Embryonic vascular establishment requires protein C receptor-expressing endothelial progenitors

Qing Cissy Yu, Lanyue Bai, Yingying Chen, Yujie Chen, Gangdun Peng, Daisong Wang, Guowei Yang, Guizhong Cui, Naihe Jing and Yi Arial Zeng
DOI: 10.1242/dev.200419

Editor: Liz Robertson

Review timeline
Original submission: 30 November 2021
Editorial decision: 22 December 2021
First revision received: 7 April 2022
Accepted: 5 May 2022

Original submission

First decision letter

MS ID#: DEVELOP/2021/200419

MS TITLE: Embryonic vascular formation requires Protein C receptor-expressing endothelial progenitors

AUTHORS: Qing Cissy Yu, Lanyue Bai, Yingying Chen, Yujie Chen, Daisong Wang, Guowei Yang, and Yi Arial Zeng

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

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

Reviewer 1

Advance summary and potential significance to field

The manuscript by Yu et al. presents results from experiments examining the developmental and lineage specific expression of Procr. The authors use a Procr-LacZ reporter mouse for initial screening of the embryonic expression pattern and then go on to do genetic lineage tracing of Procr expression in mice with a Procr-Cre and Procr-CreERT crossed with Rosa reporter mice. They show that contrary to previous studies Procr is expressed as early as E7/7.5 in the developing embryo. Examination of published scRNAseq data on whole E7.5 embryos shows Procr expression enrichment in the hematoendothelial precursor cluster.
Cell tracing experiments confirm that the Procr reporter marked cells contribute to the endothelial lineage of the embryo proper, as well as the yolk sac and placenta. Additional experiments performed by the authors involve scRNA sequencing of CD31+ cells from embryos at several developmental stages and demonstrate by pseudotime analysis with the published dataset that the Procr marked early endothelial precursors transit to both hematopoietic and endothelial lineages. Procr-DTA mediated ablation experiments suggest that Procr is necessary for vessel development and viability. The novel finding that Procr is expressed early in mouse development contributes to our understanding of the endothelial lineage during vasculogenesis and angiogenesis and the possible role Procr in vessel development as well as hematopoietic development.

Comments for the author

It is unclear in Figure S1B whether the Procr expression represents LacZ expression or actual Procr protein expression as detected by an anti-Procr antibody? These details should be provided as well as the gating strategy and controls. The data indicating that 4-5% of CD31+ cells are Procr+ are confusing and the percentages don't correspond to the what is observed in the whole mount Lacz stained or the immunostained embryos shown. A higher percentage is expected.
Have FACS experiments been performed to examine the overall in expression of the LacZ marker with the Procr protein in the LacZ transgenic mice? Similarly have FACS experiments been performed to examine reporter expression and Procr protein expression in the Proc-Cre/CreERT-Rosa reporter embryos? The overlap in expression as demonstrated by flow cytometer along with other markers would provide a more detailed understanding of the timing of surface protein expression and possible function.
The Procr-DTA ablation approach is questionable. While the transgenic embryos upon visual examination suffer from fragmentation and leakage of vessels, some CD31+ cells are being generated. What is the efficiency of recombination with the Procr Cre? Was FACS performed and were the remaining CD31+ cells tested for recombination? A detailed examination of the embryos using a conditional approach to knockout Procr in early embryonic mesoderm or endothelial precursors would provide more information.
The M and M refers to the Cdh5-Cre mouse strain but there is no data in manuscript that uses these mice.
What is IB4?
The number of embryos used/examined and the number of experiments performed is not indicated.
For the DTA experiments, was a time course for lethality performed?
The manuscript text would benefit from editing for language and grammar.

Reviewer 2

Advance summary and potential significance to field

This is a thorough and well-conducted study that reports the expression and role of Procr at the onset of vascular development. This study analyses the expression pattern and importance of Procr using a series of knocking conditional knockout, and lineages tracing. The data presented establish that Procr is expressed very early during development, that Procr-expressing cells contribute substantially to the vasculature of the developing embryo, and that Procr-expressing cells give rise to hematopoietic cells.

Comments for the author

Please find below a list of comments on the manuscript:
The manuscript would benefit from careful re-reading and English correction.
In figure 1B, the blood islands are not really formed at this stage, the labeling is somewhat misleading.
Figure 1C, 1D-D': the blue staining is extremely difficult to see.
Figure S1C: please show individual panels
Figure 2B: tdTomato staining in the primitive streak. What are the indications that these cells are within the primitive streak? This is not obvious from the data presented.
Figure S2B-D is missing.
Lineage tracing (Fig 2J-L): it is unlikely that tamoxifen injection at E8.5 is early enough to mark early endothelial progenitors. The conclusion from this set of experiments: "These lineage-tracing findings suggest that Procr labels vessel initiating endothelial progenitors that generate embryonic vasculature of the embryo proper." is not fully supported by the experimental setting.
Page 7: "We investigated the properties of Procr+ cells at early vasculature formation using single-cell sequencing (scRNA-seq) analysis." Strictly speaking, scRNA-seq cannot really reveal the "properties" of cells.
Page 7: CD45+ (primitive hematopoietic): CD45 expression cannot distinguish primitive from definitive hematopoiesis
Page 7: "endothelial and hematopoietic progenitors are known to share many molecular features" This is incorrect, especially this is untrue for CD45+ cells. It is rather the endothelial progenitors (hemogenic endothelium) of blood cells that do share many molecular features with endothelium.
In the data presented in Figures 3B and C, the cells seem to cluster more according to their stages than according to their identities, this is likely due to "batch effects". This set of data is not very credible. Endothelial cell clusters should be much closer to each other.
Page 8: What are the targets of HOXB5?

