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Mechanoinduction of lymph vessel expansion

Lara Planas-Paz, Boris Strilic, Axel Goedecke, Georg Breier, Reinhard Fässler and Eckhard Lammert

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Transaction Report:

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

1st Editorial Decision 04 August 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three reviewers express significant interest in your work, and are broadly in favour of publication. However, all also raise a number of concerns that would need to be addressed in a revised version of your manuscript.

Their reports are explicit, but I would just highlight a couple of points:
- Referee 1 asks for more direct evidence that the observed effects are VEGFR3-dependent. He/she mentions genetic manipulation: unless you already have the relevant mice available, we would not insist on your providing genetic evidence, but pharmacological disruption would be very valuable here. The same goes for analysing the effects of beta1 integrin disruption at later time points, as requested by referee 3.
- Referee 1 also makes some valuable suggestions re. additional in vitro analysis that I would strongly encourage you to follow.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

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. Also please don't hesitate to get in touch if you have any questions about the revision.

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

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

In this MS, the authors provide evidences for a mechano-dependent activation of lymphatic endothelial cells resulting in proliferation, change of cell shape and VEGFR3 activation. The authors further demonstrate that beta1 integrin is crucial in this mechanotransduction pathway. The in vitro and in vivo experiments are well planned.
COMMENTS

Fig 1. To visualize the stretching effect on LEC at E12.5 I suggest to stain cytoskeleton. This staining better support this concept and may be shown together DAPI staining. (similarly in GOF and LOF experiments).
Altogether these experiments demonstrate that the interstitial fluid accumulation modify lymphatic endothelial cell behavior. May the author provide a direct link with VEGFR3. For instance, does the genetic manipulation of VEGFR3 or the mother treatment with a VEGFR3 inhibitor modify the experimental observations reported?

Fig 2. May the author provide a demonstration that in this model H3 histone phosphorylation really reflects LEC proliferative status. On the other hand, other markers of proliferation should be considered and shown.

The authors show interesting in vitro experiments by stretching LEC. However the equipment used does not exactly mirror the in vivo situation. I suggest to plate lymphatic endothelial cells on ECM with different stiffness. This experiment may allow excluding any other possible origin of the increased VEGFR3 activation observed at E12.5 in jugular area. Then the experiments in fig s4 should be also performed by VEGFR3 IP .
However the in vitro experiments reported in Fig 4s are largely convincing.
In my opinion the in vitro approaches could improve the information about the mechanism. It is important to study whether beta1 forms a complex with VEGFR3. Is it present in basal conditions? Is it dependent by cell stretching or VEGC-C stimulation.
Are there any synergistic effect between cell stretching and VEGF-C stimulation in term of LEC proliferation and VEGFR3 phosphorylation? May the author exclude that cell stretching increase VEGF-C production?. This should be easily demonstrated

Fig 6S. Which is the pattern of beta1 expression in vascular endothelial cells in jugular area? The absence of b1integrin in LEC should be demonstrated in other anatomical districts. Do LEC isolated from these mice lack beta1?
In general a more precise and complete description of the phenotype of lymphatic system in these mice is strongly recommended.

There are increasing evidences that Tie-2/Ang system regulate LEC function. Furthermore, a fraction of Tie2 is constitutively associated with a5b1 integrin (JCB 170:993,2005). This point should be addressed in the discussion

Referee #2:

This manuscript reports that interstitial fluid pressure leads to stretched lymphatic endothelial cells (LEC), more LEC proliferation and VGFR3 phosphorylation. Whereas these effects correlated with increasing interstitial fluid pressure in mouse embryos between E11.5 and E12.5, injection of fluid and removal of fluid affected VEGFR3 phosphorylation and LEC proliferation accordingly. Endothelial specific deletion of β1-integrin reduced VEGFR3 phosphorylation and LEC proliferation and abrogated the effect of fluid injection on these two parameters. In addition, injection of an anti β1-integrin blocking antibody reduced the number of LEC in the embryo. As important controls, the manuscript shows that fluid injection into the embryo did not affect blood vessel endothelial cell proliferation; stretching of cultured EC enhanced proliferation; endothelial specific deletion of β1-integrin in vivo did not enhance LEC apoptosis; and the effect of fluid injection on VEGFR3 activation and LEC proliferation was even demonstrated in the ear of adult mice.

