Chondroitin sulfate enhances the barrier function of basement membrane assembled by heparan sulfate

Chenqi Tao, Neoklis Makrides, Jen-Zen Chuang, Yihua Wu, Steven E. Brooks, Jeffrey D. Esko, Ching-Hwa Sung and Xin Zhang
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Original submission

First decision letter

MS ID#: DEVELOP/2022/200569

MS TITLE: Chondroitin sulfates enhances the barrier function of basement membrane assembled by heparan sulfates

AUTHORS: Chenqi Tao, Neoklis Makrides, Yihua Wu, Steven E Brooks, Jeffrey E Esko, and Xin Zhang

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. 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 of Drs. Tao et al., investigates the role of HS and CS glycosylation in mouse retinal development using several mouse mutants. Initial data showed no major phenotypes in ILM formation following relative late HS deletion and a minor phenotype in the pruning of the retinal vasculature.
Challenging the retinal vasculogenesis by high oxygen led to a much greater vascular defect that was partially curable by external VEGF. Most defects were restricted to the extreme periphery of the retina.
This was experimentally addressed by a cre-mouse that led to a developmentally earlier deletion of the enzyme.
The most interesting data is the use of viruses to detect defects in the ILM following HS and CS deletion that were not detected by immunostaining. This happens to be also the most important finding for the authors as it is stated in the title of their manuscript.

Comments for the author

Just the last part needs to be expanded. I was particularly stunned that the authors did not back up these last data by high-resolution transmission (TEM) and scanning electron microscopy (SEM). The obvious question that arises from the virus experiment is whether actual breaks in the ILM are histological detectable that are not readily visible by fluorescent microscopy. Since the authors are experienced in ocular injection, an obvious experiment would have been to inject heparitinase and chondroitinase to allow viral transduction in the adult mouse.
The manuscript was not easy to read. In addition, many of the micrographs are tiny, and the indicate defects hard if not impossible to see, Examples are: Fig. 2, the empty vessel sheaths. Fig. 3, the stippled lines. Fig. 4 the spindle-shaped and round astrocytes. The ILM disruptions in Fig 5A and astrocytes and vesculature in Fig. 5 B third row. Again, SEM and TEM would really help, particularly to test for ILM disruptions. I would also expect retinal ectopias, where retinal cells bulge through holes in the ILM into the vitreous.
Another question is whether ILM defects in the development of the retina are permanent and never get repaired in the adult.

Reviewer 2

Advance summary and potential significance to field

In this paper, new and interesting information about the role of glycosaminoglycans in the assembly and function of retinal basement membranes. Several mouse models are used in the study which concentrates on the effects of halting heparan sulfate production.

Comments for the author

While the experiments are well performed, the introduction and discussion of the results need to be improved.

Importantly, it should be made clear when CS means CS/DS or CS alone. For instance, Chsy/Chpf complexes synthesize both CS and DS. In the description of GAG biosynthesis, epimerases are not mentioned, but they appear unexplained in Fig. 1A.
Furthermore, in the introduction, the consequences of inactivating Ugdh on CS and HS biosynthesis are discussed and a previous paper from the group is referenced. What about hyaluronan which also contains glucuronic acid? This issue is also discussed in the Discussion without mentioning hyaluronan. Also, the title of the paper needs to be improved; Basement membranes are not assembled by heparan sulfate and it is not appropriate to use the plural form of heparan sulfate (heparan sulfates) and chondroitin sulfate (chondroitin sulfates).

Minor points
1. Fig. 1A has many errors.
a) CS is sulfated before it encounters the sulfotransferases (upper line of CS-symbols).

b) The blue top of the hexuronic acid symbol has not been removed when glucuronic acid has been epimerized to iduronic acid (middle and bottom line of CS-symbols).
c) "GalNac" should be written "GalNAc".
d) Protein names are used for all enzymes except for Ext1 and Ext2 which are written in italics.
e) The legend needs to be rewritten to better explain the biosynthesis.

2. Please be consistent when using Vegf (mouse protein) and VEGF (human counterpart). There are also some inconsistencies between gene (italics) and protein names (regular).
3. In the discussion it should be emphasized that Chst11 is not the only CS 4-O-sulfotransferase, maybe explaining the mild phenotypes gained. In addition, biosynthesis of dermatan sulfate will not be influenced by the knockout of Chst11.

