alt, R. Rothoerl, M. Hoppert, H.G. Frank, T. Wuerfel, C. Alt and C. Schmitz

Point-by-point reply to the comments and suggestions of the editors and the reviewers:

Editor

1 Scientific quality: The manuscript describes a case report of the comprehensive immunohistochemical evaluation of human tendon repair following injection of autologous, unmodified stem cells. The topic is within the scope of the WJSC. (1) Classification: Grade A, Grade C and Grade C; (2) Summary of the Peer-Review Report: The authors found an insteresting and important report. It is well-organized and well-presented. However, the questions raised by the reviewers should be answered;

All questions raised by the reviewers were answered (outlined below).

and (3) Format: There are 2 tables and 13 figures.

This is correct. Adequately handling the comments and suggestions of the reviewers increased the number of tables to four and the number of figures to 15. Of note, reviewers asked for even more analyses that would have increased the number of figures even further (which was not necessary). The numbers of tables and figures may reflect the complexity but also the significance of the present study.

(4) References: A total of 61 references are cited, including 17 references published in the last 3 years;

Adequately handling the comments and suggestions of the reviewers increased the number of references to 72. Again, this may reflect the complexity but also the significance of the present study.

(5) Self-cited references: There are 6 self-cited references. The self-referencing rates should be less than 10%. Please keep the reasonable self-citations that are closely related to the topic of the manuscript, and remove other improper self-citations. If the authors fail to address the critical issue of self-citation, the editing process of this manuscript will be terminated;

We removed two self-citations (Winnier et al., 2019; Alt et al., 2020) in our revised manuscript. Together with the increase in the number of citations which is due to adequately handling of the comments and suggestions of the reviewers the relative

number of self-citations dropped to 4/72 =5.7% in our revised manuscript, which is below the critical border of 10%.

> and (6) References recommend: The authors have the right to refuse to cite improper references recommended by peer reviewer(s), especially the references published by the peer reviewer(s) themselves. If the authors found the peer reviewer(s) request the authors to cite improper references published by themselves, please send the peer reviewer's ID number to the editorialoffice@wjgnet.com. The Editorial Office will close and remove the peer reviewer from the F6Publishing system immediately.

Not applicable.

> 2 Language evaluation: Classification: Grade A, Grade B and Grade B. A language editing certificate issued by AJE was provided. 3 Academic norms and rules: The authors provided the Biostatistics Review Certificate, and the Institutional Review Board Approval Form. Written informed consent was waived. No academic misconduct was found in the Bing search.

We are grateful for this comment by the editor.

> 4 Supplementary comments: This is an unsolicited manuscript. No financial support was obtained for the study.

On page 2 of our initial manuscript the following was stated: "This study was in part supported by the Alliance of Cardiovascular Researchers (New Orleans, LA, USA). The sponsors of the study did not have any influence on data collection, analysis or publication. No constraints were placed on publication of the data."

We have submitted the original funding statement together with our revised manuscript.

> The topic has not previously been published in the WJSC. 5 Issues raised: The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor.

A PowerPoint file containing all original, decomposed figure documents was uploaded together with the revised manuscript.

> 6 Recommendation: Conditional acceptance.
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> **Company Editor-in-Chief:**
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> I have reviewed the Peer-Review Report, the full text of the manuscript, and the relevant ethics documents, all of which have met the basic publishing requirements of the World Journal of Stem Cells, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office's comments and the Criteria for Manuscript Revision by Authors. Before its final acceptance, the author(s) must provide the Signed Informed Consent Form(s) or

Document(s). For example, authors from China should upload the Chinese version of the document, authors from Italy should upload the Italian version of the document, authors from Germany should upload the Deutsch version of the document, and authors from the United States and the United Kingdom should upload the English version of the document, etc.

The Signed Informed Consent Form was uploaded together with the revised manuscript.

Reviewer's code: 02446101

This manuscript is really an interesting and important report. The results of this study firstly suggested that treating an injured human supraspinatus tendon with fresh, uncultured, autologous, adipose derived regenerative cells (UA-ADRCs) prepared at the point of care enables regenerative healing of an injured tendon. It provides some new and important ideas and references to the readers and other scholars in the field of stem cell medicine. I pay special tribute to the dedication of the paper's lead author, Dr. Eckhard U. Alt.

We are grateful for these comments by the reviewer.

There're only two issues which should be addressed. 1. It's only a case report instead of RCT.

This is correct and mentioned in both title ("… a first-in-human case report") and abstract ("Case summary:…") of our manuscript.

Accordingly, we have not modified our manuscript based on this comment by the reviewer.

