nature portfolio

Peer Review File

Notch3 regulates pericyte phenotypic plasticity in colorectal cancer

Corresponding Author: Dr Vasiliki Koliaraki

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)
In their manuscript “Notch3 regulates pericyte phenotypic plasticity in colorectal cancer”, Chalkidi, Stavropoulou et al. describe the functional heterogeneity of tumor-associated pericytes in colorectal cancer and the important regulatory role of Notch3 signaling in pericyte plasticity. The study is highly interesting as pericytes are a component of the tumor microenvironment, which has an important role for tumor progression and metastasis in colorectal cancer. Targeting Notch3 signaling might therefore be a promising therapeutic target for colorectal cancer.

The authors used performed single-cell RNA-sequencing analysis to investigate pericyte heterogeneity in mouse colitis-associated cancer. Using published datasets, they validated their observations in human colorectal cancer samples. To investigate pericyte proliferation during carcinogenesis, they used a lineage tracing approach. Furthermore, the role of Notch3 was analyzed using in vivo genetic manipulation as well as an orthotopic transplantation model.

The manuscript is very well written and the line of thought is clear to follow throughout the manuscript. The figures are clearly presented. However, I have some suggestions for further optimization:

Major points:
1) The authors present in detail how Notch3 impacts pericyte plasticity. The text abstract summarizes these findings well. To allow the reader to have an easily accessible overview of the findings, a graphical abstract would be helpful.
2) The concept of phenotypic plasticity in general should be described in the introduction.
3) The authors should describe in more detail how the heterogeneous pericyte functions/phenotypes are related to pericyte morphology.

Minor points:
1) In ll. 78-80, context-dependent effects of Notch3 signaling are mentioned. As this aspect is highly interesting, the authors should give examples and comment on this in a bit more detail.
2) The results part starts very technical (p. 5). To facilitate reading, the aim/purpose should be described first.
3) The published single-cell RNA-sequencing datasets (ll. 126-127) used in this study should be described with basic information like number of samples, etc.
4) In figure 1, it should become more clear which parts are based on mouse and which ones based on human samples. It would be helpful to have matching color assignments for the human and mouse subfigures.
5) For all figures, the authors should ensure that all figure/graph elements are described, e.g., for the violin plots.
6) The transition of the text towards Notch3 (l. 165) requires more connecting details.
7) The figures are put together well and show the different findings. The sections describing the results for each figure should always have its own subheading to allow the user to navigate through the text (e.g., l. 204)
8) Expression of Heyl is shown in supplementary figure 4, but there is no introduction on why Heyl is interesting and also no description of the results depicted.
9) When statistical tests were performed, precise p-values should be added. This is also the case for differences that are not significant.
10) The reference to subfigure 4f in the text is missing (p. 10).
11) In some figures, “one of two experiments” is shown. Are the shown experiments representative?
12) In addition to figure 5f, also the number of tumors should be plotted (l. 263).

13) The authors claim that “Notch3 signaling plays a crucial role in advanced stages of colorectal cancer progression” (ll. 266-267). It would be interesting to explore associations of Notch3 using TCGA.
14) The authors should describe the relevance or role of Acta2 when mentioning it in the results (ll. 275-276).
15) The authors should show representative images for the quantifications performed in figures 5j-k.
16) The FACS tSNE plots in figure 6c need more explanation. What does it show? How was it generated?
17) The authors should describe the availability of used code.
18) A similar figure setup for supplementary figures 1 and 2 would be beneficial.
19) The legend should be phrased differently for supplementary figure 3a.

Reviewer #2

(Remarks to the Author)
The authors presented data to show the role of Notch3 signaling in tumor pericytes phenotype and ultimately tumor progression in the context of CRC tumors.
The authors used lineage tracing and single-cell sequencing to show the abundance and origin of the tumor pericytes. In vivo knockdown and overexpression experiments were utilized to confirm the causative relationship between Notch3 signaling and vascular cell proliferation/properties. Lastly, an in-depth analysis of single-cell sequencing data was performed to show the pericyte heterogeneity. The manuscript shows a significant increase in vascular cells and a decrease in fibroblasts in the tumor model, indicating a change in the stroma landscape during tumor progression. Based on the abundance of proliferating pericytes and lineage tracing using reporter mice, the authors determined the residential pericyte as a source of tumor pericytes. Overexpression of N3ICD in PDGFRβ+ cells stimulated pericyte proliferation but decreased αSMA expression, suggesting the role of Notch 3 signaling in shifting pericyte cell fate. KO of Notch3 resulted in reduced endothelial cell proliferation, but no changes in α-SMA expression or tumor progression were observed. Orthotopic implantation of AKP organoids resulted in a significant reduction in tumorigenicity. Finally, single-cell sequencing data revealed several distinct populations of tumor pericytes, including ECM-related, myogenic-related, and inflammatory pericytes. While most of the in-vivo experiments are well-organized and thought through, the in-vitro experiments are superficial, and not all results support the hypothesis, thereby weakening the work. Lineage tracing to identify the source of the tumor pericyte is logical, leading to a clear result. While the functional study of Notch3 signaling is not novel, considering the known function of Notch3 in cellular proliferation, it suggests a potential angle toward vascular normalization through shifting the cell fate from proliferation to differentiation. Orthotopic implantation of AKP organoids in Notch3 KO mice doesn’t seem to make a clear connection between Notch3 signaling and pericyte property or vascular integrity, as there was no data to support the hypothesis. In addition, implanting organoids doesn’t seem to simulate late-stage CRC, and therefore, the interpretation requires revision. Lastly, the expression of αSMA does not necessarily mean that the pericytes are mature and functional. Normal capillary pericytes lack αSMA expression; therefore, these pericytes are unlikely to be intact or functional. Single-cell sequencing data provide valuable insights into pericyte heterogeneity and can inform future research. Statistical analysis and material and methods sections are provided in detail, allowing the work to be reproduced.

