nature portfolio

Peer Review File

Endothelial stem cells of the retinal vasculature reside in the optic nerve

Corresponding Author: Professor Kohji Nishida

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the submitted manuscript, the authors propose that endothelial stem cells in the retinal vasculature reside in the optic nerve. The authors use a variety of approaches to support this assertion including quantification followed by single cell sequencing of CD34 positive CD45 negative retinal and retinal plus optic nerve cells and lineage tracing to provide evidence for a population of progenitor cells that migrate from the optic nerve to the retina. These are studied further after damage such at oxygen induced retinopathy. Many of the experiments are well executed and provide quality data. However, it is not clear to me that the central hypothesis has been proven. The data from figure 2 provides single cell sequencing information from retinal and retinal+optic nerve vascular endothelial cells and figure 2d provides a velocity estimate of RNA expression on a UMAP. The figure does not provide compelling evidence for a set of genes that clearly demonstrate a differentiation from a population located in the optic nerve to a population located in the retina. Figure 2J uses tdTomata and Cd201 Cre for gene lineage analysis and while the images are beautiful, they do not provide information on where the tdTomato positive cells came from, only the time course of deposition in optic nerve then retina. Finally, that stem cell locate in the retina after OIR is well established but the authors do not provide any information as to whether these stem cells differentiate into functional endothelial cells. Collectively, the central hypothesis was not demonstrated.

Additional comments

In figure 2J
There are many dtTomato positive cells not in the vasculature. The relative amount should be quantified.
What are the non-vascular dtTomato+ cells?

Fig 3 panel f: statistics needed.

Figure 4: without staining for CD157 no conclusion about that marker can be made. No comment about the origin of the stem cells can be made from this data. Instead, this shows a nice time course of where the CD201+ stem cells fill or target.

This sentence is very confusing and needs to be rewritten,
In the OIR model, CD201-positive cells represent less than 5% of cells in the retina plus optic nerve samples at P28, the vascular repair phase, with more than 50% of cells found to be derived from CD201-positive cells (Extended Data Fig. 2c).

The experiment in fig 5 is interesting. The authors use the CD201 CRE for stem cell targeted deletion of ATF3 floxed mice. ATF3 was identified as a transcription factor changed in the single cell sequencing. However, the result was a modest reduction in vaso-obliteration with no change in neovessel formation. The authors provide no information as to why this particular result would occur. Stem cells presumably are needed for neovascularization so why no change despite elevated hypoxia with increased vaso-obliteration? Also, why would ATF deletion lead to changes in vaso-obliteration?

Reviewer #2

(Remarks to the Author)

Overall, this is a very interesting and novel study with important implications for the field of vascular biology. The manuscript is a bit sloppy and the figures, figure legends and the narrative (results and discussion) all need to be improved.

Major points:
Fig. 2g, k should be turned into a separate figure. The text in the results does not match the images, e.g. none of the peripheral retina image are really peripheral. More appropriate images should be shown. Also, there are red dots in e.g. 7 dpi or 14 dpi, I cannot see whether they are associated with small vessels or capillaries. It seems the labelled cells are moving from the veins to the arteries via the capillaries. This journey should be demonstrated much better. Also, is there evidence of clones (e.g. 28 dpi and 2 mpi peripheral retina)? Some of the red patches seem rather large (i.e. could be clones), higher magnification images at cellular resolution should be shown.

Fig. 3a: I cannot see the difference in between retina and retina+ON the way it is displayed in different colours. To me all three time points seem to have similar amounts of red in population 2. Either way, this difference should be quantified. BTW, there are no letters on the panels. Also, it is very difficult to see differences in the blue/orange UMAP plots. This data should be displayed in a better way.

Fig. 4: Why are there no neovascular tufts visible shown in the images at P17? They seem to there in the figure 5. It would be interesting to see how the red cells integrate into the tufts.

Fig. 4: Again, as above, there should be some images shown at single cell resolution. BTW, the magnification factors in Retina and ON are not given. Also, claims about lineage in panel g are not well supported. Double stains of tdTomato/CD157 and tdTomato/CD201 should be shown.

Fig. 4: The authors should show an immuno stain of CD157/CD201 in addition to the panels shown. They have shown earlier that there are hardly any CD201 positive cell in the normal adult retinal vasculature (8 weeks) but the same cannot be assumed at P12. Admittedly, there are not many red cells at P13 but the number of red cells P17 seems to be very high and I struggle to imagine that they have all come from the optic nerve in just 4 days and it is possible the CD201::Cre may have also activated cells that were already in the retina. It would also be useful to see whether the spread at P15 is less peripheral.

Fig. 5: Why do the authors think there is no difference in N.S. but one in avascular area? Is there a suggestion that VESCs do not contribute to N.S. If that is the case, we should not see any read cell on neovascular tufts in Fig. 4. Either way, I actually don't think this line of investigation has added much to the manuscript (and wouldn't mind it placed into supplemental data). If find the other aspects of the study far more interesting and convincing, such as migrating VESCs/ECs in the retina from veins to capillaries to arteries and the remarkable contribution of tdTomato cells to the regenerating plexus, which are truly impressive

Minor points:
L85: This should probably be CD31+/CD45- cells.
L86: onwards: The numbers and notation in this section don't match the figures, e.g. Fig. 1e, f show 1.53% and 12.4% for double positive CD201/157 cells.
Fig.1p: the images of retina and retina+ON make no sense if they were not used (they should show live and heart.
Fig. 1r: I don't think we can draw any lineage conclusion (as suggested by the arrows in Fig. 1r) based on the FACS data, which only shows the size of different subpopulations but not lineage relationships.
Fig. 2a: do you mean 3'/5' scRNAseq transcriptomics analysis? I don't see where genes and enhancers come into this.
Figure 2 legend: "444,358 total" should be 44,358
Fig. 1c: Italics is typically used for genes (you used it here for proteins). Also, the use of e.g. CD157 and Bst1 is inconsistent. The authors should pick on term and use it throughout all the figures.