This paper describes an elegant and most direct approach of the question what drives the enlargement of the primary lymph sacs in the mouse embryo. By manipulating interstitial fluid pressure via injecting and removal of fluid of short term cultured embryos, the authors succeed in giving clear answers to this question exploiting a demanding technology.

Detailed comments:

1) My major concern is directed to the very short time interval of 30 min between fluid injection or removal and the effects on LEC proliferation as measured by phospho-histone-3 staining. The authors should comment on this and discuss examples in the literature that show similarly rapid effects using the same read out.

2) How was VEGR3/p-Tyr PLA staining quantified? Was it number of stained dots per Lyve-1 positive cell area? This is not described in the methods section, but it is one of the central read outs.

3) Determining β1-integrin activity by antibody staining (9EG7) is in my eyes not very convincing. With the strong overall staining for this ubiquitous group of integrins, I think that quantifying this staining for LEC is certainly very hard to do. Was this analysis done in a truly blinded fashion? I am skeptical on such measurements, in my eyes it is a too messy read out. The paper is not really strengthened by these data and they could be removed.

4) Figure 4 shows a clear example of edema formation in β1-integrinΔ/Δ mice. Could the authors give numbers in how many cases this was seen?

Referee #3:

In this manuscript Planas-Paz et al propose that lymphatic vessel development is controlled by mechanical signals that regulate activation of VEGFR3/beta1 integrin signaling. Using novel and innovative methods to measure and control fluid pressure in mouse embryo they first show that the amount of fluid present within the interstitium correlates with VEGFR3 phosphorylation and lymphatic EC proliferation. They further show that genetic deletion of beta1 integrins or their inhibition using function-blocking antibodies leads to defective lymphatic development due to failure of LECs to respond to increased fluid pressure by enhancing VEGFR3 signaling. Based on these sophisticated experiments the authors conclude that increased interstitial fluid volume leads to LEC stretching, which activates beta1 integrins, leading to VEGFR3 phosphorylation and LEC proliferation. Upon expansion of lymphatic sacs the fluid volume then decreases in E12.5 embryos, which is suggested to lead to reduced LEC proliferation and slowing down of the growth of the lymphatic vasculature. The proposed hypothesis is very interesting, however, the conclusions are not fully supported by the experimental data. The following points should be addressed:

1. As the authors point out, from E13.5 onwards there is extensive sprouting of vessels from the lymphatic sacs to peripheral tissues, rather than slowing down of lymphatic growth, which seems to be in controversy with the proposed model. Do the authors suggest that fluid pressure is involved regulating only the early but not the later stages of lymphatic development? Can they test this experimentally, for example by investigating the requirement of beta1 integrins for the later

sprouting phase of lymphatic development?

2. The authors demonstrate and quantify increased interstitial fluid pressure histologically by measuring the length of LEC nuclei, which is used as indicative of the extent of cell stretching. To my mind this phenomenon has not been shown convincingly. In several occasions schematic illustrations rather than original data are provided, and where original images are shown the differences in cell/nuclei morphology are not obvious (for example Fig 3F vs 3I). Specific staining of LEC nuclei using Prox1 antibodies would provide much clearer picture and should be used to verify key data. Images taken from corresponding areas of lymphatic sacs of embryos in different treatment groups should be shown.

3. Activation of VEGFR3 signaling in vivo is demonstrated by visualizing VEGFR3 phosphorylation using proximity ligation assay. Since VEGFR3 phosphorylation levels are used throughout the study as a key demonstration of LEC activation, it would be important to show that this method provides a reliable (and specific) readout of VEGFR3-pTyr levels and/or verify the key findings using another method. For example, in vivo or in vitro experiments showing increased staining upon VEGF-C (but not VEGF) stimulation and immunoprecipitation/western blotting for VEGFR3 and p-Tyr could be employed. In addition, it is not clear how it was concluded that the staining in Figure 1C' and 1G' is on plasma membrane vs cytoplasm.

