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The blood vasculature instructs lymphatic patterning in a SOX7-dependent manner.

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you again for the submission of your manuscript entitled "The blood vasculature instructs lymphatics patterning in a SOX7 dependent manner" and for your patience during the review process. We have now received the reports from the referees, which I copy below.

As you can see from their comments, all three referees are very positive towards your work but point out to some concerns that will require your attention before your manuscript can be published in The EMBO Journal.

Based on the overall interest expressed in the reports, I would like to invite you to address the comments of all referees in a revised version of the manuscript. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. I believe the concerns of the referees are reasonable and addressable, but we are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, so please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

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: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

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

Referee #1:

In this article Chiang et al report that the transcription factor SOX7 regulates lymphatic vascular patterning by inhibiting VEGF-C expression in arterial endothelial cells. Furthermore, SOX7 inhibits VEGF-C expression by directly associating with its regulatory elements. Additionally, SOX7 promotes the expression of HEY1, which is another inhibitor of VEGF-C expression.
The authors have used numerous elegant approaches and the data is quite convincing. I have just a few questions that I think are important to be addressed.

Major comments

1. I think it is a bit too strong to state that SOX7 is not expressed in LECs. In Supp Fig 2B SOX7 appears to be modestly expressed in the PROX1+LECs. The authors should remove the asterisk and provide a higher magnification image of this picture as a main figure. Their statement could be revised appropriately.
2. An extensive expression analysis for SOX7 (especially mesenteric lymphatic vessels, lymphatic valves) could be more convincing. Likewise, the lymphatic phenotype of Sox7-KO embryos could be more thoroughly analyzed.
3. Supp Fig 4A is difficult to comprehend. I would prefer to see NRP2 in Green and SOX7 in red. Provide PROX1 in red with SOX7 in green in a separate panel. Higher magnification images will also be helpful.
4. In Figures 2A and B V5 appears to be expressed in the cardinal vein. Is there any reason why SOX7 may not be directly inhibiting LEC specification in the cardinal vein?
5. Have the authors attempted to delete Sox7 in LECs by using Lyve1-Cre or Prox1-CreERT2? This could be a definitive approach to exclude any LEC-specific contribution of Sox7.
6. Consistent with Karkkainen et al (Nat Immunology 2004) VEGF-C is expressed potently in the mesenchymal cells around the cardinal vein of E11.5 embryos (Fig 2C). Therefore, I am a bit troubled by the conclusion that VEGFC overexpression in the BECs is responsible for the phenotypes of Sox7 KO embryos. I am not sure if a modest increase in BEC-expressed VEGF-C will be sufficient to cause such a dramatic phenotype. Could there be changes in mesenchymal VEGF-C as well? Please use in situ hybridization to compare and quantify Vegfc expressions in control and Sox7 KO.
7. Please determine if VEGFC overexpression in BECs results in lymphatic vascular defects that are similar to Sox7 KO embryos.

Minor comments
1. Does Sox7 play a time-specific role in lymphatic vascular development?
2. What are the other VEGFC+ cell types in Fig 2B?

Referee #2:

In this study Chiang and colleagues have investigated the role of the blood endothelium in patterning the dermal lymphatic vasculature at the level of Sox7 acting directly on Vegfc to control the number and distribution of Prox1+ LECs. They use a combination of an endothelial cell specific knock-out of Sox7, snRNA-Seq, protein-protein interaction and ChIP-Seq analyses to conclude that Sox7 interacts directly with the transcriptional repressor Hey1 at the Vegfc locus (promoter and distal elements) to repress Vegfc in BECs and as such regulate the appropriate number of LECs that contribute to the forming dermal lymphatics. This is a very interesting study and one which adds significant insight into how the blood vasculature functions to regulate lymphatic vessel growth and patterning. Whilst hinted at previously in conjunction with BEC chemotactic guidance cues, a detailed molecular characterisation as to how blood vessels might guide lymphatic patterning is currently lacking. Moreover, we know very little about the upstream regulation of Vegfc as a key factor in lymphatic vessel development; as such, the insights provided here around the direct role of Sox2-Hey1 provide important mechanistic insight. That said, there are some issues with the study in its current form that warrant attention and some suggestions that might improve the manuscript:

1. The authors have focused their analysis exclusively on the lymphatics of the skin and yet both blood and lymphatic endothelium are known to be heterogeneous across lineages and organ systems. An open question, therefore, is whether the mechanism of BEC and LEC interaction described herein translates to other vascular beds(?). This is an important issue to support some of the more general statements and conclusions herein (for eg. end of the Introduction: " . . . SOX7-mediated fine-tuning of VEGFC expression in BECs, a process essential for correct lymphatic growth and patterning."). Whilst it is not expected the authors repeat their study in its entirety, it would be highly informative if they could provide some analysis of Sox7-V5 expression in other (non-dermal) blood endothelium and investigate at least one other lymphatic bed (eg. mesenteric, cardiac) in the context of a potential gross lymphatic patterning phenotype in the Sox7-ECKO mutant embryos.

2. The authors rely on a Sox7-V5 transgenic reporter line to determine SOX7 expression in BECs- the assumption here is that there is no suitable SOX7 antibody to support these studies; please clarify in the text. If so, then it would be helpful to include some RNAScope or HCR to at least show endogenous Sox7 mRNA expression and to provide confidence that the Sox7-V5 (or indeed the additional beta-galactosidase analysis in the Sox7-KI line) appropriately reflects endogenous SOX7 expression.

3. The lymphatic phenotype in the Sox7 ECKO mutant embryos (Fig. 1) is one of ectopic and increased numbers of Prox1+ LECs, which as the authors later conclude is consistent with over-expression of VEGFC- that said, how do the authors explain the other aspect of the mutant phenotype, ie severely dilated, lacked inter-vessel branches and showed migration defects (Fig. 1C-F), given the known role of VEGFC in lymphangiogenesis?

4. The snRNA-Seq analysis presented in Fig. 2 is a nice study and clearly indicates appropriate Sox7 and Vegfc expression in BECs, however, what would be highly informative here is an snRNA-seq assessment of Sox7-ECKO whole skin, to potentially

identify dysregulated pathways. Assuming this has not been carried out, and as such represents an extensive amount of work, the authors could select candidate genes and pathways from the wild type snRNA-Seq dataset, including Sox7 and Vegfc, and test expression in Sox7 ECKO cells (analogous to the Vegfc/Vegfr3 focused analysis in Fig. 2D).

5. In the analysis to test whether Sox7 cell autonomously regulates Vegfc transcript levels the authors down-regulated Sox7 using a siRNA-based approach in HUAECs and HUVECs. Both cell lines showed reduced Vegfc expression following siRNA KD- how does this relate to the in vivo situation, given the restricted expression of Sox7 (Sox7-V5) to arterioles (and low levels in mature arteries) and no expression in veins/venules (Supp. Fig. 2)?

6. The data to demonstrate Sox7 interaction with Hey1 in Fig. 3E and 3F is based on a cell-free interaction assay and over-expression in HEK293 cells; this is not in vivo proof nor is it carried out in an appropriate EC line. As such the protein-protein interaction analysis needs improving to make a relevant and meaningful claim on Sox7-Hey1 interaction in the developing blood endothelium.

7. To test whether the identified Vegfc-255 and Vegfc-152 regions act as enhancers of Vegfc activity, the authors generated a series of Vegffc-luciferase reporter constructs that harbor a combination of different putative regulatory regions from the mouse or human locus (Fig. 4C, E). This is an artificial system and could be significantly improved by a more elegant in vivo approach, to establish transient Fo transgenic reporter lines of the VEGFC regulatory regions in a wild type versus Sox7 ECKO background; the latter serving to further demonstrate that the identified regulatory regions are also Sox7-dependent.

Referee #3:

In this study, the authors investigated blood endothelial cell (BEC)-mediated paracrine mechanisms involved in the regulation of lymphangiogenic growth factor (VEGFC) expression and lymphatic patterning. Using various state-of-the-art methodologies (including transgenic reporter mice, conditional gene knockout mice, single nucleus RNA-seq, luciferase reporter mediated promoter studies, ChIP-seq and ChIP PCR, protein-protein interaction assays, etc.), they show that the BEC-specific transcription factor SOX7 modulates BEC expression of VEGFC via a dual mechanism: by upregulating Hey1 which represses VEGFC expression, and by directly binding to the VEGFC promoter and repressing its expression, alone or in interaction/combination with HEY1 to which SOX7 was also found to bind. BEC-selective knockout of Sox7 in mouse embryos resulted in a defective dermal lymphatic phenotype and in ectopic supernumerous LEC progenitors in the cardinal vein.
The study is interesting and revealed novel mechanisms. It also left a few straightforward questions unanswered that could be addressed or discussed. Some points for clarification of specific data, and a few recommendations for further strengthening of the data (e.g. missing controls) and the conclusions are listed below.

MAJOR COMMENTS
- The authors analyzed the consequences of loss of Sox7 expression in BECs on lymphatic patterning around the time of lymph sac formation in the jugular region, but no data are shown on lymph sac formation. What is the phenotype? Is it abnormally formed, or normally formed but enlarged?
- In control conditions, the newly revealed SOX7-HEY1-VEGFC axis acts in a polarized manner in the cardinal vein (cf Fig. 5A,B). Is there any clue how this polarization is regulated?
- A few questions are raised related to Figure 1:
{degree sign} Fig. 1A: the quantification in the top right panel is somewhat confusing as it does not show whether all peripheral and all DA and all CV ECs are positive for SOX7 for the control genotype. Although indeed unmistakenly low, the results shown for the ECKO embryos are therefore also confusing. Showing the data per EC type should be considered.
{degree sign} Fig. 1C,D: Is it correct to name this parameter migration? Likely the closure of the 'gap' between the lymphatic front and the midline normally relies on a combination of migration and proliferation?
{degree sign} Figure 1I,J: the results are interpreted as hyperproliferation and/or defective remodeling (page 6). If possible, a proliferation staining could be done to verify this interpretation further, if feasible to be performed (it is away from the blood vascular front, so no/less problems to be expected with superposition of proliferating BECs).
{degree sign} Figure 1J: this data needs to be recalculated per embryo rather than per cluster not to bias the statistics; there are only n=3 and n=5 biological repeats.
{degree sign} Fig. 2C: The images are not entirely convincing. Replacement with better images and quantification of the RNA scope signal is recommended.
- Related to the findings from Fig. 2E-G and Fig. 3B-D on the axis SOX7-HEY1-VEGFC wherein SOX7 induces Hey1 expression and HEY1 represses the VEGFC promoter: to what extent is Notch activation involved in this effect? Is the siSox7 effect of enhanced VEGFC expression in EC cultures (Fig. 2E-G) lost upon Notch inhibition (DAPT, Dll4-Fc)? And is it lost upon silencing of Hey1?
- Fig. 3B-D: The inhibitory effect on the human promoter construct is close to 55%, but on the mouse promoter construct at most ca 15%. Do the authors have an explanation for this difference? Hey1 overexpression was done using human HEY1; could it be

a (partial) species incompatibility? Furthermore, Hey1 overexpression is not verified by PCR, which is best included (baseline expression, extent of overexpression).
- Fig. 4A, VEGFC promoter-luciferase assay in HeLa cells: the result is interpreted as supporting the notion that SOX7 has the potential to act as a direct negative regulator of VEGFC transcription. Was it verified that it is solely direct and not perhaps also via HEY1? HeLa cells can express HEY1. Its expression could be tested to verify baseline expression and potential induction/upregulation upon SOX7 overexpression.
- Fig. 8A currently is not entirely clear. Which genotypes are shown in panel A and B? Regarding the markers in panels C-E, what does low/-, +/low and +/- refer to?
- Tamoxifen treatment: It is not clear why in some experiments another tamoxifen treatment regimen was used. For instance in Sup. Fig. 4C and Fig. 2D. The experiment in Sup. Fig. 4C was included to exclude that the observed phenotype was due to or influenced by potential Cre toxicity, and thus should be done using the same tamoxifen regimen as used in the phenotyping experiment. Also, the quantification of LV branching and LV dilation in this experiment is missing.

MINOR COMMENTS
- Abstract and elsewhere in the text: the BEC effect is referred to as 'lymphangiocrine'. I think this should be 'angiocrine'.
- Page 9, the description of the results of Fig. 3F and the legend to Fig. 3F are not in accordance.
- Page 13, line 8: including the reference for the Tie2Cre;VegfcGOF data is recommended.
- Material and Methods, Mice and breeding: The Schimmel et al reference should be the Muzumdar et al reference.
- Page 25, Expression constructs, top: the species indication needs to be corrected.
- Some English writing or wording could be optimized (e.g. (auxiliary) verbs are often missing; snapped freeze should be snap-frozen; etc)
- Legend to Figure 1A: the tamoxifen injection is not really correctly described.

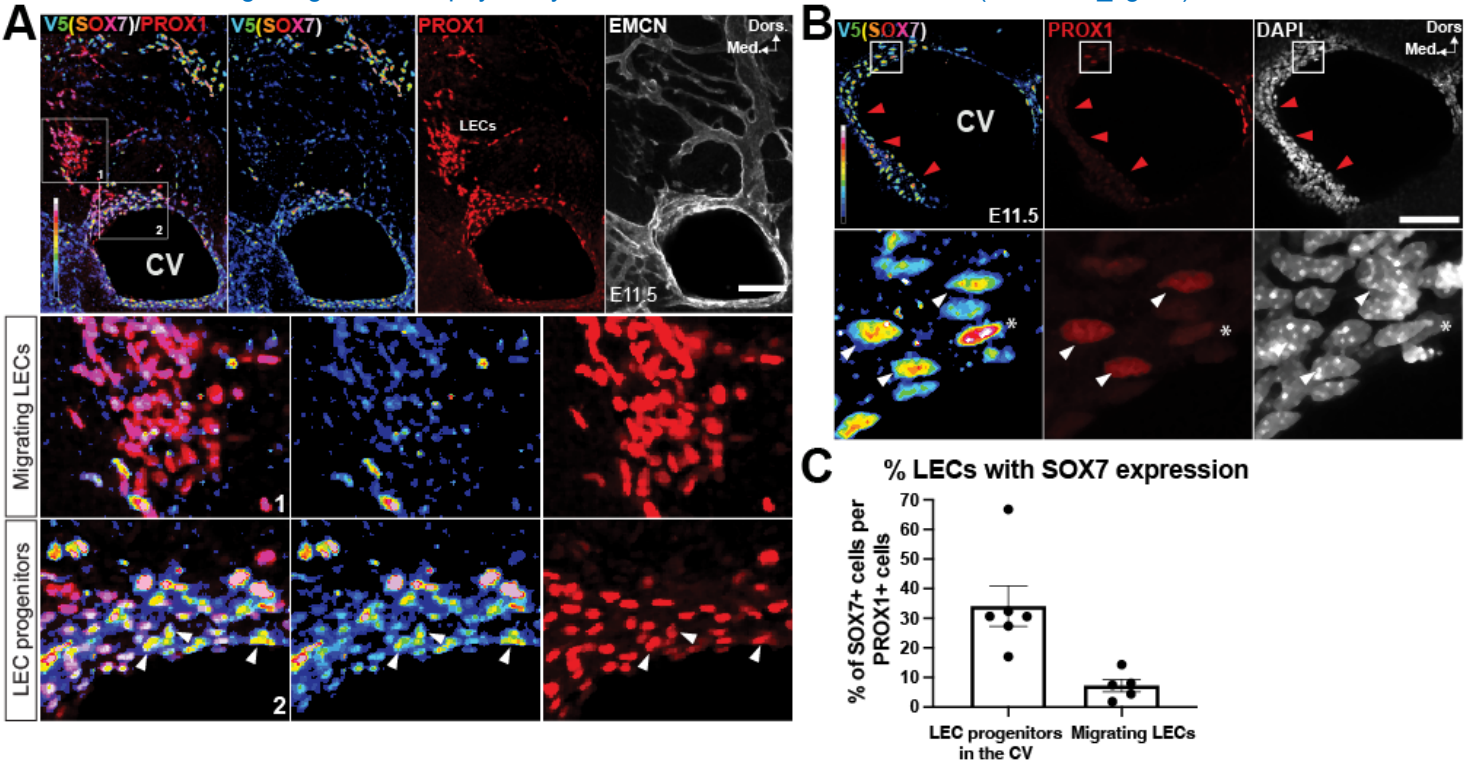
The team would like the thank the reviewers for their insightful and supportive comments which overall have contributed to significantly improve the scientific quality of this manuscript. Please find appended below a point-by-point response that address the reviewers' queries.

