Osteopontin attenuates aging-associated phenotypes of hematopoietic stem cells

Novella Guidi, Mehmet Sacma, Ludger Ständker, Karin Soller, Gina Marka, Karina Eiwen, Johannes Weiss, Frank Kirchhoff, Tanja Weil, Jose Cancelas, Maria Carolina Florian, and Hartmut Geiger

Corresponding author: Hartmut Geiger, University of Ulm

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 04 October 2016

Thank you for the submission of your manuscript (EMBOJ-2016-94969) to The EMBO Journal, and my apologies again for the unusual delay in the processing of this manuscript, which was caused by delayed feedback from the referees due to travel commitments. Your study has been sent to three referees, and we have received reports from all of them, which I copy below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of major concerns that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail, while referee #1 is overall more positive, referee #2 points out that some conclusions are not sufficiently well supported by the current data, and is concerned about some of the assays utilized (ref #2, pts. 2 to 6). In particular, this referee states the need for you to provide further characterization of link between niche factors and cell polarity in the aged phenotype (ref #2, pts. 2,3), and asks you to investigate aged HSC’s homing (ref #2, pts. 4). Referee #3 agrees in that some of the current data are overstated in his/her view (ref #3, pts. 4,6,8), and requests you to adjust the claims made. In addition, referees #2 and #3 list a number of technical issues and controls regarding the in vitro reconstitution and other points, that would need to be addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments.

Please contact me if you have any questions or need further input on the referee comments or if you want to discuss the revisional work.
Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

This report provides new insight into hematopoietic stem cell aging; an area to which they have previously made important contributions. Here, they report a stromal impact on the aging phenotype. It is in contrast to the prevailing wisdom from work by others that HSC aging is predominantly HSC-autonomous. However, the experiments are well conducted and the data are convincing; the conclusions are well tempered and the mechanistic information builds on prior publications that add credibility.

I have previously reviewed this manuscript and felt it was strong then and should be published. In the interim the authors have added some further definition about the processing of OPN that further strengthens the manuscript. Overall, I think this paper will add substantially to our thinking about the aging phenotype and provoke further investigation by others. It dovetails nicely with emerging work on stromal contributions to age related hematologic diseases and points to future directions of research.

If there are minor issues that could be improved there are only two: 1. the abundant expression of Rantes raises the possibility that the HSPC are not well retained in the marrow and it would be helpful to determine whether splenic HSPC are increased in age that may partially account for some of the overall changes. 2. citations about HSPC aging should be on the primary literature, not reviews.

Referee #2:

This article by Guidi et al, describe the age associated change of osteopontin (OPN) in the BM niche in association with the alternations of stem cell function of hematopoietic stem cells (HSCs). The article delicately describes OPN as one of the significantly decreased niche secreted molecules upon age and provides evidence that thrombin-cleaved OPN may rejuvenate HSCs. Functions of OPN are supported through analysis of OPN KO mice. Although the authors display an interesting study of the alterations in niche components during age, some aspects of analysis, especially those regarding stem cell polarity in relation to stem cell potential and assays reflecting 'youthful' stem cells seem rather vague and insufficient.

(1) Regarding the novel aspect of the findings of the manuscript. As the defective hematopoiesis phenotype of OPN KO mice have been reported, the concepts that the manuscript presents are not strikingly novel. Yet the authors do display novel findings in the role of OPN in aged mice and associate it with cell polarity which has not been previously described.

(2) The authors utilize cell polarity as a feature to monitor stem cell aging phenotype. Although loss of polarity is noted in Cdc42 KO mice in association with aging, it is questionable whether it is a specific feature of aged HSCs. Also, as the manuscript is based on analysis of the aged microenvironment and its effect on HSCs, a fundamental question arises whether cell polarity is altered with niche interaction. The authors should provide data on how cell polarity is affected in vivo. In conjunction, the authors should provide data using IHC in Fig 1, showing the distribution of HSCs in BMT recipients.

(3) Another parameter that the authors utilize to assess what they term 'youthful' stem cell quality is the lower number of HSCs engrafted. Although controversial, HSCs from aged mice display an increase in self-renewal due to a decrease in clonal diversity. Therefore, assessing the change in number of the engrafted cells may not sufficiently demonstrate the aged phenotype of HSCs. The authors should attempt to assay for symmetric and asymmetric division patterns. Also, they should display progenitor cell reconstitution (CLP, CMP, GMP etc) to show myeloid skewing. Gene expression assay of aged associated genes may also be helpful.
(4) As increased mobilization and less retention to the BM of HSCs is a characteristic of aged hematopoiesis, the authors should analyze whether HSC homing was affected in Y to O or Y to OPN KO BMT.