4. The conclusion that beta1 integrins are activated upon fluid injection (Supp Fig S3) is based on higher intensity of immunofluorescence staining, but it is difficult to distinguish which signal is from LEC and which is from the surrounding cells. Higher magnification images should be provided to show this more clearly. The data would be much stronger if a more quantitative method (for example FACS) could be used. Do activated beta1 integrins co-localize with phosphorylated VEGFR3 in vivo?

1st Revision - authors' response 27 October 2011

Referee #1:

In this MS, the authors provide evidences for a mechano-dependent activation of lymphatic endothelial cells resulting in proliferation, change of cell shape and VEGFR3 activation. The authors further demonstrate that beta1 integrin is crucial in this mechanotransduction pathway. The in vitro and in vivo experiments are well planned.

COMMENTS

Fig 1. To visualize the stretching effect on LEC at E12.5 I suggest to stain cytoskeleton. This staining better support this concept and may be shown together DAPI staining. (similarly in GOF and LOF experiments).

To better visualize the elongation of LECs, we performed immunostainings of LEC nuclei and plasma membrane for Prox-1 (as referee 3 suggested) and Lyve-1, respectively. We showed these stainings in Figure 1B' and 1F', 2C' and 2C'', and 3C' and 3C''. Since it is impossible to quickly fix the F-actin cytoskeleton in the rather large E12.5 mouse embryos, we cannot preserve well the F-actin cytoskeleton. Thus a good F-actin immunostaining is difficult. We therefore stained cultured LECs for F-actin to reveal the presence of focal sites when the LECs were stretched (Suppl. Figure S8L-O).

Altogether these experiments demonstrate that the interstitial fluid accumulation modify lymphatic endothelial cell behavior. May the author provide a direct link with VEGFR3. For instance, does the genetic manipulation of VEGFR3 or the mother treatment with a VEGFR3 inhibitor modify the experimental observations reported?

To elucidate the link between interstitial fluid accumulation and VEGFR3, we injected VEGFR3-Fc fusion proteins into the jugular region of E11.5 mouse embryos. Subsequently, we analysed the phosphorylation of VEGFR3 in LECs and quantified LEC proliferation (Figure 5). VEGFR3-Fc significantly reduced the fluid-induced increase in

VEGFR3 phosphorylation as well as LEC proliferation. To genetically address this point, we knocked down VEGFR3 in LECs and observed a significant reduction in cell proliferation upon cell stretching compared to the stretched controls (Suppl. Figure S11).

Fig 2. May the author provide a demonstration that in this model H3 histone phosphorylation really reflects LEC proliferative status. On the other hand, other markers of proliferation should be considered and shown.

To investigate whether fluid removal reduced the numbers of LECs, we performed additional experiments where we cultivated aspirated embryos for 5 hours in WEC. We then quantified the numbers of LECs in these 'loss-of-fluid' experiments (Figure 4A-C). Decreasing the fluid volume was found to significantly reduce the number of LECs (Figure 4C). Conversely, we asked whether an increased amount of interstitial fluid resulted in an increased number of LECs after 5 hours WEC (Figure 4D-F). In these 'gain-of-fluid' experiments, this was found to be the case (Figure 4F). Therefore, the amount of interstitial fluid directly influences the absolute number of LECs and not just phospho-histone H3 as a proliferation marker.

The authors show interesting in vitro experiments by stretching LEC. However the equipment used does not exactly mirror the in vivo situation. I suggest to plate lymphatic endothelial cells on ECM with different stiffness. This experiment may allow excluding any other possible origin of the increased VEGFR3 activation observed at E12.5 in jugular area.

