EMBO reports

Notch-inducing hydrogels reveal a perivascular switch of mesenchymal stem cell fate

Ulrich Blache, Queralt Vallmajo-Martin, Edward R Horton, Julien Guerrero, Valentin Djonov, Arnaud Scherberich, Janine T Erler, Ivan Martin, Jess G Snedeker, Vincent Milleret, Martin Ehrbar

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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 3 April 2018

Thank you for the submission of your research manuscript to EMBO reports. I have to apologize for the delay in getting back to you, but one of the referees we asked to assess the manuscript delivered his report severely delayed. In addition, there was the Easter break that slowed down things further. But, we have now received reports from three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think the manuscript is of interest, but requires a major revision to allow publication in EMBO reports. All three referees have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript. As the reports are below, I will not detail them here. We feel, however, that in particular the two major points by referee #2 (differences depending on the angiogenic factor employed, expression of typical marker proteins), and referee #3 (demonstrating that the niche behaviour described is physiologically relevant) need to be addressed experimentally. Also the minor points by referee #1 need attention.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can

submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature.

For more details please refer to our guide to authors:
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Important: All materials and methods should be included in the main manuscript file.

See also our guide for figure preparation:
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Please also format the references according to EMBO reports style. See:
http://embor.embopress.org/authorguide#referencesformat

Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable. See:
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We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)

Please also note that we now mandate that all corresponding authors list an ORCID digital identifier that is linked to their EMBO reports account!

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Summary:

In this manuscript by Blache, et al., the authors have applied an impressive array of tools to better understand the regulation of mesenchymal stem cell (MSC) fates by their location in the perivascular niche. MSCs were encapsulated in RGD-modified MMP-degradable PEG hydrogels, either in monoculture or in co-culture with endothelial cells (ECs), and their phenotype examined

with transcriptomics (with key transcripts confirmed via qRT-PCR). The PEG hydrogel system has been previously described, and involves conjugation of FXIIIa substrates to multi-arm PEG and either adhesive or degradable peptides, which can then be cross-linked together in the presence of FXIIIa and calcium. MSCs were found to support vascular morphogenesis in the presence of FGF-2 in a manner that was dependent on MSC-secreted fibronectin and MMPs. Co-culture of MSCs with ECs led to an upregulation of vascular basement membrane proteins and Notch signaling components in the MSCs. Strikingly, the conjugation of the Notch ligand Jagged1 to the PEG hydrogel appeared to be able to effectively replace the ECs in terms of the altered gene expression profiles of the MSCs, and the effects of Jagged1 on MSC were reversible.

Overall this work is an excellent demonstration of the power of synthetic hydrogel biomaterial platforms to ask mechanistic questions in cell biology, in this case involving the regulation of MSC fate by elements in the perivascular niche. The results from the FN knock-down experiments (Fig. 1C and Fig. S2C) are very important. It is already well established that FN is required for vascular development, but most efforts with synthetic gels typically involve conjugating RGD only. The finding that MSCs secrete FN, and that it is essential for vascular morphogenesis is consistent with the developmental biology literature, but to my knowledge never before demonstrated in an engineered hydrogel system.

The TEM images in Fig. 2F are beautiful, and the transcriptomic analyses are thorough, well done, and insightful. The use of multiple types of MSCs in Fig. 4, and of MSCs from multiple donors in Fig. S4, was very helpful to the overall arguments.

The manuscript is well-written, logical, high quality, and the conclusions and interpretations are largely supported by the data. Overall, I am very favorably impressed. I have just a few minor questions/comments.

Minor questions:

1. The results from the PEG gels containing immobilized Jagged1 suggests that BM-MSC up-regulation of key genes is not quite as complete as the case of EC/MSC co-culture. COL4A1 in particular shows an increased expression in the Jagged1 gels, but the levels are much lower than those induced by ECs. To me, this suggests that Jagged1 by itself is insufficient to completely control MSC phenotype in the perivascular space, which is also somewhat satisfying given the complexity of the niche interactions and the huge number of differentially regulated genes. However, I think this point was pretty much glossed over by this statement in the Results section (page 8): "Moreover, all tested ECM genes that were induced by BM-MSCs by endothelial cells (Fig. 4A) were also induced in BM-MSCs by the matrix immobilized Jagged1." While this statement may be factually true, the relative amounts of the expressed genes were different, in particular the COL4A1 transcript. This could be more explicitly articulated or discussed.

