Heterogeneous *pdgfrb*$^{+}$ cells regulate coronary vessel development and revascularization during heart regeneration

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MS TITLE: Heterogeneous pdgfrb+ cells regulate coronary vessel development and revascularization during heart regeneration

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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 interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript prior to consideration for publication. For instance, they consider that the current RNAseq analysis is too superficial and needs much more comprehensive, robust analysis. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript.

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 revised manuscript by Kapuria et al focuses on pdgfrb cells during coronary vessel development and revascularization during heart regeneration. They show that zebrafish mural cells are derived from the epicardium and that pdgfrb mutants show coronary mural cell and angiogenic defects. They also describe heterogeneity in pdgfrb mural cell population and analyze pdgfrb role during cardiac regeneration. This is supplemented by scRNA-seq analysis of cardiac mural cells in wt, pdgfrb mutant and regenerating embryos.

The authors have attempted to address some of the previous concerns by providing new scRNA-seq data. However, the analysis of scRNA-seq results is somewhat limited, and there are some major concerns regarding methodology used as listed below. While the study provides novel findings (at least in zebrafish) regarding the origin of cardiac mural cells and analysis of cardiac mural cell coverage in pdgfrb mutants and in the context of cardiac regeneration, it still suffers from some of the major issues brought by the original reviewers, including limited mechanistic insight, and limited characterization of pdgfrb+ mural cell populations; I am not even convinced that there are multiple distinct pdgfrb+ mural cell populations based on the data provided. On the other hand, a broader scRNA-seq analysis could be a strength of this manuscript but this would require further analysis as listed below.

Comments for the author

Specific points:

1. Please provide information regarding all differentially expressed genes (marker genes) for each cluster identified in the two scRNA-seq experiments (wt, pdgfrb mutants, and during cardiac regeneration). This could be provided as a supplementary table. This is useful to confirm cluster annotation, and as a resource to discover other genes co-expressed in the same clusters.
2. It is not clear that single cell data distinguished between pdgfrb+cxcl12b+ and pdgfrb+ only mural cells.

As authors mentioned, cluster 3 seems to correspond to epicardial cells and not mural cells. It is positive for epicardial marker (tcf21 and tbx18) expression. Based on confocal imaging, 82% of mural cells were double positive for both reporters while 13% were pdgfrb+ only. However, cluster 3 has clearly more cells than cluster 6 suggesting that these are not the same pdgfrb+ mural cells as observed by confocal imaging. It is very likely that both pdgfrb+ cxcl12b+ and pdgfrb+ only mural cells cluster together in cluster 6. However they cannot be separated because only a fraction of cells in cluster 6 show pdgfrb or cxcl12b expression due to dropouts common with scRNA-seq. Therefore I am not clear that this scRNA-seq analysis has provided any information regarding heterogeneity of cardiac mural cells.

3. Instead of (or in addition to) ridge plots for selected genes shown in Fig. 3D,E and Suppl. Fig. 3.2, it would be better to perform differential expression analysis between selected clusters, such as clusters 6 in wild-type and pdgfrb mutant embryos. Such analysis will provide an unbiased list of differentially expressed genes (which should be shown in the manuscript), and will provide quantifiable values for the difference in expression for selected genes of interest which are difficult to quantify from ridge plots. Similarly, differences in pdgfrb expression in subclusters 0 and 3 (Fig. 5B) should be quantified using differential expression analysis.
4. It is difficult to appreciate the significance of heterogeneity among epicardial clusters described in Fig.
5. Some of them could be an artifact of sublcustering. The study would benefit from better characterization of these subclusters (sucg as, where are the cells in subclusters 0-3 located in the heart, are there biological or functional differences between them) although this would require extensive new experiments.
5. It appears from the Methods that clustering analysis in Fig. 5 between uninjured and inured heart ventricles was performed separately, and then they were merged together. Ideally the data from both control and injured samples would be combined before clustering so that the same clustering is performed on both samples, and subsequently the samples can be separated for

subsequent analysis. Otherwise it is difficult to say that the cluster 0 in control samples is the same as cluster 0 in experimental samples. Are the differences in the number of cells between the two samples described in Fig. 5A' statistically significant?
6. Fig. 5A and Suppl. Fig. 5.2A are identical and show the same plots 7. Please show marker genes (or all differentially expressed genes) for each subcluster identified in Fig. 5 8. Please show all induced (or reduced) genes during cardiac regeneration and fold change / p-values for each gene. The text mentions selected genes (mdka, hapln1a, col1a1) but a complete list and more extensive analysis should be provided. Ideally, this should be performed not only for epicardial subclusters but for other cardiac related clusters (myocardial and endocardial) that were identified in the analysis (Suppl. Fig. 5.1).
What are the genes induced or downregulated in different cell types during cardiac regeneration? scRNA-seq data from wild-type and regenerating hearts would be an important resource for the entire community and it could be one of the strengths of this manuscript. Currently the UMAP plot is provided but only very limited analysis has been performed.
9. Analysis of mdka mutants seems unrelated to the main story of the manuscript. It would be best to remove this section. There are multiple questions related to these mutants (is allele null, could there be a redundancy or genetic compensation), but it may be better to address these in a separate study.
10. scRNA-seq datasets need to be deposited into one of public databases so that they are accessible to the community.
11. A minor point: The numbering of supplemental figures 3.1, 3.2 and 5.1, 5.2. is confusing. I would recommend using only whole numbers.

