Regenerating vascular mural cells in zebrafish fin blood vessels are not derived from pre-existing mural cells and differentially require Pdgfrb signalling for their development

Elvin V. Leonard, Ricardo J. Figueroa, Jeroen Bussmann, Nathan D. Lawson, Julio D. Amigo and Arndt F. Siekmann
DOI: 10.1242/dev.199640

Editor: Steve Wilson

Review timeline

Original submission:	29 March 2021
Editorial decision:	10 November 2021
First revision received:	31 January 2022
Editorial decision:	22 February 2022
Second revision received:	22 February 2022
Accepted:	24 February 2022

Original submission

First decision letter

MS ID#: DEVELOP/2021/199640

MS TITLE: Regenerating vascular mural cells in zebrafish fin blood vessels are not derived from pre-existing ones and differentially require pdgfrb signaling for their development

AUTHORS: Arndt F Siekmann, Elvin Vincent Leonard, Ricardo Figueroa, Jeroen Bussmann, Julio D Amigo, and Nathan D Lawson

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have criticisms and suggestions for improvements. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Leonard et al focuses on regeneration of vascular mural cells in zebrafish fin. The authors perform detailed characterization of mural and other pdgfrb-expressing cells in zebrafish larvae and adult fins. They identify the precursor cells that give rise to pericytes in zebrafish fins, and demonstrate the role of pdgfrb signaling in mural cell formation in the fin. Furthermore, the authors argue that newly formed mural cells during regeneration are not derived from pre-existing cells.
Overall this study is well performed, data are convincing and sufficiently novel. Although the study is largely descriptive, the results are significant and are expected to be of interest to researchers in developmental vascular and regenerative biology.

Comments for the author

Specific points:
1. Tg(pdgfrb:H2B dendra2) lineage tracing shows cuboidal shaped cells transition into elongated mural-like cells. It would be great to confirm this lineage tracing with the change in mural marker expression using myh11:YFP transgene. Do Dendra2 labeled cells initiate myh11:YFP expression?
2. Do cuboidal shaped cells express Tg(pdgfrb:H2B-dendra) and do they also get photoconverted in the experiment shown in Fig. 6? If new mural cells are not derived from existing mural cells in regenerating fin, are they derived from cuboidal shaped cells? It seems that all tools are available to answer this question.

Minor points:
3. The text on page 8 (3rd line) says that “YFP expressing cuboidal cells did not express mCherry (fig. 2I’)”.
Please check this statement, it seems that it should be the other way around.
4. The authors state on page 8 that ‘mural cells on aISVs co-expressed both transgenes, while those on venous ISVs only expressed pdgfrb transgene’. However, Suppl. Fig. 3 shows the presence of pdgfrb-only cells on both aISVs and vISVs, while double positive cells were also observed in both types of vessels although the number of double positive vISVs was lower than single pdgfrb-positive vISVs. Please clarify this point.
5. In fig. 5b artery is already present at 3 dpi. Did it regenerate so quickly? It would be useful to show an image of vascular and mural marker expression right after injury, at 0 dpi.

Reviewer 2

Advance summary and potential significance to field

This manuscript describes the acquisition of mural cells in the vessels of the zebrafish tail and ontogeny of mural cells in the regenerating tail. The tail is a structure often used to look at both developmental and regenerative processes.
Transgenic lines to trace different mural cell and endothelial markers are employed at different stages to characterize morphology, gene expression of mural cells, with a comparison to the more well studied trunk mural cells. The results show that the smooth muscle marker and Myh11 and pdgfrb are largely co-expressed with a few exceptions. The authors describe pdgfrb expressing cuboidal and ovoid mural cells in the tail that are spatially distributed. Using a photoconvertible nuclear dendra protein under pdgfrb, the authors observed transformation of cuboidal cells into mural cells, as well as proliferation .
They used the same construct in a regenerative experiment to look for origins of pericytes in regenerated tissue. The novelty in the manuscript lies in both the description of the distribution of the mural cells and their markers at different developmental stages, but also in the tracing of development and regenerative properties. The authors attempt to address an open question of whether pericytes have stem cell-like properties, and what the source of the pericytes is during injury. One interesting observation is that in fish is that myh11 is co-expressed with pdgfrb and does not correlate with a mature mural cell morphology, which had been expected.

Comments for the author

1. Two very strong claims are made in the abstract that a) newly formed mural cells are not derived from pre-existing ones in regeneration and b) there is a limited capacity of mural cells to self-renew or contribute to other cell types during tissue regeneration. This data is based on a single labelling technique that has caveats. The dendra conversion experiment relies on a transgenic line, and photoconversion of a fluorophore.
   a. Based on Figure 6, it is clear that not every cell has complete photoconversion of dendra b. Transgenic lines are almost invariably mosaic.

These two caveats mean that there is some possibility that not all mural cells express dendra and that not all dendra-expressing cells are photoconverted. For this reason, the authors claims cannot be fully supported by the data. The authors have not used a lineage tracing technique which would be a definitive and complementary method to support their claim.This is a major point that must be addressed if the authors wish to address whether perictyes have stem-cell like properties and regenerate tissue etc. Without full experimental demonstration that the regenerated mural cells do or do not come from pdgfrb-expressing cells, this remains a speculative question. To turn this around, can the authors demonstrate which tissue they do come from? This would also be an acceptable experimental second approach needed to support the most important conclusion of the paper.

