nature portfolio

Peer Review File

Integrating spatial and single-cell transcriptomics to characterize mouse long bone fracture healing process

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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)
This paper presents the use of spatial transcriptomics to assess the progression of fracture healing in mice. Two times points (one at day five after fracture when the endochondral soft callus is forming and one at day 15 when it has transitioned to coupled remodeling and hard callus formation. A new method of decalcification using formic acid method (Morse's solution) is shown to provide superior performance maintaining transcript integrity compared to EDTA. While the authors are to be commended in being one of the first groups to apply spatial transcriptomic data to analyze fracture healing, both the text and graphic presentation of the data in the figures is extremely hard to follow and the quality of the figures is poor diminishing the ability to assess both the validity of the data and understand the author's interpretation of the data. Some of the major deficiencies are identified

1) Two examples of figure legends either being incomplete or not labeled correctly are Figure 2 in which the legend does not have descriptions for two of the panels and the and figure 4 that indicates there is a panel B showing day 15 samples.

2) For images throughout, the overlay of the ontology color coding on top of H&E sections makes it impossible to correlate simple histology to the spatial mapping. The two should be presented side by side and H&E sections should be of sufficient quality and size to identify by simple cell morphology tissue tissues like cartilage and bone.

3. For figure two it unclear how the authors related their spatial mapping and nomenclature of spatial clusters in the U MAP in panels D to the hierarchy and composition bar graphs in panels B and C. The text is also confusing relative to explaining the figure. As an example it's clear there are four related spatial cluster groups in B, but how these relate to D and E is not clear since cell data is mixed up with cluster data.

4) A general note as well is that closely related colors groups should be resolved by including a line patterning in the so the cell types in the spatial grouping can be sorted apart

5) Figure four is mislabeled on data is missing for day 15

Reviewer #2

(Remarks to the Author)
Here the authors detail technical advances in tissue handling that allow for enhanced spatial transcriptomic analyses of skeletal tissue. A considerable amount of data are included to demonstrate the improved methodologies however several questions remain:

1. The authors perform these studies using an n=2 and the data suggest variability (see gene expression trajectories as an example) so it is difficult to know the variability is due to technical differences or animal variation in healing responses. A suggestion would be to increase the number of animals investigated so as to determine the strength of the technical gains.

2. The authors chose day 5 and day 15 for analyses and compared their results to previously published data, thus providing a deeper analysis of already reported events. This seems like a missed opportunity as the enhanced techniques could be

used to examine earlier stages of fracture repair where current spatial techniques have been unable to capture important information.

Other points:
1. In the first paragraph of the Introduction, the authors state that chondrocytes and osteoblasts are essential for bone remodeling. This is inaccurate and should be reworded for clarity.
2. The description of the spatial transcriptomics protocol is very difficult to understand, especially paragraphs 2. Since this MS is based on highlighting a new protocol, clarifying the exact procedure should be a priority.

Reviewer #3

(Remarks to the Author)
The authors present a study utilizing spatial transcriptomics to examine the mouse fracture callus at Days 5 and 10 post-injury. While this manuscript holds promise as a valuable resource for the field, the scientific message and novel insights derived from these data are currently not well-articulated. A significant portion of the figures is devoted to standard quality control and cluster identification, at the expense of biological findings that give new information about fracture repair. Clarification on the specific contributions of this study to our understanding of fracture repair would greatly enhance its impact.

Major Concerns:

