EMBO reports

Manuscript EMBO-2015-41019

Endothelial Primary Cilia Inhibit Atherosclerosis

Colin Dinsmore and Jeremy F. Reiter

Corresponding author: Jeremy F. Reiter, University of California, San Francisco

Review timeline:

Submission date:	14 July 2015
Editorial Decision:	18 August 2015
Revision received:	15 October 2015
Editorial Decision:	17 November 2015
Revision received:	24 November 2015
Accepted:	27 November 2015

Editor: Esther Schnapp

Transaction Report:

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

1st Editorial Decision 18 August 2015

Thank you for the submission of your research manuscript to our journal. I apologize for the slight delay in getting back to you; we have only now received the full set of referee reports that is copied below.

As you will see, all referees acknowledge that the findings are interesting, and I think that they are a good fit for our journal. However, they also suggest several experiments to strengthen the study, and given the limited number, I think that all of them should be addressed, except for point 4 by referee 3, who is asking for further insight into mechanism. While such data would be most welcome, they are not strictly required for publication of the study by EMBO reports. Please discuss the comment in the manuscript text though. Please let me know in case you think that any of the other points cannot be addressed in a reasonable time frame.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that the concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 35,000

© European Molecular Biology Organization

characters (including spaces and references) and 5 main plus 5 expanded view figures. The results and discussion section must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. Commonly used materials and methods can be moved to the supplementary information, however, please note that materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file. For a normal article there are no length limitations, but the results and discussion section must be separate and the entire materials and methods included in the main manuscript file. We now integrate expanded view figures and tables inline into the main manuscript text where they expand when clicked. Please label these figures EV1, 2, etc and include the figure legends at the end of the main manuscript text. At the moment, we can only offer this for 5 EV figures. If you have more supplementary figures they need to be in the Appendix file (please see our guide to authors for further information).

Regarding data quantification, please always specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images. I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

In this study, Dinsmore and Reiter address the function of primary cilia in the vascular endothelium using a mouse genetic approach. Ciliopathies such as polycystic kidney diseases display conditions in the vascular system including intracranial aneurisms and hypertention. Therefore, this study is relevant to human health.

This is the first study directly addressing the role of cilia in the vascular endothelium in mice. The authors conditionally ablate two floxed ciliogenic genes (Ift88 and Kif3A) using a constitutive Cre expressing mouse line that targets the vascular endothelium. Among the several lines available for this purpose the authors adopted the Tek-Cre line which, broadly targets angioblast as early as e7.5. Because in this line Cre is also expressed in blood cells, some experiments included the Mix-Cre line, which drives Cre expression exclusively in blood cells.

Surprisingly, the authors did not detect any morphological changes or defects in the vasculature of the mutant mice due to absence of cilia. This, as discussed in the study, is in contrast with results obtained in zebrafish. Because of this discrepancy future studies should include additional Cre lines such as those listed in Circ Res. 2015;116:e99-e132 to validate these data. This, however, does not hamper the main finding of this study that is the function of the cilium in protecting from atherosclerosis. In addition, even though there is no mechanism proposed, the authors suggest a critical role of the cilium in the eNox pathway. Therefore, this reviewer recommends publication of this study. However, several major points need to be addressed before publication.

In several instances the authors did not provide important experimental information. Moreover, given the modest differences between mutant and control mice for some of the experiments carried out in this study, more sensitive approaches need to be considered.

- The author stated that the mice were viable and lived to adult age without noticeable anomalies. How long the mice were monitored for?

-For experiments displayed in Fig2 there is no indication of how many mice per genotype were analyzed (n=?).

In figure 2B, aortas from Mx1-Cre controls (last two panels) appear more stained than the aortas from the mutant (second panel from left). Moreover, the percent of ORO-positive areas in Mxl-Cre mice mutant or control are similar to those detected in the Tek-creIFT88 mutant male, raising concerns of reproducibility.

© European Molecular Biology Organization

This reviewer feels that if these measurements were done only on one mouse for each genotype and sex, additional mice should be added to this study and included in statistics. In addition, a close-up of stained atherosclerotic lesions (ORO-positive areas) should be provided for different regions of the aorta.

