nature portfolio

Peer Review File

Complementing Muscle Regeneration: Fibro-Adipogenic Progenitor and Macrophage-Mediated Repair of Elderly Human Skeletal Muscle

Corresponding Author: Professor Jean Farup

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Brorson et al reports the spatial transcriptomic profiling of muscle regeneration in humans and derives new biological conclusions for the understanding of cellular crosstalks during muscle repair in humans. The main conclusions are validated using independent technologies (FACS, cellular co-culture)and a specific cross-talk between FAPs and macrophages via complement C3 is further explored. While several studies have profiled human muscle at single cell level and many rodent studies have characterized the single cell response to muscle regeneration, this work is the first study reporting human muscle regeneration with spatial resolution and cellular cross-talk information. It will be an important resource for the field both for data mining and cellular understanding of human muscle regeneration, provided that some important points explained below can be addressed, in particular linked to the age of subjects (point 2) and resolution of injured vs non injured myofibers in the muscle biopsies (point 4 below).

1/ The study design comes with some limitations since only 3 subjects per condition were profiled. In addition the samples come from a larger intervention CT with 32 participants (NCT03754842). While I understand the limitations of running the spatial analyses on a broad number of samples, it would be important that the authors explain in the methods how the samples analyzed were selected. Also were any of the NR/pterostilbene group analyzed?

2/ One part that is surprising in the research methodology is that the analysis was ran in older people. This is a major limitation as it is unclear if the conclusions relate to healthy productive muscle regeneration or impaired muscle regeneration during aging. A validation of the main conclusions in a model of human muscle repair in young subjects would be important, this can be done on existing samples of other studies.

3/ It is unclear how statistical analyses of the spatial transcriptomics time course were performed. Were analyses paired by subject?

4/ Muscle injury in human muscle has a heterogenous response with some fibers undergoing necrosis/regeneration (typically losing dystrophin and re-expressing embryonic myosin during regeneration) while others remain uninjured. Does the resolution of the spatial transcriptomics allow to differentiate the response around injured fibers vs non injured fibers of the same muscle? In Fig.1B-C it would be important to dissociate the direct response of a regenerating fiber from the indirect response of an uninjured myofiber.

5/ Fig 1: Typical controls for muscle regeneration by histology and/or FACS should be provided for the 3 donors (extent of injury, Pax7 response, MyoD activation…)

6/ Fig 2B & D: muscle stem cells which are the driving cells for regeneration are under-explored. Can the authors separate quiescent and activated muscle stem cells? There is also a risk that the correlation between FAPs and immune cells in Fig 2D is driven by the cell types that have the highest response to injury while the muscle stem cell response is diluted by quiescent cells. Are the correlations with niche cells similar for quiescent and activated muscle stem cells?

7/ In Fig 2C, it is unclear how relative amounts of cells were quantified as results are presented as fold change before injury.

It is important to report if quantification is per mg of tissue or per total number of cells in homogenates as number of mononucleated cells will change during regeneration with immune infiltration. Also it would be important to validate the main conclusions on tissue sections by immunostaining

8/ Line 275 & Figure S5B: why is C3 present in injured myofibers if secreted soecifically by FAPs? Is this soluble C3 in the extracellular space after fiber clearance?

9/ Fig.4F: while effects on clearance of apoptotic myoblasts in vitro are interesting, the groups of Benedicte Chazaud and Michael Kjaer have demonstrated that macrophage polarization controls myogenic fate of muscle stem cells, including in human muscle regeneration. It would be important to test how C3 treated macrophages influence myogenic fate in a coculture or conditioned medium assays with muscle stem ceéés

Minor:
-Figure 4G: the labels of the figure with and without phagocytosis conditions should be added to facilitate understanding

-Line 29: the authors should remove single cell transcriptomic from the abstract as it is misleading with single cell sequencing after isolation. What the authors have done here is in silico single cell deconvolution of spatial transcriptomics.

Reviewer #2

(Remarks to the Author)
The capacity to regenerate skeletal muscle function after injury requires a complex and well-coordinated cellular response, which is challenged in aged skeletal muscle. In this paper, the authors combined spatial, temporal, and single cell transcriptomics to reveal new information about the dynamics of elderly human skeletal muscle regeneration.

The major strengths of the manuscript are:

1) Using spatial RNA sequencing to the expression of human protein-coding genes in elderly human skeletal muscle biopsies before and 2-, 8-, and 30-days post injury.

2) Authors used single Cell-Spatial deconvolution analysis to determine that monocytes/macrophages and fibro-adipogenic progenitors (FAPs) are central players in human muscle regeneration.

3) Authors also provided intriguing data from Spatial correlation analysis to show FAPs and monocytes/macrophages co-localization and intercellular communication, mediated by complement factor C3.

4) They used Immunostaining approaches to confirm C3 expression in FAPs and FAP secretion of C3 and concluded that FAP secretion of C3 suggested a role in phagocytosis of necrotic muscle cells.

5) Authors used specific functional assays to demonstrate C3's impact on human monocyte metabolism, survival, and phagocytosis, and concluded its involvement in skeletal muscle regeneration.
Authors concluded that their studies elucidate FAP-macrophage interplay in aged human muscle with perspectives for future therapeutic interventions to reduce the aged-induced decline in regenerative capacity.

The work in this manuscript is extremely important for the area of aging and muscle regeneration. The studies are indeed state of the art and should advance our understanding of muscle regeneration during aging. Some weaknesses noted are the following:

A) One of the major problems of the manuscript is that while the study is about aged human muscle regeneration, only one figure shows very limited data of the regeneration process in the human cells (Fig. 1).

B) In Fig. 1, only H&E staining is shown for the muscle cell regeneration.

C) Throughout the manuscript, the authors attempt to demonstrate the role of monocytes, macrophages, and FAPs, but in exploring their roles, there was not concomitantly data on muscle cell regeneration.

D) In the field of muscle of muscle regeneration, it is widely accepted that DAPI and Myosin Heavy Chain are labeled for determination of Fusion Index (FI), which is never determined under any condition in this manuscript.

E) While the work is obviously valid and very important as it is, it could add some value if the authors could contrast some of their findings with known databases in young muscle cells or conduct at least a basic overall experiment in biopsies from young donors.

F) Supplementary Figure 6: These experiments are conducted with monocytes from human peripheral blood, not from any cells directly obtained from the muscle biopsies. Authors are trying to prove a point that C3 changes the function of monocytes. Why then not add C3 as well to young muscle cells to observe a potential phenotype?

G) Statistical analyses: Authors do not describe if all data was parametric. They just mentioned ANOVA and T-test. They do not include any Post correction tests, such as Tukey's or Bonferroni.