1. Data Availability and Consistency: Not all the data described in the manuscript text are presented in the figures. It seems that an earlier version of the figures may have been submitted by mistake, making the review process challenging due to missing information and misaligned figure references and legends.
2. Clarity of Scientific Message: The manuscript lacks a clear and focused scientific message. This is particularly evident in the introduction and abstract, where key findings are vaguely described without a concise summary. The authors should more clearly define and emphasize the main scientific contributions of their study.
3. Contextualization of Data: The manuscript does not adequately situate its findings within the current landscape of skeletal stem and progenitor cell research. Various cell populations have been characterized by different markers (e.g., Lepr, Nestin, aSma, Pdgfra, Sca1, Edil3, Thy1). A thorough analysis of these markers, including their spatial expression patterns in the fracture callus, would provide crucial context for understanding the significance of MSC, PPC1, and PPC2 cells as described by the authors.
4. Lineage and Marker Analysis: Related to the former point, the manuscript would benefit from a more comprehensive analysis of lineage markers for different cell types within the fracture callus e.g. osteoblasts, chondrocytes, adipocytes. The current analysis references only a few markers, which limits the understanding of the data, particularly regarding the authors' conclusion that their dataset includes adipocytes. Specifically, the authors use 2 markers to define adipocytes, one of which (Adipoq) is known to be expressed in a variety of cell types in bone and its spatial expression is not shown. Studies have also shown that Plin1, the other marker used, is also expressed in pre-adipocytes. Therefore, a greater number of adipocyte markers should be assessed to increase confidence in these data.
5. Sample Size and Experimental Limitations: The study analyzes only two male mice per time point. While spatial transcriptomics remains costly, this limited sample size and the lack of female samples restrict the robustness of the conclusions. This limitation should be discussed.
6. Lack of Baseline Uninjured Control: The absence of a Day 0, uninjured sample for comparison is a significant limitation. Statements regarding the onset or increase in gene expression at Day 5 cannot be substantiated without a baseline. This is also relevant for the Cell Chat analysis, where the suggestion that MSC interactions are essential for bone healing requires comparison with uninjured controls to validate. For example “early chondrocyte markers such as Col2a1 begin to emerge signifying...”, “Significant upregulation of hypoxia-inducible markers.” This is also relevant in the Cell Chat analysis as the authors suggest that the high number of MSC interactions indicates their essential role in bone healing. However, it is not clear from these spatial data alone that there is an increase in MSC interactions during healing without the control.
7. Statistical Analysis: There are several instances where the authors refer to "significant" changes without providing statistical evidence to support these claims (e.g., "significant upregulation of hypoxia-inducible markers" and “significantly increased vascular distribution”. “elevation in Postn, Mif, Mdk, and Cxcl12 signaling”). The manuscript would benefit from the inclusion of appropriate statistical analyses to validate these observations.
8. Assignment of MPCs: The logic behind assigning spatial spots to "MPCs" is unclear, particularly since they do not appear to constitute the majority of the cluster. The reasoning behind this classification needs to be clearly articulated.
9. Endothelial Marker Expression: The manuscript references "elevated expression of endothelial markers" between Days 5 and 15, but this is not visually evident from the data. The authors should present these findings in the form of violin plots or other visual aids to clarify the data, especially since no endothelial clusters are identified.
10. Differential Expression and Pathway Analysis: A basic analysis of differentially expressed genes (DEGs) and gene set enrichment analysis (GSEA) in the various spatial clusters over time is notably absent. This is a minimal requirement to enhance the biological insights of the study.
11. Trajectory Analysis Bias: Figure 4 raises several questions, including the rationale for the trajectory analysis and the exclusion of PPC2s and osteoblasts. The data suggest an increase in chondrocyte markers and a decline in progenitor markers along this trajectory, which is expected given the setup. The manuscript should clearly explain what new insights are gained from this analysis and also use the day 15 sample as well as day 5 for this analysis.

Minor Concerns:

1. Selection Criteria for Cross-Section: The authors mention the "meticulous selection" of a central cross-section showcasing maximal callus development but do not elaborate on the criteria used. Further explanation of these criteria would be helpful.
2. RNA Quality and Decalcification Protocol: The authors claim that their decalcification protocol significantly improves RNA quality compared to other studies. However, without a direct comparison of different methods, it is difficult to attribute this improvement solely to the decalcification protocol.
3. Data Analysis Pipeline: The text refers to Fig. 1A as a pipeline illustrating comprehensive data analysis, but the figure does not clearly convey the role of each software package. This should be clarified to aid the reader's understanding.
4. H&E Staining Labels: The H&E stainings in Figure 1 should be labeled to indicate key features such as the callus, fracture line, cortical bone, growth plate, and proximal/distal femur. This is particularly important since one of the fractures appears more proximal, which could affect the role of the growth plate in repair.
5. RNA Quality and Ossification: The manuscript states that "areas undergoing ossification showed enhanced RNA quality." However, no data are provided to link specific regions with RNA quality, and there should not be a direct correlation between RNA quality and biological activity. This statement should be clarified or removed.
6. PPCs and Proliferative Capacity: The authors state that "PPCs are distinguished by their robust proliferative capacity." However, at Day 5, a large proportion of cells are in G2/M, which does not seem to uniquely distinguish PPCs from other cell types. This point requires further clarification.
7. Necrotic Cells and Cell Cycle: The authors refer to necrotic cells within the callus but the data also suggest that these cells are in G2/M phase. The interpretation that these cells are necrotic is inconsistent with their active division. This discrepancy should be addressed.
8. Changes in Cell Populations Over Time: The manuscript briefly presents changes in cell populations over time in Fig. 2F but does not discuss these changes in the text. The authors should elaborate on these observations and their biological implications.
9. Bioinformatics Pipeline: A detailed bioinformatics pipeline for the analysis in Figure 2 would clarify the progression of the analysis. The logic behind simplifying 17 cell types into 12 groups, particularly the inclusion of adipocytes not present in the scRNA sequencing data, needs further explanation.
10. Mechanosensitive Genes in PPCs: The enrichment of mechanosensitive genes in PPCs, particularly for genes other than Ccn1, is not clearly demonstrated from the plots shown. Violin plots showing gene expression within different clusters would better illustrate these findings. The authors should also include Day 15 data for a more comprehensive analysis.
11. GSEA Analysis: The manuscript mentions GSEA results showing enrichment of hypoxia and glycolysis pathways but does not clarify whether this analysis is specific to certain clusters or applies to all clusters combined. A cluster-specific GSEA would provide deeper biological insights.
12. MSC and PPC Relationship: The relationship between MSCs and PPCs is unclear—is one simply a proliferating version of the other? Cell cycle normalization could help clarify this, and projecting cell cycle information onto the UMAP in Fig. 2A would provide additional context.
13. Macrophage Analysis: The manuscript states that "a significant increase of macrophages within the MPC spot" was observed near the fracture site but does not present statistical evidence. This should be plotted as a graph, and significance should be assessed.
14. Biological Interpretation of Data: The manuscript would benefit from a more in-depth discussion of the biological meaning behind the data. For example, Fig. 3B shows distinct clusters for MPCs and PPCs, but the reasons for this clustering—whether due to clonal expansion, differential adhesion, or other factors—are not discussed.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript presents a detailed spatial transcriptomic analysis of murine fracture healing over three time points of healing.