First revision

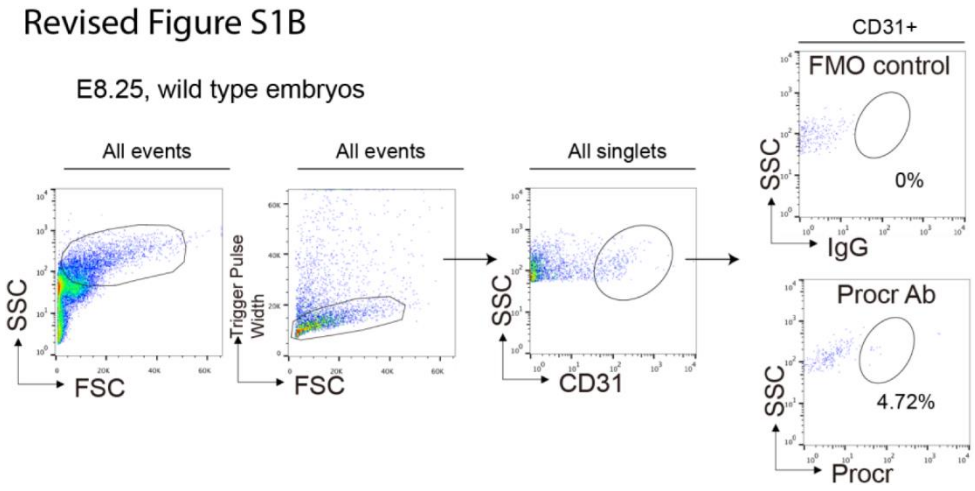
Author response to reviewers' comments

We thank all reviewers for their helpful comments and suggestions, which had provided invaluable guidance for our resubmission. We have revised the manuscript and figures accordingly.

Reviewer 1 Advance Summary and Potential Significance to Field:
The manuscript by Yu et al. presents results from experiments examining the developmental and lineage specific expression of Procr. The authors use a Procr-LacZ reporter mouse for initial screening of the embryonic expression pattern and then go on to do genetic lineage tracing of Procr expression in mice with a Procr-Cre and Procr-CreERT crossed with Rosa reporter mice. They show that contrary to previous studies Procr is expressed as early as E7/7.5 in the developing embryo. Examination of published scRNAseq data on whole E7.5 embryos shows Procr expression enrichment in the hematoendothelial precursor cluster. Cell tracing experiments confirm that the Procr reporter marked cells contribute to the endothelial lineage of the embryo proper, as well as the yolk sac and placenta. Additional experiments performed by the authors involve scRNA sequencing of CD31+ cells from embryos at several developmental stages and demonstrate by pseudotime analysis with the published dataset that the Procr marked early endothelial precursors transit to both hematopoietic and endothelial lineages. Procr-DTA mediated ablation experiments suggest that Procr is necessary for vessel development and viability. The novel finding that Procr is expressed early in mouse development contributes to our understanding of the endothelial lineage during vasculogenesis and angiogenesis and the possible role Procr in vessel development as well as hematopoietic development.

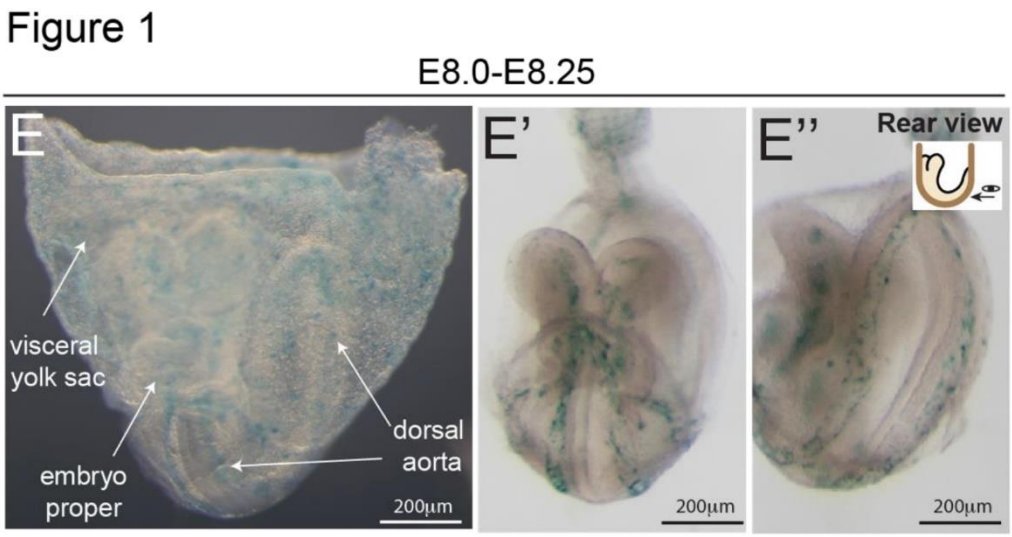
Reviewer 1 Comments for the Author:
1. It is unclear in Figure S1B whether the Procr expression represents LacZ expression or actual Procr protein expression as detected by an anti-Procr antibody? These details should be provided as well as the gating strategy and controls.