Detailed comments:
Detailed/Specific Comments:
1. Figure 1e, please move the aSMAhi SMCs percentage inside the positive quadrant (it’s confusing with where it currently is). Figure 1j, please include a color annotation for this bar graph. Figure 1c: Does the individual cell type include both normal and tumor? This data is confusing. If fibroblasts include both normal fibroblasts and CAFs, then shouldn’t the ACTA2 level be higher, as that’s the classic indication of CAFs?
2. There seems to be quite a difference in stromal composition between mouse AOM/DSS (fig. 1d) and human tumor tissue (fig 1j). For example, the majority of stroma in the normal mouse colon is fibroblasts, but that’s not the case in the patient sample. Also, fibroblast levels decrease in mouse tumors but increase in human cases. The same applies to all other types of cells, unless the color annotation differs between mouse and human.
3. “Notch3 is predominantly expressed in vascular smooth muscle cells (vSMC) and pericytes…” according to this sentence in the introduction, but later the authored indicated Notch3 as a specific pericyte marker. Additionally, throughout the manuscript, αSMA was used as a pericyte marker. While tumor pericytes tend to misexpress αSMA, vascular smooth muscle cells are the ones that predominantly express αSMA. Therefore, the interpretation of the results is ambiguous.
4. Figure 2c and h, please label the representative FACS plot with either normal or AOM/DSS, and move the cell percentage inside the box/gate for clarification. Fig 2f and 2k, is it normal or tumor tissue image? Please label all the images/data appropriately.
5. Figure 4d: How did the author determine if GFP+ proliferative cells are specifically pericytes, not CAFs? Fig. 4f representative FACS plot is missing.
6. As the author claims, there is an increased proliferation of pericytes and endothelial cells in CRC tumors, but there is decreased coverage of pericytes as compared to normal. Please elaborate on this hypothesis.
7. Figure 4, while there is an increase in pericyte and endothelial cells proliferation in the tumor microenvironment, there were no changes in tumor size and multiplicity. What is the explanation for this? Were there actually a greater number of vessels? This is an important piece of data missing for this experiment. For the Notch3 signaling-mediated vascular cell proliferation to have a functional effect on tumor progression, there should be a greater number of vessels, not just the cells, and the perfusion assay should confirm whether those vessels are functional.
8. While quantifying proliferating pericytes, Ki67+PDGFRβ+/Ki67+DAPI+ were counted, how CAFs were differentiated from pericytes, as both cell types express the same marker- PDGFRβ.
9. For the quantification of αSMA+ signal around blood vessels, how did you differentiate bigger blood vessels from capillaries? Larger blood vessels have vascular smooth muscle cells surrounding them, even in a healthy situation, to provide contractile function, whereas healthy capillaries do not. αSMA expression is acquired in a tumor condition. If this is not taken into consideration, then quantification could be misleading.

10. As per lines 320-321, capillary endothelial cells are the main source of Jag1 and D1l4 in TME, but in Fig. 4d and Fig. 5c, αSMA expression around the blood vessels by pericyte is presented around artery endothelial cells.
11. No data is presented to show that deleting Notch 3 in tumor pericytes affects the tumor microenvironment or tumor vasculature. Suggested conditions include hypoxia, vasculature leakage, and metastasis, among others.
12. I am not so sure what AKP organoids in the Notch3 KO mice experiment suggest. In this condition, Notch3 is absence from the whole body, making me wonder what the vessels look like in even normal mice without a tumor. Then, after organoid implantation, if the Notch3 KO prevents endothelial cell and pericyte proliferation, tumor angiogenesis will not occur, making the tumor avascular, if the hypothesis is correct. In this case, I am unsure whether this actually tests the role of Notch3 in tumor progression at the advanced stage. Please provide some explanation on this.
13. Missing the rationale behind how the author determined high and low for PDGFRαhi and PDGFRαlow, similarly for αSMAhi and αSMAlow.

While there are several comments that need to be addressed, if the authors can provide a revision in a timely manner with clarifications, the quality of the work appears suitable for publication in Communications Biology.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
In this manuscript, Chaldiki et al. investigated the phenotypic heterogeneity of pericytes in colorectal cancer and specifically the role of Notch3 signalling. Using mouse models of colitis-associated CRC and published human CRC data, they identified heterogeneous pericyte subpopulations with different Notch3 activity. In both the colitis model and a more advanced AKP CRC model, they showed that Notch3 deletion leads to reduced endothelial proliferation and a potential shift toward a contractile pericyte phenotype. In the AKP model, Notch3 KO also leads to decrease in tumorigenesis.

The data is of very good quality and the analyses are robust. The results about Notch3 regulating endothelial cell proliferation are also very convincing. However, even though activation of Notch3 showed decreased aSMA expression, in Notch3 KO in the colitis CRC models there was no difference in aSMA staining around blood vessels. The difference in Acta2 was more convincing in the AKP models, however Acta2 is also highly expressed in fibroblasts and smooth muscle cells. In our opinion, there is no direct evidence to support the claim that Notch3 can induce a shift towards a contractile phenotype and that it is a key regulator of pericyte plasticity, and the text should be modified to reflect that or additional experiments should be performed to validate a transition in phenotype.

Major comments:

In Figure 1, could you please define define ** and *** in 1(f). It was also not clear why the authors used one-way ANOVA in 1(f) when in subsequent similar data used t-test? e.g. 2(d)

Figure 2 e-f - how are the authors sure that these are Notch3 expressing pericytes, are they inferring this based on location/morphology? Are there other cell types expressing Notch3 e..g epithelial cells?

Figure 4e, in the legend the authors write “Results were analysed using Mann-Whitney test and unpaired two-tailed t-test”. Which of the two was specifically used for the corresponding figure?

Figure 6, the myPCS have a very low expression of Rgs5 (and the same in Figure 7 for the human pericytes), how confident are the authors that these are pericytes and not smooth muscle cells? Related to this, line 304, it would be very helpful to include the pericyte markers mentioned (Notch3, Pdgfrb, and Ndufa4l2) and other general pericyte markers in the dot plot in figure 6b to confirm that those are pericytes.

For the trajectory analyses, why was ecmPC used as a root?

Line 316, the authors wrote that proliferation markers are also increased in ecmPCs, but from the plots this is not obvious. Could they confirm this with more genes or a cell cycle score?

Figure 7a,b - A reasonable proportion of the cluster named ecmPC_N is tumour cells so it could be better to called ecmPC_mixed?

Figure 7e - can the authors show an additional marker gene for the inflammatory pericytes, to make it more convincing?

Figure 7c - HEYL seems to have the highest expression in the intermediate cluster and not ecmPCs, how do the authors interpret this?

For the quality control analyses, it was not clear why a lower cut-off of 3% and 2% for ribosomal gene expression was used?

Minor:

I think the annotation “synthetic” ecmPC maybe could be renamed to just ecmPC?

In the abstract, the authors wrote “metastatic orthotopic model” - Advanced might be a better term here? There wasn’t anything mentioned of whether metastases were formed in the AKP model, only primary tumours.

Line 569 - anti-CD45 and anti-CD326 antibodies should be the other way around - for immune and epithelial cells respectively

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed the majority of my suggestions regarding the original manuscript.

However, regarding my previous comment #5 (description of all graph elements), the authors should double check for every subfigure that all required information is included and correct (see also in the data presentation paragraph in the editorial policy checklist). n should be described for the different plotted subgroups, clusters etc. Examples: How many pericytes are in each individual cluster in Figure 7? What do the different lines in Figure 5d indicate? Do the error bars really always depict the mean +- SD as stated (sometimes there are asymmetric error bars, e.g. Figure 5b)... This information allows the reader to better evaluate the data.