2. What is the identity of the ovoid cells? This is not discussed even though this is one of the 4 populations in the fin. Further, what is the identity of the cuboid cells? The authors demonstrate that they are able to differentiate into mural cells, but what is there identity when they in the cuboidal morphology and are not attached to vessels. Some additional maker exploration (co-expression of collagens and other fibroblast genes for instance) to define these cells would strengthen conclusions about the origins of the fin mural cells. It would be unusual to find cuboidal fibroblasts, but if they are not of the fibroblast lineage, this would make the results even more interesting.

3. On page 8, the authors show that fin mural cells differentiate from local precursors that express pdgfrb, but the authors do not show this is the only source. It is possible that the cuboidal cells are only one of several sources (see comment 1 above). The current experiments only show that cuboidal turn into pdgfrb wrapping cells but does not rule out that other cells don't turn into mural cells as well. A lineage experiment as suggested in point 1 would show this.

4. Experiments involving dendra photoconversion are key to the arguments of the paper. Figure 3 shows that dendra photoconversion marks precursor cells transitioning into mural cells. It is essential that these experiments are supported by quantitative data.
   a. How did the authors ensure that only 'cuboidal cells' were photoconverted? Since pdgfrb would also be expressed in the mural cells, how did the authors pick 'cuboidal' cells out of a field to convert as both cell types would express the transgene.
   b. There is no description in the methods of how single cell photoconversion was achieved. The images look fairly convincing, but were examples where more than one cell was photoconverted discarded?
   c. There is no quantitation of the photoconversion data. How many cells were converted? How many became mural cells? What proportion of converted cells divided (i.e. estimation of proliferative ability). It is hard to see the morphology of the differentiated cells in a,b,c.
   d. In figure 6, the dendra conversion experiment is the sole evidence that regenerating mural cells do not come from pdgfrb precursors and that regenerated mural cells do not come from pre-existing cells. Given caveats of the dendra technique, this conclusion cannot be made confidently. A second line of evidence is needed here to support this strong (and interesting if it was true) statement.

5. If cuboidal cells are mural cell precursors, it is perhaps not surprising that the number of cuboidal cells increases in pdgfrb knockouts while mural cells goes down as pdgfrb is expressed in both.
   a. This could be a differentiation block, but it is also possible that they have proliferated vs. control. The authors should test proliferation of the cuboidal cells in the mutants.
   b. Also the comment is made that they increase in citrine expression. No quantification is provided (either image intensity or qPCR) to support this.

6. The discussion is poorly written in sections with the writing flipping between discussing vSMCs and pericytes, almost interchangeably. In the sentences starting with 'On note, we did not detect cells with a similar SMC morphology in the zebrafish fin', this is confusing because pericytes

were being discussed in the prior sentence. Then the discussion changes again to pericyte morphology in the mouse context with alpha smooth muscle actin. (mouse vs. fish). Please re-read and simplify this discussion logic.
7. Since mouse scRNA seq supports that there are myh11 mural cells without alpha SMA, this would make sense if myh11 is expressed in alpha SMA negative pericytes. So what are these cells in the tail? Pericytes or SMCs.?
8. Page 11. That these cells might have different unique functions is completely speculative and should be removed. No functional work ha been done with the cells.
9. The idea that there is no capillary bed in this area is interesting, and perhaps should be highlighted earlier in the manuscript.
10. The authors note proximal and distal differences in mural cell morphology but do not tie it to how the vessels are different in terms of diameter and flow, and whether this could underlie different mural cell morphologies? If flow does not control mural cell morphology, can the authors suggest what else would control the proximal-distal morphological changes.

Reviewer 3

Advance summary and potential significance to field

The study by Leonard et al explores the origins of pericytes and their contributions during fin regeneration in zebrafish. Previous studies in pericyte biology over many years have variously suggested that pericytes contribute during wound healing and regeneration, from proposing mechanisms of trans-differentiation to give rise to multiple different cell types, to promoting angiogenesis, to contributing in fibrosis. The role of pericytes in regeneration is still unclear and even controversial. The origin of new pericytes in regeneration and wound healing is also understudied. Thus, the approach of the authors here to use a well-established model of regeneration to study the role of pericytes is clever and timely.

The authors describe the different anatomical features of mural cells of the different fin segments. They developed a new transgenic tool to visualise smooth muscle cells that takes advantage of the myh11a promoter. They find that mural cells develop from pdgfrb-expressing cells in the fin and require pdgfrb for their expression. Furthermore, they show that during regeneration of ablated fin segments the pericytes regenerate in a pdgfrb-dependent manner. They show in elegant and simple lineage tracing experiments using kaede photoconversion that pdgfrb cells do not form from pre-existing pericytes in regeneration but are formed from other cells in the regenerating tissue. This observation is important because it suggests that pericytes don’t serve as tissue resident stem cell in damaged tissue.

Overall, the study is well written, presents clear and carefully analysed data and offers new insights in pericyte biology that are informative and useful. In this reviewers opinion the study is suitable for publication in development but should be improved for clarity with some essential minor revisions.

