The growth and expansion of meningeal lymphatic networks are affected in craniosynostosis

Phillip S. Ang, Matt J. Matrongolo and Max A. Tischfield
DOI: 10.1242/dev.200065

Editor: Patrick Tam

Review timeline
Original submission: 2 August 2021
Editorial decision: 3 September 2021
First revision received: 11 November 2021
Accepted: 2 December 2021

Original submission

First decision letter

MS ID#: DEVELOP/2021/200065

MS TITLE: The growth and expansion of meningeal lymphatic networks are affected in craniosynostosis

AUTHORS: Phillip Ang, Matt Matrongolo, and Max Tischfield

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript by Ang and colleagues, they demonstrate that meningeal lymphatic development and function is perturbed in the Twist1 mutant mouse model of the congenital cranial suture defect craniosynostosis. This work is important as it provides needed insight into development of dorsal meninges lymphatics, a research area that is largely overlooked as most of the focus has been dysfunction is adult disease or injury. Further, it has potential clinical implications as meningeal lymphatics may be impaired in craniosynostosis patients. The authors also provide an important technical protocol with the calvarium wholemounts that better preserve the lymphatic organization. The manuscript is well written, and the discussion does a good job of outlining potential mechanisms for altered meningeal lymphatic development.

Comments for the author

My comments are primarily to improve data presentation (higher magnification images, more labels, different colors, etc), the most significant critique is there needs to be more validation of the dura hypoplasia/loss in the mutants
(see point #6).
Comments in the order they appear in the manuscript:
1) In figures, please label (as appropriate) the genotype of the tissue presented in the panels (similar to labels used in Fig. 3, 4).
2) Ln 104-107: The author's introduce data in Fig 2B in their description of data in Fig 1A and then go back to Fig 1 data. I would recommend for ease of reading to either move example of 'hardened, think translucent material' into figure 1 or wait to describe this feature until the text in figure 2.
3) Ln: 110 'regionalized bone loss' – is this loss (formed then goes away) or is never formed? If the authors don't have data to support loss rephase to say 'regionalized absence of bone'.
4) Figure 1: C, D: please add additional detail in the text, the figure legend as to the labeling (is this a cre fluorescent reporter Ai14?) and needs higher magnification images and include more labels/annotations of the regions of interest (ex: skin, calvarium mesenchyme, presumptive meninges) with arrows.
For example, 'DM' is the only label but this is not defined in the figure legend. In its current state, it is hard for the reader to tell which regions of the calvarium mesenchyme/condensation and meninges are hypoplastic. Given that Sma22-cre recombines quite a bit, this is important. One suggestion (up the author if they want to use this) is to put the control and mutant high magnification images side by side and slightly rotated so that the the pial surface is parallel to the bottom of the page, that may be easier for readers to compare and see the hypoplasia and along with annotations to better appreciate the phenotype.
5) Ln 125-126: should be Fig. 1G referring to fibronectin?
6) Figure 1G, H, I: Similar to #3 above, these images would benefit from higher magnification and better annotations to indicate regions of interest, in particular comparing analogous regions in control vs mutant. One of the claims here is that the dura is hypoplastic/absent, based primarily on fibronectin expression. However fibronectin is not specific, at least not based on the data presented here. I think their data is convincing the arachnoid is still present but it is less convincing of the absence of the dura layer. In a recent paper by Farmer et al., single cell of the fetal mouse suture mesenchyme (https://www.nature.com/articles/s41467-021-24917-9#Fig1) identified several new markers of dura subpopulations that were validated by RNAscope. Utilizing more specific markers such as these would go a long way to support dura hypoplasia in the mutants. It would also help support the regionality of the loss (dorsal-lateral and dorsal midline) as this is currently not well supported by the H&E staining in Fig. 1 E&F.
7) Fig. 2 – please add
1) arrows to indicate Lyve1+ positive lymphatic vessels in TVS and SSS samples (consider outlining the sinus in 'SSS' with dashed lines so it is easier to appreciate the adjacent brown Lyve1+ staining).
2) labels to indicate the antibody or reporter being shown
3) select different colors for C as the merged image with red-green will be difficult for color-blind readers to see the different channels.