- An important result is that the ratio between the total eNos and the activated eNos is reduced in mutant mice compared to the control (Fig. 3). However, it is not clear by how much. In the text it is reported to have been reduced by 20%, but in the figure legends it changes to 80%. In any case this conclusion lays exclusively on quantification of confocal images such as the one displayed in Fig. 2. Therefore, a more sensitive approach such as western-blot should be applied to compare control and mutant aortas. If not possible, a more stringent quantitative microscopy analysis should be applied. For example, a quantitative approach should include a normalization of the image background, which could be of different intensities in different samples. This would take into account the signal-to-noise ratio (See: Waters JC, Wittmann T. Concepts in quantitative fluorescence microscopy. Methods Cell Biol. 2014;123:1-18) or Waters JC. Accuracy and precision in quantitative fluorescence microscopy. J Cell Biol. 2009 Jun 29;185(7):1135-48.)

- In the retina vascular assay branching should also be considered and quantified as parameters of vasculogenesis.

Referee #2:

In this manuscript Dinsmore and Reiter investigate the in vivo role of primary cilia on endothelial cells. Primary cilia are important signaling organelles present on almost all mammalian cell types. Several signaling pathways and cellular processes are regulated through cilia and ciliary proteins. Defects in ciliary structure and function are found in a number of hereditary diseases, which are summarized as ciliopathies. Interestingly, the authors find endothelial cilia to be dispensable for mammalian vascular development. This is shown in mice using floxed alleles for either IFT88 or KIF3a, both encoding for proteins essential for ciliogenesis, combined with an endothelial cre line. Although to this point negative data, this a very interesting and important finding for the cilia community. Strikingly, the authors demonstrate, that atherosclerosis develops more severely, when endothelial ciliogenesis is disrupted in Apoe-/- mice. The presence of primary endothelial cilia seems to be protective in terms of inflammation and the maintenance of endothelial function as measured by eNOS expression and phosphorylation.

Taken together, this is an important study on a highly relevant topic relevant to a very frequent human pathology. Both novelty and quality of the data is very high and the manuscript is written very clear. Although I really like the story and the concept, I have some concerns that should be addressed by the authors.

1. Findings in Fig. 1 A/B are currently very similar to the results published by Van Der Heiden et al. (cited as ref. 12; see Fig. 3). Therefore I would suggest to present this as suppl. information showing that the model is working. Moreover, I would suggest to cite ref.12 already in the introduction when giving the overview on cilia and endothelial cells.

2. It is the policy of EMBO Reports to not permit citation of "Data not shown". Therefore - and because it would be important info for the readers - a couple of additional data points should be provided by the authors as supplemental figures or as part of the main figures: (1) general data on viability of Tek-Cre Ift88C/- and Tek-Cre KIF3aC/- mice, (2) data on Tek-cre SmoC/C mice (viability and vasculature) as well as Tek-Cre Pkd2C/- mice, (3) data from the Tek-Cre Kif3a C/-Pecam1Gt/Gt mice.

3. The finding that loss of cilia increases the size of plaques is very central in this study. It would be terrific if this important finding could be confirmed in Tek-Cre Kif3a mice to exclude that the effect is caused by loss of non-ciliary functions of IFT88.

Minor point:
- Fig. S3: data presented (n=1/n=2) should be confirmed and presented with n=3 to allow stats.

© European Molecular Biology Organization

Referee #3:

In the manuscript entitled "Endothelial Primary Cilia Inhibits Atherosclerosis", Dinsmore and Reiter showed that the genetic ablation of Ift88 in ECs results in enhance atherosclerosis in Apoe-/- mice. Mechanistically, the authors found a marked reduction of eNOS phosphorylation and inflammation markers in the artery wall. Even thought these results could be of interest, many key experiments are missing to support author's conclusions.

Specific comments.

1- The authors only assessed atherogenesis by performing on face Oil Red O staining. While this approach represent the neutral lipid deposition in the arterial wall, the authors should analyze the atherosclerotic lesion in the aortic root and brachiocephalic artery.

2- The authors should also measured plaque composition in the aortic root and brachiocephalic artery because the increase in inflammatory markers (IL1b, etc) observed in mice lacking Ift88 might be explained by different amount of macrophage infiltrated in the arterial wall. This is the reason why the analysis of plaque composition is important to support author's conclusions.