Comments for the author

Suggested revisions
1. The new myh11a transgenic line will be a very useful tool for the zebrafish vascular community. The authors state in the discussion that it overlaps with previously reported expression of the established SMC marker transgenic line using acta2 (from Whitesell et al 2014) but they discuss that contractile protein encoding genes are expressed heterogeneously in mural cell populations in sc-Seq data. Of note, this marker is expressed on intersegmental vessels (Supp Figure 2), while acta2 is not (Whitesell et al 2014).

To fully understand the results that take advantage of this new line, it is important to better understand how unique this line is by direct comparison with previously described markers. In the fin and perhaps embryo, does expression of the myh11a transgenic line overlap completely with, or only partially with, the previously reported smooth muscle acta2 line? Examining overlap with acta2 should be easily achievable and will show if the new line is an SMC marker strain or a more general mural cell marker.

2. It is interesting in the pdgrb mutants that there is still reasonably normal development of myh11a-expressing mural cells in S1. However, these cells don't look the same morphologically as the wt S1 cells. Are there defects in the number of cellular extensions that are formed around the blood vessels, even in the mural cells that do form? Can the authors resolve this with higher resolution imaging to improve the clarity of the result?

3. Did the authors assess whether fin regeneration is impaired in the absence of mural cells and pericytes in the pdgfrb mutants? If they performed traditional distal fin amputations, it would be useful for them to describe whether there was impaired regeneration or equally important for them to describe if there was no impact on regeneration. Such negative data would be informative and could be included in the supplementary material or described in the discussion.

4. What is the identity of the "cuboidal" cells that differentiate into pericytes in development and regeneration? Are they perivascular fibroblasts as very elegantly suggested by Rajan et al 2020 (PloS Genetics)? This previous study seems highly relevant to the claims being made here but is not cited. It would be suitable to add a discussion of the identity of these cells to the discussion in light of the recent published findings.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

The manuscript by Leonard et al focuses on regeneration of vascular mural cells in zebrafish fin. The authors perform detailed characterization of mural and other pdgfrb- expressing cells in zebrafish larvae and adult fins. They identify the precursor cells thatgive rise to pericytes in zebrafish fins and demonstrate the role of pdgfrb signaling in mural cell formation in the fin. Furthermore, the authors argue that newly formed muralcells during regeneration are not derived from pre-existing cells. Overall this study is well performed, data are convincing and sufficiently novel. Although the study is largelydescriptive, the results are significant and are expected to be of interest to researchersin developmental, vascular and regenerative biology.

Reviewer 1 Comments for the Author:

Specific points:
1. Tg(pdgfrb:H2B dendra2) lineage tracing shows cuboidal shaped cells transition intoelongated mural-like cells. It would be great to confirm this lineage tracing with the change in mural marker expression using myh11:YFP transgene. Do Dendra2 labeledcells initiate myh11:YFP expression?

Response: We would like to thank the reviewer for this suggestion. We attempted to perform the lineage tracing experiments using *Tg(pdgfrb:H2Bdendra2)*^{mu148}; *Tg(myh11a:YFp)*^{mu125} fish. However, this experiment was technically challenging as the *myh11a:YFP* transgene requires higher magnification (40x) and averaging, leadingto increased imaging time during and after photo conversion, resulting in the death of sedated juvenile fish. From our observations of the overlap of pdgfrb and myh11a cellsdescribed in Fig. 2g, l, we show that elongated mural cells express both pdgfrb and myh11a and there are no other pdgfrb positive cells with elongated morphology in the region. This suggests that the elongated pdgfrb cells derived from cuboidal cells, wouldalso express myh11a.

2. Do cuboidal shaped cells express Tg(pdgfrb:H2B-dendra) and do they also get photoconverted in the experiment shown in Fig. 6? If new mural cells are not derived from existing mural cells in regenerating fin, are they derived from cuboidal shapedcells? It seems that all tools are available to answer this question.

Response: This is a very interesting question, and we would like to thank the reviewer for raising

this point. To better characterize cuboidal cells, we obtained an additional transgenic line expressing EGFP under the control of the col1a2 promoter (*Tg(col1a2:GFP)*^{ca103}), previously shown to be expressed in fibroblasts. We crossed thisline to *Tg(pdgfrb:mcherry)*^{ncv23} animals and subsequently analysed expression of EGFP and mCherry (Figure 5). We find that in distal regions cuboidal shaped cells and mural cells expressed both transgenes. Towards more proximal regions, col1a2 expression remained high in cuboidal shaped cells while it became progressively downregulated in mural cells. In the most proximal regions, cuboidal shaped cells were devoid of pdgfrb expression, which was strongly expressed in mural cells. These were in turn devoid of col1a2 expression. Together, these results suggest that initially cuboidal shaped cells express both, the fibroblast marker col1a2 and pdgfrb. After differentiation into the muralcell lineage, expression of pdgfrb intensifies, while col1a2 is being downregulated.