8) Fig. 3: 1) please change colors so that red-green color-blind readers can visualize the two fluorescent signal. 2) The Lyve1+ macrophages vs lymphatics are hard to appreciate in the image Fig. 3D – a high magnification inset to show these are individual cells with macrophage morphology would help.
Also, I don't believe that Figure S2 is mentioned in this section, please incorporate into the description.
9) Fig. 4D, Ln 223-224 – please indicate in the figure with an arrow of a different color what supports the statement 'growth of mLV along the sigmoid sinus was also more robust'.
10) Ln 236-239 – I am a little confused by this sentence regarding hypoplastic dcLNs and how this relates to the meningeal lymphatics. Also without data to support it seems out of place – I would suggest removing this statement. Fig. 4E – please put genotype above representative image of lymph node.

Reviewer 2

Advance summary and potential significance to field

Overall, these findings in the Twist1 craniosynostosis model make a significant and novel contribution to our understanding of developmental mechanisms.

Comments for the author

The manuscript by Ang et al shows that venous malformations and dural structural changes caused by Twist1 craniosynostosis model perturbs the growth and expansion of the meningeal lymphatic vessel (mLV) development.
The manuscript is generally well written, and the findings support the conclusions. Of course, it would have been interesting to investigate if VEGFC overexpression could rescue the development and functionality of intracranial lymphatic vessels in Twist1 craniosynostosis model.
Perhaps a further comment should be included about the intracranial pressure in this model.
The authors should also mention if similar findings could apply also to FGFR-associated craniosynostosis.
Overall, these findings make a significant and novel contribution to our understanding of developmental mechanisms.
The other comments below are mostly related to the writing of the manuscript and presenting the data.

Major comments:
1) Authors state multiple times that their staining and imaging technique is novel (lines 16, 80, 139-144, 158-167, 200-205, and 291-300. However, Aspelund et al (2015) and Antila et al (2017) already described the whole mount staining and stereomicroscopic imaging of the whole skull as well as decalcification, whole-mount staining, flat-mounting, and confocal microscopy of the skull. In these papers, no scraping of dural tissue was used to visualize the mLVs. Please refer to these papers when discussing the technique and tone down the novelty of the technique used in this paper.
2) It is not always clear how many animals were used and how many times experiments were repeated. Please mark the used n-numbers and experimental repetition information in the figure legends. For proper statistical analysis, it would be good to use three mice per group, with repetition of experiments.
3) Lines 234-237: Please show the data.
Citations:
1) Line 35: when talking about the macromolecular transport into mLVs, please cite also Aspelund et al (2015), showing uptake of intracranially injected macromolecular tracers into mLVs.
2) Line 34-38: please note that the connection between meningeal lymphatic and glymphatic systems is not fully understood, Louveau et al (2015) did not show the connection between mLVs and the glymphatic system, Xie et al (2013) and Lundgaard et al (2016) studied only glymphatic system and Da Mesquita et al (2018) did not show transportation of amyloid plaques into dcLNs. please correct the sentences.

3) Line 43-44: Louveau et al (2015) and Antila et al (2017) showed mLVs in humans and primates, respectively. The citation should be made to these papers when talking about the discovery of mLVs in primates.
4) Line 280-282: Antila et al (2017) showed that aging affects mLVs, please add a citation.
Recommendations for improving the figures
5) Figure 1 and 2: please mark the ages of the mice and the fluorescent protein signals and stainings in the panels using matching colors.
6) Figure 3: please mark the age of mice as well as the stainings in the panels with suitable colors. Please show the quantification also for P60 animals and indicate p-values and the statistical test used in the figure legend.
7) Figure 4: please mark the ages of the mice and the stainings in the panels with suitable colors and indicate the individual values of the mice as dots in panel E. Please indicate p-values and the statistical tests used in the figure legend.
8) Supplemental Figure 1: please mark the age of mice and indicate mice as dots in the quantification.
9) Supplemental Figure 2: please mark the ages of the mice.
10) Supplemental Figures 3, 4: please mark the ages of the mice as well as the staining in the panels using suitable colors.
11) Supplemental Figure 4: please mark the ages of mice as well as the staining in the panels with suitable colors.

