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Identification and characterization of a resident vascular stem/progenitor cell population in preexisting blood vessels

Hisamichi Naito, Hiroyasu Kidoya, Susumu Sakimoto, Taku Wakabayashi and Nobuyuki Takakura

Corresponding author: Nobuyuki Takakura, Research Institute for Microbial Diseases, Osaka University

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1st Editorial Decision 18 July 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

Should you be able to address these criticisms in full, we could consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses in this revised version. I do realize that addressing all the referees' criticisms will require a lot of additional time and effort and be technically challenging. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know so we can withdraw it from our system.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

Naito at al. have studied CD31+CD45- SP cells and claim them to constitute an endothelial progenitor/ stem cell population which is predominantly dormant and has the ability to produce endothelial cells and reconstitute to blood vessels in ischemic models. The idea presented is potentially interesting for both vascular biology and adult stem cell biology. However, the data actually presented is very much less obvious and clear than the authors' interpretation and conclusions, and thus at present much too preliminary.

More robust and definite additional experiments addressing the identity, origin, true in vitro and in vivo differentiation capacity, and proliferative and clonogenic potential (versus "ordinary" ECs) of the studied cells are needed to prove the conclusions and to justify the publication of this report.

MAJOR COMMENTS:

1. The authors claim the cells: to be resident vascular cells". However, there are no direct evidence to show these cells reside on the vessel wall. This needs to be studied. If these indeed are true vessel wall cells, do these cells exist in all vessels (vein, artery and capilaries, lymphatics ?) ? More interestingly, do they have some spesific locations on the vessels wall like epithelial stem cell niche on the intestine ?

2. The authors provide a variety of FACS analysis of the SP cells in different organs, which confirmed the dissue distribution of the population. However as the CD31+CD45- SP population is very heterogenous, is the ability of the SP the authors studied here tissue specific ? Could the same population from other tissue origin share the same colony forming ability? Moreover, quantitation is missing. What proportion of CD31+CD45-SP cells have the progenitor/stem cell characteristics? Does this vary between different tissues and/or different vessel types ?

3. The identity/purity of the studied CD31+CD45- SP population remains undefined:
The authors need to provide comprehensive cell-surface marker analyses (IHC and/or FACS) to study the cellular identity and possible hematopoietic contamination of the CD31+CD45- SP population cells.

The authors also need to study mRNA expression (RT-PCR) of the targeted population to detect more rare transcripts and contaminating cells.
Do the target cells express SMA or other mesenchymal markers? Do they express hematopoietic lineage related mRNAs? There can well be a contamination of hematopoietic cells and/or mesenchymal cells (fibroblasts, pericytes etc) here, the authors have not properly studied this possibility.

4. The identity/purity of the colonies formed in colony forming assays is not well defined, and the colonies migh well be formed by hematopoietic cells which are well known to both take up LDL and also occasionally express various "endothelial" surface markers in vivo and especially in vitro .

Besides L DL uptake and N/C ratio characteristics, do the EC-SP cells form identical cobblestone EC colonies? Moreover, the cordlike colonies the authors described, what exact phenotype they are other than CD31+ ? Do they express other endothelial cell markers, e.g Flk-2, Ve-Cadherin, vWF , CD34, CD105, and ZO-1 ? Additional morphological characterization of a true endothelial phenotype is missing (glycocalyx, Weibel-Palade bodies,etc.)

5. It is disingenuous to say that a single cell form colonies in vitro, when in reality equal proportions of EC-SP cells are cultured. To what extent do the EC-SP cells actually proliferate? Statistics other than a vague description must be provided by here.

6. Stem/progenitor cells are defined as cells that are able to produce high numbers of daughter cells. The authors do not prove this fundamental stem cell quality.

On Page 9, limiting dilution analysis and culturing equal proportion of EC-SP from normal and

EGFP mice actually did not reveal exactly to what extent the stem/progenitor population could proliferate. (2 quotes from the manuscrip: "To assess clonal expansion of ECs from single cells, equal proportions of sorted EC-SP cells from normal mice and EGFP mice were mixed and cultured. " "The frequency to form colonies with a cordlike structure was significantly higher in EC-SP cells by a factor of ten")

7. In the manuscript, the authors mentioned SP population peaked on D3 and declined to steady state after 2 weeks. How many of these cells will retain a progenitor/ stem cell ability? Or it might be the case that the ability of proliferation of the cells in SP population or MP population is turned on and off in response to enviromental conditions? Because SP population authors defined is still a heterogenous population, it would be interesting to know the hierarchy between SP population and MP. For instance, is the stem/progenitor population give rise to other EC subpopulations and maintain their abilities in vivo.

8. In fig. 6E, both SP and MP form colonies and blood vessels.
This indicates that -in contrast to authors' conclusions- actually both populations contain endothelial cells that may have the capacity to contribute to angiogenesis under certain circumstances. If this is the case, then EC progenitors do not actually exist, but all ECs migh have an equal capacity to proliferate and produce daughter cells under certain conditions.

9. Indeed, I would be more enthusiastic if the authors identified more powerful markers for the selection of the proposed EC progenitors. For instance, in the manuscript, authors mentioned CD44 low expression and E-selectin on EC-SP cells. Could these provide a functional selection marker for the potential progenitor population?