2. Is there any indication that changing the amount of immobilized Jagged1 changes the response of the MSCs? Perhaps this is related to the differential gene expression levels in MSCs cultured within Jagged1-functionalized gels vs. co-culture with ECs?

3. The quantification scheme for vascular morphogenesis involves characterization of the length of CD31+ structures in 2D projections, but it seems like there is quite significant z-directionality to the vascular morphogenesis (Fig. 2E) that might be missed via this method. Was a full 3D (i.e., length/volume) quantification considered?

4. The authors some MMP inhibitors with specificity to MMP-2 and -9, and show in Fig. S2D that inhibiting either of these completely blocks vascular morphogenesis. I found this result somewhat surprising given the evidence that the membrane-type MMPs (MT-MMPs, and MT1-MMP) are reportedly essential for vascular development, while MMPs-2 and -9 are not required. Is it possible the inhibitors used also affect MT-MMPs, especially at the higher doses?

5. In the Methods section describing the rheometry (page 11), the authors indicate gels of 100 uL were made. But what volume of gels were used for the EC:MSC co-cultures? I don't believe the final volume was specified (only that a final cell concentration of 1, 1.5, or 3 million cells/mL was used). The casting volume would be helpful to know.

Referee #2:

Comments:

Blanche et al very elegantly describe how endothelial cells actively orchestrate the behavior of perivascular MSCs that all can differentiate into pericytes to form stable new blood vessels. The work is based on a hydrogel system, which allows 3D cell culture. The production of these hydrogels has been published by these groups before. Here they use it for the first time to culture endothelial cells and MSCs. It is absolutely astonishing to see how this system can be used to generate luminized vascular structures in which the endothelial tubes are well covered with pericytes. There have been numerous attempts to achieve this in the past and I believe that this system is outstanding. The images are absolutely beautiful. Secondly, the authors use this system to study the impact of the Notch ligand Jag1 in MSC differentiation. It is well known that endothelial cells signal to pericytes and smooth muscle cells via Jag1. This can activate Notch3 receptors and thereby alter their behavior e.g. the synthetic vs. the contractile phenotype. Here the authors show that interaction of Jag1 with Notch receptors in MSCs is controlling their differentiation. Recombinant Jag1 bound to the hydrogels was sufficient for this process. The most interesting remark is that this process is reversible, explaining why this commitment of MSCs is restricted to those cells in close proximity with ECs.

This study may clarify the controversial discussion regarding how MSCs were committed to the perivascular program. Most importantly, the 3D hydrogels will provide a very valuable tool for researchers in the field of vascular biology to generate mature vessels in vitro and to manipulate them with recombinant proteins. It could also be a highly valuable tool for researchers in the Notch signaling field to test impact of different ligands in 3D cell differentiation assays.

I am quite enthusiastic about this work and I do see only a few points that need to be addressed:

1) The Authors used FGF-2 to induce angiogenesis. This is an important growth factor but of course it is essential to test how the most potent vascular growth factor VEGF-A acts in this system. It would be interesting to see whether or not there are differences in some of these processes depending on the angiogenic factor employed. This is especially important considering the close relation between VEGF-A and Notch signaling in endothelial cells.

2) In Fig. 1 the authors used FN for their experiments, however, there is not an explanation for why this particular ECM protein was used.

3) Electron microscopy shows clear indication that MSC turn into pericytes. It would be interesting to see if these cells also express some of the typical marker proteins.

Minor points:
1) Abstract: "The fate of mesenchymal stem cells (MSCs) in the perivascular niche, as well as factors controlling their fate, is not understood." This is obviously wrong. One should at least say "poorly understood".

2) Scale bar in Figure 2B is missing.

3) Will the RNAseq data be fully available in a public database?

Referee #3:

Overall, the questions the authors address and the methods are interesting. The formation of vascular networks and the support of the MSC in this process is well documented and impressive. The role of Notch in modifying MSC behaviour and perivascular cells is not novel, but the ability to mimic the effects of endothelial cells in this 3D environment is worth reporting, in my view.