Reviewer 2

Advance summary and potential significance to field

Overall, this is a very interesting project with the key points being the role of pdgfrb cells in coronary vessel development and repair, as well as the role of the pdgfrb gene within these cells. The authors have responded to reviews with some new data, particularly the addition of the midkine knockout in heart regeneration (unfortunately, a negative result) and new analysis of RNAsequencing data. The authors have tried to improve quantitation. The additions and revisions answer most of my points from my previous review but there are a few remaining small things that I think need to be addressed.

Comments for the author

1) New quantification of phenotypes now appears in figure 1 and figure 2, but there are still bar graphs for figures 2a', 3'''' and 4a'. Please graph in a format to show the underlying data and add statistical significance where appropriate. I am concerned that there is not statistical significance for some of the phenotypes that are being presented, but it is impossible to know from the current graph format (Supplemental Figure 6C for instance).
2) It is unfortunate that midkine did not turn out to be important for heart regeneration. I appreciate the work that went into this gene and it is good quality data. I suggest that this data could be moved to the supplement but leave it to the authors to decide this.
3) Figure 6- the image of the wildtype with AFOG staining is not in the same plane as the mutant (or the midkine) showing the AV valve. Presenting a similar view is ideal.
4) The scRNAseq analysis in this version is more thorough but is presented confusingly and lacks secondary validation.
- The text describing the scRNAseq data is very poorly written, particularly the description of the clustering and reclustering of the regenerating heart. Please take a look at this text and make it clearer. It is not clear whether the clusters in this analysis match the first clustering that was done, and if not why not (6 clusters vs. 15, but then 2 of 6 are reclustered into 5, but are these the same 5 that are examined in the regenerating hearts?).
-Speculation on the more 'smooth muscle like' character of the double positives are supported only by scRNAseq analysis in the form of ridge and violin plots which don't tell the significance of the data and changes in gene expression.
Speculations that pdgfrb cells regulate angiogenesis, but pdgfrb mutants cells are stressed are also only supported by scRNAseq data of a relatively small number of cells is supported only by GO

analysis. To make this data really robust either a fluorescent in situ technique or qPCR should be used to shore up the most important markers. The authors do have fish with both transgenic lines available for qPCR that could be used for sorting.
5) Figure 3D' and 3E show the wrong data. The text says that they show how many cells in the double positive cluster express notch 3 vs. single positive according to the text, but the data presented is about pdgfrb mutant vs. wildtype. The same occurs for 3E where data for mutant vs. wildtype is shown instead of single vs. double positive.

Reviewer 3

Advance summary and potential significance to field

This study from Kapuria et al investigated the role of pdgfrb in cardiac development and regeneration. They take advantage of the zebrafish model, using both transgenic lines that report expression of pdgfrb and a pdgfrb mutant line for functional studies. They show that pdgfrb is expressed in multiple cell types during regeneration, is required for normal coronary vascularization and they show that pdgfrb mutants have reduced regenerative capacity in the heart. Finally, they also profile the pdgfrb-positive cells in wt and injured hearts as well as wt and mutant animals using single cell sequencing. Of note the paper includes impressive use of ex vivo imaging of heart regeneration.

Overall, the role of pdgfrb in coronary vascularization and in regeneration is of great interest to the cardiovascular biology community, however much has already been shown before and while this is the first-time many phenotypes are shown in zebrafish, it is unclear if significant new insights are offered. Several of the experiments shown here require improvement and there are conclusions drawn that do not appear to be fully supported by the data.

In particular, it is unclear what the reduced regeneration in pdgfrb mutants really means and which pdgfrb-expressing cell type is essential for regeneration.
The authors establish nicely that these mutants have defects in coronary vascularization and it has been well established that coronary vascularisation is essential for normal cardiac regeneration (Marin-Jues et al 2016. PNAS). So could the defect in regeneration be exclusively caused by vessel defects that exist prior to injury? There does not seem to be convincing evidence that either mural cells or epicardial cells contribute to the phenotype. Furthermore, the analysis of pdgfrb expression and function does not at any stage address a potential role in fibroblasts. Pdgfrb is well established to be expressed in perivascular and tissue fibroblasts in many tissues. Rajan et al (2020, PLoS Genetics) recently showed that pdgfrb-expressing fibroblasts differentiate into pericytes during zebrafish development. Could the cells identified upon wounding here be activated fibroblasts? This is not addressed and could significantly impact the conclusions being drawn (see comments below). In addition, the scSeq analysis is potentially informative but current analysis if cursory and does not allow for generalisable conclusions about how pdgfrb cells change upon wounding or how pdgfrb mutants change the developmental landscape of the heart. The same analysis shows expression of EGFP transcripts in the endothelial cell cluster (but not expression of the endogenous pdgfrB transcripts), which may suggest that the overlapping expression with cxcl12b is not in mural cells. There are many questions such as these and further analysis is needed throughout.