Reviewer 3

Advance summary and potential significance to field

This is a follow-up study of the authors' previous finding that loss of Twist1 in bone and meningeal progenitors results in defects in cranial venous development like those observed in humans with craniosynostosis, and that Twist1 regulates BMP2/4 expression in these progenitors and BMP2/4 serves as a paracrine signal to regulate cranial venous development (Tischfield et al. Dev Cell 2017). Here the authors identify defects in meningeal lymphatic vasculature in the conditional Twist1 mutants. Such meningeal lymphatic vasculature in the mutants fails to function as lymphatic drainage.

Comments for the author

Although the findings are interesting, this study is descriptive and lacks sufficiency and thoroughness of the data to convincingly support their claims.
Probably the most important question is what affects meningeal lymphatic development in the mutants. Does defective cranial venous development lead to abnormal meningeal lymphatic development? If so, the authors need to define whether the varying degree of cranial venous defects (size of vessels, amount of vascular smooth muscle coverage, etc.) is closely associated with that of cranial lymphatic defects. Alternatively, the authors need to consider whether the bone and meningeal progenitors may secrete pro-lymphangiogenic VEGF-C to control cranial lymphatic development, independently of cranial venous development. Is it possible that the progenitors-derived BMP2/4 directs both cranial venous and lymphatic development?

Specific comments
- All figures contain high-quality images but no indication of genotypes (Figs. 1 and 2) and fluorescence markers (Figs. 1-4).
- In Fig. 1, the authors need to perform quantification measurements of hypoplastic meningeal structure (C-D, G-I, control versus mutants). In C and D there is no description about arrowheads in figure legend. It is not clear what is the difference between E and F.
- In Fig. 2, the authors need to perform quantification measurements of lymphatic defects (A versus B). the authors also need to examine cranial venous development and carry out a side-by-side anatomical comparison between venous and lymphatic phenotypes. In the text, there is no description about Fig. 2E and F.
- In Fig. 3, the authors need to measure the size of TVS and amount of vascular smooth muscle coverage. It is puzzling that the authors indicate the diagram illustrating the meningeal

development at P60 but the fluorescent images of the meningeal development in C and D are taken from control and mutant animals at P90.

• In Fig, 4A-D, the authors need to perform quantification measurements of venous structure and show a side-by-side anatomical comparison between venous and lymphatic phenotypes.

First revision

Author response to reviewers' comments

We are pleased to resubmit our revised manuscript entitled "*The growth and expansion of meningeal lymphatic networks are affected in craniosynostosis*" for consideration as a Research Report. We appreciate the reviewers' thoughtful critiques, as they have helped strengthen our findings and main conclusions. Below, we first detail key changes found in the revised manuscript before providing a point- by-point response to the reviewers' suggestions and concerns.

- Reviewer #1 only had one significant critique, as she/he felt that there needs to be "*more validation of the dura hypoplasia/loss in the mutants*" and suggested we test a molecular marker specific to dura mater in lieu of fibronectin staining. We agree with this critique. Following the suggested reference that identified dural markers via RNAseq, we now provide high magnification images showing that dura in *Twist1*^{CS} mutants is hypoplastic at and adjacent to the dorsal midline according to Crabp2 and Connexin-43 co-staining (Figs. 1C-E, S1C). Thus, we chose to omit the fibronectin staining results originally found in figure panels 1C, D, G and, in its place, provide our new data points.
- Reviewer #3 also felt that we needed to provide measurements of the hypoplastic dura to validate our claim. We now quantify loss of dura mater, and also a reduction in the thickness of condensed osteogenic mesenchyme, at the dorsal midline where bone development is delayed/absent (Fig. 1D').
- Reviewer #3 also felt that we "*need to define whether the varying degree of cranial venous defects (size of vessels, amount of smooth muscle coverage) is closely associated with the cranial lymphatic defects*". In Fig. 3, panels A-B, we provide side-by-side images (and quantifications) showing that smooth muscle coverage is not significantly affected in juvenile *Twist1*^{CS} animals, despite hypoplasia of the transverse sinus. In panels 3C/D, we also quantify smooth muscle coverage in P60 adult animals and measure the size of the transverse sinus. By contrast to juvenile animals, the intact transverse sinus has "caught-up" in size and does not show significant hypoplasia. Smooth muscle coverage is also comparable to control littermates, with the exception of severely affected animals, which can show patchy coverage and less striation in regions along the transverse sinus. Given that smooth muscle coverage and the size of the intact transverse sinus is not significantly affected across the *whole* population (at least in adults), this leads us to conclude that hypoplastic dura exerts a stronger effect on the lymphatic phenotypes across the spectrum, versus changes to the size of the transverse sinus and/or smooth muscle coverage (which may have a stronger and/or additive role in severely affected animals).