We apologize for the confusion. In Figure S1B, the Procr expression represents Procr protein expression. Per suggestion, we included gating strategies and controls in revised Figure S1B. We FACS-analysed wild-type E8.25 embryo endothelium using PECAM (CD31) and Procr antibodies.



2. The data indicating that 4-5% of CD31+ cells are Procr+ are confusing and the percentages don't correspond to the what is observed in the whole mount Lacz stained or the immunostained embryos shown. A higher percentage is expected.

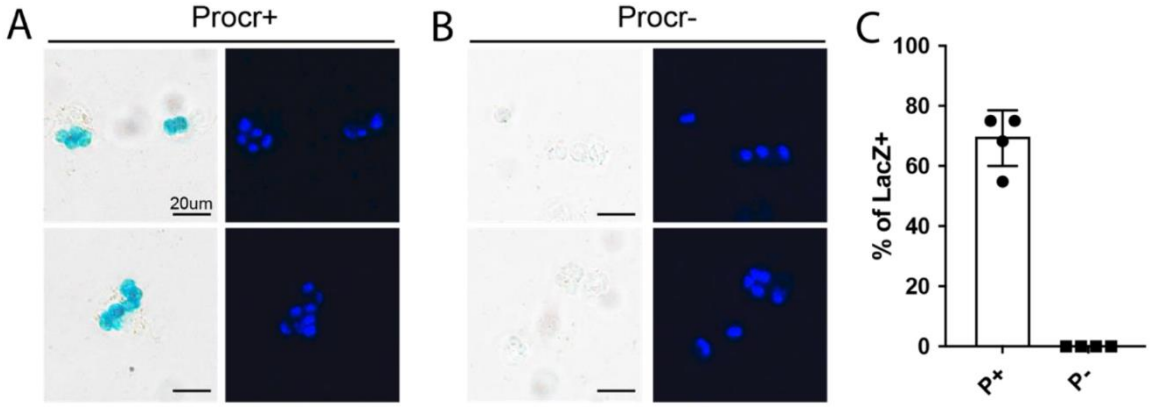
We understand the reviewer's concern. Figure 1E shows LacZ+ cells in both the yolk sac and embryo proper, therefore, it might appear more frequent than 4-5%. To better visualize the embryo proper, we removed the yolk sac and performed X-gal staining (Panel E' and E"), which clearly illustrate the localization and proportion of LacZ+ cells in the embryo proper. We have added these data into the revised Figure 1. To quantify the proportion of Procr + cells, we rely on FACS analysis, combining endothelial markers (CD31) and Procr, which indicates that Procr+ CD31+ cells are 4-5% of total CD31+ cells.