2. UA-ADRCs hadn't been labeled.

This is correct. By definition, UA-ADRCs cannot be labeled because this would render them modified. As a result, UA-ADRCs cannot be unequivocally identified in the host tissue. Therefore, histological regeneration of injured human tendons after injection of UA-ADRCs must be assessed using comprehensive, immunohistochemical and microscopic analysis of biopsies taken from the treated tendon a few weeks after injection of UA-ADRCs, as done for the first time in our study.

We have incorporated this important information in the *Abstract* and *Multidisciplinary Expert Consultation* sections of our revised manuscript.

The exact mechanism of UA-ADRCs is also unclear and much more studies should be performed in the future.

We fully agree with the reviewer. We have made this point more clear in the *Multidisciplinary Expert Consultation* section of our revised manuscript.

> Although the above issues occur. Considering the outstanding innovation and important research significance of this manuscript, I still suggest that it should be published as soon as possible.

We are grateful for this comment by the reviewer.

> **Reviewer's code: 02446101**
>
> The authors discussed the biopsy of the rotator cuff tissue after 10 weeks of treatment with UA-ADRCs. It was examined to indirectly prove the theraputic effect of UA-ADRCs through relatively comprehensive immunohistochemical staining. This is the first case report that treating an injured human supraspinatus tendon with UA-ADRCs.The work of this paper is practical and logical.

We are grateful for this comment by the reviewer.

> However, there are some issues to be further improved as follows. 1. Regarding UA-ADRCs: Did the authors have direct proof that UA-ADRCs are indeed injected into the tendon

We have specified this. UA-ADRCs were injected (controlled by biplanar X-ray imaging) adjacent to the injured supraspinatus tendon immediately after isolation. Injection directly into the tendon would not made have sense because injection into a combined partial-thickness tear of the supraspinatus tendon (PASTA) would bear the risk that the cells evade into the joint space. The results of the present study demonstrate the validity and significance of this approach.

We have incorporated this information in the *Abstract* and *Treatment* sections of our revised manuscript.

> and evidence that UA-ADRCs can survive, proliferate and differentiate for a long time? Since UA-ADRCs are difficult to be labeled on the cell surface, intracellular labeling can be performed with fluorescent probes to track the behavior of cells in tendon. This is an important theory basis for this study.

This statement by the reviewer may reflect a general misconception of UA-ADRCs. As stated above, UA-ADRCs can by definition not be labeled because this would render them modified. What is actually addressed here by the reviewer are adipose derived stem cells (ADSCs) that can be derived from UA-ADRCs by culturing (c.f., e.g., the comprehensive discussion of the difference between UA-ADRCs and ADSCs in Section 2.3 of our publication Alt et al. (Cells 2020;9;1097; doi:10.3390/cells9051097) titled "*Towards a comprehensive understanding of UA-ADRCs (uncultured, autologous, fresh, unmodified, adipose derived regenerative cells, isolated at point of care) in regenerative*

medicine"). In contrast to UA-ADRCs, ADSCs can indeed be intracellularly labeled with fluorescent probes, as demonstrated by ourselves (Fig. 6 in Alt et al., 2020) and many others. Furthermore, ADRCs have been demonstrated to survive, proliferate and differentiate for a long time in the host tissue (discussed in detail in Section 4.3 of our publication Alt et al., 2020). Because UA-ADRCs cannot be labeled, it is in principle not possible to provide the same evidence for UA-ADRCs as has be done in the literature for ADCS.

We have incorporated this important information in the *Discussion* section of our revised manuscript.

> 2. Regarding physical examination: Before treatment, the authors showed the ASES score and the physical assessments of the patient related to rotator cuff injury, but why did the authors not mention the improvement of ASES score and related physical examinations after 10 weeks of treatment? The ultimate goal of this therapy is to improve the clinical manifestations after treatment.

We are grateful for this comment by the reviewer. Indeed, the ASES total score improved from 12 at baseline to 79 at ten weeks post treatment, indicating clinical efficacy of the treatment.

We have incorporated the corresponding information in the *Treatment* section of our revised manuscript.

> 3. Regarding histological assessment: A standardized histological scores should be performed for tissues in different regions, including cell density, cell morphological changes, collagen arrangement, neovascularization, ground substance and calcification. The authors can refer to the Bonar scores which is widely used in tendon histological and pathological assessment. A semi-quantitative standardized assessment is helpful to establish a unified histological criterion for future clinical trials.

The Bonar score is a well-established scoring system to classify the histopathological findings of tendinopathy and tendon degeneration (Cook et al., J Orthop Res 2004;22(2):334-338; Maffulli et al., Clin Orthop Relat Res 2008;466(7):1605-1611; Fearon et al., J Sci Med Sport 2014;17(4):346-350). This was not addressed in our study, and was the reason why we did not apply the Bonar score in our manuscript.