3-The conclusion that eNOS activation is reduced is based in p-eNOS immunostaining in vivo. The authors should provide additional evidences that eNOS activity is reduced. Measurement of blood pressure and plasma NO will strength this conclusion. Additionally, the authors could also measure NO production and EC inflammation in vitro.

4- The molecular mechanism associated with reduced eNOS phosphorylation is poorly characterized. Is Akt phosphorylation reduced in aortas isolated from Ift88-deficient mice?. If yes, which is the link between the cilia and Akt activation?.

1st Revision - authors' response 15 October 2015

We deeply appreciate your helpful comments. We were heartened to learn that referee 1 recommended the publication of the work, that referee 2 described the work as “interesting and important,” “an important study on a highly relevant topic relevant to a very frequent human pathology,” and that “both novelty and quality of the data is very high and the manuscript is written very clear.” Your suggestions have led to additional experiments that have significantly strengthened the manuscript. We were instructed by the editor to not respond to point 4 by referee 3, as it was deemed beyond the requirements for publication, and point 3 of referee 2, which asks for an analysis of atherosclerosis in Tek-Cre Kif3a conditional mice. This referee described this experiment as “it would be terrific if” it could be done. We agree, and given the time required to complete this analysis, we will include it in a subsequent paper. We have also added detailed descriptions regarding data quantification. We discuss how we have attended to your comments point by point, below, with your comments in bold:

Referee #1:
In this study, Dinsmore and Reiter address the function of primary cilia in the vascular endothelium using a mouse genetic approach. Ciliopathies such as polycystic kidney diseases display conditions in the vascular system including intracranial aneurisms and hypertention. Therefore, this study is relevant to human health.
This is the first study directly addressing the role of cilia in the vascular endothelium in mice. The authors conditionally ablate two floxed ciliogenic genes (Ift88 and Kif3A) using a constitutive Cre expressing mouse line that targets the vascular endothelium. Among the several lines available for this purpose the authors adopted the Tek-Cre line which, broadly targets angioblast as early as e7.5. Because in this line Cre is also expressed in blood cells, some experiments included the Mix-Cre line, which drives Cre expression exclusively in blood cells.
Surprisingly, the authors did not detect any morphological changes or defects in the vasculature of the mutant mice due to absence of cilia. This, as discussed in the

study, is in contrast with results obtained in zebrafish. Because of this discrepancy future studies should include additional Cre lines such as those listed in Circ Res. 2015;116:e99-e132 to validate these data. This, however, does not hamper the main finding of this study that is the function of the cilium in

protecting from atherosclerosis. In addition, even though there is no mechanism proposed, the authors suggest a critical role of the cilium in the eNox pathway. Therefore, this reviewer recommends publication of this study. However, several major points need to be addressed before publication.

In several instances the authors did not provide important experimental information. Moreover, given the modest differences between mutant and control mice for some of the experiments carried out in this study, more sensitive approaches need to be considered.

- The author stated that the mice were viable and lived to adult age without noticeable anomalies. How long the mice were monitored for?
The mice were monitored throughout a year of life.

-For experiments displayed in Fig2 there is no indication of how many mice per genotype were analyzed (n=?).
The number of mice analyzed, separated by genotype and gender, is now included in the Figure 2 legend. Briefly, 24 experimental *Tek-Cre Apoe*$^{-/-}$ *Ift88*$^{C/-}$ mice and 30 control mice were analyzed for the investigation into the effect of loss of endothelial Ift88 on atherosclerosis. 14 *Mx1-Cre Apoe*$^{-/-}$ *Ift88*$^{C/-}$ mice and 16 control mice were analyzed for the investigation into the effect of loss of blood cell Ift88 on atherosclerosis.

In figure 2B, aortas from Mx1-Cre controls (last two panels) appear more stained than the aortas from the mutant (second panel from left). Moreover, the percent of ORO-positive areas in Mxl-Cre mice mutant or control are similar to those detected in the Tek-creIFT88 mutant male, raising concerns of reproducibility. This reviewer feels that if these measurements were done only on one mouse for each genotype and sex, additional mice should be added to this study and included in statistics. In addition, a close-up of stained atherosclerotic lesions (ORO-positive areas) should be provided for different regions of the aorta.
We have replaced the images in Figure 2B to depict truly representative data, indicated by the red dot from whisker graphs of Figure 2E and 2F, indicating the quantitation from the shown aortae. An increase in lesion size in the Mx1-Cre mice as compared to the Tek-Cre mice is likely due to the method of Cre induction. Mx1-Cre is induced using pIpC, which activates inflammatory responses and may contribute to atherosclerosis. All measurements were not done on only one mouse for each genotype and sex: at least seven mice were included in each gender and genotype specific group. As requested, we have included close up photos of the stained atherosclerotic lesions in the aortic arch in the new Figure 2C.

