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Identification of a clonally expanding hematopoietic compartment in bone marrow

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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 08 May 2012

Thank you very much for submitting your research paper reporting on the identification of a presumably unique hematopoietic (stem-) cell compartment for consideration to The EMBO Journal editorial office.

I did receive two sets of rather complementary reports enabling a decision that should avoid further delay to the proceedings of your study.

As you will recognize, both scientists appreciate the timeliness and technical quality of the paper. They also conclude that significant further molecular characterization and possibly functional corroboration on the reported morphological structures would be needed to reach the level of general interest and detail they would expect from a rather conceptual and still molecular -oriented journal such as ours. Accordingly, ref#1 frankly suggests to either shorten for report paper OR experimentally establish the intriguing proposal to overcome the current relatively descriptive/preliminary state of analyses.

On balance, we would be willing to offer the chance to appropriately address their concerns. As this entails time-consuming experimentation as outlined from both referees, we would fully understand if you might find it easier to seek more rapid publication elsewhere.

In case you embark on a potential revision, I urge you to take the demands into careful consideration to not waste your time and avoid unpleasant disappointments much later in the process. Please do not hesitate to contact me in case of further questions or indeed outlining possible experiments and

timeline for revisions of the study (preferably via E-mail).

I am sorry that I cannot be more encouraging at this stage of analysis but I hope that clearly communicating our demands and expectations facilitates efficient proceedings for this exciting line of research.

Sincerely yours,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1:

This is an interesting paper that extends previous observations that different types of hematopoietic cells are not homogeneously distributed throughout the bone marrow of adult mice. The major innovation in this paper is the use of state-of-the-art gene marking tools to characterize specific cell types in vivo and to reveal associations between hematopoietic and stromal phenotypes that have not been previously documented with such precision.
However, the authors do not adequate recognize significant previous related findings, which leads them to overstate the novelty of their findings. These are also overinterpreted as explained in more detail below. In particular, it is unfortunate that data used to support the involvement of HSCs were so weak. Given the minimal new information, I would suggest this either be shortened to a brief note or include additional data to flesh out the possibilities inferred.

1. The authors should reference the early studies of Lord et al who first demonstrated a non-homogeneous distribution of primitive hematopoietic cells throughout the bone marrow (eg Blood 46:65-72, 1975) and many subsequent later such descriptions including a number of papers by Blazsek et al characterizing an aggregate cell structure within the marrow that they called the "hematon" because it could be isolated and shown to represent an enriched concentration of stromal sells, perivascular lipocytes, endothelial cells, macrophages and functionally defined primitive hematopoietic cells with longterm lympho-myeloid differentiation potential. Given this historical background as well as more recent literature that is cited, it is clearly an overstatement to say: "In fact, there is currently no evidence for specialized partitions and structures related to hematopoiesis." (page 4, lines 13-14).

2. Similarly, the statement in the Abstract (line 4-5) "it is currently unclear whether bone marrow is a unitary compartment without structural or functional partitions" does not convey any meaning to this reviewer. What is a unitary compartment? It is well known from parabiosis experiments that HSCs are constantly recirculating and, even from transplant experiments, that small input populations reconstitute sites of HSC localization in muscle and liver as well as bone marrow. Conversely, it is also well known from initial histological studies of the bone marrow of irradiated mice transplanted with small numbers of cells and later from vector marking studies that localized clonal growth is a frequent event post-transplant. The interesting questions are then: which cells move around and at what rate in an unperturbed mouse, how is this affected when a transplant is performed, and what boundary function do the mT+ cells delimiting the hemispheres described here serve? Unfortunately, none of these issues are addressed here.

3. Fig 3B and C are difficult to understand. What exactly are the numerators and denominators used to show the values shown? Please convey the proportion of different phenotypes in both compartments. (Note also that Fig C and D seem to be exchanged given the corresponding legends).