10. The work and the idea would be remarkably benefited by an unequivocal marker of endothelium such as use of the VE-cadherin-Cre X tamoxifen induced by ROSA lac Z or expressing beta-gal under VEGFR2 promoter mouse, where LacZ expresses in arterial, venous and capillary enodthelial cells. Isolation of CD31+CD45- SP poplation from these endothelial specific reporter animals and functional assayed in vivo would greatly increase confidence in the data.

MINOR COMMENTS:

1 If this is a endothelial progenitor/stem cell population residing on the preexisting blood vessels, how can this population compensate for the endothelial cell loss during normal lifelong cellular turnover and how are they induced to generate novel ECs in angiogenic situations?.

2. The author showed that the SP population does not differentiate into smooth muscle cells in vivo in ischemic models. However, Most stem/progenitor cells have the ability to differentiate into other cell type in vitro, depending on the conditions. Can tthe cells studied here give rise to any other cell types, e.g vascular smooth muscle cells or pericytes in vitro?

3. Images in Fig. 6G is not sufficient magnification to determine the anatomice location of the GFP cells.

4. FACS analysis in Fig7 H is not informative, what is the statistic for this identical experiment?

5. Page 10 typo: Sham

Referee #2

The authors provide evidence that the side population subset of CD31+CD45- cells from different mouse organs shows features of endothelial stem and progenitor cells both in vitro and in vivo, as indicated by their contribution to vascular endothelium regeneration after experimental ischemia. The work is extremely interesting and nicely executed but would benefit from further clarifications:
1. Which are the ABC transporters that mediate the behavior as side population of these cells?
2. The absence of a marker for CD31+SP cells does not allow their localization. The CD31+ SP cells were purified from whole dissociation of different tissues, not specifically from vessels. Thus, it is theoretically possible that this rare population resides also in non-vascular organs. I am not sure

that the experiments shown in Fig. 7 can prove that CD31+SP cells are exclusively present in peripheral vascular endothelium.
3. Although these cells do not express CD45, it would be good if the hematopoietic potential could be tested by analyzing the hematopoietic cell repopulation in subletally irradiated mice. This could be easily done by using GFP mice.

1st Revision - authors' response 10 November 2011

Response to Reviewers' Comments to Authors:

Referee #1

We thank the reviewer for the constructive criticisms and positive comments on our manuscript. In response, we have added further experiments and discussion.

Naito at al. have studied CD31+CD45- SP cells and claim them to constitute an endothelial progenitor/ stem cell population which is predominantly dormant and has the ability to produce endothelial cells and reconstitute to blood vessels in ischemic models. The idea presented is potentially interesting for both vascular biology and adult stem cell biology. However, the data actually presented is very much less obvious and clear than the authors' interpretation and conclusions, and thus at present much too preliminary.

More robust and definite additional experiments addressing the identity, origin, true in vitro and in vivo differentiation capacity, and proliferative and clonogenic potential (versus "ordinary" ECs) of the studied cells are needed to prove the conclusions and to justify the publication of this report.

MAJOR COMMENTS:

1. The authors claim the cells: to be resident vascular cells". However, there are no direct evidence to show these cells reside on the vessel wall. This needs to be studied. If these indeed are true vessel wall cells, do these cells exist in all vessels (vein, artery and capilaries, lymphatics ?) ? More interestingly, do they have some spesific locations on the vessels wall like epithelial stem cell niche on the intestine ?

According to the suggestion of the referee, we performed lectin perfusion analysis to ensure that the $CD31^{+}CD45^{-}$ EC-SP cells reside at the vessel wall (intra-luminal cavity). This analysis revealed that approximately 91% of EC-SP cells are lectin-positive, suggesting that most of the EC-SP cells do reside at the inner surface of the blood vessels. Although it may well be that not all of the intraluminal endothelial cells were labeled with lectin, this result raises the possibility that a small population of EC-SP may reside deeper within the blood vessel wall or at other sites altogether. We have now added these results in Fig. 1E-G and sentences in Results (p6-7, lines 12-1, underlined). The localization of EC-SP cells is of great interest, but the absence of a marker for these cells does not allow their exact localization and niche to be determined. We have now re-written the paragraphs on localization and niche in the Discussion (p21, lines 10-16 and p22, lines 1-2, underlined).

The referee asked what types of vessels harbor such EC-SP cells if they are true vessel wall cells. Accordingly, we have attempted to study this issue in several ways. Firstly, we found that EC-SP cells are not present in LYVE1-positive lymphatic endothelium (Supplementary Figure S4 and sentences in Results p8, lines 15-16, underlined). Although we have tested all commercially available Ephrin B2 and EphB4 antibodies, we could not distinguish between arterial or venous endothelium in this respect by FACS analysis. Moreover, we used Ephrin B2-LacZ mice (kindly provided from the laboratory of David J. Anderson, California Institute of Technology) to identify arterial endothelium and stained dispersed cells with FluoReporter-LacZ (Molecular probes) and Hoechst dye. However, we could not detect the EC-SP pattern in ephrinB2-positive cells. This might be caused by the FluoReporter-LacZ method damaging the cell membrane to enable loading of FDG into the cells, but at the same time this might also make it impossible to maintain the Hoechst effluxing ability of the cells. Moreover, most of the LacZ-positive cells disappeared after Hoechst staining, because according to the manufacturer's protocol, cells stained with FDG (tuning to highly fluorescent fluorescein) must be kept on ice to avoid leakage of the fluorescein from the cell - but

Hoechst staining has to be performed at 37°C (Figures for the Referee, Figure R1; presented at the end of this letter). Therefore, we had to abandon efforts to show the EC-SP pattern in ECs fractionated by their expression of ephrinB2 or EphB4.