Reviewer #3

(Remarks to the Author)
In this study Brorson et al. perform spatial single-cell RNAseq on skeletal muscle, before, 2-, 8-, and 30-days post injury. They identify immune-enriched and regenerative clusters at 8 dpi. Through deconvolution they highlight the role of FAPs and monocytes in regeneration. With receptor-ligand analysis they identify complement C3 as pivotal player.

Major:
- As the regenerative capacity of human muscle declines with age it would have been interesting to compare the regeneration after injury between young and old muscle. The authors could use publicly available single cell datasets to study if the genes involved in regenerating old muscles are also differentially expressed in young vs. old muscle.
- In Figure 1B, why aren't the clusters well separated and common cell types identified like in Figure 3D? In this sense it could have been interesting to perform the same experiment with a more classical single-cell RNA-seq (not spatial).
- Figure 1F. Instead of visualizing GO modules, the authors should try to visualize actual genes.
- It would be interesting combine H&E, immuno-staining and spatial transcriptomics on the same samples, as theoretically possible in the Visium technology.
- It is not completely clear how the authors took advantage of the spatial technology compared to classical single cell RNAseq.

Minor:
- Figure 1C. The changes are from which time point compared to which?
- The author may use a pseudo-bulk approach to understand the broad dynamics before going into the single cell.
- Figure 1B. What are the top genes in each cluster?
- Could the authors confirm by immuno-staining at different timepoints some of the top genes they observe in Figure 1?
- Can the authors comment on the lack of correlation between Figure 2 B and C, especially regarding the FAPs. Here also there could be a time-resolved immuno-staining to confirm.
- The authors should explain more what is behind the receptor-ligand analysis, since this is not common practice.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have performed a serious and solid revision and addressed all my comments.
I just could not find the new supplementary figure 2B mentioned in the manuscript and rebuttal

Reviewer #2

(Remarks to the Author)
The authors have made a major effort to address all major criticisms and added additional analyses requested by all reviewers. The manuscript rigor was also enhanced by the increased N (sample size) and enhanced statistical analyses, as well as some of the added clarifications to the results and methods.

Reviewer #3

(Remarks to the Author)
Major:
- As the regenerative capacity of human muscle declines with age it would have been interesting to compare the regeneration after injury between young and old muscle. The authors could use publicly available single cell datasets to study if the genes involved in regenerating old muscles are also differentially expressed in young vs. old muscle.

Ok. Here I would have thought more about a direct comparison of DEGs involved in regeneration vs. DEGs in Y vs. O. Might also want to look at [PMID: 36516485], in addition to Kedlian, V.R., et al.

- Figure 1F. Instead of visualizing GO modules, the authors should try to visualize actual genes.

I understand but I still believe that visualizing the spatial expression of a few selected genes deemed important by the authors would add a lot of value to the paper.

- It is not completely clear how the authors took advantage of the spatial technology
compared to classical single cell RNAseq.

I was referring to Figure 1F. I believe this figure, together with additional genes should be able to demonstrate the value of spatial transcriptomics. The author should be able to identify patterns of colocalization or differential expression. Although the authors do mention some patterns, I would say that they are not striking. I am providing some examples below. I do not know if the fact that the authors do not observe such clear patterns is due to the technology used or the biological context.

1. https://embed-ssl.wistia.com/deliveries/716c1856c750044a946968defe719a56.jpg?
image_play_button_size=2x&image_crop_resized=960x540&image_play_button=1&image_play_button_color=54bbffe0
2. https://bpb-us-e1.wpmucdn.com/sites.dartmouth.edu/dist/a/2122/files/2020/11/Spatial-Transcriptomics.jpeg
3. https://media.springernature.com/lw1200/springer-static/image/art%3A10.1038/s41576-021-00370-8/MediaObjects/41576_2021_370_Fig3_HTML.png

- Figure 1C. The changes are from which time point compared to which?
I think it would also be interesting to present the DEG results of 0 vs. 2, 0 vs. 8, 0 vs. 30.

- The author may use a pseudo-bulk approach to understand the broad dynamics
before going into the single cell.
Same as above.

Reviewer #4

(Remarks to the Author)
I have reviewed this manuscript with the advantage of getting to read the three prior reviews and the authors' responses. I concur with those reviews that this paper presents novel, important findings. I found the responses to reviewer critiques satisfactory. I will focus on my one main concern and share the typos/errors I saw.

Main concern: The Discussion did not cover the study's limitations. Notably, I didn't see a discussion around the spatial transcriptomics analysis being based on only 3 subjects, which is the paper's biggest limitation. In order for an analysis to find anything in 3 subjects, it must rely on parametric assumptions and there needs to be a fairly big signal relative to the measurements' variance. Figure 1 panel D is a nice example of this. The 3 pre measurements hang together closely and certain post measurements clearly deviate from the pre. Put another way, this study will only pick up on "hit you between the eyes" signals. The paper needs to be read with that caveat in mind. The paper should be cautious in trying to interpret anything other than those big signals, e.g. avoid stating two things appeared similar to each other. In addition, the sample sizes should be made clear throughout the manuscript, including stating them in the abstract where possible and repeating them wherever it helps with clarity.

Typos, etc.:

Sometimes precise p-values < 0.05 are presented, e.g. Figure 1 panel D, and sometimes the star system is used, e.g. Figure 3 panel F.

Lines 578-580 are unclear: "Pearson analysis was used to analysis the cell type correlation in the spot, and p-values and confidence were calculated with a significance test using cor.mtest function in corrplot package (v0.89)." I believe what was intended was: "Pearson correlation analysis was used to analyze the cell type correlation in the spot, and p-values and confidence intervals were calculated in R using cor.mtest from the corrplot package (v0.89)."

Supplementary Figure 4.
* The letters and figure descriptions are not lining up. It looks like a description for C was missed and everything is off by one letter from that point on.
* The sample size for elderly is not stated in the sentence: "The content of FAPs in healthy young subjects
(n=8) shows similar total FAP content compared to elderly."
* In addition, the authors should be cautious when claiming similarity with very small sample sizes. That statement should be toned down if possible.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
I'm generally satisfied with the overall response provided, and I also see that the other reviewers are satisfied.

The new Supplementary figure 2B, as I expected, brings a lot of value to the paper; and I would personally move it to the main figures.

Some of my requests were not fully addressed by the authors (the fact that the data is available on the app does not justify it). But I think generally this is okay.

Reviewer #4

(Remarks to the Author)
The revisions have addressed my remaining concerns. I do not need to see the corrections for the typos below.