The manuscript is greatly improved from the prior submission. The text and figures now show good correspondence and the figures are now much clearer, so that they are more easily understood.
The authors have been very responsive to the reviewer’s question’s

It is unclear how to interpret the data in figure 6 between the spatial and single cell data. Is the proximity of cell types as represented by colored dots based on if they are clustered as group together? If so this should be stated in the text.

The only concern to be noted it that the study assessed only two mice and it is unclear the degree of variability of these data even between replicates. In this regard it is hard to assess the accuracy of assigning a cells position to another cell or likely hood of the assigned communication between cell types. How is this enumerated?

Reviewer #2

(Remarks to the Author)

This revised MS by Yao et al provides an analysis of the use of enhanced methodologies to examine fracture repair. While the MS is improved from the original submission, substantial concerns remain to be addressed.
1. There are numerous typos and grammatical errors throughout the MS.
2. In the Introduction, the authors state that there is a need to develop novel targeted therapies to improve fracture repair and that the new data from this study will inform these therapies. However, the study only focuses on normal healing and does not compare that to fractures that do not heal so it is unclear how the study meets this goal.
3. The study does not focus on the initial stages of healing where our knowledge is most limited and new information would be most useful for the goal of the project (see point 2). As BMP2 is known to be required to initiate healing, and no mention is made about BMP2 in this study, it suggests that the time point of D=5 is too late to achieve the goal of finding new factors that influence the healing response.
4. While the authors added D=0, the data are obtained on a different platform from D=5 and D=15; it would have been simple to collect D=0 and analyze using both platforms to demonstrate compatibility of results. Additionally, the number of D=0 samples could have been increased as this was a new set of experiments. As presented, concerns remain about the robustness of the results.
5. The descriptions for how the histological samples were prepared is only for D=5, with no mention of how D=0 or D=15 were handled.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
Please check spelling and punctuation as many errors are still present.

I suggest you change our very broad statements such as "comprehensive analysis of fracture healing" to statements that more accurately reflect your work which focused only on 2 specific stages of healing.

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Dear Editors and Reviewers,

Thank you for your comprehensive review of our manuscript, "Integrating spatial and single-cell transcriptomics to characterize mouse long bone fracture healing process." We appreciate the constructive feedback provided by all three reviewers and have made substantial revisions to address each of the concerns raised. Below, we provide detailed responses to the comments and describe the modifications we have made to our manuscript.

General Revisions Across the Manuscript:

We have incorporated a Day 0 (uninjured) timepoint to enhance the distinction between homeostatic and regenerative changes, as suggested. We utilized the Visium HD platform for sequencing of day 0 samples. The adoption of the HD platform was timely, as it became officially available during the submission period of our manuscript. This addition was crucial not only to supplement the dataset at this specific timepoint but also to enable more precise detection of the thin layers of periosteal cells.

We chose the HD platform for its ability to merge bins, which offers a resolution of 60 μm. This resolution is comparable to that of the traditional Visium platform which has a resolution of 55 μm, allowing for consistent cross-comparison between the platforms.

We have also improved clarity and data presentation, including enhancing figure quality and revising legends for better understanding.

Although adding a third sample for each group was challenging due to resource constraints, we have discussed the limitation of having an n=2 and its impact on data interpretation explicitly in the revised manuscript.

We believe that the revisions made in response to the reviewers' comments have significantly strengthened our manuscript, making it a valuable contribution to the field of bone biology. The revised manuscript, along with a point-by-point response and updated figures, has been resubmitted for your consideration. We hope that the changes meet your expectations and look forward to the possibility of our work being published in Communications Biology.

Thank you once again for the opportunity to revise our manuscript. Please do not hesitate to contact us if further information is required.

Specific Responses to Reviewer Comments:

Reviewer #1:

1) Two examples of figure legends either being incomplete or not labeled correctly are Figure 2 in which the legend does not have descriptions for two of the panels and the and figure 4 that indicates there is a panel B showing day 15 samples.

Response: We have revised all figure legends to ensure completeness and accuracy. New high-resolution figures have been prepared to improve visual clarity and data assessment.

2) For images throughout, the overlay of the ontology color coding on top of H&E sections makes it impossible to correlate simple histology to the spatial mapping. The two should be presented side by side and H&E sections should be of sufficient quality and size to identify by simple cell morphology tissue tissues like cartilage and bone.

Response: We now present H&E stained sections side by side with ontology color coding to better correlate histological features with spatial mapping.

3) For figure two it unclear how the authors related their spatial mapping and nomenclature of spatial clusters in the U MAP in panels D to the hierarchy and composition bar graphs in panels B and C. The text is also confusing relative to explaining the figure. As an example it's clear there are four related spatial cluster groups in B, but how these relate to D and E is not clear since cell data is mixed up with cluster data.

