FOXO1 represses sprouty 2 and sprouty 4 expression to promote arterial specification and vascular remodeling in the mouse yolk sac

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Original submission

First decision letter

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MS TITLE: FOXO1 represses Sprouty2 and Sprouty4 expression in endothelial cells to promote arterial specification and vascular remodeling in the mouse yolk sac

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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 BenchPress and 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. One of the issues raised by Reviewer 2 concerns the specificity of the Tie2Cre line and whether or not your analyses include embryos which are effectively FOXO1 null rather than endothelial cell nulls. Can you provide some data to this point?

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

The manuscript “FOXO1 represses Sprouty2 and Sprouty4 expression in endothelial cells to promote arterial specification and vascular remodeling in the mouse yolk sac” by Li-Villarreal et al builds off previous reports about roles for the FOXO1 transcription factor in early murine vascular development. Global knockouts for Foxo1 display vascular remodeling defects by E9.5—a time at which circulatory blood flow has commenced—but the authors were interested in determining whether FOXO1 influenced endothelial cells (ECs) before the onset of circulation and hemodynamic signaling, since such signaling influences vascular remodeling and EC fate specification. Here they generated Foxo1-ECKO embryos and analyzed their ECs for transcripts at E8.25 before the onset of blood flow. They found decreased transcripts and protein for Flk1 in mutant yolk sacs although other pan-endothelial, proliferation, and apoptosis markers of mutant ECs were unaffected. The authors also found transcripts for arterial Notch pathway markers (i.e. Dll4) downregulated in Foxo1 mutant yolk sac ECs but not in embryonic ECs. Because Flk1 and Dll4 gene regulatory regions do not contain known binding sites for FOXO1, the authors sought other direct target genes that might explain these transcriptional changes. They discovered that Spry2 (a known FOXO1 target gene) and Spyr4 are directly suppressed by FOXO1 in yolk sac ECs. Transient endothelial overexpression of Spry4 in transgenic mouse embryos led to yolk sac remodeling defects and downregulation of many transcripts at E8.25, including several that are likewise seen in Foxo1 mutant ECs (i.e. Dll4 and Flk1). Altogether, the authors have accomplished their goal of defining molecular consequences of endothelial Foxo1 deletion prior to the onset of circulation, but caution is advised about over-interpreting the results.

Comments for the author

-The PECAM staining in Fig. 1J helps to substantiate the light microscopy images in 1F,G. Similar PECAM stains would be beneficial for E8.5 Foxo1-ECKO control/mutant embryos, so that the claim that yolk sac patterning is unaffected at this timepoint in mutants is clearly demonstrated. Alternatively, larger image fields of the FLK1 immunostaining/reporter? shown in Fig. 2A/B would suffice. This is important if the subsequent molecular analyses on mutants are meant to be performed pre-phenotypically (prior to patterning defects).
-Line 182 is imprecise in stating that “flow abnormalities are not detected until after defects in vessel remodeling are evident.” We are presented with normal vessel remodeling and normal flow at E8.5 but abnormal vessel remodeling and abnormal flow at E9.5 (Fig. 1 and 2), so it’s still hard to know which comes or to substantiate the title of the first results section: “Defective yolk sac vascular remodeling in Foxo1-ECKO embryos is not due to abnormalities in hemodynamic force.” Nevertheless, this critique does not undercut the main focus of the paper, which is to define molecular alterations in Foxo1 mutant yolk sac ECs prior to flow (the experimental approach of focusing on transcripts at E8.25 is sound).
-The Spry4 transgenics would also benefit from a PECAM stain to more easily visualize the branching defects implied in Fig. 8B.
-One of the first molecular phenotypes described in the manuscript is the downregulation of Flk1 expression in Foxo1-ECKO yolk sacs. What about in the embryo proper? It would be nice to comment on the embryonic/extraembryonic specificity of this phenotype, particularly in light of the yolk sac-specific effect of Foxo1 depletion on arterial gene expression and the yolk sac-specific downregulation of Flk1 in the Spry4 transgenics. These data might help support the speculative comment in the discussion that Flk1 downregulation in Foxo1 mutants could be secondary to arterial specification [lines 465-467]. Is this speculation supported by other mutants with arterial specification defects (i.e. Dll4-ECKO)?

-Similarly, Spty2/4 are upregulated in Foxo1-ECKO yolk sacs, but what about in the embryo proper? This would be nice evidence to use when interpreting the impact of the Spry4 transgenics on arterial and venous genes in the yolk sac (Fig. 8C) and the embryo proper (Fig. 8D).
-The fact that the Spry4 transgenics downregulated almost all genes analyzed in the yolk sac (except Cx40), makes it hard to draw a linear connection between upregulation and the arterialization defects seen in the Foxo1-ECKO yolk sacs—as is implied in the title and abstract. Be careful with the language that endothelial Spry4 overexpression "phenocopies conditional loss-of-function FoxO1 mutants" (title of last results section) because the Spry4 transgenic seems more widely impactful on endothelial genes than your FoxO1 mutant does. Also, the lack of a direct/quantitative comparison between the yolk sac remodeling defects in Spry4 transgenics vs. Foxo1-ECKO mutants makes the title/conclusion hard to accept.
-All bar graphs would benefit from showing individual data points, including on controls so that variation can be appreciated better and so that "n" biological or technical replicates are apparent.