Hence, it was a challenge to find out whether sorted EC-SP cells are from vein, artery or capillaries; we approached this issue by assessing the expression of arterial and venous markers by qRT-PCR. We had initially found that the RNA expression level of EphrinB2 and EphB4 tended to be lower in EC-SP cells than EC-MP cells but this did not reach significance due to wide interindividual variability. However, we have repeated these experiments and do now find significant differences in ephrinB2 expression; we have now edited the p value in the revised Figure 2B. Moreover, we compared EC-SP cells with the whole EC fraction, which contains a mixture of arterial, venous and capillary ECs. The results of qRT-PCR for EphB4 and Ephrin B2 were in line with our original data showing that the expression levels of EphB4 and EphrinB2 in EC-SP cells were lower than in the whole EC fraction. EphrinB2 was significantly lower ($p<0.01$) (comparison of the expression levels of SP vs MP and SP vs EC are shown in Figures for the Referee, Figure R2; presented at the end of this letter). Additionally, RNA expression levels of the arterial markers Neuropilin-1, Hey1 and Hey2 and the venous marker Coup-TFII were also evaluated. We have added Supplemental Figure (Figure S5) and sentences in the Results on this issue (p8-9, lines 16-1,underlined).

In summary, the RNA expression levels of arterial markers, except for Hey2, were lower than in the whole EC fraction and venous markers were comparable. This indicates that EC-SP cells are present predominantly in capillaries and veins.

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According to this suggestion, we performed quantitative colony-forming assays for EC-SP cells from different tissues. In summary, the colony-forming ability of liver EC-SP cells is comparable to limb muscle EC-SP cells, but lung and heart EC-SP cells possess about 60% of that. However, when compared to the MP fraction in each tissue, EC-SP cells possess higher colony-forming ability. We have added these results in Supplementary Figure (Figure S7) that shows that the EC-SP cells in different organs do possess colony-forming ability. We describe these results in Results (p10, lines 8-12). We are sorry that we could not further fractionate EC-SP cells from arteries, veins, or capillaries, as described above, and therefore we could not compare colony-forming ability of EC-SP from different vessel types.

3. The identity/purity of the studied CD31+CD45- SP population remains undefined: The authors need to provide comprehensive cell-surface marker analyses (IHC and/or FACS) to study the cellular identity and possible hematopoietic contamination of the CD31+CD45- SP population cells.

Thank you for this comment. As shown in Figure 2A, we initially showed that EC-SP and EC-MP cells are negative for hematopoietic lineage markers (mixture of anti-CD4, -CD8, -B220, -Ter119, -Mac1 and -Gr1 antibodies), but we have now added the FACS analysis of EC-SP cells to document that these cells are not contaminated with hematopoietic cells. We have now added a new Supplementary Figure S1 showing that EC-SP cells do not express hematopoietic markers and have added new sentences in Results (p7, lines 7-10). In summary, we compared hematopoietic cell-surface lineage markers between EC-SP cells and $CD45^+$ hematopoietic cells from digested muscle and ruled out the possibility of hematopoietic cell contamination in $CD31^+CD45^-$ SP population cells.

The authors also need to study mRNA expression (RT-PCR) of the targeted population to detect more rare transcripts and contaminating cells.
Do the target cells express SMA or other mesenchymal markers? Do they express hematopoietic lineage related mRNAs? There can well be a contamination of hematopoietic cells and/or mesenchymal cells (fibroblasts, pericytes etc) here, the authors have not properly studied this possibility.

As depicted in Figure 2A, we originally showed that EC-SP cells are negative for PDGFR-beta, but according to this suggestion, we further checked the possibility of contamination by qRT-PCR. PDGFR-beta, NG2 and Desmin were tested as pericyte markers, Fsp1 as a fibroblast marker and CD45 as a hematopoietic cell marker. In this way, we have been able to exclude contamination by hematopoietic cells and mesenchymal cells. Now we have added the new supplementary Figure S2 and new sentences in Results (p7, lines 7-10). The hind limb-derived $CD31^{-}CD45^{-}$ cell fraction (negative fraction) was used as a control.

4. The identity/purity of the colonies formed in colony forming assays is not well defined, and the colonies migh well be formed by hematopoietic cells which are well known to both take up LDL and also occasionally express various "endothelial" surface markers in vivo and especially in vitro .

To exclude the possibility that colonies generated by EC-SP cells are of the hematopoietic lineage, we performed immunostaining of EC-SP-derived EC colonies for hematopoietic lineage markers (Supplementary Figure S9, Results in p10-11, lines 14-1). As a comparison, we used KSL hematopoietic stem cells as a positive control to form hematopoietic colonies on OP9 cells. This experiment revealed that colonies generated by EC-SP cells were negative for hematopoietic markers and different from those generated by hematopoietic stem cells. As suggested by the reviewer, hematopoietic lineage cells sometimes express CD31 in vitro and in vivo; however, under our culture conditions, hematopoietic cells do not form CD31-positive colonies on OP9 stromal cells.