4. Text related to Fig 3 and elsewhere should not refer to CD48-CD150+ cells as putative HSCs. The authors should assay the cells obtained by FACS using the same staining strategy as used for the in situ staining (i.e. without additional Abs) and document the HSC purity in the suspensions thus obtained to provide a more accurate measure of what is being recognized by the in situ staining.

5. The cycling data are confusing. Pulse labelling experiments of others have shown that primitive

hematopoietic cells (all the way from HSCs down to primitive subsets of clonogenic progenitors) under homeostatic conditions are quiescent in mice at 6 weeks of age when these experiments were performed according to the Methods. Thus the high cycling rate of cells within hemospheres documented in Fig 4 suggests that this refers to the bulk intermediate-end production of blood cells, not particularly to HSC-initiated events. This is important as the confetti data do not discriminate the stage of differentiation where clonal growth was initiated. This could (?should) be examined by transplanting HSCs marked with one of 2 or 3 different colours and asking the same intra-hemosphere clonality question.

6. The fact that injected bone marrow cells concentrate in hemispheres is uninformative without additional phenotypic or functional data.

7. It would be interesting to know whether hemispheres can be found in the spleen, particularly post-transplant where a fair amount of the regenerative activity also takes place.

8. Page 11, line 20 - Radioprotection (Fig 9B) is not a likely indicator of functional CD48-CD150+ HSC numbers, but more likely of surviving short term repopulating cells/CFU-S. The text is misleading on what this data means/implies.

Referee #2:

This is an interesting paper demonstrating the existence of discrete locations in the bone marrow with a specialized architecture in which expansion of hematopoietic cells occur. The data are mostly of high quality and the topic of great interest in view of current efforts in defining hematopoietic "niches". Some aspects of the study and some inferences made are however less than compelling. There are two main points that should be addressed

1. The specific cellular architecture of the "hemospheres" should be better defined. In particular, clear data should be shown on the comparative localization of nestin and CD146 within the hemospheres and within the remaining marrow tissue. The authors make an effort to identify distinct vascular compartments (with or without an adventitial coating) but the identity (or phenotype) of the coating cells is relevant.

2. The claim that the hemospheres are the sites of clonal expansion is too strong. The data only show circumstantial evidence, as the occurrence of multiple (2 at least) recombination events cannot be ruled out.

Other points

The authors should provide more formal data on the relative distribution of LSK CD150+ CD48- in the region where most of the "hemosphere" concentrate and the rest of the endosteal surface. This is important in view of the widespread notion that "endosteal" cells are enriched in HSCs. The authors seem to have data showing that this is not the case, unless one would consider the primary spongiosa an endosteal surface.

The overall topography of the hemospheres is not very clear. Are ALL of them located next to the growth plate, and if not, what is their relative frequency in different regions. The implications are obvious in view of the fact that adult human bone marrow would not include a growth plate.

Figure 4C. Why would one see "hemospheres" after intrafemoral transplantation in the same region where one sees them in steady state hematopoiesis? This does not seem intuitive. Also, what kind of changes in the bone marrow architecture are induced by intrafemoral injection per se?

Figure 7A. From the images it is not clear that the two regions shown are indeed equivalent and both demonstrating a growth plate. Overall, the use of multicolor fluorescence images is not optimal to grasp histology, and histology is important in this context. The authors should include brightfield images for orientation.

Finally, I would suggest to drop the term "hemosphere". It evokes the many "spheres" introduced in the stem cell jargon to denote putative stem cell derived clones in a variety of systems, but in no system has any "sphere-making" capacity been ever formally linked to anything related to stemness.

1st Revision - authors' response 12 October 2012

We would like to thank the reviewers for their time and useful comments, which have allowed us to improve the manuscript. While we are providing a detailed point-by-point response below, I would like to highlight the most important pieces of new data:

We show that CD150+ CD48- cells in hemospheres were negative for lineage markers. This pattern, in accordance with the current literature (see response to reviewer #1 below), is a strong indicator of putative HSCs (Suppl. Figure 6A). However, since the nature of the CD150+ CD48- cells in hemospheres cannot be directly determined by transplantation experiments or other functional assays, we have avoided overly strong statements and only occasionally refer to these cells as putative HSCs (according to the SLAM code established Kiel et al. 2005; Cell 121:1109-21).