Reviewer 2

Advance summary and potential significance to field

The manuscript "FOXO1 represses Sprouty2 and Sprouty4 expression in endothelial cells to promote arterial specification and vascular remodeling in the mouse yolk sac" by Li-Villarreal et al., present interesting data regarding the role of the transcription factor FoxO1 in the arterial specification of yolk sac vasculature. The authors note that FoxO1 global mutants and endothelial cell conditional mutants were analyzed previously by other labs. However, the presence of severe heart defects confounded conclusions from prior studies, since defective heart function leads to defects in yolk sac vascular development. Li-Villarreal et al., performed careful measurements of blood flow to determine the time frame for their analyses. The authors found defects in the arterial specification of yolk sac vasculature in FoxO1 EC conditional KOs. However, the conclusion that FoxO1 regulates pre-flow arterial EC specification is overstated because a) it is not clear whether the analyses were done before the flow was initiated, b) the time point when arterial markers begin to be expressed in the yolk sac was not assessed, and c) the data does not distinguish whether or not FoxO1 is important for the initiation or maintenance of arterial marker expression. A major part of the paper is devoted to the role of FoxO1 in the regulation of Sprouty 4 expression and its role in modulating arterial-venous fate. The data are interesting but the role of the FoxO1-Sprouty 4 axis during arterial specification is overstated.

Comments for the author

The manuscript "FOXO1 represses *Sprouty2* and *Sprouty4* expression in endothelial cells to promote arterial specification and vascular remodeling in the mouse yolk sac" by Li-Villarreal et al., present interesting data regarding the role of the transcription factor FoxO1 in arterial specification of yolk sac vasculature. The authors note that FoxO1 global mutants and endothelial cell conditional mutants were analyzed previously by other labs.
However, the presence of severe heart defects confounded conclusions from prior studies, since defective heart function leads to defects in yolk sac vascular development. Li-Villarreal et al., performed careful measurements of blood flow to determine the time frame for their analyses. The authors found defects in arterial specification of yolk sac vasculature in FoxO1 EC conditional KOs. However, the conclusion that FoxO1 regulates pre-flow arterial EC specification is overstated because a) it is not clear whether the analyses were done before flow was initiated, b) the time point when arterial markers begin to be expressed in the yolk sac was not assessed, and c) the data does not distinguish whether or not FoxO1 is important for the initiation or maintenance of arterial marker expression. A major part of the paper is devoted to the role of FoxO1 in the regulation of Sprouty 4 expression and it's role in modulating arterial-venous fate. The data are interesting but the role of FoxO1-Sprouty 4 axis during arterial specification is overstated.

Major:

1. The transgenic Tie2Cre strain is known for a substantial activity of the Cre transgene in the germline (Physiol Genomics. 2008 Sep; 35(1): 1–4). Therefore, the analyses presented in this manuscript are compounded by the uncertainty of whether or not each of the embryos analyzed are actually globally null for FoxO1. At the least, the expression of a

Cre reporter in each embryo is necessary to rule out potential germline deletion, especially when using Tie2Cre strain for endothelial deletion.

2. The authors state that phenotypes observed in their studies are consistent with those observed by prior studies. However, prior studies were done either using global deletion or done using Tie2Cre transgenic mice, and the latter ones were not controlled for possible germline deletions.
3. Even though the analyses were performed a day earlier than in the published literature, the conclusion that FoxO1 is important for the pre-flow establishment of arterial EC identity in ys ECs is not supported. There is no time course showing when arterial identity is first evident in yolk sac ECs, and whether arterial identity is never established in mutant ys ECs, or whether it is not maintained, is not examined.
4. qRT-PCR results are not confirmed by any other means. Either IF or in situ hybridization would be helpful to ensure endothelial-specificity of the observed changes in gene expression.
5. Data in Figure S3 is not convincing. It is not clear whether Cx37 and Cx40 seen in the micrographs is specific staining and whether the observed staining is in endothelial cells.
6. The fact that the expression of AFP was not changed among yolk sacs of controls and mutants does not mean that the changes in gene expression noted are endothelial-specific. Especially, since the expression of many of the markers tested is not confined to the endothelium.
7. The authors do not state how they staged embryos. Since there is a substantial variability in embryo development at early stages, staging by somite counts is more appropriate. This point is important because difference in 1 – 2 somites may indicate whether there is and there isn't blood flow in a particular embryo being analyzed, and impact authors' claims that FoxO1 regulates arterial specification pre-flow.
8. Figures 3, 4 and others. The authors do not mention statistical tests used in their analyses, or how the analyses were performed, e.g., correction for multiple testing. In the case of Student's t tests, were tests 2-sided, unpaired? In the case of qRT-PCR analyses, how the data was analyzed or statistical analyses were not described
9. Please used SD instead of S.E. in the analyses and plots, as this is a better metric to evaluate variability.
10. EC-specificity of changes in the expression of Sprouty 2 and 4 needs to be confirmed by either in situ hybridization or IF with co-staining of an EC marker.
11. The authors state that levels of the assayed arterial and venous markers in Fig. 8 were normalized to the levels of endogenous Sprouty 4, however, it is not clear how this was done in transgenic embryos where Sprouty 4 was overexpressed. Why not normalize to GAPDH as in other qRT-PCR analyses in this paper?
12. Overexpression of Sprouty 4 led to decreased expression of not only arterial EC markers but also venous, as well as to the decrease in the expression of pan-EC markers. These results are not consistent with the role of Sprouty 4 in FoxO1ECKO and suggest gain-of-function effects.
13. Compound FoxO1 / Sprouty 2/4 knockout mutants are necessary to test the hypothesis that the suppression of these Sprouty genes by FoxO1 regulates EC arterial- venous specification.