Thus, while the study is potentially of interest, it does not go far enough in its current form and does not appear to offer clear new insights that would be required for publication at this journal.

Comments for the author

Major comments
1) Can the authors provide evidence that the regeneration phenotype in pdgfrB mutants is caused by mural cell or epicardial cell function of pdgfrb and not caused by the pre-existing coronary vascular phenotype in these mutants?
Identifying the cell type responsible for the role of pdgfrB in regeneration would provide significant novelty over previous publications (such as those mentioned in the previous reviewer 1 comment 1 assessment).

2) The authors need to exclude the possibility that the cells they are imaging and analysing in single cell sequencing data are not perivascular or tissue resident fibroblasts. Pdgfrb is expressed in fibroblasts and even activated in pathological settings in fibroblasts (eg. CAFs) and so the expression imaged in accompanying movies would not be inconsistent with fibroblast, rather than epicardial, expression. Current scSeq expression shows that pdgfrb cells here described as epicardial also express col1a1, postnb, twist genes and snai genes all consistent with fibroblasts. Do they express characterised fibroblast markers such as Vimentin or those characterised in Rajan et al 2020 (Pdgfra, Col1a2 Nkx3.1). a comprehensive analysis of the scSeq data with UMAPs and dot plots of expression for fibroblast markers is needed.

3) The authors need to clearly demonstrate that the cxcl12b and the pdgfrb EGFP expression imaged in Figure 3A is not in endothelial cells. High power imaging with dual markers that include ECs is needed, especially given EC expression of the EGFP transcripts shown in the supplement.

4) The analysis of scSeq data provided is generally cursory and should be improved throughout.
The plots used to display expression differences in Figure 3 would be easier to interpret if presented as a simple dot plots of transcript abundance that takes into account also the number of cells in the clusters expressing each marker.
Other markers should also be shown to prove cluster identity in the main paper.
Dot plots of markers for ECs, cardiomyocyte markers, fibroblast markers as well as the main cells of interest, would demonstrate specificity in cluster assignment while also allowing the authors to display mutant wt differences in a more digestible manner.
The only point in the main paper where the scSeq data is analysed to provide any general, high level conclusions is in Figure 5C. However, this is not a deep analysis. The supplementary material only shows the reclustering and cluster selection and some of the markers used for this.
Improving the analysis of mutant vs wildtype and of injured versus uninjured sc data using a broad analysis of cluster associated GO-terms, GSEA, pathways enrichment (eg. reactome) and analysis of differences between conditions for the above is needed for the study to fully benefit from the inclusion of the scSeq data. The authors can then in an unbiased manner ask broader questions such as – what pathways and processes are most changed between mutant and wildtype in different cell populations? What pathways and processes are most activated or repressed upon the wound response in the different cell types? Improving the depth of analysis here would offer important novelty to this study.

5) The Figures at several points are confusing. There is often no set rule for the ordering of panel labelling (eg. labels A,B,C,D do not simply go from left to right and top to bottom). This makes it very hard to follow the figures in general. Please modify all figures to make it easier for readers.

6) Zebrafish development is highly variable post-7dpf, as they rely on nutrients from feeding. Post-7 dpf, zebrafish length should be used as a measure of developmental stage and not days post fertilisation. Please replace dpf with zebrafish lengths in figures and clearly state how developmental stages were standardised.

7) Figure 2A – were heart sizes matched? Different fish ages and heart sizes may result in variable coronary vessel density.

8) Figure 4A: Please provide an image of 14 dpa. This is essential as it is when pdgfrB expression in wound site is the highest according to quantification.

9) Movies: I would recommend changing the pseudo colour of the movie to magenta (or another colour-blind friendly colour) and green, to make the red channel more visible. It would also be helpful to combine the movie with a pdgfrB expression only movie (perhaps with grey pseudo colour). As of now, it is very difficult to see mural cell migration.

10) Movie 2: Unfortunately, the heart has moved between 11 and 11:30. Was this intentional or was this unexpected? It would be better to use another example which did not move during the duration of the movie.

Minor
1) Figure 1C'' – please provide images of individual colours to clearly show co-expression.
2) Page 7: line 4 – should be Tg(pdgfrB:Citrine;fli1a:DsRed)
3) To an untrained eye, it is not clear where the cxcl12b/pdgfrB-positive mural cells are in both wild type and pdgfrB mutant coronary artery in Figure 3B.
Please provide a single colour image of pdgfrB expression and clearly label these cells.
4) Figure 3C: Please standardise graph size.
5) Movies: no scale bar provided.
6) Grammatical errors can be spotted quite frequently. Please proof read carefully.

First revision

Author response to reviewers' comments

Rebuttal Letter
We appreciate the reviewers' insightful and helpful comments, especially that the reviewers recognize the study provides novel findings regarding the origin of cardiac mural cells and analysis of cardiac mural cell coverage in pdgfrb mutants and in the context of cardiac regeneration. We have provided extensive new analyses based on the reviewers' comments. We highlighted all the changes in the text and addressed point-by-point the reviewer's comments below.