Reviewer #2

(Remarks to the Author)
The authors addressed most of the comments and concerns that significantly impact the quality and accuracy of the manuscript. Therefore, satisfied with the revised version of the manuscript with no further comments.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
The authors have addressed our comments and the manuscript is much improved.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Note from Editor*: You will see from the referees' comments copied below that while they find your work of potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the manuscript for publication but would be interested in considering a revised version that addresses these concerns. We hope you will find the referees' comments useful as you decide how to proceed. Should further experimental data or analysis allow you to address these criticisms, we would be happy to look at a substantially revised manuscript. In particular, in terms of additional experiments, we ask that you focus on adding the data requested by Reviewers #2/#3 in their comment 7. Also, if additional data are not added to support other claims that Reviewers #2/#3 and #4/#5 pointed out as unsupported by the current data (e.g., Notch 3 affecting the tumor microenvironment or functioning as a key regulator of pericyte plasticity), such claims should be thoroughly toned down/altered to address the relevant concerns from these reviewers.*

We would like to thank the editors and reviewers for their positive and insightful comments, which have helped improve our manuscript. We have paid particular attention to comments 7 and 11 by Reviewers #2/3 and provided additional data to better describe the effects of Notch3 genetic manipulation on the tumor microenvironment. We have also made several modifications to increase the clarity of our manuscript and draw only conclusions that are robustly supported by our experimental evidence. All reviewers' comments are addressed in our point-by-point response below.

Reviewer #1 (Remarks to the Author):

In their manuscript "Notch3 regulates pericyte phenotypic plasticity in colorectal cancer", Chalkidi, Stavropoulou et al. describe the functional heterogeneity of tumor-associated pericytes in colorectal cancer and the important regulatory role of Notch3 signaling in pericyte plasticity. The study is highly interesting as pericytes are a component of the tumor microenvironment, which has an important role for tumor progression and metastasis in colorectal cancer. Targeting Notch3 signaling might therefore be a promising therapeutic target for colorectal cancer. The authors used performed single-cell RNA-sequencing analysis to investigate pericyte heterogeneity in mouse colitis-associated cancer. Using published datasets, they validated their observations in human colorectal cancer samples. To investigate pericyte proliferation during carcinogenesis, they used a lineage tracing approach. Furthermore, the role of Notch3 was analyzed using in vivo genetic manipulation as well as an orthotopic transplantation model. The manuscript is very well written and the line of thought is clear to follow throughout the manuscript. The figures are clearly presented.

However, I have some suggestions for further optimization:

We thank the reviewer for their careful review and encouraging feedback on our manuscript. We appreciate their positive assessment and constructive suggestions that helped improve our manuscript.

Major points:

1) The authors present in detail how Notch3 impacts pericyte plasticity. The text abstract summarizes these findings well. To allow the reader to have an easily accessible overview of the findings, a graphical abstract would be helpful.

We thank the reviewer for this suggestion. We have prepared a graphical abstract based partly on Figure 7j that summarizes the experimental approach and key findings regarding Notch3's role in pericyte plasticity. This has been submitted now along with the revised manuscript.

2) The concept of phenotypic plasticity in general should be described in the introduction.

We thank the reviewer for this comment. To address it, we have expanded the introduction to include a general description of phenotypic plasticity (lines 41-43), along with a relevant reference to provide readers with the necessary background (reference 5).

3) The authors should describe in more detail how the heterogeneous pericyte functions/phenotypes are related to pericyte morphology.

Pericyte phenotypes are indeed linked to their morphology, as changes in shape and structure reflect their functional state. Quiescent, mature pericytes typically display an elongated morphology with slender cytoplasmic processes extending along and around capillaries. These cells possess a cytoskeleton enriched in contractile filaments including α-smooth muscle actin (α-SMA), enabling their vasomodulatory functions. In contrast, synthetic or proliferative pericytes exhibit a broader, rounded appearance. They show reduced contractile filament content and instead contain extensive rough endoplasmic reticulum, Golgi apparatus, and ribosomes that support their proliferative and matrix-secreting functions (references 55-56). To address the reviewer's comment, we have now added a short paragraph in our Discussion to provide readers with a clearer understanding of how pericyte morphology reflects their functional state and how this may be relevant to our study (Lines 309-312, references 55-56).

Minor points:

1) In ll. 78-80, context-dependent effects of Notch3 signaling are mentioned. As this aspect is highly interesting, the authors should give examples and comment on this in a bit more detail.

We agree that this is an important aspect of Notch3 signaling. Since our focus is on CRC, we have rephrased this sentence (now Lines 80-81) to place greater emphasis on Notch3's tumor-promoting functions that are most relevant to CRC and specifically analyzed in our study. We note that the cited reference at the end of this sentence is an excellent review on the role of Notch3 in cancer that contains examples of both pro- and anti- tumor functions. These revisions provide better context for understanding the specific Notch3 functions we investigate in the current work.

2) The results part starts very technical (p. 5). To facilitate reading, the aim/purpose should be described first.

We have rephrased the opening paragraph of the Results section (now p.4, line 99) to begin with the study's aim and rationale before presenting the technical details, as recommended by the reviewer.

3) The published single-cell RNA-sequencing datasets (ll. 126-127) used in this study should be described with basic information like number of samples, etc.

We have added more clear information about the published single-cell RNA-sequencing datasets in the Methods section (Lines 523-525), including sample number and type (normal, tumor, border). For complete patient-related information and detailed clinical metadata, we have directed readers to the original publications, in which these datasets were first reported.

4) In figure 1, it should become more clear which parts are based on mouse and which ones based on human samples. It would be helpful to have matching color assignments for the human and mouse subfigures.

We acknowledge that the different color annotations used in the single-cell analysis of mouse and human datasets may have been confusing. In response to comments from both reviewers #1 and #2/3, we have now standardized the color scheme across both analyses to ensure consistent representation of stromal clusters between species (updated in Figures 1i-j). In addition, we have added labels in Figure 1 to explicitly indicate the mouse- and human related analysis, as suggested by the reviewer.

5) For all figures, the authors should ensure that all figure/graph elements are described, e.g., for the violin plots.

We have carefully reviewed all figures in the manuscript and have ensured that all graphical elements are properly described in the figure legends and that all figures are cited in the revised manuscript.

6) The transition of the text towards Notch3 (l. 165) requires more connecting details.

We added more connecting detail in line 143 to facilitate the transition of the text towards Notch3, as suggested by the reviewer.

7) The figures are put together well and show the different findings. The sections describing the results for each figure should always have its own subheading to allow the user to navigate through the text (e.g., l. 204)

We have now added subheadings that correspond to each figure, as suggested by the reviewer. These additions can be found in Lines 156 and 245 of the revised manuscript.

8) Expression of Heyl is shown in supplementary figure 4, but there is no introduction on why Heyl is interesting and also no description of the results depicted.