Figures:

1. The authors state the morphology of FoxO1^{ECKO} embryos was fine at E8.5. However, the picture shows embryos inside yolk sacs obscuring embryonic morphology.

2. The legend to Figure 3 does not described the panel. The panels and numbering in the figure and legend do not correspond. The genotypes of samples portrayed by the black and gray bars need to be revisited, it is not clear what "floxdel" designation means.

3. Figure 4. Please state embryonic stage samples used in panels A and B.

4. Please describe in more detail or cite Farsight software used for segmentation

5. Figure 5. Dll4-LacZ expression in FoxO1-null embryo is not evident in any tissue. How

D..4 expression was quantified in 5C and F is not stated.

6. The color coding for Figure 6C and D makes it hard to tell different greys apart

7. Legend for Fig. 7 is in accurate in some places and incomplete in others (e.g., not all panels are mentioned in the legend). It is not clear what is shown in the last 4 bars of Fig, 7C.

First revision

Author response to reviewers' comments

Response to reviewers:

We would like to start by thanking the conscientious reviewers for their thoughtful and thorough review of our manuscript. Overall, the comments were extremely helpful and improve the presentation and interpretation of the data for the readers. Detailed responses to the comments are provided below:

Reviewer 1:

-The PECAM staining in Fig. 1J helps to substantiate the light microscopy images in 1F, G. Similar PECAM stains would be beneficial for E8.5 Foxo1-ECKO control/mutant embryos, so that the claim that yolk sac patterning is unaffected at this timepoint in mutants is clearly demonstrated. Alternatively, larger image fields of the FLK1 immunostaining/reporter? shown in Fig. 2A/B would suffice. This is important if the subsequent molecular analyses on mutants are meant to be performed pre-phenotypically (prior to patterning defects).

Response: We have provided PECAM1 staining data in Fig. 1 L, M. These data indicate no obvious discernable difference between mutant and wild type yolk sacs at this stage. These data are very similar to PECAM stains of E8.5 germline null mutants shown in Furuyama et al 2004, Fig. 3 I and J who concluded that the mutant yolk sacs had a normal vascular plexus but showed subsequent abnormal YS vascular remodeling.

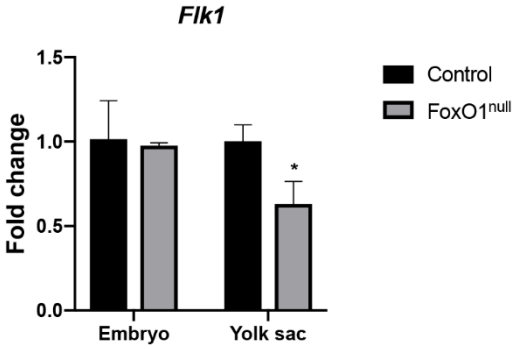
-Line 182 is imprecise in stating that “flow abnormalities are not detected until after defects in vessel remodeling are evident.” We are presented with normal vessel remodeling and normal flow at E8.5 but abnormal vessel remodeling and abnormal flow at E9.5 (Fig. 1 and 2), so it’s still hard to know which comes or to substantiate the title of the first results section: “Defective yolk sac vascular remodeling in Foxo1-ECKO embryos is not due to abnormalities in hemodynamic force.” Nevertheless, this critique does not undercut the main focus of the paper, which is to define molecular alterations in Foxo1 mutant yolk sac ECs prior to flow (the experimental approach of focusing on transcripts at E8.25 is sound).

Response: We understand the reviewer’s concern and have changed that statement as follows:
Thus, flow initiates normally in FoxO1^{ECKO} embryos, but defects in both vascular remodeling and blood flow abnormalities were observed at E9.5.
We wanted to ensure that we could study molecular events prior to the disruption in blood flow to ensure we were looking at events upstream of those that might be triggered by the force exerted by blood flow. Hopefully, this is clearer.

-The Spry4 transgenics would also benefit from a PECAM stain to more easily visualize the branching defects implied in Fig. 8B.