Response: We have clarified the relationship between spatial clusters identified in the UMAPs and the hierarchical data presented in the manuscript. A new supplementary figure now explicitly maps these relationships.

4) A general note as well is that closely related colors groups should be resolved by including a line patterning in the so the cell types in the spatial grouping can be sorted apart

Response: To resolve issues with closely related color groups, we have introduced different patterning in the figures, making it easier to distinguish between cell types.

5) Figure four is mislabeled on data is missing for day 15

Response: This mistake has been corrected.

Reviewer #2

1. The authors perform these studies using an n=2 and the data suggest variability (see gene expression trajectories as an example) so it is difficult to know the variability is due to technical differences or animal variation in healing responses. A suggestion would be to increase the number of animals investigated so as to determine the strength of the technical gains.

Response: We acknowledge the limitation due to the small sample size and have included a more detailed discussion on the variability and its potential impacts.

2. The authors chose day 5 and day 15 for analyses and compared their results to previously published data, thus providing a deeper analysis of already reported events. This seems like a missed opportunity as the enhanced techniques could be used to examine earlier stages of fracture repair where current spatial techniques have been unable to capture important information.
Response: We appreciate the suggestion and have now included data from day 0, allowing us to conduct a comprehensive reanalysis that strengthens our findings.

3. In the first paragraph of the Introduction, the authors state that chondrocytes and osteoblasts are essential for bone remodeling. This is inaccurate and should be reworded for clarity.
Response: We are sorry for this oversight, bone remodeling has been changed to fracture healing.

4. The description of the spatial transcriptomics protocol is very difficult to understand, especially paragraphs 2. Since this MS is based on highlighting a new protocol, clarifying the exact procedure should be a priority.
Response: The spatial transcriptomics protocol section has been extensively revised for clarity. We have included step-by-step descriptions to ensure comprehensibility.

Reviewer #3
1. Data Availability and Consistency: Not all the data described in the manuscript text are presented in the figures. It seems that an earlier version of the figures may have been submitted by mistake, making the review process challenging due to missing information and misaligned figure references and legends.
Response: We are so sorry for the carelessness, all missing data have now been included, and references to figures and datasets in the manuscript have been corrected to ensure consistency.

2. Clarity of Scientific Message: ***The manuscript lacks a clear and focused scientific message.*** *This is particularly evident in the introduction and abstract, where key findings are vaguely described without a concise summary. The authors should more clearly define and emphasize the main scientific contributions of their study.*
Response: We have extensively revised the manuscript to sharply define and highlight our study's core scientific contributions. Our research uniquely integrates spatial and single-cell transcriptomics to map cellular interactions during bone healing, offering novel insights into the spatial organization and temporal dynamics of cellular activities within the periosteum. We specifically emphasize the critical role of mesenchymal progenitor cells (MPCs) and their transition to regenerative MPCs (rMPCs), pivotal in the healing process. Additionally, we detail methodological enhancements in spatial transcriptomics that improve data resolution and accuracy, allowing for deeper understanding of the molecular mechanisms driving bone regeneration.

3. Contextualization of Data: The manuscript does not adequately situate its findings within the current landscape of skeletal stem and progenitor cell research. Various cell populations have been characterized by different markers (e.g., Lepr, Nestin, aSma, Pdgfra, Sca1, Edil3, Thy1). A thorough analysis of these markers, including their spatial expression patterns in the fracture callus, would provide crucial context for understanding the significance of MSC, PPC1, and PPC2 cells as described by the authors.
Response: Thanks to the suggestion, we have expanded our analysis of lineage-specific markers in the revised manuscript. We now include a detailed examination of the spatial expression patterns of key markers of reported periosteal progenitors. Among them, *Pdgfra*, *Ctsk*, *Ly6a*, *Pi16*, and *Edil3* are prominently displayed in the periosteal stem cell regions. Our spatial transcriptomic analysis reveals that these markers are preferentially expressed in areas identified as periosteal stem cell niches, suggesting their critical roles in defining the identity and function of periosteal progenitors during bone repair and regeneration.

4. Lineage and Marker Analysis: Related to the former point, the manuscript would benefit from a more comprehensive analysis of lineage markers for different cell types within the fracture callus e.g. osteoblasts, chondrocytes, adipocytes. The current analysis references only a few markers, which limits the understanding of the data, particularly regarding the authors' conclusion that their dataset includes adipocytes. Specifically, the authors use 2 markers to define adipocytes, one of which (Adipoq) is known to be expressed in a variety of cell types in bone and its spatial expression is not shown. Studies have also shown that Plin1, the other marker used, is also expressed in pre-adipocytes. Therefore, a greater number of adipocyte markers should be assessed to increase confidence in these data.
Response: A comprehensive analysis of lineage-specific markers is now included, detailing spatial expression patterns and their relevance to identified cell populations in the fracture callus. Enhanced visualizations through violin plots now illustrate the expression patterns of these markers across different cell clusters in Figure 3E, providing a detailed view of their distribution and intensity. Furthermore, we have compiled a list of the top 10 lineage-specific genes for each cell cluster in Table S2, which supports the robust classification and understanding of the diverse cellular responses during bone healing.