We thank the reviewer for the opportunity to clarify the use of Heyl in our analysis. *Heyl* was initially introduced at line 165 as a Notch3 downstream target, and a marker of Notch3 pathway activation. Throughout the manuscript, we use *Heyl* expression as a readout of Notch3 activity in multiple figures (Figures 3d, 6b/e/g, 7c/g/i, and S5). We have now revised the text to ensure this rationale is clearly stated when *Heyl* is presented in Supplementary Figure 4 (now 5) (Line 174).

9) When statistical tests were performed, precise p-values should be added. This is also the case for differences that are not significant.

We have added precise p-values in all Figures, as suggested by the reviewer.

10) The reference to subfigure 4f in the text is missing (p. 10).

We apologize for this omission. We have cited the subfigure 4f that was missing from the text (Line 185).

11) In some figures, "one of two experiments" is shown. Are the shown experiments representative?

The shown experiments are indeed representative. We have now added the word "representative" in the figure legend (Figure legend 4 and 5) for clarity.

12) In addition to figure 5f, also the number of tumors should be plotted (l. 263).

We appreciate the reviewer's suggestion. We have updated Figure 5f to clearly indicate the number of mice that developed a tumor per genotype.

13) The authors claim that "Notch3 signaling plays a crucial role in advanced stages of colorectal cancer progression" (ll. 266-267). It would be interesting to explore associations of Notch3 using TCGA.

We thank the reviewer for this comment. We would like to clarify that the correlation between Notch3 expression and advanced tumor stage in colorectal cancer is addressed in the Introduction (Lines 82-86), where we cite relevant studies (references 24, 26, 27). Notably, reference 27 provides a detailed analysis of Notch3 expression using TCGA data specifically for colorectal cancer, demonstrating the association between elevated Notch3 levels and advanced disease stages. This existing literature supports our statement and provides the TCGA-based evidence the reviewer mentions.

14) The authors should describe the relevance or role of Acta2 when mentioning it in the results (ll. 275-276).

In this section we referred to *Acta2*, the gene encoding for αSMA, as a marker of contractility and pericyte maturation, since the quantification is at the RNA level. Following Reviewer #2/3 suggestions, we have now removed this data for the revised manuscript.

15) The authors should show representative images for the quantifications performed in figures 5j-k.

As mentioned above, in response to comments by reviewer #2/3, we have now removed the associated in vitro data from the revised manuscript.

16) The FACS tSNE plots in figure 6c need more explanation. What does it show? How was it generated?

The FACS tSNE plot in Figure 6c is a way to visualize FACS data by reducing multiple parameters into two dimensions, so that cells with similar marker expression patterns cluster together, making it easier to identify distinct populations. This visualization provides an unbiased view of cellular heterogeneity in a sample in a similar way to a single-cell RNA sequencing tSNE or UMAP visualization. This plot was generated using the tSNE plugin in FlowJo. We specifically refer now to this plugin in the Methods section (Line 445) of the revised manuscript.

17) The authors should describe the availability of used code.

All scripts used to generate the figures are now available on Github [https://github.com/AthanasiaSt/Notch3-regulates-pericyte-phenotypic-plasticity-in-colorectal-cancer.git]. A code availability statement is also added in the revised manuscript (Line 551-554).

18) A similar figure setup for supplementary figures 1 and 2 would be beneficial.

To address the reviewer's comment, we have revised Supplementary Figure 1 to align with the presentation format of Figure 1. Specifically, we have replaced the heatmap and violin plots previously shown in Supplementary Figure 1b-c with a marker gene dot plot. In response also to Reviewers 2/3 and given our focus on pericytes and the changes in how we present human single-cell RNA-seq data in relation to the mouse data, we have now omitted the fibroblast subpopulation data that was previously shown in Figures 1i-j, Supplementary Figure 1, and Supplementary Figure 3c. This revision maintains focus on pericytes and prevents potential confusion or misinterpretation.

19) The legend should be phrased differently for supplementary figure 3a.

We have rephrased Supplementary Figure 3a (now 2a), as suggested by the reviewer.

Reviewer #2 (Remarks to the Author):

The authors presented data to show the role of Notch3 signaling in tumor pericytes phenotype and ultimately tumor progression in the context of CRC tumors. The authors used lineage tracing and single-cell sequencing to show the abundance and origin of the tumor pericytes. In vivo knockdown and overexpression experiments were utilized to confirm the causative relationship between Notch3 signaling and vascular cell proliferation/properties. Lastly, an in-depth analysis of single-cell sequencing data was performed to show the pericyte heterogeneity. The manuscript shows a significant increase in vascular cells and a decrease in fibroblasts in the tumor model, indicating a change in the stroma landscape during tumor progression. Based on the abundance of proliferating pericytes and lineage tracing using reporter mice, the authors determined the residential pericyte as a source of tumor pericytes. Overexpression of N3ICD in PDGFRβ+ cells stimulated pericyte proliferation but decreased αSMA expression, suggesting the role of Notch 3 signaling in shifting pericyte cell fate. KO of Notch3 resulted in reduced endothelial cell proliferation, but no changes in α-SMA expression or tumor progression were observed. Orthotopic implantation of AKP organoids resulted in a significant reduction in tumorigenicity. Finally, single-cell sequencing data revealed several distinct populations of tumor pericytes, including ECM-related, myogenic-related, and inflammatory pericytes. While most of the in-vivo experiments are well-organized and thought through, the in-vitro experiments are superficial, and not all results support the hypothesis, thereby weakening the work. Lineage tracing to identify the source of the tumor pericyte is logical, leading to a clear result. While the functional study of Notch3 signaling is not novel, considering the known function of Notch3 in cellular proliferation, it suggests a potential angle toward vascular normalization through shifting the cell fate from proliferation to differentiation. Orthotopic implantation of AKP organoids in Notch3 KO mice doesn't seem to make a clear connection between Notch3 signaling and pericyte property or vascular integrity, as there was no data to support the hypothesis. In addition, implanting organoids doesn't seem to simulate late-stage CRC, and therefore, the interpretation requires revision. Lastly, the expression of αSMA does not necessarily mean that the pericytes are mature and functional. Normal capillary pericytes lack αSMA expression; therefore, these pericytes are unlikely to be intact or functional. Single-cell sequencing data provide valuable insights into pericyte heterogeneity and can inform future research. Statistical analysis and material and methods sections are provided in detail, allowing the work to be reproduced.

We thank the reviewer for this thorough and insightful evaluation of our manuscript. We appreciate the recognition of our robust experimental approaches and the constructive feedback provided. Regarding in vitro studies, following the reviewer's concern, we have removed them from Figure 5. These experiments were performed using total intestinal mesenchymal cells rather than purified pericytes, which could lead to ambiguity in interpreting the observed effects. The in vivo pericyte-specific data provide a more focused and convincing narrative. In relation to αSMA stainings and

the AKP model, we have addressed the reviewer's concerns in detail in our specific responses below. We provide detailed point-by-point responses below, outlining all revisions made. We believe these changes have significantly strengthened the manuscript.