Referee #1:

Major comments

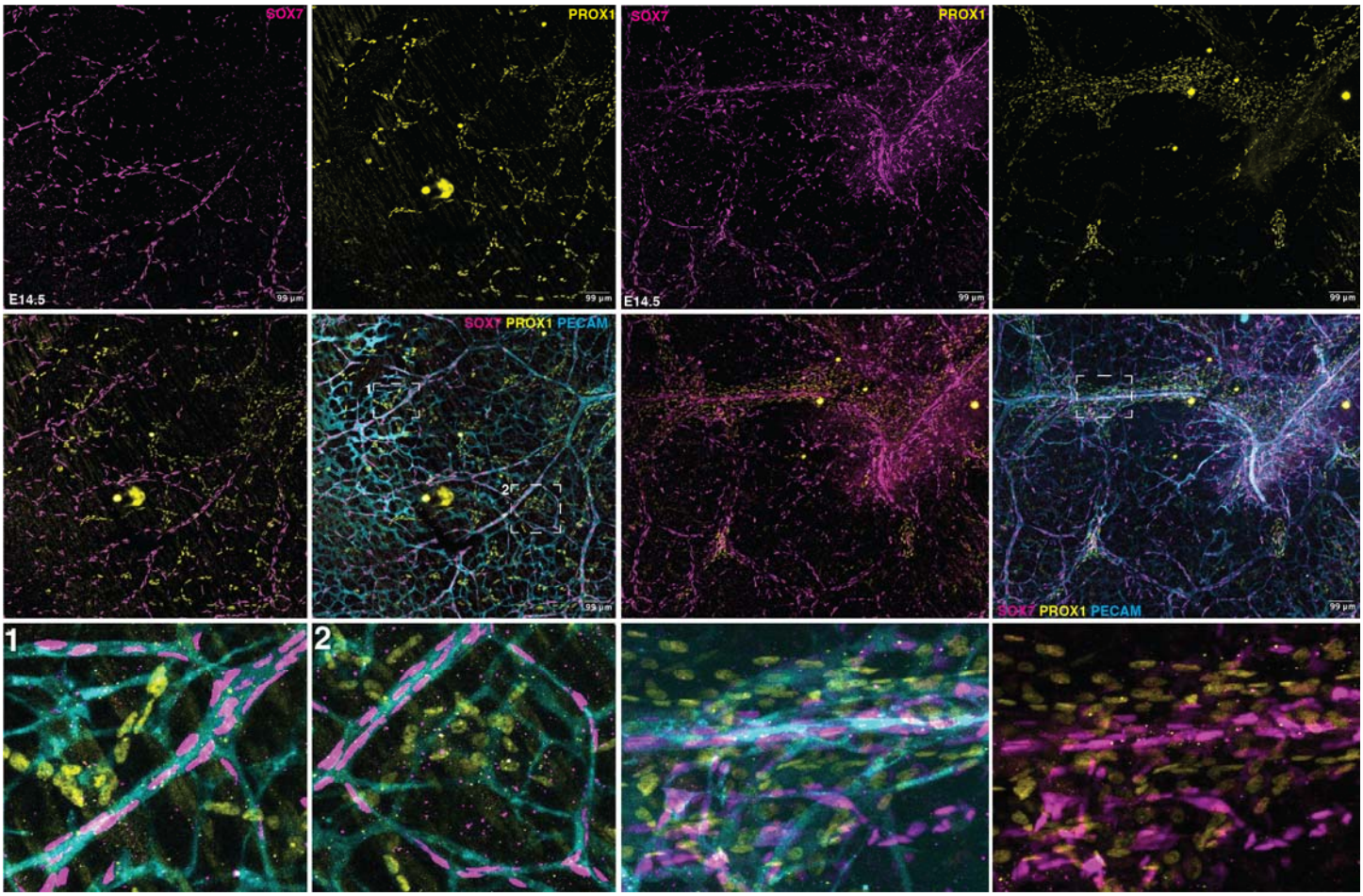
1. I think it is a bit too strong to state that SOX7 is not expressed in LECs. In Supp Fig. 2B (now Fig. EV1B) SOX7 appears to be modestly expressed in the PROX1+LECs. The authors should remove the asterisk and provide a higher magnification image of this picture as a main figure. Their statement could be revised appropriately.

This is a fair comment and we agree that there are some PROX1-positive LECs that express SOX7 specifically in endothelial cell progenitors of the cardinal vein (CV) at the early steps of lymphangiogenesis (E10.5). Later, during skin organogenesis (E13.5-E15.5), SOX7 expression in not detected in the more mature PROX1-positive LECs.
We have quantified the number of LECs in the CV as well as in migrating LECs after these cells have disconnected from the vessels walls. Both these cell populations show SOX7 expression with various degrees of intensity (Reviewer_fig1). A color scale is used to represent the range of SOX7 expression as a heatmap in endothelial cells nuclei: with no to low expression labeled in blue, green or yellow, and more robust expression levels shown in orange, red and white (Reviewer_fig1, indicator range). As observed in Reviewer_fig1A, LEC progenitors in the cardinal vein typically express a higher level of SOX7 (inset 2, yellow, orange and red signal intensity according to the heatmap color scale) when compared to the migrating LECs in the mesenchyme (inset 1, blue and green signal intensity), where SOX7 expression is low and almost undetectable. Regardless of SOX7 and PROX1 intensity level, in the cardinal vein, there is around 34% of endothelial cells that are detected as double positive cells for these two TFs. This means that a large proportion of PROX1-positive cells in this vessel wall are negative for SOX7 (>60%) (Reviewer_fig 1C). In the 34% of SOX7/PROX1 double positive cells, it is possible to distinguish two distinct populations: either SOX7^{low}/PROX1^{high} (Reviewer_fig1B, white arrowheads) or SOX7^{high}/PROX1^{low} endothelial cells (Reviewer_fig1B, asterisk). This double positivity rate drops to only 7.3 %, once the migrating LECs are physically disconnected from the vessel wall (Reviewer_fig 1C).



Reviewer_figure 1. Relative expression of SOX7 and PROX1 in endothelial cells at the early steps of lymphangiogenesis. (**A** and **B**) Transverse sections of a E10.5 wild type embryo stained for SOX7 (intensity levels shown as a heat map), PROX1 (red) and Endomucin (white). High levels of SOX7 are indicated by red to white signals, and weak expression (limit of detection), by a blue to yellow signal according to a 16 color scaling system. Arrowheads indicate cells that have high expression level for PROX1 and low for SOX7. Asterisks indicate cells with high level of SOX7 but low level of PROX1. (**C**) Quantitation of SOX7/PROX1 double positive cells, either in the cardinal vein wall (n = 6) or in the mesenchyme (n = 5). Mean ± SEM, scale bars = 100 µm.
This new result is now shown as Fig. EV1 in the manuscript.

2. An extensive expression analysis for SOX7 (especially mesenteric lymphatic vessels, lymphatic valves) could be more convincing. Likewise, the lymphatic phenotype of Sox7-KO embryos could be more thoroughly analyzed.
By E14.5, there is no more expression of SOX7 in LECs (Reviewer_fig2), this new result is also shown as Fig. EV1 in the manuscript. Therefore, we did not pursue the analysis of SOX7 expression in lymphatic valve at later developmental stages. The initial stages of lymphatic valves formation start during mid-organogenesis at around E15.5 in different tissue types. We do appreciate that valve formation is an important feature of lymphatic vessels, but this biological question is really different from the general vessel network assembly, which is the focus of this study.



Reviewer_figure 2. Wholemount immune staining on skin at E14.5 using SOX7 (magenta), PROX1 (yellow) and PECAM (CD31, blue). During mid-organogenesis, SOX7 expression is not observed in the developing lymphatic endothelial cells (PROX1/PECAM double positive cells), but often, SOX7 positive cells are found in close vicinity of PROX1-positive LECs.

Regarding development of the mesentery, with the current schedule of tamoxifen injection to deflox *Sox7* (eg injection at E9.5 and E10.5), the *Sox7*-null embryos die at around E14.5 and SOX7 constitutive knockout embryos die at E10.5. This, therefore, excludes the possibility of analysing *Sox7* loss of function in the lymphatic vasculature of the mesentery under these experimental conditions. We have performed loss of *Sox7* function (*Cdh5-CreERT2: Sox7fl/fl*) using a different tamoxifen treatment (eg injection at E11.5 and E12.5) to delay the onset of the phenotype, this gave us the capability to study the growing lymphatics in the mesentery at a later time point (eg E16.5). Of note, we show in the manuscript that this time window (E11.5 and E12.5) to deflox *Sox7* also causes a profound dermal lymphatic phenotype (Fig. EV2I). Full results for this experiment are shown in this report, under point#1 reviewer 2 (Reviewer_fig6). Please refer to that section for more information.
Briefly, this approach revealed that developing lymphatic vessels of the mesentery in the $Sox7^{iECKO}$ assemble as a vascular network, but show gross remodeling defects. Wholemount immuno-staining of the mesentery with NRP2 antibody reveals there is no quantitative difference in vessel area coverage, but some lymphatics in the $Sox7^{iECKO}$ appears severely enlarged and mis-patterned. Further, quantitation of the NRP2 fluorescent signal normalized to vessel area in the $Sox7^{iECKO}$ embryos reveals an increase in signal intensity, albeit not significant. Therefore, it seems that the mechanism we reported here in this study is broadly relevant to organ-specific lymphangiogenesis, and not a specific mode of action restricted only to the dermal lymphatics. This new result is now shown as Appendix Fig. S4 in the manuscript.

3. Supp Fig 4A (now Fig. EV2A) is difficult to comprehend. I would prefer to see NRP2 in Green and SOX7 in red. Provide PROX1 in red with SOX7 in green in a separate panel. Higher magnification images will also be helpful.

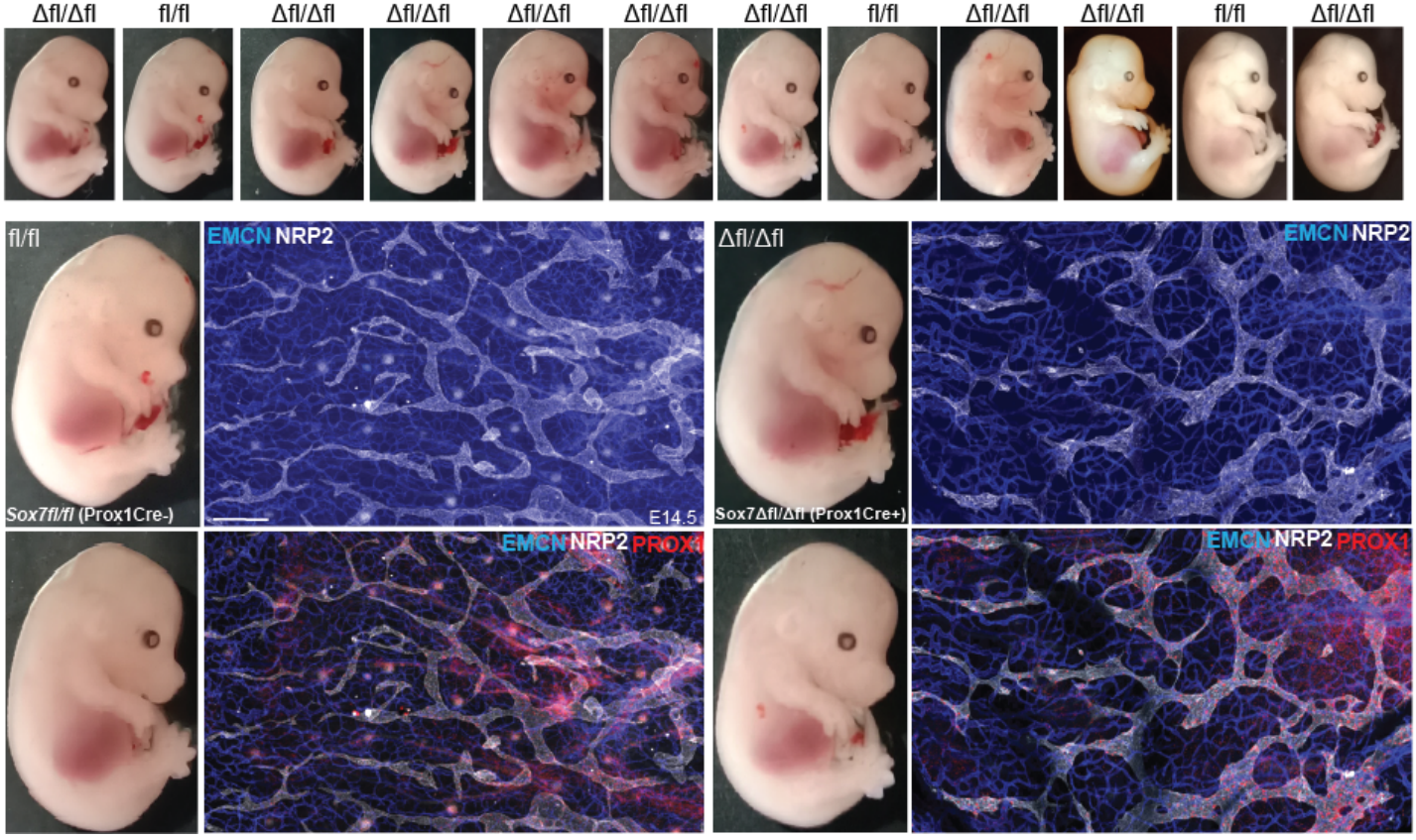
Unfortunately due to species incompatibility, we had to use the same secondary fluorophore conjugated antibody (anti-goat) to detect SOX7 (nucleus) and NRP2 (membrane), which are both pseudo-colored in white. It is not possible to change the color display of the current image.
The main message of Supp Fig 4A (now Fig. EV2A) is to show the dramatic change in lymphatic vessels morphogenesis (NRP2 staining) along with the severe loss of SOX7 expression (decreased nuclear staining).