Fig. 2d: this panel is of very poor quality, the numbers a not explained and I can see how lineage is implied. The figure legend mentions velocity, but that is neither explained in the text nor in the figure. Furthermore, this analysis should be expanded (e.g. with a pseudo time analysis). Furthermore, population 10 (Lrg1) should be characterised in a more systematic way, i.e. what other genes are uniquely expressed in these cells, is Lrg1 the only one? Fig. 2h is far too small and should contain the annotation of the most extreme genes (and not just 3 TFs).

Reviewer #3

(Remarks to the Author)
The work led by Sakimoto and colleagues characterized a population of vascular endothelial stem cells (VESCs) residing in the optic nerve that may serve as a reservoir to supply damaged vasculature in the inner retina. The approaches are highly interdisciplinary, including lineage tracing, colony-forming assays, as well as scRNA-seq analyses. This sounds like an attractive and novel concept for vascular repair conceptually.

I have multiple major concerns that need to be addressed in the revised format. These concerns are regarding key molecular

and cellular mechanisms, which need to be demonstrated to support the major conclusions and offer transparency to general readers -

1. Developmental vs. Adult - Figures 1 and 2 were launched in adults, 8 weeks or older, for both the cell marker analyses and lineage marking using CD201; Tamoxifen. However, the later Figures (Fig 3-5) dominantly used one model that focused on retinas during the first 2-3 weeks of their lives. There is a clear separation between the two parts of the study, especially with the claim that these adult VESCs may serve as reservoirs for adult vascular repairs. In order to further enhance the significance of the study, the authors should consider carrying out the analyses using adult ischemic and vascular insult models, such as AION (anterior ischemic optic neuropathy), and should conduct the adult blockade/ischemic conditions. The current pathological studies are more akin to OIR (premature retinopathy); during this stage, the vasculature is also growing and patterning.

2. Related to point 1, if the authors carried out the Figure 2I-k experiments in neonatal development (say P7 or P12 without OIR), what would the retina vasculature look like? Will the retina vasculature be heavily populated with TdTomato? In other words, what is the control/baseline condition for Figure 4b that also needs to be quantified?

3. Cellular events associated with cell migration and repair: sample images in Figure 4 b-c are very small, only representing a small area of the retina, and the data were NOT quantified. Thus, it is very hard to judge the efficacy of such repair/repopulation of the retina vasculature subjected to the OIR/CD201 tracing. The authors need to quantify the area/% of PECAM that are converted to TdTomato over the time course, and also across the different regions/zones, as the authors did in Figure 2k. Additional sample images should be included as extended figures, and the quantifications should include not only the number of cells but also reflect the % changes over time (i.e., density, fractions, and changes across time over a larger window).

4. Similar critiques apply to Figure 5 (in terms of Atf3 mutants). The authors should characterize the basal level of activity changes of CD201, Atf3 F/F, and R26-CAG-TdTMT in adults at 8 weeks, as shown in Figure 2i-k, before proceeding with Figure 5 OIR experiments. At this moment, it is not very clear how the VESCs are selectively affected in ATF3 mutants or whether the conditional mutants selectively affect the baseline of VESCs.

5. Instead of Atf3 mutants, can the authors generate CD201 creer, R26 -DTA. cross and induce specific VESC ablation mutants and re-examine the phenotypes again in the OIR setting; in other words, how specific and important are the lineage of the CD201 VESC population?

6. Proportion of each population in the scRNA-seq analysis (Figure 3F) - it is not clear how the experiments were done and what the size of each column is. As we all know, scRNA-seq is highly fluctuating in terms of capture rate and batch effects. Was the current analysis quantified as one reaction in each condition? In this case, it is really hard to quantify and compare experiments across all these conditions. The authors should consider justifying these points using biological replicates and ways to normalize/compare the conditions, especially considering the experiments' highly fluctuating survival/recovery rates.

7. It is highly advised that the authors provide a report on the N-size (animal numbers, cells counted, areas covered) in each of the imaging experiments throughout the study. Unless the authors included a report/table somewhere, I found such information generally missing from the methods/figure legends.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors propose that endothelial progenitor cells exist in the optic nerve that act to support retinal endothelial renewal. The authors use a variety of approaches to support this assertion including quantification followed by single cell sequencing of CD34 positive CD45 negative retinal and retinal plus optic nerve cells and lineage tracing to provide evidence for a population of progenitor cells that migrate from the optic nerve to the retina. These are studied further after damage such at oxygen induced retinopathy. The data is of high quality and the authors have responded to my previous critiques well. However, there remain two significant issues.

First, the authors do not have any experiments demonstrating that the CD157+ CD201+ optic nerve populations serves as the source of cells filling the retinal vasculature (Fig 5). Softening the conclusion to include the possibility that these cells originate from a separate source (potentially bone marrow) is needed.

Second, figure 6 remains perplexing and the authors rebuttal does not clear the question. The experiment essentially shows that targeting Procr (CD201) cells with diptheria toxin does not reduce neovascularization in the OIR model. This suggests this stem cell population is not part of the retinal angiogenesis in OIR. This needs to be clearly stated. Importantly, why OIR induced vessel obliteration increases in the Procr targeted deletion remains unclear.
This data and the data in suppl fig 5 with ATF gene deletion under Procr Cre is indeed fascinating but there is no explanation for why vasoobliteration increases.