To confirm the mechanoinduction of VEGFR3 signalling, LECs were grown on substrates with increasing stiffness as suggested. Consistent with our stretching experiments, an increased stiffness enhanced VEGFR3 tyrosine phosphorylation (Suppl. Figure S9F).

Then the experiments in fig s4 should be also performed by VEGFR3 IP .
However the in vitro experiments reported in Fig 4s are largely convincing.

We performed the measurement of VEGFR3 tyrosine phosphorylation using two different assays. Firstly, we analysed VEGFR3 tyrosine phosphorylation using a phospho-VEGFR3 ELISA (Suppl. Figure S9E, F). Secondly, we analyzed VEGFR3 phosphorylation using PLA (Suppl. Figure S10). Both experiments gave similar results.

In my opinion the in vitro approaches could improve the information about the mechanism. It is important to study whether beta1 forms a complex with VEGFR3. Is it present in basal conditions? Is it dependent by cell stretching or VEGC-C stimulation.

We investigated whether β1 integrin formed a complex with VEGFR3 using different approaches. *In vitro*, we stretched the LECs in the presence or absence of VEGF-C, and analysed the interaction of VEGFR3 and β1 integrin using proximity ligation assays (Suppl. Figure S8H-K). Stretching the LECs significantly increased the PLA signals indicating an increased interaction between VEGFR3 and β1 integrin (Suppl. Figure S8K). Furthermore, we showed in mouse embryos an increased colocalisation of VEGFR3 with activated β1 integrin upon 34 nl fluid injection compared to injection of a small amount of fluid or no fluid injection (Suppl. Figure S7D, H, and see Suppl. Figure S7N). Proximity ligation assays also indicated that the interaction between VEGFR3 and β1 integrin was increased when a large amount of fluid was injected (Suppl. Figure S7IL, and see Suppl. Figure S7O).

Are there any synergistic effect between cell stretching and VEGF-C stimulation in term of LEC proliferation and VEGFR3 phosphorylation?

Mechanical stretching and VEGF-C stimulation alone significantly increased both VEGFR3 tyrosine phosphorylation and LEC proliferation (Suppl. Figures S10 and S11). However, the combination of mechanical stretching and VEGF-C stimulation appeared to synergistically increase LEC proliferation (Suppl. Figure S11E).

May the author exclude that cell stretching increase VEGF-C production?. This should be easily demonstrated

To exclude that the stretching of LECs leads to an increase in VEGF-C mRNA expression, we performed RT-PCR on non-stretched and stretched LECs (Suppl. Figure S12). VEGF-C expression was not changed when LECs were stretched compared to non-stretched LECs (Suppl. Figure S12).

Fig 6S. Which is the pattern of beta1 expression in vascular endothelial cells in jugular area?

We analysed the expression of β1 integrin in lymphatic (LECs) and vascular endothelial cells (VECs) in the jugular area of E12.0 embryos (Suppl. Figure S4). β1 integrin was expressed in both LECs and VECs in the jugular area (Suppl. Figure S4, red staining). However, VEGFR3 was mainly expressed in LECs (Suppl. Figure S4I-N).

The absence of b1integrin in LEC should be demonstrated in other anatomical districts. Do LEC isolated from these mice lack beta1?
In general a more precise and complete description of the phenotype of lymphatic system in these mice is strongly recommended.

To demonstrate the absence of β1 integrin from LECs, we isolated LECs from β1 integrinΔ/Δ and heterozygous control embryos by magnetic-activated cell sorting (MACS) (Suppl. Figure S13K, L). In agreement with the immunohistochemistry data (Suppl. Figure S13E-J), β1 integrinΔ/Δ LECs sorted from E12.0-E12.5 mice presented a reduction in the β1 integrin gene expression of around 75 % compared to the LECs of heterozygous controls (Suppl. Figure S13K-L).
To depict the lymphatic phenotype in the β1 integrinΔ/Δ mice, we immunostained LECs in the skin and mesenteric region of β1 integrinΔ/Δ mouse embryos and control embryos. We showed that β1 integrinΔ/Δ embryos lacked lymphatic vessels in both regions (compare Suppl. Figure S14B-D with S14E-G).