Typos:

Figure 2 - For consistency in the footer, there should be a "D:" before "Cell-cell proportion correlation" instead of having the "(D)" at the end of that sentence.

Figure 3 - It's similarly disorienting to switch to using "(D)" at the end of the sentence in this figure. You could still maintain the idea flow while separating sentences and keeping the style consistent, e.g. "C: ... communication. D: This is also shown by ...".

Figure 4 - "F:" is missing before "Macrophage phagocytosis".

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript by Brorson et al reports the spatial transcriptomic profiling of muscle regeneration in humans and derives new biological conclusions for the understanding of cellular crosstalks during muscle repair in humans. The main conclusions are validated using independent technologies (FACS, cellular co-culture)and a specific cross-talk between FAPs and macrophages via complement C3 is further explored. While several studies have profiled human muscle at single cell level and many rodent studies have characterized the single cell response to muscle regeneration, this work is the first study reporting human muscle regeneration with spatial resolution and cellular cross-talk information. It will be an important resource for the field both for data mining and cellular understanding of human muscle regeneration, provided that some important points explained below can be addressed, in particular linked to the age of subjects (point 2) and resolution of injured vs non injured myofibers in the muscle biopsies (point 4 below).

Response: Thank you for highlighting the novel and important aspects of our work. Moreover, we appreciate the constructive critasism. Please find our comments and changes to comments below.

1/ The study design comes with some limitations since only 3 subjects per condition were profiled. In addition the samples come from a larger intervention CT with 32 participants (NCT03754842). While I understand the limitations of running the spatial analyses on a broad number of samples, it would be important that the authors explain in the methods how the samples analyzed were selected. Also were any of the NR/pterostilbene group analyzed?

Response: We agree with the reviewer that selecting samples from only three subjects is of course a limition. That is why we have carefully complemented the study with many independent validation experiments. However, given the type of analysis, the cost of spatial sequencing and the scale of data generated, we reasoned that three subjects, with repeated biopsies for each (i.e. 12 samples in total) would be adequate. Moreover, the cell analysis using flow cytometry was performed on the full sample set and these data nicely confirmed the predictions made by spatial transcriptomics. We should stress that *only the placebo* group from the clinical trial was included, to avoid any potential effects of the supplemement ingested in the intervention group. This is now highlighted in the results (**Line 114-115**) and methods description (**line 581-584**). Furthermore, we have extended the information in the three subjects included in the spatial analysis on measures such as MuSC response, embryonic MHC etc (**Supplementary Figure 2b**) to illuminate how these specific subjects responded to the injury protocol.

2/ One part that is surprising in the research methodology is that the analysis was ran in older people. This is a major limitation as it is unclear if the conclusions relate to healthy productive muscle regeneration or impaired muscle regeneration during aging. A validation of the main conclusions in a model of human muscle repair in young subjects would be important, this can be done on existing samples of other

studies.

Response: Thank you for highlighting the age aspect and the suggestion to include published data sources. Firstly, while the subjects were elderly, they were also recruited as healthy elderly, not requiring chronic medication that could potentially affect muscle regeneration. None-the-less, we cannot exclude that the age of the subjects affected the outcomes, although previous evidence suggest that regeneration in healthy elderly humans is intact [1].

To validate the main conclusions on human muscle repair in young subjects, we have now analyzed publicly availiable data-sets from single nuclei seq of human skeletal muscle in an age-span from 20 to 75 years [2]. Here we identified FAPs by unique PDGFRa expression. In agreement with our single cell data, this population was the major population expressing Complement 3 (**Supplementary Figure 4 A**), suggesting that local C3 production is mediated specifically by FAPs. Moreover, when analysing by age (grouped into young [<40 years] and elderly [>60 years]), there was no difference in C3 expression, suggesting a conserved mechanism over this age-span (**Supplementary Figure 4 B**). This supports that the data in our regeneration of elderly muscle is present in healthy young subjects as well. Unfortunately, we were unable to find data (RNA seq, single cell/nuclei RNA seq or spatial RNA seq) from young human muscle during muscle regeneration.

Since no studies have investigated FAP functions in healthy young human skeletal muscle, we decided to further confirm the role of FAP activation and FAP C3 expression in healthy young subjects. We isolated FAPs from healthy young men (**Supplementary Figure 4 C**) as described in our manuscript and previous work. Here we find that 1) the content and function (cell cycle entry, EdU incorporation, **supplementary Figure 4 D+E**), upon tisse injury (mechanical and enzymatic digestion of the muscle tissue), is retained and 2) FAP specifically express the C3 protein (**supplementary Figure 4 F**) in freshly isolated FAPs (please note that since C3 is rapidly downregulated upon activation, we could only perform this in freshly isolated FAPs).

Collectively, these data support that upon tissue damage (either in vivo or upon cell isolation) both young and old FAPs enter the cell cycle and FAPs uniquely secret C3 which play a critical role in the early phase of muscle regeneration. We have now included these data in **supplementary Figure 4** and in the results section (**line 252-263**).

3/ It is unclear how statistical analyses of the spatial transcriptomics time course were performed. Were analyses paired by subject?
Author Response: To avoid of the batch effect from library generation, the 4 sections/time points from same object were attacted on the same visium slide and processed at the same time. The statistical analysis was a repeated measures one-way ANOVA, Holm-Sidak's multiple comparisons test, with a single pooled variance. We have now included this information in the statistical section.

4/ Muscle injury in human muscle has a heterogenous response with some fibers

undergoing necrosis/regeneration (typically losing dystrophin and re-expressing embryonic myosin during regeneration) while others remain uninjured. Does the resolution of the spatial transcriptomics allow to differentiate the response around injured fibers vs non injured fibers of the same muscle? In Fig.1B-C it would be important to dissociate the direct response of a regenerating fiber from the indirect response of an uninjured myofiber.

Response: Thank you for this excellent point. This is exactly why spatial transcriptomics is particular suitable for understanding physiological human muscle regeneration at the molecular level. Moreover, while we have taken advantage of the data in this study, these data could be utilized for many other questions, which is why we have made them available through a user-friendly web-interface (https://dreamapp.biomed.au.dk/HuMdb/) for other researchers to search and utilize the data. Link for this is provided in the methods section.