On the general questions relating to MSCs and their niche, I believe this work falls short of

providing relevant new insights. In my view the current literature shows there is limited evidence of stem cell behaviour in the perivascular niche in vivo, and therefore this "reversible switch" may in fact be another in vitro artefact.

In the absence of demonstrating that this is indeed a niche behaviour that is relevant, I don't agree with the authors that they provide a model to study the niche in vitro. Many papers demonstrate that different stiffness of matrix affect MSC in vitro, and this paper is another one to demonstrate it, yet with a nice 3D component added.

Overall, it may be appropriate for publishing in EMBO reports, but the conclusions on the true niche should be strengthened, or moderated.

1st Revision - authors' response 16 May 2018

Referee #1:

Summary:
In this manuscript by Blache, et al., the authors have applied an impressive array of tools to better understand the regulation of mesenchymal stem cell (MSC) fates by their location in the perivascular niche. MSCs were encapsulated in RGD-modified MMP-degradable PEG hydrogels, either in monoculture or in co-culture with endothelial cells (ECs), and their phenotype examined with transcriptomics (with key transcripts confirmed via qRT-PCR). The PEG hydrogel system has been previously described, and involves conjugation of FXIIIa substrates to multi-arm PEG and either adhesive or degradable peptides, which can then be cross-linked together in the presence of FXIIIa and calcium. MSCs were found to support vascular morphogenesis in the presence of FGF-2 in a manner that was dependent on MSC-secreted fibronectin and MMPs. Co-culture of MSCs with ECs led to an upregulation of vascular basement membrane proteins and Notch signaling components in the MSCs. Strikingly, the conjugation of the Notch ligand Jagged1 to the PEG hydrogel appeared to be able to effectively replace the ECs in terms of the altered gene expression profiles of the MSCs, and the effects of Jagged1 on MSC were reversible.
Overall this work is an excellent demonstration of the power of synthetic hydrogel biomaterial platforms to ask mechanistic questions in cell biology, in this case involving the regulation of MSC fate by elements in the perivascular niche. The results from the FN knock-down experiments (Fig. 1C and Fig. S2C) are very important. It is already well established that FN is required for vascular development, but most efforts with synthetic gels typically involve conjugating RGD only. The finding that MSCs secrete FN, and that it is essential for vascular morphogenesis is consistent with the developmental biology literature, but to my knowledge never before demonstrated in an engineered hydrogel system. The TEM images in Fig. 2F are beautiful, and the transcriptomic analyses are thorough, well done, and insightful. The use of multiple types of MSCs in Fig. 4, and of MSCs from multiple donors in Fig. S4, was very helpful to the overall arguments. The manuscript is well-written, logical, high quality, and the conclusions and interpretations are largely supported by the data. Overall, I am very favorably impressed. I have just a few minor questions/comments.
We thank the reviewer for assessing our manuscript and for his/her overall very encouraging and positive verdict. Based on his/her constructive comments, we have added further experimental data and revised the manuscript as detailed below. Additionally, based on the reviewers comment we moved the co-culture FN knockdown experiments from supplementary data (old Fig. S2C) to the main manuscript (now in Figure 3D).

Minor questions:

1. The results from the PEG gels containing immobilized Jagged1 suggests that BM-MSC up-regulation of key genes is not quite as complete as the case of EC/MSC co-culture. COL4A1 in particular shows an increased expression in the Jagged1 gels, but the levels are much lower than those induced by ECs. To me, this suggests that Jagged1 by itself is insufficient to completely control MSC phenotype in the perivascular space, which is also somewhat satisfying given the complexity of the niche interactions and the huge number of differentially regulated genes. However, I think this point was pretty much glossed over by this statement in the Results section (page 8): "Moreover, all tested ECM genes that were induced by BM-MSCs by endothelial cells