However, we have now incorporated Bonar scores in our revised manuscript (Table 3). Bonar scores were determined by Mr. Tobias Wuerfel, MS, a specialist in quantitative histology at the Department of Anatomy at LMU Munich who was not involved in the treatment of the patient. Mr. Wuerfel is listed as co-author of our revised manuscript. It should be mentioned that the results of the analyses performed by Mr. Wuerfel have not changed the general conclusions of our study.

> 4. Regarding immunohistochemical evaluation: (1) Scar tissue is mainly composed of disordered type III collagen which generally considered to represent higher hardness but lower strength. It is suggested to supplement the immunohistochemical staining of type III collagen and a semi-quantitative analysis of the content of type I/III collagen, so that

the content and arrangement of collagen in regenerative and degenerative parts can be compared more intuitively.

We are grateful for this comment by the reviewer.

Immunohistochemical detection of type III collagen is shown in Figure 11 and addressed in the *Outcome and Follow-up* section of our revised manuscript. Type III collagen was found in the degenerative tissue but not the regenerative tissue in the second part of the biopsy investigated in our study. Of note, the patterns of type I procollagen and type I collagen detection (Figures 9 and 10 in our revised manuscript) were inverse compared to the pattern of type III collagen detection (Figure 11 in our revised manuscript). This finding further corroborated our hypothesis that treatment of symptomatic, partial-thickness rotator cuff tears with UA-ADRCs results in regenerative healing without scar formation.

（2）Macrophages play an important role in inflammation and immunoregulation after tendon injury. Different phenotypes of macrophages and their different duration of continuous aggregation at the injured tissue have different or even opposite effects on tendon repair. It is suggested that different phenotypes of macrophages should be further detected to clarify the role of macrophages in tendon repair.

In general, we agree with the reviewer in this point. However, for the following reasons this is beyond the scope of the present study: (i) our study did not address inflammation and immunoregulation after tendon injury but histological regeneration of an injured human tendon after injection of UA-ADRCs; (ii) only a single timepoint was investigated; (iii) no control treatment was performed and no spontaneous course after tendon injury was investigated, and (iv) immunolabeling for CD68 was only found in a few cells in the investigated biopsy ten weeks after injection of UA-ADRCs (Figure 13 in our initial manuscript and Figure 15 in our revised manuscript). Detection of different phenotypes of macrophages to clarify the role of macrophages in tendon repair and their interaction with UA-ADRCs would require controlled trials with repeated taking of biopsies. This may be achieved in animal studies in the future.

Accordingly, we have not modified our manuscript based on this comment by the reviewer.

(3) Ki-67 often is often considered to indicate the activity of cell proliferation. But why the cells with high expression of Ki-67 outside the blood vessel could indicate the presence of injected UA-ADRCs and their non-endothelial Descendants which is mentioned in your manuscript. Please further explain it with proper experiments or literature.

We agree with the reviewer that mechanistic proof of concept that cells outside blood vessels that are immunopositive for Ki-67 are indeed injected UA-ADRCs and their non-endothelial descendants would be highly desirable. However, corresponding investigations would require to label the injected cells, which is in principle not possible

in case of UA-ADRCs (as outlined in detail above). Accordingly, experiments as proposed by the reviewer cannot be performed.

Accordingly, we have not modified our manuscript based on this comment by the reviewer.

Reviewer's code: 03840803

This interesting report describes a case of symptomatic, partial-thickness rotator cuff tear resulting in painfully restricted mobility of the right shoulder treated with a single injection of fresh, uncultured, autologous adipose-derived regenerative cells (UA-ADRCs) into the supraspinatus tendon. As highlighted by the authors, the main strength of this study is the fact that it is the first report demonstrating the regenerative healing of a partial-thickness tear in a human tendon following local injection of UA-ADRCs. To this aim, the authors have carried out a comprehensive histological and immunohistochemical analysis of the biopsy taken from this tendon 10 weeks post UA-ADRC injection. In particular, the injection of fresh UA-ADRCs was executed within the same interventional procedure that comprised harvesting of autologous abdominal adipose tissue, isolation of cells and transcutaneous injection within 2 hours without cell culturing. Histology and immunohistochemistry assessment of the supraspinatus tendon taken after 10 weeks during open revision procedure of the injured infraspinatus tendon, which had not been treated with UA-ADRCs, revealed that the injured and partially ruptured supraspinatus tendon was significantly improved after injection of UA-ADRCs through growth of new tendon tissue, which was in line with the overall significant improvement of clinical symptoms. Collectively, the results of this study are relevant because indicate, for the first time, that treatment of an injured human tendon with UA-ADRCs can enable regenerative healing. It is of special importance that these beneficial effects were achieved without prior manipulation, stimulation and/or reprogramming of the autologous cells injected. Even if the study has intrinsic limitations being single case-based, such an approach described for the first time in a human patient has the potential to pave the way for the development of novel regenerative treatment options for tendon injuries. Overall, the study is well-organized and well-presented.