One possibility is that basally in both transgenic models there is reduced blood vessels and thus the OIR model leads to increased area of vaso-obliteration. But there remains a need for some explanation.

Reviewer #2

(Remarks to the Author)
The authors have sufficiently addressed my concerns and have improved the manuscript's quality and clarity substantially. The most interesting phenomena in my eyes (reverse migration) is now demonstrated more convincingly. A comment for future extensions of this study, the injury work uses OIR (a developmental model); adult injury models would be a interesting to see in followup work.

Reviewer #3

(Remarks to the Author)
The revision was thorough and fully addressed my questions. It is a great study and carries enough novelty - should be published with Nature Comm timely and highly suitable for broad audience in neurobiology, vascular biology and retina biology studies.

AUTHORS' RESPONSES

Reviewer #1

In the submitted manuscript, the authors propose that endothelial stem cells in the retinal vasculature reside in the optic nerve. The authors use a variety of approaches to support this assertion including quantification followed by single cell sequencing of CD34 positive CD45 negative retinal and retinal plus optic nerve cells and lineage tracing to provide evidence for a population of progenitor cells that migrate from the optic nerve to the retina. These are studied further after damage such at oxygen induced retinopathy. Many of the experiments are well executed and provide quality data. However, it is not clear to me that the central hypothesis has been proven.

RESPONSE: We appreciate the reviewer's constructive comments and hope that or additional work, as described, helps support our central hypothesis.

Figure 2J uses tdTomata and Cd201 Cre for gene lineage analysis and while the images are beautiful, they do not provide information on where the tdTomato positive cells came from, only the time course of deposition in optic nerve then retina.

RESPONSE: Thank you for this insightful suggestion. Obtaining direct evidence via live cell imaging in our model system to pinpoint the precise origin of tdTomato$^+$ cells is technically very challenging and beyond current capabilities. However, we would argue that the fact that CD201$^+$ cells are overwhelmingly concentrated in the optic nerve strongly suggests that the tdTomato$^+$ population arises from this region.

Finally, that stem cell locate in the retina after OIR is well established but the authors do not provide any information as to whether these stem cells differentiate into functional endothelial cells. Collectively, the central hypothesis was not demonstrated.

RESPONSE: On this point, we are not in full agreement with the reviewer. As now shown in new Supplementary Fig Fig S4a, at P28, during the repair phase of the OIR model, hypoxyprobe is no longer detectable in the vascular areas that are constructed by clones of tdTomato-positive cells, i.e. CD201-positive cells. This indicates that the hypoxic state has been resolved to normoxic and is sufficient evidence to show that functional blood vessels have been reconstructed. Therefore, the sentence "In addition, it was also shown that the blood vessels reconstructed by tdTomato-positive cells improved ischemia and were functional (Supplementary Fig. S4a)" has been added to page 11, line 188.

The data from figure 2 provides single cell sequencing information from retinal and retinal+optic nerve vascular endothelial cells and figure 2d provides a velocity estimate of RNA expression on a UMAP. The figure does not provide compelling evidence for a set of genes that clearly demonstrate a differentiation from a population located in the optic nerve to a population located in the retina.

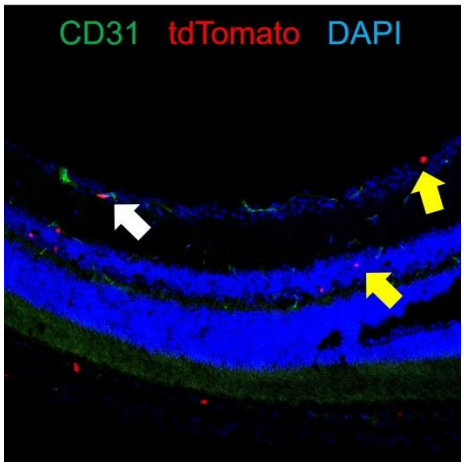
RESPONSE: In response to this point and some comments by Reviewer 2, we have now improved the image quality of Fig 2d and added a pseudo-time analysis that illustrates the differentiation process from the stem cell cluster. Furthermore, we do not claim that retinal endothelial cells differentiate from the optic nerve endothelial stem cell population based solely on the results of single-cell RNAseq analysis. Nevertheless, it is true that the number of Bst1(CD157)-positive cells clearly increases and cells form a distinct cluster when the optic nerve is included in the sampling (Fig 2 and the new Fig 4), which is an interesting and novel finding.

Additional comments

In figure 2J

There are many dtTomato positive cells not in the vasculature. The relative amount should be quantified. What are the non-vascular dtTomato+ cells?

RESPONSE: To clarify, we actually counted CD31+tdTomato+ cells not "Tomato+ cells", and have corrected this in the revised mauscript, Fig 3c. As shown by the retinal section (below) from an 8wk-old CD201-tdTomato mouse 14 dpi tamoxifen injection, the tdTomato signal is present in the inner retina and appears to be expressed in the cytoplasm of retinal ganglion cells, horizontal cells and amacrine cells.



Retinal section from *8wk-old CD201-tdTomato mouse 14 dpi tamoxifen injection*, following DAPI staining and immunostaining for CD31. tdTomato+CD31+ (white arrow)

cells are also present, with tdTomato+CD31neg cells (yellow arrows) also present in the ganglion cell layer and inner granular layer.

Fig 3 panel f: statistics needed.

RESPONSE: We used Fisher's exact test to compare the proportions of CD157negCD201+ cells versus all other cells in control and OIR at each time point (P12, P17, P25) for both Retina only and Retina + ON. The following results are incorporated into Fig 4e.