There are increasing evidences that Tie-2/Ang system regulate LEC function. Furthermore, a fraction of Tie2 is constitutively associated with a5b1 integrin (JCB 170:993,2005). This point should be addressed in the discussion

We refer to this interaction in the discussion on page 20.

Referee #2:

This manuscript reports that interstitial fluid pressure leads to stretched lymphatic endothelial cells (LEC), more LEC proliferation and VGFR3 phosphorylation. Whereas these effects correlated with increasing interstitial fluid pressure in mouse embryos between E11.5 and E12.5, injection of fluid and removal of fluid affected VEGFR3 phosphorylation and LEC proliferation accordingly. Endothelial specific deletion of β1-integrin reduced VEGFR3 phosphorylation and LEC proliferation and abrogated the effect of fluid injection on these two parameters. In addition, injection of an anti β1-integrin blocking antibody reduced the number of LEC in the embryo. As important controls, the manuscript shows that fluid injection into the embryo did not affect blood vessel endothelial cell proliferation; stretching of cultured EC enhanced proliferation; endothelial specific deletion of β1-integrin in vivo did not enhance LEC apoptosis; and the effect of fluid injection on VEGFR3 activation and LEC proliferation was even demonstrated in the ear of adult mice.

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technology.

Detailed comments:

1) My major concern is directed to the very short time interval of 30 min between fluid injection or removal and the effects on LEC proliferation as measured by phosphohistone-3 staining. The authors should comment on this and discuss examples in the literature that show similarly rapid effects using the same read out.
To investigate whether fluid removal reduced the numbers of LECs, we performed additional experiments where we cultivated aspirated embryos for 5 hours in WEC. We then quantified the numbers of LECs in these 'loss-of-fluid' experiments (Figure 4A-C). Decreasing the fluid volume was found to significantly reduce the number of LECs (Figure 4C). Conversely, an increased amount of interstitial fluid resulted in an increased number of LECs after 5 hours WEC (Figure 4D-F). Therefore, the amount of interstitial fluid directly influences the absolute number of LECs and not just phospho-histone H3 as a proliferation marker.

There are other examples in the literature showing that phospho-histone H3 can be activated as early as 15 to 30 minutes after treatment (Illi B et al., 2003, Circ Res 93:155; Bertran-Gonzalez J et al., 2009, Neuropsychopharmacology 34 (7):1710; Park GY et al., 2006, J Biol Chem 281:18684).

2) How was VEGR3/p-Tyr PLA staining quantified? Was it number of stained dots per Lyve-1 positive cell area? This is not described in the methods section, but it is one of the central read outs.

VEGFR3/p-Tyr was quantified as the total number of red dots divided by the total number of Lyve-1 positive cells. We added a sentence in the Materials & Methods on pages 25-26.

3) Determining β1-integrin activity by antibody staining (9EG7) is in my eyes not very convincing. With the strong overall staining for this ubiquitous group of integrins, I think that quantifying this staining for LEC is certainly very hard to do. Was this analysis done in a truly blinded fashion? I am skeptical on such measurements, in my eyes it is a too messy read out. The paper is not really strengthened by these data and they could be removed.

To quantify the staining intensity of activated β1 integrin in our raw images, a threshold mask of the VEGFR3+ area occupied by LECs was determined using the Fiji/ImageJ image analysis software. Subsequently, this mask was superimposed on the activated β1 integrin channel (Suppl. Figure S7C, G). Finally, the software was used to measure the fluorescence intensity of this specific LEC area (Suppl. Figure S7A-C, S7E-G, and see Suppl. Figure 7M). In addition, the image analysis software was used to measure the colocalisation between VEGFR3 and activated β1 integrin (Suppl. Figure S7D, H, and see Suppl. Figure S7N).

4) Figure 4 shows a clear example of edema formation in β1-integrinΔ/Δ mice. Could the authors give numbers in how many cases this was seen?