(5) The authors should show whether HSCs in OPN KO mice (not BMT) have aged phenotype.

(6) The authors should use HSC populations for BMT assays rather than whole BM cells.

(7) In Fig 2 the authors assess the niche cell content in old mice by using the bone region BM. They present that there was no difference in the MSC cell population. Yet MSCs are distributed through the BM thus the authors should provide data on the cell populations in the flushed (central) BM as well.

(8) In Fig 3, the authors show that OPN KO mice have an overall decrease in OBL numbers. Could the decline in OPN KO mice to harbor HSCs depend on a decrease of the number of OBL and not directly on OPN expression. The authors should attempt to rescue the phenotype with addition of OPN in vivo.

Referee #3:

The manuscript by Guidi et al focuses on the role of the niche as a mediator of hematopoietic stem cells aging, and suggests that the matrix glycoprotein osteopontin is a mediator of the effect of the aging microenvironment on HSCs. The authors describe a decline in osteopontin in the microenvironment of aged mice, which is not surprising and consistent with the loss of osteolineage populations that has previously been described. They find that the HSCs of young mice with global genetic osteopontin loss partially recapitulate the aging phenotype. The paper adds importantly to the aging HSC field, where only very few data support a role for the microenvironment. The authors importantly establish the importance of the microenvironment in inducing or reversing aging HSC characteristics. Notably, the author convincingly demonstrate that treatment of aged HSCs with the thrombin activated fragment of OPN rejuvenates certain aspects of aged HSCs. However, there are numerous major and minor limitations, both experimental and conceptual, including overinterpretation of some of their data, that diminish the enthusiasm of this reviewer:

Major issues:

1) Figure S1a: The authors should temper their claim that age does not differentially impact the osteoblastic lineage: for example, there appears to be a more drastic reduction of both CFU-Fs and CFU-OB in young compared to aged mice.

2) Page 5 and Fig S1b: CD45- should not be used as an indicator of non-hematopoietic stromal cells, since this population includes CD45- Ter119+ erythroid cell populations.

3) Page 6 and Figure 2F: This experiment appears underpowered to detect a difference in osteopontin expression in MSCs.

4) Figure 2G: These images are not convincing. The old OB appears to be in metaphase, showing the 2 prominent nucleoli vs the multiple nucleoli of the young OB. Is this a consistent finding or is it a common characteristic of aged OBs?

5) Figure 3C: the authors should highlight more clearly the fact that exposure of young HSCs to an OPN KO niche recapitulates only certain features of aged HSCs. For example, while the expansion in LT-HSCs is recapitulated, the myeloid shifting is not.

6) Figure 4: In general these data are important to demonstrate that the young microenvironment can improve the function of aged HSCs, however the role of OPN in this case is confusing with respect of the lineage allocation of the transplant(panel D).
7) Page 9: The authors incorrectly state that the young microenvironment increases T cells engraftment compared to Old into Old and Old into OPN KO.

8) Figure 4C. The Western blot is not convincing.

9) Figure 5E: The authors state that treatment of aged HSCs with thrombin activated OPN fragments functionally rejuvenates aged HSCs, however their total cell engraftment remains defective. They should tone down their claim.

10) Figure 5F: How do the authors explain that the activated OPN fragment could block aged HSC myeloid while this was not a characteristic of the OPN effect in all other experiments?

11) Figure S3C (and others). The authors use the term stroma inconsistently, do they mean endosteal cells, CD45- endosteal cells, or CD45- Ter119- endosteal cells. They should clarify in the figure legends or labels.

12) P4: myeloid skewing is attributed here to increased myeloid contribution and the presence of an aged thymus. However, others (see Flach et al) have previously demonstrated that there is significantly decreased lymphoid lineage contribution by sorted aged HSCs. This should be discussed by the authors and begs the discussion of the impact of OPN KO on lymphoid contribution on aged HSCs.

1st Revision - authors' response 08 December 2016

Point-to-point response to reviewer comments.