Point-by-Point response to reviewers:

Reviewer 1: We are pleased that reviewer 1 finds the work to be important by providing "*needed insight into meningeal lymphatic development*" and "*clinical implications as meningeal lymphatics may be impaired in craniosynostosis patients*." We thank the reviewer for his/her suggestions to improve data presentation, and to provide a more accurate assessment of the hypoplastic dura in the mutant animals. Our response to each individual critique is listed below.

1. We have now labeled the genotypes in figure panels.
2. We now show data for "mineralized bone instead contained a thin hardened, semi-translucent material" in Fig. S1A
3. Thank you for this clarification. We have substituted "absence of bone" for bone loss in lines 96- 101 as our previous studies indicate the bone fails to develop and/or properly

mineralize in late gestation embryos.

4. We have removed the former Fig. 1C, D panels (showing fibronectin staining) in lieu of new data (panels C-E) more specific to dura in order to address critique 6 below.
5. We have removed the former Fig. 1G panel (fibronectin staining) in lieu of new data that addresses critique 6 below.
6. Thank you for this great suggestion. We now show dural hypoplasia in co-labeled images using Crabp2 to mark dura and arachnoid, and connexin-43 to label arachnoid tissue (with control and mutant images placed side by side). We chose Crabp2 because it was shown in the suggested reference (Farmer et al., Nature Comm) to label dura, and this marker was confirmed to label dura (and arachnoid) by a separate single cell transcriptomic analysis of meningeal tissue published by DeSisto et al., Dev Cell 2020. By assessing the degree of Crabp2 labeling above the connexin-43 signal, we show that dura is hypoplastic in *Twist1*^{CS} animals at or adjacent to the dorsal midline (Figs. 1C, D, D' and 1E, respectively). We also now provide higher magnification images for e-cadherin/n-cadherin labeling.
7. For figure 2, we have provided arrowheads to label Lyve1 positive lymphatic vessels. We have also now labeled figure panels for the *Sm22a-Cre:ai14* reporter cross. Thank you for reminding us to change the colors to magenta and green so color-blind readers can properly assess the data.
8. For figure 3, we have changed the red-green color scheme to magenta-green to aide color blind readers. In panel 3B, we also added images from a more mildly affected animal detailing similar phenotypes. We have provided a higher magnification image detailing the Lyve-1 positive macrophages (inset, Fig. 3D). Figure S2 is now incorporated into this section of the manuscript text, and provides additional images showing smooth muscle coverage in juvenile and adult animals, as well as unilateral absence of the TVS in affected animals, as mentioned in the text.
9. For space limitations, we have decided to omit the statement "the growth of mLVs along the sigmoid sinus was more robust" because it is hard to see in the figure crops that were chosen. The growth appears to be more robust at the posterior aspect of this vessel as it leaves to skull casing. This data can be added if necessary.
10. As requested (and also due to space limitations), we have removed the sentence stating "the dCLNs showed unilateral hypoplasia in some animals, suggesting lymphatic drainage was ipsilateral or passaged through alternative routes". This was seen in 2/5 animals (1 of which was not included in our analysis of tracer coverage in the dCLNs) and at the present time, we are not exactly sure what this phenotype indicates and how prevalent it is among a larger population. Thus, we agree it is more prudent to omit this data in the present study. Instead, we added data and pictures to show that tracer accumulation in the sCLNs was comparable between control and mutant animals (Fig. 4E).