We are grateful for this comment by the reviewer.

However, the following points deserve consideration by the authors in order to improve the clarity of presentation and the relevance of the findings/conclusions:
- Table 1 (antibodies used for immunohistochemistry) is not sufficiently clear and explicative in its current form. Please reorganize this table by using different columns with appropriate headings. Moreover, antibody "name" should be "catalog no.".

We have reorganized Table 1 in our revised manuscript as proposed by the reviewer.

- Table 2 – scoring of immunostaining. Please use "+/-" instead of "(+)" to indicate "minimal presence of staining".

We have modified Table 2 in our revised manuscript as proposed by the reviewer.

- Since you are referring to "uncultured, autologous adipose-derived regenerative cells" with the acronym "UA-ADRCs", "ADSCs" should be used for "adipose-derived stem cells" instead of "ASCs".

Both "ASCs" and "ADSCs" are used in the literature as abbreviation of "adipose derived stem cells".

Nevertheless, we have replaced "ASCs" by "ADSCs" in our revised manuscript as proposed by the reviewer.

> - Please carefully check the text for misspelling and typing errors. For instance, “intermittend” at page 14.

We have checked our revised manuscript for misspelling and typing errors as proposed by the reviewer.

> - Figures 2 and 3 should be merged in a single figure in order to improve the clarity of presentation. Moreover, a same panel illustrating type I collagen staining is present in both figures.

We would like to disagree with the reviewer in this (minor) point. It is correct that both Figures 2C and 3A illustrate type I collagen staining. However, both figures have a different focus. Figure 2 shows adjacent sections that were processed for detection of type I collagen, aggrecan and type II collagen. In contrast, Figure 3 demonstrates the appearance of type I collagen in brightfield and polarized light microscopy. The conclusions that can be drawn from Figure 2 cannot be drawn from Figure 3, and vice versa.

Accordingly, we have not modified our manuscript based on this comment by the reviewer.

> - In the Results section, it is not sufficiently clear the description of differences in basic histology among the six different regions from the second part of the tendon biopsy illustrated in Figure 4. This is crucial, since such a biopsy has been used for all subsequent immunohistochemical analyses with much emphasis on differences in immunostaining among the six different regions.

Unfortunately the reviewer did not specify what "not sufficiently clear" actually means; this topic was not addressed by the other reviewers.

Nevertheless, we have performed a quantitative histological analysis of all investigated sections, the results of which are summarized in Table 4 in our revised manuscript.

> - Results section – The authors state the following: “CD34 is considered a common progenitor cell marker and is expressed by a wide range of cell types, including bone marrow hematopoietic stem cells, mesenchymal stem cells (MSCs) and endothelial progenitor cells”. This statement is not correct. Indeed, the CD34 antigen is commonly expressed by fully differentiated endothelial cells of blood vessels in nearly every tissue, not only in endothelial progenitor cells as affirmed by the authors.

Unfortunately this statement by the reviewer came without reference to the corresponding literature. In contrast, a review by Müller et al. (Exp Mol Pathol 2002;72:221-229) came to the conclusion that *CD34 is primarily expressed by small or newly formed vessels and endothelial cells of endothelial tumors… while endothelial cells of larger veins, the placenta and lymphatic tissue have been reported to be CD34 negative….* A more recent review by Sidney et al. (Stem Cells 2014;32:1380-1389) concluded that *CD34 is widely regarded as a marker of vascular endothelial progenitor cells* and *There is a subset of noncirculating adult endothelial cells that are also CD34+, most notably located within smaller blood vessels, while most endothelial cells in larger veins and arteries are CD34-*. These reviews clearly support the description of the role of CD34 in our manuscript, not the statement by the reviewer (without reference to the literature).

We have added references to the reviews by Müller et al. (2002) and Sidney et al. (2014) in our revised manuscript.

> Furthermore, the immunopositivity for CD34 in the endothelium cannot be considered a marker of angiogenesis as commented by the authors in different parts of the Results section. For instance, please see the following sentences: "Accordingly, the presence of CD34+ immunolabeling in endothelial cells of microvessels in Regions D and F of the second part of the investigated biopsy (Figure 5D and F) indicate ongoing angiogenesis in highly specific regions of the investigated biopsy ten weeks post injection of UA-ADRCs", and "The presence of immunolabeling for Ki-67 in cells inside microvessel walls in Region D of the second part of the investigated biopsy (Figure 6D) is in line with the presence of immunolabeling for CD34 in endothelial cells of microvessels in this region (Figure 5D)". In order to verify the presence of an ongoing angiogenic process, other markers need to be employed. For instance, VEGF? This would seem appropriate, especially considering that in Figure 13 the authors refer to the presence of CD68+ macrophages which are known to produce different molecules orchestrating tissue remodeling/regeneration and angiogenesis, among which VEGF.