Reviewer 1 Comments for the Author:

Specific points:
1. Please provide information regarding all differentially expressed genes (marker genes) for each cluster identified in the two scRNA-seq experiments (wt, pdgfrb mutants, and during cardiac regeneration). This could be provided as a supplementary table. This is useful to confirm cluster annotation, and as a resource to discover other genes co-expressed in the same clusters.

Response. The differentially expressed genes for each cluster are provided as supplemental Table S.3.1 for Fig. 3 comparing WT and pdgfrb mutants and supplemental Table S5.1 for Fig. 5 comparing uninjured control and 7 days post amputation for heart regeneration.

2. It is not clear that single cell data distinguished between pdgfrb+cxcl12b+ and pdgfrb+ only mural cells.
As authors mentioned, cluster 3 seems to correspond to epicardial cells and not mural cells. It is positive for epicardial marker (tcf21 and tbx18) expression. Based on confocal imaging, 82% of mural cells were double positive for both reporters while 13% were pdgfrb+ only. However, cluster 3 has clearly more cells than cluster 6 suggesting that these are not the same pdgfrb+ mural cells as observed by confocal imaging. It is very likely that both pdgfrb+ cxcl12b+ and pdgfrb+ only mural cells cluster together in cluster 6. However, they cannot be separated because only a fraction of cells in cluster 6 show pdgfrb or cxcl12b expression due to dropouts common with scRNA-seq. Therefore I am not clear that this scRNA-seq analysis has provided any information regarding heterogeneity of cardiac mural cells.

Response. We understand the concerns raised by the reviewers and apologized that we did not explain the data clearly. In Fig. 3A-A'''', only the coronary arterial mural cells were shown in the confocal images and quantified. In addition to these cells, there are many more other *pdgfrb*+ mural cells on other coronary vessels and epicardium derived cells and all of them are *pdgfrb*+ only cells. In scRNAseq data all *pdgfrb*+ cells were considered. Cluster 3 is composed of *pdgfrb*+ only epicardial cells and thus has more cells than cluster 6 which contain *pdgfrb*+; *cxcl12*+ double positive and *pdgfrb*+ and *cxcl12*+ mural cells. We have provided new data (Fig. 3D and Fig. S3.4) to further demonstrate the heterogeneity of the mural cells (cluster 6), epicardial cells/EPDCs (cluster 3) and gene expression signatures in subclusters (Table S3.5, S3.6).

3. Instead of (or in addition to) ridge plots for selected genes shown in Fig. 3D, E and Suppl. Fig. 3.2, it would be better to perform differential expression analysis between selected clusters, such

as clusters 6 in wild-type and pdgfrb mutant embryos. Such analysis will provide an unbiased list of differentially expressed genes (which should be shown in the manuscript), and will provide quantifiable values for the difference in expression for selected genes of interest which are difficult to quantify from ridge plots. Similarly, differences in pdgfrb expression in subclusters 0 and 3 (Fig. 5B) should be quantified using differential expression analysis.

Response. We have provided new data of differential expression analysis with new dot plots in the new Fig. 3C', Fig. S3.1 and Supplemental Table S3.1, S.3.2, S.3.3 and S3.4 (Differentially expressed gene list of cluster 6 cells, between WT and *pdgfrb* mutant). Differences in pdgfrb expression are also provided in new Fig. 5B and Fig. S5.1C and Supplemental Table S5.1 and S5.2.

4. It is difficult to appreciate the significance of heterogeneity among epicardial clusters described in Fig.

Response. We have provided new data to demonstrate the heterogeneity of the epicardial/EPDC cluster, cluster 3 (of developing heart) (in Fig. 3C' and S3.4. C, D, Table S3.6), and cluster 5, 6, 10 (of regenerating heart) (in Fig. S5.1B, S5.4, Table S5.1).

5. Some of them could be an artifact of subclustering. The study would benefit from better characterization of these subclusters (such as, where are the cells in subclusters 0-3 located in the heart, are there biological or functional differences between them) although this would require extensive new experiments.

Response. We made more/new analyses and more GO-term analysis was done for the differentially expressed genes (DEG) for the biological functions in *pdgfrb*+ epicardial/ mural cell clusters (Fig. S3.5, S5.4).

It appears from the Methods that clustering analysis in Fig. 5 between uninjured and inured heart ventricles was performed separately, and then they were merged together. Ideally the data from both control and injured samples would be combined before clustering so that the same clustering is performed on both samples, and subsequently the samples can be separated for subsequent analysis. Otherwise it is difficult to say that the cluster 0 in control samples is the same as cluster 0 in experimental samples. Are the differences in the number of cells between the two samples described in Fig. 5A' statistically significant?

Response. We tested samples merged in cell ranger before clustering and after clustering and could not find difference since we used Seurat V3 integration workflow which find 'anchors' (represents a similar biological state) for integration.
By identifying 'anchors' between pairs of datasets the method finds pairwise correspondences between individual cells (one in each dataset). This method only requires some overlaps in cell populations between datasets. We integrated datasets from same tissue (zebrafish adult heart ventricle) with different conditions. Thus, significant overlap was there among cell types and same cell types across samples formed the clusters.