Detailed comments:

Detailed/Specific Comments:

1. Figure 1e, please move the aSMAhi SMCs percentage inside the positive quadrant (it's confusing with where it currently is). Figure 1j, please include a color annotation for this bar graph. Figure 1c: Does the individual cell type include both normal and tumor? This data is confusing. If fibroblasts include both normal fibroblasts and CAFs, then shouldn't the ACTA2 level be higher, as that's the classic indication of CAFs?

We thank the reviewer for these helpful suggestions to improve the clarity of Figure 1. In more detail:

- Figure 1e: we have moved the SMC percentage label inside the positive quadrant.

- Figure 1j: we have clarified in the figure legend that we use the same color annotations used in Figure 1i.

- Figure 1c: this displays the markers of the whole dataset, as shown in the UMAP of Figure 1a. The differences between the control and tumor sample are presented in the UMAP of Figure 1b, while the quantification of cell subsets in each sample is shown in Figure 1d. This represents a standard approach for presenting single-cell analysis of heterogenous datasets.

- Regarding CAFs, in the mouse data our annotation does not include CAFs as a distinct population because we did not detect a clearly differentiated CAF subset in our mouse dataset based on classical CAF markers. We believe this is most likely because fully differentiated CAFs are not present in this adenoma model. This also explains the absence of elevated *Acta2* expression. Since the primary focus of this paper is on pericytes, we have not provided an extended discussion on CAFs. In the human data, CAFs are a distinct cluster showing high ACTA2 along with other established CAF markers (*FAP*, *INHBA*, *THBS2*) (Supplementary Figure 2).

2. There seems to be quite a difference in stromal composition between mouse AOM/DSS (fig. 1d) and human tumor tissue (fig 1j). For example, the majority of stroma in the normal mouse colon is fibroblasts, but that's not the case in the patient sample. Also, fibroblast levels decrease in mouse tumors but increase in human cases. The same applies to all other types of cells, unless the color annotation differs between mouse and human.

We acknowledge that the different color annotations used in the single-cell analysis of mouse and human datasets may have been confusing, contributing to misinterpretation of the stromal composition. In response to comments from both reviewers, we have now standardized the color scheme across both analyses to ensure consistent representation of stromal clusters between

species (updated in Figures 1i-j). With the corrected color annotations, it is now clear that the stromal composition between mouse and human samples is well aligned, especially regarding the relative increase of pericytes in tumors.

3. "Notch3 is predominantly expressed in vascular smooth muscle cells (vSMC) and pericytes…" according to this sentence in the introduction, but later the authored indicated Notch3 as a specific pericyte marker. Additionally, throughout the manuscript, αSMA was used as a pericyte marker. While tumor pericytes tend to misexpress αSMA, vascular smooth muscle cells are the ones that predominantly express αSMA. Therefore, the interpretation of the results is ambiguous.

The reviewer is correct to note that Notch3 is reported to be expressed in both vascular smooth muscle cells (vSMC) and pericytes, as described in the introduction. However, in our results section, we present findings from the mouse intestine and intestinal adenomas, showing that Notch3 appears to be a specific marker for pericytes in these particular tissues (Figures 1c, 2e). Our lineage tracing data using Notch3-CreERT2 mice further support this conclusion (Figures 2g-j). Importantly, this pericyte-specific expression pattern is also observed in the human intestine and intestinal tumors (Supplementary Figure 2c). These findings likely reflect tissue-specific differences in Notch3 expression patterns.

We agree with the reviewer's concern about relying on aSMA alone as a pericyte marker, given the overlapping expression between pericytes and vascular smooth muscle cells. For this reason, we have employed a combination of markers throughout the manuscript to ensure that pericyte identification is as accurate as possible: We use MCAM in combination with aSMA for FACS analysis (Fig 1e, 2c,h), PDGFRβ lineage in combination with aSMA and perivascular location (Figure 4d-e), MCAM for cell sorting and quantifications in Notch3 KO mice (Figure 4f; Figure 5c). This combinatorial strategy addresses the potential ambiguity raised by the reviewer and ensures confident identification of pericyte populations.

4. Figure 2c and h, please label the representative FACS plot with either normal or AOM/DSS, and move the cell percentage inside the box/gate for clarification. Fig 2f and 2k, is it normal or tumor tissue image? Please label all the images/data appropriately.

We thank the reviewer for these suggestions to improve the clarity of Figure 2. We made the following changes to address these points: In Figure 2, we added the missing labels indicating "normal" or "AOM/DSS" samples in the representative FACS plots, we moved the cell percentage values inside the gate, and we clarified in the figure legend that the images in Figures 2f and 2k represent tumor tissue.

5. Figure 4d: How did the author determine if GFP+ proliferative cells are specifically pericytes, not CAFs? Fig. 4f representative FACS plot is missing.

We acknowledge that PDGFRβ-CreERT2 mice can also target fibroblasts in the intestine, in addition to pericytes. We recognize that the specificity of Cre lines is not absolute, and this limitation should be considered when interpreting the results. For this reason, we have measured GFP+ cells with characteristic perivascular positioning, consistent with pericyte identity. We have also been careful to describe "…increased proliferation of GFP+ cells (line 179)…", stating that our findings "…suggest that Notch3 plays a role in pericyte in balancing proliferation and activation of pericytes" (lines 181-182), rather than making definitive claims about pericyte-specific effects. To address this potential ambiguity, we had included in vitro validations using isolated pericytes overexpressing Notch3, which showed increased proliferation (Figure 4h). Together, these approaches strengthen our conclusions about the role of Notch3 in pericyte proliferation.

In response to the reviewer, we have also now included the representative FACS plot in the revised Figure 4f.

6. As the author claims, there is an increased proliferation of pericytes and endothelial cells in CRC tumors, but there is decreased coverage of pericytes as compared to normal. Please elaborate on this hypothesis.

The point raised by the reviewer refers to the phenotype of PDGFRβ-Cre-N3ICD tumors, which show increased proliferation of both pericytes and endothelial cells but reduced pericyte coverage (Figure 4d-e). We acknowledge that this finding may sound contradictory at first. However, this phenomenon – increased pericyte proliferation along with less contractility, reduced pericyte coverage and leakier vessels – has been previously documented in other studies (references 9, 10, 46). Our and others' results show that increased proliferation of both cell types does not necessarily result in proportional coverage. If endothelial cell proliferation surpasses pericyte recruitment and maturation, the net result is reduced pericyte-to-endothelial cell ratios, leading to inadequate vessel coverage and compromised vessel integrity. To clarify this important point in the manuscript, we revised the original text (Lines 193-198) as follows:

"… This apparent paradox can be explained by disproportionate proliferation rates. If endothelial cell proliferation surpasses pericyte recruitment and maturation, the net result is reduced pericyte-to-endothelial cell ratios and inadequate vessel coverage. This phenomenon along with reduced contractility can further compromise vessel integrity in CAC tumors (9, 10, 46)."