(Fig. 4A) were also induced in BM-MSCs by the matrix immobilized Jagged1." While this statement may be factually true, the relative amounts of the expressed genes were different, in particular the COL4A1 transcript. This could be more explicitly articulated or discussed.
We agree with the reviewer that, given the complexity of the niche, Jagged1 by itself probably only partially controls the perivascular commitment and ECM switch of MSCs. Additionally, the level of gene induction in MSCs depends on the amount of hydrogel-immobilized Jagged1 and can be increased by more Jagged1 (see also point 2 of #1).
Since it is very difficult to know a) how much immobilized Jagged1 is actually sensed by the MSCs and b) how much Jagged1 is presented on the cell surface of ECs in co-cultures, the levels of gene induction between both systems need to be compared with caution. We believe that these experiments rather show the mechanism behind than allow for directly comparing the levels of gene induction.
➔ New data now presented as Figure 5F
➔ Text re-worded for clarification in results and discussion section

2. Is there any indication that changing the amount of immobilized Jagged1 changes the response of the MSCs? Perhaps this is related to the differential gene expression levels in MSCs cultured within Jagged1-functionalized gels vs. co-culture with ECs?

To address this very important question we immobilized increasing concentrations of Jagged1 and control IgG (10, 70, 500 nM) into the hydrogels (while keeping the concentration of the TG-ZZ linker constant). Indeed, the amount of immobilized Jagged1 does change the response of the MSCs in a dose-dependent manner. We agree with the reviewer that this might be related to the differential gene expression levels in MSCs cultured within Jagged1-hydrogels vs. co-cultured with ECs.
➔ New data now presented as Figure 5F
➔ Text added in results and materials section

3. The quantification scheme for vascular morphogenesis involves characterization of the length of CD31+ structures in 2D projections, but it seems like there is quite significant z-directionality to the vascular morphogenesis (Fig. 2E) that might be missed via this method. Was a full 3D (i.e., length/volume) quantification considered?
It is correct that there is some Z-directionality of the vascular structures, which might be missed by 2D projections. Given the huge number of hydrogels we scanned by confocal microscopy for quantification (>200) and given their sensitivity to heat, we made the compromise to scan for quantification at a Z-resolution that is too low to apply an appropriate full 3D quantification. However, we are confident that the quantification of 2D projections accurately describe the differences between the conditions (see example images used for quantification in Figure EV2 and Figure EV3).

4. The authors some MMP inhibitors with specificity to MMP-2 and -9, and show in Fig. S2D that inhibiting either of these completely blocks vascular morphogenesis. I found this result somewhat surprising given the evidence that the membrane-type MMPs (MT-MMPs, and MT1-MMP) are reportedly essential for vascular development, while MMPs-2 and -9 are not required. Is it possible the inhibitors used also affect MT-MMPs, especially at the higher doses?
We thank the reviewer for the comment. According to the manufacturer, the MMP-Inhibitors we used against MMP-2 and MMP-9 exhibit selectivity over other MMPs by three orders of magnitude (IC_{50} values). However, it cannot be ruled out that at higher concentrations these inhibitors affect other MMPs such as MT-MMPs or MMP-1. Moreover, the PEG system depends strongly on MMP-mediated degradation of the hydrogel backbone and therefore our system might be more sensitive to MMPs (such as MMPs-2 and -9) than other models.
➔ Text re-worded for a more careful interpretation of the data (page 6).

5. In the Methods section describing the rheometry (page 11), the authors indicate gels of 100 uL were made. But what volume of gels were used for the EC:MSC co-cultures? I don't believe the final volume was specified (only that a final cell concentration of 1, 1.5, or 3 million cells/mL was used). The casting volume would be helpful to know.
We thank the reviewer for this suggestion. The casting volume was adjusted to the technical requirements of the read-out experiments. 100 uL was the minimum volume that could be used on the rheometer. For cell experiments used for microcopy, we made hydrogels of 10 or 20 uL. For experiments that required higher absolute cell numbers (FACS and PCRs) we made hydrogels of 40

uL. Importantly, we kept the thickness/height of resulting hydrogel discs constant at 0.95 mm throughout the study. We have added the gel volumes used in the different experiments to the Methods section.
➔ Text added in the materials and methods section.