The quantification of the relative abundance of each sample Fig. 5A' (now S5.1A') in a cluster was performed to identify the clusters which are more represented for a condition (condition: tissue injury, for having more cells from the injured heart). This was done to find the affected clusters by the condition. In this way we found cluster 6 to be more represented by injured heart cells and by GO-term analysis of differentially expressed genes was found promoting regeneration more than other clusters (e.g.: cluster 5) (Fig. S5.4).

6. Fig. 5A and Suppl. Fig. 5.2A are identical and show the same plots

Response. New data analyses were done and Fig. S5.2A were removed.

7. Please show marker genes (or all differentially expressed genes) for each subcluster identified in Fig. 5

Response. The differentially expressed genes are now shown in Table S5.1 (for different epicardial clusters: cluster 5, 6, 10 and the mural cell cluster: cluster 12).

8. Please show all induced (or reduced) genes during cardiac regeneration and fold change / p-values for each gene. The text mentions selected genes (mdka, hapln1a, col1a1) but a complete list and more extensive analysis should be provided. Ideally, this should be performed not only for epicardial subclusters but for other cardiac related clusters (myocardial and endocardial) that were identified in the analysis (Suppl. Fig. 5.1).
What are the genes induced or downregulated in different cell types during cardiac regeneration? scRNA-seq data from wild-type and regenerating hearts would be an important resource for the entire community and it could be one of the strengths of this manuscript.
Currently the UMAP plot is provided but only very limited analysis has been performed.

Response. We have provided the list of differentially expressed genes (upregulated and downregulated in the *pdgfrb*+ clusters in response to injury), fold change and p values in Table S5.2.

9. Analysis of mdka mutants seems unrelated to the main story of the manuscript. It would be best to remove this section. There are multiple questions related to these mutants (is allele null, could there be a redundancy or genetic compensation), but it may be better to address these in a separate study.

Response. The study of mdka is included as an example of highly differentially expressed genes during heart regeneration and it remains as one of the top differentially expressed genes in pdgfrb+ cells after the new analyses. The mdka allele used in this study has been published (Nagashima et al.) and showed phenotypes in retinal regeneration. We included this study here as a proof of principle to show how the scRNAseq data will be followed up although unfortunately this mdka allele does not show a strong phenotype in heart regeneration after amputation. As the reviewer 2 suggested, we moved the mdka mutant data to the supplemental figure 6. And we discussed and compared allelic differences and injury model utilized.

10. scRNA-seq datasets need to be deposited into one of public databases so that they are accessible to the community.

Responses. We have deposited the scRNAseq datasets into the public databases (GEO).

11. A minor point: The numbering of supplemental figures 3.1, 3.2 and 5.1, 5.2. is confusing. I would recommend using only whole numbers.

Response. We thank the reviewers for the suggestions. We decided to keep this numbering since there will be too many figures of using the whole numbers.

Reviewer 2 Comments for the Author:
1) New quantification of phenotypes now appears in figure 1 and figure 2, but there are still bar graphs for figures 2a', 3a'''' and 4a'. Please graph in a format to show the underlying data and add statistical significance where appropriate. I am concerned that there is not statistical significance for some of the phenotypes that are being presented, but it is impossible to know from the current graph format (Supplemental Figure 6C for instance).

Response. We have replaced all the bar graphs in Fig. 2a', 3"" and 4a' as scatter plots to show the data points.

2) It is unfortunate that midkine did not turn out to be important for heart regeneration. I appreciate the work that went into this gene and it is good quality data. I suggest that this data could be moved to the supplement but leave it to the authors to decide this.

Response.
We thank the reviewers for the suggestion. We have moved the midkine data to the supplemental Figure 6.

3) Figure 6- the image of the wildtype with AFOG staining is not in the same plane as the mutant (or the midkine) showing the AV valve. Presenting a similar view is ideal.

Response. We have provided new AFOG staining images in the new Fig. S6.D.

4) The scRNAseq analysis in this version is more thorough but is presented confusingly and lacks secondary validation.
- The text describing the scRNAseq data is very poorly written, particularly the description of the clustering and reclustering of the regenerating heart. Please take a look at this text and make it clearer. It is not clear whether the clusters in this analysis match the first clustering that was done, and if not, why not (6 clusters vs. 15, but then 2 of 6 are reclustered into 5, but are these the same 5 that are examined in the regenerating hearts?).

Response. We thank the reviewer for the comment and suggestion. Instead of re-clustering, we have maintained the same clusters for the new analyses of the differentially expressed genes for the regenerating heart vs uninjured controls.

-Speculation on the more 'smooth muscle like' character of the double positives are supported only by scRNAseq analysis in the form of ridge and violin plots which don't tell the significance of the data and changes in gene expression. Speculations that pdgfrb cells regulate angiogenesis, but pdgfrb mutants cells are stressed are also only supported by scRNAseq data of a relatively small number of cells is supported only by GO analysis. To make this data really robust either a fluorescent in situ technique or qPCR should be used to shore up the most important markers. The authors do have fish with both transgenic lines available for qPCR that could be used for sorting.