Besides LDL uptake and N/C ratio characteristics, do the EC-SP cells form identical cobblestone EC colonies? Moreover, the cordlike colonies the authors described, what exact phenotype they are other than CD31+ ? Do they express other endothelial cell markers, e.g Flk-2, Ve-Cadherin, vWF , CD34, CD105, and ZO-1 ? Additional morphological characterization of a true endothelial phenotype is missing (glycocalyx, Weibel-Palade bodies,etc.)

With regard to cobblestone EC colony formation, upon culturing hind limb EC-SP cells on OP9 stromal cells, most of the EC colonies show cord-like characteristics, but approximately 1% are indeed cobblestone-like. We have now added a new Supplementary Figure S6 and rewrote the Results (p10, lines 7-8).

Moreover, according to the reviewer's suggestion, we performed immunostaining of these colonies. We performed all the stainings listed by the reviewer: Flk-1 (we assume that the referee meant Flk1, not Flk-2, suggested in the comment above), VE-Cadherin, vWF, CD34, CD105 and ZO-1, and we found that colonies generated by EC-SP cells express all these molecules. We have now added a new Supplementary Figure S8 and rewrote the Results (p10, lines 11-14).

In terms of morphological characterization of a true endothelial phenotype, we performed transmission electron microscopy (TEM) with ECs from EC-SP cells cultured on OP9 stromal cells. Unfortunately, we could not identify ECs on OP9 stromal flat layers by TEM. It was technically too difficult. Therefore, to assure ourselves that original EC-SP cells are morphologically true EC, we performed TEM analysis with sorted EC-SP cells, directly. This revealed a Glycocalyx on sorted EC-SP cells (Figures for the Referee, Figure R3; presented at the end of this letter). However, due to the technical problem that cells are kept out of fixation medium when they are cytospun onto the slide glass, the cell membrane observed by TEM is damaged, the Glycocalyx is not so clear, and Palade bodies are lost. The appearance might therefore not exactly reflect the true phenotype of EC-SP cells. For this reason, we abandoned efforts to show the morphological characterization of EC-SP cells. If the reviewer recommends showing the image in Figure R3, we can certainly insert it as a supplemental figure.

5. It is disingenuous to say that a single cell form colonies in vitro, when in reality equal proportions of EC-SP cells are cultured. To what extent do the EC-SP cells actually proliferate? Statistics other than a vague description must be provided by here.

Following this comment, we performed three independent experiments. 1) Time-lapse analysis to show that a single EC-SP cell can form an EC colony. This is now added as a new part in Figure 5, and new Supplementary Movie S1. We have re-written the Results accordingly (p11-12, lines 17-1). 2) To find out to what extent ECs actually proliferate, we stained nuclei of EC colonies and counted the number that constitute a single colony. We have now added a new part to Figure 4 and rewrote the Results (p11, lines 6-9). 3) We had shown the results representing EC numbers at day 8 after

culturing of EC-SP cells, but in this revised version we report that EC colonies continue to proliferate**,** using time-lapse analysis. Therefore, to estimate to what extent EC-SP cells proliferate, we performed modified long-term culture-initiating cell (LTC-IC) assays on OP9 stromal cells. Because the results of the LTC-IC assay are relevant to comment 6, we will respond to this in reply to that comment. We found that ECs derived from EC-SP cells have the ability to proliferate more persistently than those from EC-MP cells. This result has been added to Figure 5D and the Results p12, lines 9-11.

6. Stem/progenitor cells are defined as cells that are able to produce high numbers of daughter cells. The authors do not prove this fundamental stem cell quality.

On Page 9, limiting dilution analysis and culturing equal proportion of EC-SP from normal and EGFP mice actually did not reveal exactly to what extent the stem/progenitor population could proliferate. (2 quotes from the manuscrip: "To assess clonal expansion of ECs from single cells, equal proportions of sorted EC-SP cells from normal mice and EGFP mice were mixed and cultured. " "The frequency to form colonies with a cordlike structure was significantly higher in EC-SP cells by a factor of ten")

We apologize for the ambiguous wording, but in the manuscript we cultured equal numbers of EC-SP from normal and EGFP mice together to show that a single EC-SP cell can form a colony, not to measure proliferation. We have also done limiting dilution analysis to establish the frequency of colony-forming cells in the EC-SP fraction (which we calculate to be one in 6.6 EC-SP cells) compared to the EC-MP fraction (only one in 66**)** and stated *"The frequency to form colonies with a cordlike structure was significantly higher in EC-SP cells by a factor of ten"* in the manuscript. In other words, we performed these two experiments to show clonal expansion of ECs from a single EC-SP cell and to estimate the frequency of the ECs with colony-forming ability, not to examine the proliferative capacity of the EC-SP cells. We showed proliferation only by the number of EC colonies (Figure 4C) and the number of ECs formed on OP9 cells after culturing 10 days (Figure 4D). According to this suggestion by the referee, we now show the proliferative capacity of ECs derived from EC-SP cells by performing time-lapse analysis, nuclear staining of ECs cultured on OP9 cells, and a modified LTC-IC assay, as described in the response to comment 5. Moreover, we tried to identify the SP phenotype again in ECs derived from EC-SP cells cultured on OP9. However, the SP phenotype of EC-SP cells disappeared soon after culture on OP9 cells. Therefore, maintenance of EC-SP cells with an immature phenotype in vitro is difficult at present. As stem cell factor (SCF), Wnt3a, Notch-L and other factors for the maintenance and expansion of hematopoietic stem/progenitor cells have now been identified, isolation of maintenance factor(s) for endothelial stem/progenitor cells may enable us to demonstrate clear differences in the stem/progenitor activity of EC-SP cells for expansion of daughter cells in LTC-IC assays. However, at present, it is difficult to show fundamental qualities of stemness in EC-SP cells. We wish to isolate molecules that support the maintenance of the immature phenotype of EC-SP cells in the future and hope that the reviewer comprehends this point at present.