To more specifically address the question raised by you, we defined an injury enrichment score based on following criteria (Macrophage gene score calculated based on the gene list ("PTPRC","CD163", "AIF1", "CD14", "CSF1R", "CD68", "MSR1", "FCGR3A", "HLA-DRA", "HLA-DRB1", "MARCO", "MRC1", "SIGLEC1", "MS4A6A", "FCER1G", "TYROBP", "LAPTM5", "LILRB2", "HLA-DPB1", "HLA-DMB", "HLA-DPA1") > 0, and MYH3 expression > 0, MYOG expression > 0). This effectively allowed us to clearly identify areas of muscle regeneration, which we could verify histologically on the H&E stainings. Specifically, none (or very few) spot were present in the pre-injury biopsy, but displayed a large increase at day 2 and 8, while returning almost to baseline at day 30. Moreover, the spots with high gene-set expression also had histological evidence (from the H&E image) of muscle damage. When interrogating the spots with high injury gene-set expression this revealed marked differences compared to non-injured areas (supplementary table 1, Supplementary figure 6). Interestingly, amongst the most highly differentially expressed genes was C3, underscoring the role of C3 in muscle regeneration. In addition, the comparison of injured versus non-injured areas will provide great data for future studies. We have now included a brief section on this in the results section (**line 305-313**) and in supplementary figure 6. Moreover, we have included a supplementary table 1, providing DEG between injured versus non-injured areas.

5/ Fig 1: Typical controls for muscle regeneration by histology and/or FACS should be provided for the 3 donors (extent of injury, Pax7 response, MyoD activation…)

Response: Yes, we agree and as described in repsonse to Comment 1, we have now provided all available immunohistochemical, FACS and biochemical blood-analyses data for these specific subjects (Supplementary 2B).

6/ Fig 2B & D: muscle stem cells which are the driving cells for regeneration are under-explored. Can the authors separate quiescent and activated muscle stem cells? There is also a risk that the correlation between FAPs and immune cells in Fig 2D is driven by the cell types that have the highest response to injury while the muscle stem cell response is diluted by quiescent cells. Are the correlations with niche cells similar for quiescent and activated muscle stem cells?

Response: This is an important and interesting topic that certainly deserves more focus, particularly in human skeletal muscle. Unfortunately, the combination of quiescent muscle stem cell having a low RNA content [3] and the resolution of the spatial transcriptomics platform in the present study (50um in diameter), precludes this type of detailed analysis between activated versus quiescent muscle stem cells. In general, our previously published data suggest a very robust proliferative response in the MuSC in this study [4] with increases in EdU incorporation from baseline to day two at around 600%. Thus, if anything, the proliferative response of the MuSC in this study seems to be more effective than the FAPs.

7/ In Fig 2C, it is unclear how relative amounts of cells were quantified as results are presented as fold change before injury. It is important to report if quantification is per mg of tissue or per total number of cells in homogenates as number of mononucleated cells will change during regeneration with immune infiltration. Also it would be important to validate the main conclusions on tissue sections by immunostaining

Response: Yes, we apologize for not being clear. The initial cell counts are based on cell counts per mg of tissue to avoid the potential challenges with major alterations in infiltrating cells etc. Afterwards, the percentage changes in cell counts were calculated based on the initial amount of cell/mg. We have now clarified this in the figure legend of 2C.

8/ Line 275 & Figure S5B: why is C3 present in injured myofibers if secreted soecifically by FAPs? Is this soluble C3 in the extracellular space after fiber clearance?

Response: After cellular secretion, C3 protein is often cleaved in the blood or the interstitial space by enzyme complexes (convertases) to form C3a and C3b. C3a exerts chemotactic properties as shown by decreased macrophage trafficking after injury in C3aR ablated animals [5]. Conversely, C3b often appear surface-bound to form a C3/C5-covertase and can act as an effective opsonization agent to target apoptotic/necrotic cells for phagocytosis [6]. Our C3 antibody detects the N-terminal part of C3 (which forms C3b after cleavage). When C3b is bound to the cell membrane, complement factor Bb is often co-present. We found that factor Bb was highly present on injured fibers which (combined with surrounding macrophages) indicates opsonization of injured myofibers with C3b. In addition, from our sequencing data C3 transcript appears to be specific for FAPs and the murine sc-RNA seq data supports this [7].

We have tried to clarify this observation in the main text (line 322-323).

9/ Fig.4F: while effects on clearance of apoptotic myoblasts in vitro are interesting, the groups of Benedicte Chazaud and Michael Kjaer have demonstrated that macrophage polarization controls myogenic fate of muscle stem cells, including in human muscle regeneration. It would be important to test how C3 treated macrophages influence myogenic fate in a coculture or conditioned medium assays

with muscle stem cells

Response: We agree that this aspects is clearly interesting in the context of muscle regeneration in general, meanwhile we believe this is somewhat outside the scope of the manuscript. None-the-less, we have now treated human primary macrophages with C3 to investigate how this affect macrophage polarization. Interestingly, we do see a small effect of C3 on polarization towards an anti-inflammatory phenotype (judged by expression CD206), although all the macrophages (CD68+) remained positive for the pro-inflammatory marker CD11c. We then investigated how the conditioned media from macrophages treated with or without C3 potentially affect human myoblast proliferation. Interestingly, we observed a suppression of myoblast proliferation when exposed to the macrophage medium, irrespective of C3 treatment. This suggest that the human CD11c+ macrophages suppress myoblast proliferation. To confirm this in relation to human skeletal muscle, we isolated CD11c postitive and negative macrophages from surgical biopsies to generate conditioned medium. We then exposed human MuSC to the conditioned medium and confirmed that indeed CD11c+ macrophages indeed suppress MuSC proliferation compared to CD11c- macrophages. In sum, although no major effect of C3 in vitro, we do confirm a functional effect of CD11c+ macrophages on human MuSC/myoblasts, suggesting that C3 may, at least indirectly in vivo, affect myoblast proliferation. We have now included these findings in **supplementary figure 9** and in the results section (line 361-374).

Minor:
-Figure 4G: the labels of the figure with and without phagocytosis conditions should be added to facilitate understanding
This has been corrected

-Line 29: the authors should remove single cell transcriptomic from the abstract as it is misleading with single cell sequencing after isolation. What the authors have done here is in silico single cell deconvolution of spatial transcriptomics.
This has been removed.

Reviewer #2 (Remarks to the Author):

The capacity to regenerate skeletal muscle function after injury requires a complex and well-coordinated cellular response, which is challenged in aged skeletal muscle. In this paper, the authors combined spatial, temporal, and single cell transcriptomics to reveal new information about the dynamics of elderly human skeletal muscle regeneration.

The major strengths of the manuscript are:

1) Using spatial RNA sequencing to the expression of human protein-coding genes in elderly human skeletal muscle biopsies before and 2-, 8-, and 30-days post injury.

2) Authors used single Cell-Spatial deconvolution analysis to determine that monocytes/macrophages and fibro-adipogenic progenitors (FAPs) are central players in human muscle regeneration.