Response. We intended to use qRT-PCR to validate the gene expression but the FACS experiments using cxcl12b+ cells and pdgfrb+ cells from pdgfrb mutant fish were hampered by the low numbers of pdgfrb mutant fish we have left and extremely low numbers of cxcl12b+ cells. We have observed that cxcl12b+ cells are the top 1% brightest pdgfrb+ cells. Using this gate to sort pdgfrb high vs pdgfrb low cells and perform qRT-PCR, we were able to validate the differential gene expression of acta2 and other mural cell markers also showed a trend of increased expression. Furthermore, we now provide new notch activity reporter data to validate that notch 3/Notch signaling are strongest in the arterial (cxcl12b/pdgfrb double positive) mural cells.

5) Figure 3D' and 3E show the wrong data. The text says that they show how many cells in the double positive cluster express notch 3 vs. single positive according to the text, but the data presented is about pdgfrb mutant vs. wildtype. The same occurs for 3E where data for mutant vs. wildtype is shown instead of single vs. double positive.

Response. We thank the reviewer for the comments. We have now provided new data analyses and changed the text and figures. There is a separate analysis now for wildtype cxcl12b+ mural cells vs cxcl12b- (pdgfrb only) mural cells in Fig. 3 D, Table S3.3.

Reviewer 3 Comments for the Author:

Major comments

1) Can the authors provide evidence that the regeneration phenotype in pdgfrB mutants is caused by mural cell or epicardial cell function of pdgfrb and not caused by the pre-existing coronary vascular phenotype in these mutants? Identifying the cell type responsible for the role of pdgfrB in regeneration would provide significant novelty over previous publications (such as those mentioned in the previous reviewer 1 comment 1 assessment).

Response. Unfortunately this is not feasible currently due to our fish loss during the pandemic. The fish lines to overexpress dnPdgfrb were not breeding and growing up in time.

2) The authors need to exclude the possibility that the cells they are imaging and analysing in single cell sequencing data are not perivascular or tissue resident fibroblasts. Pdgfrb is expressed in fibroblasts and even activated in pathological settings in fibroblasts (eg. CAFs) and so the expression imaged in accompanying movies would not be inconsistent with fibroblast, rather than epicardial, expression. Current scSeq expression shows that pdgfrb cells here described as epicardial also express col1a1, postnb, twist genes and snai genes, all consistent with fibroblasts.

Do they express characterised fibroblast markers such as Vimentin or those characterised in Rajan et al 2020 (Pdgfra, Col1a2, Nkx3.1). a comprehensive analysis of the scSeq data with UMAPs and dot plots of expression for fibroblast markers is needed.

Response. We have included new analyses and the fibroblast makers in Fig. S3.4. and Fig. S5.1 as the reviewer suggested. Some epicardial but not mural cell clusters (subclusters) do express fibroblast markers. This is consistent with our confocal imaging data.

3) The authors need to clearly demonstrate that the cxcl12b and the pdgfrb EGFP expression imaged in Figure 3A is not in endothelial cells. High power imaging with dual markers that include ECs is needed, especially given EC expression of the EGFP transcripts shown in the supplement.

Response. We have provided new high power images in Fig. 3B.

4) The analysis of scSeq data provided is generally cursory and should be improved throughout. The plots used to display expression differences in Figure 3 would be easier to interpret if presented as a simple dot plots of transcript abundance that takes into account also the number of cells in the clusters expressing each marker.
Other markers should also be shown to prove cluster identity in the main paper. Dot plots of markers for ECs, cardiomyocyte markers, fibroblast markers as well as the main cells of interest, would demonstrate specificity in cluster assignment, while also allowing the authors to display mutant wt differences in a more digestible manner.

Response. We thank the reviewers for the comments and suggestions. We have now changed the ridge plots to the dot plots. Furthermore, we have provided the cluster markers in Fig. S3-1.

The only point in the main paper where the scSeq data is analysed to provide any general, high level conclusions is in Figure 5C. However, this is not a deep analysis. The supplementary material only shows the reclustering and cluster selection and some of the markers used for this. Improving the analysis of mutant vs wildtype and of injured versus uninjured sc data using a broad analysis of cluster associated GO-terms, GSEA, pathways enrichment (eg. reactome) and analysis of differences between conditions for the above is needed for the study to fully benefit from the inclusion of the scSeq data. The authors can then in an unbiased manner ask broader questions such as – what pathways and processes are most changed between mutant and wildtype in different cell populations? What pathways and processes are most activated or repressed upon the wound response in the different cell types? Improving the depth of analysis here would offer important novelty to this study.

Response. We thank the comments and suggestions from the reviewer. As the reviewer1 suggested, we have focused our analyses on differentially expressed gene between uninjured control and regenerating hearts (7 days post amputation). We then performed cluster associated GO-terms analysis.

5) The Figures at several points are confusing. There is often no set rule for the ordering of panel labelling (eg. labels A,B,C,D do not simply go from left to right and top to bottom). This makes it very hard to follow the figures in general. Please modify all figures to make it easier for readers.

