nature portfolio

Peer Review File

Discovery of an embryonically derived bipotent population of endothelial-macrophage progenitor cells in postnatal aorta

Editorial Note: This manuscript has been previously reviewed at another journal. This document only contains reviewer comments and rebuttal letters for versions considered at *Nature Communications*.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I think that the authors have answered adequately the reviewer concerns and have modified the manuscript in an acceptable way. They have also toned down their claims.

Reviewer #4 (Remarks to the Author):

I agree with Reviewers 1 and 3 on their very insightful and detailed comments. To me it is also worrisome that authors overlooked on the source of Flt3-Cre mice taking into account the importance on their conclusions regarding the embryonic cell of origin of this endothelial-macrophage progenitors and only after Reviewers' comments they started thinking on the specificity of this mouse strain.

Still, I think that Authors have addressed Reviewers' comments correctly in their revised version of the manuscript.

Regarding the embryonic cell of origin of adult CFU-M activity, it is important that authors have now changed the way they phrased it as Flt3 independent and not from definitive HSCs.

There are two main points in the manuscript:
1) Determining the cell of origin of those progenitors and
2) Demonstrating that they are actually bipotent (i.e. endothelial and macrophage potential).

I think it is still possible that phenotypically Flt3+ (CD135+) cells which don't get marked as GFP+ in the Flt3-Cre TmGFP reporter mouse could be the source of these progenitors.
Hence, It would be very helpful if authors could show the actual efficiency and specificity of GFP+ labelling of phenotypic Flt3+ cells in the Flt3-Cre model at E10.5.
Additionally, if they could sort CD135+ and CD135- cells from E10.5 embryos and perform CFU-M. Considering the concerns raised with the Flt3-Cre model this would support their findings.

NCOMMS-23-61362-T "Discovery of an embryonically derived bipotent population of endothelial-macrophage progenitor cells in postnatal aorta"

We thank the reviewers for reviewing our manuscript and for providing constructive comments. We have revised the manuscript to address the raised comments. Please find below our point-by-point responses to the comments. We have indicated below the changes made to the text in the manuscript and have also uploaded a version with changes highlighted in blue text. The raw data related to the new data presented in the revised manuscript has been included in the Source Data file.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

I think that the authors have answered adequately the reviewer concerns and have modified the manuscript in an acceptable way. They have also toned down their claims.

Response:
We thank the reviewer for their favourable remarks on our submission.

Reviewer #4 (Remarks to the Author):

I agree with Reviewers 1 and 3 on their very insightful and detailed comments. To me it is also worrisome that authors overlooked on the source of Flt3-Cre mice taking into account the importance on their conclusions regarding the embryonic cell of origin of this endothelial-macrophage progenitors and only after Reviewers' comments they started thinking on the specificity of this mouse strain.

Still, I think that Authors have addressed Reviewers' comments correctly in their revised version of the manuscript.

Response:
We thank the reviewer for accepting the changes made in the revised version of our manuscript transferred to *Nature Communications*.

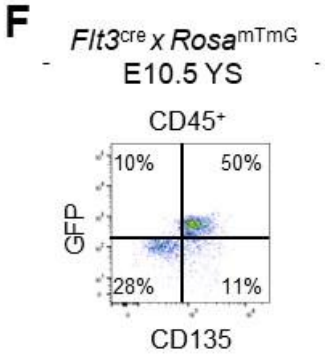
Regarding the embryonic cell of origin of adult CFU-M activity, it is important that authors have now changed the way they phrased it as Flt3 independent and not from definitive HSCs.

There are two main points in the manuscript:
1) Determining the cell of origin of those progenitors and
2) Demonstrating that they are actually bipotent (i.e. endothelial and macrophage potential).

I think it is still possible that phenotypically Flt3+ (CD135+) cells which don't get marked as GFP+ in the Flt3-Cre TmGFP reporter mouse could be the source of these progenitors.
Hence, It would be very helpful if authors could show the actual efficiency and specificity of GFP+ labeling of phenotypic Flt3+ cells in the Flt3-Cre model at E10.5.

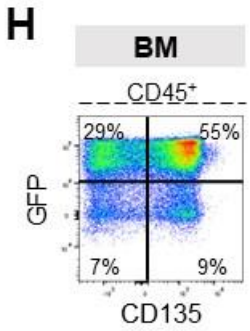
Response:
As suggested by the reviewer, we have performed flow cytometry on yolk sac (YS) cells from E10.5 *Flt3*^{Cre} *x Rosa*^{mTmG} embryos. This shows that 81.4±3.9% of CD45^{+}Flt3/CD135^{+} cells express GFP (N=5), indicating high labeling efficiency. Specificity of labeling is also high as 83.6±6.0% of CD45+GFP+ cells express CD135. We have included the labeling efficiency data from this experiment in **Extended Data Fig. 5F** and described it in the 2nd paragraph on page 11 in the revised manuscript.