3) Authors also provided intriguing data from Spatial correlation analysis to show FAPs and monocytes/macrophages co-localization and intercellular communication, mediated by complement factor C3.

4) They used Immunostaining approaches to confirm C3 expression in FAPs and FAP secretion of C3 and concluded that FAP secretion of C3 suggested a role in phagocytosis of necrotic muscle cells.

5) Authors used specific functional assays to demonstrate C3's impact on human monocyte metabolism, survival, and phagocytosis, and concluded its involvement in skeletal muscle regeneration.
Authors concluded that their studies elucidate FAP-macrophage interplay in aged human muscle with perspectives for future therapeutic interventions to reduce the aged-induced decline in regenerative capacity.

The work in this manuscript is extremely important for the area of aging and muscle regeneration. The studies are indeed state of the art and should advance our understanding of muscle regeneration during aging. Some weaknesses noted are the following:

Response: We thank the reviewer for the positive words, feedbacks, and acknowledgement of the work performed in vivo in humans.

A) One of the major problems of the manuscript is that while the study is about aged human muscle regeneration, only one figure shows very limited data of the regeneration process in the human cells (Fig. 1).
Response: Thank you for pointing out the important of human muscle regeneration. We have now included more detailed and elaborate information of muscle injury and subsequent muscle regeneration (**Supplementary 2B**). This allows for a detailed view into the injury and regeneration process. Moreover, both we [4] and others [1, 8, 9] have previously, in details, described the time-course of regeneration using this injury model. The current study provides another angle for understanding the injury-induced muscle regeneration process through spatial transcriptomics.

B) In Fig. 1, only H&E staining is shown for the muscle cell regeneration.
Response: As described above, we have included more information, including morphological information, on the muscle regeneration (Supplementary 2B). Moreover, we refer to our previous work wherein we have detailed phenotypic analysis on the injury and regeneration.

C) Throughout the manuscript, the authors attempt to demonstrate the role of monocytes, macrophages, and FAPs, but in exploring their roles, there was not concomitantly data on muscle cell regeneration.

Response: As detailed above, we have now included this. Thanks for raising this critical concern.

D) In the field of muscle of muscle regeneration, it is widely accepted that DAPI and Myosin Heavy Chain are labeled for determination of Fusion Index (FI), which is never determined under any condition in this manuscript.
Response: We understand that Myosin Heavy Chain and nuclei (DAPI) labeling are utilized for determination of fusion index in vitro in culture systems. However, we have not performed this in our manuscript as we are not exploring this aspect of myogenesis and muscle regeneration. The role of FAP secreted C3 is, as explained in the manuscript, important for the early regeneration, while fusion of myoblasts are occurring later in regeneration. Since our manuscript is focusing on understanding the early molecular and cellular interactions in regulating muscle regeneration, the late stages of myoblasts fusion are not investigated. Also, as related to the previous comments, the muscle regeneration hallmarks and features for this clinical trial have been documented in our previous study [4].

E) While the work is obviously valid and very important as it is, it could add some value if the authors could contrast some of their findings with known databases in young muscle cells or conduct at least a basic overall experiment in biopsies from young donors.
Response: We agree with the reviewer that information on the role of FAPs and C3 in healthy young regeneration would be valuable (as also detailed in the response to Reviewers 1 and 3).

Firstly, while the subjects were elderly, they were also recruited as healthy elderly, not requiring chronic medication that could potentially affect muscle regeneration. None-the-less, we cannot exclude that the age of the subjects affected the outcomes, although previous evidence suggest that regeneration in healthy elderly humans is intact [1].

To validate the main conclusions on human muscle repair in young subjects, we have now analyzed publicly availiable data-sets from single nuclei seq of human skeletal muscle in an age-span from 20 to 75 years [2]. Here we identified FAPs by unique PDGFRa expression. In agreement with our single cell data, this population was the major population expressing Complement 3 (Supplementary Figure 4 A), suggesting that local C3 production is mediated by specifically by FAPs. Moreover, when analysing by age (grouped into young [<40 years] and elderly [>60 years]), there was no difference in C3 expression, suggesting a conserved mechanism over this age-span (Supplementary Figure 4 B). This support that the data in our regeneration of elderly muscle is present in healthy young subjects as well. Unfortunately, we were unable to find data (RNA seq, single cell/nuclei RNA seq or spatial RNA seq) from young human muscle during muscle regeneration.

Since no studies have investigated FAP function in healthy young human skeletal muscle, we decided to further confirm the role of FAP activation and FAP C3 expression in healthy young subjects. We isolated FAPs from healthy young men (Supplementary Figure 4 C) as described in our manuscript and previous work. Here we 1) find that the content and function (cell cycle entry, EdU incorporation,

supplementary Figure 4 D+E), upon tisse injury (mechanical and enzymatic digestion of the muscle tissue), is retained and 2) that FAP specifically express the C3 protein (supplementary Figure 4 F) in freshly isolated FAPs (please note that since C3 is rapidly downregulated upon activation, we could only perform this in freshly isolated FAPs).

Collectively, these data support that upon tissue damage (either in vivo or upon cell isolation) both young and old FAPs enter the cell cycle and FAPs uniquely secret C3 which play a critical role in the early phase of muscle regeneration. We have now included these data in **supplementary figure 4** and in the results section (**line 252-263**).

F) Supplementary Figure 6: These experiments are conducted with monocytes from human peripheral blood, not from any cells directly obtained from the muscle biopsies. Authors are trying to prove a point that C3 changes the function of monocytes. Why then not add C3 as well to young muscle cells to observe a potential phenotype?

Response: As detailed in response to reviewer 1 (comment 9), we have now treated human primary macrophages with C3 to investigate how this affect macrophage polarization. Interestingly, we do see a small effect of C3 on polarization towards an anti-inflammatory phenotype (judged by expression CD206), although all the macrophages (CD68+) remained positive for the pro-inflammatory marker CD11c. We then investigated how the conditioned media from macrophages treated with or without C3 potentially affect human myoblast proliferation. Interestingly, we observed a suppression of myoblast proliferation when exposed to the macrophage medium, irrespective of C3 treatment. This suggest that the human CD11c+ macrophages suppress myoblast proliferation. To confirm this in relation to human skeletal muscle, we isolated CD11c postitive and negative macrophages from surgical biopsies to generate conditioned medium. We then exposed human MuSC to the conditioned medium and confirmed that indeed CD11c+ macrophages indeed suppress MuSC proliferation compared to CD11c- macrophages. In sum, although no major effect of C3 in vitro, we do confirm a functional effect of CD11c+ macrophages on human MuSC/myoblasts, suggesting that C3 may, at least indirectly in vivo, affect myoblast proliferation. We have now included these findings in supplementary figure 9 and in the results section (line 361-374).