First revision

Author response to reviewers' comments

Reviewer: 1
The manuscript of Drs. Tao et al., investigates the role of HS and CS glycosylation in mouse retinal development using several mouse mutants. Initial data showed no major phenotypes in ILM formation following relative late HS deletion and a minor phenotype in the pruning of the retinal vasculature. Challenging the retinal vasculogenesis by high oxygen led to a much greater vascular defect that was partially curable by external VEGF. Most defects were restricted to the extreme periphery of the retina. This was experimentally addressed by a cre-mouse that led to a developmentally earlier deletion of the enzyme.

The most interesting data is the use of viruses to detect defects in the ILM following HS and CS deletion that were not detected by immunostaining. This happens to be also the most important finding for the authors as it is stated in the title of their manuscript.

Response: We thank the reviewer for positive comments on our work.

Just the last part needs to be expanded. I was particularly stunned that the authors did not back up these last data by high-resolution transmission (TEM) and scanning electron microscopy (SEM). The obvious question that arises from the virus experiment is whether actual breaks in the ILM are histological detectable that are not readily visible by fluorescent microscopy. Since the authors are experienced in ocular injection, an obvious experiment would have been to inject heparitinase and chondroitinase to allow viral transduction in the adult mouse.

Response: We performed TEM as the reviewer suggested and indeed observed breaks in the basement membrane (Fig. 7A). Interestingly, we also noticed a band of intermediate electron density materials above the basement membrane, which was disrupted by Ext1/C4st1 knockout. We also performed ocular injection of heparitinase and chondroitinase, which indeed led to viral transduction in the adult retina (Fig. 7B).

The manuscript was not easy to read. In addition, many of the micrographs are tiny, and the indicate defects hard if not impossible to see, Examples are: Fig. 2, the empty vessel sheaths. Fig. 3, the stippled lines. Fig. 4, the spindle-shaped and round astrocytes. The ILM disruptions in Fig 5A and astrocytes and vesculature in Fig. 5 B third row. Again, SEM and TEM would really help, particularly to test for ILM disruptions. I would also expect retinal ectopias, where retinal cells bulge through holes in the ILM into the vitreous. Another question is whether ILM defects in the development of the retina are permanent and never get repaired in the adult.

Response: Enlarged images are now presented in these figures. We did not observe obvious retinal ectopias as shown in the new Fig. 1C, which was likely because the holes in the ILM were quite small. As shown in the updated Fig. 5A, ILM breaks became more severe in our mutants from P7 to P28, suggesting that ILM defects were not repaired in adult animals.

Reviewer: 2
In this paper, new and interesting information about the role of glycosaminoglycans in the assembly and function of retinal basement membranes. Several mouse models are used in the study which concentrates on the effects of halting heparan sulfate production.

Response: We appreciate the reviewer's encouraging comments on our work.

While the experiments are well performed, the introduction and discussion of the results need to be improved. Importantly, it should be made clear when CS means CS/DS or CS alone. For instance, Chsy/Chpf complexes synthesize both CS and DS. In the description of GAG biosynthesis, epimerases are not mentioned, but they appear unexplained in Fig. 1A.
Furthermore, in the introduction, the consequences of inactivating Ugdh on CS and HS biosynthesis are discussed and a previous paper from the group is referenced. What about hyaluronan which also contains glucuronic acid? This issue is also discussed in the Discussion without mentioning hyaluronan. Also, the title of the paper needs to be improved; Basement membranes are not assembled by heparan sulfate and it is not appropriate to use the plural form of heparan sulfate (heparan sulfates) and chondroitin sulfate (chondroitin sulfates).

Response: We made these clarifications as the reviewer suggested in the Introduction and Discussion. Based on our previous study and the data in this paper, we believe that HSPGs act as cellular receptors to assemble the retinal basement membrane, so we would like to keep the current title to emphasize this model.

1. Fig. 1A has many errors.
a) CS is sulfated before it encounters the sulfotransferases (upper line of CS-symbols).
b) The blue top of the hexuronic acid symbol has not been removed when glucuronic acid has been epimerized to iduronic acid (middle and bottom line of CS-symbols).
c) "GalNac" should be written "GalNAc".
d) Protein names are used for all enzymes except for Ext1 and Ext2 which are written in italics.
e) The legend needs to be rewritten to better explain the biosynthesis.

Response: We apologize for these mistakes. They have all be corrected.

2. Please be consistent when using Vegf (mouse protein) and VEGF (human counterpart). There are also some inconsistencies between gene (italics) and protein names (regular).

Response: We have carefully checked the consistency of these gene and protein names in the manuscript.