7. Figure 4, while there is an increase in pericyte and endothelial cells proliferation in the tumor microenvironment, there were no changes in tumor size and multiplicity. What is the explanation for this? Were there actually a greater number of vessels? This is an important

piece of data missing for this experiment. For the Notch3 signaling-mediated vascular cell proliferation to have a functional effect on tumor progression, there should be a greater number of vessels, not just the cells, and the perfusion assay should confirm whether those vessels are functional.

We thank the reviewer for this important point. To address the question of functional vascular changes, we have added blood vessel diameter measurement and albumin staining (Figure 4d, e), which show increased vessel size and permeability in tumors overexpressing Notch3 in PDGFRb+ cells. These functional assessments confirm that the observed changes in cell proliferation translate to functional vascular effects. Regarding tumor multiplicity and size, our studies were performed using an early-stage tumor model. It is possible that differences in tumor burden are not yet apparent, as vascular changes require time to manifest on tumor growth. We have expanded our discussion of these aspects in our revised manuscript (Lines 193-198) to provide a more comprehensive interpretation of the data.

8. While quantifying proliferating pericytes, Ki67+PDGFRβ+/Ki67+DAPI+ were counted, how CAFs were differentiated from pericytes, as both cell types express the same marker- PDGFRβ.

This concern relates to the important point raised by the reviewer in comment 5, where we have addressed it in detail.

9. For the quantification of αSMA+ signal around blood vessels, how did you differentiate bigger blood vessels from capillaries? Larger blood vessels have vascular smooth muscle cells surrounding them, even in a healthy situation, to provide contractile function, whereas healthy capillaries do not. αSMA expression is acquired in a tumor condition. If this is not taken into consideration, then quantification could be misleading.

We acknowledge that our quantification does not differentiate between larger blood vessels and capillaries, which is a limitation of our analysis. This is further complicated by the difference in BV size between different genotypes. Nevertheless, we would like to clarify that our comparisons are made exclusively between tumor conditions. The major downside of this limitation is that our analysis may actually underestimate the true effect size. We have now added a statement in the revised manuscript to acknowledge this limitation and ensure appropriate interpretation of our findings (Lines 179-181).

10. As per lines 320-321, capillary endothelial cells are the main source of Jag1 and Dl14 in TME, but in Fig. 4d and Fig. 5c, αSMA expression around the blood vessels by pericyte is presented around artery endothelial cells.

We apologize for this mistake. As shown in Figure 6h-i, arterial endothelial cells are the main producers of Jag1 and Dll4. We have corrected this mistake in the revised manuscript.

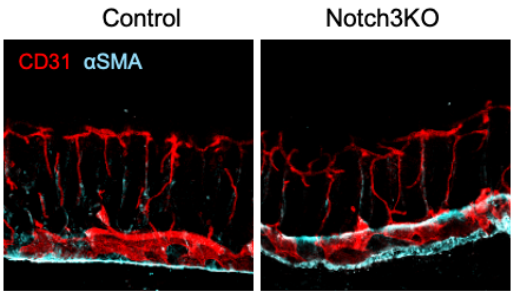
11. No data is presented to show that deleting Notch 3 in tumor pericytes affects the tumor microenvironment or tumor vasculature. Suggested conditions include hypoxia, vasculature leakage, and metastasis, among others.

We thank the reviewer for raising this important point. To address the reviewer's comment, we have now added albumin permeability staining, which demonstrates reduced vascular leakiness in Notch3-deleted tumors, indicating improved vessel integrity. We have also included measurements of BV diameter, which along with proliferating blood endothelial cells provides a more comprehensive characterization of the tumor microenvironment in Notch3 KO mice (Figure 5c-d). The effect of Notch3 on the metastatic potential was assessed using the AKP orthotopic transplantation model, which represents the most reproducible model system for advanced colon cancer. Our results showed significantly reduced tumor volume in Notch3 KO mice (Figure 5g), suggesting that Notch3 plays an important role in advanced cancer progression (*See also the comment below*). We did not observe liver metastasis in either the controls or KO mice, and as such we have revised the text to avoid drawing conclusions about metastatic spread. To our knowledge, no other model can reproducibly drive metastasis in CRC.

12. I am not so sure what AKP organoids in the Notch3 KO mice experiment suggest. In this condition, Notch3 is absence from the whole body, making me wonder what the vessels look like in even normal mice without a tumor. Then, after organoid implantation, if the Notch3 KO prevents endothelial cell and pericyte proliferation, tumor angiogenesis will not occur, making the tumor avascular, if the hypothesis is correct. In this case, I am unsure whether this actually tests the role of Notch3 in tumor progression at the advanced stage. Please provide some explanation on this.

We thank the reviewer for the opportunity to clarify the rationale and interpretation of the AKP organoid transplantation experiments. The AKP organoid transplantation model represents a powerful tool for studying colorectal cancer progression and metastasis, which is absent in the AOM/DSS model that results mainly in adenoma development. As such, we used this model to assess Notch3 function in a more advanced cancer setting.

First, regarding the reviewer's questions about Notch3 expression and vascular phenotypes in the normal intestine, Notch3 KO mice are viable and fertile, demonstrating that Notch3 is not essential for normal development or basal vascular function in healthy intestinal tissue. In addition, staining for blood vessels in normal intestinal tissue did not reveal gross defects in Notch3KO mice.



Second, our findings demonstrate that AOM/DSS tumors develop in Notch3 KO mice with comparable multiplicity and size to wild-type controls, and blood vessels are present despite reduced size and endothelial cell proliferation. These observations indicate that Notch3 deletion does not prevent tumor initiation or the establishment of a functional vascular network (Figure 5a-

d). The presence of blood vessels in AKP tumors in Notch3KO mice despite the significantly reduced tumor volume further supports the idea that Notch3 and its downstream regulation of blood vessel function may phenotypically affect more advanced cancers (Figure 5g). In summary, our data suggest that Notch3 contributes to tumor progression and optimal vascular function rather than being essential for tumor initiation or basic angiogenesis, which addresses both tumor progression and maintenance aspects raised by the reviewer.

13. Missing the rationale behind how the author determined high and low for PDGFRαhi and PDGFRαlow, similarly for αSMAhi and αSMAlow.

We are happy to provide more information on gating strategies used in our analysis and clarify that the distinction between high and low expression cells is based on both FACS analysis and single cell transcriptomics data. For PDGFRa, the distinction between PDGFRahi and PDGFRalo is well established for fibroblast subpopulations in the intestine (e.g. reference-PMID: 32884148). This is also evident in FACS, where staining for PDGFRa results in distinct PDGFRahi and PDGFRalo cells. However, given our focus on pericytes and the changes in how we present human single-cell RNA-seq data in relation to the mouse data, we have now omitted the fibroblast subpopulation data that was previously shown in Figures 1i-j, Supplementary Figure 1, and Supplementary Figure 3c. This revision maintains focus on pericytes and prevents potential confusion or misinterpretation.