7. In the manuscript, the authors mentioned SP population peaked on D3 and declined to steady state after 2 weeks. How many of these cells will retain a progenitor/ stem cell ability? Or it might be the case that the ability of proliferation of the cells in SP population or MP population is turned on and off in response to enviromental conditions? Because SP population authors defined is still a heterogenous population, it would be interesting to know the hierarchy between SP population and MP. For instance, is the stem/progenitor population give rise to other EC subpopulations and maintain their abilities in vivo.

This is a very interesting point that the reviewer raises. First, as suggested by the reviewer, we performed colony-forming assays using EC-SP and EC-MP cells on day 3 and 14 after induction of ischemia, and showed that EC-SP cells in the ischemic environment possess similar colony-forming ability as under non-ischemic conditions. However, EC-MP cells in the ischemic environment did not acquire colony-forming function. We have added these results in Supplementary Figure S15 and added new sentences in the Results (p13, lines 5-7).

The origin of EC-SP cells which are found to be increased after ischemia is still unknown. It is possible that EC-SP cells proliferate or some of the EC-MP cells acquire the EC-SP phenotype; or perhaps EC-SP cells originate from unidentified multipotent tissue stem cells that can produce true EC. This issue can be addressed by finding new markers of EC-SP cells and generating Tg mice

expressing marker molecules such as EGFP or LacZ under transcriptional control of EC-SP-specific genes that would enable us to trace the development of EC-SP cells in vivo. However, at present, we do not have any available molecule to identify EC-SP cells specifically. We are currently engaged in a major effort to isolate candidate molecules and would like to be able to document the development of EC-SP cells in the future.

In terms of investigations of the hierarchy of EC-SP cells and EC-MP cells, analyzing transplanted cells would be required. Although this is very important issue and certainly a challenging problem, we could not address this here using the hind limb ischemia model. When EC-SP cells from GFP mice were injected in this model, we confirmed that ECs derived from EC-SP cells generate enlarged mature blood vessels. We expected that EC-SP cells could again be isolated from GFP-positive ECs from the hind limb, but we were unable to detect any such cells. This suggests that EC-SP cells differentiate immediately after injection into ischemic muscle. However, we confirmed that EC-SP cells can differentiate into EC-MP cells in vivo, but the reviewer may not expect this kind of hierarchy. A niche is required to support stemness in vivo. Therefore, we need other models maintaining stemness of EC-SP cells in order to investigate hierarchy. Thus far, we regret that we have not been able to overcome this difficult problem.

8. In fig. 6E, both SP and MP form colonies and blood vessels.
This indicates that -in contrast to authors' conclusions- actually both populations contain endothelial cells that may have the capacity to contribute to angiogenesis under certain circumstances. If this is the case, then EC progenitors do not actually exist, but all ECs migh have an equal capacity to proliferate and produce daughter cells under certain conditions.

Again, we apologize for any ambiguous wording. In the manuscript we did not clearly state that we do think there are colony forming cells in the EC-MP population. It is well known that in the hematopoietic side population assay, hematopoietic stem cells are included in the MP fraction (Morita et al. 2006 Blood: 108, 2850-6) and the same may be the case with the EC side population assay. Indeed, although the frequency is significantly lower, some EC-MP cells do form colonies in vitro and in vivo. We think that the side population assay enriches progenitor/stem cell populations of EC in the SP region but does not completely exclude progenitor/stem cells from the EC-MP region. We have now added some considerations of this problem to the Discussion (p19, lines 1-5).

However, as shown in our experiments, we would like to say that the EC-MP population does indeed contain cells able to form small colonies in vitro but they do not clearly contribute to mature blood vessel formation when injected in vivo.

9. Indeed, I would be more enthusiastic if the authors identified more powerful markers for the selection of the proposed EC progenitors. For instance, in the manuscript, authors mentioned CD44 low expression and E-selectin on EC-SP cells. Could these provide a functional selection marker for the potential progenitor population?