5. Sample Size and Experimental Limitations: The study analyzes only two male mice per time point. While spatial transcriptomics remains costly, this limited sample size and the lack of female samples restrict the robustness of the conclusions. This limitation should be discussed.
Response: We have acknowledged these constraints within our revised manuscript. We recognize that the limited sample size, consisting of only two male mice per time point, and the absence of female samples, might impact the robustness and generalizability of our conclusions. This limitation has been clearly discussed in the manuscript, including the potential variability due to individual differences and the gender-specific responses that could influence the healing process.

We have also emphasized the need for future studies to include a larger and more diverse sample pool, encompassing both genders, to enhance the reliability and applicability of our findings in bone healing research.

6. Lack of Baseline Uninjured Control: The absence of a Day 0, uninjured sample for comparison is a significant limitation. Statements regarding the onset or increase in gene expression at Day 5 cannot be substantiated without a baseline. This is also relevant for the Cell Chat analysis, where the suggestion that MSC interactions are essential for bone healing requires comparison with uninjured controls to validate. For example "early chondrocyte markers such as Col2a1 begin to emerge signifying...", "Significant upregulation of hypoxia-inducible markers." This is also relevant in the Cell Chat analysis as the authors suggest that the high number of MSC interactions indicates their essential role in bone healing. However, it is not clear from these spatial data alone that there is an increase in MSC interactions during healing without the control.

Response: We have added a Day 0, uninjured sample to our experimental design in the revised manuscript. This addition allows for a direct comparison of gene expression and cellular interactions before and after the fracture. We have thoroughly analyzed the Day 0 control data, which has provided crucial insights into the baseline state of cellular and molecular activities within the bone. Notably, the inclusion of this baseline has enabled us to substantiate claims regarding the onset and increase of gene expressions, such as early chondrocyte markers like Col2a1 and hypoxia-inducible markers, from a quiescent to an active state post-injury. Furthermore, the updated analysis using Cell Chat has highlighted the significant changes in regenerative mesenchymal progenitor cell (rMPC) interactions post-fracture, confirming their essential role in the healing process. This comparison has made it evident that the interactions among rMPC and other cellular constituents of the bone microenvironment intensify significantly during the repair process,

7. Statistical Analysis: There are several instances where the authors refer to "significant" changes without providing statistical evidence to support these claims (e.g., "significant upregulation of hypoxia-inducible markers" and "significantly increased vascular distribution". "elevation in Postn, Mif, Mdk, and Cxcl12 signaling"). The manuscript would benefit from the inclusion of appropriate statistical analyses to validate these observations.

Response: We have carefully reviewed our manuscript and amended the language to ensure that it accurately reflects the data presented. We have removed any references to "significant" where statistical comparisons were not performed.

8. Assignment of MPCs: The logic behind assigning spatial spots to "MPCs" is unclear, particularly since they do not appear to constitute the majority of the cluster. The reasoning behind this classification needs to be clearly articulated.

Response: We have reevaluated our results and clarified the classification process in the revised manuscript. We conducted a detailed reanalysis of the Day 0 data, focusing specifically on manually selecting cells located in the periosteal region for further examination.
This reanalysis involved integrating spatial transcriptomic data with single-cell deconvolution techniques to accurately identify the cell types present at the periosteum on Day 0. Our findings indicate that these periosteal cell spots predominantly consisted of MPCs and osteoprogenitor cells (OsteoP), which correspond to the fibrous and cambium layers of the periosteum, respectively. This identification is supported by both the spatial and single-cell transcriptomic profiles.
Furthermore, we tracked these cell types to Day 15, where they reappeared in the outermost layer of the regenerating callus, indicative of newly formed periosteal cells.

9. Endothelial Marker Expression: The manuscript references "elevated expression of endothelial markers" between Days 5 and 15, but this is not visually evident from the data. The authors should present these findings in the form of violin plots or other visual aids to clarify the data, especially since no endothelial clusters are identified.
Response: We have reassessed our presentation and focus concerning endothelial cell involvement in the bone healing process. While our initial manuscript did not clearly visualize the expression of endothelial markers, we have shifted our approach in the revision.
Rather than emphasizing individual endothelial cell clusters, which were not distinctly identified in our data, we have enhanced our analysis of angiogenesis pathways through Gene Set Variation Analysis (GSVA). Our revised data analysis reveals that several clusters exhibit a significant increase in angiogenic pathway activity post-fracture, indicative of vascular remodeling and new vessel formation in the healing tissue.

10. Differential Expression and Pathway Analysis: A basic analysis of differentially expressed genes (DEGs) and gene set enrichment analysis (GSEA) in the various spatial clusters over time is notably absent. This is a minimal requirement to enhance the biological insights of the study.
Response: We thank the reviewers for their insightful comments and suggestions. In response to the concerns raised about the absence of differential gene expression (DEG) analysis and gene set enrichment analysis (GSEA), we have incorporated these crucial analyses into our revised manuscript. we have conducted a comprehensive DEG analysis focusing primarily on the comparison between regenerative mesenchymal progenitor cells (rMPCs) and MPCs, This analysis has identified key genes that are differentially expressed between these important cell types during fracture healing.
Additionally, we have performed GSEA to provide insights into the biological pathways that are significantly enriched or suppressed in these clusters over time. This analysis helps elucidate the molecular mechanisms and biological processes driving bone repair and regeneration.