Referee #1:
This report provides new insight into hematopoietic stem cell aging; an area to which they have previously made important contributions. Here, they report a stromal impact on the aging phenotype. It is in contrast to the prevailing wisdom from work by others that HSC aging is predominantly HSC-autonomous. However, the experiments are well conducted and the data are convincing; the conclusions are well tempered and the mechanistic information builds on prior publications that add credibility.

I have previously reviewed this manuscript and felt it was strong then and should be published. In the interim the authors have added some further definition about the processing of OPN that further strengthens the manuscript. Overall, I think this paper will add substantially to our thinking about the aging phenotype and provoke further investigation by others. It dovetails nicely with emerging work on stromal contributions to age related hematologic diseases and points to future directions of research.

We are honored by the referee’s encouraging comments on our data and MS with respect to novelty and strength of our data.

If there are minor issues that could be improved there are only two: 1. the abundant expression of Rantes raises the possibility that the HSPC are not well retained in the marrow and it would be helpful to determine whether splenic HSPC are increased in age that may partially account for some of the overall changes.

Novel data demonstrate an increase in HSPCs in spleen in old mice compared to young. The number of HSPCs in old mice is increased not only in the bone marrow but also in the spleen, suggesting that the pro-inflammatory microenvironment contributes also to an augmented HSPCs mobilization out of the niche. These data are now shown in Figure EV1G, H.

2. Citations about HSPC aging should be on the primary literature, not reviews.

We have addressed the referee’s concern and modified our manuscript accordingly: citations on HSPC aging refer now to primary literature.
Referee #2:

This article by Guidi et al, describe the age associated change of osteopontin (OPN) in the BM niche in association with the alternations of stem cell function of hematopoietic stem cells (HSCs). The article delicately describes OPN as one of the significantly decreased niche secreted molecules upon age and provides evidence that thrombin-cleaved OPN may rejuvenate HSCs. Functions of OPN are supported through analysis of OPN KO mice. Although the authors display an interesting study of the alterations in niche components during age, some aspects of analysis, especially those regarding stem cell polarity in relation to stem cell potential and assays reflecting 'youthful' stem cells seem rather vague and insufficient.

Our manuscript identifies thrombin-processed OPN as a critical niche-derived factor for regulating aging of HSCs. We further analyzed commonly accepted (“canonical”) parameters of HSCs aging. To determine the status of HSCs with respect to aging (young or aged) we analyzed canonical and thus published phenotypes of aging, plus our recently established novel aging phenotype of HSCs, polarity ex vivo. We demonstrate how an aged BM niche is directly responsible for the loss of polarity in young HSCs (fig. 1E and 3J-K). We also provide a whole set of ex vivo and in vivo (BMT) experiments demonstrating how the changes in HSC polarity correlate with changes in stem cell function (fig.5), consistently with previous publications (1,2). A loss of polarity of HSCs is thus a valid aging marker.

(1) Regarding the novel aspect of the findings of the manuscript. As the defective hematopoiesis phenotype of OPN KO mice have been reported, the concepts that the manuscript presents are not strikingly novel. Yet the authors do display novel findings in the role of OPN in aged mice and associate it with cell polarity which has not been previously described.

The laboratory of Susi Nilsson has previously reported changes in hematopoiesis in OPN KO animals (3,4). Our data on changes in HSCs polarity and HSCs numbers for example in these animals are novel, not yet published data. Most importantly, previous work did not connect changes in OPN in the niche to aging of HSCs. Aging of HSCs by loss of OPN in stroma upon aging is thus a very novel concept.

2) The authors utilize cell polarity as a feature to monitor stem cell aging phenotype. Although loss of polarity is noted in Cdc42 KO mice in association with aging, it is questionable whether it is a specific feature of aged HSCs. Also, as the manuscript is based on analysis of the aged microenvironment and its effect on HSCs, a fundamental question arises whether cell polarity is altered with niche interaction. The authors should provide data on how cell polarity is affected in vivo. In conjunction, the authors should provide data using IHC in Fig 1, showing the distribution of HSCs in BMT recipients.

Our heterochronic transplant experiment (Y into Y, Y into O and Y into young OPN KO) show that HSC polarity is altered as a consequence of the interaction with a heterochronic niche in vivo (see fig.3J). Multiple sets of data from our laboratory demonstrate that a change in the percentage of polar HSCs upon aging is a very robust phenotype of HSC aging. We feel that analyzing HSCs polarity directly in vivo in young and aged animals, and not ex vivo as done in this MS as well as detailed distribution of HSCs in vivo are beyond the current scope of the manuscript, as such data will not further contribute to a more detailed understanding of mechanisms of action of OPN on HSCs.