Addressing the nature of the fast-cycling cells, we show that the population labeled by a short EdU pulse did not express lineage markers or CD150. Instead, a substantial fraction (around 30%) of EdU+ cells showed CD48 immunostaining, which suggests these represent transient amplifiying progenitors (Suppl. Figure 7).

We also show that the targeted inactivation of endothelial VEGFR2 altered the oxygenation of hemospheres and led to the loss of the fast-cycling, EdU+ cell population (Suppl. Figure 11).

Point-by-point response to all comments:

Referee #1: This is an interesting paper that extends previous observations that different types of hematopoietic cells are not homogeneously distributed throughout the bone marrow of adult mice. The major innovation in this paper is the use of state-of-the-art gene marking tools to characterize specific cell types in vivo and to reveal associations between hematopoietic and stromal phenotypes that have not been previously documented with such precision. However, the authors do not adequate recognize significant previous related findings, which leads them to overstate the novelty of their findings. These are also overinterpreted as explained in more detail below. In particular, it is unfortunate that data used to support the involvement of HSCs were so weak. Given the minimal new information, I would suggest this either be shortened to a brief note or include additional data to flesh out the possibilities inferred.

Answer: We are grateful for the positive but also the critical comments.

Referee #1: 1. The authors should reference the early studies of Lord et al who first demonstrated a non-homogeneous distribution of primitive hematopoietic cells throughout the bone marrow (eg Blood 46:65-72, 1975) and many subsequent later such descriptions including a number of papers by Blazsek et al characterizing an aggregate cell structure within the marrow that they called the "hematon" because it could be isolated and shown to represent an enriched concentration of stromal sells, perivascular lipocytes, endothelial cells, macrophages and functionally defined primitive hematopoietic cells with longterm lympho-myeloid differentiation potential. Given this historical background as well as more recent literature that is cited, it is clearly an overstatement to say: "In fact, there is currently no evidence for specialized partitions and structures related to hematopoiesis." (page 4, lines 13-14).

Answer: We were indeed unaware of the 1975 publication on “hematons”, but we now mention this interesting study in the Introduction. Looking at the Blazsek et al. data, it is, however, impossible to tell where “hematons” would be located *in situ*. As “hematons” were isolated by aspiration, it cannot be ruled out that these clusters might have even been generated by this process. Conversely, we find it very unlikely that “hemospheres” could be isolated by aspiration and, in fact, we have noted that even the most distal part of the bone marrow cannot be readily displaced from the spaces between bone trabeculae.
Although we appreciate the criticism (“overstatement”) raised by the reviewer, it is fair to say that the literature in the field is paying little attention to the structural organization of the bone marrow. For example, recent work by the Morrisson group (Ding et al. 2012, Nature 481:457-62) has nicely demonstrated that HSCs are maintained by endothelial and perivascular SCF expression, but it is not clear whether this occurs uniformly throughout the BM or is confined to specific, specialized sites. Our own study is certainly limited by the fact that we can currently not isolate hemospheres and functionally characterize the cells contained in these structures. On the other hand, what we report is very novel and certainly not appreciated in the field.

Referee #1: 2. Similarly, the statement in the Abstract (line 4-5) "it is currently unclear whether bone marrow is a unitary compartment without structural or functional partitions" does not convey any meaning to this reviewer. What is a unitary compartment? It is well known from parabiosis experiments that HSCs are constantly recirculating and, even from transplant experiments, that small input populations reconstitute sites of HSC localization in muscle and liver as well as bone marrow.

Answer: The term “unitary” refers to the bone marrow as a single and uniform structure lacking functional substructures or compartmentalization. In other words, is all bone marrow the same and, for example, equal in its capacity to harbor certain hematopoietic cells and functional microenvironments? Our data on the clonal expansion of hematopietic cells suggests that this is not the case for hemospheres.
Based on the comment made by the referee, we have decided to avoid the term “unitary” and have rephrased the relevant sentence in the revised manuscript.