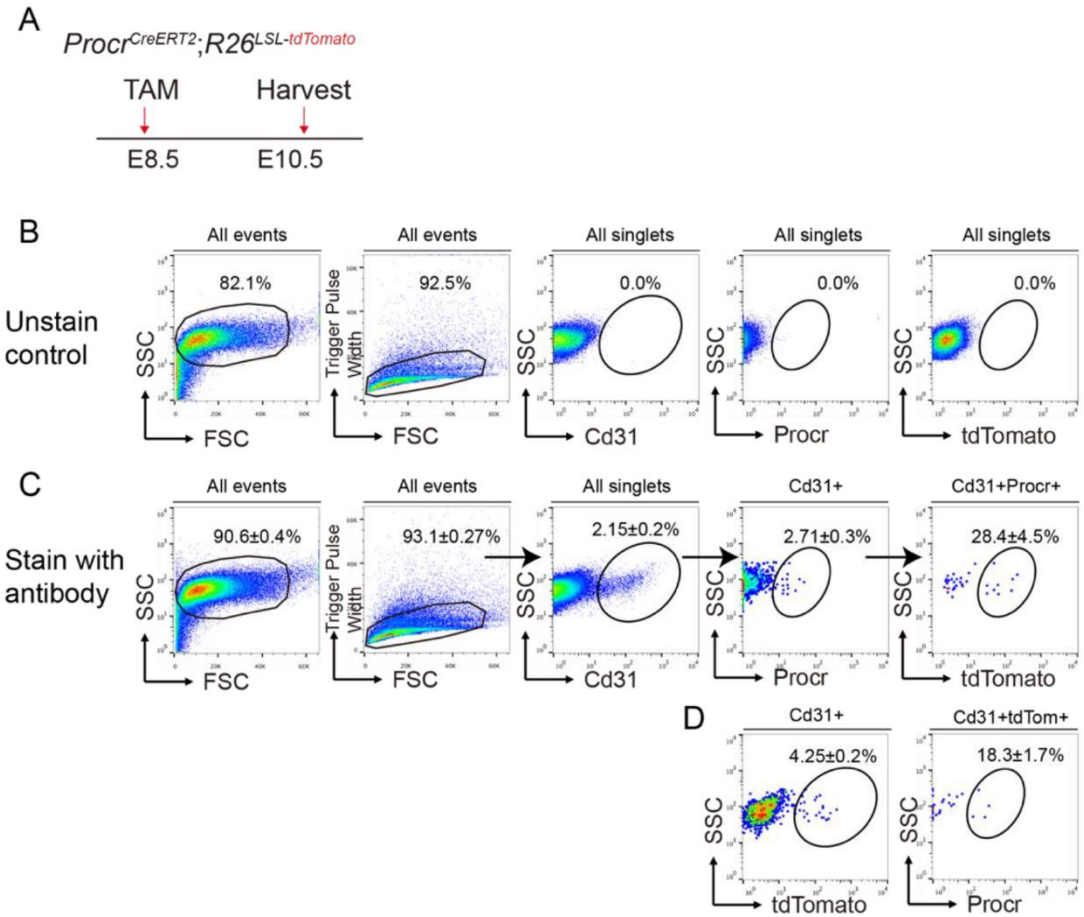
3. Have FACS experiments been performed to examine the overall in expression of the LacZ marker with the Procr protein in the LacZ transgenic mice? Similarly have FACS experiments been performed to examine reporter expression and Procr protein expression in the Proc-Cre/CreERT-Rosa reporter embryos? The overlap in expression as demonstrated by flow cytometer along with other markers would provide a more detailed understanding of the timing of surface protein expression and possible function.

Following the suggestion, we FACS sorted Procr+ and Procr- endothelial cells from E9.5 *Procr*$^{mGFP\text{-}2A\text{-}LacZ}$ embryos, followed by cytospin and X-gal staining. As shown in panel A, Procr+ cells were

positive for LacZ staining (about 70%, panel C), whereas no LacZ staining was observed in sorted Procr- cells (panel B). n>5 views and >50 cells in each group. The validation of the *Procr*$^{mGFP\text{-}2A\text{-}LacZ}$ model has been published in our previous paper (Wang et al, Cell 2020), thus we do not include these data in the current manuscript.



As to the *Procr*^{CreERT2}; *R26*$^{LSL\text{-}tdTomato}$ model, Tamoxifen was administered to the pregnant female at timed E8.5, and the embryos were harvested at E10.5 (panel A). After genotyping, *Procr*^{CreERT2}; *R26*$^{LSL\text{-}tdTomato}$ embryos were individually digested and analysed by FACS. As shown below, 1) 28.4±4.5% of CD31+ Procr+ cells were tdTomato+ (panel C), reflecting the *Procr*^{CreERT2} labelling efficiency; 2) 18.3±1.4% CD31+ tdTomato+ cells were Procr+ (panel D), suggesting that during the 2-day period (E8.5 to E10.5), 81.7% of Procr- CD31+ tdTomato+ cells were generated from 18.3% of Procr+ CD31+ tdTomato+ cells (about 4-folds). These data have been included in revised Figure S3C-F



5. The Procr-DTA ablation approach is questionable. While the transgenic embryos upon visual examination suffer from fragmentation and leakage of vessels, some CD31+ cells are being generated. What is the efficiency of recombination with the Procr Cre? Was FACS performed and were the remaining CD31+ cells tested for recombination?

Respectfully, we would like to point out that the Procr-DTA model is driven by inducible *Procr*^{CreERT2}, not Procr-Cre. As data presented in the last question, the labelling efficiency of *Procr*^{CreERT2} after a single Tamoxifen injection is 28.4±4.5%, analysed by FACS. Therefore, the remaining CD31+ cells likely resulted from non-ablated Procr+ progenitors.

6. A detailed examination of the embryos using a conditional approach to knockout Procr in early embryonic mesoderm or endothelial precursors would provide more information.

We appreciate the reviewer's suggestion. We agree with the reviewer that knockout Procr will provide more information on the function of Procr. These experiments require mesoderm or endothelial precursors specific CreER mouse models and time-consuming genetic crosses, which we won't be able to finish during the revision period. I hope the reviewer agrees with us that the current manuscript is focused on the biological significance of Procr+ cells, and the DTA experiments have made the point.

7. The M and M refers to the Cdh5-Cre mouse strain but there is no data in manuscript that uses these mice.

We apologize for the mistake; we have corrected this error in the revised manuscript Material and Method section (Page 12).