- An important result is that the ratio between the total eNos and the activated eNos is reduced in mutant mice compared to the control (Fig. 3). However, it is not clear by how much. In the text it is reported to have been reduced by 20%, but in the figure legends it changes to 80%. In any case this conclusion lays exclusively on quantification of confocal images such as the one displayed in Fig. 2. Therefore, a more sensitive approach such as western-blot should be applied to compare control and mutant aortas. If not possible, a more stringent quantitative microscopy analysis should be applied. For example, a quantitative approach should include a normalization of the image background, which could be of different intensities in different samples. This would take into account the signal-to-noise ratio (See: Waters JC, Wittmann T. Concepts in quantitative fluorescence microscopy. Methods Cell Biol. 2014;123:1-18) or Waters JC Accuracy and precision in quantitative fluorescence microscopy. J Cell Biol. 2009 Jun 29;185(7):1135-48.)
We appreciate the suggestion and apologize for the confusion regarding how we reported the results.

© European Molecular Biology Organization

Following your suggestion, the eNos staining was quantified using confocal microscopy and normalization of the image background as recommended by Waters and Wittmann. This improved approach increased the statisitical significance of the result. To avoid confusion regarding the magnitude of the result, we now state the finding in terms of percent of the control level, and not percent reduction

- In the retina vascular assay branching should also be considered and quantified as parameters of vasculogenesis.
We quantified retinal vascular branching in five experimental and six control animals, and found that removal of endothelial cilia did not affect this parameter. These data are now included in the new Figure EV3C.

Referee #2:
In this manuscript Dinsmore and Reiter investigate the in vivo role of primary cilia on endothelial cells. Primary cilia are important signaling organelles present on almost all mammalian cell types. Several signaling pathways and cellular processes are regulated through cilia and ciliary proteins. Defects in ciliary structure and function are found in a number of hereditary diseases, which are summarized as ciliopathies. Interestingly, the authors find endothelial cilia to be dispensable for mammalian vascular development. This is shown in mice using floxed alleles for either IFT88 or KIF3a, both encoding for proteins essential for ciliogenesis, combined with an endothelial cre line. Although to this point negative data, this is a very interesting and important finding for the cilia community. Strikingly, the authors demonstrate, that atherosclerosis develops more severely, when endothelial ciliogenesis is disrupted in Apoe-/- mice. The presence of primary endothelial cilia seems to be protective in terms of inflammation and the maintenance of endothelial function as measured by eNOS expression and phosphorylation.

Taken together, this is an important study on a highly relevant topic relevant to a very frequent human pathology. Both novelty and quality of the data is very high and the manuscript is written very clear. Although I really like the story and the concept, I have some concerns that should be addressed by the authors.

1. Findings in Fig. 1 A/B are currently very similar to the results published by Van Der Heiden et al. (cited as ref. 12; see Fig. 3). Therefore I would suggest to present this as suppl. information showing that the model is working. Moreover, I would suggest to cite ref.12 already in the introduction when giving the overview on cilia and endothelial cells.
After consulting with the editor, we have kept the confirmation of the Van Der Heiden et al. data in the results section as it confirms that the model is working with the mice of our age and background. We have included reference to this important work in our introduction.

2. It is the policy of EMBO Reports to not permit citation of "Data not shown". Therefore - and because it would be important info for the readers - a couple of additional data points should be provided by the authors as supplemental figures or as part of the main figures: (1) general data on viability of Tek-Cre Ift88C/- and Tek-Cre KIF3aC/- mice, (2) data on Tek-cre SmoC/C mice (viability and vasculature) as well as Tek-Cre Pkd2C/- mice, (3) data from the Tek-Cre Kif3a C/- Pecam1Gt/Gt mice.
We have included these data on these mice in the new Figure EV2A. We include new data about the *Tek-Cre Kif3a*$^{C/C}$ *Pecam1*$^{Gt/Gt}$ vasculature in the new Figure EV2B.