Referee #2:

Comments:
Blanche et al very elegantly describe how endothelial cells actively orchestrate the behavior of perivascular MSCs that all can differentiate into pericytes to form stable new blood vessels. The work is based on a hydrogel system, which allows 3D cell culture. The production of these hydrogels has been published by these groups before. Here they use it for the first time to culture endothelial cells and MSCs. It is absolutely astonishing to see how this system can be used to generate luminized vascular structures in which the endothelial tubes are well covered with pericytes. There have been numerous attempts to achieve this in the past and I believe that this system is outstanding. The images are absolutely beautiful. Secondly, the authors use this system to study the impact of the Notch ligand Jag1 in MSC differentiation. It is well known that endothelial cells signal to pericytes and smooth muscle cells via Jag1. This can activate Notch3 receptors and thereby alter their behavior e.g. the synthetic vs. the contractile phenotype. Here the authors show that interaction of Jag1 with Notch receptors in MSCs is controlling their differentiation. Recombinant Jag1 bound to the hydrogels was sufficient for this process. The most interesting remark is that this process is reversible, explaining why this commitment of MSCs is restricted to those cells in close proximity with ECs.
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I am quite enthusiastic about this work and I do see only a few points that need to be addressed:
We thank the reviewer for assessing our manuscript and for his/her extremely enthusiastic feedback. Based on his/her constructive comments, we have added further experimental data and revised the manuscript as detailed below.

1) The Authors used FGF-2 to induce angiogenesis. This is an important growth factor but of course it is essential to test how the most potent vascular growth factor VEGF-A acts in this system. It would be interesting to see whether or not there are differences in some of these processes depending on the angiogenic factor employed. This is especially important considering the close relation between VEGF-A and Notch signaling in endothelial cells.
We thank the reviewer for the very interesting suggestion. We have followed the suggestion and conducted several experiments to test and apply VEGF-A 165 as vascular inducing factor. In our system, VEGF-A 165 seems to be less potent than FGF-2, probably because both ECs and MSCs need to be stimulated and VEGF-A 165 in contrast to FGF-2 acts only on ECs. Nevertheless, micro-capillary networks can be induced with VEGF-A 165 (Figure EV2B); which seems to be saturated at 200 ng/ml. To examine whether or not the relation between VEGF-A and Notch signaling would alter the Notch-based ECM switch of MSCs, we did the full set of experiments including co-culture-based micro-capillary network formation in presence of VEGF-A, FACS-isolation of BM-MSCs from these micro-capillary networks and qRT-PCR of the full set of genes investigated in our study. Importantly, we found that the ECM switch of BM-MSCs occurs also when VEGF-A 165 was used as vascular inducing factor (Figure EV6). Overall, the levels of gene induction are similar (intensity, pattern, top six induced genes) with the minor exception that COL4A1 and COL18A1 swapped their position as the most and the second most strongly induced gene, respectively.
➔ New data now presented as Figure EV2B and EV6
➔ Text expansion in results section

2) In Fig. 1 the authors used FN for their experiments, however, there is not an explanation for why this particular ECM protein was used.
We thank the reviewer for raising this point. We had briefly explained in the text why we focused our experiments on FN ("Based on the very early appearance of FN, we asked whether the cell-derived ECM influences the interaction of cells with the engineered microenvironment. We knocked

down FN in BM-MSCs… "). Additionally, we used FN because it is a key protein of mesenchymal cells; because it provides cell-adhesion sites and might be important for initial cell events that would influence tissue morphogenesis long-term; and because it is considered a templating ECM-component for many other ECM proteins (Kubow et al Nature Communications 2015; Kadler et al. Current Opinion in Cell Biology). Finally, as FN is a well-studied protein and present in many cell types, we believe that FN is optimal to a) exemplarily visualize our message of cell-endogenous ECM in 3D matrices and b) to make our finding important to scientists in other fields of research.
➔ Text expansion in results and discussion

3) Electron microscopy shows clear indication that MSC turn into pericytes. It would be interesting to see if these cells also express some of the typical marker proteins.
We have conducted stains of typical marker proteins (PDGFRB, MCAM/CD146, ACTA2/SMA) and added the images to the new Figure 3.
➔ New data now presented as Figure 3A

Minor points:
1) Abstract: "The fate of mesenchymal stem cells (MSCs) in the perivascular niche, as well as factors controlling their fate, is not understood." This is obviously wrong. One should at least say "poorly understood".
The reviewer is absolutely right and we have toned down the statement accordingly.
➔ Text change in the abstract

2) Scale bar in Figure 2B is missing.
Thanks. Now present. 3) Will the RNAseq data be fully available in a public database?
Yes, RNAseq data are deposited and will be available on one of data base recommended by EMBO press (ArrayExpress accession E-MTAB-6849).