We could have performed anti-VEGF immunohistochemistry, but considering the limited number of sections that were left from the second part of the biopsy we decided to rather detect type IV collagen. The macromolecular network of type IV collagen provides the scaffold for basement membranes (Kühn, Matrix Biol 1995;14:439-445); type IV collagen is the most abundant member of the basement membrane (Rhodes and Simons, J Cell Mol Med 2007;11:176-205). Thus, immunohistochemical detection of type IV collagen is suitable for detecting vessels in connective tissue independent of endothelial markers.

Figure 6 in our revised manuscript shows immunohistochemical detection of type IV collagen in a section of the second part of the biopsy that was investigated in this study. As expected, immunolabeling for type IV collagen was found in the basement membrane of microvessels in all regions (black arrows in Figure 6), except for Region C in which no microvessels were found. Of note, by immunohistochemical detection of type IV collagen many vessels were found in the degenerative tendon tissue (i.e., those areas that included Regions B and C in Figures 4-15), whereas no immunolabeling for CD34 was found in the degenerative tendon tissue. This finding demonstrates that the

majority of endothelial cells in the second part of the biopsy that was investigated in this study was not immunopositive for CD34, which is in line with the aforementioned reviews by Müller et al. (2002) and Sidney et al. (2014) and clearly contrast the view of the reviewer. The additional finding of cells in the walls of small vessels at the putative side of injection of UA-ADRCs that were immunopositive for Ki-67 (Figure 7D in our revised manuscript) support our hypothesis that the presence of CD34+ immunolabeling in endothelial cells of microvessels in Regions D and F of the second part of the investigated biopsy (Figure 5D and F in our manuscript) indicate ongoing angiogenesis in highly specific regions of the investigated biopsy ten weeks post injection of UA-ADRCs.

We have incorporated this information in the Outcome and Follow-up section of our revised manuscript.

> - Another crucial point concerning CD34 – The authors state that "More than 50% of freshly isolated MSCs express the CD34 cell marker; however, human cultured MSCs are commonly immunonegative for CD34 [27]" and "On the other hand, anti CD34 immunohistochemistry could not be used to assess the potential presence of injected UA-ADRCs and their non-endothelial descendants in the investigated biopsy". In my opinion, the point of view of the authors is rather questionable and represent a major weakness of the study. Indeed, CD34 expression is known to be progressively lost after multiple passages in culture, but the authors should carefully consider that in the present study they have used freshly isolated cells without cell culturing. Therefore, one would expect to detect the injected stem cells by CD34 immunostaining, if they have really stably integrated in the host tissue.

This statement by the reviewer is based on her/his view on CD34 immunostaining that is neither supported by the literature nor by the outcome of the present study (outlined in detail above). This statement would be correct if all (or almost all) endothelial cells would express CD34. However, as outlined in detail above this is not the case.

Accordingly, we have not modified our manuscript based on this comment by the reviewer.

> Moreover, it is well-known that CD34+ stromal cells (also recently referred to as telocytes) reside in the stromal compartment of nearly every organ. Besides CD34, why the authors have not used other possible markers to analyze the presence of the injected UA-ADRCs in the host healing tissue? This seems very important in order to clarify whether the reported beneficial effects may be due to an effective integration of the injected cells in the host tissue microenvironment or rather they are due to paracrine effects. This point is of crucial importance and deserves particular attention.

The inability to label UA-ADRCs was already addressed above.

With respect to paracrine effects of injected stem cells, it is correct that mesenchymal stem cell-derived exosomes have been shown to promote tendon regeneration by facilitating the proliferation and migration of endogenous tendon stem and progenitor cells (e.g., Yu et al., 2020). Thus, one cannot exclude that paracrine effects may play a

certain role in histological regeneration of injured human tendon tissue after injection of UA-ADRCs. On the other hand, the latter would only limit the significance of our results if histological regeneration of injured human tendon tissue after injection of UA-ADRCs would be fully due to paracrine effects (only in this case one could replace injection of UA-ADRCs by injection of exosomes or secretomes of mesenchymal stem cells). However, there is no evidence in the literature supporting this view. In this regard a recent systematic review (Rhatomy et al., Stem Cells Transl Med 2020;9:895-902) summarized five studies in which the potential of stem cells conditioned medium (secretome) in ligament and tendon healing was investigated. Of note, in none of these studies injection of stem cells conditioned medium was compared to injection of UA-ADRCs or ADSCs. In summary, the question about potential contribution of paracrine effects to the histological regeneration of injured human tendon tissue after injection of UA-ADRCs as described in this study cannot be answered at this time. Clarifying the role of paracrine effects in tendon repair after injection of UA-ADRCs would require controlled trials with repeated taking of biopsies. This may be achieved in animal studies in the future.