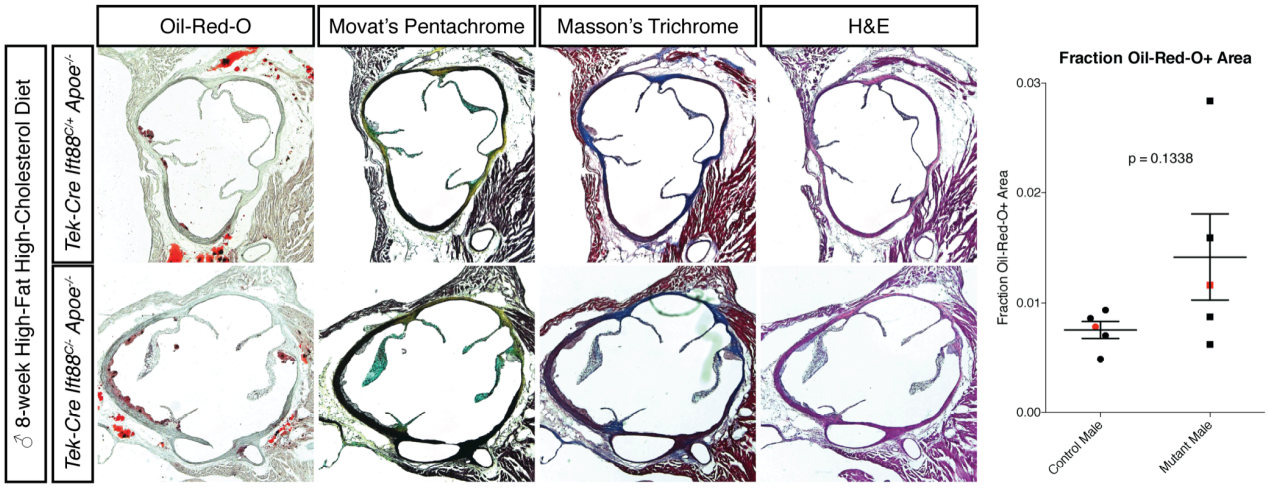
3. The finding that loss of cilia increases the size of plaques is very central in this study. It would be terrific if this important finding could be confirmed in Tek-Cre Kif3a mice to exclude that the effect is caused by loss of non-ciliary functions of IFT88.
Please see above.

Minor point:

- Fig. S3: data presented (n=1/n=2) should be confirmed and presented with n=3 to allow stats.
For the analysis of endothelial cell polarity (Figure EV3), we have included additional mice and applied statistical tests to the data (Figure EV3G).