We then analysed col1a2 expression during tissue regeneration (Fig. 9f). We detected cells that initially expressed both col1a2 and pdgfrb in the tissue regenerate, while mural cells with high pdgfrb expression at later stages of regeneration were devoid of col1a2 expression. Thus, these results suggest that also during tissue regeneration, mural cell precursors initially display characteristics of cuboidal cells before differentiating into the mural cell lineage. They also suggest that these cuboidalcells are not being derived from pre-existing cuboidal cells, as they would also be photoconverted. Thus, while the transition from cuboidal to mural cell appears to occurduring tissue regeneration, we still do not know which progenitor population within the fin would give rise to either cuboidal or mural cells.

Minor points:

3. The text on page 8 (3rd line) says that "YFP expressing cuboidal cells did not express mCherry (fig. 2I')". Please check this statement, it seems that it should be the other way around.

Response: Thanks for pointing it out, we have made the correction.

4. The authors state on page 8 that 'mural cells on aISVs co-expressed both transgenes, while those on venous ISVs only expressed pdgfrb transgene'. However, Suppl. Fig. 3 shows the presence of pdgfrb-only cells on both aISVs and vISVs, while double positive cells were also observed in both types of vessels, although the number of double positive vISVs was lower than single pdgfrb-positive vISVs. Please clarify thispoint.

Response: We would like to thank the reviewer for directing our attention to this oversight and requesting clarification. We reanalysed our data to determine the distribution of pdgfrb only or pdgfrb/myh11a double positive cells on arterial and venousintersegmental vessels (Supplementary Fig. 3). Based on this new analysis we find thatpdgfrb only expressing mural cells are enriched on vISVs, while pdgfrb/myh11a double positive mural cells are enriched on aISVs.

5. In fig. 5b artery is already present at 3 dpi. Did it regenerate so quickly? It would be useful to show an image of vascular and mural marker expression right after injury, at 0dpi.

Response: We agree that the speed of regeneration is quite remarkable. There is somevariability in the degree of regeneration within the first days after tissue removal.
However, in all examples we have imaged, the artery regenerated to some degree within 3 to 4 days post injury. We have now provided more images of artery regeneration using repetitive imaging (Figure 9). In this figure, we also include an imageat one day after tissue removal, where no artery is present.

Reviewer 2 Advance Summary and Potential Significance to Field:

This manuscript describes the acquisition of mural cells in the vessels of the zebrafish tail and ontogeny of mural cells in the regenerating tail. The tail is a structure often used to look at both developmental and regenerative processes. Transgenic lines to trace different mural cell and endothelial markers are employed at different stages to characterize morphology, gene expression of mural cells, with a comparison to the more well studied trunk mural cells. The results show that the smooth muscle marker and Myh11 and pdgfrb are largely co-expressed with a few exceptions. The authors describe pdgfrb expressing cuboidaland ovoid mural cells in the tail that are spatially distributed. Using a photoconvertible nuclear dendra protein under pdgfrb, the authors observed transformation of cuboidal cells into mural cells, as well as proliferation .

They used the same construct in a regenerative experiment to look for origins ofpericytes in regenerated tissue. The novelty in the manuscript lies in both the description of the distribution of the mural cells and their markers at different developmental stages, but also in the tracing of development and regenerative properties. The authors attempt to address an open question of whether pericytes have stem cell-like properties, and what the source of the pericytes is during injury. One interesting observation is that in fish is that myh11 is co-expressed with pdgfrb and does not correlate with a mature muralcell morphology, which had been expected.

Reviewer 2 Comments for the Author:

1. Two very strong claims are made in the abstract that
    a) newly formed mural cells are not derived from pre-existing ones in regeneration and b) there is a limited capacity of mural cells to self-renew or contribute to other cell types during tissue regeneration. This data is based on a single labelling technique that has caveats. The dendra conversion experiment relies ona transgenic line, and photoconversion of a fluorophore.

        a. Based on Figure 6, it is clear that not every cell has completephotoconversion of dendra
        b. Transgenic lines are almost invariably mosaic.

        These two caveats mean that there is some possibility that not all mural cells express dendra and that not all dendra-expressing cells are photoconverted. For this reason, the authors claims cannot be fully supported by the data. The authors have not used a lineage tracing technique which would be a definitive and complementary method to support their claim. This is a major point that mustbe addressed if the authors wish to address whether perictyes have stem-cell like properties and regenerate tissue etc. Without full experimental demonstration that the regenerated mural cells do or do not come from pdgfrb- expressing cells, this remains a speculative question. To turn this around, can the authors demonstrate which tissue they do come from? This would also be anacceptable experimental second approach needed to support the most importantconclusion of the paper.

Response: We would like to thank the reviewer for addressing this shortcoming of our work. We fully agree that lineage tracing using photoconvertible fluorophores cannot provide definite answers concerning lineage relationships. To back our findings, we attempted two additional lineage tracing approaches. As suggested by the reviewer, weobtained the recently published *Tg(pdgfrb:CreERT2)*^{mps6}; *Tg(hsp70l:LOXP-DsRed2- LOXPEGFP)*^{tud107} fish (Tsata et al. 2020) to perform genetic lineage tracing.
Unfortunately, despite varying tamoxifen concentrations and heat shock duration, we were unable to reliably obtain sufficiently large numbers of labelled cells in the fin of juvenile zebrafish to trace them. The reason for the low number of labelled cells (in thebest scenario, only about 5 cells were labelled) is not clear now. However, sometimes transgenic lines that show good expression in embryos can be silenced in older animals, as this might have been the case in our experiment. We hope to obtain other transgenic lines that show good expression in juvenile and adult fins to perform geneticlineage tracing studies in the future in to obtain definite results. As a reflection of our inability to do so at this moment, we have softened our statements concerning the lineage relationships between pre-existing mural cells and newly forming ones during regeneration and have pointed out the caveats the reviewer mentions.