We appreciate your kind suggestion. Accordingly, we performed FACS analysis of $CD31^{+}CD45^{-}$ EC with CD44 and CD62E. As shown in the (Figures for the Referee, Figure R4; presented at the end of this letter), in $CD31^{+}CD45^{-}$ gated cells, there is no clear population in the $CD44^{-}CD62E^{+}$ region. But when cells gated as in Figure R3 (black box) are stained with Hoechst, approximately 10% are in the SP fraction. This percentage is higher than when gating with CD44 alone, showing our results from microarray analysis to be accurate. However, we think that these results are too preliminary to suggest that the $CD44^{-}CD62^{+}$ phenotype represents a true marker of EC progenitor/stem cells. Indeed, in some organs we have good evidence that the $CD44^{-}CD62E^{+}$ EC fraction makes up a new population by FACS analysis. Many studies must be done, including establishing the relationship between EC-SP cells, cells active in long-term blood vessel reconstitution assays, cells present in different organs, and their surface markers - at least for all the experimental approaches that we have pursued here. More intensive investigations of this problem are now ongoing, and we plan to report on this in a separate manuscript in the near future. At the present time we would like just to add some words on this to the Discussion (p22, lines 11-13).

10. The work and the idea would be remarkably benefited by an unequivocal marker of endothelium such as use of the VE-cadherin-Cre X tamoxifen induced by ROSA lac Z or expressing beta-gal under VEGFR2 promoter mouse, where LacZ expresses in arterial, venous and capillary enodthelial cells. Isolation of CD31+CD45- SP poplation from these endothelial specific reporter animals and functional assayed in vivo would greatly increase confidence in the data.

We thank the reviewer for this suggestion. We crossed the VE-Cadherin-Cre X tamoxifen mouse with a loxP-CAT-EGFP reporter mouse and isolated $CD31^{+}CD45^{-}$ EC-SP cells from these endothelial-specific reporter mice. We have now added data in the new Supplementary Figure S11 showing the presence of $CD31^{+}CD45^{-}$ EC-SP cells in the GFP^{+} (VE-$cadherin^{+}$) population having in vitro colony-forming ability (Figure 4 H) and blood vessel constituting ability in vivo (Supplementary Figure S11). Results are now described on p11, lines 9-15, p15, line 4-9 and p19, lines 12-13.

MINOR COMMENTS:

1 If this is a endothelial progenitor/stem cell population residing on the preexisting blood vessels, how can this population compensate for the endothelial cell loss during normal lifelong cellular turnover and how are they induced to generate novel ECs in angiogenic situations?

Thank you for this comment. The relationship between EC-SP cells and normal lifelong cellular turnover is not clear, because we showed merely the role of these cells in ischemia. We suggest the importance of the endogenous function of EC-SP cells for the maintenance of the vasculature and for neovascularization during angiogenesis; however, we do not know the key regulator of EC-SP cell survival at present. Therefore, we cannot deplete EC-SP cells in vivo by using exogenous factors or by disrupting the function of EC-SP cells for new vessel formation. However, we were able to evaluate whether differentiation or proliferation of ECs from EC-SP cells depends on VEGF signaling. In summary, we performed colony-forming assays using sorted EC-SP cells in the presence of soluble VEGFR2-Fc and found that ECs are produced in a VEGF-dependent manner, as with other ECs. Furthermore, we tried to block bFGF by adding PD166866 or SU 5402; however, these inhibitors caused destruction of the OP9 feeder cells, so we could not evaluate whether development or proliferation of ECs from EC-SP cells is bFGF-dependent or not. Therefore, we have now added these results showing that the growth or survival of the EC-SP cells also depends on VEGF in Supplementary Figure S10 and described this in Results (p11, lines 1-3).

2. The author showed that the SP population does not differentiate into smooth muscle cells in vivo in ischemic models. However, Most stem/progenitor cells have the ability to differentiate into other cell type in vitro, depending on the conditions. Can tthe cells studied here give rise to any other cell types, e.g vascular smooth muscle cells or pericytes in vitro?

Following this suggestion, we have cultured EC colonies in the presence of TGF-beta. We checked more than 300 colonies, i.e. > 9000 ECs, but we did not find any evidence for endothelial mesenchymal transition (EndMT) using this OP9 co-culture system. Moreover, as detailed in our response to Referee #2, Comment #,3 we performed several experiments to test the hematopoietic potential of EC-SP cells, but failed to induce hematopoietic cells in vitro and in vivo. The results are now shown in Supplementary Figure S12 and discussed in Results (p11, lines 15-16) and Discussion (p20, lines 13-17).

3. Images in Fig. 6G is not sufficient magnification to determine the anatomice location of the GFP cells.

As suggested by the reviewer, we have now changed the size of the figure to show GFP-positive cells more clearly (now Figure 7G)

4. FACS analysis in Fig7 H is not informative, what is the statistic for this identical experiment?

The FACS analysis shown in Fig. 7H (now Fig. 8H in the revised manuscript) is representative of data showing the relationship between CD44 expression and the percentage of EC-SP cells. We give the statistics in Fig. 7I (now Fig. 8 in the revised manuscript). We apologize for the absence of statistical evaluation, which is now included in Fig. 8I.

5. Page 10 typo: Sham

"Sham" is not a typo. It refers to the control, sham, operation, a normal term.

This paper has been carefully reviewed by an experienced medical editor whose first language is English and who is specialized in the editing of papers written by physicians and scientists whose native language is not English.

Referee #2

The authors provide evidence that the side population subset of CD31+CD45- cells from different mouse organs shows features of endothelial stem and progenitor cells both in vitro and in vivo, as indicated by their contribution to vascular endothelium regeneration after experimental ischemia. The work is extremely interesting and nicely executed but would benefit from further clarifications:

Thank you very much for your review comments and valuable suggestions for improving our manuscript. In response, we have added further experiments and discussion.