For aSMA, FACS analysis also showed aSMAhi and aSMAlo expressing cells, which correlated well with our single cell RNA sequencing data and the concept of pericyte phenotype switching. To improve clarity and transparency, we have added an additional supplementary figure showing FACS gating strategies, including representative FACS plots with the specific gates used to define high and low expressing populations for aSMA (Supplementary Figure 4). These gates were set based on the natural separation observed in the fluorescence intensity distributions. For αSMAhi cells, we selected the very high-expressing population, based also on the corresponding gate for smooth muscle cells.

While there are several comments that need to be addressed, if the authors can provide a revision in a timely manner with clarifications, the quality of the work appears suitable for publication in Communications Biology.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewer for their contributions to the evaluation of our manuscript through the co-review training program. We appreciate the time and effort invested in this review and are grateful for the constructive feedback that has helped improve our work.

Reviewer #4 (Remarks to the Author):

In this manuscript, Chaldiki et al. investigated the phenotypic heterogeneity of pericytes in colorectal cancer and specifically the role of Notch3 signalling. Using mouse models of colitis-associated CRC and published human CRC data, they identified heterogeneous pericyte subpopulations with different Notch3 activity. In both the colitis model and a more advanced AKP CRC model, they showed that Notch3 deletion leads to reduced endothelial proliferation and a potential shift toward a contractile pericyte phenotype. In the AKP model, Notch3 KO also leads to decrease in tumorigenesis.

The data is of very good quality and the analyses are robust. The results about Notch3 regulating endothelial cell proliferation are also very convincing. However, even though activation of Notch3 showed decreased aSMA expression, in Notch3 KO in the colitis CRC models there was no difference in aSMA staining around blood vessels. The difference in Acta2 was more convincing in the AKP models, however Acta2 is also highly expressed in fibroblasts and smooth muscle cells. In our opinion, there is no direct evidence to support the claim that Notch3 can induce a shift towards a contractile phenotype and that it is a key regulator of pericyte plasticity, and the text should be modified to reflect that or additional experiments should be performed to validate a transition in phenotype.

We thank the reviewer for their comprehensive and constructive evaluation of our manuscript. The insightful comments have significantly improved the quality and clarity of our work. We have carefully addressed each comment and provide detailed point-by-point responses below, outlining the specific revisions made to the manuscript. We believe these changes have improved our manuscript.

Major comments:

1. In Figure 1, could you please define define ** and *** in 1(f). It was also not clear why the authors used one-way ANOVA in 1(f) when in subsequent similar data used t-test? e.g. 2(d)

We thank the reviewer for bringing this to our attention. Upon reviewing our statistical analysis, we identified we had incorrectly written that the statistical test in Figure 1f was a one-way ANOVA, while we had used a Welch's t test. We have now corrected the description of statistical analysis in the Methods section of our revised manuscript. Following reviewer's 1 recommendation, we have also included the exact p-values in the figures rather than using asterisk notation, which improves clarity and transparency of our statistical reporting.

2. Figure 2 e-f - how are the authors sure that these are Notch3 expressing pericytes, are they inferring this based on location/morphology? Are there other cell types expressing Notch3 e..g epithelial cells?

We thank the reviewer for raising this important question about cellular identity. We have used multiple complementary approaches to confirm that Notch3+ cells are predominantly pericytes.

First, Figure 3e is based on our single cell analysis and the annotation of cell types, which identifies Notch3-expressing cells as pericytes based on their transcriptional profiles. Figure 3f was based on the location of Notch3+ cells around blood vessels. To further validate these findings, we performed lineage tracing analysis demonstrating that Notch3+ cells in colorectal tumors are primarily pericytes based on the FACS analysis (Figure 2h-i). This is now supported by FACS analysis showing that Notch3CreER mice target a minimal number of Lin+ (Epcam+CD45+) cells (~0.03%). We have now included these FACS data in the newly added Supplementary Figure 3b. To further expand our validation, we also analyzed a single cell dataset from AOM/DSS tumors and normal colon, which further verified Notch3's specificity for pericytes in cancer. These data are now included in Supplementary Figure 4.

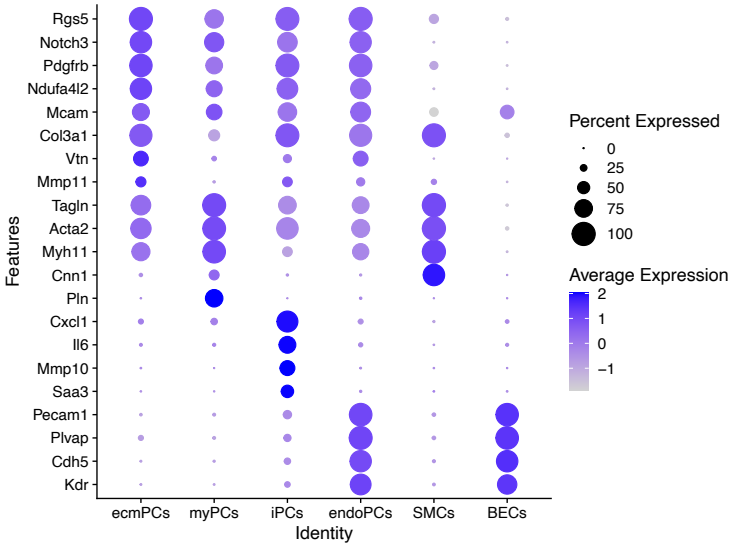
3. Figure 4e, in the legend the authors write "Results were analysed using Mann-Whitney test and unpaired two-tailed t-test". Which of the two was specifically used for the corresponding figure?

We apologize for the confusion regarding statistical analysis. We have now reviewed all statistical analyses, added exact p-values -as suggested by reviewer 1-, and rephrased the related part in the Methods section to ensure clear and correct description of our statistical analysis (Lines 556-560).

4. Figure 6, the myPCS have a very low expression of Rgs5 (and the same in Figure 7 for the human pericytes), how confident are the authors that these are pericytes and not smooth muscle cells? Related to this, line 304, it would be very helpful to include the pericyte markers mentioned (Notch3, Pdgfrb, and Ndufa4l2) and other general pericyte markers in the dot plot in figure 6b to confirm that those are pericytes.

We thank the reviewer for this important question. We would like to clarify that the subclustering analysis presented in Figure 6 was performed exclusively on cells already identified as pericytes based on canonical marker gene expression from our initial clustering. Therefore, smooth muscle cells were not included in this analysis.