<Results>

P12	***Retina***	***control VS OIR***	***(p = 0.9147)***
	Retina + ON	***control VS OIR***	***(p = 0.0001297)***
P17	***Retina***	***control VS OIR***	***(p < 2.2e-16)***
	Retina + ON	***control VS OIR***	***(p < 2.2e-16)***
P25	***Retina***	***control VS OIR***	***(p < 2.2e-16)***
	Retina + ON	***control VS OIR***	***(p < 2.2e-16)***

Figure 4: without staining for CD157 no conclusion about that marker can be made. No comment about the origin of the stem cells can be made from this data. Instead, this shows a nice time course of where the CD201+ stem cells fill or target.

RESPONSE: We described the cell hierarchy based on the results of the colony assay, single-cell RNAseq and CD201 lineage tracing. We appreciate the reviewer's comment about the nice time course, and in based on their assessment we have now added "Proposed" to the legend of Fig 6f to tone down our conclusion.

This sentence is very confusing and needs to be rewritten,
In the OIR model, CD201-positive cells represent less than 5% of cells in the retina plus optic nerve samples at P28, the vascular repair phase, with more than 50% of cells found to be derived from CD201-positive cells (Extended Data Fig. 2c).

RESPONSE: We thank the reviewer for this comment and have rewritten the text as follows. "In the OIR model at P28 when the blood vessels are undergoing repair, an analysis of retinal and optic nerve samples showed that less than 5% of the $CD31^{+}CD45^{neg}$ endothelial cells were CD201-positive (i.e. expressing CD201 by FACS analysis). However, at this juncture more than 50% of the cells are clones of CD201-positive cells (i.e. tdTomato-positive ones) (Supplementary Fig Fig S4b)."

The experiment in fig 5 is interesting. The authors use the CD201 CRE for stem cell

targeted deletion of ATF3 floxed mice. ATF3 was identified as a transcription factor changed in the single cell sequencing. However, the result was a modest reduction in vaso-obliteration with no change in neovessel formation. The authors provide no information as to why this particular result would occur. Stem cells presumably are needed for neovascularization so why no change despite elevated hypoxia with increased vaso-obliteration? Also, why would ATF deletion lead to changes in vaso-obliteration?

RESPONSE: As shown in P17 of new Fig 5b and Supplementary Fig Fig S3c (which is added in accordance with the comments of reviewer 2), there were relatively few tdTomato-positive cells in neovascular tufts compared to large veins. Furthermore, with regards to the reason why vaso-obliteration did not change, we recently reported that in ATF3 pan-endothelial cell knockout mice, the area of vascular occlusion expands and vascular repair is delayed but no change in neovascular tufts in the OIR model (Ueda et al. iScience. 2024 Dec 2;28(1):111516. doi: 10.1016/j.isci.2024.111516. PMID: 39790557). As shown in Fig 4b, the vessels repaired in the OIR model were mostly clones of CD201-positive cells, suggesting that ATF3 deletion prevented vascular repair.

Reviewer #2 (Remarks to the Author):

Overall, this is a very interesting and novel study with important implications for the field of vascular biology. The manuscript is a bit sloppy and the figures, figure legends and the narrative (results and discussion) all need to be improved.

RESPONSE: We appreciate the reviewer's positive evaluation of our work. As suggested, we have revised the manuscript including figures, legends, and the narrative in accordance with the reviewer's comments below.

Major points:

Fig. 2g, k should be turned into a separate figure. The text in the results does not match the images, e.g. none of the peripheral retina image are really peripheral. More appropriate images should be shown.

RESPONSE: As requested, we have separated Fig. 2i, j, and k into a new figure (designated as Fig 3) and corrected the corresponding panels. Regarding imaging the "periphery," we would like to respectfully point out that there is no standard definition for the periphery of the mouse retina. It appears the reviewer is referring to the distal periphery, where relatively large arteries and veins are often obscured. Our images include regions in which larger vessels are clearly visible, therefore we have

changed the term "periphery" to "mid-periphery" in response to the reviewer's comment.

Also, there are red dots in e.g. 7 dpi or 14 dpi, I cannot see whether they are associated with small vessels or capillaries. It seems the labelled cells are moving from the veins to the arteries via the capillaries. This journey should be demonstrated much better. Also, is there evidence of clones (e.g. 28 dpi and 2 mpi peripheral retina)? Some of the red patches seem rather large (i.e. could be clones), higher magnification images at cellular resolution should be shown.

RESPONSE: As requested, we have made significant changes to Fig 3, enlarging all the images and including a signal image of tdTomato to make it easier to see the tdTomato-positive cells. We have also added enlarged images of the retina. To clarify the directionality of cell movement, we have added a simplified schematic (new Fig. 3d) depicting proposed labelled cells migrating from veins to arteries through the capillary network (i.e., reverse migration). In the text we have now stated that 'These data suggest that tdTomato-labeled cells undergo reverse migration—migrating against the direction of blood flow[17]—to traverse from veins to arteries via the capillary network' (page 9, line 142 when referring to Fig 3d) to indicate the migration of endothelial cells.

Fig. 3a: I cannot see the difference in between retina and retina+ON the way it is displayed in different colours. To me all three time points seem to have similar amounts of red in population 2. Either way, this difference should be quantified. BTW, there are no letters on the panels. Also, it is very difficult to see differences in the blue/orange UMAP plots. This data should be displayed in a better way.