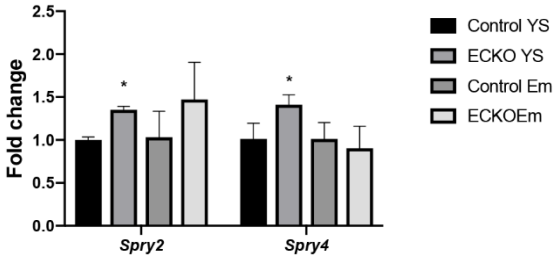
Response: Unfortunately, we would have to recreate this transient overexpression experiment as we collected whole yolk sacs for the qRT-PCR analysis, so we would not be able to show a PECAM stained yolk sac and the subsequent qRT-PCR data. We included YFP in the overexpression construct to be able to show the pattern of EC nuclei (shown in Fig 8B) as well as to be able to

choose individual embryos likely to have significant expression of Sprouty4 (as in Fig S7).
-One of the first molecular phenotypes described in the manuscript is the downregulation of Flk1 expression in Foxo1-ECKO yolk sacs. What about in the embryo proper? It would be nice to comment on the embryonic/extraembryonic specificity of this phenotype, particularly in light of the yolk sac-specific effect of Foxo1 depletion on arterial gene expression and the yolk sac-specific downregulation of Flk1 in the Spry4 transgenics. These data might help support the speculative comment in the discussion that Flk1 downregulation in Foxo1 mutants could be secondary to arterial specification [lines 465-467]. Is this speculation supported by other mutants with arterial specification defects (i.e. Dll4-ECKO)?



Response: We have included the data above in Fig. S2A. *Flk1* mRNA expression is only significantly affected in the YS and expression is not significantly different in nulls compared to controls for the embryo proper. We ran this from the germline null in order to compare *Flk1* expression between yolk sac and embryo in the strongest deletion of *FoxO1*. The reviewer is correct that both *Flk1* and *Dll4* mRNA expression seem to be more profoundly affected in the YS of *FoxO1* mutants. We don't know if this is true in Dll4-ECKOs. Future experiments would be needed to understand more about the extra-embryonic vs embryonic mechanisms in ECs This is now discussed more in the revised penultimate paragraph of the discussion.

-Similarly, Spty2/4 are upregulated in Foxo1-ECKO yolk sacs, but what about in the embryo proper? This would be nice evidence to use when interpreting the impact of the Spry4 transgenics on arterial and venous genes in the yolk sac (Fig. 8C) and the embryo proper (Fig. 8D).



Response: We have included the data above in Fig. S5. The ECKO yolk sac shows the only significant changes in each of the Sprouty gene transcript levels.

-The fact that the Spry4 transgenics downregulated almost all genes analyzed in the yolk sac (except Cx40), makes it hard to draw a linear connection between upregulation and the arterialization defects seen in the Foxo1-ECKO yolk sacs—as is implied in the title and abstract. Be careful with the language that endothelial Spry4 overexpression "phenocopies conditional loss-of-function FoxO1 mutants" (title of last results section) because the Spry4 transgenic seems more widely impactful on endothelial genes than your FoxO1 mutant does. Also, the lack of a direct/quantitative comparison between the yolk sac remodeling defects in Spry4 transgenics vs. Foxo1-ECKO mutants makes the title/conclusion hard to accept.

Response: We understand and have clarified our interpretation in this section. The Sprouty overexpression experiment did show that Sprouty4, when overexpressed in ECs could prevent normal vascular remodeling Figure 8B. This is the same phenotype seen in FoxO1 knockouts Figure

1G. From a molecular perspective, it is true that more gene expression is altered in the Sprouty4 overexpression yolk sacs than in the FoxO1 ECKOs. We do not know if this is due to the levels of expression, overexpression in a broader group of cells than FoxO1 functions in, additional functions of Sprouty4 independent of FOXO1, or if there are other considerations. We agree that it is not the perfect experiment but was informative as it showed that Sprouty overexpression is sufficient to disrupt vascular remodeling and endothelial gene expression although it was not a perfect match. Together with ChIP and Luciferase assays in the paper, these data show that Sprouty 4 is downstream of FOXO1.

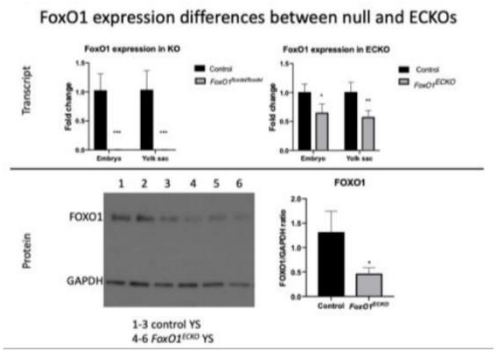
-All bar graphs would benefit from showing individual data points, including on controls so that variation can be appreciated better and so that "n" biological or technical replicates are apparent.

Response: We have added individual data points for the controls as well as the experimental samples and repeated our statistical analysis using the average of the controls for each target as the reference sample to compare individual data points. The graphs now all reflect this approach. You will notice that some of the previously significant values are no longer significant but it is still clear that there is a significant effect on arterial marker expression in FoxO1 CKOs.