11. Trajectory Analysis Bias: Figure 4 raises several questions, including the rationale for the

trajectory analysis and the exclusion of PPC2s and osteoblasts. The data suggest an increase in chondrocyte markers and a decline in progenitor markers along this trajectory, which is expected given the setup. The manuscript should clearly explain what new insights are gained from this analysis and also use the day 15 sample as well as day 5 for this analysis.
Response: We appreciate the reviewers' critical insights regarding our initial presentation of the trajectory analysis in Figure 4. We have revised our trajectory analysis using the Monocle tool to include comprehensive data from Day 0, Day 5, and Day 15, enhancing the temporal depth of our study. This new analysis primarily focuses on elucidating the critical transcription factors that regulate the transition of MPCs into osteogenic and chondrogenic lineages across the different stages of bone healing.

Minor Concerns:

1. Selection Criteria for Cross-Section: The authors mention the "meticulous selection" of a central cross-section showcasing maximal callus development but do not elaborate on the criteria used. Further explanation of these criteria would be helpful.
Response: We thank the reviewers for their review and helpful suggestions, we have updated the manuscript to include a detailed explanation of our methodology. For each femur, we section the entire bone continuously and select the cross-section that demonstrates the greatest area of callus development.
2. RNA Quality and Decalcification Protocol: The authors claim that their decalcification protocol significantly improves RNA quality compared to other studies. However, without a direct comparison of different methods, it is difficult to attribute this improvement solely to the decalcification protocol.
Response: We thank the reviewers for their insightful observations regarding our decalcification protocol. In our study, we have observed that the Morse's solution decalcification method results in higher DV200 values, which indicates better preservation of RNA integrity compared to the traditional EDTA decalcification. Furthermore, compared to published studies using similar tissues and techniques, our sequencing results have demonstrated superior quality.
It is important to note that while other aspects of our sample processing, such as fixation and sectioning, were standardized and comparable to conventional methods, the significant improvement in RNA quality in our samples suggests that the decalcification step might plays a critical role in preserving RNA integrity during spatial transcriptomics.

3. Data Analysis Pipeline: The text refers to Fig. 1A as a pipeline illustrating comprehensive data analysis, but the figure does not clearly convey the role of each software package. This should be clarified to aid the reader's understanding.

Response: We thank the reviewers for pointing out the need for clarity regarding the data analysis pipeline presented in Fig. 1A. In response, we have revised the figure to include brief descriptions of each software package used in our analysis.

4. H&E Staining Labels: The H&E stainings in Figure 1 should be labeled to indicate key features such as the callus, fracture line, cortical bone, growth plate, and proximal/distal femur. This is particularly important since one of the fractures appears more proximal, which could affect the role of the growth plate in repair.
Response: We appreciate the reviewers' suggestions for improving the clarity of our H&E staining images in Figure 1. We have now added labels to these images to clearly indicate key anatomical features such as the callus, fracture line, growth plate, and the proximal parts of the femur.

5. RNA Quality and Ossification: The manuscript states that "areas undergoing ossification showed enhanced RNA quality." However, no data are provided to link specific regions with RNA quality, and there should not be a direct correlation between RNA quality and biological activity. This statement should be clarified or removed.
Response: Thank you for your insightful comments regarding our description of transcriptional activity or RNA quality in different bone regions during the healing process. We have revised the manuscript to clarify these observations. We now specify that "gene expression" rather than "transcriptional activity" or "RNA quality" is being discussed to more accurately reflect changes in the number and diversity of detected genes across various time points.

6. PPCs and Proliferative Capacity: The authors state that "PPCs are distinguished by their robust proliferative capacity." However, at Day 5, a large proportion of cells are in G2/M, which does not seem to uniquely distinguish PPCs from other cell types. This point requires further clarification.
Response: We appreciate the feedback regarding the need for clarification on the proliferative capacity of rMPC. To address this, we have included new figures (Figures 3F and G) that display the cell cycle stages at different time points post-fracture using barplot.

7. Necrotic Cells and Cell Cycle: The authors refer to necrotic cells within the callus but the data also suggest that these cells are in G2/M phase. The interpretation that these cells are necrotic is inconsistent with their active division. This discrepancy should be addressed.
Response: We appreciate the comment regarding the potential inconsistency in identifying necrotic cells within the callus. Upon reevaluation, we have adjusted our analysis and classifications. The cells initially indicated as necrotic are now reclassified as clusters 0 and 9, which represent osteocytes or low quality cells. This refined analysis and updated classification have been incorporated into the revised manuscript to better reflect the observed cellular states.

8. Changes in Cell Populations Over Time: The manuscript briefly presents changes in cell populations over time in Fig. 2F but does not discuss these changes in the text. The authors should elaborate on these observations and their biological implications.
Response: Thank you for your observation regarding the changes in cell populations over time. We have now included a detailed explanation of these changes and their biological implications in the results section of the revised manuscript.

9. Bioinformatics Pipeline: A detailed bioinformatics pipeline for the analysis in Figure 2 would clarify the progression of the analysis. The logic behind simplifying 17 cell types into 12 groups, particularly the inclusion of adipocytes not present in the scRNA sequencing data, needs further explanation.
Response: Thank you for your insightful comments. We have completely revised Figure 2 and have thoroughly addressed the new findings and their implications within the manuscript.