Referee #1: Conversely, it is also well known from initial histological studies of the bone marrow of irradiated mice transplanted with small numbers of cells and later from vector marking studies that localized clonal growth is a frequent event post-transplant. The interesting questions are then: which cells move around and at what rate in an unperturbed mouse, how is this affected when a transplant is performed, and what boundary function do the mT+ cells delimiting the hemispheres described here serve? Unfortunately, none of these issues are addressed here.

Answer: Agree. It is indeed currently not known which cells move around in the BM and at what rates. This undoubtedly important and interesting question was, however, not the aim of our study. Of course, we are by no means the first to provide evidence for clonal expansion of hematopoietic cells – there is a rich body of literature here using retroviruses and other techniques, which we have also cited in the manuscript – but we are the first to show that there are a few select sites where this expansion is visible by genetic fate mapping.

Referee #1: 3. Fig 3B and C are difficult to understand. What exactly are the numerators and denominators used to show the values shown? Please convey the proportion of different phenotypes in both compartments. (Note also that Fig C and D seem to be exchanged given the corresponding legends).

Answer: The graph in 3B provides a count of CD150+ CD48- for a certain area. The x axis shows the number of CD150+ CD48- cells within this area, the y axis refers to the frequency of such structures. For example, about 30% of the hemospheres contained 4 CD150+ CD48- cells, whereas almost no such sectors were seen in the BM cavity.
Panel C shows that about 90% of the CD150+ CD48- cells in hemospheres and about 60% in the BM cavity were located on the abluminal surface of vessels. As the figure legends for B and C were

obviously not clear and precise enough, we have revised these text passages. It is also correct that the legends for C and D were inversed and needed to get swapped.

Referee #1: 4. Text related to Fig 3 and elsewhere should not refer to CD48- CD150+ cells as putative HSCs. The authors should assay the cells obtained by FACS using the same staining strategy as used for the in situ staining (i.e. without additional Abs) and document the HSC purity in the suspensions thus obtained to provide a more accurate measure of what is being recognized by the in situ staining.

Answer: It is appreciated that CD150 is not an exclusive stem cell marker but also labels a fraction of other hematopoietic cells such as T, B and dendritic cells. Nevertheless, it has been nicely demonstrated by Kiel et al (2005; Cell 121:1109-21) that HSCs from the BM "were highly purified as CD150(+) CD244(-) CD48(-) cells". Moreover, anti-CD150 immunostaining in combination with other markers (CD48, CD41 or lineage cocktail) is frequently used in the recent literature to determine the location of putative stem cells in marrow. Examples include Mendez-Ferrer et al. 2010, Nature 466:829-83; Yamazaki et al. 2011, Cell 147:1146-58; or Omatsu et al. 2010, Immunity 33:387-99. Such spatial information can obviously not be obtained after isolating the cells from their natural environment. Thus, the experiment proposed by the reviewer would still not prove that we would characterize the very same CD150+ CD48- cells seen in hemospheres and not cells from elsewhere in the BM.
To make clear that we have used the CD150/CD48 marker combination with high stringency, we have added new data showing CD150 immunofluorescence in combination with CD48/lineage cocktail double staining (Suppl. Figure 6A). This approach labels putative HSCs according to the current standards established by the recent literature in the field (see above).

Referee #1: 5. The cycling data are confusing. Pulse labelling experiments of others have shown that primitive hematopoietic cells (all the way from HSCs down to primitive subsets of clonogenic progenitors) under homeostatic conditions are quiescent in mice at 6 weeks of age when these experiments were performed according to the Methods. Thus the high cycling rate of cells within hemospheres documented in Fig 4 suggests that this refers to the bulk intermediate-end production of blood cells, not particularly to HSC-initiated events. This is important as the confetti data do not discriminate the stage of differentiation where clonal growth was initiated. This could (?should) be examined by transplanting HSCs marked with one of 2 or 3 different colours and asking the same intra-hemosphere clonality question.