We have now included some higher magnification images to illustrate the morphogenic defects (enlarged vessels), this also helps to visualize specific expression patterns of SOX7 (PECAM positive, white and green) and NRP2 (PROX1 positive, red and white). Please refer to inset 1 and inset 2 in the updated Fig. EV2A.

4. In Supp Figures 2A and B (now Appendix Fig. S2A and Fig. EV1B) V5 appears to be expressed in the cardinal vein. Is there any reason why SOX7 may not be directly inhibiting LEC specification in the cardinal vein?
Yes this observation is accurate, there is a subset of venous endothelial in the CV that do express SOX7. Not all these endothelial cells are PROX1-positive. As shown above in Reviewer_fig1, there is a fraction (around 30%) of PROX1-positive LEC progenitors in the CV that co-express SOX7, some are $SOX7^{low}/PROX1^{high}$ or $SOX7^{high}/PROX1^{Low}$.
It is important to note that in the cardinal vein, not all PROX1-positive cells will turn into LECs. For instance some PROX1-positive endothelial cells will contribute to venous valve formation. It is possible that SOX7 negatively regulates PROX1 directly or indirectly in the context of venous valve formation. As shown in the next point below, this seems not be the case during lymphangiogenesis, since the genetic disruption of *Sox7* in PROX1-positive LECs does not cause a lymphatic phenotype.

5. Have the authors attempted to delete Sox7 in LECs by using Lyve1-Cre or Prox1-CreERT2? This could be a definitive approach to exclude any LEC-specific contribution of Sox7.

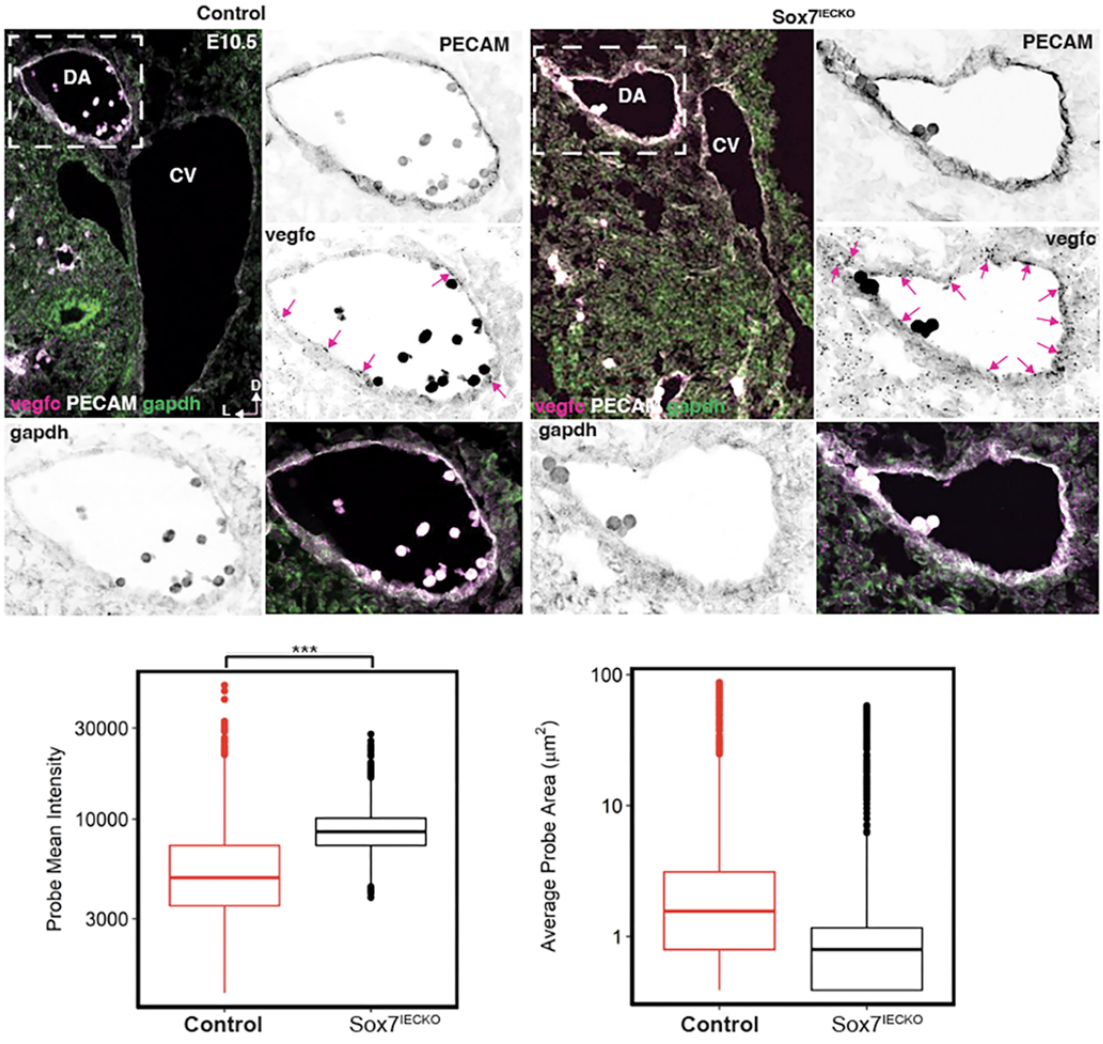
In order to address cell autonomy and to investigate further the cell of origin driving the lymphatic vascular phenotype, we generated a new conditional knock-out mouse line using *Prox1-CreERT2:Sox7 fl/fl* genetic background.
The tamoxifen treatment regimen was similar to that used in the *Cdh5-CreERT2* line (Injection at E9.5 and E10.5, harvest at E14.5). We have analysed in total, 7 control and 12 homozygous embryos at E14.5 using gross morphological comparison and skin wholemount with PROX1, Endomucin (EMCN) and Neuropilin 2 (NRP2) (Reviewer_Figure 3). When the *Sox7* gene is deleted by the *Prox1-CreERT2* driver in lymphatic endothelial cell progenitor (E9.5 and E10.5) and embryonic skin (analysed at E14.5), neither an obvious oedemic phenotype nor any gross morphogenetic abnormalities of the lymphatics were found. This finding suggests that the *Cdh5-CreERT2: Sox7 fl/fl* lymphatic phenotype is non-cell autonomous and is caused by the loss of SOX7 function in blood vascular endothelial cells. Further, we have let the *Prox1-CreERT2: Sox7 fl/fl* embryo developed to E16.5 and did not observe any phenotype at that time point either (data not shown). This result is now shown as a new figure in the manuscript (Fig. EV3).



Reviewer_figure 3. Conditional deletion of *Sox7* gene in lymphatic endothelial cells using *Prox1-CreERT2: Sox7 fl/fl* genetic background. Genetic disruption of *Sox7* exon 2 in lymphatic endothelial cells was performed upon tamoxifen treatment at E9.5 and E10.5. Skin lymphatics were analysed at E14.5. At that time point, control and mutant embryos display no significant morphological difference (eg. lack of edema).
The lymphatic vasculature in the *Sox7*-null embryos appears patterned properly and do not reproduce the phenotypic outcome obtained when using the *Cdh5-CreERT2* driver to delete *Sox7* function.

6. Consistent with Karkkainen et al (Nat Immunology 2004) VEGF-C is expressed potently in the mesenchymal cells around the cardinal vein of E11.5 embryos (Fig 2C). Therefore, I am a bit troubled by the conclusion that VEGFC overexpression in the BECs is responsible for the phenotypes of Sox7 KO embryos. I am not sure if a modest increase in BEC-expressed VEGF-C will be sufficient to cause such a dramatic phenotype. Could there be changes in mesenchymal VEGF-C as well? Please use in situ hybridization to compare and quantify Vegfc expressions in control and Sox7 KO.

We do agree with the comment stating that VEGFC expression is not restricted to the vascular endothelium and is clearly detectable in the mesenchyme. Our observation using single molecule Fluorescent *in situ* Hybridysation (smFISH) further validate this observation (Reviewer_fig 4). It is possible that in fact *Sox7* loss of function in endothelial cells also impact a cross talk between the endothelium and the mesenchyme leading to a change of mesenchymal Vegfc expression but also other lymphangiocrine molecules. This is beyond the scope of this work and we have modified the discussion accordingly to be more flexible with a potential mechanism at play (eg other cells types involved and / or other molecules having an additive effect to Vegfc loss of function).
In order to quantify Vegfc transcripts *in vivo* using smFISH, we have developed an automated and unbiased analysis pipeline, to process images post-acquisition in order to quantify the change in fluorescence intensity of the probe signal intensity. Details on this method are available in the material and method section of the manuscript. Serial sections on both control (n=5) and *Sox7*^{iECKO} (n=7) embryos were performed at E10.5 and *Vegfc* probe signal intensity was measured specifically in endothelial cells of the dorsal aorta. This measurement was performed systematically in an unbiased manner over dozen of images from a similar anatomical location in the control and *Sox7*^{iECKO} embryos. Hundreds of cells and thousands of molecular events were measured to compare signal intensity (eg ctrl :10535 probes within vessel membranes from 37 images and *Sox7*^{iECKO} 4055 probes within vessel membranes from 18 confocal images). This new result is now shown as Fig 4G in the manuscript.



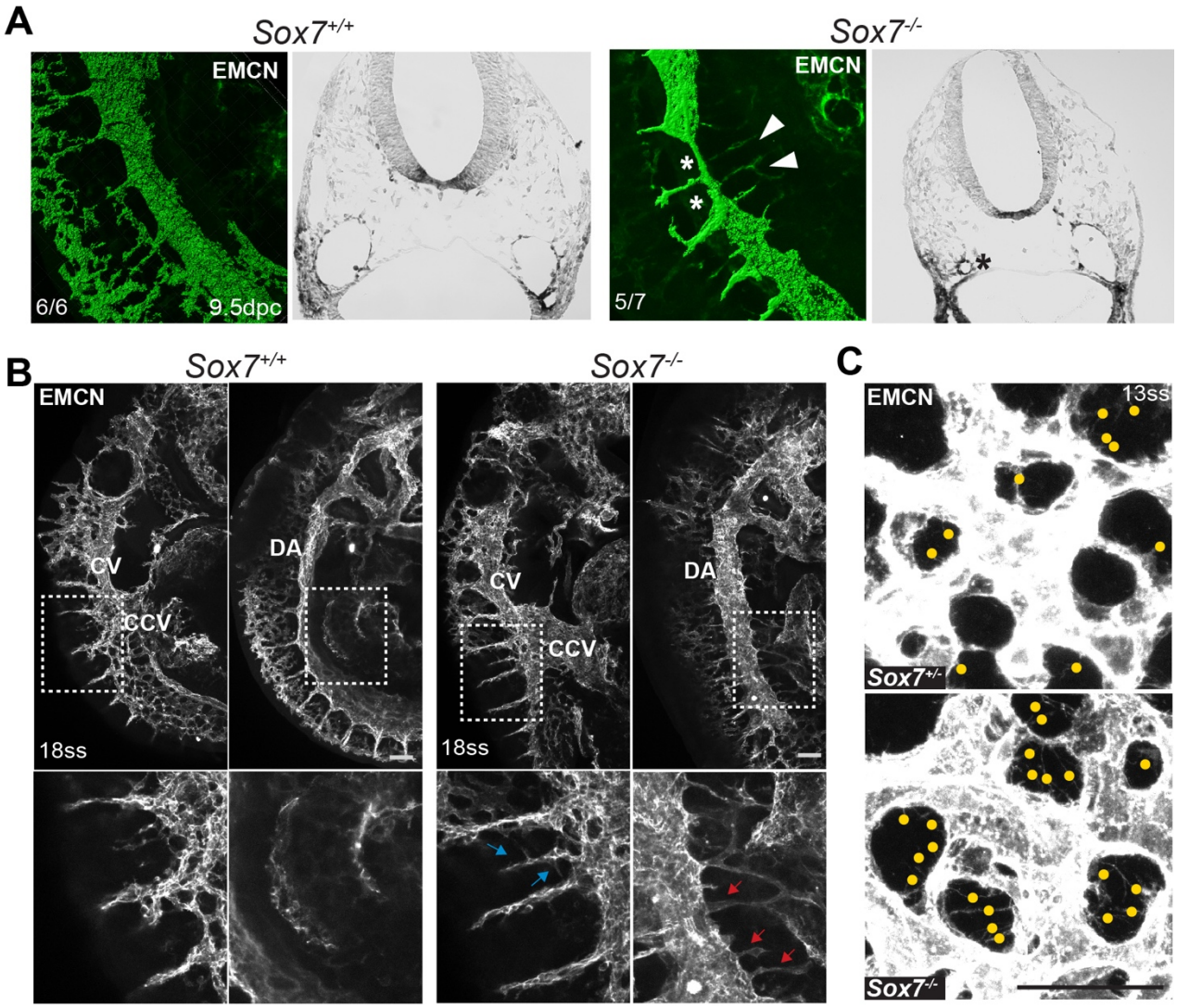
Reviewer_figure 4. Quantitation of *Vegfc* transcript using single molecule RNA FISH *in vivo*. Trunk transverse sections were performed at E10.5 for both control and *Sox7*^{iECKO} and immune-stained for PECAM along with *Gapdh* and *Vegfc* fluorescent *in situ*. Individual molecule or *Vegfc* transcript (pink arrows) is detectable within the cytoplasm of PECAM positive cells of the dorsal aorta. The mean grey scale intensity of these probes in Control was 5845. The mean grey scale intensity of these probes in *Sox7*^{iECKO} was 8956. Mean probe intensity was significantly different between *Sox7*^{iECKO} and Control (P Value <2e-16, Test = *t*-test, control embryos, n=5; *Sox7*^{iECKO}, n=7). No differences were observed for the average probe area, meaning that distribution of the probe across the DA remained unchanged between control and *Sox7*^{iECKO}.