1. Which are the ABC transporters that mediate the behavior as side population of these cells?

As shown in Figure 2B, we originally reported that EC-SP cells express higher ABCB1a (MDR1a) RNA than EC-MP cells. ABCB1a is a member of the ABC transporter gene family correlating with the SP phenotype (Bunting et al. 2000 Blood 96:902-909, Lin et al. 2006 Cell Research 16:857-871). Moreover, the expression of ABCG2, which is the ABC transporter most often correlated with the SP phenotype, tended to be higher (but the difference did not reach significance).

Following this suggestion, we have now also evaluated the expression of several other ABC transporters that are reported to correlate with SP phenotype, notably ABCA3, ABCB2, ABCC7 and ABCA5 (Chiba et al. 2006 Hepatology 44:240-251, Norwood et al. 2004 Leuk Res 28:295-299) and have re-evaluated ABCB1a and ABCG2. Of these, ABCB2 and ABCA5 were also higher in EC-SP cells. The results of re-evaluation of ABCB1a was comparable to our original data, but for ABCG2, we have done qRT-PCR several times more (previously n=7 and this time we added 4 experiments and now n=11, see Figures for the Referee, Figure R5; presented at the end of this leter) and found that ABCG2 was also significantly higher in the EC-SP population (p=0.018) (In the original manuscript we stated “Expression level of the ABC transporter ABCG2 was higher in EC-SP cells but did not reach significance due to wide interindividual variability” in the legend of Figure 2). Now we have added a new Supplementary Figure S3, corrected the results of ABCG2 in Figure 2B and rewrote the Results (p7, lines 12, 14-15, underlined).

In summary, we think that the difference in expression of each transporter gene is not marked, but that several ABC transporters (notably ABCB1a, ABCB2, ABCA5 and ABCG2) could mediate effects in a synergistic manner and could determine the functionality of the EC-SP phenotype.

2. The absence of a marker for CD31+SP cells does not allow their localization. The CD31+ SP cells were purified from whole dissociation of different tissues, not specifically from vessels. Thus, it is theoretically possible that this rare population resides also in non-vascular organs. I am not sure that the experiments shown in Fig. 7 can prove that CD31+SP cells are exclusively present in peripheral vascular endothelium.

Thank you for this comment. We do agree that staining with Glycam1 is not sufficient for showing that EC-SP cells reside at vessel walls. Therefore, we performed lectin perfusion analysis to confirm that the $CD31^{+}CD45^{-}$ EC-SP cells do reside at the vessel wall (intra-luminal cavity). We have now added new results in Fig. 1E-G and new sentences in the Results (p6-7, lines 12-1, underlined). This analysis revealed that approximately 91% of EC-SP cells are lectin-positive, suggesting that most do reside at the inner surface of blood vessels. Although it is possible that not all of intraluminal endothelial cells were labeled with lectin, this result indicates that a small population of EC-SP cells may reside deeper within the blood vessel wall or entirely elsewhere. Therefore, we have added a new paragraph in the Discussion on this (p21, lines 10-16). As also detailed in our response to Referee#1, Major comment #10, we performed EC-SP analysis with VE-cadherin-Cre ERT X LoxP-CAT-EGFP mice and found that EC-SP cells are GFP-positive (Fig. 4H, Supplementary Fig. S11 and the Results p11, lines 9-13). This suggests that EC-SP cells are found within a population already committed to ECs. We are sure that almost all, but not entirely all, EC-SP cells are indeed ECs based on those experiments as described above.

3. Although these cells do not express CD45, it would be good if the hematopoietic potential could be tested by analyzing the hematopoietic cell repopulation in subletally irradiated mice. This could be easily done by using GFP mice.

Thank you for this suggestion. We are very interested in the hematopoietic potential of EC-SP cells and have done several experiments on this issue, but could not induce hematopoietic cells to develop from EC-SP cells. As suggested by the reviewer, we observed a contribution of EC-SP cells derived from GFP mice to BM hematopoiesis, as revealed by transplantation to sublethally irradiated mice (4 Gy). We could not identify any contribution of EC-SP cells to hematopoietic cells at 2, 4 and 8 weeks after transplantation. Moreover, we performed CFU-S assays (Spangrude et al. Science 241, 58-62, 1988) and found no colonies in the spleen after 12 days. We also performed in vitro colony-forming unit in culture (CFU-c) assays, but again failed to detect any hematopoietic colony formation from EC-SP cells. We have now added these data in Supplementary Figure S13 and in the Results (p11, lines 15-16) and Discussion (p20, lines 13-17).