Reviewer 2:

Major:

-The transgenic Tie2Cre strain is known for a substantial activity of the Cre transgene in the germline (Physiol Genomics. 2008 Sep; 35(1): 1–4). Therefore, the analyses presented in this manuscript are compounded by the uncertainty of whether or not each of the embryos analyzed are actually globally null for FoxO1. At the least, the expression of a Cre reporter in each embryo is necessary to rule out potential germline deletion, especially when using Tie2Cre strain for endothelial deletion.



Response: We are very aware of this complication, and we have instituted several controls. This problem is encountered when the Tie2-cre passes through the female germline (Physiol Genomics. 2008 Sep; 35(1): 1–4). All of our crosses are set up so the Tie2-cre runs through the male germline. We have carefully monitored the cre-excision and FoxO1 expression resulting from cre excision throughout the work (ie FoxO1 expression was analyzed for each set of qRT- PCR experiments). We pooled YS from several 4-7 to increase robustness of the data collected. We have added the figures above Fig 1 A,B,C and S1C to show the difference between FoxO1 expression after Tie2-cre excision vs. germline nulls. These data show that the difference is obvious and we have included data points for qRT-PCRs to better show variance between biological samples. In addition, we have provided data from sorted endothelial cells taken from germline nulls, in several instances, to confirm the differences that we observe are in fact due to *FoxO1* deletion in ECs (Fig 3C,D,E; Fig 6D) and these experiments confirmed our CKO data.
Finally, for *Dll4* expression, we have compared CKOs to germline nulls embryos so the difference is clear and for the YS, similar results are observed although the germline knockouts show a slightly stronger effect. We are as confident as we can be that our results are well controlled.

14. The authors state that phenotypes observed in their studies are consistent with those observed by prior studies. However, prior studies were done either using global deletion or done using Tie2Cre transgenic mice, and the latter ones were not controlled for possible germline deletions.

Response: We have identified where our observations appear consistent with what others have seen. Our data support and hopefully strengthen those of others who have attributed the vascular phenotype to a requirement of FoxO1 in the endothelium.

15. Even though the analyses were performed a day earlier than in the published literature, the conclusion that FoxO1 is important for the pre-flow establishment of arterial EC identity in ys ECs is not supported. There is no time course showing when arterial identity is first evident in yolk sac ECs, and whether arterial identity is never established in mutant ys ECs, or whether it is not maintained, is not examined.

Response: Our data indicate a significant down regulation in arterial markers in 4-7 somite FoxO1 CKOs. Using the *Dll4* reporter, we describe a significant reduction in *Dll4* expression in both CKOs and germline null embryos. *Dll4* is thought to be one of the earliest, if not the earliest arterial markers, and it is clear that normal levels require FoxO1. We do not know and do not assert that *FoxO1* initiates arterial gene expression. Our data support a model where FoxO1 represses *Sprouty2* and *4* expression and that repression is necessary for normal arterial gene expression. Thus, it is likely that another mechanism is required for initiating arterial gene expression but we do not have any insight into that mechanism as of yet. A greater understanding of these mechanisms will require future studies.

16. qRT-PCR results are not confirmed by any other means. Either IF or in situ hybridization would be helpful to ensure endothelial-specificity of the observed changes in gene expression.

Response: While we did not confirm all markers by other means, we have provided Immunohistochemistry for Flk1, PECAM, eNOS, cx37, c40 and we have used a published reporter to provide spatial information about *Dll4* expression. Further, we have sorted endothelial cells from *FoxO1* germline nulls using CD31 to ensure the changes in *Flk1*, *PECAM* and *Sprouty 2 and 4* mRNA could be repeated using an approach independent of the FoxO1 ECKO and all results were in alignment.

17. Data in Figure S3 is not convincing.

Response: We have added a lower magnification view of these data and added data from E9.5. Both additions clearly show endothelial cells based on morphology and a reduction in expression is evident (Figure S3A and B). Connexin expression is often difficult to colocalize with other markers but the experiments were performed in the FoxO1 cKOs which showed a reduction in connexin expression and FoxO1 expression so we feel that these data support the conclusion that there is indeed a reduction in connexin 37, 40 expression in ECs.

18. The fact that the expression of AFP was not changed among yolk sacs of controls and mutants does not mean that the changes in gene expression noted are endothelial-specific. Especially, since the expression of many of the markers tested is not confined to the endothelium.

Response: True, this is just one marker of the endoderm that is not affected. We have adjusted our description too report the data but have a removed a clause so that we are more cautious about the conclusion.

19. The authors do not state how they staged embryos. Since there is a substantial variability in embryo development at early stages, staging by somite counts is more appropriate. This point is important because difference in 1 – 2 somites may indicate whether there is and there isn't blood flow in a particular embryo being analyzed, and impact authors' claims that FoxO1 regulates arterial specification pre-flow.

Response: We apologize for the oversight. We have clarified this in the text and methods. 4-7 somite embryos were used.