G) Statistical analyses: Authors do not describe if all data was parametric. They just mentioned ANOVA and T-test. They do not include any Post correction tests, such as Tukey's or Bonferroni.
Response: We have now included updated this in the statistical section of the methods (line 819-830). Thank you for highlighting this.

Reviewer #3 (Remarks to the Author):

In this study Brorson et al. perform spatial single-cell RNAseq on skeletal muscle, before, 2-, 8-, and 30-days post injury. They identify immune-enriched and regenerative clusters at 8 dpi. Through deconvolution they highlight the role of FAPs

and monocytes in regeneration. With receptor-ligand analysis they identify complement C3 as pivotal player.

Major:

- As the regenerative capacity of human muscle declines with age it would have been interesting to compare the regeneration after injury between young and old muscle. The authors could use publicly available single cell datasets to study if the genes involved in regenerating old muscles are also differentially expressed in young vs. old muscle.

Response: We agree with the reviewer (as well as similar points made by reviewer 1 and 2) that this would be an important add on, while also acknowledging that access to human skeletal muscle biopsies during regeneration is difficult. The suggestion to look into available database is greatly appreciated.

To validate the main conclusions on human muscle repair in young subjects, we have now analyzed publicly availiable data-sets from single nuclei seq of human skeletal muscle in an age-span from 20 to 75 years [2]. Here we identified FAPs by unique PDGFRa expression. In agreement with our data, this population was the major population expressing Complement factor 3 (Supplementary Figure 4 A), suggesting that local C3 production is mediated specifically by FAPs. Moreover, when analysing by age (grouped into young [<40 years] and elderly [>60 years]), there was no difference in C3 expression, suggesting a conserved mechanism over this age-span (Supplementary Figure 4B). This supports that the data in our regeneration of elderly muscle is present in healthy young subjects as well. Unfortunately, we were unable to find data (RNA seq, single cell/nuclei RNA seq or spatial RNA seq) from young human muscle during muscle regeneration.

Since no studies have investigated FAP function in healthy young human skeletal muscle, we decided to further confirm the role of FAP activation and FAP C3 expression in healthy young subjects. We isolated FAPs from healthy young men (Supplementary Figure 4 C) as described in our manuscript and previous work. Here we 1) find that the content and function (cell cycle entry, EdU incorporation, supplementary Figure 4 D+E), upon tisse injury (mechanical and enzymatic digestion of the muscle tissue), is retained and 2) that FAP specifically express the C3 protein (supplementary Figure 4 F) in freshly isolated FAPs (please note that since C3 is rapidly downregulated upon activation, we could only perform this in freshly isolated FAPs).

Collectively, these data support that upon tissue damage (either in vivo or upon cell isolation) both young and old FAPs enter the cell cycle and FAPs uniquely secret C3 which play a critical role in the early phase of muscle regeneration. We have now included these data in **supplementary figure 4** and in the results section (**line 252-263**).

- In Figure 1B, why aren't the clusters well separated and common cell types

identified like in Figure 3D? In this sense it could have been interesting to perform the same experiment with a more classical single-cell RNA-seq (not spatial).
Response: This is related to the resolution of the Visium spatial RNA sequencing technologies. Each spatial spot capture approximately 3-10 cells depending on the tissue regions. That is why annotation to each cluster in figure 1 was performed according to enriched functions rather than cell types. We agree that it would be more interesting if both single cell RNA sequencing and spatial RNA sequencing could be performed on these samples. Unfortuantely, with the limited amout of muscle biopsies that we could uptain from the human subjects, we could only perform spatial RNA sequencing, filling gap of lacking such data by the field. We complement this by carrying out single cell RNA sequencing based deconvolution analysis (Figure 2).

- Figure 1F. Instead of visualizing GO modules, the authors should try to visualize actual genes.
Response: This is also possible and can be performed on the user-friendly web-interface we have created to access the data (https://dreamapp.biomed.au.dk/HuMdb/). In this manuscript we aimed to look more broadly, initially, to gain insight into the overall major patterns within muscle regeneration. This approach supported our interest in the role of FAPs and their prominent role in communicating with the inflammatory infiltrates, in this case particular the macrophages.

- It would be interesting combine H&E, immuno-staining and spatial transcriptomics on the same samples, as theoretically possible in the Visium technology.
Response: Thank you for the suggestion and we completely agree that complementing such techniques would be valuable. Unfortuantely, the V1 visium chip used for this study is not compatible for having both H&E and immuno-staining on the same section.

- It is not completely clear how the authors took advantage of the spatial technology compared to classical single cell RNAseq.
Response: We have now rephrased to enhance the clarity of why we choose to utilize spatial transcriptomics. In short, the main reasons for utilizing this technology was:

1) Gain insight into the spatial dynamics of muscle regeneration to understand which cell types communicate with FAPs during this process. The purpose of this was to understand for which processes FAPs are needed during regeneration, as this is yet unclear, even in animal models such as mice
2) Given that the injury was performed in human skeletal muscle, this creates a physiological injury where focal areas of the muscle are undergoing necrosis and later new fiber formation, while other areas seem completely intact and unaffected [9]. This makes it challenging to dissect which cell are actually engaged in the injury process and communicating to the niche in this context. Spatial transcriptomics allowed us to dissect this further and predict which cell types are spatially in a position to communicate. Recent evidence from Rossi lab, has highlighted that the spatial positioning during regeneration is critical

to understand how factors realeased from a specific cell type affect muscle regeneration [10].

To more specifically address the question raised by you, we defined an injury enrichment score based on following criteria (Macrophage gene score calculated based on the gene list ("PTPRC","CD163", "AIF1", "CD14", "CSF1R", "CD68", "MSR1", "FCGR3A", "HLA-DRA", "HLA-DRB1", "MARCO", "MRC1", "SIGLEC1", "MS4A6A", "FCER1G", "TYROBP", "LAPTM5", "LILRB2", "HLA-DPB1", "HLA-DMB", "HLA-DPA1") > 0, and MYH3 expression > 0, MYOG expression > 0). The effectively allowed us to clearly identify areas of muscle regeneration. Specifically, none (or very few) spot were present in the baseline biopsy, but displayed a large increase at day 2 and 8, while returning almost to baseline at day 30. Moreover, the spots with high gene-set expression also had histological evidence (from the H&E image) of muscle damage. When interrogating the spots with high injury gene-set expression this revealed marked differences compared to non-injured areas (supplementary table 1 and supplementary figure 6). Interestingly, amongst the most highly differentially expressed genes was C3, underscoring the role of C3 in muscle regeneration. In addition, the comparison of injured versus non-injured areas will provide great data for future studies. We have now included this information in **Supplementary figure 6** and line **line 305-313** in the results section.