RESPONSE: In accordance with the reviewers' comments, we re-analyzed scRNA-seq data, and the Bst1+ stem cell cluster is now defined as cluster 1. Since this cluster was previously difficult to identify, we have added stacked bar charts to the revised Fig. 4d to more clearly show the distribution of cluster 1 cells across the conditions. Statistical analysis showed that cluster 1 cells were significantly more abundant in retina+ON than in retina-only under OIR conditions at all of P12, P17 and P25. In addition, to address the problem that the difference between the blue/orange dots was hard to distinguish, we have removed the original UMAP plots.

Fig. 4: Why are there no neovascular tufts visible shown in the images at P17? They seem to there in the figure 5. It would be interesting to see how the red cells integrate into the

tufts.

RESPONSE: Neovascular tufts often form in the peripheral area and just happened not to be in the selected image. In accordance with the reviewer's suggestion, we have now replaced the image with one showing neovascular tufts. As shown in the new Fig 5b and Supplementary Fig Fig S3c, tdTomato-positive cells were found in the tufts during the vascular repair phase after P17, but their proportion was lower than that in retinal blood vessels.

Fig. 4: Again, as above, there should be some images shown at single cell resolution. BTW, the magnification factors in Retina and ON are not given. Also, claims about lineage in panel g are not well supported. Double stains of tdTomato/CD157 and tdTomato/CD201 should be shown.

RESPONSE: As the reviewer requests, we have now added high-resolution images to Supplementary Fig S3c. To the best of our knowledge, except for the following example, there are no reported cases of successful CD157 or CD201 staining in flat-mount retinal sections. We also attempted immunostaining using antibodies we had created ourselves, but none of these worked. Neither the CD157 staining protocol of Wakabayashi et al. (Invest Ophthalmol Vis Sci. 2022 Apr 1;63(4):5. doi: 10.1167/iovs.63.4.5; PMID: 35394492) nor the CD201 method described by Yu et al. (Cell Res. 2016 Oct;26(10):1079–1098. doi: 10.1038/cr.2016.85; PMID: 27364685) has yielded successful staining in flat-mount retinal sections. Related to magnification factors, we have added several scale bars throughout the manuscript. Regarding the claims about lineage, it is difficult to prove the hierarchy shown in the panel using only this OIR experiment and CD201cre-tdTomato mice. However, since CD201-positive cells gave rise to CD201-negative cells in this experiment, the correct hierarchy is shown when combined with the results of the experiment shown in Fig 1p-r.

Fig. 4: The authors should show an immuno stain of CD157/CD201 in addition to the panels shown. They have shown earlier that there are hardly any CD201 positive cell in the normal adult retinal vasculature (8 weeks) but the same cannot be assumed at P12. Admittedly, there are not many red cells at P13 but the number of red cells P17 seems to be very high and I struggle to imagine that they have all come from the optic nerve in just 4 days and it is possible the CD201::Cre may have also activated cells that were already in the retina. It would also be useful to see whether the spread at P15 is less peripheral.

RESPONSE: As mentioned above, immunostaining for CD157/CD201 in retinal

flatmounts is technically very difficult. Although there are very few, it cannot be asserted that all of the tdTomato-positive cells in retina are of optic nerve origin. In the CD201cre-tdTomato mice with OIR at P15, because cells are present on the optic nerve side of the large veins but not in peripheral in the retina it is assumed that most of the cells are of optic nerve origin. We have added these results to S Fig 3d. The OIR model uses mice at developmental stages, and while it is not possible to rule out the possibility that some of the tdTomato-positive cells remain in the retina and become active again, we believe that progenitor cells derived from the optic nerve play a major role in vascular reconstruction.

Fig. 5: Why do the authors think there is no difference in N.S. but one in avascular area? Is there a suggestion that VESCs do not contribute to N.S. If that is the case, we should not see any read cell on neovascular tufts in Fig. 4. Either way, I actually don't think this line of investigation has added much to the manuscript (and wouldn't mind it placed into supplemental data). If find the other aspects of the study far more interesting and convincing, such as migrating VESCs/ECs in the retina from veins to capillaries to arteries and the remarkable contribution of tdTomato cells to the regenerating plexus, which are truly impressive

RESPONSE: Thank you for your positive evaluation of the results of our research into the supply of VESCs/ECs from the optic nerve to the retina and their ability to repair blood vessels. Perhaps NS is related to neovascular tufts. As shown in CD201creERT2-tdTomato mice, tdTomato-positive cells were also expressed in tufts, but the number was not large. Furthermore, as we showed in Ueda et al. (***iScience. 2024 Dec 2;28(1):111516. doi: 10.1016/j.isci.2024.111516. PMID: 39790557) there were no changes in tufts even in the pan-endothelial specific deletion of ATF3 mutant. In conclusion, although there were significant changes in vascular obliteration in both this study and that of Ueda et al., it is difficult to say that these were dramatic, which may be due to the fact that ATF3 gene activity is not high. Accordingly, following the reviewer's advice, we have moved the ATF3 KO results to Supplementary Fig S5.***

Minor points:

L85: This should probably be CD31+/CD45- cells.

RESPONSE: This has been corrected to "it was found that about 1% of CD157-positive cells were seen in CD45negCD31+ endothelial cells of the retina."

L86: onwards: The numbers and notation in this section don't match the figures, e.g. Fig. 1e, f show 1.53% and 12.4% for double positive CD201/157 cells.

RESPONSE: Thank you for spotting this error. The numbers and notation have been corrected throughout this section.

Fig.1p: the images of retina and retina+ON make no sense if they were not used (they should show live and heart.

RESPONSE: This has been corrected in Fig 1p.

Fig. 1r: I don't think we can draw any lineage conclusion (as suggested by the arrows in Fig. 1r) based on the FACS data, which only shows the size of different subpopulations but not lineage relationships.