8. What is IB4?

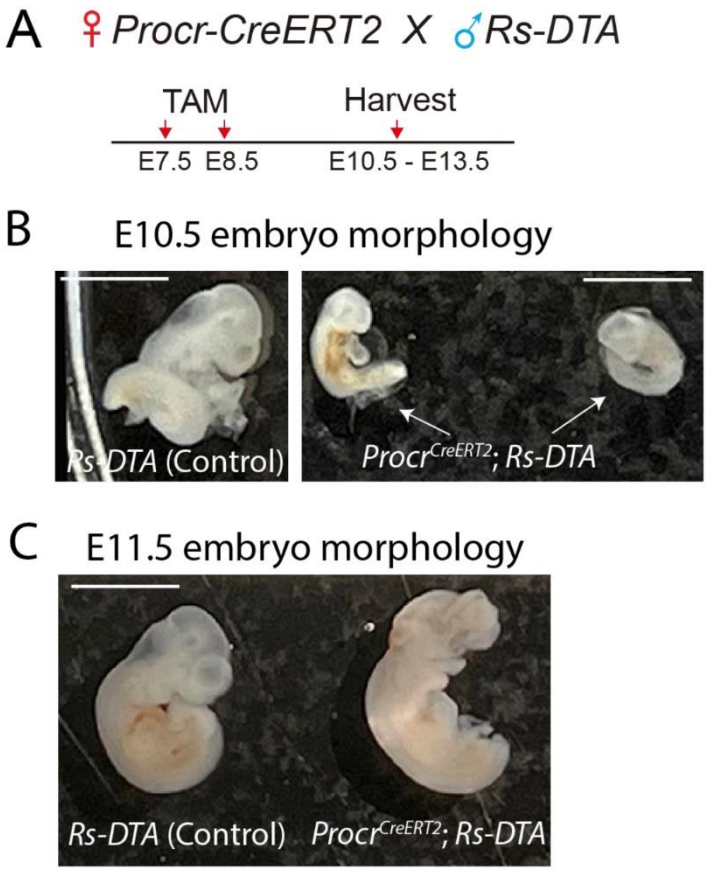
We apologize for not making it clear. IB4 refers to Isolectin B4, a common marker for the endothelial vessels. We have added the description of IB4 in the revised manuscript (Page 22).

9. The number of embryos used/examined and the number of experiments performed is not indicated.

We apologize for this mistake. We have added the information (the number of pregnant female mice or embryos used) into the revised figure legend.

10. For the DTA experiments, was a time course for lethality performed?

As suggested, we performed timed ablation of Procr+ cells by administering tamoxifen to pregnant mice at E7.5 and E8.5, and collected embryos at different time courses (panel A). We found that at E10.5, *Procr*^{CreERT2};*R26*^{DTA} embryos appeared to be drastically smaller in size compared to control (panel B). By E11.5, the *Procr*^{CreERT2};*R26*^{DTA} embryos turned opaque and displayed deformity at multiple sites, including head, limb development, as well as a lack of active circulation (panel C). These features are similar to those observed in Figure 4. These results suggest that the lethality time window is at E10.5 – E11.5. We have included these data in revised Figure S6.



11.The manuscript text would benefit from editing for language and grammar.

We thank the reviewer for this suggestion. We have carefully edited our revised manuscript.

Reviewer 2 Advance Summary and Potential Significance to Field:
This is a thorough and well-conducted study that reports the expression and role of Procr at the onset of vascular development. This study analyses the expression pattern and importance of Procr using a series of knocking, conditional knockout, and lineages tracing. The data presented establish that Procr is expressed very early during development, that Procr-expressing cells contribute substantially to the vasculature of the developing embryo, and that Procr-expressing cells give rise to hematopoietic cells.

Reviewer 2 Comments for the Author:
Please find below a list of comments on the manuscript:
1. The manuscript would benefit from careful re-reading and English correction.

We thank the reviewer for this suggestion. We have carefully edited our revised manuscript.