Minor:
- Figure 1C. The changes are from which time point compared to which?
Author Response:
The log2FC are comparison of the target cluster against all remaining clusters. We have now included this information in the figure legend.

- The author may use a pseudo-bulk approach to understand the broad dynamics before going into the single cell.
Response: Thank you for the suggestion. The analysis presented in Figure 1C and 1E is similar to pseudo-bulk. We then go directly to more single cell based strategy to immidiatly get into the cellular dynamics, in particular to understand the role of FAPs in muscle injury.

- Figure 1B. What are the top genes in each cluster?
These top marker genes are highlighted in 1C. Furthermore, all data can be easily accessed throught the provided web-app.

- Could the authors confirm by immuno-staining at different timepoints some of the top genes they observe in Figure 1?
Response: This would be interesting, although we are of course not expecting that all genes that alter their expression are mirrored in protein expression. Unfortunately, we no longer have tissue available form these samples, so its not possible to perform in the present project.

- Can the authors comment on the lack of correlation between Figure 2 B and C, especially regarding the FAPs. Here also there could be a time-resolved immuno-staining to confirm.

Response: The very early response of FAPs in the time-resolved cell prediction based on spatial seq is unlikely to represent a change in cell number. This is based on the fact that it takes both FAPs and also MuSCs at least 48h and often longer (in particular in elderly muscle) to divide. Therefore, the increase in FAPs early on in 2 B, is likely reflecting a marked increase in gene expression activity in the already present FAPs. This is consistant with the marked increased in cell size already at day 2 post injury (Supplementary Fig 5 D). Unfortunately, we don't have more tissue to perform immunostainings. However, even if tissue was available, performing stainings and quantifying FAPs is very difficult (PDGFRa staining in human skeletal muscle is highly challenging), and hence the quality of such quantification is, in our opinion, questionable. Using flow-cytometry to quantify FAP content where several positive/negative markers can be included is currently the best option.

- The authors should explain more what is behind the receptor-ligand analysis, since this is not common practice.

Response: We have included more explaination in the use of ligand-receptor analysis in the revised manuscript (**line 637-650**).

References

1. Karlsen, A., et al., *Preserved capacity for satellite cell proliferation, regeneration, and hypertrophy in the skeletal muscle of healthy elderly men.* FASEB J, 2020. **34**(5): p. 6418-6436.
2. Kedlian, V.R., et al., *Human skeletal muscle aging atlas.* Nat Aging, 2024. **4**(5): p. 727-744.
3. van Velthoven, C.T.J. and T.A. Rando, *Stem Cell Quiescence: Dynamism, Restraint, and Cellular Idling.* Cell Stem Cell, 2019. **24**(2): p. 213-225.
4. Jensen, J.B., et al., *A randomized placebo-controlled trial of nicotinamide riboside and pterostilbene supplementation in experimental muscle injury in elderly individuals.* JCI Insight, 2022. **7**(19).
5. Zhang, C., et al., *Complement C3a signaling facilitates skeletal muscle regeneration by regulating monocyte function and trafficking.* Nat Commun, 2017. **8**(1): p. 2078.
6. Ricklin, D., et al., *Complement component C3 - The "Swiss Army Knife" of innate immunity and host defense.* Immunol Rev, 2016. **274**(1): p. 33-58.
7. Tabula Muris, C., et al., *Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris.* Nature, 2018. **562**(7727): p. 367-372.
8. Mackey, A.L. and M. Kjaer, *The breaking and making of healthy adult human skeletal muscle in vivo.* Skelet Muscle, 2017. **7**(1): p. 24.
9. Mackey, A.L., et al., *Human skeletal muscle fibroblasts stimulate in vitro myogenesis and in vivo muscle regeneration.* J Physiol, 2017.
10. Groppa, E., et al., *Spatial compartmentalization of signaling imparts source-specific functions on secreted factors.* Cell Rep, 2023. **42**(2): p. 112051.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have performed a serious and solid revision and addressed all my comments. I just could not find the new supplementary figure 2B mentioned in the manuscript and rebuttal

Dear Reviewer, thank you for the constructive feedback to our manuscript and the revision. Moreover, thank you for pointing out the figure reference mistake. This should read “Supplementary Figure 1B” and has now been corrected (Line 114, Page 5).

Reviewer #2 (Remarks to the Author):

The authors have made a major effort to address all major criticisms and added additional analyses requested by all reviewers. The manuscript rigor was also enhanced by the increased N (sample size) and enhanced statistical analyses, as well as some of the added clarifications to the results and methods.

Dear Reviewer, thank you for the constructive feedback to our manuscript and the changes made in the revision.

Reviewer #3 (Remarks to the Author):

Major:
- As the regenerative capacity of human muscle declines with age it would have been interesting to compare the regeneration after injury between young and old muscle. The authors could use publicly available single cell datasets to study if the genes involved in regenerating old muscles are also differentially expressed in young vs. old muscle.

Ok. Here I would have thought more about a direct comparison of DEGs involved in regeneration vs. DEGs in Y vs. O. Might also want to look at [PMID: 36516485], in addition to Kedlian, V.R., et al.

Dear Reviewer, thank you for this suggestion and for pointing out another relevant paper. We have now included this in our discussion on the relevancy to human muscle aging (Line 406-407, Page 17). Indeed, it would be possible to cross-correlate DEG in aging (from publicly available data) with DEG in regeneration (obtained from our data in the current study). In fact, this approach could generate exciting new hypothesis to be tested. For the very same reason, we have made our data easily accessible through a web-app to allow researchers to utilize our data for this purpose: https://dreamapp.biomed.au.dk/HuMdb/.

- Figure 1F. Instead of visualizing GO modules, the authors should try to visualize actual genes.

I understand but I still believe that visualizing the spatial expression of a few selected genes deemed important by the authors would add a lot of value to the paper.

In addition to our web-app, which provides an easy, user friendly access to plot all genes in a spatial and temporal manner, we have now provided a few examples of spatial/temporal genes expression of COL1A1/MYH3 (embryonic myosin heavy chain)/CD68/CDKN1A(p21) in supplementary figure 2 B.

- It is not completely clear how the authors took advantage of the spatial technology compared to classical single cell RNAseq.

I was referring to Figure 1F. I believe this figure, together with additional genes should be able to demonstrate the value of spatial transcriptomics. The author should be able to identify patterns of colocalization or differential expression. Although the authors do mention some patterns, I would say that they are not striking. I am providing some examples below. I do not know if the fact that the authors do not observe such clear patterns is due to the technology used or the biological context.