7. Please determine if VEGFC overexpression in BECs results in lymphatic vascular defects that are similar to Sox7 KO embryos.

It has been previously published by Lohela et al (Am J. Pathol 2008) that VEGFC over-expression by the endothelium (*Tie1-TetR-VP16:tetOS-mVegfc* double transgenic animals) give rise to lymphatic vessels hyperplasia and angiogenic sprouting. At E10.5 peripheral lymphatic vessels have not yet developed, and most blood vessels still express VEGFR-3, hence the angiogenic defects in presence of *Vegfc* over-expression.

We have appended results that illustrate ectopic angiogenic response in the dorsal aorta at E10.5 (Reviewer_fig5).

Dysmorphic lymphatic vessels are thoroughly characterized in the manuscript and display comparable morphology to what is described in Lohela et al 2008: “The lymphatic vasculature in the skin was hyperplastic and in many cases integrity of the endothelium had been lost, with individual lymphatic endothelial cells no longer tightly attached to each other to form tubes”



Reviewer_figure 5. Deletion of *Sox7* cause ectopic angiogenic sprouts at E10.5. (A) *Sox7*$^{-/-}$showed asymmetrical vessel constriction in the dorsal aorta (asterisks), and sprouts (white arrowheads) were frequently observed in this constricted region, which was never observed in *Sox7*$^{+/+}$. **(B)** Sagittal view of the 18ss wild type and *Sox7*$^{-/-}$ at the level of cardinal vein (CV) and dorsal aorta (DA). The *Sox7*$^{-/-}$ mutant shows protrusion of random sprouts (blue arrow) perpendicular to the venous intersomitic vessels and the abnormal medial vessel sprouting (red arrow) towards the heart cavity from the DA of *Sox7*$^{-/-}$. **(C)** High resolution imaging at 13ss revealed multidirectional hyper sprouting in *Sox7*$^{-/-}$ cephalic plexus. Filipodia are indicated as yellow dots. CV, cardinal vein; DA, dorsal aorta. Scale bar 100 µm.

Minor comments

1. Does Sox7 play a time-specific role in lymphatic vascular development?

According to SOX7 expression pattern that is mostly observed in blood vascular endothelial, and weakly expressed in a sub-population of PROX1 positive cells in the wall of the cardinal vein, it might have a role really early on, however the *Prox1-Cre* deletion of *Sox7* suggests that it is a non-cell autonomous function.
It is likely that SOX7 plays a role in time-specific fashion indirectly via the arterial endothelium at time points where BEC and LEC interplay is required to shape the lymphatic vasculature.

2. What are the other VEGFC+ cell types in Fig 2B?

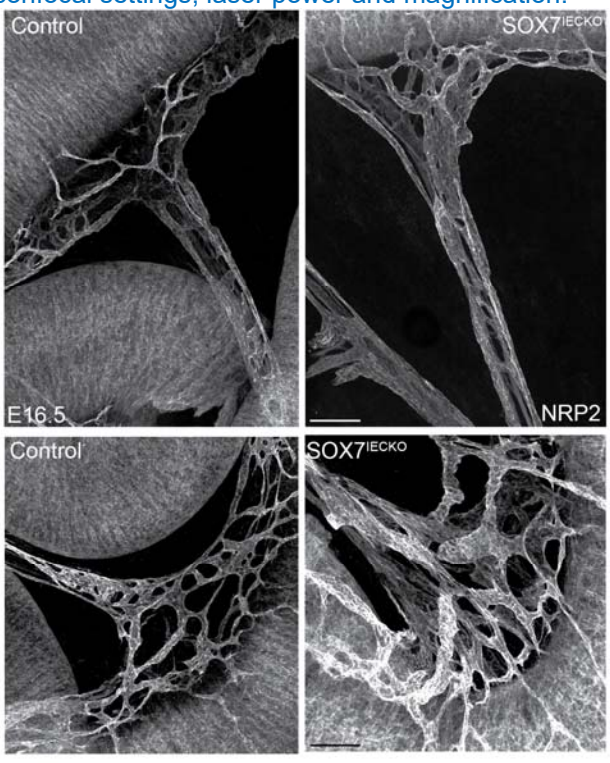
These cells are mesenchymal cells., likely fibroblasts and possibly neurons but we have not further characterized these Vegfc expressing cells types *in vivo* using combination of specific molecular markers. Nebulosa plots from single-nuclei RNA-Seq shows that some of these cells are Npas3+ neurons and Acta2+ smooth muscle cells (Fig. EV4).

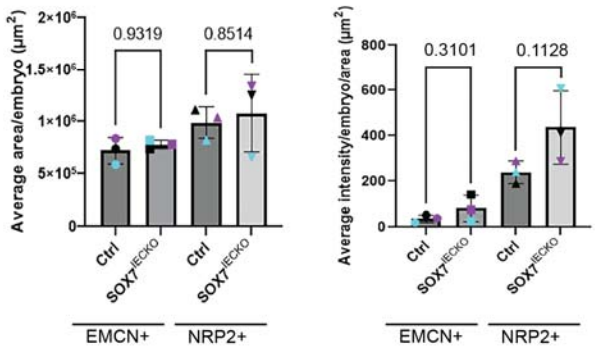
Referee #2:

In this study Chiang and colleagues have investigated the role of the blood endothelium in patterning the dermal lymphatic vasculature at the level of Sox7 acting directly on Vegfc to control the number and distribution of Prox1+ LECs. They use a combination of an endothelial cell specific knock-out of Sox7, snRNA-Seq, protein-protein interaction and ChIP-Seq analyses to conclude that Sox7 interacts directly with the transcriptional repressor Hey1 at the Vegfc locus (promoter and distal elements) to repress Vegfc in BECs and as such regulate the appropriate number of LECs that contribute to the forming dermal lymphatics. This is a very interesting study and one which adds significant insight into how the blood vasculature functions to regulate lymphatic vessel growth and patterning. Whilst hinted at previously in conjunction with BEC chemotactic guidance cues, a detailed molecular characterisation as to how blood vessels might guide lymphatic patterning is currently lacking. Moreover, we know very little about the upstream regulation of Vegfc as a key factor in lymphatic vessel development; as such, the insights provided here around the direct role of Sox2-Hey1 provide important mechanistic insight. That said, there are some issues with the study in its current form that warrant attention and some suggestions that might improve the manuscript:

1. The authors have focused their analysis exclusively on the lymphatics of the skin and yet both blood and lymphatic endothelium are known to be heterogeneous across lineages and organ systems. An open question, therefore, is whether the mechanism of BEC and LEC interaction described herein translates to other vascular beds(?). This is an important issue to support some of the more general statements and conclusions herein (for eg. end of the Introduction: " . . . SOX7-mediated fine-tuning of VEGFC expression in BECs, a process essential for correct lymphatic growth and patterning."). Whilst it is not expected the authors repeat their study in its entirety, it would be highly informative if they could provide some analysis of Sox7-V5 expression in other (non-dermal) blood endothelium and investigate at least one other lymphatic bed (eg. mesenteric, cardiac) in the context of a potential gross lymphatic patterning phenotype in the Sox7-ECKO mutant embryos.

We have now performed an analysis of the developing lymphatics in the mesentery comparing control and *Sox7*^{iECKO} embryos at E16.5 (Reviewer_fig6). The most striking difference between the two genotypes when comparing neuropilin-2 (NRP2) signal is at the level of vessel network remodeling. Images of all samples were taken with the exact same confocal settings, laser power and magnification.





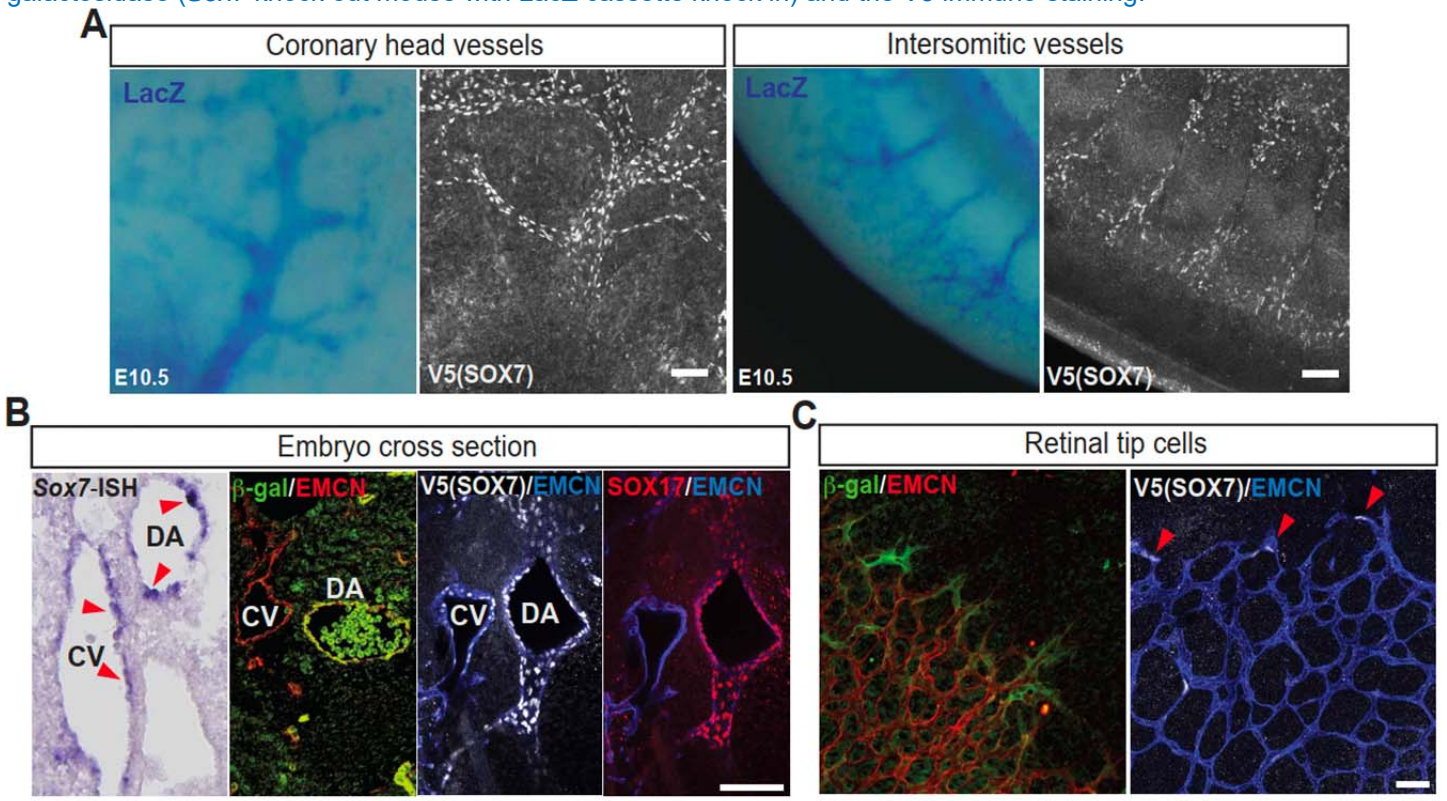
Reviewer_Figure 6. Developing lymphatic vessels of the mesentery in the *Sox7*^{iECKO} assemble as a vascular network but show gross remodeling defects. Wholemount immuno-staining of the mesentery with NRP2 antibody reveals the lymphatic vascular network that connects to the gut. The left graph reports the total area of endomucin-positive (IF not shown) and NRP2-positive vessels (upper and lower panels). There is no quantitative difference in vessel area coverage, but some lymphatics in the *Sox7*^{iECKO} appears severely enlarged and mis-patterned. Further, quantitation of the NRP2 fluorescent signal normalized to vessel area in the *Sox7*^{iECKO} embryos reveals an increase in signal intensity, albeit not significant (right graph). Control, n=3; *Sox7*^{iECKO}, n=3), *p*-value one-way Anova. Each embryo is colour-coded.

During embryonic development at the early steps of lymphangiogenesis (E10.5), two sub-populations of LECs have been reported that are either LYVE1^{high}; NRP2^{low} or LYVE1^{low}; NRP2^{high}. The former establishes the developing lymph sacs, whereas the latter migrates as individual stream of cells towards the dorso-lateral part of the embryo in response to Vegfc signalling (Francois et al Dev Biol 2012). In the *Sox7*^{iECKO} we show that *Vegfc* is over-expressed *in vivo* (please see Reviewer_figure4). It is therefore expected to observe an increased response of the NRP2-positive population as it is observed in the developing gut at E16.5.

It is important to note that organ-specific mechanisms of lymphatic vessel assembly are different in the skin and in the gut. Dermal lymphatics assemble via the connection of pre-existing lymphatics with local progenitors arising from a non-venous vasculature. By contrast mesentery lymphatics grow from pre-existing lymphatics, which connect with Pitx2 positive progenitors cells from the arterial endothelium (Mahadevan et al Dev Cell 2014). Given the role of SOX7 in A/V specification and the hemogenic endothelium differentiation, the interpretation of the data in the mesentery has to be considered carefully.

2. The authors rely on a Sox7-V5 transgenic reporter line to determine SOX7 expression in BECs- the assumption here is that there is no suitable SOX7 antibody to support these studies; please clarify in the text. If so, then it would be helpful to include some RNAScope or HCR to at least show endogenous Sox7 mRNA expression and to provide confidence that the Sox7-V5 (or indeed the additional beta-galactosidase analysis in the Sox7-KI line) appropriately reflects endogenous SOX7 expression.

When we initially developed the *Sox7*-V5 transgenics (2013), due to the lack of reliable SOX7 antibody available; but since then, a trustworthy reagent became available (Zhou et al PlosOne 2015). We have used this antibody to detect endogenous SOX7 expression on skin samples (Reviewer_figure 2, under reviewer 1 request #2).
In order to verify that the V5 epitope knock in reporter system provide a faithful labeling of SOX7 similar to the endogenous SOX7, we performed a comparison of the expression pattern between *Sox7 in situ* hybridization, beta-galactosidase (*Sox7* knock out mouse with LacZ cassette knock in) and the V5 immuno-staining.