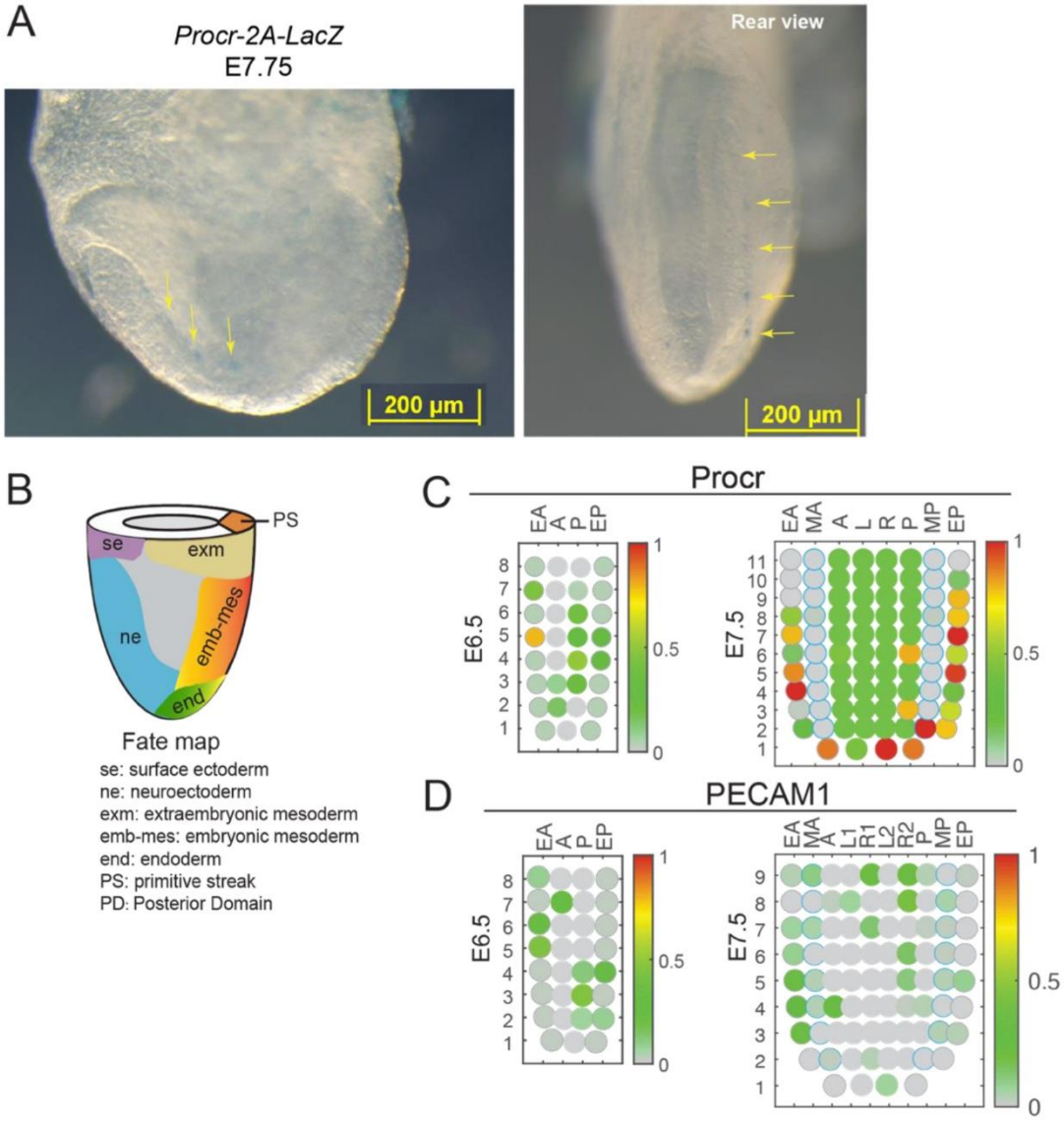
2. In figure 1B, the blood islands are not really formed at this stage, the labeling is somewhat misleading.

We thank the reviewer for pointing this out. We carefully checked the original publication on blood islands formation (Ferkowicz and Yoder, 2005) and realize that it was indeed mislabelled in our manuscript. We have removed this labelling to avoid confusion.

3. Figure 1C, 1D-D': the blue staining is extremely difficult to see.

We apologize for the image quality. Due to the restraint in file size, we could only upload the resized, low-resolution images in the initial submission. The X-gal staining in the original image is clearer (panel A). We also added arrows in Figure 1D-D' to indicate the staining.

We apologize for the weak signal in Figure 1C. Weak signals are especially difficult to capture under the microscopic camera due to light deflection. To further validate Procr spatial expression, we included a corn-plot generated using online spatial transcriptome analysis (GEO-seq) showing the mRNA expression of Procr and CD31 (CD31 as a comparison) at E6.5 and E7.5 (panel B-D). In GEO-seq, cells at a defined anatomical position of E6.5 and E7.5 embryos were captured by laser microdissection, followed by transcriptome analyses (Peng et al, Dev Cell, 2016). With spatial gene expression displayed in a 2D formation in the corn plot, we found that the very first Procr-expressing cells within embryo proper emerge at the migrating posterior mesoderm, prior to the onset of mature endothelial lineage marker PECAM1 (CD31) expression. We have included this information into the revised manuscript (Figure S2B-D).



4. Figure S1C: please show individual panels

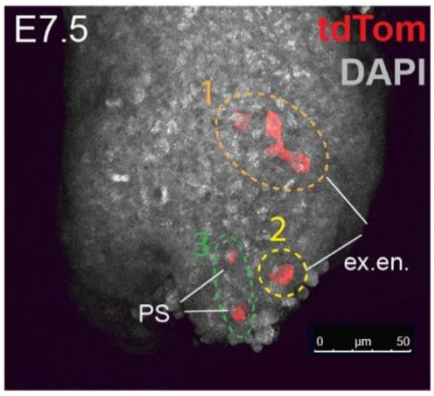
Following the suggestion, we have corrected it in the revised manuscript.

5. Figure 2B: tdTomato staining in the primitive streak. What are the indications that these cells are within the primitive streak? This is not obvious from the data presented.