Answer: We agree with the assessment made by the reviewer and have performed additional experiments addressing the nature of the fast-cycling cells. New data has been added (Suppl. Figure 7) showing that the fast-cycling cells are not differentiated (Lin+) blood cells and do not overlap with the CD150+ population. In contrast, a substantial fraction of the EdU-labeled population expressed the marker CD48 suggesting that these cells might potentially represent transient amplifying progenitors (Kiel et al. 2005, Cell 121:1109-21). Thus, our findings suggest that hemospheres harbor putative HSCs and enable the rapid expansion of committed progenitors (e.g. transient amplifying cells).

To further investigate the issues raised by the reviewer, we have also transplanted bone marrow cells isolated from Vav1-Cre x Rosa26-Confetti double transgenics. In these experiments, we were able to observe clonal expansion events in hemosphere-like structures (see Suppl. Figure 8). Nevertheless, there were also certain limitations with this approach: First, as we had to preserve the endogenous fluorescence of the labeled hematopopoietic cells, it was not possible to perform immunohistochemistry at the same time. Second, even though we have analyzed a large number of sections, capturing sites of clonal expansion was a rare event. Finally, the colonization of bone marrow by transplanted cells is unlikely to reflect HSC engraftment at the relatively early time points investigated. We are not making this claim in the manuscript.

Despite of these limitations, our new data shed more light on the function of hemospheres and, in particular, the nature of the fast-cycling cells.

Referee #1: 6. The fact that injected bone marrow cells concentrate in hemispheres is uninformative without additional phenotypic or functional data.

Answer: All we are saying with this data is that an initial step of transplant homing and expansion involves hemospheres, which raises the question as to whether they are required for this process. We do not have functional data that would allow the selective disruption of hemospheres. However, we show that hemospheres are severely compromised after the loss of endothelial VEGFR2 expression. Previous work by the group of Shahin Rafii (Hooper et al. 2009; Cell Stem Cell. 4:263-74) has already established that BM engraftment and reconstitution of hematopoiesis requires VEGFR2 function.

Referee #1: 7. It would be interesting to know whether hemispheres can be found in the spleen, particularly post-transplant where a fair amount of the regenerative activity also takes place.

Answer: We currently have no data concerning this interesting question. Would this really strengthen the main message of the manuscript or rather lead towards a different story?

Referee #1: 8. Page 11, line 20 - Radioprotection (Fig 9B) is not a likely indicator of functional CD48-CD150+ HSC numbers, but more likely of surviving short term repopulating cells/CFU-S. The text is misleading on what this data means/implies.

Answer: We are clearly referring to this data as a short-term survival experiment (see page 11). However, we are prepared to remove the data if the referee thinks that it is confusing or irrelevant.

Referee #2: This is an interesting paper demonstrating the existence of discrete locations in the bone marrow with a specialized architecture in which expansion of hematopoietic cells occur. The data are mostly of high quality and the topic of great interest in view of current efforts in defining hematopoietic "niches". Some aspects of the study and some inferences made are however less than compelling. There are two main points that should be addressed

Answer: We are grateful for the positive but also the critical comments.

Referee #2: 1. The specific cellular architecture of the "hemospheres" should be better defined. In particular, clear data should be shown on the comparative localization of nestin and CD146 within the hemospheres and within the remaining marrow tissue. The authors make an effort to identify distinct vascular compartments (with or without an adventitial coating) but the identity (or phenotype) of the coating cells is relevant.

Answer: This comment is very much appreciated and we have added Nestin staining to Suppl. Figure 1B. However, it should be mentioned that the precise nature of the Nestin+ mesenchymal cells seems still very much a matter of debate. Following the publication by Mendez-Ferrer et al. (2010, Nature 466:829-83), Yamazaki et al. (2011, Cell 147:1146-58) have shown that there is also an important role of Nestin+ non-myelinating Schwann cells in the bone marrow. Ding et al. (2012, Nature 481:457-62) state that HSCs do not require SCF from Nestin+ cells.