We have incorporated this information in the *Discussion* section of our revised manuscript.

> - Figure 5 legend: “the red arrows in Panel B indicate cells inside a microvessel”. Such a microvessel is not stained by CD34. Are the authors sure that these are cells inside a microvessel? If so, why the microvessel is not stained by CD34? Perhaps it could be a lymphatic vessel (lymphatic vessels do not express CD34 at variance with blood vessels). It is strongly recommended the use of an additional, reliable endothelial cell marker such as CD31/PECAM1 that is a pan-endothelial marker expressed in both blood and lymphatic vascular endothelial cells. In addition, to discriminate the presence of lymphatics, the authors may further use a lymphatic endothelial cell specific marker, like PDPN or LYVE-1.

This statement by the reviewer is based on her/his view on CD34 immunostaining that is neither supported by the literature nor by the outcome of the present study (outlined in detail above). This statement would be correct if all (or almost all) endothelial cells would express CD34. However, as outlined in detail above this is not the case.

In order to make this point more clear we have replaced Figure 5B by an even more appropriate panel. The microvessels that are shown in this panel were found in the degenerative tissue and were clearly immunonegative for CD34; the cells found inside these microvessels show the typical morphological appearance of erythrocytes with no staining of cell nuclei (which are not present in erythrocytes).

> - Results section - “The abundant intracellular and extracellular presence of tenomodulin in Region D of the second part of the biopsy (Figure 7D) is in line with the hypothesis that tendon regeneration observed in the investigated biopsy was 'orchestrated' from this region, further supporting the hypothesis that Region D in the second part of the investigated biopsy hosted injected UA-ADRCs and their descendants (c.f. Figure 4D)” and “Thus, the presence of intracellular and extracellular immunolabeling for laminin in

> Regions D and E of the second part of the investigated biopsy (Figure 10D and E) are in line with the hypothesis that precursors of endothelial cells and tenocytes migrated from Region D (where they were generated) via Region E to Region F where tendon regeneration took place". Again, in order to support the conclusions of the authors it would be of crucial importance the identification of the injected UA-ADRCs within the host tissue. Otherwise, it is not possible to affirm with certainty that the injected cells have stably integrated in the host tissue giving rise to tenocytes. The observed effects could be given to paracrine effects exerted mainly in the first days after local cell injection, without stably integration and differentiation processes of the injected stem cells.

This has been addressed in detail above (labeling of UA-ADRCs, potential paracrine effects of UA-ADRCs).

Accordingly, we have not modified our manuscript based on this comment by the reviewer.

> - Please carefully check results on MMP-2 and MMP-9. There is much confusion in legends of Figures 11 and 12. The authors are referring to MMP-9 in the legend of Figure 12 titled "MMP-2" and the other way around for Figure 13.

We are grateful for this comment by the reviewer. The description of MMP-2 and MMP-9 in Figures 11 and 12 in our initial manuscript was indeed confused.

We have corrected this in the legends of Figures 13 and 14 in our revised manuscript.

> - Discussion section – The authors state: "Our comprehensive immunohistochemical analysis of the biopsy with a broad number of antibodies (Tables 1 and 2) allow the conclusion that Region D in the second part of the biopsy (Panels D in Figures 4-13) represented the site of injection of UA-ADRCs from where tendon regeneration started. It further indicates that endothelial precursors and tenocytes might have migrated from Region D (where they were generated) via Region E to Region F, in which tendon regeneration took place". As already commented, the current study design does not allow these strong conclusions. In order to support such strong conclusions, it would be of crucial importance the identification of the injected UA-ADRCs within the host tissue. Otherwise, it is not possible to affirm with certainty that the injected cells have stably integrated in the host tissue giving rise to tenocytes. The observed effects could be given to paracrine effects exerted mainly in the first days after local cell injection, without stably integration and differentiation processes of the injected stem cells.

This has been addressed in detail above (labeling of UA-ADRCs, potential paracrine effects of UA-ADRCs).

Accordingly, we have not modified our manuscript based on this comment by the reviewer.