As an alternate approach, we performed consecutive imaging of regenerating blood vessels at daily intervals. This allowed us to follow individual mural cells over time and address their contribution to the mural cell population on regenerating blood vessels (New Fig.9). This analysis, while unfortunately not allowing us to determine thesource of new mural cells, provided additional evidence that pre-existing mural cells were relatively static on neighbouring pre-existing blood vessels.

2. What is the identity of the ovoid cells? This is not discussed even though this is one of the 4 populations in the fin. Further, what is the identity of the cuboid cells? The authors demonstrate that they are able todifferentiate into mural cells, but what is there identity when they in the cuboidal morphology and are not attached to vessels. Some additional maker exploration (co-expression of collagens and other fibroblast genes for instance)to define these cells would

strengthen conclusions about the origins of the fin mural cells. It would be unusual to find cuboidal fibroblasts, but if they are not of the fibroblast lineage, this would make the results even more interesting.

Response: We would like to thank the reviewer for raising these interesting points. We have now performed additional analysis to better characterize these cell populations. Todo so, we obtained an additional transgenic line that expresses EGFP under the controlof the col1a2 promoter (*TgBAC(col1a2:GFP)*^{ca103}), a marker for cells of the fibroblast lineage in the zebrafish trunk (Rajan et al., 2020, Plos Genetics). We then crossed these fish to *TgBAC(pdgfrb:mcherry)*^{ncv23} animals and examined double transgenic animals (New Figure 5). This analysis revealed the presence of cuboidal cells expressing pdgfrb and col1a2 in distal fin segments, suggesting that cuboidal cells might belong to the fibroblast lineage. As suggested by the reviewer, we also sorted either pdgfrb only expressing cells (potential cuboidal cells) or pdgfrb/myh11a expressing cells (potential mural cells) from zebrafish fins and analysed the expression of a set of mural and fibroblast markers (New Fig. 5g). This analysis revealed that double transgenic cells expressed higher levels of the mural cell markers myh11a, acta2 and transgelin, while fibroblast markers were expressed to a lesser degree.

Together, these findings suggest that cuboidal cells can express pdgfrb and col1a2,potentially belonging to the fibroblast lineage.
We fully agree that it would be of interest to determine the identity of the oval-shaped cells located between the fin ray joints. Based on their location, they might belong to thecartilage lineage. However, to fully define these cells an in-depth marker analysis of chondrocyte and osteoblast markers would be necessary. At this moment we feel that such an analysis would be beyond the scope of our current study.

3. On page 8, the authors show that fin mural cells differentiate from local precursors that express pdgfrb, but the authors do not show this is theonly source. It is possible that the cuboidal cells are only one of several sources (see comment 1 above). The current experiments only show that cuboidal turninto pdgfrb wrapping cells but does not rule out that other cells don't turn into mural cells as well. A lineage experiment as suggested in point 1would show this.

Response: We fully agree with this and have modified the manuscript accordingly to reflect the possibility that other cell types in addition to cuboidal cells might contribute to the mural cell population (results section, last sentence on page 10 and discussion page14).

4. Experiments involving dendra photoconversion are key to the arguments ofthe paper. Figure 3 shows that dendra photoconversion marks precursor cells transitioning into mural cells. It is essential that these experiments are supported by quantitative data.

Response: We would like to thank the reviewer for this suggestion and have nowprovided quantifications in Fig. 4f, g.

a. How did the authors ensure that only 'cuboidal cells' were photoconverted? Since pdgfrb would also be expressed in the mural cells, how did the authors pick 'cuboidal' cells out of a field to convert as both cell typeswould express the transgene.

Response: Our results show that in distal fin regions, only very few mural cells with elongated morphologies are present (Figure 1j, k). We therefore chose thoseregions for our photoconversion experiments. In addition, we made sure that the photoconverted cells did not show long cellular extensions characteristic of the mural cell lineage. To do so, we combined the *TgBAC(pdgfrb:H2B-dendra2)*^{mu158} transgenic line with *TgBAC(pdgfrb:gal4ff)*^{ncv24}; *Tg(UAS:GFP)*^{nkuasgfp1a} fish to obtain robust cytoplasmatic GFP expression in mural cells. This approach enabled us to readily determine cell morphologies.

b. There is no description in the methods of how single cell photoconversion was achieved. The images look fairly convincing, but wereexamples where more than one cell was photoconverted discarded?