10. Mechanosensitive Genes in PPCs: The enrichment of mechanosensitive genes in PPCs, particularly for genes other than Ccn1, is not clearly demonstrated from the plots shown. Violin plots showing gene expression within different clusters would better illustrate these findings. The authors should also include Day 15 data for a more comprehensive analysis.
Response: We have added additional analyses to further highlight the upregulation of mechanosensitive pathways within the regenerative mesenchymal progenitor cells (rMPCs) post-fracture. This is now clearly illustrated in our dot plots, which show significant upregulation of mechanosensitive marker genes across all examined time points.

11. GSEA Analysis: The manuscript mentions GSEA results showing enrichment of hypoxia and glycolysis pathways but does not clarify whether this analysis is specific to certain clusters or applies to all clusters combined. A cluster-specific GSEA would provide deeper biological insights.
Response: We have performed a cluster-specific gene set variation analysis (GSVA) to provide detailed insights into the biological pathways active within each cluster. This refined analysis highlights the enrichment of hypoxia and glycolysis pathways in specific clusters, enhancing our understanding of their distinct roles in fracture healing.

12. MSC and PPC Relationship: The relationship between MSCs and PPCs is unclear—is one simply a proliferating version of the other? Cell cycle normalization could help clarify this, and projecting cell cycle information onto the UMAP in Fig. 2A would provide additional context.
Response: We have addressed the relationship between MPCs and PPCs (rMPCs) by applying cell cycle regression during our analysis to prevent biases. Our UMAP and Monocle pseudotime analysis, aligned with our prior single-cell data, supports that PPCs or rMPCs derive from MPCs.

Following injury, MPCs transition into a more proliferative state, undergoing biological functional changes towards characteristics of myofibroblasts, which is a focal point of our study.

13. Macrophage Analysis: The manuscript states that "a significant increase of macrophages within the MPC spot" was observed near the fracture site but does not present statistical evidence. This should be plotted as a graph, and significance should be assessed.
Response: Thank you for your insightful feedback and suggestions. We have conducted a correlation analysis of the proportion of macrophages within MPC spots relative to their distance from the fracture line, revealing statistically significant differences. This finding is further substantiated by histological staining, confirming the correlation analysis results.

14. Biological Interpretation of Data: The manuscript would benefit from a more in-depth discussion of the biological meaning behind the data. For example, Fig. 3B shows distinct clusters for MPCs and PPCs, but the reasons for this clustering—whether due to clonal expansion, differential adhesion, or other factors—are not discussed.
Response: Thank you for your valuable comment. In our revised manuscript, we have expanded the discussion to clarify the transformation from MPCs to rMPC (PPCs) as an activation process. We detail how resting stem cells, upon receiving injury signals—mechanical and inflammatory—activate pathways such as TGF-beta and engage transcription factors like En1 and Meox2. These factors play a crucial role in the activation of myofibroblasts, influencing both intracellular structures and extracellular factors. Acta2, a key marker of myofibroblasts, is regulated by these transcription factors, illustrating the complex interplay of signaling that governs cell state transitions during bone healing. This refined explanation enhances our biological interpretation of the data.

Dear Editors and Reviewers,

Thank you for your comprehensive review of our manuscript, "Integrating spatial and single-cell transcriptomics to characterize mouse long bone fracture healing process." We appreciate the constructive feedback provided by all reviewers and have made substantial revisions to address each of the concerns raised. Below, we provide detailed responses to the comments and describe the modifications we have made to our manuscript.

General Revisions Across the Manuscript:

We therefore invite you to revise and resubmit your manuscript, taking into account the points raised. In particular, please provide:

- *a restructured introduction to illustrate the focus of the work on in depth characterization of the healing process rather than focusing on novel therapies*

We appreciate the editor's suggestion and have revised the introduction accordingly. The introduction now clearly emphasizes our study's primary aim: providing an in-depth characterization of the cellular and molecular processes involved in fracture healing, rather than focusing on novel therapeutic strategies.

- *provide a comparative analysis of the duplicates to show reproducibility*

We thank the editor for this suggestion. To demonstrate reproducibility, we have included correlation analyses, differential gene expression heatmaps, and split-view UMAP plots, all of which illustrate strong consistency between biological replicates at each time point.

- *provide details on how histology samples were prepared for D=0 or D=15*

We thank the editor for this suggestion. We have revised the histology methods section to clearly indicate that immunofluorescence staining for macrophages was specifically performed on day 5 samples. Additionally, the preparation procedures for spatial transcriptomics were identical across all time points (days 0, 5, and 15), and these details have been explicitly stated in the methods section.

- *add a discussion showing the limitations of the study (duplicates use).*

We thank the editor for this suggestion. We have now clearly addressed this limitation in our discussion, acknowledging that although ideally three replicates would enhance robustness, the high reproducibility between our duplicates supports the reliability of our findings.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript presents a detailed spatial transcriptomic analysis of murine fracture healing over three time points of healing.