Response. We have re-arranged the figure panels.

6) Zebrafish development is highly variable post-7dpf, as they rely on nutrients from feeding. Post-7 dpf, zebrafish length should be used as a measure of developmental stage and not days post fertilisation. Please replace dpf with zebrafish lengths in figures and clearly state how developmental stages were standardised.

Response. We regularly use length matched fish to compare WT and mutant. We have included info of representative fish length used in the figure 1.

7) Figure 2A – were heart sizes matched? Different fish ages and heart sizes may result in variable coronary vessel density.

Response. We usually use fish of comparable length to compare mutants and controls. Some of the *pdgfrB* mutant hearts do appear smaller than the sibling controls, likely due to the coronary vessel defects and these defects do not recover.

8) Figure 4A: Please provide an image of 14 dpa. This is essential as it is when pdgfrB expression in wound site is the highest according to quantification.

Response. We have included the 14 dpa image in Fig. 4A.

9) Movies: I would recommend changing the pseudo colour of the movie to magenta (or another colour-blind friendly colour) and green, to make the red channel more visible. It would also be helpful to combine the movie with a pdgfrB expression only movie (perhaps with grey pseudo colour). As of now, it is very difficult to see mural cell migration.

Response. We have changed the pseudo color of the movie to magenta and green. Minor 1)Figure 1C'' – please provide images of individual colours to clearly show co-expression. We have now provided single channel images for Fig. 1C".

2)Page 7: line 4 – should be Tg(pdgfrB:Citrine;fli1a:DsRed).

Response. We thank reviewer for catching these typos. We have made corrections.

3) To an untrained eye, it is not clear where the cxcl12b/pdgfrB-positive mural cells are in both wild type and pdgfrB mutant coronary artery in Figure 3B. Please provide a single colour image of pdgfrB expression and clearly label these cells.

Response. We have provided single channel images for Fig. 3B.

4) Figure 3C: Please standardise graph size.

Response. We have made corrections.

5) Grammatical errors can be spotted quite frequently. Please proof read carefully.

Response. We thank reviewer for catching these errors. We have corrected the typos and grammatical errors.

Second decision letter

MS ID#: DEVELOP/2021/199752

MS TITLE: Heterogeneous pdgfrb+ cells regulate coronary vessel development and revascularization during heart regeneration

AUTHORS: Subir Kapuria, Haipeng Bai, Juancarlos Fierros, Ying Huang, Feiyang MA, Tyler Yoshida, Antonio Aguayo, Fatma Kok, Katie Wiens, Joycelyn Yip, Megan McCain, Matteo Pellegrini, Mikiko Nagashima, Peter F. Hitchcock, Naoki Mochizuki, Nathan Lawson, Michael Harrison, and Ching-Ling Lien

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 differ in their opinion on the suitability of your manuscript for publication in Development. Although two reviewers are happy with your revisions, one is not. This reviewer considers that the phenotypes you describe are potentially an indirect consequence of disrupted vasculature. As a role for vasculature in regeneration is already established, this

reviewer considers that there is insufficient novelty to justify publication in this journal. I would like you to respond to this concern as it is obviously important to consider all reasons why a phenotype may arise. Given the support from the other two reviewers, I am less concerned about whether the manuscript is a sufficient advance on the published literature; however, I do consider it critical that the conclusions drawn from the data are robust and that limitations and alternative interpretations are discussed.

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 revised manuscript by Kapuria et al focuses on pdgfrb cells during coronary vessel development and revascularization during heart regeneration. They show that zebrafish mural cells are derived from the epicardium and that pdgfrb mutants show coronary mural cell and angiogenic defects. They also describe heterogeneity in pdgfrb mural cell population and analyze pdgfrb role during cardiac regeneration. This is supplemented by scRNA-seq analysis of cardiac mural cells in wt, pdgfrb mutant and regenerating embryos.

Comments for the author

The revised manuscript has been greatly improved overall, and the authors addressed most of my concerns.

There are a few relatively minor points remaining.
1. The authors have not answered whether the differences in the percentage of cells present in different clusters in the injured and uninjured hearts (Fig. S5.1A, A') are statistically significant. This is important to clarify because authors argue that percentage of cells in certain clusters (such as cluster 6 and 10) are increased in injured (or uninjured) hearts. If the differences are not statistically significant, then such claims should not be made.
2. On page 7, text says "…tcf21:CreRET2 labeled cells that did not express pdgfrb (fig. 1C'', blue arrow).
Please check, it should be fig. 1C''' instead of fig. 1C''
3. Please check the panel label in Fig. 3A, it reads 'cx and cl12b' instead of 'cxcl12b'

Reviewer 2

Advance summary and potential significance to field

This paper identifies different populations of different coronary endothelial cells and the effects of different pathways (largely pdgf signalling but also touches on notch and chemokine) required for both differentiation and later injury repair.

Comments for the author

Most of my concerns have been addressed. Please note the typo in the transgene name in Figure 3A. Also the term 'gene expressions' is used frequently. This should always be 'gene expression', not plural.