RESPONSE: While it's true that FACS data primarily quantifies the distribution of cell subpopulations, taken in combination with data from complementary experiments such as colony forming assays, it can help inform lineage relationships. In our study, the second FACS wasn't used in isolation, but was combined with the colony assay and evaluated expression patterns of well-characterized stem cell surface markers that correlate with distinct differentiation states. These markers, when analyzed collectively, provide a proxy for cell lineage. Thus, in this integrated framework, FACS data does aid in mapping the cellular hierarchy, rather than just depicting static subpopulation sizes.

Fig. 2a: do you mean 3'/5' scRNAseq transcriptomics analysis? I don't see where genes and enhancers come into this.

RESPONSE: To clarify, we sorted CD45^{neg}CD31+ endothelial cells from the retinas and retinas and optic nerves of 8-week-old wild-type mice, and performed 3' and 5' scRNAseq on them. In particular, for the 5' scRNAseq, we identified transcribed candidate enhancer regions and performed motif analysis. The results are shown in Supplementary Fig Fig 1a and Fig 2h, respectively.

Figure 2 legend: "444,358 total" should be 44,358

RESPONSE: Thank you. This has been corrected in the legend to Fig. 2.

Fig. 1c: Italics is typically used for genes (you used it here for proteins). Also, the use of e.g. CD157 and Bst1 is inconsistent. The authors should pick on term and use it throughout all the figures.

RESPONSE: If the reviewer is referring to Fig. 2c, these are the results of scRNAseq, and they are genes, not proteins. Regarding the distinction between CD157 and Bst1, we use CD markers such as CD157 and CD201 at the protein level. In contrast, we use gene symbols such as Bst1 and procr at the gene level of scRNAseq experiments. We have confirmed that this notation policy is consistent throughout the manuscript.

Fig. 2d: this panel is of very poor quality, the numbers a not explained and I can see how lineage is implied. The figure legend mentions velocity, but that is neither explained in the text nor in the figure. Furthermore, this analysis should be expanded (e.g. with a pseudo time analysis). Furthermore, population 10 (Lrg1) should be characterised in a more systematic way, i.e. what other genes are uniquely expressed in these cells, is Lrg1 the only one? Fig. 2h is far too small and should contain the annotation of the most extreme genes (and not just 3 TFs).

RESPONSE: As requested, we have improved the image quality of Fig 2d and performed a pseudotime analysis, presented the results in Fig 2e. These results show that population 10 is upstream. We have added following "The velocity analysis (Fig. 2d) and pseudo-time analysis (Fig. 2e) estimate indicated a potential differentiation pathway where Procr-low endothelial cells might emerge from Procr-positive or Bst1-positive cells (Fig 2d and e)." in line 126, page 8. We also now describe the genes expressed in particular in population 10 and characterized the pathway more detail (see Supplementary Data Fig 1b and 1c). We also enlarged Fig 2h, as requested.

Reviewer #3 (Remarks to the Author):

The work led by Sakimoto and colleagues characterized a population of vascular endothelial stem cells (VESCs) residing in the optic nerve that may serve as a reservoir to supply damaged vasculature in the inner retina. The approaches are highly interdisciplinary, including lineage tracing, colony-forming assays, as well as scRNA-seq analyses. This sounds like an attractive and novel concept for vascular repair conceptually.

I have multiple major concerns that need to be addressed in the revised format. These concerns are regarding key molecular and cellular mechanisms, which need to be demonstrated to support the major conclusions and offer transparency to general readers -

RESPONSE: We sincerely appreciate your positive evaluation of our paper.

Following your suggestions, we conducted additional experiments, rewrote the necessary parts, and modified the paper to support the major conclusions and improve readability.

1. Developmental vs. Adult - Figures 1 and 2 were launched in adults, 8 weeks or older, for both the cell marker analyses and lineage marking using CD201; Tamoxifen. However, the later Figures (Fig 3-5) dominantly used one model that focused on retinas during the first 2-3 weeks of their lives. There is a clear separation between the two parts of the study, especially with the claim that these adult VESCs may serve as reservoirs for adult vascular repairs. In order to further enhance the significance of the study, the authors should consider carrying out the analyses using adult ischemic and vascular insult models, such as AION (anterior ischemic optic neuropathy), and should conduct the adult blockade/ischemic conditions. The current pathological studies are more akin to OIR (premature retinopathy); during this stage, the vasculature is also growing and patterning.
RESPONSE: As the reviewers points out, in our paper, we used adult mice in Figs 1-3 and the OIR model in Figs 4-6. As state, the first half of the paper describes the supply of endothelial cells involved in vascular homeostasis in the steady state, whilst the second half describes the repair mechanism by stem cells during damage. Although many models of ischemic retinopathy in adulthood have been examined, there is no model that surpasses the OIR model in terms of reproducibility and reliability. Also, a number of groups, ours included, consider acute ischemic optic neuropathy to be an ischemic disease of the optic nerve. With regard to the issue of the relevance of the models we used to disease observe in adults, we respectfully disagree with the reviewer. While we appreciate the fact that there is no perfect animal model to study adult ischemic retinopathies, the OIR model is one of the most reproducible, reliable and established models used not only in ophthalmology, but in vascular biology, too.