(3) Another parameter that the authors utilize to assess what they term 'youthful' stem cell quality is the lower number of HSCs engrafted. Although controversial, HSCs from aged mice display an increase in self-renewal due to a decrease in clonal diversity. Therefore, assessing the change in number of the engrafted cells may not sufficiently demonstrate the aged phenotype of HSCs. The authors should attempt to assay for symmetric and asymmetric division patterns. Also, they should display progenitor cell reconstitution (CLP, CMP, GMP etc) to show myeloid skewing. Gene expression assay of aged associated genes may also be helpful.

The increase in the number of HSCs upon aging is a well-established hallmark of aging and confirmed independently by multiple laboratories (5–8). We agree with the reviewer that assessing the change in number of engrafted donor cells is not enough to demonstrate the aging phenotype. In fact we show, in addition to HSC numbers, changes in chimersim in peripheral blood, altered BM
lineage differentiation, frequency of LT, ST-HSC and MPPs and HSC protein polarity for each of the experimental group. As the reviewer suggests, we have now displayed novel CLP, CMP and GMP data in the revised version to better inform the reader on additional aging-associated changes in hematopoiesis influenced by OPN.

Whether aging-associated changes in polarity are linked to changes in symmetric/asymmetric pattern of division of HSC has not been established/published. Such a novel concept on HSCs aging has been recently presented by our laboratory as preliminary yet not finally verified and published data. This can thus not serve as a validated marker for aging, and such experiments are beyond the scope of the current manuscript, which focuses on OPN.

(4) As increased mobilization and less retention to the BM of HSCs is a characteristic of aged hematopoiesis, the authors should analyze whether HSC homing was affected in Y to O or Y to OPN KO BMT.

We agree with the reviewer that this is a relevant aspect to consider. Because aged HSCs are found at a greater distance from the endosteum within the BM and present with a reduced lodging ability (9), we have tested the contribution of loss of OPN to these aging-associated phenotypes by performing additional experiments to address homing in Y, O or OPN KO niches. These data suggest that an OPN KO microenvironment resembles an old microenvironment with respect to harboring more HSPCs upon homing. These data are now shown in Fig. EV3M-O.

(5) The authors should show whether HSCs in OPN KO mice (not BMT) have aged phenotype.

HSCs from OPN KO mice show an aged phenotype with an increase in HSC number, a decrease in protein polarity and an increase in cdc42 activity (fig EV3 E-I). Moreover we see accelerated changes in differentiation in BM of intermediate aged OPN-/-- mice (18 month). They present with reduced frequency of B-cells and an increase in the number of myeloid cells compared to age matched littermates (fig EV3 J-L).

(6) The authors should use HSC populations for BMT assays rather than whole BM cells.

Transplanting bone marrow cell populations that contain HSCs and analyzing chimerism 4-6 month post transplant is an accepted and valid assay in the field with respect to the analysis of stem cell potential. Primarily stem cells contribute to chimerism at that time point post transplantation (10). Highly purified HSCs should be anyhow transplanted alongside bone marrow competitor cells to ensure the survival of the irradiated recipients. Finally, there is no robust published data demonstrating that transplantation of purified HSCs is in general superior to determine stem cell potential compared to transplantation of BM cells that contain HSCs.

(7) In Fig 2 the authors assess the niche cell content in old mice by using the bone region BM. They present that there was no difference in the MSC cell population. Yet MSCs are distributed through the BM thus the authors should provide data on the cell populations in the flushed (central) BM as well.

We share the interest of the reviewer in the cellular stroma composition of the central bone marrow although we focus throughout our MS on a stroma population enriched for endosteal cells (stated on page 5). We now provide in the revised version novel experimental data also on the frequency of different stromal cell types (OBs, CD31+, MSCs, CARs) in central bone marrow which do not show significant differences between young and aged mice. Thus, the stroma cell composition in the central part of the BM is interestingly not affected by aging (Fig. EV1D).

(8) In Fig 3, the authors show that OPN KO mice have an overall decrease in OBL numbers. Could the decline in OPN KO mice to harbor HSCs depend on a decrease of the number of OBL and not directly on OPN expression. The authors should attempt to rescue the phenotype with addition of OPN in vivo.