> - Discussion section – The authors state: "Conclusions of this study are presented on the basis of a single time point analysis of molecular and cellular events. As such, limitations consist in the fact that only a single patient was investigated; no control biopsy was analyzed, and the scientists who analyzed the biopsy were not blinded". According to my

above comments on the unidentified fate of the injected UA-ADRCs and based on the current study design, clearly the limitations of the study are not only those discussed by the authors.

This has been addressed in detail above (labeling of UA-ADRCs, potential paracrine effects of UA-ADRCs; expression of CD34 by endothelial cells).

Accordingly, we have not modified our manuscript based on this comment by the reviewer.

Re-review
Reviewer's code: 03840803
The reviewer respectfully disagree with the authors with reference to previous comments related to the use of the CD34 marker. CD34 is widely used and validated as pan-endothelial cell marker in immunohistochemical analyses. CD34 is expressed in the adult with some heterogeneity by blood endothelial cells in most vascular beds. It is a pan-endothelial marker of microvascular endothelial cells that is not expressed by most large vessel endothelial cells. Blood capillary endothelial cells are CD34+ in nearly every tissue. For instance, please see: - Baumhueter S, Dybdal N, Kyle C, Lasky LA. Global vascular expression of murine CD34, a sialomucin-like endothelial ligand for L-selectin. Blood. 1994;84:2554–2565. - Fina L, Molgaard HV, Robertson D, Bradley NJ, Monaghan P, Delia D, Sutherland DR, Baker MA, Greaves MF. Expression of the CD34 gene in vascular endothelial cells. Blood. 1990;75:2417–2426. Moreover, please see The Human Protein Atlas for reference: https://www.proteinatlas.org/ENSG00000174059-CD34/tissue Therefore, all relevant concerns on CD34/vessel staining/angiogenesis that have not been addressed by the authors should be carefully reconsidered.

This statement by the reviewer raises a number of important concerns with respect to reviewing a manuscript submitted to an academic journal:

- The reviewer did not refer to the new data in our revised manuscript (Fig. 5B (lack of immunohistochemical detection of CD34 in certain vessels, including capillaries) and Fig. 6 (immunohistochemical detection of type IV collagen).
- The reviewer did not comment on the literature cited by us related to CD34. We would like to highlight the most important quotes from this literature (references to other publications in this literature was removed):
    - Marvasti et al., Can J Cardiol 2019; 35: 1311-1321 (reference #26 in our revised manuscript):
      "*CD34 is a conserved marker of human and murine endothelial progenitor cells* [EPCs]. *Examination of cell surface markers expressed by human EPCs demonstrate that human EPCs are found within the CD34+/CD45-/VEGFR2+ population. Isolation and characterization of CD34+ cells from human peripheral blood demonstrated that these cells are capable of endothelial cell colony forming activity in-vitro and incorporate into active sites of angiogenesis when injected in rodent models of hind limb ischemia. CD34 is also considered a marker of EPCs in mice. Early studies in mice used Sca-1 as well as CD34 expression to identify EPCs capable of promoting blood vessel formation following*

endothelial or ischemic injury. Recent studies have also demonstrated that mouse bone marrow CD34+ cells possess endothelial colony forming ability and can incorporate into newly formed blood vessels post-MI."

- Müller et al., Exp Mol Pathol 2002; 72: 221-229 (reference #27 in our revised manuscript):
  "*CD34 is primarily expressed by small or newly formed vessels and ECs* [endothelial cells] *of endothelial tumors…while ECs of larger veins, the placenta and lymphatic tissue have been reported to be CD34 negative... CD34 is assumed to play a role in the formation of endothelial adherens junctions, which are key components of angiogenesis… In embryogenesis the sprouting of new capillaries from existing vessels is associated with high CD34 levels… After birth CD34 mRNA decreases but increases again in newly formed blood vessels in wound healing and tumor growth… Therefore, the strong expression in capillaries as the starting point of new vessel sprouts would be explainable."*
- Sidney et al., Stem Cells 2014; 32: 1380-1389 (reference #28 in our revised manuscript):
  "*CD34 is widely regarded as a marker of vascular endothelial progenitor cells …These BM* [bone marrow] *-derived cells are found circulating in peripheral blood…and their usefulness in proangiogenic therapies has been extensively researched… There is a subset of noncirculating adult endothelial cells that are also CD34+, most notably located within smaller blood vessels, while most endothelial cells in larger veins and arteries are CD34-... It has been proposed that this CD34+ subpopulation is homologous to sprouting tip cells, a specialized type of endothelial cell present at the leading edge during in vivo angiogenesis… CD34 is strongly expressed on the filopodia of these tip cells at sites of active angiogenesis and evidence yet again emphasizes the important functional role for CD34 in progenitor cell activity."*

Taken together, this literature fully supports our findings and their interpretation in our revised (as well as in our original) manuscript.