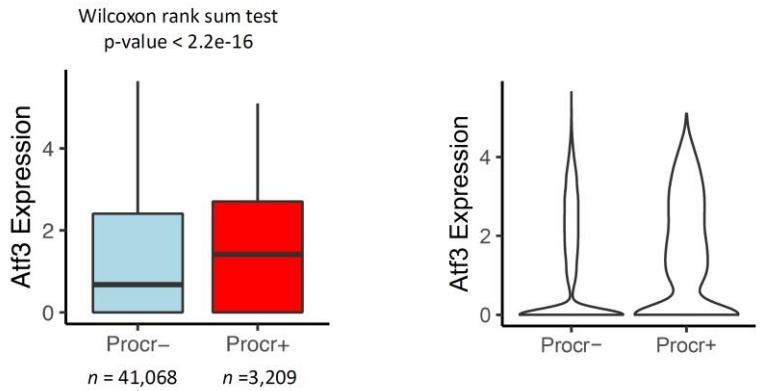
Below, we refer to several publications that use the OIR model for studying "proliferative diabetic retinopathy".
1. Crespo-Garcia S et al. Cell Metab 2021, PMID: 33548171. 2. Usui-Ouchi A et al. PNAS 2020, PMID: 33106407. 3. Binet F et al. Science 2020, PMID: 32820093. 4. Lee, J et al. Sci Transl Med 2013, PMID: 24048525. 5. Okuno, Y et al. Nat Med 2012, PMID: 22797809. There are numerous articles the that similarly use the OIR model as a model of diabetic retinopathy: Year 2024: 80 publications; Year 2023: 90 publications; Year 2022: 70 publications.

2. Related to point 1, if the authors carried out the Figure 2I-k experiments in neonatal development (say P7 or P12 without OIR), what would the retina vasculature look like? Will the retina vasculature be heavily populated with TdTomato? In other words, what is the control/baseline condition for Figure 4b that also needs to be quantified?
RESPONSE: We take the reviewer's point and recognize that it is important to analyze the developmental phase without OIR. Accordingly, and as requested, we have conducted the suggested experiment and shown the results in Supplementary Fig Fig S3a, b.

3. Cellular events associated with cell migration and repair: sample images in Figure 4 b-c are very small, only representing a small area of the retina, and the data were NOT quantified. Thus, it is very hard to judge the efficacy of such repair/repopulation of the retina vasculature subjected to the OIR/CD201 tracing. The authors need to quantify the area/% of PECAM that are converted to TdTomato over the time course, and also across the different regions/zones, as the authors did in Figure 2k. Additional sample images should be included as extended figures, and the quantifications should include not only the number of cells but also reflect the % changes over time (i.e., density, fractions, and changes across time over a larger window).
RESPONSE: As requested, we have enlarged Fig 4b (now the new Fig 5b). Furthermore, as suggested, we have quantified the area/percentage of PECAM converted to TdTomato over time and across different regions/zones, and have added quantified tdTomato+ Area / CD31+ Area (%) data in a new Fig 5c. However, we cannot respond to the second part of the comment. For the number of cells, as shown in Extended S2b, during the developmental period (P13–28) cell proliferation occur irrespective of ischemic injury, resulting in concomitant increases in both tdTomato$^+$ cell numbers (numerator) and vascular endothelial cell numbers (denominator). Moreover, in the OIR model—where overall vascular density is reduced—it is technically challenging to compare absolute cell counts between groups. For these reasons, we believe that reporting tdTomato$^+$ cells as a percentage of CD31$^+$ endothelial cells is the most appropriate metric; while one could in principle count every green (endothelial) cell as the denominator, this is not feasible in practice, so we instead use area% as a robust proxy. In contrast, for the adult analyses in Figure 3, the total number of CD31$^+$ endothelial cells remain essentially constant, allowing us to present absolute numbers of tdTomato$^+$ cells directly. Moreover, the area/% of tdTomato/PECAM is also equivalent to "density" and

"fraction".

4. Similar critiques apply to Figure 5 (in terms of Atf3 mutants). The authors should characterize the basal level of activity changes of CD201, Atf3 F/F, and R26-CAG-TdTMT in adults at 8 weeks, as shown in Figure 2i-k, before proceeding with Figure 5 OIR experiments. At this moment, it is not very clear how the VESCs are selectively affected in ATF3 mutants or whether the conditional mutants selectively affect the baseline of VESCs.
RESPONSE: It would be interesting to see whether ATF3 affects the behavior of progenitor cells, and as shown in the following scRNAseq dataset, ATF3 is indeed expressed in Procr-positive cells. In line with the critique of reviewer 2, however, the importance of ATF3 is downplayed by this reviewer, so results of the ATF3 KO experiments are now presented in the Supplementary Fig section.



Comparison of ATF3 expression level in scRNAseq. Box plot and violin plot of ECs identified by scRNAseq of sorted 44,358 total (retina and retina plus optic nerve) cells (CD45negCD31+) at 8-weeks of age.

5. Instead of Atf3 mutants, can the authors generate CD201 creer, R26 -DTA. cross and induce specific VESC ablation mutants and re-examine the phenotypes again in the OIR setting; in other words, how specific and important are the lineage of the CD201 VESC population?
RESPONSE: As requested, we created Procr-ROSA26iDTR-tdTomato mice and examined these using the OIR model. In this experiment, P28 was analyzed, and while neovascularization did not change, vascular opacity (VO) was increased in mice with Procr-ROSA26iDTR-tdTomato mice. We have added these results Fig 6a-c.