Regarding the low Rgs5 expression in myogenic pericytes (myPCs), this observation is consistent with the published literature showing that Rgs5 deletion leads to myogenic/contractile gene expression and phenotype (reference 10). As such, the relatively lower Rgs5 expression in myPCs, compared to other tumor-associated pericyte subpopulations, supports our interpretation that these cells represent a more differentiated, contractile pericyte state. To further confirm the pericyte identity of all subclusters and address the reviewer's concern, we have expanded Figure 6b to include additional pericyte markers (*Notch3*, *Pdgfrb*, *Ndufa4l*). Additionally, we have included a figure here for the reviewer showing the expression of these markers, along with smooth muscle cell and endothelial cell markers, in our initial clustering analysis, which clearly demonstrates the distinct separation of pericytes from smooth muscle and endothelial cells.



5. For the trajectory analyses, why was ecmPC used as a root?

We acknowledge that this an important issue regarding trajectory analysis. Pseudotime trajectory analysis requires the designation of a root cell population as a starting point for inferring transitional paths. We selected ecmPCs as the root because this state is characterized by lower expression of differentiation markers and considered the most immature in the literature (references 9-11). In our approach, we use trajectory analysis to reveal potential cellular transitions and plasticity rather than definitively establishing the cellular origin or directionality of differentiation. To address this important distinction and improve clarity, we have now revised the text to explicitly explain our rationale for selecting ecmPCs as the root and to emphasize that the trajectory analysis indicates potential phenotypic transitions between pericyte subpopulations rather than confirming specific cellular sources or lineage relationships (Lines 238, 253-254, 511-519).

6. Line 316, the authors wrote that proliferation markers are also increased in ecmPCs, but from the plots this is not obvious. Could they confirm this with more genes or a cell cycle score?

We thank the reviewer for bringing this issue to our attention. Upon careful re-examination, we agree that the difference in proliferation markers shown in Figure 6e is minimal and does not sufficiently support the statement that proliferation is increased in ecmPCs. We have, therefore, removed this part from our revised manuscript. One reason for the absence of robust data to support the proliferation of ecmPCs could be that highly proliferating PCs are clustered separately in our dataset (Figure 1). Due to the low abundance of this subpopulation, we did not proceed to further analysis and have been cautious in drawing strong conclusions about these cells in the revised text.

7. Figure 7a,b - A reasonable proportion of the cluster named ecmPC_N is tumour cells so it could be better to called ecmPC_mixed?

We agree with the reviewer that this cluster contains a heterogeneous population. While the designation ecmPC_mixed could be appropriate, we have retained ecmPC_N because these cells clustered with normal tissue pericytes based on their transcriptional profiles. To clarify this complexity, we have added an explanation in the text (Line 248) stating that this cluster includes both normal tissue pericytes and normal-like pericytes found in tumors. Additionally, we have expanded the figure legend to include the abbreviations and definitions used for all cluster names and improve transparency.

8. Figure 7e - can the authors show an additional marker gene for the inflammatory pericytes, to make it more convincing?

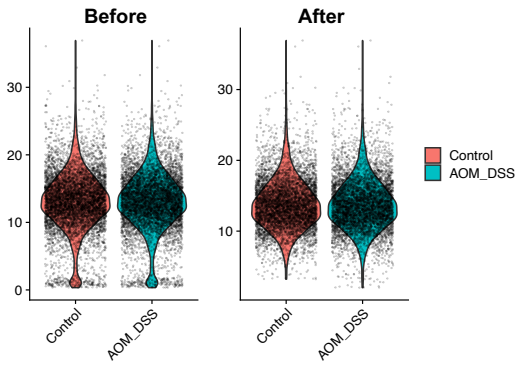
We have now added additional markers of inflammation-activated pericytes in Fig 7e, specifically feature plots for *CCL8* and *CXCL1*, which further support our observation that inflammatory-activated pericytes do not form a distinct cluster in this analysis (Line 251-253).

9. Figure 7c - HEYL seems to have the highest expression in the intermediate cluster and not ecmPCs, how do the authors interpret this?

We thank the reviewer for raising this point. HEYL expression is indeed high in intermediate pericytes (intPCs) but is also elevated in ecmPCs and proliferative pericytes compared to myogenic pericytes and normal ecmPCs. We interpret this pattern as reflecting pathway activation during the transition from myogenic to tumor-associated pericyte phenotypes, with intPCs representing an intermediate transitional state. To improve visualization and clarity, we have added a feature plot showing Heyl expression across all pericyte clusters (Figure 7g). We have also added a sentence in the text (Line 255-257) to discuss this issue.

10. For the quality control analyses, it was not clear why a lower cut-off of 3% and 2% for ribosomal gene expression was used?

During quality control, we visually inspected cell distribution in our dataset and applied lower cut-off of 3% and 2% for ribosomal content to filter out outliers. In the accompanying image, the initial and final cell distribution based on ribosomal protein expression is shown.



Minor:

1. I think the annotation "synthetic" ecmPC maybe could be renamed to just ecmPC?

We appreciate this suggestion. We have now removed this descriptor and refer to this population simply as ecmPC to avoid confusion.

2. In the abstract, the authors wrote "metastatic orthotopic model" - Advanced might be a better term here? There wasn't anything mentioned of whether metastases were formed in the AKP model, only primary tumours.

We agree that "advanced" is more accurate given that we evaluated primary tumors rather than distant metastases. We have revised the abstract accordingly.

3. Line 569 - anti-CD45 and anti-CD326 antibodies should be the other way around - for immune and epithelial cells respectively

We thank the reviewer for catching this error. We have corrected the antibody assignments in Line 462 (anti-CD45 for immune cells and anti-CD326 for epithelial cells).

Point-by-point response to Reviewer comments

Reviewer #1

The authors have adequately addressed the majority of my suggestions regarding the original manuscript.

However, regarding my previous comment #5 (description of all graph elements), the authors should double check for every subfigure that all required information is included and correct (see also in the data presentation paragraph in the editorial policy checklist). n should be described for the different plotted subgroups, clusters etc. Examples: How many pericytes are in each individual cluster in Figure 7? What do the different lines in Figure 5d indicate? Do the error bars really always depict the mean +- SD as stated (sometimes there are asymmetric error bars, e.g. Figure 5b)... This information allows the reader to better evaluate the data.

We would like to thank Reviewer #1 for their positive assessment of our revised manuscript and their attention to detail, which can help with the better presentation of our data. Based on the reviewer's comment and the editorial policy checklist, we have ensured that all information for each subfigure is included and correct in this final submission. Specifically,

- "n" is now included in the figure legend of all relevant plots.

- The number of cells per cluster in Figures 6 and 7 is also included.

- All bar plots are represented as mean ± SD. But we have also used boxes (lines represent median values, 25%, and 75% percentiles) and whiskers (min to max), or violin plots (large dotted lines represent median values, small dotted lines represent 25% and 75 % percentiles). These types of data presentation are now described in the Statistics paragraph of the Methods section.

- The Supplementary Data file with all raw data is also submitted in accordance with editorial guidelines.