Suppl. Figure S14 A shows the total numbers of analysed mice, the numbers of mice with homozygous deletion of β1 integrin, as well as the numbers of β1 integrinΔ/Δ mice that contain oedema. At E13.5 and E15.5, 65% and 80% of the β1 integrinΔ/Δ embryos had oedema, respectively.

Referee #3:

In this manuscript Planas-Paz et al propose that lymphatic vessel development is controlled by mechanical signals that regulate activation of VEGFR3/beta1 integrin signaling. Using novel and innovative methods to measure and control fluid pressure in

mouse embryo they first show that the amount of fluid present within the interstitium correlates with VEGFR3 phosphorylation and lymphatic EC proliferation. They further show that genetic deletion of beta1 integrins or their inhibition using function-blocking antibodies leads to defective lymphatic development due to failure of LECs to respond to increased fluid pressure by enhancing VEGFR3 signaling. Based on these sophisticated experiments the authors conclude that increased interstitial fluid volume leads to LEC stretching, which activates beta1 integrins, leading to VEGFR3 phosphorylation and LEC proliferation. Upon expansion of lymphatic sacs the fluid volume then decreases in E12.5 embryos, which is suggested to lead to reducedLEC proliferation and slowing down of the growth of the lymphatic vasculature. The proposed hypothesis is very interesting, however, the conclusions are not fully supported by the experimental data.

The following points should be addressed:

1. As the authors point out, from E13.5 onwards there is extensive sprouting of vessels from the lymphatic sacs to peripheral tissues, rather than slowing down of lymphatic growth, which seems to be in controversy with the proposed model. Do the authors suggest that fluid pressure is involved regulating only the early but not the later stages of lymphatic development? Can they test this experimentally, for example by investigating the requirement of beta1 integrins for the later sprouting phase of lymphatic development?

In Figure 1I, we showed that the fluid pressure peaked at E12.0, coinciding with a burst in LEC proliferation (Figure 1L). However, at E12.5 the fluid pressure still was higher when compared to E11.0, explaining why LEC proliferation continued. Whether the fluid pressure was increased at locations of lymphatic sprouting in the E13.5 or later mouse embryo was not investigated in this study. However, to show that fluid accumulation also induced LEC proliferation in sprouting lymph vessels, we applied 'gain-of-fluid' experiments to the dorsal skin of E15.5 mouse embryos (Figure 9A-D). Injection of 100 nl PBS induced LEC proliferation in dermal sprouting lymph vessels, showing that sprouting lymph vessels were also affected by the fluid volume (compare Figure 9A with 9B, and see Figure 9D). In contrast, blocking β1 integrins inhibited this enhanced LEC proliferation in response to an increased fluid volume (compare Figure 9B with 9C, and see Figure 9D). These results show that the accumulation of fluid also induces the expansion of sprouting lymph vessels in the skin in a β1 integrin-dependent manner.

2. The authors demonstrate and quantify increased interstitial fluid pressure histologically by measuring the length of LEC nuclei, which is used as indicative of the extent of cell stretching. To my mind this phenomenon has not been shown convincingly. In several occasions schematic illustrations rather than original data are provided, and where original images are shown the differences in cell/nuclei morphology are not obvious (for example Fig 3F vs 3I). Specific staining of LEC nuclei using Prox1 antibodies would provide much clearer picture and should be used to verify key data. Images taken from corresponding areas of lymphatic sacs of embryos in different treatment groups should be shown.

To better visualize the stretching of LECs, we performed immunostainings of the LEC nuclei using antibodies against Prox-1. We included these images in Figures 1B' and 1F', 2C' and 2C", and 3C' and 3C".