OPN KO mice present with a lower number of OBLs (Fig. EV3C) while they show actually an elevated number and not a reduced number (decline) of HSCs (fig. EV3E). We have again repeated these experiments to see whether initially listed trends become significant. We could confirm as
initially stated that the number of OBs in OPN KO is significantly decreased. The in vivo treatment of mice with thrombin cut OPN requires, due to the so far unknown stability and unknown bioavailability of this protein in vivo, quite a large number of additional experiments before unequivocal statements can be made with respect to interpreting the outcome. We therefore consider such experiments beyond the scope of the current MS.

Referee #3:
The manuscript by Guidi et al focuses on the role of the niche as a mediator of hematopoietic stem cells aging, and suggests that the matrix glycoprotein osteopontin is a mediator of the effect of the aging microenvironment on HSCs. The authors describe a decline in osteopontin in the microenvironment of aged mice, which is not surprising and consistent with the loss of osteolineage populations that has previously been described. They find that the HSCs of young mice with global genetic osteopontin loss partially recapitulate the aging phenotype. The paper adds importantly to the aging HSC field, where only very few data support a role for the microenvironment. The authors importantly establish the importance of the microenvironment in inducing or reversing aging HSC characteristics. Notably, the authors convincingly demonstrate that treatment of aged HSCs with the thrombin-activated fragment of OPN rejuvenates certain aspects of aged HSCs. However, there are numerous major and minor limitations, both experimental and conceptual, including overinterpretation of some of their data that diminish the enthusiasm of this reviewer:

We share the view of this reviewer that our data adds to the aging HSC field in which only few publications address the role of aging of the microenvironment in inducing/ reversing aging HSC phenotypes. The reviewer also states that treatment with OPN fragments convincingly rejuvenates many characteristics of aged HSCs.

1) Figure S1a: The authors should temper their claim that age does not differentially impact the osteoblastic lineage: for example, there appears to be a more drastic reduction of both CFU-Fs and CFU-OB in young compared to aged mice.

Our data support, as the reviewer states, that aging actually differentially influences the osteoblastic lineage as demonstrated by a decreased in the number of CFU-F and OB in aged stroma (Fig. EV1C,D). Irradiation affects the young and aged stroma compartment both in a similar fashion, with a decrease of about 40 to 50% in the ability of fibroblast and osteoblast progenitor cells to form colonies.

2) Page 5 and Fig S1b: CD45- should not be used as an indicator of non-hematopoietic stromal cells, since this population includes CD45- Ter119+ erythroid cell populations.

CD45-BM stroma cells include the erythroid population Ter 119+. In fact we have used the Ter119 exclusion marker to exclude this population and gate for the non-hematopoietic subset. However, isolating the endosteal fraction with collagenase after BM flushing brought the population of CD45-Ter119+ cells already to zero (see gating strategy here below). Ter119+ cells were thus “naturally depleted” due to our stroma isolation process. We will thus include in the revised version Ter119- in the graph in Fig. EV1B and in all the legends defining the endosteal stroma population.
3) Page 6 and Figure 2F: This experiment appears underpowered to detect a difference in osteopontin expression in MSCs

We agree with the reviewer that the current number of experimental repeats, while conclusively showing a difference in OPN expression in OBs, did not result in a statistical difference in the expression of OPN between young and aged MSCs. While these data do not fully exclude that also MSCs present with a small difference in OPN expression upon aging, they confirm though that aged OBs present with a relatively large difference in expression and are so far the only stroma cell population analyzed that showed a significant difference.

4) Figure 2G: These images are not convincing. The old OB appears to be in metaphase, showing the 2 prominent nucleoli vs the multiple nucleoli of the young OB. Is this a consistent finding or is it a common characteristic of aged OBs?

The images shown in Figure 2G are confocal images. The 2 prominent nucleoli are most likely condensed heterochromatin. This nuclear structure is not a common characteristic of aged OBs since only 20% of the old OBs analyzed show this altered nuclear architecture, and for this reason we now have changed the image of old OBs with a more representative one. In the revised version we now have also included a 3D video to better inform the reader on the nuclear structure of these cells (movie EV1, movie EV2).

5) Figure 3C: the authors should highlight more clearly the fact that exposure of young HSCs to an OPN KO niche recapitulates only certain features of aged HSCs. For example, while the expansion in LT-HSCs is recapitulated, the myeloid shifting is not.