We apologize for not making it clear. Results from both LacZ reporter model and GEO-seq analysis (Figure 1C, Figure S2B-D?) indicate that at E7.5, Procr expression is detected at both extraembryonic tissue and migrating primitive streak. In Figure 2B, we reason that it is possible that there is leftover extra-embryonic tissue attached to the embryo. At this stage (E7.5), cells of

the extra embryonic tissues appear to be flatter and cobble stone-like, while cells of the embryonic proper are smaller and round in appearance due to fast migration occurring. Therefore, based on cell location and morphology, we think cells circled in orange and yellow are extra embryonic endoderm while cells circled in green are from primitive streak.

Figure 2B



6. Figure S2B-D is missing.

We apologize for the mistake; we have corrected this error in revised manuscript.

7. Lineage tracing (Fig 2J-L): it is unlikely that tamoxifen injection at E8.5 is early enough to mark early endothelial progenitors. The conclusion from this set of experiments: "These lineage-tracing findings suggest that Procr labels vessel initiating endothelial progenitors that generate embryonic vasculature of the embryo proper." is not fully supported by the experimental setting.

We thank the reviewer for pointing it out. We have rephrased it to “These lineage-tracing findings suggest that Procr labels endothelial progenitors that generate embryonic vasculature of the embryo proper” in the revised manuscript (Page 6).

8. Page 7: "We investigated the properties of Procr+ cells at early vasculature formation using single-cell sequencing (scRNA-seq) analysis." Strictly speaking, scRNA-seq cannot really reveal the "properties" of cells.

We thank the reviewer for pointing it out. We have rephrased it to “we investigated the transcriptome characteristics of Procr+ cells at early vasculature formation using single-cell sequencing (scRNA-seq) analysis” in the revised manuscript (Page 7).

9. Page 7: CD45+ (primitive hematopoietic): CD45 expression cannot distinguish primitive from definitive hematopoiesis.

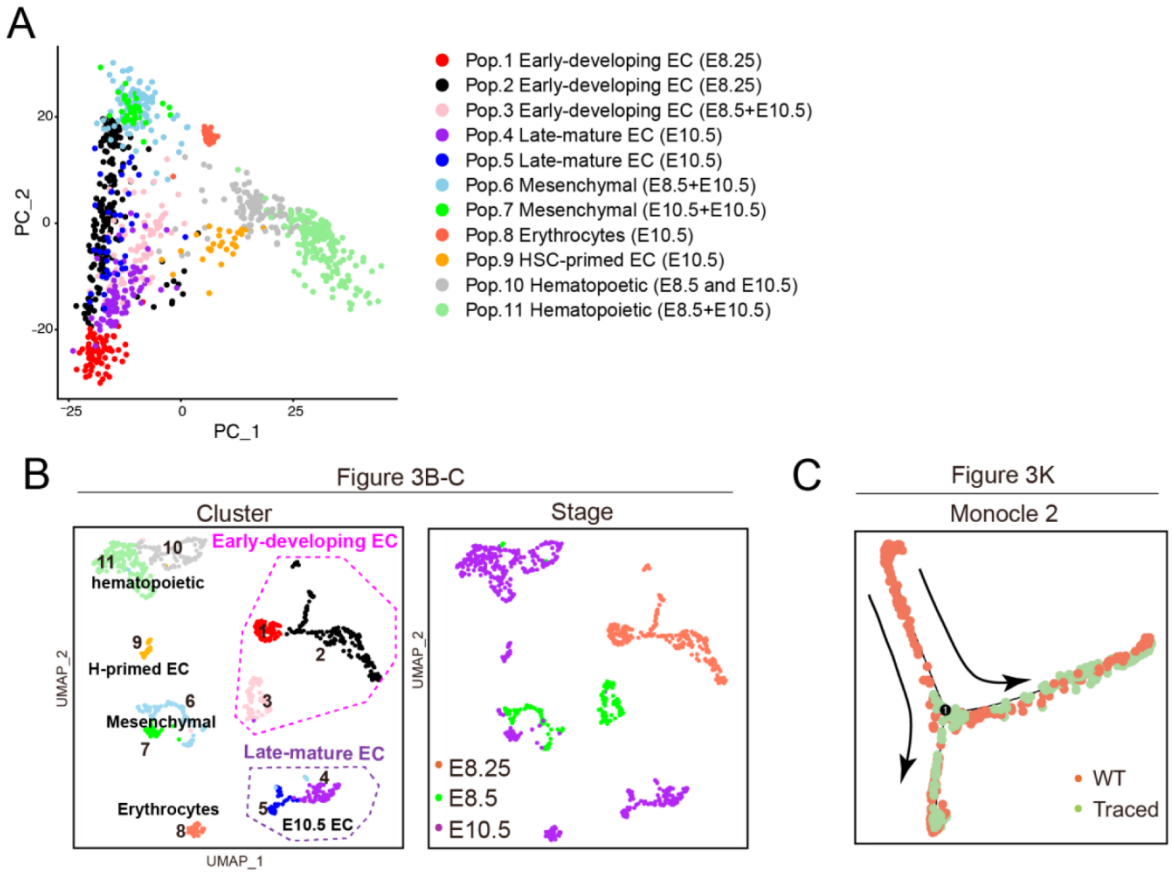
We appreciate the reviewer for pointing it out. We have rephrased “CD45+ (primitive hematopoietic) cells” to “CD45+ hematopoietic cells” in the revised manuscript.