3. Activation of VEGFR3 signaling in vivo is demonstrated by visualizing VEGFR3 phosphorylation using proximity ligation assay. Since VEGFR3 phosphorylation levels are used throughout the study as a key demonstration of LEC activation, it would be important to show that this method provides a reliable (and specific) readout of VEGFR3-pTyr levels and/or verify the key findings using another method. For example, in vivo or in vitro experiments showing increased staining upon VEGF-C (but not VEGF) stimulation and immunoprecipitation/western blotting for VEGFR3 and p-Tyr could be employed. In addition, it is not clear how it was concluded that the staining in Figure 1C' and 1G' is on plasma membrane vs cytoplasm.

To confirm that the PLA is a reliable readout of VEGFR3 tyrosine phosphorylation, we injected a specific activator of VEGFR3, VEGF-C (C156S), in the jugular region of wild type embryos (Suppl. Figure S1). As expected, the PLA signals in LECs increased when VEGF-C was injected compared to control injections (compare Suppl. Figure S1A, B with S1C, D, and see Suppl. Figure S1E). Moreover, we performed *in vitro* experiments to further confirm the reliability of the PLA method (Suppl. Figure S10). Stimulation of human LECs with VEGF-C significantly up-regulated VEGFR3 phosphorylation as indicated by an increased number of PLA signals (compare Suppl. Figure S10A with S10B, and see Suppl. Figure S10E). In addition, *in vitro*, the results of the PLA were corroborated using a phospho-VEGFR3 ELISA (Suppl. Figure S9E, compare first and third column). Finally, we showed that phosphorylation of VEGFR2 was not affected by fluid accumulation (Suppl. Figure S5). As regards localisation of the PLA signals, we removed this sentence, since indeed we cannot distinguish between the plasma membrane and cytosol in these stainings.

4. The conclusion that beta1 integrins are activated upon fluid injection (Supp Fig S3) is based on higher intensity of immunofluorescence staining, but it is difficult to distinguish which signal is from LEC and which is from the surrounding cells. Higher magnification images should be provided to show this more clearly. The data would be much stronger if a more quantitative method (for example FACS) could be used. Do activated beta1 integrins co-localize with phosphorylated VEGFR3 in vivo?

To quantify the staining intensity of activated β1 integrin in our raw images, a threshold mask of the VEGFR3+ area occupied by LECs was determined using the Fiji/ImageJ image analysis software. Subsequently, this mask was superimposed on the activated β1 integrin channel (Suppl. Figure S7C, G). Finally, the software was used to quantify the fluorescence intensity of this specific LEC area (Suppl. Figure S7A-C, S7E-G, and see Suppl. Figure 7M).

The colocalisation between VEGFR3 and activated β1 integrin was also measured using the same software (Suppl. Figure S7D, H, and see Suppl. Figure S7N). We showed an increased colocalisation of VEGFR3 with activated β1 integrin upon 34 nl fluid injection compared to injection of a small amount of fluid or no injection (Suppl. Figure S7N). Proximity ligation assays (PLA) also indicated that the interaction between VEGFR3 and β1 integrin significantly increased when a large amount of fluid was injected (Suppl. Figure S7I-L, and see Suppl. Figure S7O).

Additional Correspondence 10 November 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2011-78737R to the EMBO Journal. It has now been seen again by referees 1 and 3, whose comments are enclosed below. As you will see, both referees find the manuscript substantially improved, and are fully supportive of publication.

Referee 1 does just have one remaining concern re. the interaction between beta1 and VEGFR3, and requests that you try coIPs between the two proteins. I realise that you have the PLA data, but if you can do the IPs here, then I agree that it would strengthen your conclusions. Perhaps you can let me know whether you have tried IPs, and/or whether you have the tools to do so without too much trouble. To be clear, we are positive about publishing the paper (with or without these data), but if you can do the experiment easily, then I think it would be valuable.

I look forward to hearing from you.

REFEREE REPORTS

Referee 1:

All my previous criticisms have been properly addressed. As previously requested, I think Co-IP experiments should further improve and reinforce the demonstration that beta1 and VEGFR3 forms a complex in stretched
LEC

Referee 3:

The authors have adequately addressed all my concerns. This is an elegant study that provides novel conceptual insight into the regulation of early lymphatic development.