We have now emphasized this point (certain features) to the reader in the revised version, and commented again on our data like on page 8: Y→O animals presented with an increase in the frequency of myeloid cells over lymphoid T cells, while B-cell frequencies were not affected (Fig. 3I, see also Fig. 1). Y→OPN KO animals though did not yet recapitulate an aged phenotype in differentiated cells, showing a normal contribution to myeloid, T and B cells in PB similar to Y→Y controls (Fig. 3I).

6) Figure 4: In general these data are important to demonstrate that the young microenvironment can improve the function of aged HSCs, however the role of OPN in this case is confusing with respect of the lineage allocation of the transplant(panel D).

6. Figure 4 panel D shows that young OPN KO recipients do not present with a myeloid cell
frequency that is similar to a normal young wt level. T cells instead show a normal young level, which may be due to a young thymus in young recipients. Our data thus demonstrate that a young wt environment is able to rescue some of the aging HSC phenotypes, while a young OPN KO environment is not able to do so.

7) Page 9: The authors incorrectly state that the young microenvironment increases T cells engraftment compared to Old into Old and Old into OPN KO.

We have now corrected this statement in the revised version according to the suggestion of the reviewer.

8) Figure 4C. The Western blot is not convincing.

Figure 4C is actually not a Western Blot, so we assume that the comments refer in general to Western Blots shown in Figure 4 (like 4F and 4G). As already mentioned in the text, OPN is extensively modified and cut in vivo, resulting in a mixture of distinct “protein entities”. The antibody has been verified on OPN KO BM supernatants and these now shown in a novel western blot image in Fig.4F in a novel biological repeat of the assay.

9) Figure 5E: The authors state that treatment of aged HSCs with thrombin activated OPN fragments functionally rejuvenates aged HSCs, however their total cell engraftment remains defective. They should tone down their claim.

We now emphasize this point (certain features are rejuvenated) to the reader in the revised version, for example on page 12: “While the overall level of engraftment after 22 weeks was similar in recipients transplanted with aged HSCs and aged HSCs treated with fraction D (Fig. 5E), aged fraction D treated HSCs presented with an increase in contribution to the T cell compartment, no change in the B-cell compartment and a significantly reduced contribution to the myeloid compartment in PB compared to aged, un-treated controls (Fig. 5F).

And again in page 12: “Although the overall level of engraftment remained unchanged compared to non-treated aged controls, treatment of aged HSCs ex vivo with thrombin-activated OPN fragments resulted in HSCs youthful for polarity and frequency in vivo and also changed the frequency of myeloid and T cell to a youthful level”.

10) Figure 5F: How do the authors explain that the activated OPN fragment could block aged HSC myeloid while this was not a characteristic of the OPN effect in all other experiments?

A shift towards increased myeloid cells production is actually visible in an accelerated fashion in middle-aged OPN KO mice (fig. EV3 J-L). We have also included novel data on changes in myeloid progenitor cells and discussed these findings in more detail in the revised version.

11) Figure S3C (and others). The authors use the term stroma inconsistently, do they mean endosteal cells, CD45- endosteal cells, or CD45- Ter119- endosteal cells. They should clarify in the figure legends or labels.

We now list the stroma term consistently as “CD45-Ter119- endosteal enriched stroma population” in the revised version.

12) P4: myeloid skewing is attributed here to increased myeloid contribution and the presence of an aged thymus. However, others (see Flach et al) have previously demonstrated that there is significantly decreased lymphoid contribution by sorted aged HSCs. This should be discussed by the authors and begs the discussion of the impact of OPN KO on lymphoid contribution on aged HSCs.

We have now discussed this interesting point (lymphoid contribution) in more detail in the revised version of the manuscript.
References


2nd Editorial Decision 18 January 2017

Thank you for submitting the revised version of your manuscript. It has now been seen by two of the original referees, whose comments are enclosed below. Please note that have also asked the third referee to look into the revisions, however have not received back a report and decided in the interest of time to move on with our decision.

As you will see the referees find that their concerns have been sufficiently addressed and they now recommend the manuscript for publication. Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

REFEREE REPORTS

Referee #1:
The authors have responded appropriately to my concerns. I recommend acceptance for publication.

Referee #2:
They responded well to the reviewers question and suggestion
Specific subsection in the methods section for statistics, reagents, animal models and human subjects. Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Every question should be answered. If the question is not relevant to your research, please write NA (non-applicable).