Response: We did not describe the photoconversion experiments properly before and we would like to thank the reviewer for bringing this shortcoming to our attention. We didnot photoconvert individual cells, but rather groups of cells. We have now extended our description of the photoconversion experiments in the methods section. Cuboidal- shaped cells were identified based on their morphology. We then chose regions in the fin that only contained cuboidal-shaped cells and were devoid of cell with elongated mural cell morphologies. We marked these regions containing between 5 and 20 cells inthe Zeiss Zen software. The selected cells were exposed to 8% of 405 nm laser excitation wavelength with 40 iterations, for 25 cycles. Immediately after photoconversion, we imaged fins to determine the number of photoconverted cells. Thisimage also served as 0 dpc. We also determined the extent to which photoconverted cells contributed to the mural cell lineage (Fig. 4f, g).

c. There is no quantitation of the photoconversion data. How many cells were converted? How many became mural cells? What proportion of converted cells divided (i.e. estimation of proliferative ability). It is hard to see the morphology of thedifferentiated cells in a,b,c.

Response: We would like to thank the reviewer for pointing this out. We have now quantified our photoconversion experiments in Fig. 4f, g. We have enlarged images of differentiated cells in Fig. 4a'- d', to illustrate their morphology. We provide an example of a dividing mural cell in Figure 4, but after re-analysing our images, we realized that this was the only example that could directly observe because in that particular fish onlyone cuboidal cell had been photoconverted. We agree that it would be of great interest to determine the proliferative behaviour of individual mural cells and their precursors.
However, this is currently beyond our capabilities, as we very rarely achievephotoconversion of only one cuboidal cell.

d. In figure 6, the dendra conversion experiment is the sole evidence that regenerating mural cells do not come from pdgfrb precursors and that regenerated mural cells do not come from pre-existing cells. Given caveats of the dendra technique,this conclusion cannot be made confidently. A second line of evidence is needed here to support this strong (and interesting if it was true) statement.

Response: We fully agree with the reviewer's assessment of the limitations of lineagetracing based on photoconverted fluorophores that we discussed in point1. Since genetic lineage tracing also did not yield consistent results in regenerating fins, we performed daily imaging of fin blood vessel regeneration. These data reveal that pre-existing muralcells retain their position on uninjured blood vessels also during regeneration and no notstart migrating and or proliferating (new Fig. 8a). We further detect an overlap of of *TgBAC(pdgfrb:mcherry)*^{ncv23}; *TgBAC(col1a2:GFP)*^{ca103} cells in the regenerating tissue (3-5dpi) (new Fig. 9f), similar to our observations in developing juvenile caudal fins. These findings suggest that also in the regenerating artery Pdgfrb /Col1a2 doublepositive cells differentiate to mural cells that colonize the regenerated artery.

5. If cuboidal cells are mural cell precursors, it is perhaps not surprising that thenumber of cuboidal cells increases in pdgfrb knockouts, while mural cells goes down as pdgfrb is expressed in both.

a. This could be a differentiation block, but it is also possible that they have proliferated vs. control. The authors should test proliferation of thecuboidal cells in the mutants.

Response: We agree that it is an interesting question whether the increase in cuboidalcells is due to increased proliferation or impaired differentiation. We sought to addressthis by treating the juvenile fish in E3 containing BrDU. Unfortunately, we failed to achieve consistent BrDU incorporation into the fin of juvenile zebrafish. This might be due to problems with penetration of the fin tissue. As we also cannot assess proliferation of mural cell precursors using a photoconversion approach, as outlined above, we currently lack the experimental means to address this question.
b. Also the comment is made that they increase in citrine expression. No quantification is provided (either image intensity or qPCR) to support this.

Response: We have now provided intensity measurements in Supplementary Figure 6k.

6. The discussion is poorly written in sections with the writing flipping between discussing vSMCs and pericytes, almost interchangeably. In the sentences starting with 'On note, we did not detect cells with a similar SMC morphology in the zebrafish fin', this is confusing because pericytes were beingdiscussed in the prior sentence. Then the discussion changes again to pericytemorphology in the mouse context with alpha smooth muscle actin. (mouse vs. fish). Please re-read and simplify this discussion logic.

Response: We have now changed that part of the discussion according to thereviewer's suggestions.

7. Since mouse scRNA seq supports that there are myh11 mural cells without alpha SMA, this would make sense if myh11 is expressed in alpha SMA negativepericytes. So what are these cells in the tail? Pericytes or SMCs.?

Response: We agree with this interpretation and have obtained a new transgenic line expressing mCherry in acta2 positive cells (*Tg(acta2:mCherry)*^{ca8}) to investigate this possibility. Interestingly, we find mural cells in proximal regions of the fin that express myhh11a:YFP in addition to acta2:mcherry, while mural cells in more distal regions aredevoid of acta2 expression, while still expressing myh11a (new Figure 3). Thus, basedon gene expression, mural cells in proximal areas would be more similar to smooth muscle cells, while those in distal regions would be more similar to pericytes (page 9).

8. Page 11. That these cells might have different unique functions is completely speculative and should be removed. No functional work ha been donewith the cells.

Response: We have removed this statement.

9. The idea that there is no capillary bed in this area is interesting, andperhaps should be highlighted earlier in the manuscript.

Response: As we do not perform an analysis of vascular morphogenesis in our current study, we feel that this point is not covered to an extend that would warrant mentioning these differences in morphology already in the introduction.We think that it would be of great interest to perform a more detailed analysis offin vascular morphogenesis in the future. Our observation of the unique changein artery diameter from proximal to distal regions (unexpected increase in diameter, see point 10 below) that is included in this study further warrants theseanalyses.