10. Page 7: "endothelial and hematopoietic progenitors are known to share many molecular features" This is incorrect, especially this is untrue for CD45+ cells. It is rather the endothelial progenitors (hemogenic endothelium) of blood cells that do share many molecular features with endothelium.

We thank the reviewer for pointing this out. We have rephrased it to “blood-generating hemogenic endothelium and endothelial progenitors are known to share many molecular features (Zhou et al, Nature, 2016)”. (Page 8)

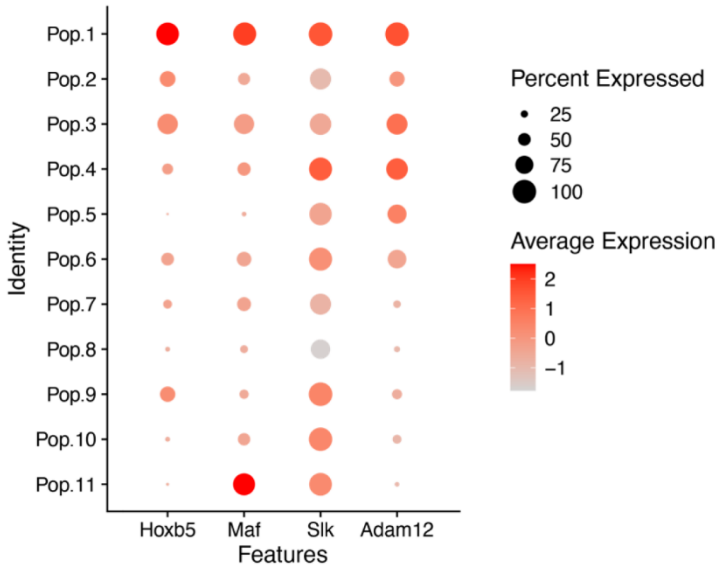
11. In the data presented in Figures 3B and C, the cells seem to cluster more according to their stages than according to their identities, this is likely due to "batch effects". This set of data is not very credible. Endothelial cell clusters should be much closer to each other.

We understand the reviewer's concern and apologize for not making it clearer. We think it is not "batch effects", based on the following analyses. 1) We performed PCA analysis with all cells (E8.25, E8.5 and E10.5), and found that population 1-5 (from 3 different batches, E8.25, E8.5 and E10.5) cluster closely, distant from population 8-11 (from 2 different batches, E8.5 and E10.5) (panel A, B). 2) In Figure 3K (shown as panel C), Monocle 2 analysis using WT cells and lineage-traced cells are two different sequencing batches. Yet, cells with similar identify cluster together. Therefore, we conclude that the separation observed in Figure 3B is due to their transcriptomic characteristics rather than due to batch variation. We have included the new data (panel A) in revised Figure S5B.



12. Page 8: What are the targets of HOXB5?

We used SCENIC workflow (SCENIC cisTarget) to predict the potential targets of Hoxb5 (Van de Sande, et al, Nat Protoco, 2020), then use RcisTarget to filter out indirect targets. *Hoxb5*, *Maf*, *Slk*, *Adam12* were predicted to be the potential target genes of Hoxb5 (see below). Their enriched expression in Procr+ progenitor cluster (Pop. 1) has been included in the revised Figure S5I.



Accordingly, we revised the text into "Given the simultaneous high activities of *Hoxb5* and its predicted target genes, including *Hoxb5*, *Maf*, *Slk*, and *Adam12* (Fig S6I), it's likely a core regulon in the molecular orchestration of endothelial specification." (Page 9)

To Editor:

We have also added Procr-GFP-Cre construction information into supplementary files (Supplementary Figure S3).

Second decision letter

MS ID#: DEVELOP/2021/200419

MS TITLE: Embryonic vascular establishment requires Protein C receptor-expressing endothelial progenitors

AUTHORS: Qing Cissy Yu, Lanyue Bai, Yingying Chen, Yujie Chen, Gangdun Peng, Daisong Wang, Guowei Yang, Guizhong Cui, Naihe Jing, and Yi Arial Zeng
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. Please note the two minor corrections required by the Reviewer - these can be altered at the proof stage.

Reviewer 1

Advance summary and potential significance to field

The novel finding that Procr is expressed early in mouse development contributes to our understanding of the endothelial lineage during vasculogenesis and angiogenesis and the possible role Procr in vessel development as well as hematopoietic development.

Comments for the author

The authors have carefully addressed all the issues that were indicated in my referee report and have included more details and figures. I have no further comments except the 2 minor points below:

1. In the Abstract line 41 – sentence should start with ‘Understanding’
2. Line 71 – tdTomato is misspelled.

Reviewer 2

Advance summary and potential significance to field

This study analyses the expression pattern and importance of Procr using a series of knocking, conditional knockout, and lineages tracing. The data presented establish that Procr is expressed very early during development, that Procr-expressing cells contribute substantially to the vasculature of the developing embryo, and that Procr-expressing cells give rise to hematopoietic cells.

Comments for the author

The authors have addressed all my comments.