Extended Data Figure 5F



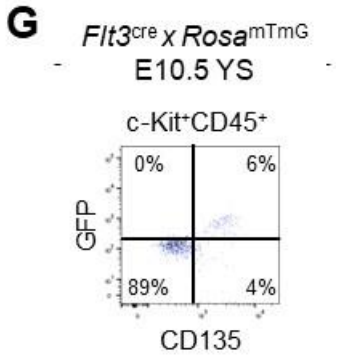
Additionally, we have further analysed the flow cytometry data from bone marrow (BM) of adult *Flt3*^{Cre} *x Rosa*^{mTmG} mice, which also shows high labeling efficiency with GFP expression in 85.9±2.7% of CD45^{+}Flt3/CD135^{+} cells. Although 65.7±1.7% of CD45^{+}GFP^{+} cells expressed Flt3/CD135, this is not surprising as GFP expression in Flt3/CD135^{-} cells is consistent with a history of Flt3/CD135 expression from ancestral CD135^{+} progenitors, rather than lower labeling specificity. We have also included the labeling efficiency data from this analysis in the manuscript in **Extended Data Fig. 4H** and described it in the 2nd paragraph on page 9.

Extended Data Figure 4H



Moreover, using the flow cytometry data from E10.5 YS from *Flt3*^{Cre} *x Rosa*^{mTmG} embryos, we examined the degree to which CFU-M producing c-Kit^{+}CD45^{+} progenitors in YS arise from a Flt3^{+} source. Most of these cells (88.9±3.5%) were Flt3/CD135^{-} and GFP^{-} and only a small proportion (6.5±3.3%) were Flt3/CD135^{+} and GFP^{+}. We have presented this data in **Extended Data Fig. 5G** and described this result in the 2nd paragraph on page 11 in the manuscript.

Extended Data Figure 5G

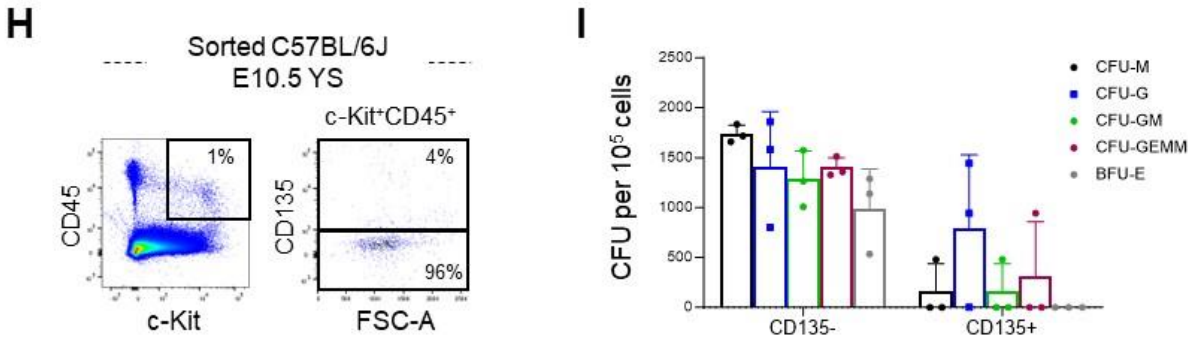


Collectively, these data support our study's finding that EndoMac progenitors largely arise independent of Flt3-mediated hematopoiesis. However, considering that the efficiency of Flt3-Cre labeling in the *Flt3*^{Cre} *x Rosa*^{mTmG} model used was not complete, we cannot rule out the possibility that a minority of progenitors arise from a Flt3^{+} source, or directly from Flt3^{-} BM LT-HSCs. We have acknowledged this in the Discussion in the 2nd paragraph on page 23 in the revised manuscript.

Additionally, if they could sort CD135+ and CD135- cells from E10.5 embryos and perform CFU-M. Considering the concerns raised with the Flt3-Cre model this would support their findings.

Response:
We thank the reviewer for suggesting this experiment. We have performed the experiment using YS cells from E10.5 C57BL/6J embryos. The CFU assay of sorted c-Kit^{+}CD45^{+}CD135^{-} and c-Kit^{+}CD45^{+}CD135^{+} cells revealed that CFU-M colonies are predominantly formed by the former CD135^{-} cell population, which further supports a Flt3-independent origin of EndoMac progenitors. We have presented the data from this experiment in **Extended Data Fig. 5H** and **I** and described the results in the 2nd paragraph on page 11 in the manuscript.

Extended Data Figure 5H,I



REVIEWERS' COMMENTS

Reviewer #4 (Remarks to the Author):

In this revised version authors have successfully addressed my concerns.