6. Proportion of each population in the scRNA-seq analysis (Figure 3F) - it is not clear how

the experiments were done and what the size of each column is. As we all know, scRNA-seq is highly fluctuating in terms of capture rate and batch effects. Was the current analysis quantified as one reaction in each condition? In this case, it is really hard to quantify and compare experiments across all these conditions. The authors should consider justifying these points using biological replicates and ways to normalize/compare the conditions, especially considering the experiments' highly fluctuating survival/recovery rates.
RESPONSE: Thank you for mentioning this very important point. To begin with, the number of retinal vascular endothelial cells that can be obtained from a single mouse is extremely small, and in our experiments, we used 10x3 pups and pooled the libraries for RNA sequence. Furthermore, in order to normalize the conditions, we randomly selected 2000 cells from each group for integrated analysis and compared them as part of this revision. As a result, Fig 3 and Extended Fig 2 have been updated. As a result of the re-analysis, the Bst1$^+$ stem cell population is now classified as cluster 1. Importantly, these revisions do not alter the overall conclusions of the study.

7. It is highly advised that the authors provide a report on the N-size (animal numbers, cells counted, areas covered) in each of the imaging experiments throughout the study. Unless the authors included a report/table somewhere, I found such information generally missing from the methods/figure legends.
RESPONSE: As suggested, we have now added information about the N size (number of animals, number of cells counted, area covered) for each imaging experiment throughout the study, in addition to the Source Data file that we will submit.

AUTHORS' RESPONSES

Reviewer #1 (Remarks to the Author):

The authors propose that endothelial progenitor cells exist in the optic nerve that act to support retinal endothelial renewal. The authors use a variety of approaches to support this assertion including quantification followed by single cell sequencing of CD34 positive CD45 negative retinal and retinal plus optic nerve cells and lineage tracing to provide evidence for a population of progenitor cells that migrate from the optic nerve to the retina. These are studied further after damage such at oxygen induced retinopathy. The data is of high quality and the authors have responded to my previous critiques well. However, there remain two significant issues.

RESPONSE: We appreciate the reviewer's positive evaluation of our work. As suggested, we have revised the manuscript in accordance with the reviewer's comments below.

First, the authors do not have any experiments demonstrating that the CD157+ CD201+ optic nerve populations serves as the source of cells filling the retinal vasculature (Fig 5). Softening the conclusion to include the possibility that these cells originate from a separate source (potentially bone marrow) is needed.

RESPONSE: We thank the reviewer for this important point. We agree that our current data do not by themselves exclude contributions from other sources such as bone marrow–derived cells. In response, we have revised the Conclusions and Discussion to soften the conclusion. Specifically, we now state that optic-nerve–resident progenitors are enriched and substantially contribute to endothelial renewal and repair, without implying exclusivity adding "by progenitors from enriched in" in Page 14, Line 234 and "While our data support a predominant contribution from optic-nerve–enriched progenitors, additional inputs cannot be excluded; for example, bone-marrow–derived cells may act in parallel under inflammatory or ischemic conditions. Future work should quantify the relative input of the optic nerve versus other sources." In Line 248. We believe these changes accurately reflect the scope of this study while maintaining the main biological message supported by our datasets.

Second, figure 6 remains perplexing and the authors rebuttal does not clear the question. The experiment essentially shows that targeting Procr (CD201) cells with diptheria toxin does not reduce neovascularization in the OIR model. This suggests this stem cell population is not part of the retinal angiogenesis in OIR. This needs to be clearly stated. Importantly, why

OIR induced vessel obliteration increases in the Procr targeted deletion remains unclear. This data and the data in suppl fig 5 with ATF gene deletion under Procr Cre is indeed fascinating but there is no explanation for why vasoobliteration increases.

One possibility is that basally in both transgenic models there is reduced blood vessels and thus the OIR model leads to increased area of vaso-obliteration. But there remains a need for some explanation.

RESPONSE: We appreciate the reviewer's request for a clearer statement and mechanistic context. We now explicitly state in the Discussion that ablation of the Procr (CD201)$^+$ lineage does not reduce neovascular tufts in OIR—new Fig. 5b and Supplementary Fig. S3c show relatively few tdTomato$^+$ cells within neovascular tufts compared with large veins at P17—indicating that this population is dispensable for pathological tuft formation. Moreover, pan-endothelial ATF3 deletion enlarges the avascular area and delays vascular repair without decreasing neovascular tufts in OIR (Ueda et al., iScience 2024, 28(1):111516; doi:10.1016/j.isci.2024.111516; PMID: 39790557). This concordance supports a model in which Procr-lineage/ATF-programmed endothelial resilience is important for survival/revascularization of the retinal capillary bed under hypoxic stress, thereby explaining why vaso-obliteration increases even when tuft area does not decline. We added those in Page 14, Line 237.

Regarding the possibility that both transgenic models might have fewer baseline vessels, in our Procr-DTR experiments, tamoxifen was administered from P12, i.e., after the hyperoxic phase and at the start of the hypoxic revascularization; thus there is no evidence of reduced baseline vascular density prior to OIR. Consistently, in Ueda et al. (2024), tamoxifen at P7 did not alter vascular density at P12, which does not support fewer baseline vascular density as the cause of the increased avascular area.

Reviewer #2 (Remarks to the Author):

The authors have sufficiently addressed my concerns and have improved the manuscript's quality and clarity substantially. The most interesting phenomena in my eyes (reverse migration) is now demonstrated more convincingly. A comment for future extensions of this study, the injury work uses OIR (a developmental model); adult injury models would be a interesting to see in followup work.

RESPONSE: Thank you for the positive assessment and for noting that reverse migration is now more convincing. We appreciate the suggestion on adult injury models and have noted this as a key direction for future work.

Reviewer #3 (Remarks to the Author):

The revision was thorough and fully addressed my questions. It is a great study and carries enough novelty - should be published with Nature Comm timely and highly suitable for broad audience in neurobiology, vascular biology and retina biology studies.

RESPONSE: Thank you for the encouraging evaluation. We're grateful that you find the study novel and well-suited for Nature Communications and a broad audience.