The manuscript is greatly improved from the prior submission. The text and figures now show good correspondence and the figures are now much clearer, so that they are more easily understood.
The authors have been very responsive to the reviewer's question's

It is unclear how to interpret the data in figure 6 between the spatial and single cell data. Is the proximity of cell types as represented by colored dots based on if they are clustered as group together? If so this should be stated in the text.

We thank the reviewer for raising this important point. To simplify visualization and interpretation of spatial interactions, certain cell clusters were merged based on their differential gene expression profiles (Figure 2B): clusters 1, 4, 6, 12, 21, and 26 were combined as skeletal muscle cells; clusters 5 and 17 as erythrocytes; clusters 8 and 25 as reparative mesenchymal progenitor cells (rMPCs); and clusters 19 and 22 as macrophages. We have highlighted this adjustment explicitly in the revised manuscript.

The only concern to be noted it that the study assessed only two mice and it is unclear the degree of variability of these data even between replicates. In this regard it is hard to assess the accuracy of assigning a cells position to another cell or likely hood of the assigned communication between cell types. How is this enumerated?

We thank the reviewer for raising this important concern. Although our study included two biological replicates per time point, we have explicitly addressed the limitation of sample size in our discussion. To demonstrate reproducibility, we performed correlation analyses, differential gene expression heatmaps, and split-view UMAP plots, which collectively showed high consistency between replicates. Additionally, our assignment of cellular positions and inferred cell-cell communication is based on robust computational methods (CellChat), which quantify interaction strengths using statistically supported ligand-receptor interaction models. While additional replicates would strengthen these conclusions, the consistency observed between existing replicates supports the reliability of our findings.

Reviewer #2 (Remarks to the Author):

This revised MS by Yao et al provides an analysis of the use of enhanced methodologies to examine fracture repair. While the MS is improved from the original submission, substantial concerns remain to be addressed.
1. There are numerous typos and grammatical errors throughout the MS.

We thank the reviewer for pointing out these issues. The manuscript has been thoroughly revised to correct all typos and grammatical errors, and has undergone careful proofreading to ensure clarity and readability.

2. In the Introduction, the authors state that there is a need to develop novel targeted therapies to improve fracture repair and that the new data from this study will inform these therapies. However, the study only focuses on normal healing and does not compare that to fractures that do not heal so it is unclear how the study meets this goal.

We appreciate the reviewer's insightful comment. We have revised the Introduction to clearly state that the primary objective of our study is to comprehensively characterize the cellular and molecular mechanisms underlying normal fracture healing. We have removed statements regarding novel therapeutic development to better reflect the scope and focus of our current analysis.

3. The study does not focus on the initial stages of healing where our knowledge is most limited and new information would be most useful for the goal of the project (see point 2). As BMP2 is known to be required to initiate healing, and no mention is made about BMP2 in this study, it suggests that the time point of D=5 is too late to achieve the goal of finding new factors that influence the healing response.

We thank the reviewer for this valuable suggestion. We have now included an analysis of BMP2 expression (Figure S9), which revealed that BMP2 was primarily expressed by regeneration-associated cell subsets and significantly upregulated after fracture, particularly at day 5. These findings have been explicitly incorporated into our results section, further highlighting the molecular events occurring in the early regenerative phase of fracture healing. Additionally, we acknowledge the importance of examining earlier stages of fracture healing, and plan to include analyses of earlier time points in future studies to more comprehensively capture the initiation and progression of the healing response.

4. While the authors added D=0, the data are obtained on a different platform from D=5 and D=15; it would have been simple to collect D=0 and analyze using both platforms to demonstrate compatibility of results. Additionally, the number of D=0 samples could have been

increased as this was a new set of experiments. As presented, concerns remain about the robustness of the results.

We appreciate the reviewer's concern regarding platform compatibility. Internal correlation analyses showed high reproducibility and consistency between the biological replicates at each individual time point, supporting the reliability of our findings. We acknowledge the reviewer's valid concern regarding sample number and platform compatibility, and recognize this as a limitation in our current study. We will address these points in future experiments to further enhance robustness and comparability.

5. The descriptions for how the histological samples were prepared is only for D=5, with no mention of how D=0 or D=15 were handled.

We thank the reviewer for raising this point. The histological preparation described (immunofluorescence staining for macrophages) was specifically performed for Day 5 samples. For spatial transcriptomics analysis, sample preparation protocols were consistent across all time points (Days 0, 5, and 15), and we have now explicitly clarified this in the revised methods section of the manuscript.

Response to Reviewer Comments

"Please address the concerns about a general statement that should be more narrow: 'comprehensive analysis of specific stages of fracture healing'."

We fully agree with the reviewer's suggestion and have now revised the manuscript accordingly. We acknowledge that our study specifically covers three selected time points (Days 0, 5, and 15 postfracture). Therefore, we replaced general statements such as "comprehensive analysis" with more precise wording.

"Please check spelling and punctuation as many errors are still present. We recommend editing the main text for English language and grammar to improve readability and clarity."

We carefully reviewed and corrected all spelling, punctuation, grammar, and readability issues throughout the manuscript.

We hope these revisions fully address the reviewers' concerns and meet the journal's high standards for publication. We thank you again for your valuable feedback and look forward to the next step in the review process.

Best regards,
Lutian Yao
On behalf of all authors