20. Figures 3, 4 and others. The authors do not mention statistical tests used in their analyses, or how the analyses were performed, e.g., correction for multiple testing. In the case of Student's t tests, were tests 2-sided, unpaired? In the case of qRT-PCR analyses, how the data was analyzed or statistical analyses were not described

Response: We had added the following in the methods section: Unpaired student's *t* -test was used to assess statistical significance and *P* values <0.05 were considered statistically significant. Additionally, standard deviation are graphed for all qTR-PCR experiments.

-Please used SD instead of S.E. in the analyses and plots, as this is a better metric to evaluate variability.

Response: We have replotted all the data as suggested.

- EC-specificity of changes in the expression of Sprouty 2 and 4 needs to be confirmed by either in situ hybridization or IF with co-staining of an EC marker.

Response: We have attempted to perform other experiments to confirm the changes in Sprouty gene expression but we have not had good luck with the Abs and expression is low in ECs normally. We were able to show clearly that mRNA expression of Sprouty 2 and 4 in sorted ECs (CD31+) from germline nulls show that both 2 and 4 are upregulated in the absence of FoxO1, but Sprouty 4 is actually downregulated in response to the loss of FoxO1 in CD31- cells. These data provide the most reliable way to examine EC specificity with available reagents.

- Overexpression of Sprouty 4 led to decreased expression of not only arterial EC markers but also venous, as well as to the decrease in the expression of pan-EC markers. These results are not consistent with the role of Sprouty 4 in FoxO1ECKO and suggest gain-of-function effects.

Response: Below is the response provided for Reviewer 1. We have added to the discussion to point out these differences. It is likely that broad, overexpression of Sprouty has a broader, perhaps stronger effect that removing the repression by FOXO1 in select cells.

Response: We understand and have clarified our interpretation in this section. The Sprouty overexpression experiment did show that Sprouty4, when overexpressed in ECs could prevent normal vascular remodeling Figure 8B. This is the same phenotype seen in FoxO1 knockouts Figure 1G. From a molecular perspective, it is true that more gene expression is altered in the Sprouty4 overexpression yolk sacs than in the FoxO1 ECKOs. We do not know if this is due to the levels of expression, overexpression in a broader group of cells than FoxO1 functions in, additional functions of Sprouty4 independent of FOXO1, or if there are other considerations. We agree that it is not the perfect experiment but was informative as it showed that Sprouty overexpression is sufficient to disrupt vascular remodeling and endothelial gene expression although it was not a perfect match. Together with ChIP and Luciferase assays in the paper, these data show that Sprouty 4 is downstream of FOXO1.

13. Compound FoxO1 / Sprouty 2/4 knockout mutants are necessary to test the hypothesis that the suppression of these Sprouty genes by FoxO1 regulates EC arterial venous specification.

Response: We attempted this experiment many times and we were never able to achieve efficient KO of FoxO1 and both Sproutys within ECs. The Tie2-cre was just not able to create efficient deletions.

-Figures: 1. The authors state the morphology of FoxO1ECKO embryos was fine at E8.5. However, the picture shows embryos inside yolk sacs obscuring embryonic morphology.

Response: We carefully examined FoxO1ECKO embryos and did not see differences in gross morphology. Additional Dll4-LacZ labeling did not show overt differences in the E8.25 embryo as seen in Figure S4A and B. Furthermore, we added PECAM staining of E8.5 YS for additional comparison, see Figure 1L and M.

-The legend to Figure 3 does not described the panel. The panels and numbering in the figure and legend do not correspond. The genotypes of samples portrayed by the black and gray bars need to be revisited, it is not clear what “floxdel” designation means.

Response: Thank you. We have addressed these to clarify.

-Figure 4. Please state embryonic stage samples used in panels A and B.

Response: We have clarified in the manuscript and in the methods that E8.25 refers to 4-7 somite embryos.

-Please describe in more detail or cite Farsight software used for segmentation

Response: We added some further description the methods. See section for: Quantification of *Flk1-H2B::YFP*$^{+}$ cell density, proliferation index and apoptotic index in whole mount YSs

-Figure 5. Dll4-LacZ expression in FoxO1-null embryo is not evident in any tissue.

Response: Fig. S4C and D shows the anterior views of embryonic expression of the Dll4-LacZ in the germline nulls.

-How was expression was quantified in 5C and F is not stated.
Response: These represent qPCR results as amended in Figure5 figure legend.

-The color coding for Figure 6C and D makes it hard to tell different greys apart Response:
This figure has been updated.

-Legend for Fig. 7 is in accurate in some places and incomplete in others (e.g., not all panels are mentioned in the legend). It is not clear what is shown in the last 4 bars of Fig, 7C.

Response: We edited to ensure the figure legend is complete.