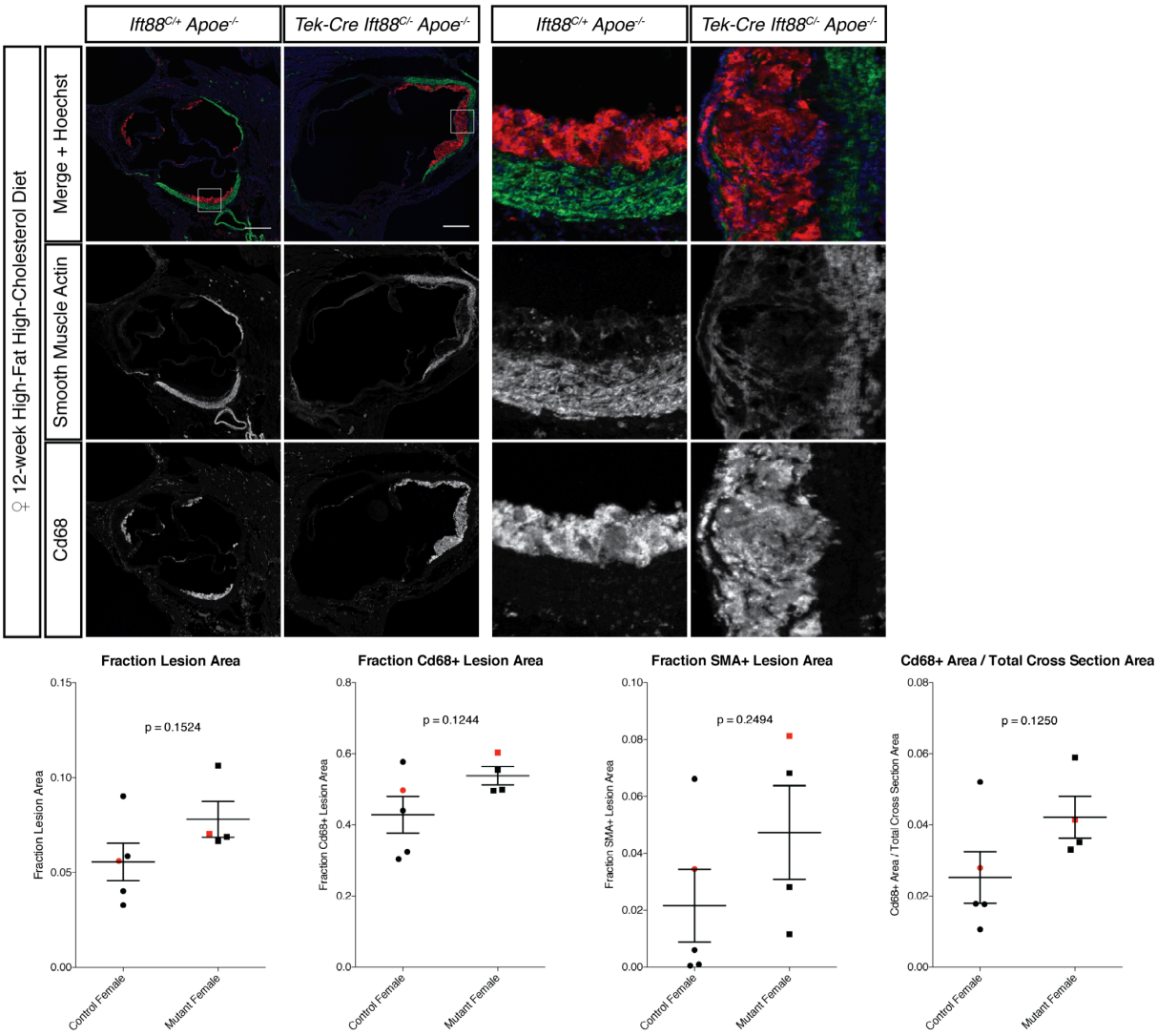
Referee #3:
In the manuscript entitled "Endothelial Primary Cilia Inhibits Atherosclerosis", Dinsmore and Reiter showed that the genetic ablation of Ift88 in ECs results in enhance atherosclerosis in Apoe-/- mice. Mechanistically, the authors found a marked reduction of eNOS phosphorylation and inflammation markers in the artery wall. Even thought these results could be of interest, many key experiments are missing to support author's conclusions.

Specific comments.
1- The authors only assessed atherogenesis by performing on face Oil Red O staining. While this approach represent the neutral lipid deposition in the arterial wall, the authors should analyze the atherosclerotic lesion in the aortic root and brachiocephalic artery.
As requested, we have analyzed the atherosclerotic lesions using a combination of Oil Red O, Movat's pentachrome, Masson's trichrome, and H&E staining. The data are included here:



2- The authors should also measured plaque composition in the aortic root and brachiocephalic artery because the increase in inflammatory markers (IL1b, etc) observed in mice lacking Ift88 might be explained by different amount of macrophage infiltrated in the arterial wall. This is the reason why the analysis of plaque composition is important to support author's conclusions.
We analyzed macrophage infiltration into the arterial wall via Cd68 staining. The data are included here:

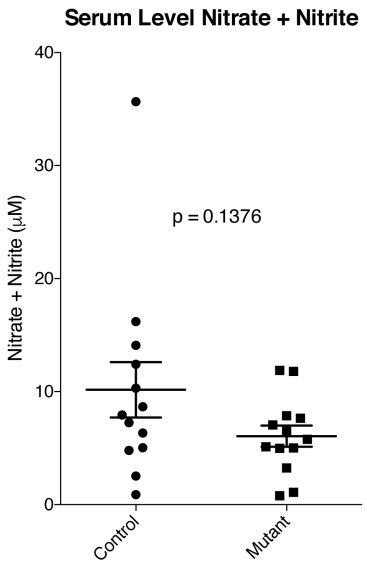
© European Molecular Biology Organization



3-The conclusion that eNOS activation is reduced is based in p-eNOS immunostaining in vivo. The authors should provide additional evidences that eNOS activity is reduced. Measurement of blood pressure and plasma NO will strength this conclusion. Additionally, the authors could also measure NO production and EC inflammation in vitro.