10. The authors note proximal and distal differences in mural cell morphology but do not tie it to how the vessels are different in terms ofdiameter and flow, and whether this could underlie different mural cell morphologies? If flow does not control mural cell morphology, can the authorssuggest what else would control the proximal-distal morphological changes.

Response: These are interesting points raised by the reviewer. We have now measuredartery diameters along the proximal-distal axis both in wildtype and pdgfrb mutants (newFig. 6i). These result show that blood vessels in distal regions have a larger diameter when compared to more distally located blood vessels and that loss of pdgfrb signalling leads to increases in distal blood vessel diameters. While we are not sure about the functional relevance of this, it is interesting to note that in mammals blood vessel diameters decrease in distal regions when the blood reaches the capillaries. However, as pointed out earlier, we do not observe a well-developed capillary bed in the fin. This specific vascular anatomy might mandate an increase in diameter of the single artery to increase blood vessel volume. In other tissues this volume increase might be achieved through an increase in the number of (smaller diameter) capillaries.

It would be very interesting to investigate how blood flow and mural cell morphologies influence each other. However, we feel that this would be beyond the scope of the current manuscript. This said, we have investigated how notch signalling isdifferently activated along the artery (Fig 3i, Supplementary Fig. 4). These results suggest that mural cells of different morphology and/or identity have different levels of notch pathway activity. We did not provide functional data

on notch signalling, which wefeel would be beyond the scope of this study but would be of great interest to pursue in future work.

Reviewer 3 Advance Summary and Potential Significance to Field:
The study by Leonard et al explores the origins of pericytes and their contributions during fin regeneration in zebrafish. Previous studies in pericyte biology over many years have variously suggested that pericytes contribute during wound healing and regeneration, from proposing mechanisms of trans- differentiation to give rise to multiple different cell types, to promoting angiogenesis, to contributing in fibrosis. The role of pericytes in regeneration is still unclear and even controversial. The origin of new pericytes in regeneration and wound healing is also understudied. Thus, the approach of the authors hereto use a well-established model of regeneration to study the role of pericytes is clever and timely.

The authors describe the different anatomical features of mural cells of the different fin segments. They developed a new transgenic tool to visualise smoothmuscle cells that takes advantage of the myh11a promoter. They find that mural cells develop from pdgfrb-expressing cells in the fin and require pdgfrb for their expression. Furthermore, they show that during regeneration of ablated fin segments the pericytes regenerate in a pdgfrb-dependent manner. They show inelegant and simple lineage tracing experiments using kaede photoconversion that pdgfrb cells do not form from pre-existing pericytes in regeneration but are formed from other cells in the regenerating tissue. This observation is importantbecause it suggests that pericytes don't serve as tissue resident stem cell in damaged tissue.

Overall, the study is well written, presents clear and carefully analysed data andoffers new insights in pericyte biology that are informative and useful. In this reviewers opinion the study is suitable for publication in development but shouldbe improved for clarity with some essential minor revisions.

Reviewer 3 Comments for the Author:

Suggested revisions
1. The new myh11a transgenic line will be a very useful tool for the zebrafish vascular community. The authors state in the discussion that it overlapswith previously reported expression of the established SMC marker transgenic line using acta2 (from Whitesell et al 2014) but they discuss that contractile protein encoding genes are expressed heterogeneously in mural cell populations in sc-Seq data. Of note, this marker is expressed on intersegmental vessels (Supp Figure 2), while acta2 is not (Whitesell et al 2014).
To fully understand the results that take advantage of this new line, it is important to better understand how unique this line is by direct comparison with previously described markers. In the fin and perhaps embryo, does expression ofthe myh11a transgenic line overlap completely with, or only partially with, the previously reported smooth muscle acta2 line? Examining overlap with acta2 should be easily achievable and will show if the new line is an SMC marker strainor a more general mural cell marker.

Response: We would like to thank the reviewer for the suggestion. We have now included data on the overlap of myh11a and acta2 in fin mural cells (New Fig. 3). Our results indicate that in proximal fin regions, myh11a and acta2 can be co-expressed in asubset of mural cells, suggesting that these cells are smooth muscle cells.

2. It is interesting in the pdgrb mutants that there is still reasonably normal development of myh11a-expressing mural cells in S1. However, these cells don't look the same morphologically as the wt S1 cells. Are there defects in the number of cellular extensions that are formed around the blood vessels, evenin the mural cells that do form? Can the authors resolve this with higher resolution imaging to improve the clarity of the result?

Response: We would like to thank the reviewer for raising this point. We have tried toexamine mural cell morphologies more closely by using *TgBAC(pdgfrb:gal4)*^{ncv24}; *Tg(UAS:GFP)*^{nkuasgfp1a}; *Tg(-0.8flt1:RFP)*^{hu5333} fish to increase the level of fluorophore expression to facilitate the imaging

cellular processes in *pdgfrb* mutants (New Fig. 6). We have also added Supplementary movies showing a three-dimensional rendering ofthe cells (Supplementary Movies 1-9). However, the level of analysis that we can currently perform revealed no differences in the wrapping cells present in the proximalsegment S1 when comparing wildtype to mutant animals.