Second decision letter

MS ID#: DEVELOP/2021/200131

MS TITLE: FOXO1 represses Sprouty2 and Sprouty4 expression in endothelial cells to promote arterial specification and vascular remodeling in the mouse yolk sac

AUTHORS: Nanbing Li-Villarreal, Rebecca Lee Yean Wong, Monica D Garcia, Ryan S Udan, Ross A. Poche, Tara L Rasmussen, Alexander M Rhyner, Joshua D Wythe, and Mary E Dickinson

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 BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development. However as you'll see one of the Reviewers still has some outstanding concerns, so I'm returning the manuscript to you so that you can make any further revisions that you think might address these. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. Your manuscript will not require any further review, rather I will look it over myself prior to acceptance.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 1

Advance summary and potential significance to field

The manuscript “FOXO1 represses Sprouty2 and Sprouty4 expression in endothelial cells to promote arterial specification and vascular remodeling in the mouse yolk sac” by Li-Villarreal et al builds off previous reports about roles for the FOXO1 transcription factor in early murine vascular development. Global knockouts for Foxo1 display vascular remodeling defects by E9.5—a time at which circulatory blood flow has commenced—but the authors were interested in determining whether FOXO1 influenced endothelial cells (ECs) before the onset of circulation and hemodynamic signaling, since such signaling influences vascular remodeling and EC fate specification. Here they generated Foxo1-ECKO embryos and analyzed their ECs for transcripts at E8.25 before the onset of blood flow. They found decreased transcripts and protein for Flk1 in mutant yolk sacs although other pan-endothelial markers, proliferation markers, and apoptosis markers of mutant ECs were unaffected. The authors also found transcripts for arterial Notch pathway markers (i.e. Dll4) downregulated in Foxo1 mutant yolk sac ECs but not in embryonic ECs. Because Flk1 and Dll4 gene regulatory regions do not contain known binding sites for FOXO1, the authors sought other direct target genes that might explain these transcriptional changes. They discovered that Spry2 (a known FOXO1 target gene) and Spyr4 are directly suppressed by FOXO1 in ECs. Transient endothelial overexpression of Spry4 in transgenic mouse embryos led to yolk sac remodeling defects and downregulation of many transcripts at E8.25, including several that are likewise seen in Foxo1 mutant ECs (i.e. Dll4 and Flk1). Altogether, the authors have accomplished their goal of defining molecular consequences of endothelial Foxo1 deletion prior to the onset of circulation.

Comments for the author

I appreciate the authors' efforts at addressing my previous comments/suggestions. I am satisfied that their revised manuscript is informative and that the text appropriately describes and interprets the data presented.

Reviewer 2

Advance summary and potential significance to field

In this first revision, "FOXO1 represses Sprouty2 and Sprouty4 expression in endothelial cells to promote arterial specification and vascular remodeling in the mouse yolk sac" the authors describe the role of the transcription factor FoxO1 in the regulation of arterial endothelial cell fate in mouse embryos.
The authors follow an interesting observation that vascular defects in yolk sacs of FoxO1 mutant embryos pre-set cardiovascular defects and lethalities seen later in mutant embryos. The authors claim that FOXO1 functions in yolk sac endothelial cells to regulate arterial specification by regulating expression levels of Sprouty 2 and Sprouty 4 genes. While these initial observations are novel and interesting, major issues raised in the previous review have not been addressed.

Comments for the author

1) The paper is based on the assumption that FoxO1 is deleted in the endothelium in the mutants in the study. However The authors do not demonstrate in any figures that FoxO1 is specifically downregulated in the endothelium. At the least, the authors should present qRT-PCR of FOXO1, as shown for Pecam1 in Figure 3D. In that figure, Pecam1 mRNA was assayed in sorted CD31-negative and CD31-positive cells. The authors should add a panel showing the expression of FoxO1 in CD31- and CD31+ cells.
This basic control is essential to interpret the experimental results in the manuscript. This issue was raised by the previous review and has remained unaddressed.

2) The title of the manuscript "FOXO1 represses Sprouty2 and Sprouty4 expression in endothelial cells to promote arterial specification and vascular remodeling in the mouse yolk sac" is not supported by the evidence presented in the manuscript. As the previous reviewers point out and the authors agree phenotypes observed in Sprouty transgenics could be interpreted as gain-of-function. Given that a multitude of genes, including VEGFR2, is downregulated in FOXO1 mutants, the causative role of Sprouty 2 and 4 downstream of FOXO1 is not convincingly shown.
3) Multiple issues with figure legends and panels outlined by the prior review remain unaddressed. For example, supplemental figure 3 is purported to support endothelial-specific downregulation of connexins in FOXO1 mutants. However neither specific nor endothelial staining is shown.

Second revision

Author response to reviewers' comments

Reviewer 2 comments:
1) The paper is based on the assumption that FoxO1 is deleted in the endothelium in the mutants in the study. However, the authors do not demonstrate in any figures that FoxO1 is specifically downregulated in the endothelium. At the least, the authors should present qRT-PCR of FOXO1, as shown for Pecam1 in Figure 3D. In that figure, Pecam1 mRNA was assayed in sorted CD31-negative and CD31-positive cells. The authors should add a panel showing the expression of FoxO1 in CD31- and CD31+ cells. This basic control is essential to interpret the experimental results in the manuscript. This issue was raised by the previous review and has remained unaddressed.