We measured blood pressure in anesthetized mice and detected no differences between those with unciliated endothelial cells and controls, but have not included these data in the manuscript because of concerns that hemodynamic effects of the anesthetic agents may mask other effects. Prior work from Nauli et al. (Circulation, 2008) demonstrated that cells with a hypomorphic mutation in *Ift88* (*Tg737 orpk*) show decreased production of NO in response to shear stress in vitro. We also measured plasma nitrate and nitrite in vivo in *Apoe* null mice with and without EC cilia, the data for which are included here:

© European Molecular Biology Organization



4- The molecular mechanism associated with reduced eNOS phosphorylation is poorly characterized. Is Akt phosphorylation reduced in aortas isolated from Ift88-deficient mice? If yes, which is the link between the cilia and Akt activation?
Please see above.

Additionally, we have complied with the EMBO Journal style and replaced data not shown with included data. Thank you again for your extensive help with this manuscript. Your efforts and suggestions have substantially deepened the discussion and improved our work. Please let me know if I can provide any additional information or answer any further questions.

2nd Editorial Decision 17 November 2015

Thank you for your patience while your manuscript was re-reviewed. We have now received the comments from two referees, and both support the publication of your revised mauscript. Referee 1 only has a minor suggestion that I would like you to address before we can proceed with the official acceptance of your manuscript.

The manusript has only 3 main figures and 5 EV figures now. It would be better to move one or two EV figures to the main manusript file, our short reports can have up to 5 figures. Please also carefully check the text again and make sure that all EV figures are cited as such. The supplementary tables need to be called expanded view tables (EV table 1 and 2). Please also combine the results and discussion sections, to comply with our short reports format.

Regarding the author checklist, is the statement about blinding (4b) also included in the manucript methods?

I have shortened the summary that you sent, but the bullet points are fine. I would use the model you provided for the synopsis image if you agree. I will also discuss your cover suggestion with my colleagues here.

I look forward to seeing a final, revised version of your manuscript as soon as possible. I think this is a very nice and perfect EMBO reports paper! Please let me know if you have any questions.

© European Molecular Biology Organization

REFEREE REPORTS

Referee #1:

In this resubmitted version of the manuscript the authors have addressed concerns regarding viability and the number of mice analyzed, added quantification analysis and dealt with issues regarding statistics raised by referee #1 and #2. These efforts added strength to the conclusions reached by the authors and complied with the standards of EMBO reports.

In addition, the authors have made a significant effort to address the points raised by referee #3 however, the results of these experiments were only shown in the rebuttal letter. New experiments were carried out in order to detect lipid deposition by alternative approaches other than just Oil Red O staining used in the previous version of the study. These results confirmed the conclusions reached in Fig2 and I agreed that it would be redundant to add to Fig2. However, I think that the experiments conducted in order to identifying the nature of plaques by staining for Cd68, a macrophage marker, would add new information to the study and should be included as an extended version or supplementary figure. I agree with referee#3 that there is limited data addressing the molecular mechanism underling the increase in the formation of plaques in ciliary mutant mice. However, the novelty of these findings justifies the publication of this study in a high visibility journal such as EMBO reports.

Referee #2:

The authors addressed all my concerns in the revised version of the MS and in their response letter. Therefore, I recommend the publication of this rev. manuscript.

2nd Revision - authors' response 24 November 2015

Referee #1 suggested that the experiments conducted in order to identifying the nature of plaques by staining for Cd68, a macrophage marker, should be included as an extended version figure. These data are now included in the new Figure EV3. The statement about blinding ("For all quantitation, all data were analyzed with only the index number of the mouse, which was not associated with a genotype until after the analysis.") is included in the Methods section under the Statistics heading. Also, we moved one of the EV figures to a main figure, as per your suggestion.

3rd Editorial Decision 27 November 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

© European Molecular Biology Organization