Referee #3:
Overall, the questions the authors address and the methods are interesting. The formation of vascular networks and the support of the MSC in this process is well documented and impressive. The role of Notch in modifying MSC behaviour and perivascular cells is not novel, but the ability to mimic the effects of endothelial cells in this 3D environment is worth reporting, in my view. On the general questions relating to MSCs and their niche, I believe this work falls short of providing relevant new insights. In my view the current literature shows there is limited evidence of stem cell behaviour in the perivascular niche in vivo, and therefore this "reversible switch" may in fact be another in vitro artefact.

In the absence of demonstrating that this is indeed a niche behaviour that is relevant, I don't agree with the authors that they provide a model to study the niche in vitro. Many papers demonstrate that different stiffness of matrix affect MSC in vitro, and this paper is another one to demonstrate it, yet with a nice 3D component added. Overall, it may be appropriate for publishing in EMBO reports, but the conclusions on the true niche should be strengthened, or moderated.
We thank the reviewer for assessing our manuscript and for his/her supportive feedback. Furthermore, we are grateful for the valuable and helpful comment on the true niche. We are very aware that future in vivo studies may be necessary to dissect the nature of the perivascular niche in physiological settings. In fact it was also not our intention to claim that our in vitro model can fully recapitulate the true in vivo niche. Rather, we provide new insights into the communication between ECs and MSCs in 3D; which is a major component of the perivascular niche. The described ECM switch to our knowledge is totally novel for MSCs, even more with Jagged1.
To address the reviewers concern we have more clearly expressed that the perivascular niche as MSC-reservoir is a *hypothesis* and that we provide and apply a novel and tunable 3D *in vitro* model/tool of the perivascular *microenvironment.* We have furthermore moderated our statements in all parts of the manuscript including title, abstract, introduction and discussion to more accurately.

2nd Editorial Decision 30 May 2018

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the report from the referee that was asked to re-evaluate your study (you will find enclosed below). As you will see, both referees now supports the publication of your manuscript in EMBO reports. Referee #3 was not able to look at the revised manuscript, but we think her/his points have been adequately addressed.

Before we can proceed with formal acceptance, I have the following editorial requests that need to be addressed in a final revised manuscript.

The title reads rather complicated. Could you provide a more simple and direct title (of not more than 100 characters including spaces)?

Please provide the abstract written in present tense throughout.

You can submit up to 5 images as Expanded View. Presently, there are 7. Thus, either try to fuse these, or supply additional supplementary material as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. I also suggest adding the antibodies table in the methods part to the Appendix.

You submitted a large file termed supplementary tables. Below the EV figure legends, there are legends for three tables in the main manuscript text. It seems these refer to the tables in the file "supplementary tables". Please upload these supplementary tables as single files called Dataset EV X (as they are very long, and should not be shown in the online version of the paper), best as excel files, with the legend on the first tab. Finally, please update the callouts for these files, and remove the table legends from the main manuscript text.

Please combine the source data for the main figures, and send one PDF file per figure (i.e. one file for Figure 2, containing the source data for B, C and D).

You further submitted a file called "Source Data for expanded view". However, it is not always clear to which EV figure the tables belong. Thus, please indicate within this file clearly to which figure the source data belongs.

It is unclear if the 2 big cells shown in Fig 1C is one of those shown in the insert, respectively. Can you indicate this? Is the big image a magnification box of the insert?

Please provide the scale bar in Fig. 2E (panel #D reconstruction) bigger, and without any writing (as this will not be legible in the online version). Please indicate the size in the respective figure legend, and not on the scale bar, or above near it.

Please add a paragraph to the M&M section explaining how the statistical testing throughout the manuscript was done, and which tests were used.

Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries (comments), we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

When submitting your revised manuscript, we will also require:
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files (main figures and EV figures) in high resolution (of those with adjusted panels or labels).

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

I am satisfied with the authors' thorough response to my original critique, and agree that this revised manuscript is a significant improvement over the original submission.