Each figure caption should contain the following information, for each panel where they are relevant:

- A specification of the experimental system investigated (e.g. cell line, species name).
- The assay(s) and method(s) used to carry out the reported observations and measurements.
- An explicit mention of the biological and chemical entity(ies) that are being measured.
- An explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- The exact sample size (n) for each experimental group/condition, given as a number, not a range.
- A statement of how many times the experiment was independently replicated in the laboratory.
- Definitions of statistical methods and measures:
  - Common tests, such as t-test (please specify whether paired or unpaired), simple p-values, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, more complex techniques should be described in the methods section;
  - Are tests one-sided or two-sided?
  - Are there adjustments for multiple comparisons?
  - Have statistical test results, e.g., P-values, been quoted as exact P-values < x or as a range?
  - How were centers values defined as median or average?
  - Definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

B- Statistics and general methods

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? In order to choose a sample size, we used GraphPad StatMate Software Version 2.0b, estimating a standard deviation between 2 and 8 (depending on the experiment and the possibility of normal distribution of data we assumed a standard deviation of 2 and a significant difference between means of at least 15).

1.b. For animal studies, include a statement about randomization even if no randomization methods were used. In our transplantation experiments we estimated a sample size of 15-20 assuming a standard deviation of 10 and a significant difference between means of at least 15.

2. Describe exclusion/inclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?

3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatments (e.g. randomization procedure)? If yes, please describe.

For animal studies, include a statement about randomization even if no randomization was used.

4.a. Were any steps taken to minimize the effects of subjective bias during group allocation of/and when assessing results (e.g. blinding of the investigators)? If yes please describe.

4.b. For animal studies, include a statement about blinding even if blinding was done. Blinding was not chosen.

5.a. For every figure, are statistical tests justified appropriately?

5.b. Are the definitions of error bars as s.d. or s.e.m. justified appropriately?

6. In the data meet the assumptions of the test(s) (e.g., normal distribution)? Describe any methods used to assess it.

7. If there is an estimate of variation within each group of data? If normal distribution of data was implied.

8. In the variance similar between the groups that are being statistically compared? Variance between the groups was similar.
D- Animal Models

1. Identify the committee(s) approving the study protocol.

2. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

3. For publication of patient photos, include a statement confirming that consent to publish was obtained.

4. Report any restrictions on the availability (and/or on the use) of human data or samples.

5. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

6. For phase I and II randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under Reporting Guidelines. Please confirm you have submitted this list.

7. In the event you are a proponent of the ARRIVE guidelines (see link list at top right), (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under Reporting Guidelines. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.

8. Provide accession codes for deposited data. See author guidelines, under Data Deposition.

9. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

10. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile e.g., Antibodypedia (see link list at top right), LitGenomics (see link list at top right).

11. Identify the committee(s) approving the study protocol.

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

E- Human Subjects

10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under Reporting Guidelines, See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.

F- Data Accessibility

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

14. Report any restrictions on the availability (and/or on the use) of human data or samples.

15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

16. For phase I and II randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under Reporting Guidelines. Please confirm you have submitted this list.

17. For phase I and II randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under Reporting Guidelines. Please confirm you have submitted this list.

18. Provide accession codes for deposited data. See author guidelines, under Data Deposition.

19. Data deposition in a public repository is mandatory for:
   a. Primer, DNA and RNA sequences
   b. Microarray and proteomics data
   c. Crystalllographic data for small molecules
   d. Functional genomic data
   e. Proteomics and molecular interaction data
   f. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View') or in a structured repository such as Dryad (see link list at top right) or Figshare (see link list at top right).

20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreements within the study, such data should be deposited in one of the major public service controlled repositories such as dbGaP (see link list at top right) or EGA (see link list at top right).

G- Dual use research of concern

12. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile e.g., Antibodypedia (see link list at top right), LitGenomics (see link list at top right).

13. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

14. For publication of patient photos, include a statement confirming that consent to publish was obtained.

15. Report any restrictions on the availability (and/or on the use) of human data or samples.

16. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

17. For phase I and II randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under Reporting Guidelines, Please confirm you have submitted this list.

18. If necessary, primary and reanalyzed data should be formally cited in a Data Availability section. Please state whether you have included this section.

19. Please provide access codes for deposited data. See author guidelines, under Data Deposition.

20. Provide accession codes for deposited data. See author guidelines, under Data Deposition.

21. In the event you are a proponent of the ARRIVE guidelines (see link list at top right), (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under Reporting Guidelines. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.