Reviewer 3

Advance summary and potential significance to field

The authors have addressed several of my concerns and improved analysis of their single cell RNA seq data and the organisation of the manuscript, but my first and major concern was not addressed. The work is informative in describing an origin of coronary mural cells and providing a single cell RNA seq profile of these populations. Unfortunately, the functional studies do not clearly offer any new insights.

Comments for the author

As raised in the original review, the coronary vasculature in pdgfrB mutants is severely impaired and the role of coronary vasculature in regeneration has been established previously (Marin-Juez et al 2016). It is therefore not a surprise to see that pdgfrB mutants have reduced cardiac regeneration in the final figure of the study. If this phenotype relates to the expression of pdgfrB in cardiac fibroblasts, mural cells or epicardium (as the authors suggest throughout) this would be a new insight. However, the authors have not been able to demonstrate if this is the case. Add to this the mdka mutants not having a phenotype and it remains very unclear from this work if any of the pdgfrB cells regulate regeneration directly, beyond leading to the previously reported coronary vessel defect.
Considering this, as it stands the study offers largely descriptive insights: that pdgfrB labels a number of heterogeneous cell types in the heart (pericytes, smooth muscle, fibroblasts and epicardial cells), that epicardium can generate some pdgfrB-expressing cells and that the distribution of the cells changes dynamically during the response to injury.

Second revision

Author response to reviewers' comments

Response to reviewers' comments

We appreciate the reviewers' insightful and helpful comments. We have addressed point-by- point the reviewer's comments below and highlighted all the changes in the text.

Reviewer 1 Comments for the Author:
1. The authors have not answered whether the differences in the percentage of cells present in different clusters in the injured and uninjured hearts (Fig. S5.1A, A') are statistically significant. This is important to clarify because authors argue that percentage of cells in certain clusters (such as cluster 6 and 10) are increased in injured (or uninjured) hearts. If the differences are not statistically significant, then such claims should not be made.

Response. We agree with the reviewer's comments. Furthermore, the purpose of the Fig. S5.1A was only to show that 15 clusters were identified. We have removed the paragraph describing the changes in cell numbers.

2. On page 7, text says "…tcf21:CreRET2 labeled cells that did not express pdgfrb (fig. 1C'', blue arrow).
Please check, it should be fig. 1C''' instead of fig. 1C''

Response. We have changed the text to Fig. 1C'''.

3. Please check the panel label in Fig. 3A, it reads 'cx and cl12b' instead of 'cxcl12b'

Response. We have changed the label in Fig. 3A.

Reviewer 2 Comments for the Author:
Most of my concerns have been addressed. Please note the typo in the transgene name in Figure 3A. Also the term 'gene expressions' is used frequently. This should always be 'gene expression', not plural.

Response. We thank the reviewers for the comments. We have made changes for Fig. 3A and "gene expression" throughout the text.

Reviewer 3 Comments for the Author:
As raised in the original review, the coronary vasculature in pdgfrB mutants is severely impaired and the role of coronary vasculature in regeneration has been established previously (Marin- Juez et al 2016). It is therefore not a surprise to see that pdgfrB mutants have reduced cardiac regeneration in the final figure of the study. If this phenotype relates to the expression of pdgfrB in cardiac fibroblasts, mural cells or epicardium (as the authors suggest throughout) this would be a new insight. However, the authors have not been able to demonstrate if this is the case.
Add to this the mdka mutants not having a phenotype and it remains very unclear from this work if any of the pdgfrB cells regulate regeneration directly, beyond leading to the previously reported coronary vessel defect.

Considering this, as it stands the study offers largely descriptive insights: that pdgfrB labels a number of heterogeneous cell types in the heart (pericytes, smooth muscle, fibroblasts and epicardial cells), that epicardium can generate some pdgfrB-expressing cells and that the distribution of the cells changes dynamically during the response to injury.

Response. We thank the reviewer for the comments. Unfortunately, the appropriate inducible fish lines to knockdown *pdgfrβ* in epicardial derived cells or mural cells to address the questions currently are not available and will take a considerably long time to generate, import and breed. Even if we could carry out a regeneration-specific knockdown of *pdgfrβ* in epicardial cells, it might still affect revascularization due to the critical role of mural cells in maintaining endothelial cell. Therefore, we have provided discussion on potential caveats in interpretating the regeneration phenotypes of *pdgfrβ* mutants. We also included caveats regarding *mdka* mutant phenotypes and suggested that different mutant alleles might reveal regeneration phenotypes in different injury models.

Third decision letter

MS ID#: DEVELOP/2021/199752

MS TITLE: Heterogeneous pdgfrb+ cells regulate coronary vessel development and revascularization during heart regeneration

AUTHORS: Subir Kapuria, Haipeng Bai, Juancarlos Fierros, Ying Huang, Feiyang MA, Tyler Yoshida, Antonio Aguayo, Fatma Kok, Katie Wiens, Joycelyn Yip, Megan McCain, Matteo Pellegrini, Mikiko Nagashima, Peter F. Hitchcock, Naoki Mochizuki, Nathan Lawson, Michael Harrison, and Ching-Ling Lien
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.