- The study by Baumhueter et al. (Blood 1994;84:2554–2565) cited by the reviewer is freely available at https://www.sciencedirect.com/science/article/pii/S0006497120713741?via%3Dihub. This study was performed on mouse tissue; differences between human and murine CD34 with respect to expression and regulation were discussed by Marvasti et al. (2019).
  The following tissues were investigated by Baumhueter et al. (1994) (Table 1 therein): lymph nodes, thymus, spleen, bone (sternum, vertebrae), brain, pituitary gland, eye, lacrimal gland, salivary gland, esophagus, stomach, small and large intestine, liver, pancreas, trachea, lung, kidney, ovary, uterus, cervix and vagina, mammary gland, skin, skeletal muscle, cardiac muscle, thyroid gland and adrenal glands.
  In this regard it is of note that Baumhueter et al. (1994)
  - did not investigate tendon tissue and, in particular, histological regeneration of injured human tendons,
  - only showed photomicrographs of immunohistochemical detection of CD34 in

brain, kidney, lymph node, thymus, pancreas, bone marrow and skin,
  - did not specify whether all endothelial cells in capillaries in the investigated tissues expressed CD34, as well as whether all capillaries in the investigated tissues showed CD34+ endothelial cells, and
  - did not investigate the role of CD34 expression in angiogenesis.

- The study by Fina et al. (Blood 1990;75:2417–2426) cited by the reviewer is freely available at https://www.sciencedirect.com/science/article/pii/S0006497120831425?via%3Dihub. This study was performed on human tissue.
  The following tissues were investigated by Fina et al. (1990) (Table 1 therein): fetal tissue: foot, kidney, heart, cerebral cortex, liver, skin; normal tissue from adults: kidney, skin, breast, spinal cord, brain (cerebellum, olivary nucleus, striatum, hypophysis); pathologic tissue: granulation tissue, angiomyosarcoma, leiomyosarcoma and hypernephroma.
  In this regard it is of note that Fina et al. (1990)
  - did not investigate tendons and, in particular, histological regeneration of injured human tendons,
  - only showed photomicrographs of immunohistochemical detection of CD34 in placenta, umbilical artery, fetal skin, breast and angioblastoma,
  - did not specify whether all endothelial cells in capillaries in the investigated tissues expressed CD34, as well as whether all capillaries in the investigated tissues showed CD34+ endothelial cells, and
  - did not investigate the role of CD34 expression in angiogenesis.

- The Human Protein Atlas cited by the reviewer is freely available at https://www.proteinatlas.org/about. According to https://www.proteinatlas.org/ENSG00000174059-CD34/tissue/primary+data endothelial cells were explicitely investigated only in the cerebral cortex and colon. Of note,
  - tendons and tendon tissue are not considered in the Human Protein Atlas (https://www.proteinatlas.org/humanproteome/tissue), and
  - neither the sections showing skeletal muscle nor the sections showing soft tissue in the Human Protein Atlas show tendon tissue (https://www.proteinatlas.org/ENSG00000174059-CD34/tissue/skeletal+muscle and https://www.proteinatlas.org/ENSG00000174059-CD34/tissue/soft+tissue),

  Accordingly, the Human Protein Atlas is not related to the topic investigated in our manuscript (histological regeneration of injured human tendons after injection of UA-ADRCs).

In summary, the studies by Fina et al. (1990) and Baumhueter et al. (1994) cited by the reviewer represent an early state of knowledge about CD34 in the literature (without consideration of tendon tissue), whereas the studies by Müller et al. (2002), Sidney et al. (2014) and Marvasti et al. (2019) cited in our revised manuscript represent the current

knowledge about CD34 in the literature. The data presented in our revised manuscript are in line with the current knowledge about CD34 outlined in Müller et al. (2002), Sidney et al. (2014) and Marvasti et al. (2019). Of note, the earlier findings by Fina et al. (1990) and Baumhueter et al. (1994) do not contradict the current knowlegde about CD34 as outlined by Müller et al. (2002), Sidney et al. (2014) and Marvasti et al. (2019) (in fact, Müller et al. (2002) cited Baumhueter et al. (1994), and Sidney et al. (2014) cited Fina et al. (1990)). Furthermore, the findings by Fina et al. (1990) and Baumhueter et al. (1994) as well as the data provided in the Human Protein Atlas do not contradict the findings in our study. Fina et al. (1990) and Baumhueter et al. (1994) did not investigate tendon tissue and histological regeneration of injured human tendons; the Human Protein Atlas is not related to the topic investigated in our manuscript.

Accordingly, we have not modified our manuscript based on this comment by the reviewer.