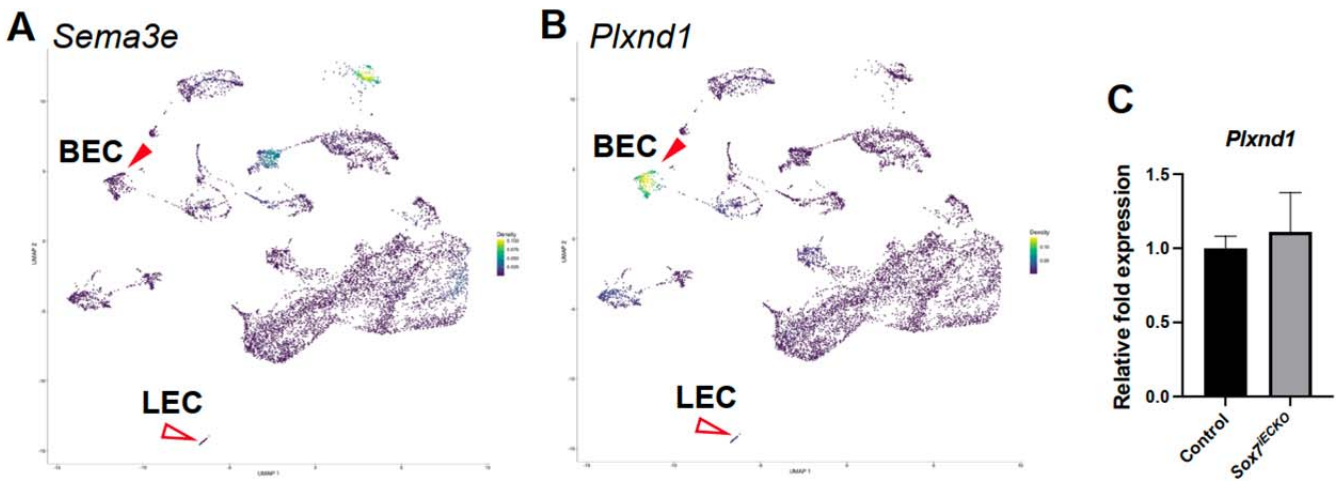
Reviewer_figure 7. *Sox7*-V5 transgenic reporter mouse line recapitulates *Sox7* endogenous expression. (A) Wholemount lacZ staining from *Sox7* knock embryos compared to V5 immunostaining at E10.5. For both the head coronary vasculature and the trunk vessel network the expression pattern of X-gal and V5 staining is similar. (B) Comparison of the dorsal aorta and cardinal vein for *Sox7 In situ* hybridization, beta-gal and V5 staining on transverse section of a E10.5 embryo. (C) Retina whole mount staining of beta-galactosidase and V5 reveals expression of SOX7 in endothelial tip cells, consistent with the expression levels of this transcription factor reported in Del Toro et al Blood 2010.

3. The lymphatic phenotype in the Sox7 ECKO mutant embryos (Fig. 1) is one of ectopic and increased numbers of Prox1+ LECs, which as the authors later conclude is consistent with over-expression of VEGFC- that said, how do the authors explain the other aspect of the mutant phenotype, ie severely dilated, lacked inter-vessel branches and showed migration defects (Fig. 1C-F), given the known role of VEGFC in lymphangiogenesis?

We do agree with the reviewer that the mechanism which underpin the lymphatic phenotype is most likely through the disruption of other genetic pathways in addition to change in *Vegfc* transcription. SOX7 has thousands of potential direct target genes and its genetic disruption may lead to multiple interferences with different regulatory networks. Our previously published work (Chiang et al 2017) and others (Kim et al Circ Res 2016) have shown that SOX7 also modulates the transcription of Notch1, Dll4 and the VEGFR2 pathways. It is therefore highly likely that perturbation of these pathways is also at play and contributes to the phenotypic outcome (eg lack of inter vessel branches).

4. The snRNA-Seq analysis presented in Fig. 2 is a nice study and clearly indicates appropriate Sox7 and Vegfc expression in BECs, however, what would be highly informative here is an snRNA-seq assessment of Sox7-ECKO whole skin, to potentially identify dysregulated pathways. Assuming this has not been carried out, and as such represents an extensive amount of work, the authors could select candidate genes and pathways from the wild type snRNA-Seq dataset, including Sox7 and Vegfc, and test expression in Sox7 ECKO cells (analogous to the Vegfc/Vegfr3 focused analysis in Fig. 2D).

We assessed Sema3e and Plxnd1, both known to be at play to regulate LEC and BEC interaction (Liu et al Cell Reports 2016 and Maruyama et al iScience 2021). We then assessed the expression of both molecules in BEC and could only detect Plxnd1 (Reviewer_figure 8). Further measuring the expression levels of Plxnd1 in BEC, comparing control and *Sox7*^{iECKO} did not reveal any significant changes for this gene. This suggests that other guidance molecules, but neither Plxnd1 nor Sema3e, are at play.

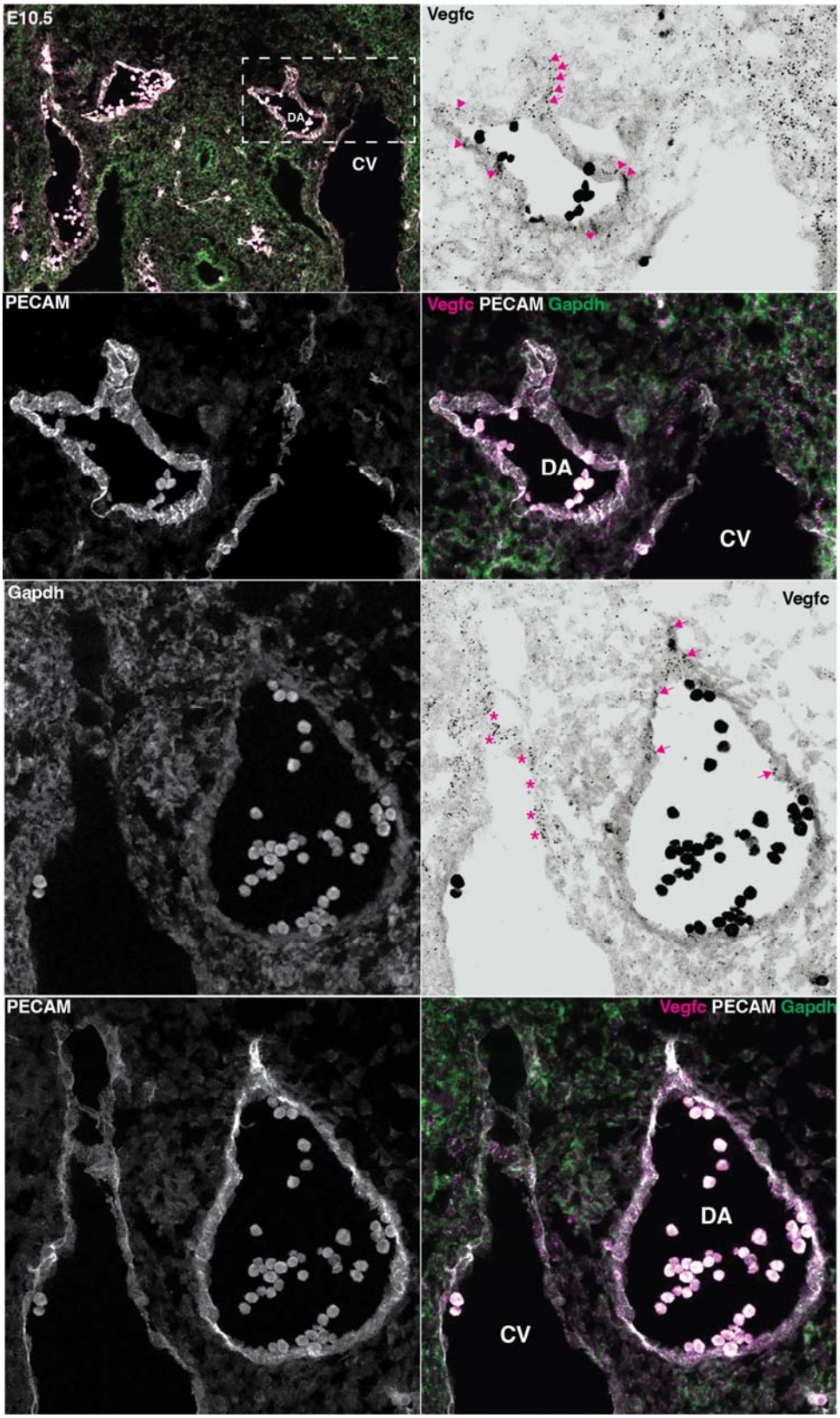


Reviewer_figure 8. Expression profiles of Sema3e and Plxnd1 in BEC and LEC during development. (**A** and **B**) Single Cell RNA sequencing reveals that *Plxnd1* but not *Sema3e* is expressed in BEC at E14.5. (**C**) qPCR analysis show that the expression levels of *Plnxd1* is not affected in the *Sox7*^{iECKO} endothelial cells. Expression is normalised to endothelial marker *Pecam* and *Cdh5*. Scored sibling controls, n=9; *Sox7*^{iECKO} mutants, n=7. Mean ± SEM; *t*-test.

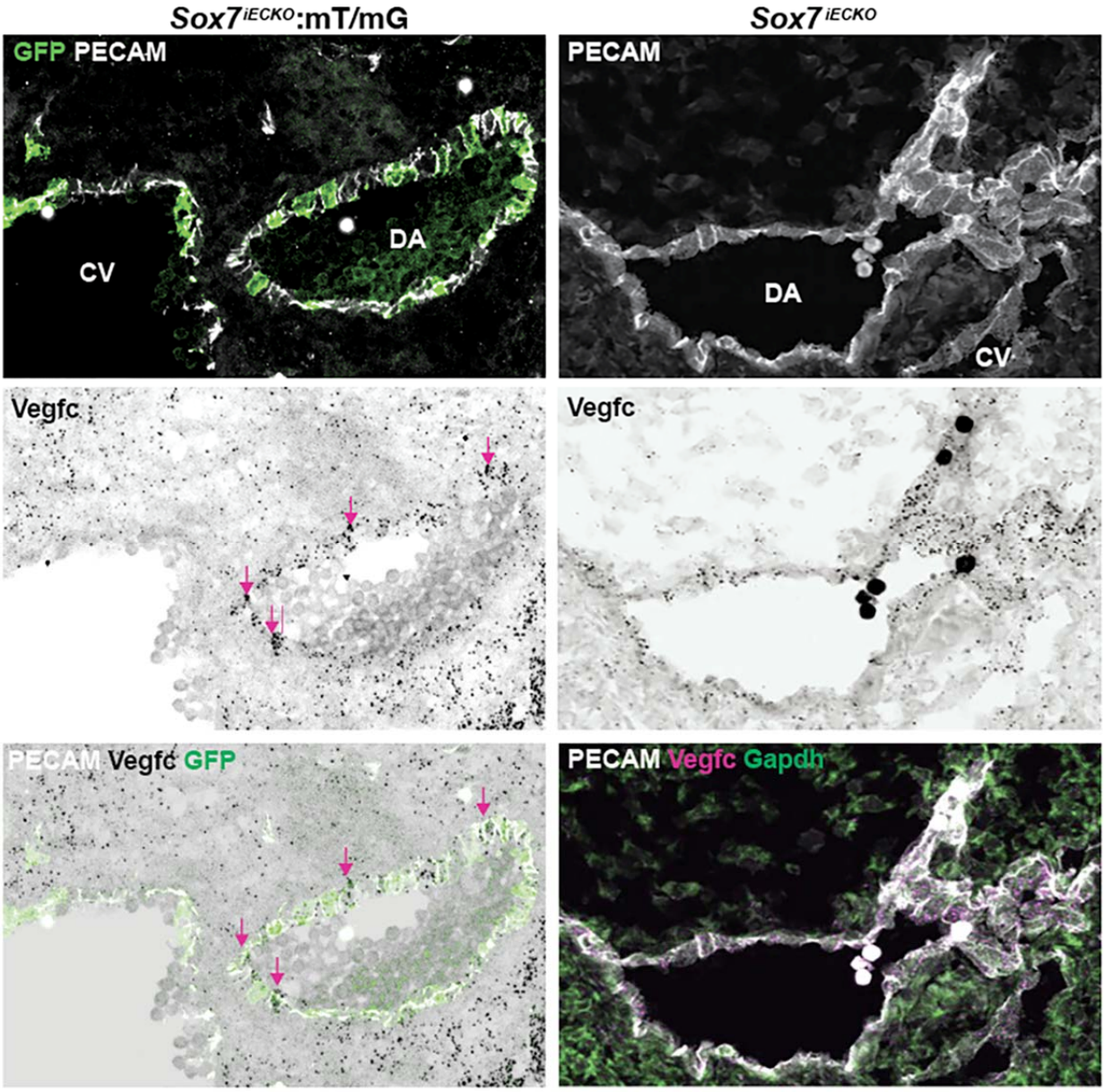
5. In the analysis to test whether Sox7 cell autonomously regulates Vegfc transcript levels the authors down-regulated Sox7 using a siRNA-based approach in HUAECs and HUVECs. Both cell lines showed reduced Vegfc expression following siRNA KD- how does this relate to the in vivo situation, given the restricted expression of Sox7 (Sox7-V5) to arterioles (and low levels in mature arteries) and no expression in veins/venules (Supp. Fig. 2) (now Appendix Fig. S2)?

We have analysed *Vegfc* transcripts with RNA smFISH *in vivo* at the early time point of lymphangiogenesis using serial transverse sections of the trunk at the level of the cardinal vein and dorsal aorta. We found that Vegfc mRNA is mostly expressed by the arterial endothelium with much lower expression levels in the venous compartment. The expression of this transcript is mostly observed in the dorsal aorta (Reviewer_fig9, pink arrowheads) and at the level of intersomitic arteries (Reviewer_fig9, pink arrows). In light of this observation, the most relevant model *in vitro* seems to be HUAECs, although in some instances venous endothelial cells positive for *Vegfc* transcripts are detected (Reviewer_fig9, pink asterisks).

The fact that *Vegfc* is up-regulated in *Sox7*-null endothelial cells of the dorsal aorta (please refer to Reviewer _fig4 under reviewer #1 and Reviewer_fig10 and Fig. 4G of the manuscript) indicates that this transcription factor is part of a negative feedback loop to maintain a certain level of *Vegfc* expression in the endothelium.



Reviewer_figure 9. *Vegfc* transcripts are differentially expressed in the dorsal aorta, cardinal vein and inter-somitic arteries in wild type embryo. Transverse section of a E10.5 wild type embryo labeled by RNA smFISH for Gapdh and Vegfc and by immuno-staining for PECAM. *Vegfc* transcripts are detectable in arterial cells (upper panels, pink arrowheads) and intersomitic artery (pink arrows) and are also expressed in a subset of cells from the venous endothelium (lower panels pink asterisks).



Reviewer-figure 10. Arterial endothelial cells in the *Sox7*^{iECKO} display high levels of *Vegfc* transcripts. *Sox7*^{iECKO} with a mT/mG genetic background (left column) reveals that GFP-positive arterial ECs (*Sox7*-null) show a robust expression of *Vegfc* transcripts (pink arrows). Similar observation is reported in the *Sox7*^{iECKO} (without the mT/mG background, right column).

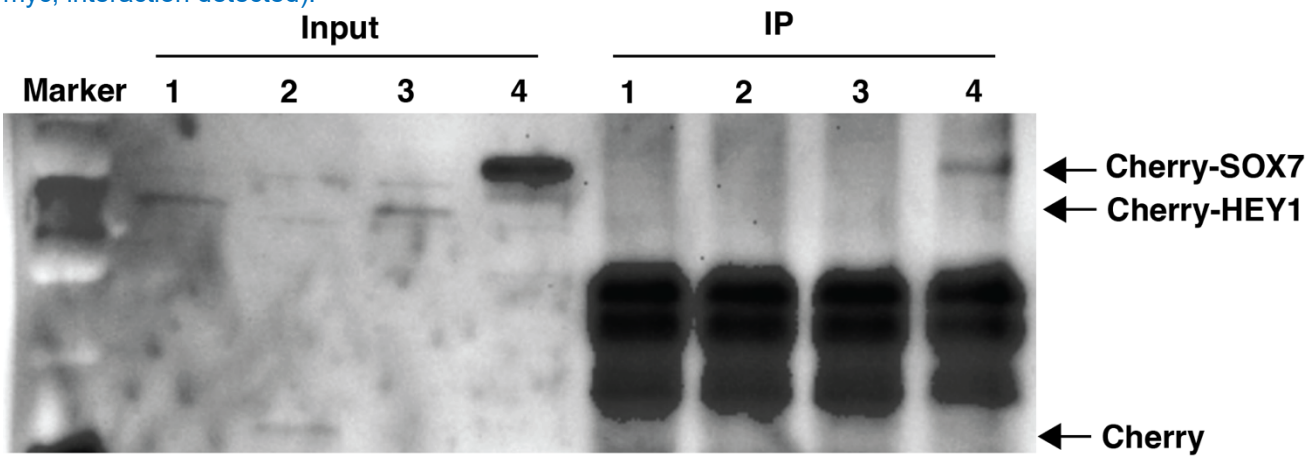
The quantitative comparison of *Vegfc* expression levels in control versus *Sox7*^{iECKO} in arterial cells of the DA is shown in Reviewer_fig4 (under reviewer 1). This analysis revealed a significant *Vegfc* up-regulation in the arterial endothelium of the *Sox7*^{iECKO}. This new result is also now in the manuscript as Fig. 4G.

6. The data to demonstrate Sox7 interaction with Hey1 in Fig. 3E and 3F is based on a cell-free interaction assay and over-expression in HEK293 cells; this is not in vivo proof nor is it carried out in an appropriate EC line. As such the protein-protein interaction analysis needs improving to make a relevant and meaningful claim on Sox7-Hey1 interaction in the developing blood endothelium.

As suggested we have performed co-immuno-precipitation (co-IP) for SOX7 and HEY 1 in HUVEC cells line (venous endothelial) used tagged version of these protein due to the lack of reliable antibody for immuno-precipitation.
This approach confirms the co-IP done in HEK293 cells. One of the limitations is the high level of endogenous SOX7 protein in HUVEC, which might compete with the tagged version therefore interfering with the pull down efficacy. Overall

we have shown using 2 different homogenous assays (ALPHAScreen and fluorescent correlation spectroscopy) combined with co-IP in two different cell lines that there is a direct protein-protein interaction between SOX7 and HEY1. The approach based on co-IP in cells does not prove a direct physical protein – protein interaction between SOX7 and HEY1. The rationale of complementing co-IP with Alpha Screen and Fluorescent Correlation Spectroscopy is to provide biophysical evidence that SOX7 and HEY1 are engaged in a direct protein-protein interaction.

We have now included the co-IP in HUVEC as Appendix Fig. S5 and this is shown here as Reviewer_fig11. Please see legend for details of the experimental conditions. Of importance the position of the HEY1 protein tag either in C-term or N-term interferes with the protein-protein interaction (IP lane 3 Cherry-HEY1, no interaction detected and IP lane 4 HEY1-myc, interaction detected).



Reviewer_figure 11. SOX7 and HEY1 protein-protein interaction in HUVEC. HUVEC were transfected with the following protein pair 1: GFP/HEY1-Cherry; 2: GFP-SOX7/Cherry; 3:GFP-SOX7/Cherry-HEY1; 4: Cherry-SOX7 /HEY1-myc.
IP was performed with either GFP (IP, lanes 1-3) or myc (IP lane 4) antibodies and western blot performed with an anti-Cherry antibody. Input conditions reveal the size of SOX7 and HEY1 Cherry-tagged proteins.

7. To test whether the identified Vegfc-255 and Vegfc-152 regions act as enhancers of Vegfc activity, the authors generated a series of Vegfc-luciferase reporter constructs that harbor a combination of different putative regulatory regions from the mouse or human locus (Fig. 4C, E). This is an artificial system and could be significantly improved by a more elegant in vivo approach, to establish transient Fo transgenic reporter lines of the VEGFC regulatory regions in a wild type versus Sox7 ECKO background; the latter serving to further demonstrate that the identified regulatory regions are also Sox7-dependent.

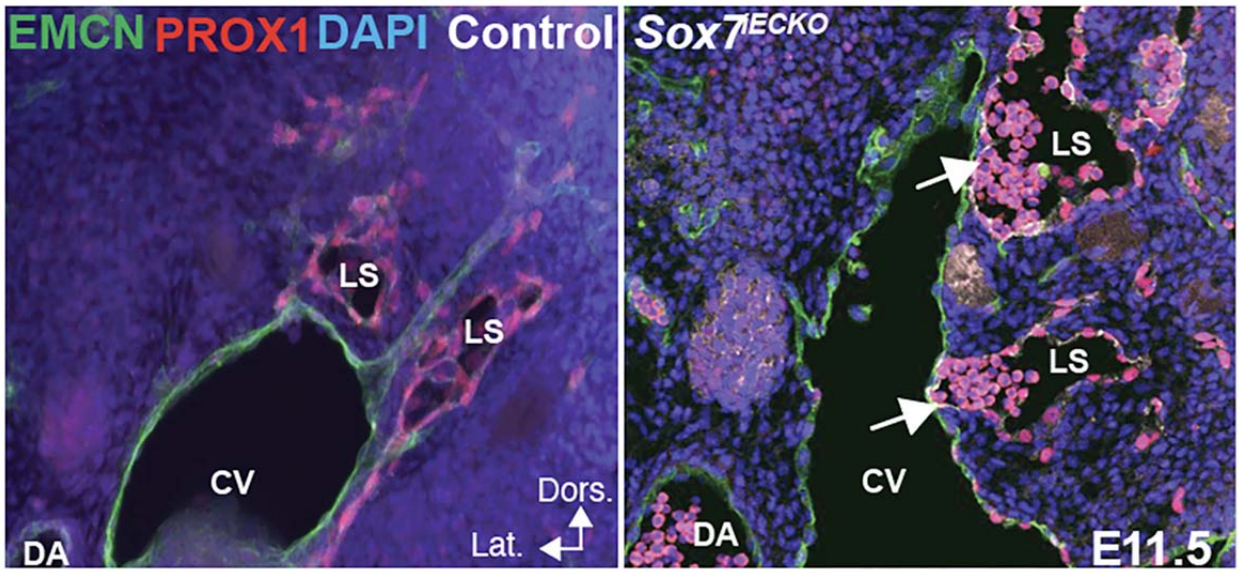
In this study, we used a synthetic *in vitro*-based assay to demonstrate Vegfc regulatory regions dependency on SOX7 activity. This approach is quantitative and leaves no doubt on the molecular mechanism at play but we agree that the limitation of such an approach is the lack of information on the chromatin context. These regions were selected based on *Sox7*-V5 ChIP-Seq dataset and overlap with ChIP-Seq histone marks from the ENCODE consortium. The presence of repressive marks on SOX7-bound region is indicative of the potential negative regulatory of SOX7 on these regions in both mouse and human.
While we appreciate the importance of *in vivo* work, using *Sox7conditional* knockout as a genetic background proved to be challenging due to the fact that blastocysts have to be genotyped, then rapidly selected to perform transient transgenic experiments. This approach also includes treating embryo-implanted pseudo-pregnant female with tamoxifen in order to induce conditional deletion of Sox7. This treatment has side effects on under these conditions. Working with F0 requires a high number of transgenic embryos to ensure reproducibility. The throughput of this of approach is too low and slow to make any robust conclusion given the difference in expression pattern of the Vegfc reporter cassette. Some of these differences could be due to either variability in plasmid copy number, or random integration sites. The combination of a low number of embryos per litter and technical limitations significantly impairs a thorough quantitative approach to compare accurately variation in reporter expression.

Referee#3:

In this study, the authors investigated blood endothelial cell (BEC)-mediated paracrine mechanisms involved in the regulation of lymphangiogenic growth factor (VEGFC) expression and lymphatic patterning. Using various state-of-the-art methodologies (including transgenic reporter mice, conditional gene knockout mice, single nucleus RNA-seq, luciferase reporter mediated promoter studies, ChIP-seq and ChIP PCR, protein-protein interaction assays, etc.), they show that the BEC-specific transcription factor SOX7 modulates BEC expression of VEGFC via a dual mechanism: by upregulating Hey1 which represses VEGFC expression, and by directly binding to the VEGFC promoter and repressing its expression, alone or in interaction/combination with HEY1 to which SOX7 was also found to bind. BEC-selective knockout of Sox7 in mouse embryos resulted in a defective dermal lymphatic phenotype and in ectopic supernumerous LEC progenitors in the cardinal vein.
The study is interesting and revealed novel mechanisms. It also left a few straightforward questions unanswered that could be addressed or discussed. Some points for clarification of specific data, and a few recommendations for further strengthening of the data (e.g. missing controls) and the conclusions are listed below.

MAJOR COMMENTS

- The authors analyzed the consequences of loss of Sox7 expression in BECs on lymphatic patterning around the time of lymph sac formation in the jugular region, but no data are shown on lymph sac formation. What is the phenotype?
Is it abnormally formed, or normally formed but enlarged?
Analysis of lymph sac formation in the *Sox7*^{iECKO} reveals that these primary structures still do form in the mutant but are filled with red blood cells (Reviewer_fig12). This is an indirect indication that either proper lymph sac assembly has been compromised or that lympho-venous valve structures may also be compromised or that potentially a hemogenic program has been reactivated in LECs. Of importance, LEC subtype presents in lymph sacs are LYVE1^{high} but neuropilin-2^{low} (Francois et al Dev Biol 2012), it is therefore likely that lymph sac morphogenesis relies on additional signalling pathways to *Vegfc* stimulation. This could explain why lymph sac patterning is not drastically affected by BEC-specific upregulation of *Vegfc*. In this study, we show that there is an ectopic activation of *Vegfc* transcription in arterial endothelial cells. This is a different scenario than a global upregulation of *Vegfc* in all cell types.



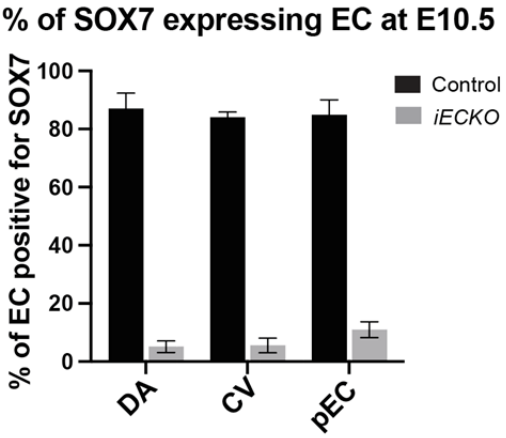
Reviewer_figure 12. Blood-filled lymph sacs in *Sox7*^{iECKO} mutant embryos. Transverse section of E11.5 control and mutant embryos stained for PROX1 (red), Endomucin (green) and DAPI (blue) show the presence of red blood cells in the developing lymph sacs of the *Sox7*^{iECKO} embryos. DA, dorsal aorta, CV, cardinal vein, LS, lymph sac. Number analysed per genotype, control, n = 4; *Sox7*^{iECKO} n = 5.

In control conditions, the newly revealed SOX7-HEY1-VEGFC axis acts in a polarized manner in the cardinal vein (cf Fig. 5A,B). Is there any clue how this polarization is regulated?

It is possible that the asymmetric genetic program that takes place in different regions of the cardinal vein to restrict LEC progenitors formation in a dorso-lateral manner originates from differential mechanical stimuli (different shear stress in a curved vessel) or exposition to distinct extra cellular matrix components. With the current level of knowledge in the field, this remains only speculative. We agree with the reviewer that understanding the molecular etiology of pattern formation is a fascinating aspect of lymphangiogenesis but remains beyond the scope of this study.

- A few questions are raised related to Figure 1:
Fig. 1A: the quantification in the top right panel is somewhat confusing as it does not show whether all peripheral and all DA and all CV ECs are positive for SOX7 for the control genotype. Although indeed unmistakenly low, the results shown for the ECKO embryos are therefore also confusing. Showing the data per EC type should be considered.
Fig. 1C,D: Is it correct to name this parameter migration?

In order to clarify the quantitation we have now display the relative abundance of SOX7 protein in the different vascular beds as follows: dorsal aorta (DA), cardinal vein (CV) and peripheral endothelial cells (pEC) (Reviewer _figure 13).
This is now shown in the graph below and Fig. 1A has been updated. Panel Fig. 1D has been also updated and labelled with “LEC-front distance to midline”



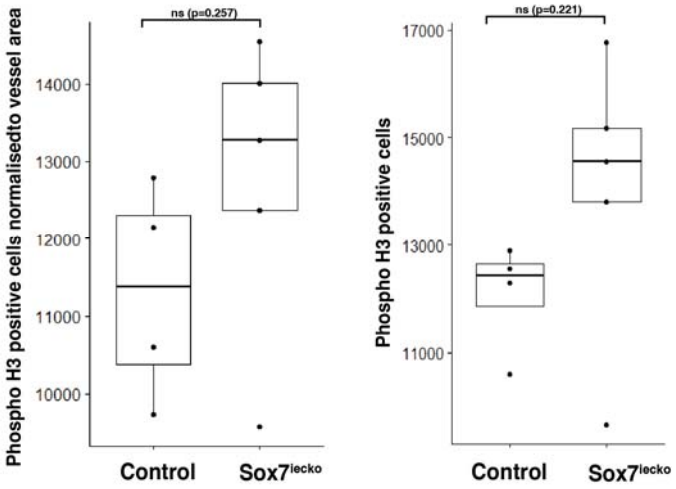
Reviewer_figure 13. Quantitation of SOX7 protein expression across different vascular beds in control and in *Sox7*^{iECKO}.

Likely the closure of the 'gap' between the lymphatic front and the midline normally relies on a combination of migration and proliferation?

Yes that is a possibility, although there could be other mechanisms at play to close this gap. For instance, the field has not investigated a “pulling” model whereby developing lymphatic vessels would anchor themselves into the underlying extra-cellular matrix and stretch out while this tissue is expanding. This would not require an excessive migration or proliferative response.

Figure 1I,J: the results are interpreted as hyperproliferation and/or defective remodeling (page 6). If possible, a proliferation staining could be done to verify this interpretation further, if feasible to be performed (it is away from the blood vascular front, so no/less problems to be expected with superposition of proliferating BECs).

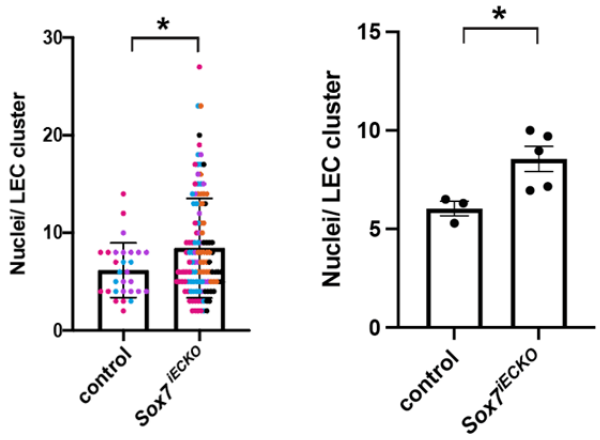
We have quantified the presence of phospho-histone H3 positive LECs in embryonic skins of the wild type and mutant embryos in order to assess whether there is a hyper-proliferation or a remodeling phenotype. The analysis was performed over 4 control and 5 homozygous mutant embryos. A trend in the increase of proliferative LECs is observed in the *Sox7*^{iECKO} samples (Reviewer_fig14), however this is not statistically significant. Please note that for the mutant condition there is an outlier sample that likely impact the statistical analysis. This observation suggests that a LEC hyperproliferative response may be contributing to the overall lymphatic phenotype.



Reviewer_figure 14. Quantitation of phospho-histone H3 positive LECs in wild-type control and *Sox7* endothelial conditional knock. Embryonic skins were immuno-stained for ERG, pH3, SOX7 and VEGFR2. A mask was applied to select mature lymphatic vessels on the basis of their VEGFR2 morphological features. SOX7-positive cells were used to exclude BEC, since this TF Is not expressed in LEC in developing skin. The selected regions of interest that were chosen for quantitation correspond to a skin territory located on both flanks of the embryo. (Control, n = 4x2 sides; *Sox7*^{iECKO}, n = 5x2 sides), *p*-value obtained with one-way Anova.

Figure 1J: this data needs to be recalculated per embryo rather than per cluster not to bias the statistics; there are only n=3 and n=5 biological repeats

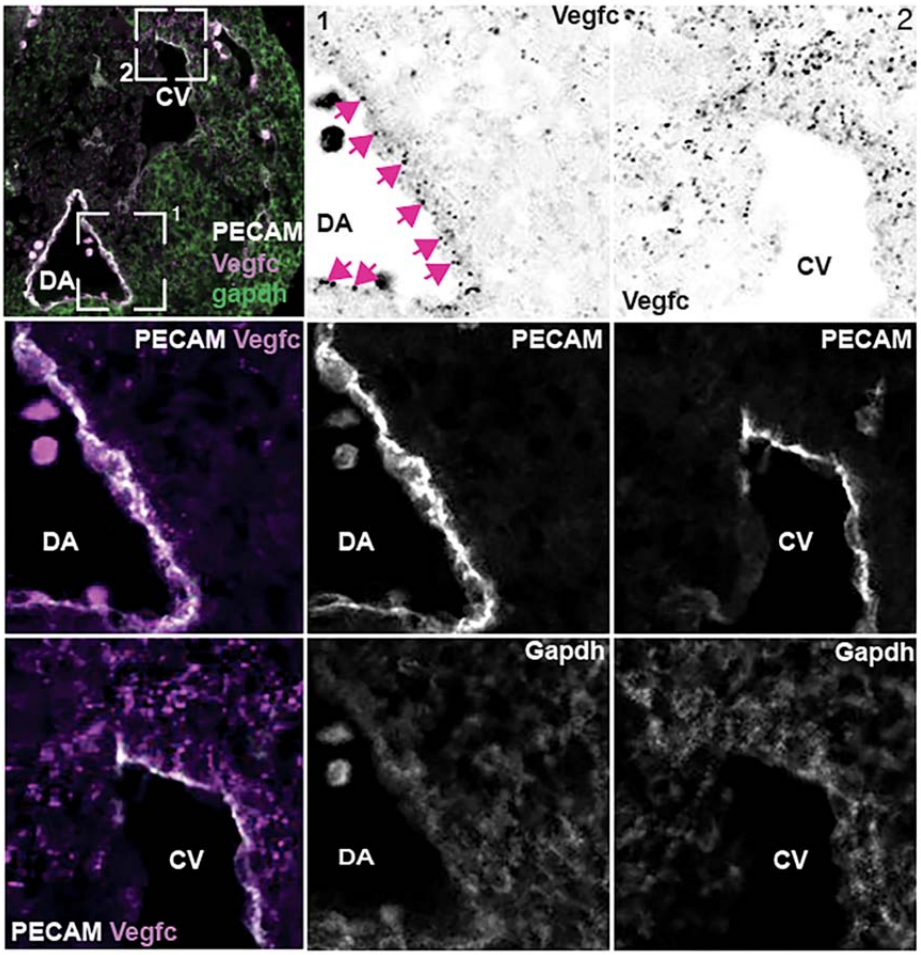
We have updated the graph in Fig. 1J (Reviewer_fig15) to represent both the result per embryos (each colour represents a different embryo) and the total number of cluster across all samples. We have also appended below for the reviewer only, another graph that shows the difference of nuclei per cluster for each genotype using the number of embryos as a "n" number.



Reviewer_figure_15 The number of PROX1 positive nuclei LEC dermal cluster at E12.5 is significantly increased in the *Sox7*^{iECKO}, when compared to the control littermate.

Fig. 2C: The images are not entirely convincing. Replacement with better images and quantification of the RNA scope signal is recommended.

As suggested, we have now updated the images in Fig. 2C to show a clear *Vegfc* staining in arterial endothelial cells. Please see Reviewer_fig16 and also refer to other figures which display *Vegfc* RNA smFISH signal in this response to reviewers (eg Reviewer_fig4 under Reviewer 1 point #6, Reviewer_fig9 and Reviewer_fig10 under Reviewer 2 point#5, and new Fig. 4G).



Reviewer_figure 16. Arterial endothelial cells express *Vegfc* transcripts. Transverse section of a E10.5 wild-type embryo stained by single molecule FISH (smFISH) for Gapdh (green) and Vegfc (magenta) and immuno-stained for PECAM (white). The first panel shows a merged version of the pseudo color signals for the 3 markers. Inset 1 is showing the expression of Vegfc in endothelial cell of the dorsal aorta (pink arrows). Inset 2 shows the high expression levels of mesenchymal Vegfc expressed in the dorso-lateral aspects of the cardinal vein.

- Related to the findings from Fig. 2E-G and Fig. 3B-D on the axis SOX7-HEY1-VEGFC wherein SOX7 induces Hey1 expression and HEY1 represses the VEGFC promoter: to what extent is Notch activation involved in this effect? Is the siSox7 effect of enhanced VEGFC expression in EC cultures (Fig. 2E-G) lost upon Notch inhibition (DAPT, Dll4-Fc)? And is it lost upon silencing of Hey1?

We understand the willingness of the reviewer to decipher further the molecular relationship between SOX7 and the Notch signaling pathways overall. We have not addressed how genetic or pharmacological disruption of the Notch pathway interferes with SOX7 activity. Establishing a hierarchical relationship between these two pathways of development in the context of lymphangiogenesis is interesting but beyond the scope of this study. Others and we have shown in the context of arterial differentiation that SOXF transcription factors are directly upstream of central effectors of the Notch pathway. Chiang et al Development 2017, uncovers a SOXF-dependent Notch1 enhancer and Sacilotto et al PNAS 2013 unveils SOXF dependent regulatory regions of Dll4.

- Fig. 3B-D: The inhibitory effect on the human promoter construct is close to 55%, but on the mouse promoter construct at most ca 15%. Do the authors have an explanation for this difference? Hey1 overexpression was done using human HEY1; could it be a (partial) species incompatibility? Furthermore, Hey1 overexpression is not verified by PCR, which is best included (baseline expression, extent of overexpression).

The mouse Vegfc promoter is only 370bp long, whereas the human VEGFC promoter is 3.4kb long. Both differences in the promoter length, besides species subtype as alluded could explain for the differences in inhibitory effects on human or mouse promoter.

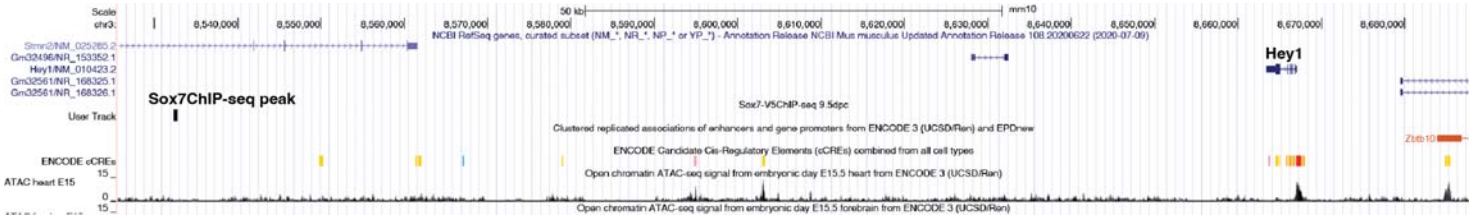
- Fig. 4A, VEGFC promoter-luciferase assay in HeLa cells: the result is interpreted as supporting the notion that SOX7 has the potential to act as a direct negative regulator of VEGFC transcription. Was it verified that it is solely direct and not perhaps also via HEY1? HeLa cells can express HEY1. Its expression could be tested to verify baseline expression and potential induction/upregulation upon SOX7 overexpression.

It might possible that SOX7 directly or indirectly regulate HEY1 transcription as part of a positive feedback loop mechanism. In this study we show that *in vivo* in the *Sox7*^{iECKO}, *Hey1* mRNA levels are reduced (Fig. 3A, sorted dermal endothelial cells). This observation supports the idea that SOX7 positively regulates its own protein partner, hence increasing the probability of protein-protein interaction to co-regulate *Vegfc* transcription.

This down-regulation of *Hey1* levels in the *Sox7*-null endothelial cells does not inform whether SOX7 directly represses *Hey1* gene transcription. In an *in vivo* setting, this transcriptional outcome in the knockout situation could due be to secondary hits caused by the *Sox7* loss-of-function. It is possible that SOX7 indirectly influence Hey1 levels through the Notch pathway because this transcription factor directly regulates Notch ligand, Dll4 and Notch receptor, Notch1 (Sacilotto et al., 2013 and Chiang et al, 2017).

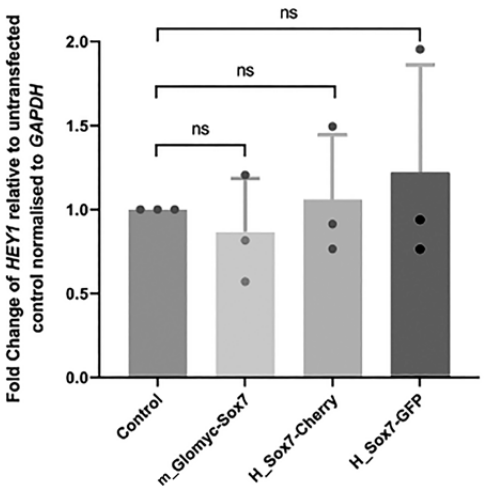
We have assessed the ChIP-Seq datasets from the *Sox7*-V5 transgenic animals at E9.5 and E10.5 to determine whether SOX7 binding locations might be assigned to the *Hey1* gene locus. Here, we used ChIP-Seq distance to the transcription start site (TSS) as a proxy for likelihood of direct transcriptional regulation by SOX7. In the E10.5 ChIP-Seq data, no peaks were assigned to the *Hey1* locus. In the E9.5 ChIP-seq data, only one ChIP-Seq peak was assigned to the *Hey1* locus (+134Kb from the TSS). Furthermore, this region of the genome is not decorated by histone marks for active enhancers according to the enhancer map from ENCODE.

Please see screen shot from UCSC browser below –This low occurrence of SOX7 binding events in potential regulatory regions of the *Hey1* locus at 2 different embryonic time points indicates that it is unlikely that Hey1 is under the direct transcriptional control of the SOX7 protein.



Screen shot of the UCSC genome browser showing *Sox7*-V5 ChIP-Seq peak (black bar) at E9.5 assigned to the Hey1 locus. Multiple tracks are shown to help determine conserved regulatory regions (cCREs, multicolor), enhancer maps (red) and ATAC-seq from embryonic heart (black peaks).

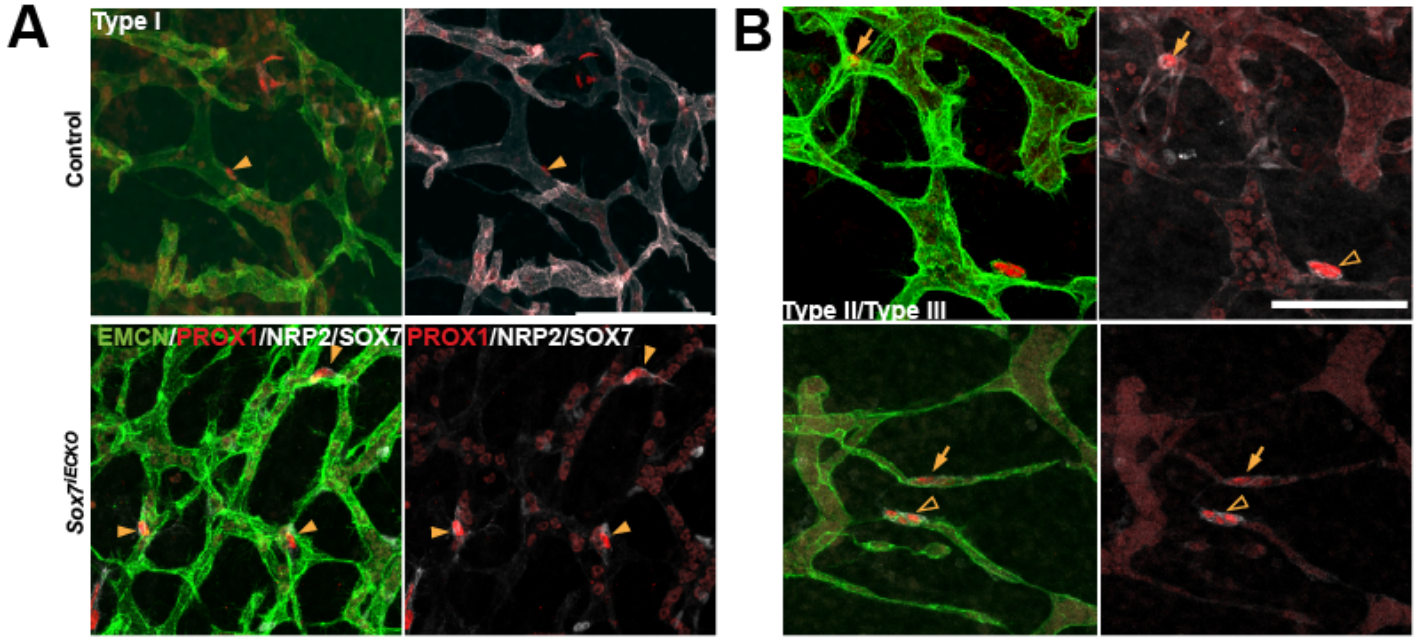
We have tested whether SOX7 over-expression in Hela cells drives changes in *Hey1* mRNA levels by qPCR analysis. This approach did not reveal any significant changes in *Hey1* transcript concentration, either in presence of the mouse, or the human SOX7 proteins. We have appended the results below (Reviewer_fig17).



Reviewer_figure17. Hey1 expression levels are not affected by SOX7 over-expression in Hela cells.
Relative expression of *HEY1* mRNA in HeLa cells transfected with three different tagged SOX7 constructs: mouse Glomyc-Sox7, Human SOX7-CherryC1 (C-terminal tag) or Human SOX7-EGFPN1 (N-terminal tag). The mean fold-change in expression is shown ± standard error from three biological independent replicates is presented, with each replicate being performed as a triplicate qPCR. Ns, $P > 0.05$
Of note *SOX7* endogenous expression in Hela cannot be detected by qPCR assay (35 cycles, no signal). Therefore, over-expression of the SOX7 protein could not be determined as a fold change compared to control. We are happy to provide raw data and analysis if necessary, to show the high level of SOX7 constructs expression 24 h post-transfection.

- Fig. 8A currently is not entirely clear. Which genotypes are shown in panel A and B? Regarding the markers in panels C-E, what does low/-, +/low and +/- refer to?

We initially included Sup Fig. 8A (now Appendix Fig. S9) to illustrate the different types of LECs clusters (type I, Type II and Type III) that we have quantified in control versus *Sox7*^{iECKO} embryos. The size of these clusters is related to their level of development (single cell in early differentiating one (E12.5) and a few cells in more mature ones (E13.5 and E14.5), Pichol-Thievend et al Development 2018 and Martinez-Corral et al Circ. Res. 2015). Importantly the quantitation shown in Appendix Fig. S9, reveals a significant increase in the number of type III LEC clusters identified in the *Sox7*^{iECKO} mutant embryo. For clarity, we have now added images representative of both the control and the mutant genotype. Please see below (Reviewer_fig18).



Reviewer_figure 18. Local lymphatic endothelial cell progenitors are increased in the immature blood vascular plexus of the *Sox7*^{iECKO}. (A and B) Embryonic skin immuno-staining for Endomucin (green), PROX1 (red), NRP2 and SOX7 (white) reveals the presence of single LEC progenitor in the EMCN-positive vascular bed at E12.5 (A) and emerging clusters at E13.5. Type I (orange arrowheads) denotes single LECs on the wall of EMCN positive vessels, type II defines individual LEC exiting the plexus (orange arrows) and type III refers to LEC cluster (empty arrowheads).

The “low/-, +/low and +/-“ indicate the different expression levels observed. All type 1, type II and type III LECs express PROX1 protein. Type 1 LECs ($EMCN^{low/-}PROX1^{+}NRP2^{+/low}$) are single LEC on blood vessel, expressing low or undetectable endomucin and various levels of the neuropilin marker (either low or positive). Type II LECs ($EMCN^{+}PROX1^{+}NRP2^{low/-}$) are single LECs exiting the blood vessel. They are typically found in the blood vessel and not outside of the blood vessel unlike type 1. Therefore, type II LECs are positive for endomucin, but have low or almost undetectable level of neuropilin. Type III LECs ($EMCN^{+/-}PROX1^{+}NRP2^{+/low}$) are emerging LECs in a cluster of two or more cells. This emerging LEC clusters may or may not express endomucin, but they express PROX1 and various levels of neuropilin-2.

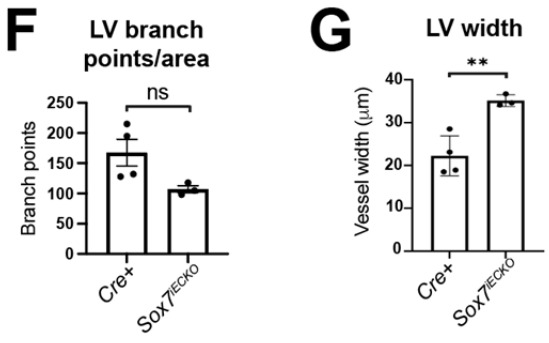
- Tamoxifen treatment: It is not clear why in some experiments another tamoxifen treatment regimen was used. For instance in Sup. Fig. 4C (now Fig. EV2C) and Fig. 2D. The experiment in Sup. Fig. 4C (now Fig. EV2C) was included to exclude that the observed phenotype was due to or influenced by potential Cre toxicity, and thus should be done using the same tamoxifen regimen as used in the phenotyping experiment. Also, the quantification of LV branching and LV dilation in this experiment is missing.

The particular experiment shown in Fig. EV2C, was designed to assess potential off-target effects of tamoxifen treatment and/or Cre toxicity. The primary objective was to rule out the possibility that the lymphatic vascular phenotype observed in Fig. 1 is neither attributable to general cardiovascular defects resulting from tamoxifen treatment, nor to Cre toxicity. The control animals with the *Cdh5-CreERT2;mT/mG* genetic background are exposed to tamoxifen and express the Cre enzyme, however they do not display any gross oedema or lymphatic phenotype. This approach clearly establishes that the phenotype we observed is caused by *Sox7* loss-of-function in the endothelium. Further, in Fig. EV2C, we show that by reducing tamoxifen dose and exposure time, we can reproducibly phenocopy the lymphatic disruption obtained when using the experimental conditions used in Fig1. This rationale is addressed in the manuscript page 6, last paragraph line 1 when we refer to the work from Brash et al 2020.

Further, the delayed genetic disruption of *Sox7* at E12.5 (Fig. EV2C, previously shown as Sup. Fig. 4C) enables us to bypass the potential consequences of a disruption during the early steps of LEC specification in the cardinal vein, which occurs between E9.5 and E11.5. Deleting *Sox7* at E12.5 and recapitulating the phenotype from *Sox7* loss-of-function at E9.5 and E10.5 argues in favor that the dermal lymphatic defects are not secondary to defects caused by a perturbation of the initial lymphatic plexus.

Regarding Fig. 2D, we used a different tamoxifen schedule (E11.5 and E12.5) to assess potential direct SOX7-regulated genes. Shortening the time between *Sox7* exon2 deletion and assessment of gene transcripts dysregulation by qPCR on sorted endothelial cells at E14.5 increases the chance of detecting specific gene dysregulation, and minimises confounding effects due to general developmental defects. The idea is to reduce the number of secondary targets, which is likely to be higher when assessing gene expression 5 days post gene deletion.

We have updated Sup. Fig. 4 and now show the quantitation of lymphatic vessels dilation and branching, please refer to the Fig. EV2, panels F and G in the manuscript, and see Reviewer_fig19 below.



Reviewer_figure 19. Reduced dose and delayed schedule of tamoxifen to induce *Sox7* loss-of-function at E12.5 in endothelial cells causes a lymphatic-specific phenotype. Quantitative analysis of the dermal lymphatics shows significant enlargement and dilation of the vessels.

MINOR COMMENTS
- Abstract and elsewhere in the text: the BEC effect is referred to as 'lymphangiocrine'. I think this should be 'angiocrine'.
- Page 9, the description of the results of Fig. 3F and the legend to Fig. 3F are not in accordance.
- Page 13, line 8: including the reference for the Tie2Cre;VegfcGOF data is recommended.
- Material and Methods, Mice and breeding: The Schimmel et al reference should be the Muzumdar et al reference.
- Page 25, Expression constructs, top: the species indication needs to be corrected.
- Some English writing or wording could be optimized (e.g. (auxiliary) verbs are often missing; snapped freeze should be snap-frozen; etc)
- Legend to Figure 1A: the tamoxifen injection is not really correctly described.

All minor comments have been addressed in the text

Thank you for submitting a revised version of your manuscript. Your study has now been seen by all original referees, who find that their major concerns have been addressed and now recommend publication of the manuscript after a minor revision. There are also a few editorial issues that have to be solved before I can extend formal acceptance of the manuscript:

1. Please address the remaining comments from reviewers #2 and #3, extend the manuscript text with the discussion as requested and add the indicated Reviewer Figures to the Appendix.

Please let me know if you have any further questions regarding any of these points. You can use the link below to upload the revised files.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

Referee #1:

In this revised manuscript the authors have addressed all my concerns and have clearly demonstrated that Sox7 regulates Vegfc expression in arteries and thereby guide lymphatic vascular growth and patterning. I have no further concerns or questions.

Referee #2:

The authors have done a thorough job in addressing reviewer comments and I do not have any further scientific issues. However, whilst they have provided robust responses in the rebuttal-letter, equivalent revisions are lacking in the actual manuscript and the responses do not always indicate if or where changes have been made to assist in review (eg page/line numbers). Similarly, for new data inclusions requested by the reviewers; these reflect a need to include additional panels/figures (supplementary or otherwise) in the actual manuscript and should not be limited to the reviewer response. The reviewer comments reflect important additions and a requirement for further investigation or clarification, that will apply to the wider readership, hence the revised manuscript should encompass the reviewer-response points and figures more comprehensively.

By way of examples, as relates to original (Reviewer 2) comments: Reviewer Figures 7-10 should be included in the manuscript as supplementary figures, with accompanying discussion in the revised text. The more expansive/informative reviewer-responses, such as under points 3) and 4) in the rebuttal letter, should be included in the revised text as opposed to no revision or a brief 1-2 line response thus far included.

Referee #3:

The authors have carefully addressed all comments by further clarification and, in many cases, additional experimentation, which has considerably improved and strengthened their study.
One remaining question and a couple of minor suggestions are listed below.

- Figure 4G and Figure S7, validation of Sox7-dependent transcriptional repression of Vegfc in vivo: Figure 4G indeed nicely confirms higher Vegfc transcript levels in de dorsal aorta endothelial cells of Sox7iECKO mice. Strangely however, in Figure S7 (mTmG column), Vegfc transcript levels are especially high in some of the GFP-negative cells (Sox7+ cells), opposite of what is expected. Why? This is rather puzzling.

Minor:
- Legend to Figure 1I, Prox+ nuclei per LEC cluster: perhaps best to omit the 'n' for the 29 and 122 clusters that were counted, so that it is more clear that is the n=3 and n=5 biological repeats that were used for the statistical analysis.
- Page 4, I assume the sentence "... emergence of endomucin (EMCN)-derived LEC progenitors" should read "... of endomucin (EMCN)+ cell-derived LEC progenitors".

The team would like the thank the reviewers for their insightful and supportive comments which overall have contributed to significantly improve the scientific quality of this manuscript. Please find appended below a point-by-point response that address the reviewers' queries.

Referee #1:

In this revised manuscript the authors have addressed all my concerns and have clearly demonstrated that Sox7 regulates Vegfc expression in arteries and thereby guide lymphatic vascular growth and patterning. I have no further concerns or questions.

Referee #2:

The authors have done a thorough job in addressing reviewer comments and I do not have any further scientific issues. However, whilst they have provided robust responses in the rebuttal-letter, equivalent revisions are lacking in the actual manuscript and the responses do not always indicate if or where changes have been made to assist in review (eg page/line numbers). Similarly, for new data inclusions requested by the reviewers; these reflect a need to include additional panels/figures (supplementary or otherwise) in the actual manuscript and should not be limited to the reviewer response. The reviewer comments reflect important additions and a requirement for further investigation or clarification, that will apply to the wider readership, hence the revised manuscript should encompass the reviewer-response points and figures more comprehensively.

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The new data requested by the reviewers have been included and updated in the actual manuscript.

Referee #3:

The authors have carefully addressed all comments by further clarification and, in many cases, additional experimentation, which has considerably improved and strengthened their study.
One remaining question and a couple of minor suggestions are listed below.

- Figure 4G and Figure S7, validation of Sox7-dependent transcriptional repression of Vegfc in vivo: Figure 4G indeed nicely confirms higher Vegfc transcript levels in de dorsal aorta endothelial cells of Sox7iECKO mice. Strangely however, in Figure S7 (mTmG column), Vegfc transcript levels are especially high in some of the GFP-

negative cells (Sox7+ cells), opposite of what is expected. Why? This is rather puzzling.

The efficacy of the CRE enzyme to deflox specific loci is variable *in vivo* and is never up to 100%, this is mostly due to chimerism. In this particular case here (*Cdh5-CreERT2: Sox7fl/fl; mT/mG*) both the *Sox7* exon2 locus and the Rosa26 locus (*mT/mG* cassette) are targeted by the CRE activity. The probability to deflox both loci in all endothelial cells cannot be expected to be 100%.
Therefore this generates different combination of genome editing, including the case where some cells are *Sox7* knock out (high levels of *Vegfc* transcripts) but show no GFP expression (lack of *mT/mG* recombination).
Importantly, we do also observe high *Vegfc* transcripts in GFP positive cells.

Minor:
- Legend to Figure 1I, Prox+ nuclei per LEC cluster: perhaps best to omit the 'n' for the 29 and 122 clusters that were counted, so that it is more clear that is the n=3 and n=5 biological repeats that were used for the statistical analysis.
- Page 4, I assume the sentence "... emergence of endomucin (EMCN)-derived LEC progenitors" should read "... of endomucin (EMCN)+ cell-derived LEC progenitors".

These points have now been addressed.

Thank you for addressing the final minor points. I am now pleased to inform you that your manuscript has been accepted for publication.

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Manuscript Number: EMBOJ-2021-109032R	EMBO Reports - Author Guidelines
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Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

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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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgement

Yes

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figures, Materials and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Figures, Materials and Methods
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Figures, Materials and Methods
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figures, Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figures, Materials and Methods
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figures, Materials and Methods

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Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

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The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	Figures, Materials and Methods
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Materials and Methods
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Materials and Methods