3. Did the authors assess whether fin regeneration is impaired in the absence of mural cells and pericytes in the pdgfrb mutants? If they performed traditional distal fin amputations, it would be useful for them to describe whether there wasimpaired regeneration or equally important for them to describe if there was no impact on regeneration. Such negative data would be informative and could be included in the supplementary material or described in the discussion.

Response: These are interesting points raised by the reviewer. We have addressed thisquestion by measuring the length of regenerated tissue and blood vessels in wildtype and *pdgfrb* mutants. Our observation did not reveal impaired regeneration of blood vessels or tissue in the mutant (Supplementary Fig. 8). However, it should be noted thatwe observed increased blood vessel connections in *pdgfrb* mutants (Supplementary Fig. 8b).

4. What is the identity of the "cuboidal" cells that differentiate into pericytes in development and regeneration? Are they perivascular fibroblasts as very elegantly suggested by Rajan et al 2020 (PloS Genetics)? This previous studyseems highly relevant to the claims being made here but is not cited. It would be suitable to add a discussion of the identity of these cells to the discussion in light of therecent published findings.

Response: We would like to thank the reviewer for raising this point. We obtained *TgBAC(col1a2:GFP)*^{ca103} transgenic fish to perform the experiments suggested by thereviewer. This analysis revealed the presence of cuboidal cells expressing pdgfrb andcol1a2 in the distal segments during juvenile development suggesting expression of fibroblast marker in the cuboidal cells. (Fig. 5). Further, we observed these double positive cells during regeneration (Fig. 9f). Thus, like the observations of Rajan et al. in embryonic zebrafish, mural cells in fins and during regeneration appear -at least in part-to be derived from cells of cuboidal morphology that express markers of the fibroblast lineage, such as col1a2.

Second decision letter

MS ID#: DEVELOP/2021/199640

MS TITLE: Regenerating vascular mural cells in zebrafish fin blood vessels are not derived from pre-existing ones and differentially require pdgfrb signaling for their development

AUTHORS: Elvin Vincent Leonard, Ricardo Figueroa, Jeroen Bussmann, Nathan D Lawson, Julio D Amigo, and Arndt F Siekmann

Iam pleased to say that the referees are happy with your revisions and there is just one minor comment for you to consider before weproceedto publication. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Leonard et al focuses on regeneration of vascular mural cells in zebrafish fin. The authors perform detailed characterization of mural and other pdgfrb-expressing cells in zebrafish larvae and adult fins. They identify the precursor cells that give rise to pericytes in zebrafish fins and demonstrate the role of pdgfrb signaling in mural cell formation in the fin. Furthermore, the authors argue that newly formed mural cells during regeneration are not derived

from pre-existing cells. Overall this study is well performed, data are convincing and sufficiently novel. Although the study is largely descriptive, the results are significant and are expected to be of interest to researchers in developmental, vascular and regenerative biology.
The authors have addressed most of my major concerns during the revision, and the manuscript has been greatly improved. I do not have any further comments except for a minor correction as listed below.

Comments for the author

Minor comment:
Please check and rephrase the discussion sentence on page 14, 1st paragraph : "This which was surprising as myh11a was previously reported...."

Reviewer 2

Advance summary and potential significance to field

The authors have done a lot of extra experiments for this revision, adding substantial new data. The reply to reviewers is detailed and admits where experiments the reviewers requrested could not be completed (failed or not possible on the timeline). I accept these reasons.

I think that the clarification of the overlaps between acta2, pdgfrb,myh11 and col1a1 cellular properties in this version has solidified the message of the paper as to the roles of the different cells, the anterior to posterior gradient and role of pdgfrb in this process. Looking at the dendra2 converted cells during regeneration also clarifies that the mural cells are normally quite static, but activated ruing repair.

Comments for the author

I am happy with the revised manuscript as it is.

Reviewer 3

Advance summary and potential significance to field

The revised manuscript is significantly improved and clearly demonstrates mural cell morphology, with key marker gene expression in zebrafish fin segments and high-quality imaging. The addition of the careful acta2 expression analysis and the col1a2 analysis significantly improves understanding of how mural cells differentiate and the relationship between fibroblast populations and mural cells in juvenile and regenerating tissues. The paper also shows a contribution of pdgfrB function in regeneration and in juvenile mural cell development. The lineage tracing experiments using photoconversion and serial imaging of individual animals appear to be robust despite small numbers of animals examined. Given the high level of photoconversion in the images and the numbers cells examined with serial imaging, it is difficult to imagine that a substantial contribution of pre-existing pdgfrB cells to regenerating fin rays would have been missed. This observation is of value to the field and will certainly lead to additional studies and work in the future.

Comments for the author

The authors have added new marker analysis, extensive new data in the form of new figures and provide careful quantification throughout. My original concerns have been well dealt with and I have no further concerns.

Second revision

Author response to reviewers' comments

We have rephrased the sentence in the discussion on page 14 as suggested by reviewer 1.

Third decision letter

MS ID#: DEVELOP/2021/199640

MS TITLE: Regenerating vascular mural cells in zebrafish fin blood vessels are not derived from pre-existing ones and differentially require pdgfrb signaling for their development

AUTHORS: Elvin Vincent Leonard, Ricardo Figueroa, Jeroen Bussmann, Nathan D Lawson, Julio D Amigo, and Arndt F Siekmann
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.