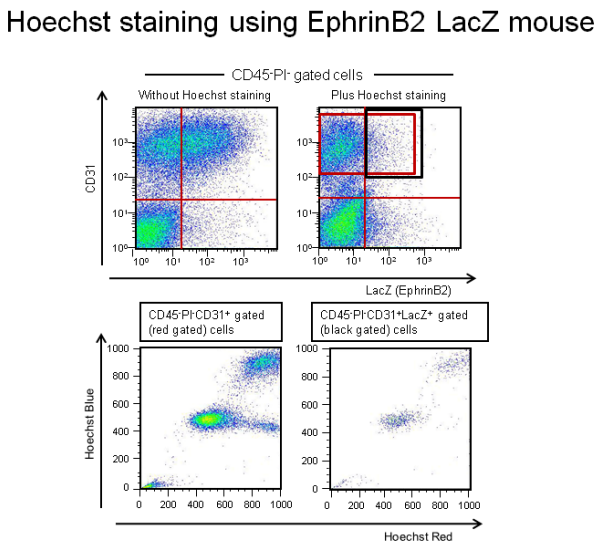

Figure R1 Naito H. et al.

Figure R1. Hoechst analysis utilizing Ephrin B2 LacZ Knock-In mouse.

FACS plots of hind limb $CD45^-$ cells from Ephrin B2 LacZ knock-In mice. Cells were analyzed for CD31 and LacZ (Ephrin B2) (left upper panel). Hoechst staining was performed after FluoReporter-LacZ method (right upper panel). LacZ positive cells were almost lost (black gate in the right upper panel). The cells in the black gated area in the right upper panel were further analyzed for the exsistance of SP cells (right lower panel). There were no SP cells in the black gated area. Furthermore, $CD31^+CD45^-$ ECs (red gate in the right upper panel) were analyzed for the exsistance of SP cells (left lower panel). There were no SP cells in the red gated area too. These results show that hoechst method and LacZ staining can not be performed simultaneously.

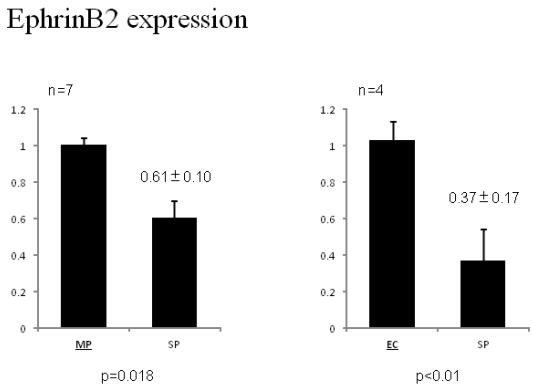

Figure R2 Naito H. et al.

Figure R2. Expression of EphrinB2 in EC-SP cells

Quantitative RT-PCR analysis of the EphrinB2 expression using total RNA from EC-SP cells (SP) and EC-MP cells (MP) is shown on the left. Quantitative RT-PCR analysis of the EphrinB2 using total RNA from EC-SP cells (SP) and total ECs (EC) is shown on the right.

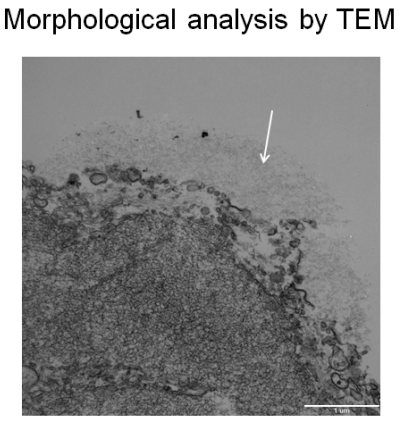

Figure R3 Naito H. et al.

Figure R3. Glycocalyx observed in EC-SP cells

EC-SP cells were sorted and cytospun onto slides. TEM analysis revealed a Glycocalyx on sorted EC-SP cells (white arrow). However, due to the technical problem that cells are kept out of fixation medium when they are cytospun onto the slide glass, the cell membrane observed by TEM is damaged, the Glycocalyx is not so clear, and Palade bodies are lost. Scale bar, 1μm.

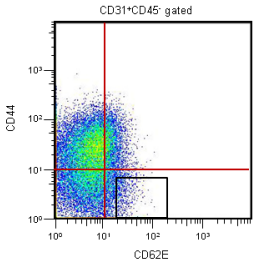

Figure R4 Naito H. et al.

Figure R4. FACS analysis of CD44 and CD62E expression in $CD31^{+}CD45^{-}$ ECs

FACS plots of hind limb $CD31^{+}CD45^{-}$ ECs. Cells were analyzed for CD44 and CD62E (E-selectin). $CD44^{-}CD62E^{+}$ ECs are shown in the black gate.

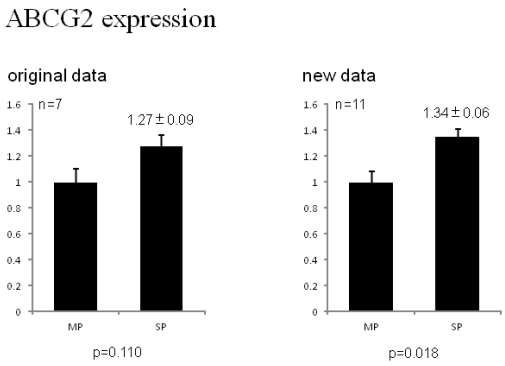

Figure R5 Naito H. et al.

Figure R5. Expression of ABCG2 in EC-SP cells

Quantitative RT-PCR analysis of the ABCG2 expression using total RNA from EC-SP cells (SP) and EC-MP cells (MP). Original data is shown on the left and new data is shown on the right.

2nd Editorial Decision 23 November 2011

The paper has been re-reviewed by one of the original referees, with no further comment.