Reviewer 2: We are again pleased that the present study was judged to "make a significant and novel contribution to our understanding our developmental mechanisms" pertaining to meningeal lymphatic vessels.

General Comments:

A. "*Perhaps a further comment should be included about the intracranial pressure in this model*"
    a. We have started measuring intracranial pressure (ICP) in these animals and preliminary findings suggest elevated pressure compared to controls. For space reasons, we omitted this data as we intend to include it with ongoing studies in the lab pertaining to FGFR2 craniosynostosis and Twist1 heterozygous loss of function models, in which we are also measuring ICP. However, we now write in lines 200-202 that "*Although not addressed in the present study, elevated intracranial pressure and changes to flow, in combination with hypoplastic dura, may affect mLV networks in Twist1*^{CS} *models*"
B. "*The authors should also mention if similar findings could also apply to FGFR-associated craniosynostosis*"
    a. We initially mentioned in the text on lines 251-253 that "Work is underway to investigate mLV phenotypes in heterozygous *Twist1*$^{FLX/WT}$*:Sm22a-Cre* animals, and other forms of craniosynostosis caused by activating gene mutations in

FGFR2". We have changed this sentence to now read "Considering venous sinus malformations are present in Apert Syndrome caused by activating mutations in *FGFR2* (Johnson and Wilkie, 2011), mLV phenotypes may be widespread in craniosynostosis" (Lines 185-187).

Major Comments:

1. We apologize for overlooking the fact that Aspelund et al (2015) and Antila et al (2017) used skull flatmounting and confocal microscopy in their seminal works to describe the development and physiology of meningeal lymphatic vessels. From our own experience working with dural scrapings versus flat mounted skulls, we agree that skull flat mounts are far superior for preserving the topology of meningeal lymphatic networks (albeit more time consuming). We have toned down our description of the technique and have referenced the previous studies.
2. We initially included our N's for each experimental group in the material and methods, but we have now added this information to the figure legends. We always aim for 3-5 animals per experiment (with littermate controls) and preferably 1-2 animals from a different set of parents.
3. For reasons concerning space limitations for a brief research report and per reviewer 1's suggestion, we have removed the text from former lines 234-237 describing hypoplastic dCLNS (point 10 above). However, as requested, we now include data to show that tracer accumulation in the sCLNs is comparable between control and mutant animals (lines 173-174, Fig. 4E).

Citations: thank you for pointing out the following inaccuracies.

1. We have now also cited Aspelund et al (2015) showing uptake of intracranially injected tracers (line 34).
2. Thank you for pointing this out. We jumped ahead of ourselves as this is a subject of interest in our lab. We have changed and simplified the sentence to read "During steady state, mLVs act to transport macromolecules (Louveau et al. 2015; Aspelund et al 2015) and dendritic cells (Louveau et al. 2018a) from the cranium to the deep cervical lymph nodes (dcLNs)" (lines 33-35).
3. We have added Louveau et al 2015 (staining in human dura) and Antila et al 2017 (confirmed presence of lymphatic vessels in marmosets) in regards to characterizing lymphatic vessels in primates (lines 40-41).
4. Line 213-215: Antila et al 2017 has been added showing aging affects lymphatic vessels

Recommendations for improving figures:

5. Mouse ages and antibody labels have been added to figures 1 and 2
6. We have marked the age of the mice, added labels for the protein staining, and have included quantifications for the P60 animals and the p value and statistical test in the fig. legend
7. Mouse ages and antibody labels have been added to Fig. 4. Individual data points are now shown in Fig.4E, with p values and statistical tests included in figure legend
8. Mouse ages have been added and dots have been included in the quantifications
9. Mouse ages have been added
10. Mouse ages and protein labeling has been added
11. mouse ages and protein labeling has been added

Reviewer 3: We are pleased the reviewer found the findings to be interesting, and we provide additional data and quantifications to help support our conclusions

Specific Comments:

1. Genotypes and antibody/staining labels have been added to the figures
2. As suggested by reviewer #1, we have substituted panels 1C, 1D, and 1G (Fibronectin staining) with new data and layer specific markers (Crabp2 and Connexin-43) to show that dura is hypoplastic in mutant animals (Figs. 1C, D, D'; fluorescent staining in panels C-F correspond to boxed regions shown on the H&E staining). We also show a reduction in dura mater at the dorsal midline in mutant animals by measuring differences in the % area coverage of Crabp2 staining above connexin-43 positive arachnoid cells within a defined ROI centered around the dorsal midline.

3. We chose not quantify the lymphatic phenotypes in Figs. 2A, B because we felt the lower resolution provided by chromogenic immunohistochemistry may lead to inaccurate results (and it's tricky to demarcate weakly stained vessels without 3D reconstructions). Instead, we quantify our findings in Fig. 3 and Fig. 4 using fluorescent markers and high resolution maximum intensity z-stacks. Also, we removed Fig. 2E, F due to repetition and space limitations (these panels were describing a procedure that is addressed in Fig. S3).
4. For Figure 3, in addition to lymphatic vessel measurements, we now provide measurements for the average size (width) of the TVS in control and mutant animals at P16 and P60, as well as the amount of smooth muscle coverage along the TVS.
    a. As shown in Figs. 3A, B, smooth muscle coverage on the intact TVS in affected juvenile mutants (P16) is comparable to control littermates. However, the intact TVS is hypoplastic
    b. In adults, the intact TVS appears to "catch-up" in size as the average overall width is comparable in affected adult animals versus controls. Smooth muscle coverage is relatively normal (also see Fig. S2 C, D) with the exception of severely affected animals. In these animals, coverage can be patchy with less striation along segments of the TVS (Fig. 3D). We summarize these findings in the results (lines 151-157) and discussion (lines 213-220)
    c. Panel 3C now reads P60 (the text in the figure legend mistakenly said P90)
5. For Figure 4, we regrettably did not co-stain with a marker (e.g. SMA) that would allow us to accurately measure the average width of the TVS in *all* mutant and control animals possessing the Prox1-tdTomato transgene. However, we show in Fig. 3 that the average width of the intact TVS is comparable between adult littermate control and mutant animals (n=7). We hope this data will be satisfactory. If the reviewer feels this data is essential, we can attempt to measure the average size of the TVS using light microscopy.

Second decision letter

MS ID#: DEVELOP/2021/200065

MS TITLE: The growth and expansion of meningeal lymphatic networks are affected in craniosynostosis

AUTHORS: Phillip S. Ang, Matt J. Matrongolo, and Max A. Tischfield
ARTICLE TYPE: Research Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. Where referee reports on this version are available (please also see Editor's Note), they are appended below.

Reviewer 1

Advance summary and potential significance to field

In this manuscript by Ang and colleagues, they demonstrate that meningeal lymphatic development and function is perturbed in the Twist1 mutant mouse model of the congenital cranial suture defect craniosynostosis. This work is important as it provides important insight into development of dorsal meninges lymphatics, a research area that is largely overlooked as most of the focus has been dysfunction is adult disease or injury. Further, it has potential clinical implications as meningeal lymphatics may be impaired in craniosynostosis patients. The authors also provide a detailed technical protocol with the calvarium whole mounts that better preserve the lymphatic organization. The manuscript is well written, and the discussion does a good job of outlining potential mechanisms for altered meningeal lymphatic development.

Comments for the author

The authors have done a very good job addressing my prior comments, in particular improving the analysis of the dura hypoplasia that was the most significant aspect of my comments in the prior review. I have no further suggested revisions.

Reviewer 3

Advance summary and potential significance to field

The authors identify defects in meningeal lymphatic structure and function in Twist1 craniosynostosis models. They have added the additional data and quantification measurements requested.

Comments for the author

The authors have not addressed this reviewer's questions about potential mechanisms underlying defective meningeal lymphatic network in Twist1 craniosynostosis models: 1) The authors need to consider whether aberrant or decreased VEGF-C expression in bone/meningeal progenitors or venous vascular smooth muscle cells results in defective meningeal lymphatic development; 2) The authors previously demonstrated that BMP2/4 regulates cranial venous development. Is it possible that BMP2/4 directs both cranial venous and lymphatic development?