1. https://embed-ssl.wistia.com/deliveries/716c1856c750044a946968defe719a56.jpg?image_play_button_size=2x&image_crop_resized=960x540&image_play_button=1&image_play_button_color=54bbffe0
2. https://bpb-us-e1.wpmucdn.com/sites.dartmouth.edu/dist/a/2122/files/2020/11/Spatial-Transcriptomics.jpeg
3. https://media.springernature.com/lw1200/springer-static/image/art%3A10.1038/s41576-021-00370-8/MediaObjects/41576_2021_370_Fig3_HTML.png

Thank you for further explaining the reasoning behind the request. The examples provided illuminates the highly specific spatial confinement within certain tissues, kidney being a prime example in which we also have generated spatial transcriptomics data. However, we should also stress that this is one of many applications of spatial transcriptomics. The nature of human muscle injury is focal areas of clear muscle regeneration with neighboring areas with only minor signs of damage. Here is the spatial transcriptomics offers a clear advantage in contrast to other technologies, as highlighted in the new supplementary figure 6. In addition, in this manuscript we focused on cellular communication, leveraging the strength of an unbiased approach to identify potential routers of communication important for the initial regenerative response. Also here, earlier papers in mouse models have highlighted the importance of taking the spatial localization and niche into consideration, when judging if a ligand has a role in certain situations (1). Finally, the analyses performed in this paper is naturally only a fraction of what could be performed and why we are providing easy access to the data.

- Figure 1C. The changes are from which time point compared to which?
I think it would also be interesting to present the DEG results of 0 vs. 2, 0 vs. 8, 0 vs. 30.

Yes, we agree, also this approach is interesting. We have presented a summary of this comparison in Fig 1D. Although, here we have clustered cluster 0-5 together, since these

displayed the same temporal behavior. Furthermore, all potential comparisons are available for further exploration in our web-app: https://dreamapp.biomed.au.dk/HuMdb/.

- The author may use a pseudo-bulk approach to understand the broad dynamics before going into the single cell.
Same as above.

Again, this approach is performed in Fig 1D. We appreciate that several different analyses strategies would yield relevant and interesting information. However, we can only cover some of this in the current manuscript, while keeping a focused storyline.

Reviewer #4 (Remarks to the Author):

I have reviewed this manuscript with the advantage of getting to read the three prior reviews and the authors' responses. I concur with those reviews that this paper presents novel, important findings. I found the responses to reviewer critiques satisfactory. I will focus on my one main concern and share the typos/errors I saw.

Main concern: The Discussion did not cover the study's limitations. Notably, I didn't see a discussion around the spatial transcriptomics analysis being based on only 3 subjects, which is the paper's biggest limitation. In order for an analysis to find anything in 3 subjects, it must rely on parametric assumptions and there needs to be a fairly big signal relative to the measurements' variance. Figure 1 panel D is a nice example of this. The 3 pre measurements hang together closely and certain post measurements clearly deviate from the pre. Put another way, this study will only pick up on "hit you between the eyes" signals. The paper needs to be read with that caveat in mind. The paper should be cautious in trying to interpret anything other than those big signals, e.g. avoid stating two things appeared similar to each other. In addition, the sample sizes should be made clear throughout the manuscript, including stating them in the abstract where possible and repeating them wherever it helps with clarity.

Dear Reviewer, thank you for the constructive feedback on our manuscript and the revision based on previous reviewer comments. We fully agree that the sample size for spatial transcriptomics is naturally a limitation and why we consider it a major strength that several analyses were performed on a larger sample size (e.g. the flow-data on cellular content) to indeed confirm the trends in the spatial transcriptomics. None-the-less, when interpreting the spatial data, the n=3 should still be kept in mind.

We have now added a limitations section at the end of the discussion to highlight this important point (line 439-443, page 18). Moreover, we have read through the manuscript/figures, to ensure that the sample size is mentioned, whenever relevant. This now also includes the abstract (Line 31 and 34, page 2).

Typos, etc.:

Sometimes precise p-values < 0.05 are presented, e.g. Figure 1 panel D, and sometimes the star system is used, e.g. Figure 3 panel F.

We have now corrected to make it uniform across the figures. Thank you for noticing.

Lines 578-580 are unclear: "Pearson analysis was used to analysis the cell type correlation in the spot, and p-values and confidence were calculated with a significance test using cor.mtest function in corrplot package (v0.89)." I believe what was intended was: "Pearson correlation analysis was used to analyze the cell type correlation in the spot, and p-values and confidence intervals were calculated in R using cor.mtest from the corrplot package (v0.89)."

Thank you. We have now corrected (Line 586-588, Page 24).

Supplementary Figure 4.
* The letters and figure descriptions are not lining up. It looks like a description for C was missed and everything is off by one letter from that point on.

Thank you for noticing. We have now corrected.

* The sample size for elderly is not stated in the sentence: "The content of FAPs in healthy young subjects
(n=8) shows similar total FAP content compared to elderly."
* In addition, the authors should be cautious when claiming similarity with very small sample sizes. That statement should be toned down if possible.

We have now removed the statement comparing the FAP content in the young cohort to the elderly cohort presented in the main paper, to tone down direct comparison.

References:
1. Groppa E, Martini P, Derakhshan N, Theret M, Ritso M, Tung LW, et al. Spatial compartmentalization of signaling imparts source-specific functions on secreted factors. Cell reports. 2023;42(2):112051.

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

I'm generally satisfied with the overall response provided, and I also see that the other reviewers are satisfied.

The new Supplementary figure 2B, as I expected, brings a lot of value to the paper; and I would personally move it to the main figures.

Some of my requests were not fully addressed by the authors (the fact that the data is available on the app does not justify it). But I think generally this is okay.

We thank the reviewer for all the helpfull comments and suggestions to our manuscript.

Reviewer #4 (Remarks to the Author):

The revisions have addressed my remaining concerns. I do not need to see the corrections for the typos below.

Thank you for the helpfull inputs and for noticing the types. These have now been corrected.

Typos:

Figure 2 - For consistency in the footer, there should be a "D:" before "Cell-cell proportion correlation" instead of having the "(D)" at the end of that sentence.

Corrected.

Figure 3 - It's similarly disorienting to switch to using "(D)" at the end of the sentence in this figure. You could still maintain the idea flow while separating sentences and keeping the style consistent, e.g. "C: ... communication. D: This is also shown by ...".

Corrected.

Figure 4 - "F:" is missing before "Macrophage phagocytosis".

Corrected.