3. In the discussion it should be emphasized that Chst11 is not the only CS 4-O-sulfotransferase, maybe explaining the mild phenotypes gained. In addition, biosynthesis of dermatan sulfate will not be influenced by the knockout of Chst11.

Response: These points are now emphasized in the Discussion.

Second decision letter

MS ID#: DEVELOP/2022/200569

MS TITLE: Chondroitin sulfates enhances the barrier function of basement membrane assembled by heparan sulfates

AUTHORS: Chenqi Tao, Neoklis Makrides, Jen-Zen Chuang, Yihua Wu, Steven E Brooks, Jeffrey E Esko, Ching-Hwa Sung, and Xin Zhang

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development. As you will see Reviewer 2 raises some very minor concerns including the suggestion to slightly modify the title of your manuscript to make it a more accurate description of your findings. You might consider this and the additional minor comment before uploading the final version of your manuscript. Your paper will not require further review, rather I will look it over myself prior to acceptance. 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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

The revised manuscript of Drs. Tao et al. addressed most if not all of the concerns raised by this reviewer.

The TEM, under refined fixation and contrasting techniques, even reveals a usually undetected extracellular

matrix layer in the vitreous that covers the vitreal side of the ILM. The authors argue that in double-mutant

mice, the thinning of this layers in conjunction with holes in the ILM are responsible for the successful viral

transduction of the neural retina after intravitreal virus injection. The authors also found a retina-wide viral

transduction after co-injection of virus with heparitinase and chondroitinase in one-month-old wild type

mice.

Comments for the author

The manuscript is clearly improved, more interesting thanks to new data, and easier to read. The tiny micrographs are still a problem for this reviewer.

Reviewer 2

Advance summary and potential significance to field

The paper highlights the specific functions of Heparan sulfate and chondroitin sulfate in the assembly and function of the retinal extracellular matrix.

Comments for the author

The manuscript has been improved by the revision. I still think the title should be changed. Heparan sulfate may be required for the assembly but "assembled by" implies an action similar to when a brick-layer builds a wall. What about writing "Chondroitin sulfate enhances the barrier function of basement membranes assembled in the presence of heparan sulfate"? A minor issue is that "CS" should be replaced by CS/DS on p. 5 l.8 and on p. 6 l.1.

Second revision

Author response to reviewers' comments

Reviewer: 1
The revised manuscript of Drs. Tao et al. addressed most if not all of the concerns raised by this reviewer.

The TEM, under refined fixation and contrasting techniques, even reveals a usually undetected extracellular matrix layer in the vitreous that covers the vitreal side of the ILM. The authors argue that in double-mutant mice, the thinning of this layers in conjunction with holes in the ILM are responsible for the successful viral transduction of the neural retina after intravitreal virus injection. The authors also found a retina-wide viral transduction after co-injection of virus with heparitinase and chondroitinase in one-month-old wild type mice.

The manuscript is clearly improved, more interesting thanks to new data, and easier to read. The tiny micrographs are still a problem for this reviewer.

Response: We thank the reviewer for suggesting the EM analysis, which revealed unexpected insights in our mutants. It is unfortunate that we can not use larger micrographs without disrupting the figure layouts, but we did include enlarged insets to highlight the phenotype.

Reviewer: 2
The paper highlights the specific functions of Heparan sulfate and chondroitin sulfate in the assembly and function of the retinal extracellular matrix.

The manuscript has been improved by the revision. I still think the title should be changed. Heparan sulfate may be required for the assembly but "assembled by" implies an action similar to when a brick-layer builds a wall. What about writing "Chondroitin sulfate enhances the barrier function of basement membranes assembled in the presence of heparan sulfate"? A minor issue is that "CS" should be replaced by CS/DS on p. 5 l.8 and on p. 6 l.1.

Response: We thank the reviewer for careful reading our manuscript. However, we feel the title suggested by the reviewer is too passive, as “in the presence of heparan sulfate” suggests a bystander effect. In contrast, we believe out data demonstrate that heparan sulfate plays an active role in the assembly of the basement membrane. Therefore, we would like to retain the original title.

The suggested changes of “CS” to “CS/DS” have been made in p. 5 and p. 6.

Third decision letter

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AUTHORS: Chenqi Tao, Neoklis Makrides, Jen-Zen Chuang, Yihua Wu, Steven E Brooks, Jeffrey E Esko, Ching-Hwa Sung, and Xin Zhang
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.