Referee #2: 2. The claim that the hemospheres are the sites of clonal expansion is too strong. The data only show circumstantial evidence, as the occurrence of multiple (2 at least) recombination events cannot be ruled out.

Answer: Genetic fate mapping with multi-color fluorescent Cre reporters is a well-established method for the identification of clonal relationships in vivo. Examples include fate mapping of developing coronary arteries in the heart (Red-Horse et al. 2010, Nature 464:549-53), the identification of lineage relationships in the regenerating mouse digit tip (Rinkevich et al. 2011,

Nature 476:409-13) or the tracking of stem cell progeny in the intestinal epithelium (Snippert et al. 2010, Cell 143: 134-44). All these studies rely critically on the same methodology that we have employed.
The chances of independently generating three clones of the same color are already 1:16, which drops to 1:64 for 4 and 1:256 for 5 clones. In fact, hemospheres typically contain cells of more than one color indicating multiple recombination events, but only cells of one color (most probably belonging to one clone) expand and therefore dominate inside these structures.

Referee #2: Other points The authors should provide more formal data on the relative distribution of LSK CD150+ CD48- in the region where most of the "hemosphere" concentrate and the rest of the endosteal surface. This is important in view of the widespread notion that "endosteal" cells are enriched in HSCs. The authors seem to have data showing that this is not the case, unless one would consider the primary spongiosa an endosteal surface.

Answer: The point is well taken. However, we are not arguing against or in favor of endosteal niches and we do not have conclusive data in this direction. In fact, we are not even stating that hemospheres are the niche or a niche enabling HSC maintenance.

Referee #2: The overall topography of the hemospheres is not very clear. Are ALL of them located next to the growth plate, and if not, what is their relative frequency in different regions. The implications are obvious in view of the fact that adult human bone marrow would not include a growth plate.

Answer: We would like to refer to the data in Suppl. Figure 4 showing the presence of hemospheres in a variety of adult skeletal elements including secondary ossification centers, skull and sternum. Secondary ossification centers represent the region where hemospheres can be detected most readily and reliably.

Referee #2: Figure 4C. Why would one see "hemospheres" after intrafemoral transplantation in the same region where one sees them in steady state hematopoiesis? This does not seem intuitive. Also, what kind of changes in the bone marrow architecture are induced by intrafemoral injection per se?

Answer: The radiation changes bone structure substantially and we think that these are newly formed, not pre-existing structures. Their formation in this area might be favored by the expression of VEGF in chondrocytes.

Referee #2: Figure 7A. From the images it is not clear that the two regions shown are indeed equivalent and both demonstrating a growth plate.

Answer: These are equivalent areas and we are prepared to provide lower magnification images demonstrating this point. In fact, the characteristic organization of the growth plate chondrocytes provides a clear tissue landmark, which ensures that we have looked at equivalent regions in both controls and mutants.

Referee #2: Overall, the use of multicolor fluorescence images is not optimal to grasp histology, and histology is important in this context. The authors should include brightfield images for orientation.

Answer: Unfortunately, it is not possibly to visualize morphological features with histological staining methods in the thick tissue sections that we are using. Thinner sections are possible but preclude the capturing of structural features of the tissue.

Referee #2: Finally, I would suggest to drop the term "hemosphere". It evokes the many "spheres" introduced in the stem cell jargon to denote putative stem cell derived clones in a variety of systems,

but in no system has any "sphere-making" capacity been ever formally linked to anything related to stemness.

Answer: It was necessary to coin a new name for these structures as we cannot constantly refer to them with the rather lengthy term novel or previously unrecognized BM compartment. However, we will be most grateful for alternative suggestions.

2nd Editorial Decision 02 November 2012

I am pleased to inform you that your manuscript has been reassessed by one of the original referees and has now been accepted for publication in the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1:

The authors have adequately addressed the concerns previously raised.