Response: Our manuscript reports the consequences of Tie2-cre induced conditional knockout of FoxO1 in embryos. Since the majority of expression of this Tie2-cre is in endothelial cells (which has been well documented and relevant papers are cited in the manuscript), we have assumed that the results that we observed do emanate, for the most part, from deletion of FoxO1 in these cells. However, we agree that it may be too strong to state that FoxO1 deletion in ECs is fully responsible for the phenotypes observed. Thus, we have deleted “In endothelial cells” from the title and we have gone back through the manuscript and better defined the cre so it is clear that we understand other cell types in the yolk sac may express Tie2-cre at E8.25. We refrain from using “endothelial-specific” deletion in the text, shifting to either “Tie2-cre deletion” or “conditional knockout” so we do not unintentionally mislead the reader. We have kept the shorthand of “ECKO” but define this clearly.

That said, we do have data that show that endothelial cells are clearly affected by the Tie-2 conditional deletion of FoxO1, as the expression levels of genes normally expressed in ECs are altered, immunostaining of Flk1 and reporter expression of Flk1 both show decreases, and Dll4 expression is decreased, as seen by both qPCR and a nuclear lacZ reporter. We have also included results from sorted CD31+ cells from the germline KO for several experiments to confirm the changes in Pecam, Flk1 and Sprouty gene expression, and finally we use an EC-specific promoter for the Sprouty overexpression experiments. Thus, we feel strongly that our description of the effects on ECs are valid and warranted in these cases.

2) The title of the manuscript "FOXO1 represses Sprouty2 and Sprouty4 expression in endothelial cells to promote arterial specification and vascular remodeling in the mouse yolk sac" is not supported by the evidence presented in the manuscript. As the previous reviewers point out and the authors agree, phenotypes observed in Sprouty transgenics could be interpreted as gain-of-function. Given that a multitude of genes, including VEGFR2, is downregulated in FOXO1 mutants, the causative role of Sprouty 2 and 4 downstream of FOXO1 is not convincingly shown.

As noted above, we have changed the title and removed “in endothelial cells”. However, our data do show that EC specific overexpression of Sprouty is capable of reducing arterial expression and we show data to support a model where the increases in Sprouty are sufficient to drive the phenotypic changes that we observe in the ECKOs. We clearly present the effects of Sprouty overexpression and do show that beyond reduced arterial gene transcripts, we do see additional gene expression alterations and we have added to the discussion of these data. We have also been

clear that there is a reduction in Flk1 in FoxO1 ECKOs but this reduction does not affect the proliferation or survival of ECs, a normal downstream consequence of Flk1 reduction. We also show that FoxO1 directly binds Sprouty regulatory regions in cells harvested from the yolk sac and that functionally, FoxO1 can repress expression of Sprouty via these regions. Thus, our data clearly support a model where direct regulation of Sprouty by FoxO1 can cause the reduction in arterial gene expression. We were not able to determine a direct connection between FoxO1 and Flk1 and also contend that while we have identified mechanism where FoxO1 normally represses Sprouty, which can repress arterial expression, other mechanisms to induce arterial gene expression are likely in place because the Sprouty mechanism is focused on repression and not activation.

3) Multiple issues with figure legends and panels outlined by the prior review remain unaddressed. For example, supplemental figure 3 is purported to support endothelial-specific downregulation of connexins in FOXO1 mutants However, neither specific nor endothelial staining is shown.
The connexin immunofluorescence images are shown in order to confirm the message reductions observed in the qPCR data. The paragraph describing those data does not suggest an endothelial-specific down regulation but rather a reduction in immunostaining. We have added lower magnification images as well as an additional stage (E9.5), to provide spatial context within the yolk sac. As we have explained in our previous response, the punctate nature of the staining makes assignment of these signals to any specific cell incredibly difficult. Thus, we can and have only concluded that there is a reduction in protein along with the reduction in mRNA.

To determine if reduced arterial-specific gene expression correlated with decreased expression of their respective proteins in FoxO1ECKO YSs, immunofluorescence was performed on sectioned YSs at E8.5 and E9.5. Confocal imaging of Cx37 and Cx40 revealed an overall reduction in the number of connexin-positive puncta in FoxO1ECKO YSs when compared to controls at both stages (Figure S3A and B). Similarly, we observed decreased eNOS expression within the vascular plexus in FoxO1ECKO yolks sacs compared to controls (Figure 4B). These data, in addition to previous gene expression analysis, indicate that FOXO1 within the developing endothelium is necessary for the regulation of arterial identity.

Third decision letter

MS ID#: DEVELOP/2021/200131

MS TITLE: FOXO1 represses Sprouty2 and Sprouty4 expression to promote arterial specification and vascular remodeling in the mouse yolk sac

AUTHORS: Nanbing Li-Villarreal, Rebecca Lee Yean Wong, Monica D Garcia, Ryan S Udan, Ross A. Poche, Tara L Rasmussen, Alexander M Rhyner, Joshua D Wythe, and Mary E Dickinson
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.