Referee #2:

The authors have nicely addressed all of my concerns.

2nd Revision - authors' response 1 June 2018

The title reads rather complicated. Could you provide a more simple and direct title (of not more than 100 characters including spaces)?
We have provided a more simple title in the manuscript document.

Please provide the abstract written in present tense throughout.
We have done this in the manuscript document.

You can submit up to 5 images as Expanded View. Presently, there are 7. Thus, either try to fuse these, or supply additional supplementary material as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. I also suggest adding the antibodies table in the methods part to the Appendix.
We chose to fuse the information from the previous 7 EV figures into now 5 EV figures. Therefore, there will be no Appendix and we would prefer anyways to have the antibodies table in the manuscript itself. However, we leave the final decision regarding the antibodies table to the Editor and the production team and are fine with either ways.

You submitted a large file termed supplementary tables. Below the EV figure legends, there are legends for three tables in the main manuscript text. It seems these refer to the tables in the file "supplementary tables". Please upload these supplementary tables as single files called Dataset EV X (as they are very long, and should not be shown in the online version of the paper), best as excel files, with the legend on the first tab. Finally, please update the callouts for these files, and remove the table legends from the main manuscript text.
Your assumption regarding the tables and legends was right and we have exactly followed your suggestion. Now these files are uploaded as Excel tables called out “Dataset EV X” with having the legends on the first tab of the Excel documents.

Please combine the source data for the main figures, and send one PDF file per figure (i.e. one file for Figure 2, containing the source data for B, C and D).
We have done this.

You further submitted a file called "Source Data for expanded view". However, it is not always clear to which EV figure the tables belong. Thus, please indicate within this file clearly to which figure the source data belongs.
We have now exactly followed the EMBO manuscript guidelines and provided the “Source Data for EV figures” as zip-file. Furthermore, all single files in the zip-file are now also sufficiently labelled to be clearly assigned to the EV figures.

It is unclear if the 2 big cells shown in Fig 1C is one of those shown in the insert, respectively. Can you indicate this? Is the big image a magnification box of the insert?
We have clarified this in the figure legend. Insets are low magnification images of fibronectin stains from a large overview of the hydrogels.

Please provide the scale bar in Fig. 2E (panel #D reconstruction) bigger, and without any writing (as this will not be legible in the online version). Please indicate the size in the respective figure legend, and not on the scale bar, or above near it.
We have done this. The scale bar is now 500 um such as the other scale bar in the sub-figure.

Please add a paragraph to the M&M section explaining how the statistical testing throughout the manuscript was done, and which tests were used.
We have done this.

Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries (comments), we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.
We have accepted all your track changes and worked on the comments. Furthermore, our changes including the re-organized EV figure call outs and legends are visible as track changes.

When submitting your revised manuscript, we will also require:
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files (main figures and EV figures) in high resolution (of those with adjusted panels or labels).
We have updated all files in the manuscript tracking system. Updated/altered figures are Fig. 2 (due to the scale bar in E), Fig. 4 (due to two small typos) and EV1-5. Adjusted figures are provided as done before and according to the EMBO manuscript guidelines as high resolution PDFs (generated from Adobe Illustrator, so that they can be opened and edited by the production team).

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Martin Ehrbar
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-45964

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg 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 description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For statistic evaluations we based our sample size based on earlier studies from our lab (Blache AHM 2016). All experiments were performed at least in triplicates. No statistical methods were used to predetermine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Wherever possible samples were quantified using non-biased quantification software/method.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, for all figures statistical tests are indicated in figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Where possible normal distribution of data was analyzed using Kolmogorow-Smirnow-Test in GraphPad Prism.
Is there an estimate of variation within each group of data?	Yes, all figures have an estimate of variation (standard deviations) as indicated in figure legend.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

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http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. 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), 1DegreeBio (see link list at top right).	Antibodies are specified in Materials and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The responsible ethical committee is indicated in Materials and Methods section.
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.	A statement confirming the ethical approval is given in Materials and Methods.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III 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.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	A data availability section has been inserted at the end of the manuscript indicating the repository where the RNA-seq data can be found.
19. 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 unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Additional datasets are made available as supplementary documents.
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 agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA