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Transcription factor FOXP2 is a flow-induced regulator of collecting lymphatic vessels

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1st Editorial Decision 17th Nov 2020

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received two reviewer reports on your manuscript, which are included below for your information.

As you will see from the comments, both reviewers appreciate the presented role of FOXP2 in lymphatic valve development, but also point out a number of issues that would have to be addressed and clarified before they can support publication of the manuscript. Given these positive evaluations from two experts of the field, I would like to invite you to address the comments of both reviewers 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. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, and I would be happy to discuss the revision in more detail via email or phone/videoconferencing.

We have extended our '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. This means that competing manuscripts published during revision period will not negatively impact on our assessment of the conceptual advance presented by your study. 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. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving the revised manuscript.

Referee #1:

Speech-linked transcription factor FOXP2 is a flow-induced regulator of collecting lymphatic vessels.

In this manuscript Hernandez Vasquez et al have identified FOXP2 as a novel regulator of collecting lymphatic vessel identity. Deletion of FOXP2 from all endothelial cells or from lymphatic endothelial cells of mice results in dilation of collecting lymphatic vessels and in the downregulation of lymphatic valve markers such as integrin-α9 and ephrin-B2. They have also identified FOXC2 as an upstream regulator of FOXP2 expression in primary human lymphatic endothelial cells. The data is novel, clear and well presented. I enjoyed reading the manuscript. I suggest a few additional experiments, which will hopefully improve the quality of this work even further.

Major comments

1. Have the authors analyzed the developing dermal lymphatic vessels (E15.5-E18.5)? When and where is FOXP2 expressed and what are the phenotypes of these vessels in mice lacking FOXP2?
2. Have they analyzed the lymphovenous valves and venous valves?
3. Was the experiment shown in Figure 6A performed under OSS condition? If not please compare LECs cultured under static and OSS conditions, with or without siRNA's for FOXC2 or FOXP2.

Please also check the expression of some additional genes that are relevant to this work- Gata2, ephrin-B2, integrin-α9, Connexin-37 and Connexin-43. In addition, perform immunohistochemistry for NFATC1 in those cells.

Minor comments

1. Was any chyle leakage observed in Prox1-CreERT2; Foxp2flox/flox mice?
2. In Figure 1A PROX1-GFP expression appears to be lower in the lymphatic capillaries when compared to collecting vessels. Is this true and does this change show up in the RNA-seq data?
3. Were there any defects in the Schlemm's canal or lacteals of Tie2-Cre;Foxp2flox/flox mice?
4. For almost 10 years OSS and LSS have been shown to be important for various aspects of lymphatic vascular development. However, detailed list of genes that are regulated by OSS and LSS is still not publically available. As the authors have generated the dataset for such an experiment I suggest that excel spreadsheets that list all the shear- (LSS and OSS) regulated genes be published. Providing the already analyzed dataset as spreadsheets will minimize the need for additional bioinformatic analysis that may not be possible for several labs. This data will be greatly appreciated and cited by the lymphatic community. Similarly, please provide the entire list of differentially expressed genes from the capillary versus collecting vessel comparison.

Referee #2:

This manuscript identifies the FoxP2 transcription factor as a transcriptional regulator of the morphogenesis of collecting lymphatic vessels. This finding is novel and presents important mechanistic insight into how lymphatic collectors acquire distinct structural and functional characteristics. Thus the manuscript will be of broad interest in the vascular/lymphatic biology field and for developmental biologists. The data fully support the conclusions made in the manuscript and are clearly presented. The text is well written and readily comprehensible. The mouse genetic models are a major strength of the study, and the results of the transcriptomic analysis of different lymphatic vessel subtypes will likely be an important resource for future research in lymphatic vascular biology. I do not have any major concerns about the manuscript but have listed my minor concerns below.

1. The Authors should present a brief one sentence explanation, in Results, of the difference between OSS and laminar flow so the Reader can immediately grasp the significance of the data relating to this aspect of the manuscript.

2. The manuscript would benefit from a schematic diagram depicting the way FoxC2, FoxP2 and downstream genes are involved in the morphogenic regulation of collecting lymphatics. I note that a Figure 6F was referred to, which may have been a schematic, but I could not find it in the manuscript accessible to me.

3. The Authors use the term "lymphatic capillaries" but in an anatomical setting the word capillary really refers to a type of blood vessel with distinct morphology and function. The more correct term may be "initial lymphatics".

4. The Authors refer to the potential for therapeutic restoration of vessel function at the end of Discussion but do so in a shallow fashion. They should address, in broad strategic terms, how this might be achieved in relation to what they have discovered.

5. There are three study groups in Figure 6A. Surely multi-group testing should have been used for statistical analysis of the data presented therein.

Response to the Reviewers
EMBOJ-2020-107192

We thank the Reviewers for their constructive comments that helped us to improve our study. We have revised the manuscript and added new experimental data. In particular, we have added data on FOXP2 expression in the developing dermal vasculature and in venous and lymphovenous valves. We have also strengthened our conclusion that FOXP2 acts downstream of FOXC2 by 1) including additional *in vitro* experiments to convincingly demonstrate downregulation of *FOXP2* upon *FOXC2* silencing but not vice versa, and 2) demonstrating unaltered expression of FOXC2 in *Foxp2* deficient vessels *in vivo*.

All specific points are addressed below (in blue, new and revised figures indicated in **bold**) and changes to the text have been highlighted in the manuscript file in red. As the number of expanded view figures is limited to five, two figures have been moved to appendix (previous Figs EV2 and EV4 are now Appendix Fig 1 and 2).

Referee #1:

Speech-linked transcription factor FOXP2 is a flow-induced regulator of collecting lymphatic vessels.

In this manuscript Hernandez Vasquez et al have identified FOXP2 as a novel regulator of collecting lymphatic vessel identity. Deletion of FOXP2 from all endothelial cells or from lymphatic endothelial cells of mice results in dilation of collecting lymphatic vessels and in the downregulation of lymphatic valve markers such as integrin-α9 and ephrin-B2. They have also identified FOXC2 as an upstream regulator of FOXP2 expression in primary human lymphatic endothelial cells. The data is novel, clear and well presented. I enjoyed reading the manuscript. I suggest a few additional experiments, which will hopefully improve the quality of this work even further.

Major comments
1. Have the authors analyzed the developing dermal lymphatic vessels (E15.5-E18.5)? When and where is FOXP2 expressed and what are the phenotypes of these vessels in mice lacking FOXP2?

Response: As suggested, we performed immunofluorescence analysis of dermal vasculature in embryonic back skin at E14-15, when a primary lymphatic capillary plexus is forming through vessel sprouting, and at E18, when remodelling into collecting vessels is initiated. FOXP2 expression was not detected at E14 or E15 (**Fig EV2**), consistent with the lack of expression in lymphatic capillaries of other tissues and developmental stages. At E18, FOXP2 was detected in the remodelling collecting vessels including developing valves (**Fig EV2**), in agreement with our previous data showing upregulation during mesenteric collecting vessel maturation (Fig 2A). However, analysis of dermal lymphatic vessels in 4-OHT-treated E18 *Foxp2*^{flox};*Prox1-CreER*^{T2} embryos did not reveal morphological differences in *Foxp2* deficient compared to control littermates (**Appendix Fig 2B**). *Foxp2* deficient vessels showed apparently normal initiation of valve formation as determined by the presence of clusters of PROX1^{high} LECs in vessel branchpoints (**Appendix Fig 2C**). Assessment of valve morphology was hampered by variation in the stage of valve formation in the developing

plexus at E18, and therefore we could not conclusively determine the effect of FOXP2 depletion on valve maturation in this tissue. Notably, analysis of dermal vasculature in the ear skin of *Foxp2*^{flox};*Tie2-Cre* mice also revealed apparently normal valves (Appendix Fig 2A), suggesting that larger collecting vessels that experience higher shear stress are more sensitive to loss of FOXP2.

2. Have they analyzed the lymphovenous valves and venous valves?

Response: Molecular mechanisms that regulate the formation of lymphatic and venous valves are indeed found to be shared ((Bazigou *et al*, 2011), reviewed in (Geng *et al*, 2017)). We have added new data showing that, similar to the lymphatic valve regulators PROX1, integrin-α9 (Bazigou *et al*, 2011) and FOXC2 (Lyons *et al*, 2017), FOXP2 is expressed in venous valve ECs (**Fig EV4A**). Genetic deletion of *Foxp2* using the *Tie2-Cre* mice did not, however, lead to apparent defects in the formation of venous valve leaflets (**Figs EV4A and EV4B**). Our assessment of valve morphology was limited to an early developmental stage and potential effect of *Foxp2* deficiency on further maturation or long-term maintenance of venous valves cannot be excluded (now clarified in the discussion, page 16).

As suggested, we also analysed FOXP2 expression in the developing lymphovenous valves (LVVs). Immunofluorescence of coronal vibratome sections of an E14 embryo showed no expression of FOXP2 in LVVs (**Fig EV4C**). Presence of FOXP2^{+} neuronal cells confirmed successful staining. Embryos with defective LVVs frequently display blood filled lymphatic vessels and edema (Geng *et al*, 2016; Martin-Almedina *et al*, 2016). Together with the absence of FOXP2 in LVVs, the lack of an overt phenotype in *Foxp2*^{flox}*;Tie2-Cre* embryos suggests presence of functional LVVs in *Foxp2* deficient mice.

3. Was the experiment shown in Figure 6A performed under OSS condition? If not please compare LECs cultured under static and OSS conditions, with or without siRNA's for FOXC2 or FOXP2. Please also check the expression of some additional genes that are relevant to this work- Gata2, ephrin-B2, integrin-α9, Connexin-37 and Connexin-43. In addition, perform immunohistochemistry for NFATC1 in those cells.

Response: The experiment was performed under static condition. Following the Reviewers suggestion, we repeated the experiment three times under static and OSS conditions and included additional genes in the analysis. We found that *FOXC2* silencing had a similar effect, and resulted in reduced *FOXP2* expression under both static and OSS conditions (**Fig 1 for the Reviewer**). As previously shown under static condition, *FOXP2* silencing did not alter *FOXC2* levels under OSS (**Fig 1 for the Reviewer**). Unexpectedly, however, we observed a generally reduced OSS-induced response in control siRNA-treated LECs, indicated by no or weak upregulation of *FOXP2*, but also the established flow-induced genes (including *GATA2*), even if *FOXC2* was modestly increased (**Fig 1 for the Reviewer)**.

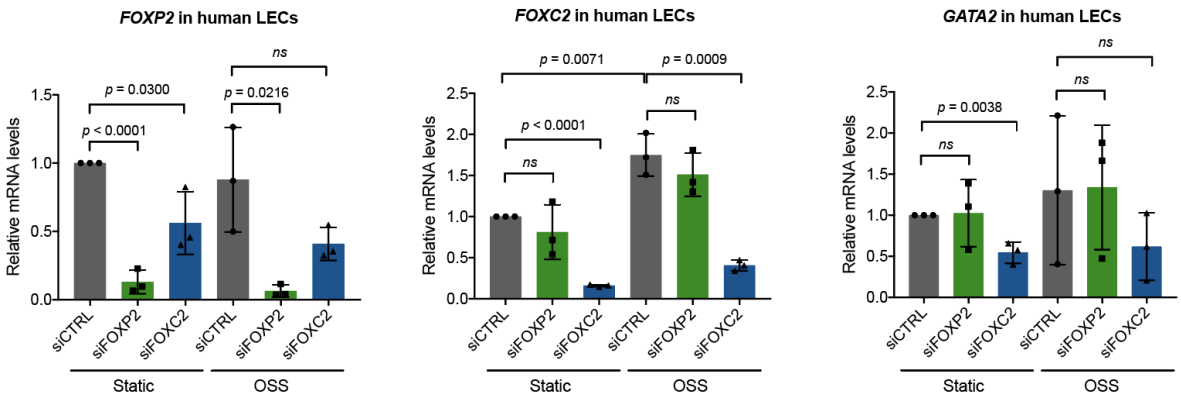

Fig 1 for the Reviewer. qRT-PCR analysis of *FOXP2*, *FOXC2* and *GATA2* expression in control (siCTRL) and *FOXP2* or *FOXC2* siRNA-treated HDLECs under static and OSS conditions. Data represent mean ± SD. (n = 3 independent experiments). P, unpaired Student's t-test. Ns = not significant.

We performed a separate experiment to compare in parallel the magnitude of OSS-induced gene expression changes in the same batch of cells treated with control siRNA or left untreated and found that OSS-induced response was reduced in the siRNA-treated in comparison to untreated cells (**Fig 2 for the Reviewer**).

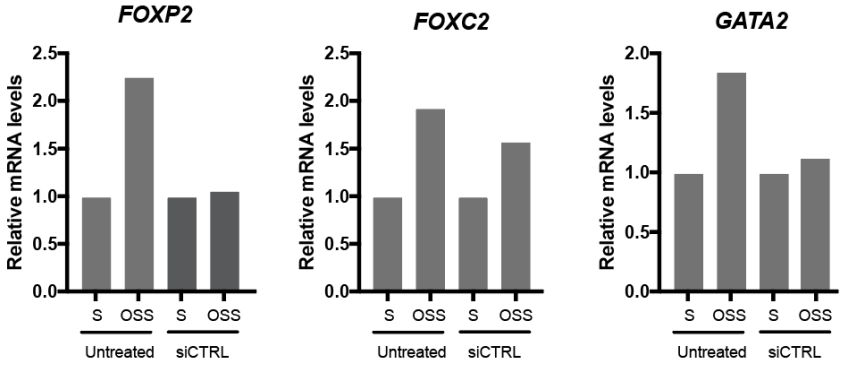

Fig 2 for the Reviewer. qRT-PCR analysis of *FOXP2*, *FOXC2* and *GATA2* expression in control (siCTRL) siRNA-treated and untreated HDLECs under static (S) and OSS conditions. Data is from one experiment using the same batch of cells that were either transfected or left untreated and analysed in parallel.

Although at this point we don't know the reason for the blunted OSS-induced response under the conditions used, we have chosen not to include data from these experiments in the manuscript. Instead, we have strengthened data in **Fig 6A** by showing downregulation of *FOXP2* upon *FOXC2* silencing now in n = 6 independent experiments (previously n = 3), performed using different batches of LECs. This is consistent with our *in vivo* data, showing downregulation of FOXP2 in *Foxc2* deficient lymphatic vessels (Fig 6B and C).

Due to the experimental challenges we encountered with assessing long-term OSS-induced responses in siRNA-treated LECs, as discussed above, it was also not possible to assess the effect of *FOXP2* knock down on OSS-induced NFATc1 nuclear translocation. We therefore induced NFATc1 activation using an alternative method, namely acute VEGF-C stimulation, in the absence or presence of FOXP2. VEGF-C promotes transient activation of

calcineurin/NFAT signaling *in vitro* (Norrmén *et al*, 2009) and was recently shown to contribute to lymphatic valve morphogenesis through the regulation of mechanonsensitive transcriptional co-factors YAP and TAZ (Cha *et al*, 2020). In agreement with previous data, we found that acute VEGF-C stimulation for 30 min induced nuclear translocation of NFATc1 in LECs (**Fig EV5B**). As previously reported (Sabine *et al*, 2012), this was abrogated in *FOXC2* silenced cells, but we also found loss of nuclear NFATc1 upon *FOXP2* silencing (**Fig EV5B**). Although further work is necessary, these results hint at the potential cross-talk of mechanosensitive and growth factor pathway signaling in lymphatic valve development. This has been now discussed on page 18.

Finally, we have strengthened our conclusion that FOXP2 acts downstream of FOXC2 by showing unaltered expression of FOXC2 in *Foxp2* deficient vessels *in vivo* (**Fig 6D**). In addition, we have added new data showing that the expression of the *FOXC2* target *GJA4* (CX37) is not reduced upon *FOXP2* silencing (under static condition) (**Fig 6A**). The expression of *GATA2*, the upstream regulator of *FOXC2* (Kazenwadel *et al*, 2015), was also not altered in *FOXP2* silenced LECs (**Fig EV5A**).

Minor comments

1. Was any chyle leakage observed in Prox1-CreERT2; Foxp2flox/flox mice?

Response: We did not observe chyle leakage in P6 *Foxp2*$^{flox/flox}$*;Prox1-CreER*^{T2} mice that were neonatally treated with 4-OHT at P1-2. Notably, we also did not observe enlargement of collecting vessels (data added in **Fig 5E**) that was seen upon constitutive EC-specific deletion in the *Foxp2*$^{flox/-}$*;Tie2-Cre* mice. This suggests that the *Foxp2* loss-induced collecting vessel defects, including barrier leakage, develop prior to birth or during early neonatal life. We have added this data and discussion in the text (page 11).

We have also analysed adult (9-week-old) *Foxp2*$^{flox/-}$*;Tie2-Cre*$^{+}$ mice to understand if collecting vessel defects and chyle reflux in these mice compromise postnatal survival and growth. This was not, however, the case. Analysis of adult mesenteries revealed presence of morphologically normal valves in the *Foxp2*$^{flox/-}$*;Tie2-Cre*$^{+}$ mice (**Appendix Fig 2D**), in agreement with the spectrum of valve defects observed in these mice at earlier stages. The phenotype in *Foxp2* deficient mice is thus similar but milder than that reported in mice lacking *Foxc2* (Sabine et al., 2012). This supports the notion that FOXP2 acts downstream of FOXC2 in regulating lymphatic (valve) development, and other FOXC2 targets can partially compensate for the loss of FOXP2 (now discussed on page 16).

2. In Figure 1A PROX1-GFP expression appears to be lower in the lymphatic capillaries when compared to collecting vessels. Is this true and does this change show up in the RNA-seq data?

Response: GFP fluorescence intensity indeed appears weaker in LECs of lymphatic capillaries compared to collecting vessels in the *Prox1-GFP* ear skin whole-mounts. We could not, however, observe differences in GFP intensity between LECs of the two vessel types by FACS analysis; both LYVE1^{low} and LYVE^{high} LEC populations showed variable levels of GFP intensity (Fig 1A) but the mean GFP fluorescence intensity (MFI) was similar in the two populations (**Fig 3 for the Reviewer**). We could also not observe differences in *Prox1* mRNA levels in the two population by RNA array analysis (Fig EV1C). It is possible

that differences in cell shape between lymphatic capillary LECs (representing large spread out cells) versus collecting vessel LECs (slender and more densely packed cells) may account for the apparent differences in fluorescent intensity in maximum intensity projection images.

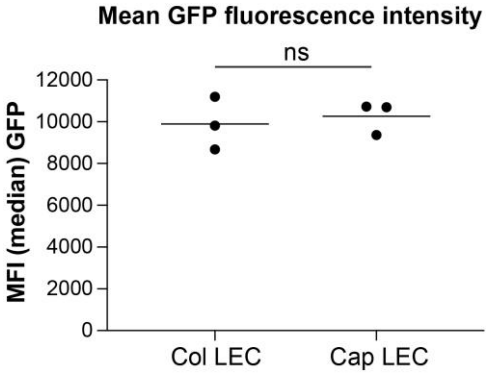

Fig 3 for the Reviewer. Mean fluorescence intensity (median) of GFP in LYVE1^{low} collecting vessel (Col) and LYVE^{high} lymphatic capillary (Cap) LEC populations in adult *Prox1-GFP*$^{+}$ ear skin, analysed by flow cytometry. Horizontal lines represent mean (n = 3). ns = not significant, unpaired two-tailed Student's t-test.

3. Were there any defects in the Schlemm's canal or lacteals of Tie2-Cre;Foxp2flox/flox mice?

Response: Whole-mount immunofluorescence did not show expression of FOXP2 in the Schlemm's canal (new data added, now **Appendix Fig 1C**), and thus we did not examine *Foxp2* deficient mice.

In agreement with the lack of expression of FOXP2 in lacteal LECs (Fig EV2B), we could not observe defects in these vessels in the *Foxp2*$^{flox/-}$*;Tie2-Cre* mice (new **Appendix Fig 2E**).

4. For almost 10 years OSS and LSS have been shown to be important for various aspects of lymphatic vascular development. However, detailed list of genes that are regulated by OSS and LSS is still not publically available. As the authors have generated the dataset for such an experiment I suggest that excel spreadsheets that list all the shear- (LSS and OSS) regulated genes be published. Providing the already analyzed dataset as spreadsheets will minimize the need for additional bioinformatic analysis that may not be possible for several labs. This data will be greatly appreciated and cited by the lymphatic community. Similarly, please provide the entire list of differentially expressed genes from the capillary versus collecting vessel comparison.

Response: Data on OSS- and LSS-regulated genes in LECs published by Sabine et al. in 2015 is not relevant in the context of our present study because gene expression changes were analyzed at 24 h after exposure to OSS when *FOXP2* expression is not yet induced (Fig EV3A).

We fully agree with the reviewer that a complete analysis of genes regulated under shear stress in LECs *in vitro* is long due. In this regard, one of us (TVP) is currently completing a manuscript with a comprehensive analysis of cultured LEC and BEC responses to different

types of fluid shear stress, where such listing will be accessible. The manuscript will be deposited on biorxiv to facilitate the access of the community.

As suggested, we now provide a full list of differentially expressed genes in capillary versus collecting vessels as a new **Table EV2**.

Referee #2:

This manuscript identifies the FoxP2 transcription factor as a transcriptional regulator of the morphogenesis of collecting lymphatic vessels. This finding is novel and presents important mechanistic insight into how lymphatic collectors acquire distinct structural and functional characteristics. Thus the manuscript will be of broad interest in the vascular/lymphatic biology field and for developmental biologists. The data fully support the conclusions made in the manuscript and are clearly presented. The text is well written and readily comprehensible. The mouse genetic models are a major strength of the study, and the results of the transcriptomic analysis of different lymphatic vessel subtypes will likely be an important resource for future research in lymphatic vascular biology. I do not have any major concerns about the manuscript but have listed my minor concerns below.

1. The Authors should present a brief one sentence explanation, in Results, of the difference between OSS and laminar flow so the Reader can immediately grasp the significance of the data relating to this aspect of the manuscript.

Response: We have added a sentence in the results section, clarifying the differences between OSS and LSS and their effects on LEC phenotype and vessel morphogenesis (page 8).

2. The manuscript would benefit from a schematic diagram depicting the way FoxC2, FoxP2 and downstream genes are involved in the morphogenic regulation of collecting lymphatics. I note that a Figure 6F was referred to, which may have been a schematic, but I could not find it in the manuscript accessible to me.

Response: We apologize for the missing figure. We have added a schematic figure summarizing the proposed molecular pathway involving FOXC2/FOXP2/NFATc1 in collecting vessel morphogenesis (**Fig 6G**).

3. The Authors use the term "lymphatic capillaries" but in an anatomical setting the word capillary really refers to a type of blood vessel with distinct morphology and function. The more correct term may be "initial lymphatics".

Response: We thank the reviewer for raising this point. Indeed both “initial lymphatics” and “lymphatic capillaries” are used in the literature, including in recent review articles (Petrova & Koh, 2020; Oliver *et al*, 2020), when referring to the blind-ended lymphatic vessel ‘terminals’. We have opted to keep the term “lymphatic capillaries”, but to avoid confusion and incorrect association with (blood) capillaries, we have carefully revised the text and now always refer to ‘lymphatic capillary’ and not ‘capillary’ alone. In addition, when first referring to lymphatic capillaries in the introduction, we have clarified that these vessels are also known as initial lymphatics (page 3).

4. The Authors refer to the potential for therapeutic restoration of vessel function at the end of Discussion but do so in a shallow fashion. They should address, in broad strategic terms, how this might be achieved in relation to what they have discovered.

Response: We have added the following sentence in discussion (page 19):

Although lymphatic vessel growth can be stimulated by pro-lymphangiogenic VEGF-C therapy, remodeling of the initially dysfunctional lymphatic capillary network into functional collecting vessels occurs with a delay (Tammela et al, 2007). Modulation of FOXP2 function to induce a collecting vessel LEC-specific transcriptional program in the newly formed lymphatic vessels could thereby provide a therapeutic strategy to promote vessel maturation and restoration of collecting vessel function.

5. There are three study groups in Figure 6A. Surely multi-group testing should have been used for statistical analysis of the data presented therein.

Response: We have now performed statistical analysis of this data using one-way ANOVA.

References

Bazigou E, Lyons OTA, Smith A, Venn GE, Cope C, Brown NA & Makinen T (2011) Genes regulating lymphangiogenesis control venous valve formation and maintenance in mice. *J Clin Invest* 121: 2984–2992

Cha B, Ho Y-C, Geng X, Mahamud MR, Chen L, Kim Y, Choi D, Kim TH, Randolph GJ, Cao X, *et al* (2020) YAP and TAZ maintain PROX1 expression in the developing lymphatic and lymphovenous valves in response to VEGF-C signaling. *Development* 147

Geng X, Cha B, Mahamud MR, Lim K-C, Silasi-Mansat R, Uddin MKM, Miura N, Xia L, Simon AM, Engel JD, *et al* (2016) Multiple mouse models of primary lymphedema exhibit distinct defects in lymphovenous valve development. *Dev Biol* 409: 218–233

Geng X, Cha B, Mahamud MR & Srinivasan RS (2017) Intraluminal valves: development, function and disease. *Dis Model Mech* 10: 1273–1287

Kazenwadel J, Betterman KL, Chong C-E, Stokes PH, Lee YK, Secker GA, Agalarov Y, Demir CS, Lawrence DM, Sutton DL, *et al* (2015) GATA2 is required for lymphatic vessel valve development and maintenance. *J Clin Invest* 125: 2979–2994

Lyons O, Saha P, Seet C, Kuchta A, Arnold A, Grover S, Rashbrook V, Sabine A, Vizcay-Barrena G, Patel A, *et al* (2017) Human venous valve disease caused by mutations in FOXC2 and GJC2. *J Exp Med* 214: 2437–2452

Martin-Almedina S, Martinez-Corral I, Holdhus R, Vicente A, Fotiou E, Lin S, Petersen K, Simpson MA, Hoischen A, Gilissen C, *et al* (2016) EPHB4 kinase-inactivating mutations cause autosomal dominant lymphatic-related hydrops fetalis. *J Clin Invest* 126: 3080–3088

Norrmén C, Ivanov KI, Cheng J, Zangger N, Delorenzi M, Jaquet M, Miura N, Puolakkainen P, Horsley V, Hu J, *et al* (2009) FOXC2 controls formation and maturation of lymphatic collecting vessels through cooperation with NFATc1. *J Cell Biol* 185: 439–457

Oliver G, Kipnis J, Randolph GJ & Harvey NL (2020) The Lymphatic Vasculature in the 21st Century: Novel Functional Roles in Homeostasis and Disease. *Cell* 182: 270–296

Petrova TV & Koh GY (2020) Biological functions of lymphatic vessels. *Science* 369

Sabine A, Agalarov Y, Maby-El Hajjami H, Jaquet M, Hägerling R, Pollmann C, Bebber D, Pfenniger A, Miura N, Dormond O, *et al* (2012) Mechanotransduction, PROX1, and FOXC2 cooperate to control connexin37 and calcineurin during lymphatic-valve formation. *Dev Cell* 22: 430–445

Tammela T, Saaristo A, Holopainen T, Lyytikkä J, Kotronen A, Pitkonen M, Abo-Ramadan U, Ylä-Herttuala S, Petrova TV & Alitalo K (2007) Therapeutic differentiation and maturation of lymphatic vessels after lymph node dissection and transplantation. *Nat Med* 13: 1458–1466

1st Revision - Editorial Decision 26th Mar 2021

Thank you for submitting a revised version of your manuscript. Your revised study has now been seen by one of the original referees, who finds that their main concerns have been addressed and recommends publication of the manuscript. Therefore, I would like to invite you to address the remaining editorial issues before I can extend the official acceptance of the manuscript.

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 manuscript Makinen and colleagues demonstrate FOXP2 as a novel regulator of lymphatic valve development. They have further shown that the expression of FOXP2 is regulated by oscillatory shear stress in a FOXC2-dependent manner.
The data is of high quality and they have addressed by concerns satisfactorily. I have no further questions or concerns.

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision 30th Mar 2021

Editor accepted the manuscript.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Taija Mäkinen
Journal Submitted to: The EMBO journal
Manuscript Number: EMBOJ-2020-107192

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

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

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen in accordance with similar previously published experiments. Data shown is generally based on a minimum of 3 mice per condition (in vivo), or 3 independent experiments (in vitro).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Data shown is based on a minimum of 3 mice per condition, from a minimum of 2 independent litters, except for Fig 1D (n= 6 mice from one litter) and Appendix Fig S2B and S2C (n = 2 embryos from 1 litter). The exact sample size (n) for each experimental group/condition is stated in the figure and/or legend and the source data.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from the analysis except for Fig 6A where one experiment was excluded due to poor efficiency of FOXC2 siRNA to decrease FOXC2 mRNA levels.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Embryos and neonatal mice were genotyped at the end of the experiment. All mice received the same treatment. Both female and male mice were included in the analysis and no differences in the phenotype between the genders were observed.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was performed. Animals of different genotypes were subjected to the same treatment.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For most experiments, no blinding was done in the data collection, analysis and quantifications. However, quantification of fluorescence intensity parameters (Figs 4B, 4D, 6C) was done in an unbiased automated fashion using ImageJ or IMARIS.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was performed but genotypes were not known at the time of treatment and some of the analysis (point 4a) was done in an automated fashion.
5. For every figure, are statistical tests justified as appropriate?	Data between two groups were compared with unpaired two-tailed Student's t-test, assuming equal variance. Ordinary one-way ANOVA Dunnett's multiple comparisons test was used to compare differences between groups (Fig 6A, Fig EV5A), and Fisher's exact test to determine association between two categorical variables (Fig 3J, Fig 6F). Differences were considered statistically significant when $P < 0.05$. The exact P values are given in the figures.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution was assumed but not tested due to small sample size.
Is there an estimate of variation within each group of data?	Each group of data are shown as mean + SD.
Is the variance similar between the groups that are being statistically compared?	Equal variance was assumed but not tested due to small sample size.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The following antibodies were used for whole mount immunofluorescence: alpha-smooth muscle actin (SMA)-Cy3 (1A4) from Sigma, cat C6198 (references for validation are provided https://www.sigmaaldrich.com/catalog/product/sigma/c6198?lang=en&region=SE); Chicken anti-mouse GFP from Abcam cat Ab13970 (references for validation are provided https://www.abcam.com/gfp-antibody-ab13970.html); Goat anti-human/mouse FOXP2 from Santa Cruz Biotechnology cat sc-21069 (references for validation are provided https://www.scbt.com/p/foxp2-antibody-n-16); Goat anti-mouse FOXP2 from Abcam, cat ab1307 (references for validation are provided https://www.abcam.com/foxp2-antibody-c-terminal-ab1307.html); Goat anti-human/mouse VE-Cadherin (C-19) from Santa Cruz Biotechnology, cat sc-6458 (references for validation are provided https://www.scbt.com/p/ve-cadherin-antibody-c-19); Goat anti-mouse NRP2 from R&D Systems, cat AF567 (references for validation are provided https://www.rndsystems.com/products/mouse-rat-neuropilin-2-antibody_af567); Goat anti-mouse VEGFR3 from R&D Systems, cat AF743 (references for validation are provided https://www.rndsystems.com/products/mouse-vegfr3-flt-4-antibody_af74); Goat anti-mouse integrin-alpha 9 from R&D Systems, cat AF3827 (references for validation are provided https://www.rndsystems.com/products/mouse-integrin-alpha9-antibody_af3827);

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumc	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

	Goat anti-human/mouse NFATc1 from R&D Systems, cat AF5640 (references for validation are provided https://www.rndsystems.com/products/human-nfatc1-antibody_af5640); Hamster anti-mouse Podoplanin (8.1.1.) from Developmental Studies Hybridoma Bank, 8.1.1. (references for validation are provided https://dshb.biology.uiowa.edu/8-1-1); Mouse anti-human/mouse FN-EIIIA (FN-3E2) from Sigma, cat. F6140 (references for validation are provided https://www.sigmaaldrich.com/catalog/product/sigma/f6140?lang=en&region=SE); Mouse anti-human NFATc1 (7A6) from BD Pharmingen cat 556602 (references for validation are provided https://www.bdbiosciences.com/us/reagents/research/antibodies-buffers/cell-biology-reagents/cell-biology-antibodies/purified-mouse-anti-nf-atc1-7a6/p/556602); Rat anti-mouse PECAM1 from BectonDickinson, cat 553370 (references for validation are provided https://www.bdbiosciences.com/eu/applications/research/stem-cell-research/cancer-research/mouse/purified-rat-anti-mouse-cd31-mec-133/p/553370); Rabbit anti-mouse LYVE1 from Reliatech, cat 103-PA50AG (references for validation are provided https://www.reliatech.de/product-storage/polyclonal-antibodies/103-pa50ag/); Rabbit anti-PROX1 from Generated against human Prox1 C-terminus (567-737aa) (Stanczuk et al, 2015); Rabbit anti- VE-cadherin from Bender MedSystems, cat BMS158 (references for validation are provided https://www.citeab.com/antibodies/2039791-bms158-anti-human-cd144-ve-cadherin-purified); Sheep anti-mouse FOXC2 from R&D Systems cat AF6989 (references for validation are provided https://www.rndsystems.com/products/mouse-foxc2-antibody_af6989); The following antibodies were used for flow cytometry: Rat anti-mouse CD16/CD32 from eBioscience, cat 14-0161-85 (https://www.thermofisher.com/antibody/product/CD16-CD32-Antibody-clone-93-Monoclonal/14-0161-82); Hamster anti-mouse PDPN (8.1.1, PE) from eBioscience, cat 12-5381-82 (references for validation are provided https://www.thermofisher.com/antibody/product/Podoplanin-Antibody-clone-eBio8-1-1-8-1-1-Monoclonal/12-5381-82); Rat anti-mouse CD31/PECAM1 (390, PE-Cyanine7) from eBioscience, cat 25-0311-82 (references for validation are provided https://www.thermofisher.com/antibody/product/CD31-PECAM-1-Antibody-clone-390-Monoclonal/25-0311-82); Rat anti-mouse CD45 (30-F11, eFluor 450) from eBioscience, cat 48-0451-82 (references for validation are provided https://www.thermofisher.com/antibody/product/CD45-Antibody-clone-30-F11-Monoclonal/48-0451-82); Rat anti-mouse CD11b (M1/70, eFluor 450) from eBioscience, cat 48-0112-82 (references for validation are provided https://www.thermofisher.com/antibody/product/CD11b-Antibody-clone-M1-70-Monoclonal/48-0112-82); Rat anti-mouse TER-119 (eFluor 450) from eBioscience, cat 48-5921-82 (references for validation are provided https://www.thermofisher.com/antibody/product/TER-119-Antibody-clone-TER-119-Monoclonal/48-5921-82); Hamster anti-mouse Podoplanin (8.1.1, APC) from BioLegend, cat 127410 (references for validation are provided https://www.biolegend.com/en-us/products/apc-anti-mouse-podoplanin-antibody-6656); Rat anti-mouse LYVE1 (ALY7, eFluor 660) from eBioscience, cat 50-0443-82 (references for validation are provided https://www.thermofisher.com/antibody/product/LYVE1-Antibody-clone-ALY7-Monoclonal/50-0443-82). All antibodies used are described in the Materials and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Human primary dermal lymphatic endothelial cells (HDLECs isolated from juvenile foreskin, cat. C-12216) were obtained from PromoCell. HDLECs tested negative for mycoplasma contamination.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mouse strains (Mus musculus) Foxp2-flox (French et al, 2007), Tie2-Cre (Koni et al, 2001), Prox1-CreERT2 (Bazigou et al, 2011) and Foxc2-flox (Sabine et al, 2015) were analyzed on a C57BL/6J background. Both female and male mice were used for analysis and no differences in the phenotype between the genders were observed. Both embryonic (E14-E18) and postnatal (P6-5 weeks) were used for experiments. The stage/age is stated in the figures and/or legends. Mice were housed in individually ventilated cages under a 12:12-h dark–light cycle (light from 07:00 to 19:00) at 22 ± 1°C with ad libitum access to food and water.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experimental procedures were approved by the Uppsala Animal Experiment Ethics Board (permit numbers C130/15 and 5.8.18-06383/2020) and performed in compliance with all relevant Swedish regulations, or by the Animal Ethics Committee of Vaud, Switzerland.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We report all relevant aspects of animal studies.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	RNA microarray data that support the findings of this study have been deposited in GEO (Gene Expression Omnibus) repository with the accession code GSE159842 and will be made available when the manuscript has been accepted for publication. Reviewers can access the data at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE159842 using the following token: qhqhkucwltujzml.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

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